Focus Issue

2nd ECS Workshop 2009

Annexins, targets and calcium-binding proteins in pathology

Smolenice, June 3–6, 2009, Slovakia

Editors

A. Breier, C. W. Heizmann and B. Uhrík

Preface

The 2nd ECS Workshop on Annexins, Targets and Calcium-Binding Proteins in Pathology

This workshop was held in the beautiful Smolenice castle in Slovakia from June 3-6, 2009 and organized by the European Calcium Society (Claus Heizmann) in cooperation with the Slovak Academy of Sciences (Albert Breier).

The village of Smolenice is located in the west of Slovakia, about 60 km from the capital city of Bratislava. The Smolenice castle is located above the village on the foothills of the Carpatian mountains.

First documents of the existence of this castle date back to the 13th century. During the Napoleon war the castle decayed after the main building and the tower had been destroyed. Reconstruction of the castle was started early in the 20th century.

In 1953 the castle was handed over to the Slovak Academy of Sciences (SAS) to become their representative International Congress Center.

65 scientists (mostly young investigators) attended this 2nd ECS workshop; the participants came from 15 different countries including Argentina, Canada, USA, Russia and Poland.

The scientific program opened with the keynote lecture of Joseph Metzger (University of Minnesota, Minneapolis) discussing the defective intracellular calcium handling in diastolic heart failure (DHF) and the gene transfer of parvalbumin restoring myocardial performance in DHF.

The next day two sessions followed discussing the structures and functions of annexins and their interactions with target proteins with the emphasis on the EF-hand calcium-binding proteins sorcin and S100 proteins.

The 3rd day was devoted to the role of the calcium-binding proteins in pathology and their application in clinical diagnostics with excellent lectures on calcineurin in leukemias, calpain in Alzheimer's disease, CaBP's in allergic diseases, S100A4 in tumor growth and metastasis and S100B in psychiatric disorders.

The final day was devoted to the calcium-binding proteins of the endoplasmatic reticulum under normal and pathological conditions. Highly interesting lectures covered the following topics: Calcium-dependent interplay between ER and multidrug resistancy, calcium-channels in health and disease, calcium–binding chaperons in the ER, calcium stores in relation to ischemia, and regulation of S100P in cancer cells.

An impressing highlight was the very lively poster session of the young investigators.

This issue of the Journal "General Physiology and Biophysics" of the Slovak Academy of Sciences, Bratislava, Slovakia contains the papers of the invited speakers. The poster abstracts will be displayed on the ECS web page and on the web page of General Physiology and Biophysics.

The organizers are especially grateful to Branislav Uhrik (SAS), the local organizers, and Roland Pochet (General Secretary of the ECS) helping in many tasks, turning this workshop into a memorable success.

More information with pictures of the lectures, poster session and of the social activities are found on our ECS webpage (www.ulb.ac.be/assoc/ecs) and the upcoming ECS Newsletter.

Summarizing I can say that this was an excellent and a well attended workshop and we are looking forward to the 11th European Symposium on Calcium which will be organized by Jacek Kuznicki and his team on the 5th-8th September 2010 in Warsaw, Poland.

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Review

Parvalbumin: Targeting calcium handling in cardiac diastolic dysfunction

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Abstract. Diastolic heart failure (DHF) is a clinical syndrome characterized by depressed myocardial relaxation performance and poor ventricular refilling. Defective intracellular calcium (Ca^{2+}) handling underlies one of the fundamental mechanisms of DHF. Manipulating the content and function of Ca^{2+} handling proteins in the heart has been the focus of intense study to develop effective therapies for DHF patients. Parvalbumin (Parv), a skeletal muscle Ca^{2+} binding protein, has been shown to facilitate myocardial relaxation both *in vitro* and *in vivo*. Parv acts as a unique "delayed" Ca^{2+} buffer and facilitates Ca^{2+} sequestration from cytosol. Here, we summarize studies employing gene transfer of Parv in cultured adult cardiac myocytes and in vivo to redress depressed diastolic function. By targeting defects in cardiac Ca^{2+} handling, Parv represents a promising therapeutic candidate for alleviating diastolic dysfunction in DHF.

Key words: Parvalbumin — Calcium handling — Diastolic heart failure

Cardiac diastolic dysfunction and calcium handling

Congestive heart failure (CHF) is a clinical syndrome of compromised pumping function of the heart. Diastolic dysfunction can dominate the early phase of CHF before significant decrease in cardiac output occurs. Moreover, about 40% of the CHF patients experience isolated diastolic heart failure (DHF) in the absence of significant systolic dysfunction (Chinnaiyan et al. 2007; Owan and Redfield 2005). Importantly, DHF is more prominent among elderly population (Kitzman et al. 2001). Mechanistically, DHF is a chronic condition that involves one or more pathological abnormalities of the myocardium, including impaired cardiac myocyte relaxation, increased ventricular wall stiffness / thickness and restricted pericardial structures. These pathological conditions are often caused by dysregulated intracellular Ca^{2+} handling, myocyte hypertrophy, extracellular collagen deposition, elevated afterload, and confined ventricular wall movements (Zile and Brutsaert 2002). Defective Ca^{2+} removal is a key factor underlying the pathogenesis of DHF. Studies in human and animals suggest that delayed Ca^{2+} sequestration is an important factor leading to slowed Ca^{2+} transient decay and myocyte relaxation as well as elevated diastolic Ca^{2+} levels (Houser et al. 2000; Zile and Brutsaert 2002).

Normal cardiac function relies on seamlessly coupled processes of myocyte contraction and relaxation driven by oscillations in cytosolic Ca^{2+}. Timely control of intracellular Ca^{2+} levels requires the released Ca^{2+} be removed promptly. The decay of the Ca^{2+} transient involves sequential processes including Ca^{2+} dissociation from troponin C, Ca^{2+} reuptake into sarcoplasmic reticulum (SR) by phospholamban (PLN) regulated sarcoplasmic (endoplasmic) reticulum Ca^{2+} ATPase (SERCA) and Ca^{2+} export through Na^{+}/Ca^{2+} exchanger (NCX). The function and abundance of these key Ca^{2+} cycling molecules are known to be altered during DHF. Such alterations include decreased SERCA2a expression and activity, decreased SERCA2a/PLN ratio, up-regulated NCX and increased ryanodine receptor (RyR) open probability and Ca^{2+} leak (Arai et al. 1994; Houser et al. 2000; Wehrens et al. 2005). These changes ultimately lead to delayed Ca^{2+} removal, diminished Ca^{2+} storage and release, elevated resting cytosolic Ca^{2+}, and increased vulnerability to arrhythmia (Minamisawa et al. 2004; Molkentin 2005; Pieske et al. 1999).

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A direct gene transfer approach that manipulates Ca^{2+} regulating proteins has been used to develop new therapies for diastolic dysfunction. Promising results in rodents have been obtained from studies such as overexpression of SERCA2a, sorcin and S100A1 or depletion of PLN, in which diastolic and systolic dysfunction are improved (del Monte and Hajjar 2003; Hoshijima 2005; Minamisawa et al. 1999; Most et al. 2004). However, several critical issues remain unsolved regarding the effectiveness of targeting endogenous Ca^{2+} cycling process in DHF. For instance, NCX has been shown to be elevated in HF, but experimental NCX overexpression exerts either beneficial or deleterious effects (Schillinger et al. 2000; Terracciano et al. 1998). The results of sorcin gene transfer in regulating contractility, relaxation and SR Ca^{2+} content are conflicting among studies (Seidler et al. 2003; Suarez et al. 2004). SERCA2a gene transfer may cause diminished responsiveness to β-adrenergic stimulation (Hirsch et al. 2004), due to imbalance between SERCA and PLN. Further, energy insufficiency, a common complication of HF (Neubauer 2007), may significantly hamper the effects of these approaches, since they rely on the energy consuming process of Ca^{2+} recycling.

Parvalbumin: Delayed Calcium Buffer Ameliorates Cardiac Diastolic Dysfunction

Parvalbumin (Parv), a 11-kDa EF-hand Ca^{2+} binding protein, has recently been identified as an energy-efficient approach to treat DHF. Parv contains three highly conserved helix-loop-helix EF-hand Ca^{2+} binding motifs with the two C-terminal motifs functioning in physiological metal binding. Both motifs show high affinity for Ca^{2+} ($K_{Ca}{}^{2+}$ = 10^7-10^9 M^{-1}) and moderate-low affinity for Mg^{2+} ($K_{Mg}{}^{2+}$ = 10^3-10^5 M^{-1}) (Pauls et al. 1996). Since Ca^{2+} competes with Mg^{2+} to bind Parv, Parv's actual Ca^{2+} binding is critically influenced by several factors, including the relative concentrations of Mg^{2+} versus Ca^{2+} and the association and dissociation rates of these metals. In mammals, abundant Parv exists in fast skeletal muscles, where Parv plays a critical role in fast twitch muscle relaxation (Muntener et al. 1995; Schwaller et al. 1999). Parv is modeled as a "delayed" Ca^{2+} buffer (Coutu P. et al. 2003) that binds Ca^{2+} in diastole rather than in the systolic phase of muscle contraction, thus facilitating removal of Ca^{2+} from the myoplasm after force generation (Schwaller et al. 1999).

We and others have employed ectopic gene transfer of Parv in cardiac muscle and evaluated Parv's role in regulating cardiac Ca^{2+} cycling, contraction and relaxation. By using adenovirus-mediated gene transfer techniques, Parv transduces adult cardiac myocytes with high efficiency (Coutu et al. 2004; Hirsch et al. 2004; Wahr et al. 1999). *In vitro* and *in vivo* functional assessments have been carried out to verify Parv's effects on cardiac performance. Parv dramatically increases the rate of Ca^{2+} transient decay and accelerates myocyte mechanical relaxation in a dose-dependent manner in normal adult cardiac myocytes from rodents and canines (Coutu et al. 2004; Hirsch et al. 2004; Rodenbaugh et al. 2007; Wahr et al. 1999). Parv expressing myocytes also retain their ability to fully respond to β-adrenergic stimulation (Hirsch et al. 2004). *In vivo* gene transfer of Parv via direct intra-myocardial injection achieves physiologically relevant concentrations of Parv in vivo (Coutu et al. 2004; Michele et al. 2004; Szatkowski et al. 2001). Parv expressing hearts exhibit increased relaxation speed measured by working heart strip preparation, *in vivo* micromanometry and echocardiography (Szatkowski et al. 2001). In aged rats, *in vivo* Parv gene transfer has a reduced efficiency, yet Parv still corrects aging-related slow cardiac relaxation (Michele et al. 2004; Schmidt et al. 2005).

Parv's beneficial effect on myocyte relaxation can be fine-tuned by altering several factors, such as the cytosolic expressing level of Parv and Parv's relative metal binding affinities. We have developed a mathematical model that incorporates Parv-based Ca^{2+} buffering into cardiac E-C coupling (Coutu et al. 2003). The model emphasizes the delayed Ca^{2+} binding feature of Parv and accurately simulates results of experimental data (Coutu and Metzger 2002). In addition to providing increased understanding of Parv's role in cardiac Ca^{2+} regulation, the model also helps to determine the optimal range of Parv's concentration in cardiac myocytes, which is estimated to be ~10-100 μM (Coutu et al. 2003; Coutu and Metzger 2002, 2005). Within this range, Parv's Ca^{2+} buffering is primarily confined within the relaxation phase with minimal impact on Ca^{2+} transient amplitude. As a result, contractile parameters remain unaffected while diastolic relaxation is accelerated (Szatkowski et al. 2001). Since the exact expression level of Parv is an important variable to control, manipulating Parv's metal binding affinities and kinetics may offer an alternative approach to optimize Parv's function. For example, two naturally existing Parv isoforms, α and β Parv, which have distinct Ca^{2+}/Mg^{2+} binding affinities, demonstrate different efficiencies in buffering Ca^{2+} and accelerating cell relaxation (Rodenbaugh et al. 2007). The higher Ca^{2+} binding affinity and lower Mg^{2+} binding affinity enables β Parv twice as potent as α Parv in accelerating Ca^{2+} transient decay and myocyte relaxation (Rodenbaugh et al. 2007). Thus, biochemical properties of Parv represent targets for determining Parv's physiological functions in cardiac myocytes. Through designing metal binding properties of Parv, gain-of-function may be achieved, in which a much lower expressing level of Parv can have similar or even better ability to improve cardiac performance.

The effectiveness of Parv in accelerating Ca^{2+} transient decay and myocyte relaxation has been tested in a number of studies using DHF animal models (Wang and Metzger

2008). First, acute Parv gene transfer accelerates the slow relaxation in senescent cardiac myocytes (Huq et al. 2004; Michele et al. 2004). A similar Parv gene transfer procedure also restored the impaired relaxation function of myocytes from hypothyroid or hypertensive DHF rats (Rodenbaugh et al. 2007; Wahr et al. 1999). In a cell model of HCM, generated by expressing mutant α-tropomyosin in cultured adult cardiac myocytes, Parv gene transfer increased Ca^{2+} removal rate and reversed the characteristic slow relaxation of HCM (Coutu et al. 2004). Further, in a canine HF model, Parv gene transfer had comparative efficacy in hastening myocyte relaxation as SERCA gene transfer (Hirsch et al. 2004). *In vivo* Parv gene transfer also demonstrated efficacy in studies using various DHF models (Michele et al. 2004; Sakata et al. 2007; Schmidt et al. 2005; Szatkowski et al. 2001). Importantly, Parv gene transfer has been shown to augment myocyte energy utilization into a more efficient state in the failing heart (Sakata et al. 2007). Taken together, Parv accelerates relaxation in a variety of cellular and animal models of diastolic dysfunction indicating its potential in translating into therapies for HF patient.

Conclusions

Gene transfer strategies can effectively introduce Ca^{2+} regulatory proteins in myocytes to remediate DHF. Ectopic expression of the skeletal muscle Ca^{2+} binding protein parvalbumin in cardiac muscle achieves improved cardiac diastolic function under both normal and disease conditions. The delayed Ca^{2+} buffering feature of wild-type Parv enables it to specifically target diastolic rather than systolic Ca^{2+} under controlled expression conditions. Alternatively, distinct Parv isoforms in cardiac myocytes demonstrates the rationale of tuning Parv's biological effects through manipulating its metal binding affinity and expression level. The low energy requirement of Parv in Ca^{2+} buffering is an additional advantage for Parv's usage in energy-deprived failing cardiac myocytes. It should be noted that buffering intracellular Ca^{2+} may exert effects beyond cell contraction and relaxation. It is possible that Parv, through regulating cytosolic free Ca^{2+} concentration, may influence overall intracellular ion regulation and/or sustained Ca^{2+} signaling, affecting diverse cell processes ranging from action potential generation to cell growth and cell death. Further studies are needed to determine whether the results obtained in adult cardiac myocytes and in small mammals can be effectively translated into the complexities of diseased heart of larger animals and humans.

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Novel protein ligands of the annexin A7 N-terminal region suggest pro-β helices engage one another with high specificity

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Abstract. The N-terminal regions of annexins A7 (synexin) and A11 consist of an extended series of short sequence repeats rich in tyrosine, proline, and glycine that provide binding sites for other proteins that may be recruited to membranes by the annexins and that may modulate the calcium and membrane binding activities of the annexin core domains. In this study two new ligands for the annexin A7 N terminal region were identified by yeast two hybrid screening: the TNFα receptor regulatory protein SODD (Suppressor Of Death Domains) and KIAA0280, a protein of unknown function. Strikingly, the sites of interaction of these proteins with the annexin also contain sequence repeats similar to those present in the N-termini of annexins A7 and A11. It was also found that the annexin A7 N-terminal region interacts with itself in the two hybrid assay. These results suggest that sequence repeats of this nature form novel structures, called YP pro-β helices, that are characterized by an ability to interact with one another. Specificity of interactions between the pro-β helices in different proteins may be encoded by the variations of residues and lengths of the sequence repeats.

Key words: Annexin A7 — SODD — KIAA0280 — Pro-β helix — Synexin

Introduction

Annexins are defined by their "core" structure, a module of about three hundred amino acids that contains four homologous domains that are responsible for binding phospholipids in a calcium-dependent fashion (Gerke et al. 2005; Creutz 1992). In addition, all annexins have a unique N-terminal region of variable length that bestows different properties on each member of the class. These regions are typically fairly short, consisting of 10 to 30 amino acids. However, much longer N-terminal regions are present in annexins A7 (synexin) and A11 (Creutz et al. 1988; Burns et al. 1989; Tokumitzu et al. 1992). These unusual N-terminal regions consist of an imperfect, variable length repeat (often 7 to 9 residues) rich in glycines, prolines, tyrosines, and glutamines that continues for 140 to 150 residues (see Fig. 1). Because of the abundance of prolines and glycines these regions are not expected to adopt classical secondary structures such as α-helices or β-strands. Previously a series of theoretical models were developed for such motifs in the annexins which were called *pro-β helices*, short for polyproline-β turn helices, that are based on repeating segments of the polyproline helix interrupted by β turns (Matsushima et al. 1990). These models are applicable to similar repeats that occur in a number of unrelated proteins including RNA polymerase II, synaptophysin, gliadin, hordein and *Octopus* rhodopsin. Although these models were proposed nineteen years ago, remarkably, the actual structure of any such domain has not yet been determined to test the models. This failure may be due to an inherent disorder of these structures that makes them resistant to crystallographic or NMR determination of a single conformation. This flexibility might be an important feature underlying their biological roles as it may enable these domains to interact reversibly with other proteins (Matsushima et al. 2008).

Although the presence of these unusual structures in only annexin A7 and A11 at first suggested these annexins are "outliers" in the annexin family, as genomic sequencing of many organisms has continued it has been found that this motif is present in at least one annexin in all animal and fungal species that have annexins. Indeed, in species that appear to have only a single annexin, such as *Neurospora* and *Dictyostelium*, the single annexin protein has this

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HUMAN A7	HUMAN A11
MS	MS
YPG	YPG
YPPTG	YPPPPGG
YPP	YPPAAPGGGPWGGAA
FPG	YPPPPSMPPIGLDNVAT
YPPAGQESS	YAGQ
FPPSGQ	FNQD
YP	YLSGMAANMSGT
YPSG	FGGANMPNL
FPPMGGGA	YPGAPGAG
YPQVPSSG	YPPVPPGG
YPGAGG	FGQPPSAQQPVPP
YPAPGG	YGM
YPAPGG	YPPPGGNPPSRMPS
YPGAPQPGGAPS	YPP
YPGVPPGQG	YPGAPVPGQPMPPPGQQPPGA
FGVPPGGAG	YPGQPPVT
FSG	YPGQPPVPLPGQQQPVPS
YPQPPSQS	YPG
YGGGPAQVPLPGG	YPGSGTVTPAVPPTQ
FPGGQMPSQ	FGS
YPGGQPT	
YPSQPATVTQVTQ	

Figure 1. Repeating amino acid sequences in the N-terminal regions of human annexin A7 and A11. The sequences have been aligned to emphasize the characteristic, imperfectly repeating tyrosine (or phenylalanine) dipeptide (YP or FP).

characteristic repeat (see Fig. 2). Sometimes the repeat is virtually perfect, as in the 15-fold repeat of YPPQQG in *Dictyostelium*, while in other species such as human, the repeat is highly variable. In contrast, N-terminal regions of this nature have not been found in the plant annexins (Mortimer et al. 2008). Fig. 2 presents a number of these tail sequences from a variety of species in a format that emphasizes the repeating tyrosine which has been suggested may play an important structural role in the repeat (Matsushima et al. 1990). It is also a signature of these annexin tail repeats that the tyrosine (sometimes substituted by a phenyalanine), is frequently followed immediately by a proline. Similar tyrosine and proline rich repeats in a number of other protein classes do not follow this rule. For example the consensus repeat in the N-terminus of RNA polymerase II is YSPTSPS, and in synaptophysin is YGPQG (Matsushima et al. 1990). Therefore, the annexin pro-β helices are members of a subclass that can be called the YP pro-β helices.

Our research group has been testing the hypothesis that these repeating sequence domains may be important sites for other proteins to bind to the annexins. Such a role would enable these annexins to recruit other proteins to membrane surfaces where they may participate in signalling or effector complexes of various kinds. By calcium-dependent affinity chromatography using the isolated recombinant N-terminal region of annexin A7 we determined that the EF-hand, calcium-binding protein sorcin binds near the N-terminus of this region in a calcium-dependent manner (Brownawell and Creutz 1997). This result reflected a common theme among the members of the annexin family – the calcium-dependent association of their N-terminal regions with members of the EF hand family of calcium-binding proteins (Gerke et al. 2005).

DICTYOSTELIUM	NEUROSPORA	C. ELEGANS	SILKWORM	ZEBRAFISH	HUMAN A7
MS	MS	G	M	MS	MS
YPPNQG	YPG	YPNQQPS	FPNQQ	YPG	YPG
YPPQSNSPQPGQ	YPPASP	YGG	FPPNVG	YPPAGGS	YPPTG
YGAPQQG	YGQPPPQGGG	YGQPPQQPG	FNNLTPQS	YPPASGP	YPP
YPPQQG	YYQQPPPQQHHQQPP	YGNGS	FSNTMNMQGSPAMRNPQVPGQG	YQQPAAG	FPG
YPPQQG	YGGPPPHAH	YDP	YPLPAQSA	YPPQPGA	YPPAGQESS
YPPQQG	YNT	YGQPQQQP	YPQPG	YPPQAGY	FPPSGQ
YPPQQG	YQQPQQG	YPGGGGQPP	YPIQQNQG	YPPQPGA	YP
YPPQQG	YGQPPPGPPPGQ	YPGSNSNQGGGG	YPQSTAQ	FPPQPGA	YPSG
YPPQQG	YGAPPPQP	YPGQGGAP	YPTQGVP	FPPQPGA	FPPMGGGA
YPPQQG	YGAPAHSPPPGT	YPGGSGG	YPTHQSQGHPQSTAQ	FPPGAG	YPQVPSSG
YPPQQG	YGAAPPPSPSY	YPQAPQ	YPTQGVP	YPPQAGG	YPGAGG
YPPQQG	YPLGPPPAG	YGFSGGQGSAPQPNQGG	YPNHQGQG	YPAAPGGG	YPAPGG
YPPQQG	YGAPPPHG	YPPQQQQ	YPQSTAQ	FPPQAGG	YPAPGG
YPPQQG	YGQPPGPPPQQS	YPPQQG	YPTQG	YPAAQPGA	YPGAPQPGGAPS
YPPQQG	YGAVPPTPTA	YQQGGQQQGGGF	FPQHAQSA	YPNMPAAGG	YPGVPPGQG
YPPQQG	YTPQP	FPNQG	YPQSSQPQTDHG	WGGHPG	FGVPPGGAG
YPPQQG	YAVQ	YGQPV	FRSHPGSI	FGAPAGGMPQG	FSG
YPPQQG	YPPTPPSPG		YGNQSHPPN	YPGVPAPGQQPMPA	YPQPPSQS
YPPQG	YGPAMNIP			YPGAPVPNPGMPG	YGGGPAQVPLPGG
YPPQQG				YGGGAPTGPTPPAP	FPGGQMPSQ
YPPVGVPVGVPVG					YPGGQPT
FAPGMVVG					YPSQPATVTQVTQ
YHQ					

Figure 2. Repeating amino acid sequences in the N-terminal regions of annexins from molds, worms, insects, fish, and humans.

It was subsequently shown that a similar, although very short, sequence motif present at the N-terminus of sorcin (see Fig. 3) is essential for the binding of sorcin to annexin A7 (Verzili et al. 2000). This raises the interesting possibility that proteins with sequence motifs of this nature may have specific affinity for other proteins containing similar sequence motifs, possibly because of the existence a novel structure underlying like-like interactions of such motifs.

An additional annexin A7-interacting protein was identified by Yu et al. (2002) who conducted a two-hybrid screen with galectin-3. Galectin-3 is a multifunctional oncogenic protein found in the nucleus and cytoplasm as well as in the extracellular milieu. It was suggested by Yu et al. that binding of galectin 3 to annexin A7 is necessary for the migration of galectin to perinuclear membranes where it regulates apoptosis. Although the sites of interaction of the two molecules was not mapped, it is striking that galectin also has a potential pro-β helix in its N-terminal portion which would seem to be an attractive candidate for a binding site for the N-terminal region of annexin A7 (Fig. 3).

The present report represents a continuation of the efforts of our group to identify other interacting partners with the annexin A7 N-terminal region using the yeast two hybrid system as a screening tool. In contrast to the affinity chromatography approach used before, this approach can potentially identify binding partners that are of low abundance or have a lower affinity interaction that would make the biochemical approach starting with crude extracts problematic. Strikingly, the two new proteins that we identify here as interactors with annexin A7 contain sequences that also suggest the presence of a pro-β helix. In addition, the interaction of the annexin A7 N-terminal region with itself is also described and mapped by deletion analysis to provide insight into the minimal size motif needed to support interactions between two pro-β helices.

SORCIN	GALECTIN 3
MA	MADN
YPGHPGAGGGY	FSLHDALSGSGNPNPQG
YPGGYGGAPGGPA	WPGAWGNQPAGAGG
FPGQTQDPL	YPGAS
YG	YPGA
	YPGQAPPGA
	YHGAPGA
	YPGAPAPGV
	YPGPPSGPGA
	YPSSGQPSAPGA
	YPATGP
	YGAPAGPLIVP
	YNLPLPGGVVPRN

Figure 3. Repeating amino acid sequences present in the N-terminal regions of two proteins that bind to annexin A7: Sorcin and Galectin 3.

Materials and Methods

The N-terminal 145 residues of human annexin A7 were used as bait in a two-hybrid screen of a murine embryonic cDNA library. The Clontech System 3 set of vectors was used for this screen. The annexin A7 bait construct was cloned into plasmid pGBKT7 that expresses a fusion with the GAL4 DNA binding domain. In control experiments the annexin A7 cDNA was shown not to cause auto-activation of the DNA binding domain. The cDNA library used has cDNAs fused to the GAL4 activation domain (Vojtek et al. 1993). The host cell AH109 was transformed with the bait vector and then with the library. The library transformants were plated directly onto medium selective for the two vectors (leu $^-$, trp $^-$) and selective for the expression of the URA3 gene and the HIS3 gene (ura $^-$, his $^-$ medium). Both the URA3 and HIS3 genes have been engineered in the host cell AH109 to be under the control of the GAL4 promoter so that the cells should only grow on this medium if there is an interaction between the fusion proteins expressed by the bait construct and a particular library construct. Colonies growing on these selective plates were harvested and grown in liquid culture under the same selection. Plasmid DNA was captured from cultures that grew under these conditions. The plasmids were then retransformed into the host cell again along with a null bait vector that expresses only the GAL4 DNA binding domain or with cells transformed with the original annexin A7 bait construct. Only clones that interacted with the annexin-GAL4 DNA binding domain fusion and did not interact with the DNA binding construct alone were considered true positives in the assay.

Results

A single screen of the murine cDNA library with the human annexin A7 N-terminal region yielded two clones that encoded repeating sequence motifs with character similar to the repeating sequence in the annexin: residues 90 to 258 of the TNFα receptor regulatory protein SODD (full length 457 residues), and residues 1 to 104 of the murine homolog of the human protein of unknown function KIAA0280 (Genbank accession number Q8BGZ2; full length 244 residues). The interactions led to strong growth signals on selective media that were easily scored (e.g., see Fig. 4 illustrating the interaction with SODD). The interacting sequences captured in the two hybrid clones are shown in Fig. 5.

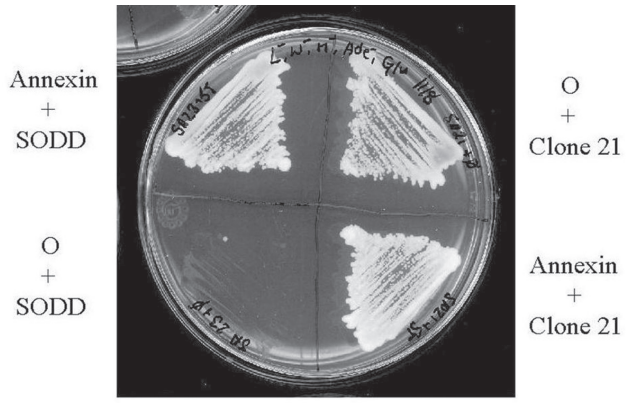

Figure 4. Yeast two hybrid evidence for an interaction between SODD (Suppressor Of Death Domains) and the N-terminal region of annexin A7. AH109 cells have been streaked on a leu$^-$, trp$^-$, his$^-$, ura$^-$ plate. Strains harboring the following plasmids are streaked in each quadrant: Upper left, annexin A7 and SODD – yeast growth indicates a strong interaction between the bait (annexin A7) and the prey (SODD); lower left, empty bait vector control ("O") and SODD – failure of yeast to grow indicates no interaction of SODD with the GAL4 DNA binding domain when it is not fused to the annexin A7 N-terminal region; upper right, empty bait vector and clone 21 (a representative false positive clone that shows activation in the absence of the annexin); lower right, annexin A7 and clone 21. In independent control experiments the annexin was found not to cause auto-activation in the absence of SODD (not shown).

SODD	KIAA0280
HQEQPP	MNPV
YPGYNSNYWNSVRPRAP	YSPGSSGVP
YPS	YANAKGIG
YSVRPELQGQSLNS	YPAG
YANGA	FPVG
YGPP	YAAAPA
YPPGPGASTAS	YSPNM
YSGA	YPGANPTFQTG
YYVPG	YTPGTP
YTQSN	YKVSCSPTSGAVPP
YSTEVPNT	YSSSPNP
YRSPGNSPTPMSRWM	YQTAV
YSQQDCPTEAPPLRGQVPG	YPVRSA
YPASQNPGMTLPH	YPQQSP
YPYGDGNRA	YAQQGTY
VPQSGGTGRPQDDA	YTQPL
WASSA	YAAPPHVIHHTTVVQPNG
YGMGAR	MPATV
YPWPS	YPAPIPPPRGSGVTMGMV
	AGTTMA

Figure 5. Amino acid sequences encoded by the inserts of two cDNA clones that interact with the annexin A7 N-terminal region in the two hybrid system: SODD and KIAA0280. The sequences have been aligned to emphasize the repeating tyrosines and prolines that are characteristic of a pro-beta helical structure.

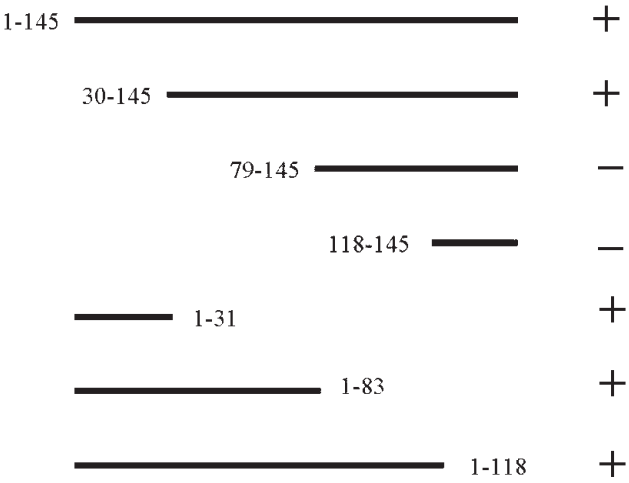

Figure 6. Deletion mapping of the binding site for SODD on the annexin A7 N-terminal region. cDNAs encoding the residues of the annexin (indicated on the left), from the full N-terminal sequence (1-145) to the fragments as labeled were cloned into the yeast two hybrid bait vector. Cells were co-transformed with the library vector encoding the portion of SODD (sequence shown in Figure 5) that binds annexin A7. Growth of the yeast cells under conditions selective for interaction of the proteins is scored on the right as + or -.

A series of N-terminal and C-terminal deletion constructs of the N-terminal portion of annexin A7 were subsequently used to map the site of interaction of SODD. The results are summarized in Fig. 6. The binding site on the annexin N-terminal region apparently involves residues between 30 and 79. However, there is an apparent contradiction in that removal of the first 30 residues does not block the interaction, while the first 31 residues alone are sufficient for the interaction. There are at least two possible interpretations of these results. The binding site might overlap regions N-terminal and C-terminal of the apparently critical residue 30 or 31 so that the presence of either residues 1 through 31 or 30 through 79 alone provide enough of the site that the interaction can still occur. Alternatively, there may be two tandem binding sites, one on each side of residue 30/31. It is of interest that the binding site for sorcin on annexin A7 has also been mapped in biochemical experiments to the N-terminal 31 residues of annexin A7 (Brownawell and Creutz 1997).

Although the two hybrid screen did not yield annexin A7 itself as an interacting protein, since annexin A7 is known to undergo self-association in the presence of calcium (Creutz et al. 1979), we tested the ability of the N-terminal region to interact with itself by cloning cDNA encoding the 145 N-terminal residues into both the bait and prey vectors. This resulted in evidence for a strong interaction of the N-terminal region with itself. The deletion mutants used for the mapping of the SODD binding site were then used to

map the site of the annexin self-association. This resulted in a deletion map as shown in Fig. 7 which is, interestingly, almost identical to the map for the interaction with SODD. In fact, the only difference between this map and the SODD map is that the interaction with the first 31 residues is significantly weaker, in terms of the growth rate of the yeast cells, so that it was scored as +/-. This striking correspondence between these two deletion maps has two interesting possible interpretations. SODD may bind the same site that is involved in the annexin self-association and could therefore potentially compete for this site in vivo, blocking annexin self-association. Alternatively, the self-association of the annexin N-terminal region may be necessary for SODD to bind a dimer or multimer of annexin A7, and what we have really mapped in the SODD deletion map is again the ability of annexin A7 to self-associate.

Although the results suggest that pro-β helix containing proteins may tend to interact with one another, there is also evidence of a degree of specificity in these interactions. SODD, the KIAA0280 protein, and the annexin A7 N-terminal region were all tested in the two hybrid assay for the ability to interact with the N-terminal region of annexin A11 which also has an apparent pro-beta helix of similar size but distinct sequence at its N-terminus (sequence shown in Fig. 1). However, no interaction was detected.

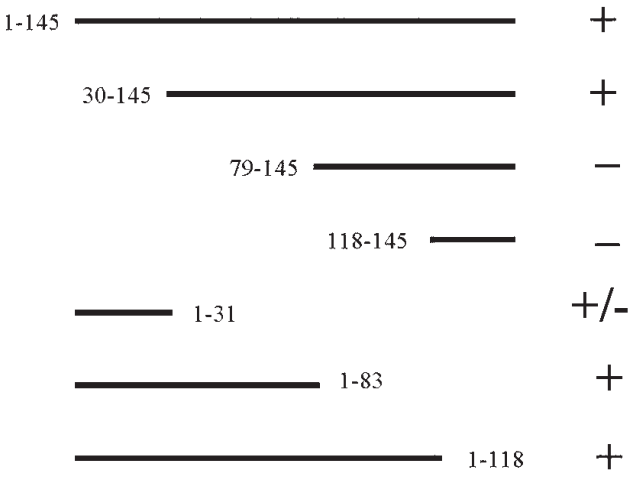

Figure 7. Deletion mapping of the interaction between the N-terminal region of annexin A7 with itself. The full N-terminal sequence (residues 1 to 145) was cloned into the prey vector and then tested in the two hybrid system with the same fragments of the N-terminal region in the bait vector as used in Figure 6. +/- indicates very slow growth of the yeast, suggesting a weak interaction with the 1 – 31 N-terminal fragment.

Discussion

Significance of the binding of SODD to annexin A7. The interaction of annexin A7 with SODD – the Suppressor Of Death Domains (Jiang et al. 1999) – has interesting implications in terms of the biological functions of annexin A7. Annexin A7 has been reported to behave as a tumor suppressor, but the mechanism of this activity is completely unknown. The evidence supporting such a role comes from several observations. In the knockout mouse model developed by the Pollard lab, the viable heterozygote develops numerous tumors in a wide variety of tissues (Srivistava et al. 2003). Human tumor cell proliferation and colony formation are reduced when the wild-type annexin A7 gene is transfected into two prostate tumor cell lines, LNCaP and DU145 (Srivistava et al. 2001). Annexin A7 protein expression in human prostate tumor microarrays reveals a significantly higher rate of loss of annexin A7 expression in metastatic and local recurrences of hormone refractory prostate cancer (Srivistava et al. 2001). Tumor expression of annexin A7 correlates with longer survival in patients with *glioblastoma multiforme*, the most common and lethal primary brain tumor in adults (Hung et al. 2003). The presence of annexin A7 expression serves as a marker for the less invasive phenotypes of malignant melanoma (Kataoka et al. 2000).

SODD is a regulator of signalling from the tumor necrosis factor receptor 1 (TNFR1) (Jiang et al. 1999; Baud and Karin 2001; Locksley et al. 2001; Harrington 2000). TNFα binds to TNFR1. The TNFα molecule is a trimer of identical subunits, and in binding to its receptor it causes the receptor to trimerize. This in turn causes the receptor to bind to signalling proteins that initiate apoptosis and activation of the NF-κB transcription factor. It appears an important initial step in signalling from the TNFR1 receptor is the binding of the TRADD protein to the so-called "Death Domain" on the cytoplasmic portion of the TNFR1 receptor. Jiang et al. (1999) discovered a 60 kDa protein that binds to the Death Domain of the resting receptor and prevents spontaneous trimerization of the receptor and ligand-free signalling. This protein was appropriately named "Suppressor Of Death Domains" (SODD) because of its ability to block the interaction of the receptor with TRADD through the death domain.

The binding of SODD to annexin A7 could be involved in the regulation of this signalling pathway. This could explain a role for annexin A7 as a tumor suppressor to the extent that the annexin might be needed for tumor cells to respond to pro-apoptotic signalling through the TNF receptor. However, several possible mechanisms for the interaction of annexin A7 with this signalling pathway can be envisaged. For example, the binding of SODD to annexin A7 might prevent it from binding to the TNFR1 receptor, leading to a higher level of activity of the receptor and a pro-apoptotic response. This raises the question of what the role of calcium activation of annexin A7 might play in this mechanism. Would calcium

promote movement of annexin A7 to the membrane so that it would be in a position to compete for SODD bound to the receptor? Or, alternatively, could calcium promote movement of an annexin A7-SODD complex to the membrane thus delivering SODD to the receptor? Further characterization the biochemistry and cell biology of the SODD/annexin A7 interaction will be required to clarify these issues.

Pro-beta helices may have a structure that promotes helix-helix interactions. The results suggest that an important property of certain pro-β helices may be the ability to interact with one another. This may be restricted to the subclass represented by a repeating motif that involves the dipeptide YP since this characterizes not only the annexins but also the four unrelated proteins that bind annexin A7 – sorcin, galectin, SODD, and KIAA280. Possibly this particular sequence feature underlies a structural constraint that allows two pro-β helices to intertwine. It will be of considerable interest to obtain structural data on one of these protein complexes. One may hope that when bound together the helices might have greater stability allowing the formation of crystals suitable for X-ray diffraction.

The interaction of the annexin A7 N-terminal region with itself may underlie its ability to undergo calcium-dependent self-association (Creutz et al. 1979). In this case, it would be of considerable interest to determine how binding of calcium in the annexin folds of the core domain is communicated into a structural change in the helices controlling their interaction. The self-association of annexin A7 is reversible when calcium is removed by chelation (Creutz et al. 1979). Therefore the regulation of the proposed intertwining of the pro-β helices must be reversible as well if it underlies the protein self-association. This should also be true for the engagement of the pro-β helices in sorcin and annexin A7 with each other since this interaction is also calcium-dependent and reversible (Brownawell and Creutz 1997).

The self-association of annexin A7 has also been suggested to underlie the ability of the annexin to promote membrane aggregation since the calcium titration curves for protein self-association and protein-mediated membrane aggregation are identical (Creutz et al. 1979). Chander and colleagues have shown that deletion of the first 29 residues of annexin A7 reduces its ability to bind to and aggregate phospholipid vesicles, supporting a possible role for N-terminal self-association in these processes (Naidu et al. 2005; Chander et al. 2006).

The results also show that not all of the "YP" subclass of pro-β helices may bind one another since the interactions reported here between annexin A7 and either SODD or KIAA0280 do not occur with the N-terminal region of annexin A11, nor do the N-terminal regions of the two annexins interact with each other. On the other hand, sorcin binds to the N-terminal regions of both annexins A7 and A11 which have identical sequence for the first 8 amino acids and a repeated GYPP motif within the first 15 residues (Brownawell and Creutz 1997). Therefore, the variations of the sequence motif lengths and residues present in various pro-β helices may represent a code providing specificity of interaction between particular helices.

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Review

Regulation of CFTR function by annexin A2-S100A10 complex in health and disease

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Abstract. Annexin A2 and S100A10 proteins form a heterotetrameric complex and belong to different families of Ca^{2+}-binding proteins. Annexins are non-EF-hand-type Ca^{2+}-binding proteins that exhibit Ca^{2+}-dependent binding to phospholipids and membranes in various tissues. They have been implicated in many Ca^{2+}-regulated processes, including regulation of membrane organization, trafficking and interact with many targets such as ion channels. S100 proteins comprise a family of small proteins characterised by the presence of two consecutive EF-hand type Ca^{2+}-binding motifs, interact with ion channels and regulate diverse processes and play a role as Ca^{2+} sensors. Several annexin–S100 complexes have been characterized and require calcium. In this regard, S100A10 binding to annexin A2 is an exception in that it is regulated by a post-translational modification of annexin A2 and occurs independently of calcium concentration. This review focuses on the regulatory mechanism behind annexin A2–S100A10 complex formation, its role in regulating chloride transport in health and cystic fibrosis and the potential of this mechanism to integrate calcium and cAMP signalling in airway epithelia. We propose that cAMP/PKA-dependent activation of chloride flux (through CFTR and ORCC) requires the mobilisation of a multi-protein complex involving calcium binding proteins from three different families (annexin 2, S100A10 and Calcineurin A).

Key words: Annexin A2 — S100A10 — cAMP — Calcium — Cystic fibrosis — CFTR

Introduction

Epithelial cells not only provide a protective barrier but play an important role in the normal function of the respiratory system. One such function is the maintenance of the fine balance between ion secretion and re-absorption across the respiratory epithelium, which is a critical determinant of the height of the fluid layer that lines the respiratory tract, the periciliary layer. This layer facilitates the function of cilia present on the apical surface of the cells, which in turn drives the movement of mucous, fluid and trapped bacteria out of the respiratory tract. Thus, the mechanisms that regulate active salt and water transport have important implications for understanding lung fluid balance under both normal and pathologic conditions. For example, the importance of epithelial ion movement is highlighted in the disease cystic fibrosis (CF) - a monogenic disorder resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP/PKA and ATP-regulated chloride channel in epithelia (Riordan et al. 1989). However, how CFTR interacts with other membrane and non-membrane proteins to control trans-epithelial transport of ions and water under normal (and pathologic conditions) remains an open question.

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Annexin A2, S100A10 and calcineurin

We have been interested in the annexin gene family and its relationship to ion transport and CFTR function for a number of years (Muimo et al. 1998; Muimo et al. 2000; Borthwick et al. 2007). Initial work, conducted in Anil Mehta's laboratory,

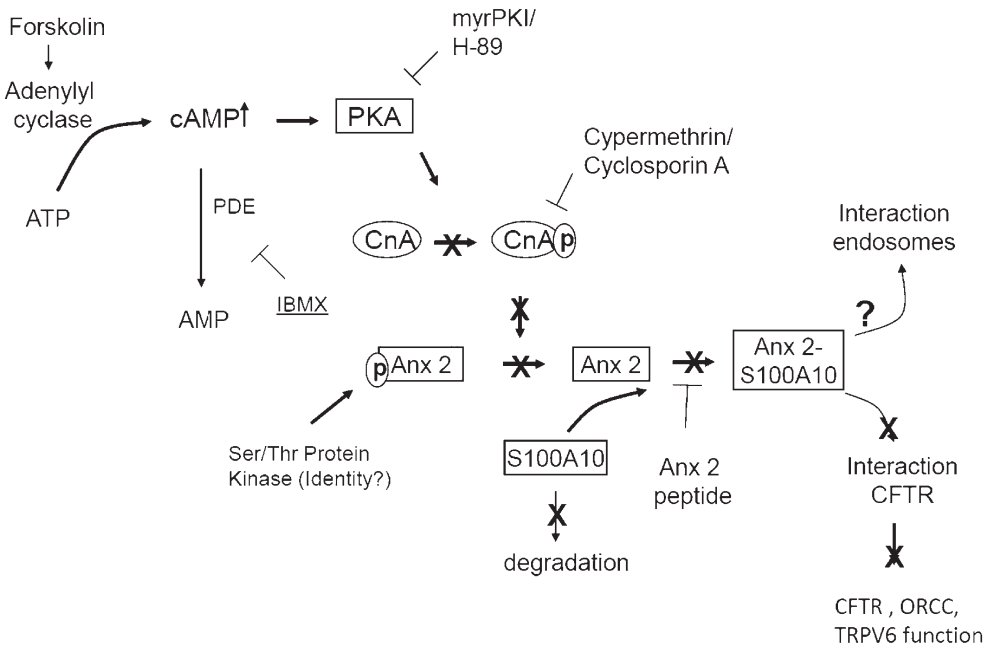

Figure 1. Schematic representation of the pathways involved in modulation of the annexin A2-S100A10/CFTR complex in airway epithelia, showing the likely components involved in modulation of CFTR ion channel activity. Potential cargo proteins and lipids bound to the annexin A2 complex are not shown for clarity. The → sign does not mean that the promoted reaction is necessarily direct. X indicates processes defective in F508del CF cells. Reproduced with permission from Cellular Signalling 20(6), 1073-1083.

indicated that chloride concentration could signal to the apical membrane by regulating phosphorylation of a number of soluble proteins within the apical membrane. We identified one of the soluble proteins as annexin 1, and established that its chloride sensitive phosphorylation occurred on histidine residue(s) (Muimo et al. 2000). Recently, we found that a cAMP/PKA/calcineurin A-dependent multi-protein complex involving annexin A2–S100A10 must be assembled with cell surface membrane CFTR before this channel can open (Borthwick et al. 2007) (see also Fig. 1). Crucially, disruption of the annexin A2-S100A10/CFTR complex in wild type cells significantly attenuates CFTR function and obtunds CFTR-regulated outwardly rectifying chloride channels (ORCC) that generate ORCC-mediated currents.

Annexin A2 is a member of the annexin family of soluble proteins that bind to certain negatively charged phospholipids in cellular membranes in a calcium-dependent manner. The annexin COOH-terminal core is conserved and contains non-EF-hand-type Ca^{2+}-binding sites, their NH_2-terminal tail is unique and enables the protein to interact with distinct cytoplasmic partners. They are involved in diverse cellular processes including inflammation, and ion transport (Perretti and Flower 2004; Gerke et al. 2005) processes that are defective in CF for obscure reasons. Interestingly, previous analysis shows annexins not only share significant sequence homology with that part of CFTR bearing the most common F508del-CFTR mutation including the (normally invariant) missing phenylalanine residue at position 508 (Chap 1991), but these family members are also associated with endosomes, caveolae, clathrin coated vesicles, and other membrane compartments, engaged in endo-/exo-cytosis (Thiel et al. 1992; Turpin et al. 1998; Gerke and Moss 2002; Zobiack et al. 2003; Gerke et al. 2005). It is worth noting that, in many cell types, full activation of CFTR depends on vesicular transport and subsequent fusion of vesicles containing mature CFTR with the plasma membrane (Bradbury et al. 1994; Bradbury 1999). This Ca^{2+} -dependent vesicle-mediated process is triggered by cAMP/PKA and requires the C-terminus of CFTR (Weber et al. 1999). Annexin A2-S100A10 regulate exocytic apical transport in polarised epithelia (Jacob et al. 2004). For unknown reasons, CFTR mutation induces defective endosome function and CFTR recycling through such compartments (Poschet et al. 2002). Interestingly, annexin A1 expression is down regulated in CF, annexin A5 binds CFTR and is overexpressed in CF epithelia from foetal trachea (Della Gaspera et al. 1995; Bensalem et al. 2005; Trouve et al. 2007). On the other hand, annexin A4 regulates Ca^{2+}-dependent Cl- conductance in epithelia (Kaetzel et al. 1994). It may also be relevant that annexin A2 is a ligand for *Pseudomonas aeruginosa* (Kirschnek et al. 2005), a leading cause of premature death in CF patients,

given that our data suggest annexin A2 is abnormally located in F508del-CFTR CF cells (Borthwick et al. 2008b).

S100A10 (previously known as p11, calpactin I light chain) belongs to the S100 protein family of small (10–14 kDa) Ca^{2+}-binding proteins that regulate various intracellular and extracellular processes and which exist as homo-/heterodimeric functional units in various tissues (Rescher and Gerke 2008). Amongst the S100 family, S100A10 is unique in that it is unable to bind Ca^{2+} because it lacks three amino acid residues in the N-terminal EF-hand motif and the crucial amino acids are substituted in the C-terminal motif (Rescher and Gerke 2008). The first fourteen N-terminal residues of annexin A2 constitute the S100A10-binding site (Kube et al. 1992). Several studies using knockout animals have suggested important biological roles for S100A10. Using conditional mouse knock out suffering a specific S100A10 deletion in nociceptive sensory neurons, Foulkes et al. (2006) have demonstrated a role in nociception, resulting from decreased sodium current. Svenningsson et al. (2006) generated a general S100A10 mouse knock out to analyse the physiological relevance of the S100A10–5-HT1B receptor interaction. They found that that S100A10–/– mice is viable but exhibits a depression-like phenotype with reduced responses to 5-HT1B agonists; this suggests that S100A10 is not required for normal development, but the lack of this complex causes a depressive disorder.

Calcineurin (Cn) is a serine/threonine protein phosphatase regulated by $[Ca^{2+}]i$ and calmodulin (CaM) (Crabtree 1999) and couples Ca^{2+}-signalling to various processes including inflammation, lymphocyte activation, gene expression, ischemic injury and apoptosis (Tong et al. 1995; Crabtree 2001; Vega et al. 2003; Borthwick et al. 2007). It is a heterodimer consisting of a 61-kDa catalytic subunit (CnA) and a calcium-binding 19-kDa regulatory subunit (CnB) (Crabtree 1999). Three isoforms of the catalytic subunit (*CnA*α, *CnA*β, and *CnA*γ) and 2 isoforms of the regulatory subunit (*CnB1* and *CnB2*) have been identified (Crabtree 1999). A rise in cytosolic Ca^{2+} concentration induces a conformational change in CaM and CnB leading to CnA activation. Compared to lymphocyte, cardiomyocytes and brain cells, the role of Cn in epithelia remains poorly characterised. Our recent data shows that in membrane of airway and gut epithelia, PKA induces serine phosphorylation of CnA and a CnA-dependent complex between annexin A2-S100A10 and CFTR or the calcium influx channel, TRPV6 (Borthwick et al. 2007; Borthwick et al. 2008a) and thus, provides new evidence for 1) CnA regulation of CFTR and TRPV6 channels and 2) a interaction between cAMP and Ca^{2+} signalling in epithelia. However, it is not known whether the PKA-dependent activation of CnA is restricted to membrane, which CnA isoform is involved and whether CnB, CaM and Ca^{2+} also play a role in the PKA-dependent activation of CnA.

Annexin A2-S100A10 complex and CFTR function

Whereas other annexins require Ca^{2+} to bind their S100 ligands, formation of the annexin A2-S100A10 heterotetrameric complex occurs independently of Ca^{2+} concentration. Instead of Ca^{2+}, the annexin A2-S100A10 complex is modulated by post-translational modifications including phosphorylation and N-terminal acetylation of annexin A2 (Johnsson et al. 1986; Chasserot-Golaz et al. 1996; Jost and Gerke 1996) but the precise regulatory mechanisms remained unclear and as a result the interaction was considered constitutive. Recent work from our laboratory has established that formation of annexin A2-S100A10 complex is regulated by cAMP/PKA and protein phosphatase (calcineurin A) and leads to a functionally important interaction with CFTR in airway and gut epithelial cells (Borthwick et al. 2007). Pharmacological agents that inhibit CnA and an acetylated peptide corresponding to the S100A10 binding domain on annexin A2, disrupt complex formation in wild type cells and significantly attenuate CFTR function. *In vitro,* PKC phosphorylates Ser-11 within the S100A10 binding domain (amino-acids 1-14) of annexin A2, and phosphorylation at this site most likely leads to a direct spatial interference with S100A10 binding (Johnsson et al. 1986; Jost and Gerke 1996). Additionally, PKC phosphorylates annexin A2 on Ser-25 but phosphorylation on this site does not affect S100A10 binding (Johnsson et al. 1986).

The annexin A2-S100A10 complex is suitably matched to link membranes and/or vesicles to cytoskeletal proteins to regulate membrane organization, mediate membrane-membrane associations and control plasma membrane receptors and ion channels. Annexin 2 preferentially binds to anionic phospholipids, such as phosphatidylinositol 4,5-bisphosphate, which is enriched in lipid rafts in the plasma membrane, while S100A10 binds and provides a bridge to link the complex to ion channels and cytoskeletal proteins, such as actin. In this regard, this complex controls the recruitment and/or function of Na^+, K^+, Ca^{2+}, and Cl^- channels (including wild type CFTR) and 5-HT1B receptors (Girard et al. 2002; Okuse et al. 2002; van de Graaf et al. 2003; Svenningsson et al. 2006; Borthwick et al. 2007; Borthwick et al. 2008a). In the epithelial membrane of the airway and gut, PKA induces formation of the CnA-dependent complex not only between annexin A2-S100A10 and CFTR but also between annexin A2-S100A10 and the calcium influx channel, TRPV6 (Borthwick et al. 2007; Borthwick et al. 2008a). S100A10 binds the TRPV5/6 Ca^{2+} channels and the background K^+ channel (TASK 1) via C-terminal VATTV and SSV motifs, respectively (Girard et al. 2002; van de Graaf et al. 2003). Thus, CFTR may also possess a similar binding motif for annexin A2-S100A10 complex. However, S100A10 also binds the tetrodotoxin-insensitive, voltage-gated Na^+ channel (Nav1.8), via the N-terminus which lacks the two

motifs described above (Okuse et al. 2002). In the annexin A2 –S100A10/CFTR complex, S100A10 acts as the bridging protein (Borthwick et al. 2007) but sequence analysis shows that CFTR lacks the above S100A10 binding motifs. Thus, the CFTR binding site for S100A10 may be novel and distinct from previously described motifs.

Annexin A2 association fails towards S100A10 and F508del-CFTR.

CF is characterised by impaired salt and water transport in several tissues including the respiratory and gut epithelia (Riordan et al. 1989). In such epithelia, the cAMP/PKA regulated and ATP-dependent CFTR chloride channel fails to control the transport of chloride, bicarbonate and hence water. However, how CFTR controls transepithelial transport and how CFTR interacts with other membrane and soluble proteins to carry out this process remain open questions. Interestingly, only a poor correlation exists between the CF genotype and lung phenotype suggesting that additional cellular factors may influence the pulmonary manifestations of the disease. In addition to defects in epithelial ion and water transport, CF is associated with multiple cellular defects including inflammation, abnormal trafficking, exocytosis and endocytosis (Yankaskas 1999; Hodson 2007).

The F508del-CFTR mutation (the most common CF mutation) causes improper folding and processing of CFTR. Delivery of F508del-CFTR to the plasma membrane is impaired and may be responsible for the lack of cAMP/PKA-dependent Cl^- conductance (Kopito 1999). Controversially, recent evidence demonstrates the presence of some of this mutant form of CFTR at the apical membrane (Kalin et al. 1999; Penque et al. 2000; Varga et al. 2004), which nevertheless remains dysfunctional. Comparative studies of purified F508del-CFTR and wt-CFTR reconstituted in planar lipid bilayers have demonstrated that Cl^- channel activity of F508del-CFTR is similar to that of wild-type CFTR (Li et al. 1993), which suggests that other regulatory elements act on CFTR within the cell. Crucially, it is increasingly evident that CFTR interacting proteins (CIP's) play an important role in regulating CFTR function (Guggino and Stanton 2006). However, most of the CIP's identified to date are inhibitory to CFTR function. In order to identify proteins that might play a role in CFTR activation, we analysed protein-protein interactions involving CFTR under conditions whereby CFTR function is activated. Our model invokes the idea that at baseline, cell surface CFTR is held inactive (in complex with proteins inhibitory to CFTR function – e.g. syntaxin 1A, syntaxin 8; AMPKα (Cormet-Boyaka et al. 2002; Hallows et al. 2003; Bilan et al. 2004), but after protein kinase A is activated, additional component plasticity occurs which is permissive for channel opening.

Disruption of the annexin A2-S100A10 interaction has implications for membrane organization and channel modulation. The significant attenuation of CFTR function following disruption of the annexin A2-S100A10/CFTR complex in wild type cells (Borthwick et al. 2007) by agents that disrupt complex (PKA/CnA inhibitors and acetylated annexin A2 N-terminal peptide), predicted defective annexin A2-S100A10/CFTR complex formation in CF cells. Correspondingly, we found that although some F508del-CFTR could be detected on the plasma membrane of CFBE41o- cells (Borthwick et al. 2008b), formation of either the annexin A2-S100A10 or the annexin A2-S100A10/CFTR complex fails in CFBE41o- cells (Borthwick et al. 2008b) (see also Fig. 1). Thus, loss of annexin A2-S100A10/CFTR complex formation may contribute to defective cAMP-induced Cl^- currents and suggests CFTR mutation affects these multi-protein interactions. Defective complex also suggests a feedback loop, whereby normal CFTR plays an important role in the function of the proteins that regulate its own activity.

S100A10 normally undergoes degradation in the absence of its partner, annexin A2, in many cell types (Rescher and Gerke 2008). The mechanisms and degradation pathway is as yet unknown. A crucial difference exists between CF and non-CF airway epithelial cells (Borthwick et al. 2008b) in that unlike non-CF cells, avidin pull down from CFBE41o- cells (homozygous F508del-CFTR expressing) (biotin surface labeled ± cAMP stimulation), co-precipitates biotin-labeled F508del-CFTR and only a small amount of S100A10, but without its normally obligate annexin A2 partner. Yet by western blot, both annexin A2 and S100A10 are detectable in the very same input fractions from CFBE41o- cells. The absence of obligate partnership between annexin A2–S100A10 in CFBE41o- cells was confirmed by immunoprecipitation of either CFTR, annexin A2 or S100A10 (Borthwick et al. 2008b) and is surprising given that S100A10 normally degrades when annexin A2 is down regulated in many cell types (Rescher and Gerke 2008). This implies that S100A10 is abnormally resistant to degradation in some way in CF cells (perhaps due to a defective degradation pathway) or binds to some other protein to promote its stability.

Conclusions

Ca^{2+}- and cAMP-dependent pathways integrate to regulate the interaction between annexin A2-S100A10 and CFTR by controlling the phosphorylation and dephosphorylation of annexin 2. Whereas the phosphorylation of annexin 2 is Ca^{2+}-dependent and interferes with S100A10 binding, dephosphorylation is cAMP -dependent and is permissive to the interaction leading to regulation of chloride flux. Given that annexin A2-S100A10 controls the recruitment and function of several other channel proteins (including Na^+,

K^+, Ca^{2+} and Cl^- channels), and CFTR regulates several other channels and proteins, this complex appears vital for epithelial ion transport and function. Future experiments are expected to characterize the interaction further, identify additional annexin A2-S100A10/CFTR interaction partners and their role in epithelial ion transport and function.

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Review

The endothelial cell annexin A2 system and vascular fibrinolysis

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Abstract. Vascular endothelial cell surface expression of annexin A2 and its binding partner p11 is a key element in maintaining fibrinolytic balance on blood vessel surfaces. In the recent decade, investigators have made significant progress toward understanding the mechanisms that regulate heterotetrameric $(A2{\bullet}p11)^2$ receptor translocation from the cytoplasm to the outer cell surface. Accumulating evidence now shows that heterotetrameric $(A2{\bullet}p11)^2$ cell surface expression is a dynamic process that modulates plasmin activation during periods of vascular stress or injury, and is independent of the classical endoplasmic reticulum-Golgi pathway. Translocation of heterotetrameric $(A2{\bullet}p11)^2$ is facilitated both by src-kinase mediated phosphorylation of A2 at tyrosine 23, and by expression of and partnering with p11. In the absence of A2 both *in vivo* and *in vitro*, p11 is expressed at very low levels in endothelial cells, because unpartnered p11 is polyubiquitinated and rapidly degraded through a proteasome-dependent mechanism. A2 directly binds and stabilizes intracellular p11 by masking an autonomous polyubiquitination signal on p11. This modulatory role of A2 binding prevents accumulation of unpartnered p11 within the endothelial cell, and ultimately suggests that the regulation of heterotetrameric $(A2{\bullet}p11)^2$ receptor surface expression is precisely attuned to the intracellular level of p11.

Key words: Annexin A2 — p11 — Fibrinolysis — Endothelial cells — Vascular homeostasis

Introduction

The fact that plasma contains one system designed to clot blood and another to dissolve thrombi is one of the teleologic wonders of vascular biology (Ratnoff and Forbes 1984). Following vessel injury, the coagulation cascade is activated and generates thrombin, which converts soluble fibrinogen into insoluble fibrin. Upon vessel healing, the process of fibrinolysis begins, whereby, the sequential activation of specific proteases culminates in production of plasmin, which cleaves fibrin into soluble, defined degradation products, and restores vascular patency.

In the last century, the principal fibrinolytic molecules, plasminogen, tissue plasminogen, and urokinase, were identified, cloned, and studied in genetic mouse models (Hajjar 2009). From the work of many investigators, it is now clear that plasminogen, an inactive zymogen, circulates in blood and can be converted to plasmin by either of two physiologic activators, tissue plasminogen activator (tPA) or urokinase (uPA). tPA is a secretory product of endothelial cells, while uPA is produced by renal epithelial cells or by endothelial cells stimulated by inflammatory cytokines (Wojta et al. 1989). Upon resolution of vascular injury, these agents dissolve mature blood clots, or thrombi, renewing the flow of blood.

Until the middle of the 20th century, fibrinolysis was thought to be confined to the surface of fibrin-containing thrombi. In 1964, however, Todd observed that fibrin gels applied directly to fresh tissue sections displayed zones of protein lysis that were specifically localized to blood vessels, and required the integrity of the endothelium (Todd 1964). Our research group has focused on vascular fibrinolysis, the generation of plasmin activity in the vicinity of the blood vessel wall, postulating that small amounts of this activity may protect the blood vessel from fibrin accumulation resulting from subliminal injury. We have hypothesized that cell surface receptors enable vascular fibrinolysis by providing a micro-environment that localizes and protects fibrinolytic activity. The relevant receptors include the urokinase receptor (uPAR), which appears to be preferentially expressed on migrating endothelial cells (Pepper et al. 1993), and the annexin A2 complex, which is expressed on both resting and activated endothelial cells (Cesarman et al. 1994; Hajjar et al. 1994).

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The endothelium and vascular fibrinolysis

Collectively, endothelial cells (ECs) constitute the vascular endothelium, a "distributed organ" whose functional properties are versatile and specific to individual organ systems (Gimbrone 2007). The endothelium's intimate association with flowing blood allows it to participate dynamically in modulating secreted activity, selective permeability, and thromboresistance (Altschul 1954; Gimbrone 1980; Gimbrone 2007). ECs are heterogeneous in terms of their structure, protein synthetic repertoire, and response to environmental stimuli (Augustin et al. 1994; Cines et al. 1998; Durr et al. 2004; Furchgott and Zawadzki 1980; Garlanda and Dejana 1997; Gimbrone 1995; Gimbrone et al. 1993; Hajjar 2006; Oh et al. 2004). In regulating hemostasis, ECs possess both antiplatelet properties, through production of nitric oxide (NO) and prostacyclin (PGI_2), and anticoagulant properties, through expression of thrombomodulin and the endothelial protein C receptor (Hajjar et al. 2001).

The fibrinolytic properties of endothelial cells are complex. Although the endothelium appears to be the major source of tPA in blood, tPA expression *in vivo* appears to be restricted to the microvasculature in specific anatomic locations (Hajjar et al. 2001), and is subject to stimulation by desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP), bradykinin, endothelin, hyperoxia, and thrombin (Diamond et al. 1989; Dichek and Quertermous 1989; Kooistra et al. 1987; Levin et al. 1989). uPA and its receptor, on the other hand, are not highly expressed by resting endothelium, but are stimulated by inflammatory cytokines, during wound repair, and in physiologic angiogenesis (Bacharach et al. 1992; Pepper et al. 1993). Similarly, the physiologic inhibitor of both tPA and uPA, plasminogen activator inhibitor-1 (PAI-1), is expressed minimally by quiescent ECs, but increases in response to angiogenic or inflammatory cytokines. Finally, thrombin bound to the integral endothelial membrane protein, thrombomodulin, can activate a protein called thrombin-activatable fibrinolysis inhibitor (TAFI), which, as a carboxypeptidase, removes C-terminal Lys and Arg residues, the primary PLG and tPA binding sites on fibrin and many receptors (Bajzar et al. 1996). Thus, the endothelial cell is equipped with a number of mechanisms for regulating vessel associated fibrinolytic activity.

Endothelial cell receptors in vascular fibrinolysis

Plasminogen Receptors

Plasminogen (PLG) receptors are a diverse group of proteins expressed on a wide array of cell types (Miles et al. 2005). The most widely recognized PLG receptors include α-enolase and histone H2B on monocytoid cells, glycoprotein IIb/IIIa complex on platelets, the Heymann nephritis antigen on renal epithelial cells, amphoterin on neuroblastoma cells, and annexin A2 (A2)/protein p11 on ECs (Barnathan et al. 1988; Das et al. 2007; Hajjar 1991; Hajjar and Hamel 1990; Kanalas and Makker 1991; Miles et al. 1991; Miles et al. 1986; Parkkinen and Rauvala 1991). These binding proteins commonly possess a C-terminal Lys residue, either in the native state, or upon proteolytic processing, which allows them to interact with the lysine-binding "kringle" domains of PLG (Das et al. 2007; Miles et al. 1991). Receptor mediated binding of plasminogen serves first to protect plasmin (PN), once it has been activated, from its principal circulating inhibitor, α2-antiplasmin, and secondly to concentrate PN activity at the cell surface (Hajjar and Francis 2006).

UPAR

uPA is expressed by ECs, as well as monocytes, macrophages, fibroblasts, and a variety of tumor cells (Cines et al. 1998). While uPA appears to be absent in quiescent endothelium (Cines et al. 1998; Wojta et al. 1989), it is present in ECs involved in wound repair or angiogenesis. This is consistent with the hypothesized importance of uPA in cell migration and tissue remodeling (Cines et al. 1998; Wojta et al. 1989). uPAR, a three-domain protein linked to cell surfaces by a glycerophosphatidyl inositol (GPI) anchor (Cines et al. 1998), binds uPA and activates PLG. While uPA appears to contribute to vascular hemostasis, since mice genetically deficient in uPA show fibrin deposition in tissues (Carmeliet et al. 1994), uPAR has yet to be shown to participate in baseline hemostasis, since mice lacking uPAR develop normally and do not exhibit spontaneous vascular occlusion. uPAR may be important for vascular repair, however, since it may be expressed mostly on the surface of migrating ECs participating in angiogenesis, rather than on quiescent ECs lining normal vessels (Bugge et al. 1996; Pepper et al. 1993).

The $(A2{\bullet}p11)^2$ receptor complex

The annexins are a family of Ca^{2+} regulated, phopholipid-binding proteins, characterized by the unique architecture of their Ca^{2+} binding sites (Gerke et al. 2005). These structures enable the annexins to interact with anionic membrane phospholipids in their Ca^{2+}-bound conformation, a property that appears to link many annexins to a range of membrane-related events, such as exocytosis, endocytosis, and the regulation of ion fluxes across membranes. Some annexins pair with members of the S100 family of Ca^{2+}-binding proteins, which appear to modulate their function.

Protein p11, a member of the S100 family (S100A10), contains two EF hand motifs, structures consisting of two nearly perpendicular α-helices flanking a roughly 12-residue loop. EF-hand proteins usually bind to cellular targets, in response

to changes in Ca^{2+} concentration (Gerke et al. 2005). p11, however, is an exception to the Ca^{2+} activation rule, since it permanently assumes a "Ca^{2+}-on" conformation and can readily target A2 subunits. Consequently, partnering of p11 appears to be regulated mainly by the concentration of each binding partner, rather than by Ca^{2+} ion fluxes (He et al. 2008). In ECs, an increase in intracellular Ca^{2+} is predicted to mobilize $(A2•p11)^2$ complexes to the inner surface of the plasma membrane (Thiel et al. 1992).

The function of A2 in fibrinolysis was discovered by members of our lab while seeking the EC surface receptor for PLG and tPA (Hajjar et al. 1994). In 1986, we reported for the first time that PLG could bind directly to cultured ECs with high affinity (K_d 300 nM) and specificity (Hajjar et al. 1986). We later found that the circulating form of PLG, N-terminal glutamic acid-plasminogen (Glu-PLG), was converted to a more readily activated form (N-terminal Lys-PLG) upon binding to ECs (Hajjar et al. 1986). These findings identified the EC surface as a profibrinolytic microenvironment. We subsequently discovered that PLG binding to human ECs could be inhibited in the presence of lipoprotein(a) (Lp(a)), a highly atherogenic lipoprotein particle whose apoprotein, apoprotein(a), is structurally homologous to PLG (Hajjar et al. 1989); these data implicated Lp(a) in atherogenesis through inhibition of EC surface fibrinolysis.

In 1987, we reported the first demonstration that tPA could bind to cultured human ECs at two independent sites with Kd's of 29 pM and 18 nM (Hajjar et al. 1987). The higher affinity site had characteristics of the physiologic plasminogen activator inhibitor type 1 (PAI-1), and could be blocked by uPA. The lower affinity site, on the other hand, appeared to be novel. Ligand blotting of an EC plasma membrane protein fraction revealed an approximately 40-kDa protein that bound tPA, but not uPA, thus distinguishing it from PAI-1 and uPAR (Hajjar and Hamel 1990). The protein was subsequently isolated from human placental membranes and shown, in both this preparation and in an EC membrane fraction, to interact specifically with both tPA and its substrate, PLG (Hajjar 1991). These findings suggested a common EC surface binding protein for tPA and PLG, somewhat reminiscent of fibrin.

Our identification of the tPA-PLG binding protein emanated from amino acid sequence analysis, which revealed a 100% match with residues 29-46 of annexin A2, then known as "annexin II" (Hajjar et al. 1994). In further investigations, antibody directed at authentic A2 blocked ~50% PLG and tPA binding to human ECs, and A2 antisense oligonucleotides blocked 50 and 40% of tPA and PLG binding to these cells, respectively. In addition, treatment of A2 with carboxypeptidase B (CPB) eliminated its ability to bind PLG, thus implicating a C-terminal Lys or Arg residue. Mutation of Lys307 to Ala specifically eliminated PLG binding as well, suggesting that PLG binding to A2 required a proteolytic processing event to liberate Lys307 at a new C-terminus.

In a companion study, native annexin A2 purified from human placenta bound tPA, PLG, and PN saturably (Cesarman et al. 1994). This A2 preparation increased the catalytic efficiency (k_{cat}/K_m) of tPA-dependent PLG activation by ~60-fold, but had no effect on uPA activation of PLG. The catalytic effect of A2 disappeared in the presence of the Lys analog ε-aminocaproic acid, or by treatment of A2 with CPB, again implicating an interaction between a C-terminal Lys or Arg of A2 and a Lys binding domain of PLG. These experiments raised the possibility of a C-terminal Lys residue for PLG binding, and demonstrated the fibrin-like cofactor-like behavior of A2 with respect to tPA-dependent PLG activation.

In follow-up studies, we explored the mechanism by which A2 interacted with the EC surface (Hajjar et al. 1996). A2 was biosynthetically labeled by cultured human ECs incubated with ^{35}S-radiolabeled methionine, indicating direct synthesis by these cells. Moreover, in the presence of Ca^{2+}, but not other divalent cations, both recombinant and native A2 showed high affinity, equilibrium binding to cultured ECs (K_d ~ 50 nM). This binding could be outcompeted by phosphatidylserine (Ptd-L-Ser)-containing vesicles, by peptides mimicking the annexin A2 repeat 2, or upon mutation of the Asp^{161} residue that coordinates with the annexin repeat (Lys-Gly-Leu-Gly-Thr) sequence. Together, this study showed that the interaction of A2 with the EC surface involved both the Ca^{2+}-dependent, anionic phospholipid-binding repeat 2 of A2, and Ptd-L-Ser moieties within the plasma membrane.

The tPA-binding domain of A2 was examined separately (Hajjar et al. 1998). While PLG bound only to native A2, suggesting the need for a proteolytic processing event to reveal a C-terminal Lys or Arg, tPA bound to both native and recombinant forms. Unlike full-length A2, however, the core fragment of A2 failed to compete for binding of tPA to full-length A2, thus implicating the N-terminal tail domain in this interaction. Indeed, residues Leu^7-Cys^8-Lys^9-Leu^{10}-Ser^{11}-Leu^{12}, and larger peptides containing this fragment, specifically blocked binding of tPA to A2. Mutation of Cys^8, but neither Cys^{133}, Cys^{262}, nor Cys^{335} prevented binding of tPA to A2; further implicating the N-terminal region. Interestingly, when A2 was incubated in a purified protein system with the atherothrombotic amino acid, homocysteine (HC), A2 was modified by HC. This treatment blocked the ability of tPA to bind to A2. Finally, incubation of cultured ECs with ^{35}S-HC led to metabolic labeling of A2 that was sensitive to protein reduction, suggesting a disulfide-mediated association between Cys^8 and HC. These data revealed a binding domain for tPA in the N-terminal tail of A2, and showed its susceptibility to modification by HC, an agent highly associated with atherothrombotic vascular disease.

Based upon these data, our working model has postulated that both PLG and tPA interact with A2 within the $(A2•p11)^2$ heterotetrameric complex at the EC surface. Our model suggests further that this assembly augments the catalytic efficiency of plasmin activation by 1-to 2-log orders-of-magnitude. The model predicts that the A2•p11 system contributes to blood vessel patency, i.e. that gain-of-function would lead to hemorrhage, and that loss-of-function would lead to vascular thrombosis. To test this hypothesis, we have carried out a series of studies both *in vitro* and *in vivo*. Using homologous recombination, we created a mouse globally deficient in A2, and discovered that it displayed both fibrin accumulation, mainly in microvessels, and defects in angiogenesis (Ling et al. 2004). Interestingly, these animals, in retrospect, also expressed very low levels of p11. In humans, overexpression of A2 in blast cells from patients with acute promyelocytic leukemia correlated with hyperfibrinolysis and hemorrhage (Menell et al. 1999). In patients with antiphospholipid syndrome, on the other hand, we found high titers of anti-A2 IgG that were associated with major thrombotic episodes. Additionally, in vascular injury models, $A2^{-/-}$ mice exhibited an increase in the degree of thrombotic vascular occlusion, compared to basal conditions, with an equivalent decrease in blood flow recovery. In rats, treatment with recombinant A2 counteracted the onset of carotid artery thrombosis (Ishii et al. 2001), and reduced cerebral infarct size secondary to increased cerebral blood flow after induced embolic stroke (Tanaka et al. 2007). Taken together, these findings support our hypothesis that the annexin A2 system functions to promote vascular fibrinolysis.

Additional studies have suggested that the proenzymatic activity of $(A2•p11)^2$ is not limited to ECs. Annexin A2 supports macrophage matrix invasion and degradation (Falcone et al. 2001). Moreover, monocytes, the major A2-expressing cells in circulating blood, express cell surface A2, and augment its expression upon differentiation into macrophages (Brownstein et al. 2004). Annexin A2 appears to support neuritogenesis of PC12 cell *in vitro* (Jacovina et al. 2001). In chick embryo, A2 promotes epithelial mesenchymal transformation during heart development by enabling plasmin-mediated activation of transforming growth factor ß3 (Krishnan et al. 2004). These data, therefore further suggest that the A2•p11 system facilitates the directed migration and/or remodeling of a spectrum of cell types.

Primary binding sites for plasmin generation

With regard to alternative models for Plg and tPA binding to the $(A2•p11)^2$ complex, another group has investigated these interactions. In 1998, it was reported that, while recombinant A2 tetramer (A2t) stimulated tPA-dependent PLG activation, it inhibited plasmin-mediated fibrinolysis in a purified system (Choi et al. 1998). This latter inhibitory effect was attributed to the *in vitro* observation of PN inactivation by autoproteolysis (Fitzpatrick et al. 2000). The same group reported that A2 binds PN, but not PLG (MacLeod et al. 2003), suggesting that p11, rather than A2, is the true binding site relevant to PN generation. It was further reported that, in a purified protein system, recombinant protein p11, which has a Lys-Gly-Lys-Lys sequence at its C-terminus, binds both tPA and PLG through Lys binding sites, whereas recombinant A2 monomer does not (Kassam et al. 1998).

While our group has not examined interactions between PLG and p11 directly, it seems quite possible that p11 could serve as a cellular PLG binding site under some circumstances. With regard to A2 binding of PLG, however, it is important to note that the studies cited above do not take into account our previous work showing that proteolytic processing of A2 appears to expose C-terminal Lys residues, converting A2 into a PLG-binding protein. This possibility has been reiterated in a study from a third group in which the authors found that, in addition to α-enolase and histone 2B, both A2 and p11 could serve as PLG receptors in murine and human macrophage-like cells (Das et al. 2007). While binding of PLG to purified, recombinant A2, required cleavage of the recombinant protein by trypsin, intact cells showed colocalization of PLG and A2 by confocal microscopy. These authors demonstrated further that this association was sensitive to carboxypeptidase B, indicating that proteolytic processing of A2 had already occurred. These data emphasize that A2 is processed to a PLG-binding form at the surface of monocytoid cells and perhaps other cells as well, and that data generated from recombinant proteins in purified systems may not reflect their full behavior in the cellular context.

Regulation of $(A2•p11)^2$ endothelial cell surface expression

Though there is a spectrum of evidence supporting the central role of the A2 system in fibrinolysis, the mechanisms governing A2 cell surface expression have only recently been addressed. It is now clear that the regulation of heterotetrameric $(A2•p11)^2$ cell surface expression is governed by intracellular signaling pathways that respond to changes in the extracellular environment. Key factors that influence EC surface expression and function of $(A2•p11)^2$ are oxidative stress (Rowan et al. 2002; Sullivan et al. 2000), heat stress (Deora et al. 2004), and thrombin stimulation (Bhattacharjee et al. 2008; Peterson et al. 2003).

Under oxidative stress, A2 becomes more susceptible to the incorporation of prothrombic amino acid homocysteine

(HC) (Hajjar and Jacovina 1998). In the presence of mid-to-high micromolar HC, there appears to be reduced EC surface potential for tPA-dependent PLG activation due to blockade of tPA binding to A2 by derivatization Cys^{8} within the N-tail domain of annexin A2 (Hajjar 1993; Hajjar et al. 1998). This hypothesis was recently confirmed in vivo in mice with diet-induced hyperhomocysteinemia; in these mice A2 was shown to be derivatized by HC, with concomitant failure of the profibrinolytic and proangiogenic activities of $(A2{\bullet}p11)^2$ (Jacovina et al. 2009). In this situation, the stress-associated modification of A2 alters the properties of the protein (Gerke et al. 2005) and impairs the function of the $(A2{\bullet}p11)^2$ complex.

On the other hand, our group has also shown, that temperature-stress stimulates translocation of A2 to the EC surface, increasing the potential for plasmin generation (Deora et al. 2004). Deora et al. injected GFP-tagged A2 cDNAs into human ECs, and noted that when the injected cells were exposed to a brief increase in temperature (42° C), there was a doubling or tripling of the cell surface expression of A2. This process required both tyrosine phosphorylation of A2 and the presence of protein p11. Interestingly, ECs treated with the protein synthesis inhibitor, cyclohexamide, exhibited enhanced, rather than repressed, translocation at both 37° C and 42° C. There was no change in steady-state mRNA levels during heat stress (Deora et al. 2004). In an additional experiment, HUVECs treated with brefeldin A, a drug that disrupts the endoplasmic reticulum (ER)-Golgi complex did not decrease the ability for A2 to be expressed

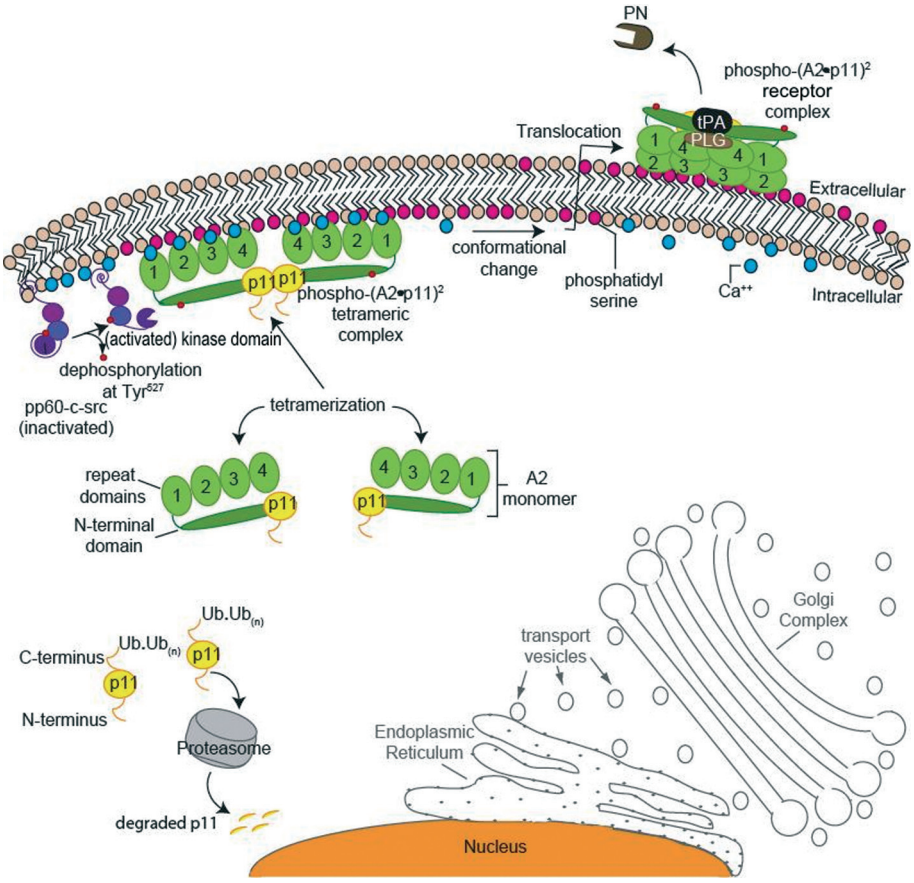

Figure. A working model for heterotetrameric $(A2{\bullet}p11)^2$ complex formation and translocation to the cell surface. $(A2{\bullet}p11)^2$ cell surface translocation and activation is independent of the endoplasmic reticulum (ER)-Golgi pathway (outlined in grey). Unpartnered p11 monomers (yellow) within the cell are polyubiquitinated and degraded by the proteasome (grey). Annexin A2 (A2) monomers (green) are bound to p11 subunits. Intracellular calcium levels increase (blue) in response to various stimuli, and this increases the affinity of the $(A2{\bullet}p11)^2$ complex for binding to anionic phospholipid (shown in dark pink) at the inner membrane surface. There, pp60-c-src kinase (purple) becomes activated following dephosphorylation of Tyr^{527} within its kinase domain, while Tyr^{416} within its SH2 domain remains phosphorylated. Once the $(A2{\bullet}p11)^2$ complex is phosphorylated (red circles) by pp60-c-src, it becomes more tightly associated with inner leaflet phosphatidyl serine (Ptd-L-Ser). The newly phospho-$(A2{\bullet}p11)^2$ complex may undergo a conformational change, making it more susceptible to translocation to the outer membrane surface. On the outer membrane surface, phospho-$(A2{\bullet}p11)^2$ associates with plasminogen (PLG) and tissue plasminogen activator (tPA), giving rise to active plasmin (PN).

on the cell surface. It was therefore concluded that A2 translocation occurs independently of the classical ER-Golgi pathway, and does not require *de novo* protein synthesis (Deora et al. 2004)

At the intracellular surface of the plasma membrane $(A2{\bullet}p11)^2$ is susceptible to phosphorylation (Bellagamba et al. 1997; Okuse et al. 2002). A2 is a known substrate for both serine/threonine and tyrosine protein kinases (Beaton et al. 2002). Previous studies in HEK 293 cells have shown that Tyr^{23} of A2 is a target for phosphorylation by the src-family kinase, pp60-c-src kinase (Glenney and Tack 1985). Src-kinase is activated when Tyr^{527} in the C-terminus kinase domain of pp60-c-src is dephosphorylated, and Tyr^{416} remains phosphorylated (Cooper and King 1986). When Tyr^{23} in the tail domain of A2 is mutated, preventing phosphorylation, neither translocation nor phosphorylation of A2 occurs. Once phosphorylated, the new phospho-$(A2{\bullet}p11)^2$ heterotetramer is predicted to couple more tightly to inner leaflet anionic Ptd-L-Ser (Montaville et al. 2002) and possibly undergo conformational changes that enhance its ability to be translocated to the outer membrane surface (Figure).

Furthermore, in a recent study focusing on p11, our group showed that p11 is unstable and rapidly degraded in the EC in the absence of A2 (He et al. 2008). When HEK 293 cells, which express very low levels of endogenous p11 and A2, were transfected with FLAG-p11, unbound p11 monomers were observed to be rapidly ubiquitinated and degraded through a proteasome-dependent mechanism (He et al. 2008). Co-transfection of A2, however, rescued p11, which bound to the 13 N-terminal amino acids in the A2 tail peptide, thus masking an autonomous p11 ubiquitination signal. Ubiquitination of p11 required residues Tyr^{86}-Gly^{95}, near its A2-binding C-terminal motif. These studies suggest that A2 directly regulates intracellular levels of p11 in endothelial cells (Figure).

Concluding remarks

Partnering of A2 and p11 to form the $(A2{\bullet}p11)^2$ complex is a cooperative process: A2 binds and stabilizes p11, thereby regulating the intracellular concentration of p11. Intracellular p11 levels, on the other hand, are likely to determine how much $(A2{\bullet}p11)^2$ translocates to the cell surface. Stress-induced stimuli, such as heat shock or the action of thrombin, can activate a cascade of events that enhance translocation to the cell surface through an ER-Golgi independent pathway (Figure). Further understanding of the regulation of the $(A2{\bullet}p11)^2$ system will be important for evaluation of the proposed functions for A2 and p11 in a wide range of cell and tissue types (Foulkes et al. 2006; Liu et al. 2003; Sharma and Sharma 2007; Tsai 2007). For instance, the role of A2 in angiogenesis and fibrin homeostasis (Ling et al. 2004) may contribute to our understanding of cancer cell biology, retinal disease, and vascular occlusion. Forthcoming investigative studies should be not only exciting, but also useful for developing future therapies.

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Characterisation of the sarcoidosis-associated variant of annexin A11

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Abstract. Recent studies on the genetic background of sarcoidosis have resulted in the discovery of a strongly associated single nucleotide polymorphism (SNP) that switches a highly conserved arginine to a cysteine at position 230 in annexin A11. The effect of the R230C SNP on the cellular distribution and Ca^{2+}-sensitivity of annexin A11 was investigated through over-expression of GFP tagged annexin A11 in A431 cells. Cells were stimulated with calcium mobilizing agonists and changes in the cellular localisation of GFP tagged annexin A11 were recorded. Neither variant of annexin 11, nor any truncation mutants, exhibited any response to EGF. In addition, there was no relocalisation of the GFP tagged C-terminal annexin A11 variants in response to ionomycin. However, both the wild type and sarcoidosis associated variants of annexin A11-GFP relocalised to the plasma membrane and then the nuclear envelope in response to ionomycin. These observations show that the sensitivity of annexin A11 to a robust, sustained rise in intracellular calcium, is not significantly affected by the sarcoidosis associated SNP. This does not rule out functional affects in the extracellular milieu, in cytokinesis, nuclear envelope breakdown or in response to other intracellular signals.

Key words: Annexin — Sarcoidosis — Calcium

Introduction

Sarcoidosis is a multisystem immune disorder, resulting in the formation of epitheloid granulomas throughout the body, particularly within the lungs, eyes and skin. The immune systems of affected individuals exhibit significant changes in cell numbers and cell signaling, with an increase in CD3 and CD4 positive T cells in the lungs. Activated T cells within sarcoid lungs have also been shown to over-express several cytokine receptors, including the interleukin-2 receptor (IL-2R), and produce increased amounts of cytokines, including interleukin-2 (Pinkston et al. 1983) and interferon-γ (IFNγ) (Robinson et al. 1997). In addition, monocytes and macrophages are heavily involved in the formation of sarcoid granulomas and also secrete a range of cytokines that further enhance the immune response. For example alveolar macrophages secrete tumor necrosis factor α (TNFα), which is a current target for sarcoidosis therapy (Toussirot et al. 2008) and interleukin-15, which has been shown to induce T cell proliferation (Agostini et al. 1996).

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Given the complex nature of the immune pathways involved and the interplay between different immune cell types, understanding the molecular aetiology of sarcoidosis has proven difficult. Although a working hypothesis for the formation of sarcoid granulomas has been put forward (Noor and Knox 2007), the initiating antigen in this process is unknown and speculated to involve both viruses and bacteria (Ezzie and Crouser 2007). A better picture is however emerging for the genetic factors linked to susceptibility to sarcoidosis.

Familial linkage studies identifying regions encoding alleles more commonly shared between affected family members uncovered a SNP in the butyrophilin-like 2 (BTNL2) gene. BTNL2 lies within the MHC class II region and is a member of the B7 receptor family. It is thought to act as a co-stimulatory molecule for T cell activation. The SNP results in a premature stop codon, which produces a truncated protein that can no longer localize to membranes. This truncation is postulated to promote a pathologically inflammatory environment, as occurs in sarcoidosis (Valentonyte et al. 2005). Genes neighbouring BTNL2 have also been implicated as they show a high linkage disequilibrium and therefore may be inherited together, such as the human leukocyte antigen DRB1 (HLA-DRB1) (Spagnolo and du Bois 2007). There-

fore it may be the HLA class II genes, like HLA-DRB1, that increase susceptibility to sarcoidosis. Several HLA genes have been implicated in association studies, with the suggestion that these mutant HLA proteins present antigen to T cells in a manner that induces a pathological immune response. These include HLA class I genes, such as HLA-B7 and 8, as well as HLA class II genes which are known to be up-regulated on alveolar macrophages from sarcoidosis patients (Spagnolo and du Bois 2007). Thus far the focus on susceptibility genes has been restricted to MHC proteins. However a recent study has identified a non-MHC protein, namely annexin A11.

The identification of annexin A11 was the result of a genome wide association study using 500 control and sarcoidosis-presenting individuals from a German population. Several disease-associated SNPs were found, including those in HLA loci and the BTNL2 gene. Excluding these known areas of association, an individual SNP within the annexin A11 gene produced the highest association signal, alongside five other neighbouring SNPs within this haplotype that were also strongly associated with sarcoidosis (Hofmann et al. 2008).

The non-synonymous SNP in annexin A11 results in the switch of a basic arginine to a polar cysteine at position 230, within a highly conserved domain called the annexin repeat in the C-terminal domain of the protein. Immunohistochemistry of control and sarcoidosis patient lung tissue showed no difference in annexin A11 expression, and in both cases expression was nuclear in epithelial and mononuclear cells and within the cilia of bronchial epithelial cells. Annexin A11 mRNA is particularly abundant in CD4, CD8, CD14 and CD19 positive immune cells. However, in normal individuals annexin A11 mRNA expression was significantly reduced in stimulated CD8 and CD19 positive cells compared to those at rest (Hofmann et al. 2008). Whether this would also be true of the SNP variant is currently unknown.

The functional significance of this SNP and so the mechanism of action of annexin A11 in sarcoidosis, is as yet unknown. Annexin A11 belongs to the family of vertebrate annexins which are calcium-dependent, phospholipid-binding proteins. Annexin A11 has been implicated in the regulation of cytokinesis (Tomas et al. 2004) and is known to bind S100A6 (calcyclin) (Tokumitsu et al. 1992) and ALG-2 (apoptosis-linked gene 2) (Satoh et al. 2002). However beyond this, little is known about the function of annexin A11. In order to better understand the effect of the sarcoidosis associated SNP on annexin A11, we have investigated the response of the wild type and the sarcoidosis-associated variant of annexin A11 to rises in intracellular calcium. We have shown that this aspect of annexin A11 biology appears unaffected by the sarcoidosis associated SNP.

Materials and Methods

Cell Culture

A431 (human epidermoid carcinoma) cells were cultured in DMEM (Dulbecco's minimal essential media, Gibco) with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine and incubated at 37°C with 5% CO_2.

Site Directed Mutagenesis

Site directed mutagenesis was carried out in plasmids containing either full-length annexin A11-GFP or C-terminal annexin A11-GFP. These wild type plasmids are as previously described (Tomas and Moss 2003). Single amino acid changes were introduced into wild type plasmids using two oligonucleotide primers that were complimentary to the target gene, but were designed to contain the appropriate mutation in the middle of the primer. (Forward primer sequence; GACTGCCTGGGGAGTTGCTCCAACAAGCAGCGG. Reverse primer sequence; GCTGCTTGTTGGAGCAACTCCCCAGGCAGTC). PCR reactions were carried out using 125 ng of each primer, 50 ng of the plasmid containing the gene insert, dNTP mix and Platinum Pfx (Invitrogen) in a 50 μl volume. The PCR reaction was performed for 18 cycles of 30 s at 95˚C (to separate template strands), 1 min at 55˚C (to anneal the primers) and finally 4 min at 68˚C (to extend from the primers). The reaction was then cooled to 4˚C and digested at 37˚C for 1 h with 1 μl Dpn1 to remove the parental methylated and hemimethylated DNA. 15 μl of this reaction was transformed into competent XL-1 Blue *E.coli*.

Transient Transfections

Cells were plated at 50% confluency on 35 mm glass bottomed microwell dishes (MatTek) in DMEM containing 10% heat-inactivated FCS without antibiotics. Following an overnight incubation at 37°C with 5% CO_2 the cells were transfected with 3 μg of plasmid DNA using 9 μl of TransIt LT1 transfection reagent (Mirus). 9 μl of TransIt LT1 transfection reagent (Mirus) was incubated with DMEM containing 10% heat-inactivated FCS without antibiotics, for 15 min at room temperature. 3 μg of plasmid DNA was then added to this solution and incubated for a further 30 min at room temperature. The volume of DMEM used in the transfection complex was such that the total volume reached 100 μl.

Cell Fixation

Cells were fixed using 4% paraformaldehyde (PFA) for 10 min at room temperature and then permeabilised for 10 min at room temperature with 0.2% Triton in PBS. Cells in 35 mm

glass bottomed microwell dishes were then mounted with Vectashield mounting medium (VectorShield Laboratories).

Live Confocal Imaging

Cells were transfected with the appropriate construct and incubated overnight at 37°C with 5% CO_2 in DMEM containing 10% heat-inactivated FCS without antibiotics. Cells were then washed once in DMEM without phenol red (Invitrogen) and imaged in DMEM without phenol red at 37°C on an inverted Leica TCS SP2 AOBS confocal microscope. Images were analysed and processed using Leica Confocal Software Version 2.6.1 and Zeiss LSM Image Browser Version 4.2.0.121.

Polyacrylamide Gel Electrophoresis and Western Blotting

Gels for SDS-PAGE were made using Acrylamide/Bis-Acrylamide (Sigma), Tris-HCl and 10% SDS and were polymerised using TEMED (Sigma) and 10% APS. 10% acrylamide was used in the resolving gel and 4% in the stacking gel. Samples were boiled in SDS-PAGE buffer before loading.

The gels were transferred onto Hybond PVDF Transfer membrane (Amersham) for Western blotting. Following transfer of the proteins, membranes were blocked with 8% milk for 1 h. Primary antibody also made up in 8% milk was then added and incubated overnight at 4°C on an orbital shaker. The membranes were washed 3 times in PBS + 0.05% Tween for 10 min each. Secondary HRP conjugated antibody made up in 8% milk was then added and incubated at room temperature on an orbital shaker for 1 h. Following 3 PBS washes for 10 min each, membranes were imaged using the ECL Western blotting detection system (GE Healthcare).

For Western blotting against phosphotyrosine-containing proteins, membranes were blocked with 5% BSA in TBS for 1 h. Primary antibody also made up in 5% BSA in TBS was then added and incubated overnight at 4°C on an orbital shaker. The membranes were washed 3 times in TBS + 0.05% Tween for 10 min each. Secondary HRP conjugated antibody made up in 5% BSA in TBS was then added and incubated at room temperature on an orbital shaker for 1 h. Following 3 TBS washes for 10 min each, membranes were imaged as above.

Antibodies

Antibodies used for Western blotting were as follows; 4G10 mouse anti-human phosphotyrosine antibody (Millipore, Upstate Biotechnology) used at 1:1000, sheep anti-human EGF receptor antibody (Fitzgerald Industries International) used at 1:2000, HRP conjugated goat anti-mouse antibody (Dako) used at 1:2000 and HRP conjugated donkey anti-sheep antibody (Dako) used at 1:2000.

Results

Characterisation of the Sarcoidosis-Associated Annexin A11 Single Nucleotide Polymorphism

A genome-wide study conducted in a German population, identified a single nucleotide polymorphism (SNP) within exon 6 of the human annexin A11 gene as the most highly associated SNP for sarcoidosis (Hofmann et al. 2008). Annexin A11, like other annexins, comprises two main domains, namely a variable N-terminal head and a conserved C-terminal core. Within the core domain are highly conserved sub-domains termed annexin repeats that are responsible for the calcium-binding properties of the annexins. Annexin A11 contains four of these repeats within its core domains, and the sarcoidosis associated SNP lies within the first 14 residues of the first annexin repeat (Fig. 1A). The mutation identified in the genome-wide study results in the substitution of a cytosine to a thymidine (Fig. 1B), which in turn results in a switch from a basic arginine to a polar cysteine at residue 230. Analysis of the protein sequence of annexin A11 in both vertebrates and invertebrates shows a high degree of homology at this residue and in the regions flanking the mutation, between species as distant as humans and zebrafish (Fig. 1C). First we investigated the cellular distribution of the sarcoidosis-associated variant of annexin A11, through the expression of GFP tagged proteins.

GFP tagged wild type (anx $A11^{WT}$-GFP) and mutant annexin A11 (anx $A11^{R230C}$-GFP) constructs were expressed in A431 cells. These cells endogenously express annexin A11 homozygous for arginine at residue 230, as determined by mRNA isolation and subsequent cDNA sequencing (data not shown). Cells were transfected with either the full length annexin A11 or the C-terminal core domain alone tagged to GFP, for both wild type and sarcoidosis associated variants. Full length anx $A11^{WT}$-GFP showed a nuclear and cytoplasmic distribution (Fig. 1D), in line with previous reports of endogenous expression patterns of annexin A11 in transformed cell lines (Tomas and Moss 2003). The C-terminal truncation mutant of this construct was predominantly cytoplasmic and mostly excluded from the nucleus, supporting previous studies identifying the N-terminal domain of annexin A11 as being essential for nuclear targeting (Mizutani et al. 1995) (Fig. 1D). The mutant constructs showed no difference in distribution to their wild type counterparts. Thus, anx $A11^{R230C}$-GFP was both nuclear and cytoplasmic, and the C-terminal truncation mutant of this construct was predominantly cytoplasmic with weak nuclear staining (Fig. 1D). Therefore the sarcoidosis associated SNP does not affect the subcellular localisation of annexin A11 in unstimulated A431 cells.

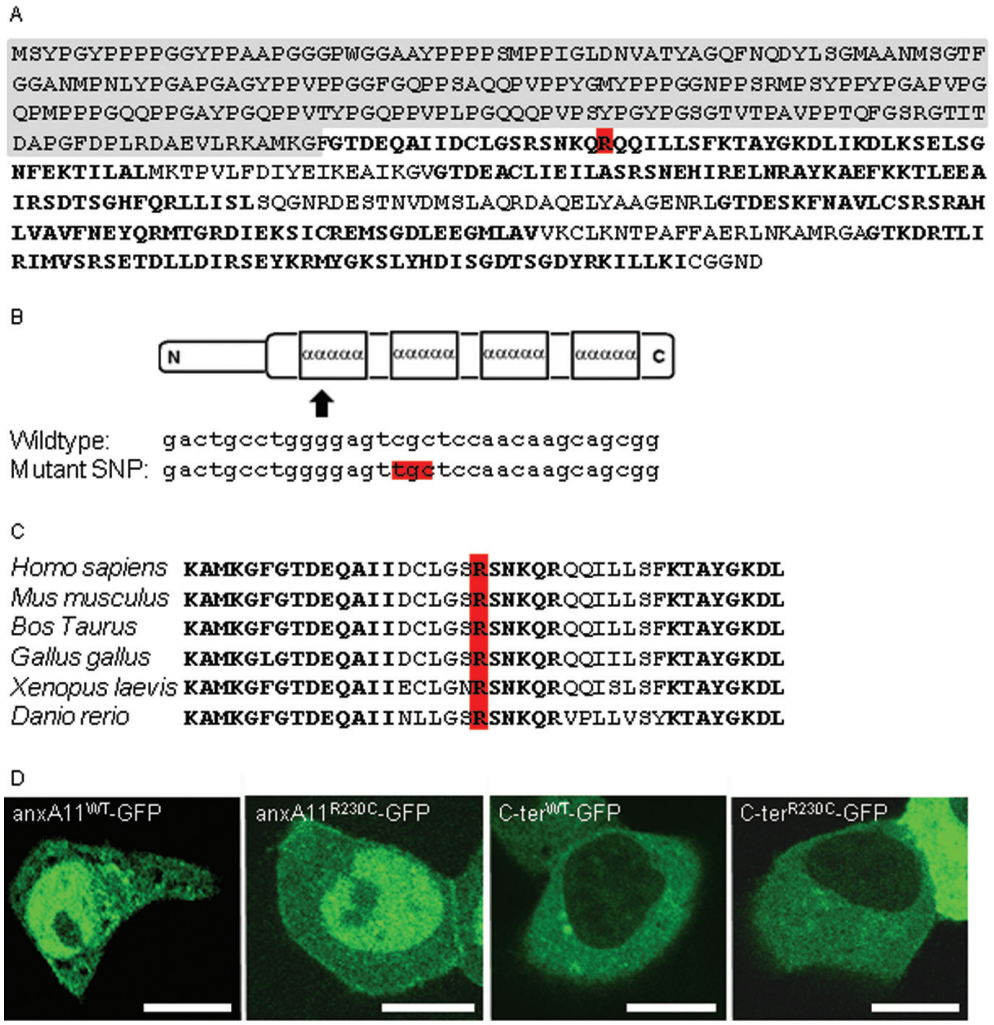

Figure 1. Annexin A11 sarcoidosis associated single nucleotide polymorphism. **A.** Full length wild type human annexin A11 protein sequence. N-terminal domain residues (grey box). Annexin repeats (bold). Sarcoidosis associated SNP results in a change in a single amino acid (red box) **B.** Schematic of annexin A11, showing that the mutation (arrow) lies within the first annexin repeat. Shown below is a section of nucleotide sequence from human annexin A11 around the sarcoidosis-associated SNP (red box) **C.** Evolutionary conservation of the amino acids surrounding the affected arginine (red box) in a range of different species. Residues conserved between all 6 species shown (bold). **D.** Expression of anx $A11^{WT}$-GFP, anx $A11^{R230C}$-GFP, anx $A11^{WT}$-Ct-GFP and anx $A11^{R230C}$Ct-GFP in A431 cells transfected with 3 µg of plasmid DNA and incubated overnight, prior to fixation in PFA. (Scale Bars 10 µm)

The Response of Wild Type and Annexin $A11^{R230C}$ to Changes in Intracellular Calcium

The SNP in annexin A11 is located within the first annexin repeat of the C-terminal domain, which as previously mentioned is responsible for the calcium-binding properties of the annexins. The change in amino acid from a basic arginine to a polar cysteine may alter the tertiary structure of the protein in a manner that could affect its calcium-binding properties. In order to investigate this possibility, the effects of elevated calcium levels on the localisation of GFP-tagged wild type and mutant annexin A11 were tested by stimulating A431 cells with ionomycin or epidermal growth factor (EGF).

The Response of Anx $A11^{WT}$-GFP and Anx $A11^{R230C}$-GFP to Ionomycin

The ionophore ionomycin was used to raise intracellular calcium levels. The addition of 1µM ionomycin to A431 cells expressing anx $A11^{WT}$-GFP resulted in a re-localisation of the tagged protein (Fig. 2A). Prior to treatment anx $A11^{WT}$-GFP was diffuse in both the nucleus and cytoplasm. Following treatment it first re-localised to the plasma

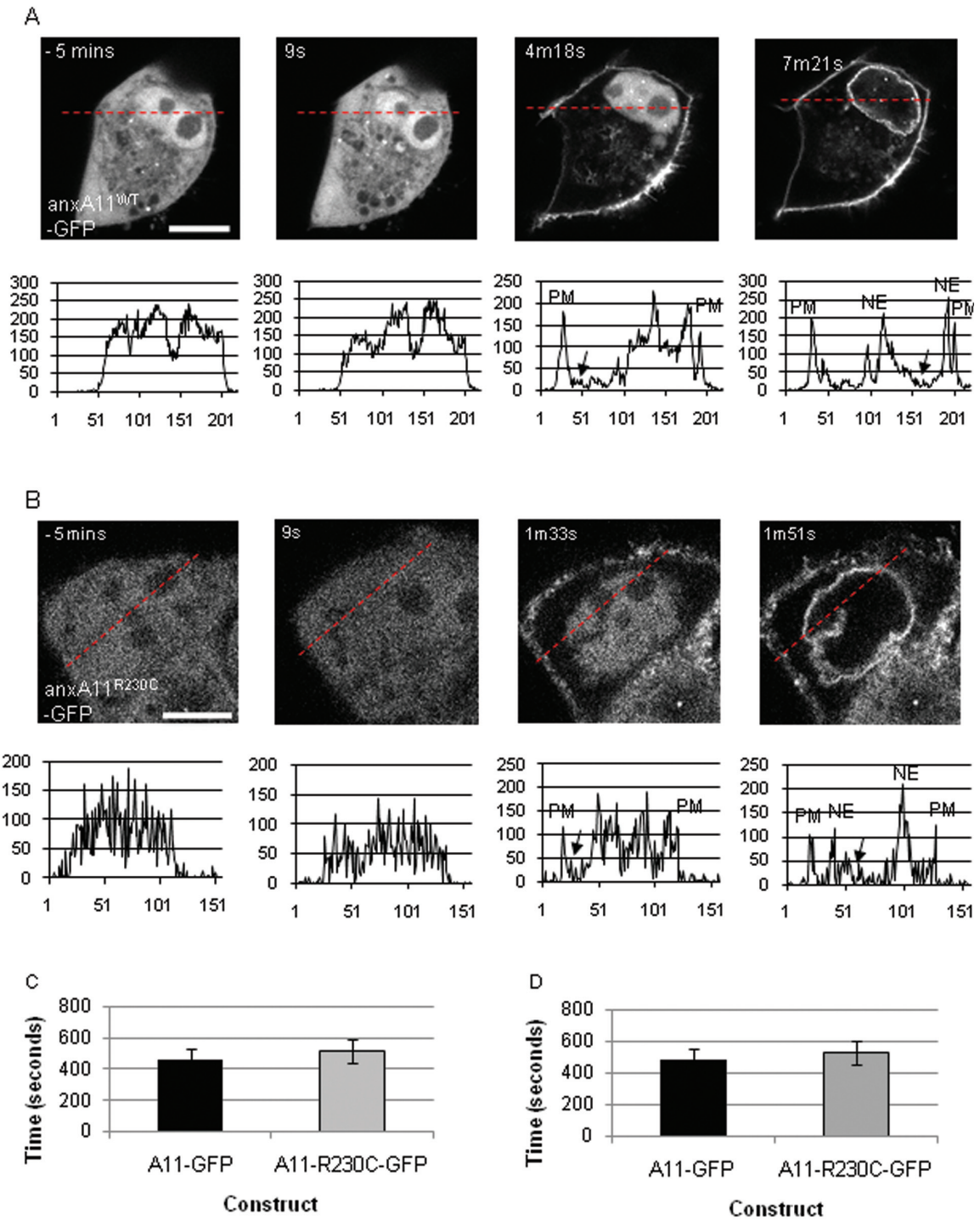

Figure 2. Full length anx A11^{WT}-GFP and anx A11^{R230C}-GFP respond to ionomycin in a similar manner. **A, B.** Real time imaging of anx A11^{WT}-GFP and anx A11^{R230C}-GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Cells were imaged in DMEM without phenol red and with treated with 1 μM ionomycin. Time pre and post treatment noted. Line scans were taken through the cell (red) and plotted in graphs below images; gray scale (y axis), position along line in pixels (x axis), plasma membrane (PM), nuclear envelope (NE), maximal cyto- or nucleo-plasmic depletion (arrow). (Scale Bars 10 μm) **C.** Bar chart of the average time taken for anx A11^{WT}-GFP and anx A11^{R230C}-GFP, expressed in A431 cells, to relocalise to the plasma membrane ($p = 0.089$) in response to 1 μM ionomycin. **D.** Bar chart of the average time taken for anx A11^{WT}-GFP and anx A11^{R230C}-GFP, expressed in A431 cells, to re-localise to the nuclear envelope ($p = 0.155$) in response to 1 μM ionomycin. Errors bars represent standard errors of the mean from 3 experiments with n = 36 cells for each construct.

membrane, depleting cytosolic GFP fluorescence whilst remaining constant in the nucleoplasm. The nucleoplasm was then also depleted of annexin A11-GFP, as the protein re-localised to the nuclear envelope. Line scans through the cell showed the appearance of clear peaks of fluorescence corresponding to these membranous accumulations; outer peaks representing the plasma membrane (PM) and inner peaks representing the nuclear envelope (NE) (Fig. 2A). The line scans also show troughs corresponding to regions of depletion of annexin A11-GFP in the cytoplasm and nucleoplasm. A similar pattern of re-localisation was observed for stimulated A431 cells expressing anx $A11^{R230C}$-GFP (Fig. 2B), with the tagged protein first becoming enriched at the plasma membrane and then the nuclear envelope, whilst concomitantly becoming depleted from the cytoplasm and nucleoplasm respectively.

The time taken for the two annexin A11 variants to re-localise to the plasma membrane and the nuclear envelope was quantified. Since the membrane accumulation of tagged protein occurred over several seconds, the time recorded for statistical analysis was defined as the point at which maximal enrichment was observed during the time course of the experiment i.e. the point at which no more GFP tagged protein is lost from the cytoplasm to the plasma membrane or the nucleoplasm to the nuclear envelope. Translocation of the tagged proteins is illustrated by line scans showing clear peaks for membrane enrichment alongside troughs of maximally depleted cytoplasm or nucleoplasm. On average the time taken for anx $A11^{WT}$-GFP to relocalise to the plasma membrane in response to ionomycin was 458s (+/- 70s, n=36) and for anx $A11^{R230C}$-GFP it was 516s (+/- 74s, n=36) (Fig. 2C). The average time taken for anx $A11^{WT}$-GFP to re-localise to the nuclear envelope in response to ionomycin was 483s (+/- 76s, n=36) and for anx $A11^{R230C}$-GFP it was 533s (+/- 75s, n=36) (Fig. 2D). Although the anx $A11^{R230C}$-GFP variant generally took approximately one minute longer than anx $A11^{WT}$-GFP to respond to the rise in Ca^{2+}, statistical analysis revealed that the difference between the wild type and mutant GFP tagged proteins fell short of significance in the time taken to re-localise to the plasma membrane (P=0.089) or the nuclear envelope (P=0.155).

Since the N-terminal domains of annexins can influence the responsiveness of these proteins to Ca^{2+}, the effects of ionomycin stimulation on mutants lacking the N-terminus was also examined. In previous work we found that removal of the N-terminus of annexin A11 rendered the protein insensitive to Ca^{2+}, despite the fact that the N-terminus lacks any Ca^{2+}-binding sites (Tomas et al. 2004). Consistent with our earlier findings, neither construct exhibited any change in subcellular localisation in response to 1μM ionomycin. Both mutant and

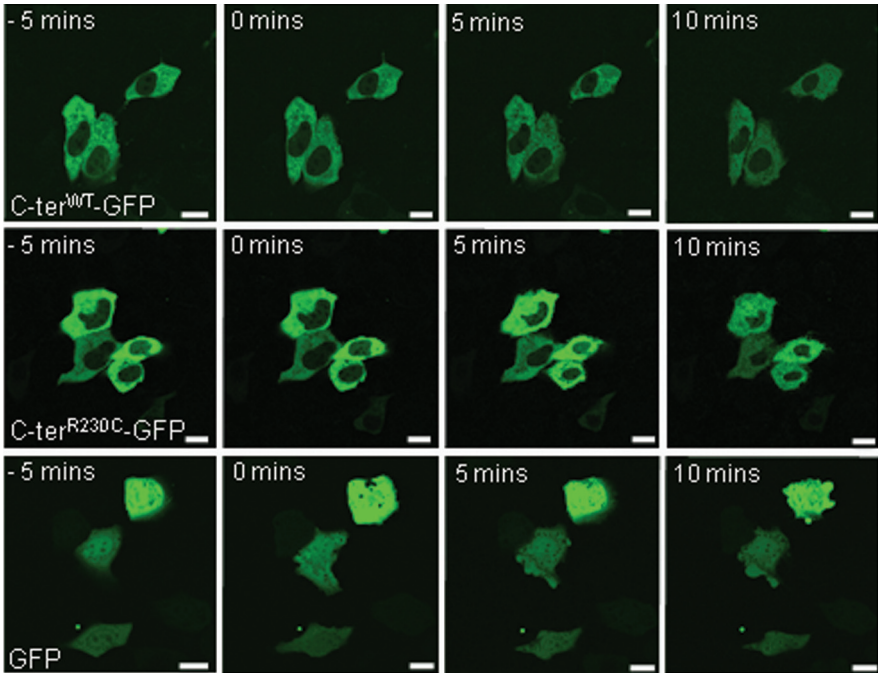

Figure 3. C-terminal wildtype and mutated annexin A11 respond to ionomycin in a similar manner. Real time imaging of anx $A11^{WT}$-Ct-GFP, anx $A11^{R230C}$Ct-GFP and GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Prior to imaging cells were loaded with Fura Red AM in DMEM without phenol red for 30 min at 30°C and then 30 min at 37°C. Cells were then imaged in DMEM without phenol red and with treated with 1 μM ionomycin. GFP construct (green), Fura Red (red). Graphs of Fura Red fluorescence over time for each experiment are shown below images. Time pre and post treatment noted. (Scale Bars 10μm).

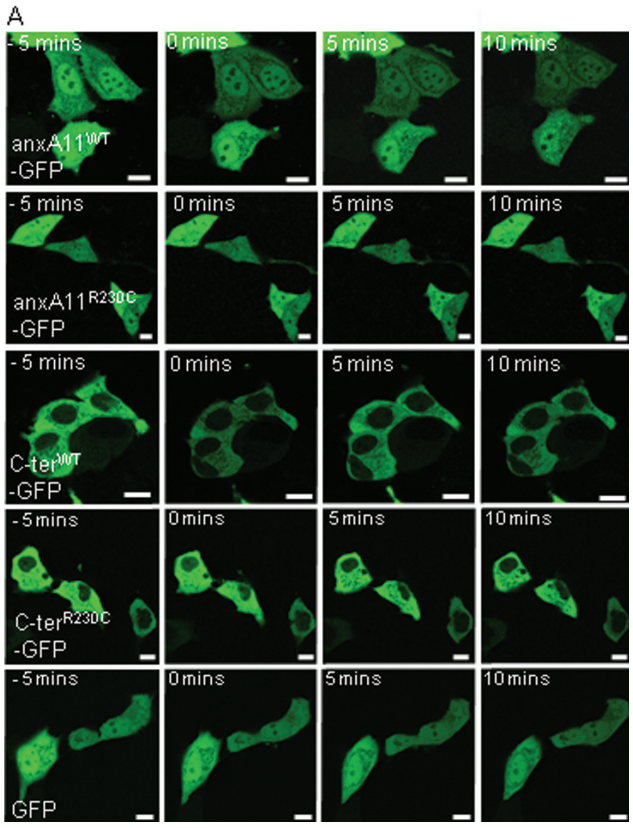

Figure 4. Wildtype and annexin A11^{R230C} do not relocalise in reponse to EGF treatment. **A.** Real time imaging of anx A11^{WT}-GFP, anx A11^{R230C}-GFP, anx A11^{WT}-Ct-GFP, anx A11^{R230C}Ct-GFP and GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Cells were imaged in DMEM without phenol red and with treated with 100 ng/ml of EGF (human epidermal growth factor). Time pre and post treatment noted. (Scale Bars 10 μm). **B.** Following imaging of the transfected cells, the cells were lysed in boiling SDS-PAGE buffer and subjected to SDS-PAGE and Western blotting. EGF receptor was blotted for using a polyclonal sheep anti-EGFR antibody (1:2000 dilution) and phosphorylated proteins using a monoclonal mouse anti phosphotyrosine antibody (1:1000 dilution). A band equivalent in size to the phosphorylated EGF receptor is shown in the phosphotyrosine blot (arrow).

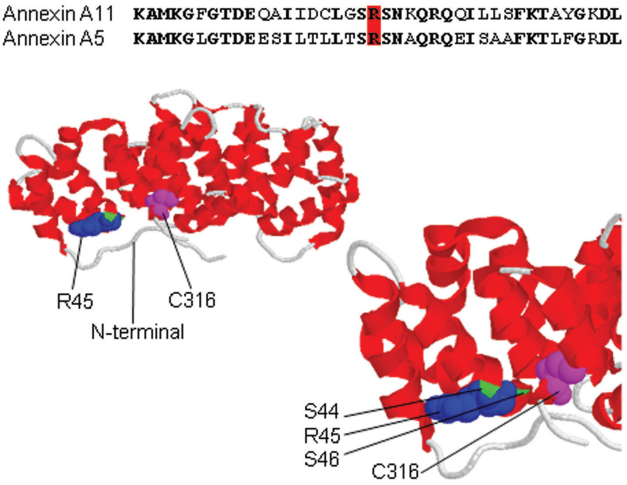

Figure 5. Structural implications of the R230C SNP in annexin A11. The crystal structure of annexin A5 was modelled in RasMol (Version 2.6). The arginine which is mutated in annexin A11^{R230C} is conserved in annexin A5 and is depicted in the crystal structure in blue. Residues either side of the arginine are shown in green. Cysteine residues which are conserved between annexin A11 and annexin A5 are shown in magenta. The crystal structure of annexin A5 highlights conserved residues S44, R45, S46 and C316. The aligned amino acid sequences of annexin A5 and annexin A11 show the conserved residues (bold), including the mutation-associated arginine (red box).

wild type proteins remained predominantly excluded from the nucleus and diffuse within the cytoplasm and showed no signs of enrichment at membranes (Fig. 3). GFP, used here as a control, also did not show any re-localisation upon ionomycin treatment. Thus, the R230C does not confer any increase in Ca^{2+}-sensitivity in the absence of the N-terminus.

The Response of Anx A11^{WT}-GFP and Anx A11^{R230C}-GFP to EGF

Ionomycin is a non-physiological agonist and is known to elicit rapid, large and sustained increases in calcium. We therefore examined the responses of the two annexin A11 variants to epidermal growth factor (EGF), which elevates intracellular calcium through activation of the EGF receptor and canonical $InsP_3$-mediated Ca^{2+}-signaling. The A431 cell line is particularly responsive to EGF as it expresses high levels of this receptor on its plasma membrane (Ullrich et al. 1984). The addition of 100ng/ml of human EGF to A431 cells transfected with anx A11^{WT}-GFP, anx A11^{R230C}-GFP, the corresponding C-terminal truncation mutants and GFP alone, failed to elicit any change in the subcellular localisation of any of these proteins during the 10 minute period imaged post-stimulation (Fig. 4A). Anx A11^{WT}-GFP and anx A11^{R230C}-GFP remained diffuse within the cytoplasm and nucleoplasm. The variants lacking N-terminal domains remained excluded from the nucleus and diffuse throughout the cytoplasm. None of the GFP tagged annexin A11 proteins or GFP alone showed membranous accumulations upon EGF treatment.

In order to verify that the cells had indeed responded appropriately to EGF, immediately following completion of the live imaging time course the cells were lysed and samples prepared for western blotting. Control samples of transfected, imaged, but unstimulated A431 cells were also prepared. Western blotting confirmed that the EGF receptor is expressed by these cells, and a phosphotyrosine blot revealed an increase in the strength of a protein band at 170kDa in stimulated cells but not in unstimulated cells (Fig. 4B). This band size corresponds to the phosphorylated EGF receptor, which upon binding EGF autophosphorylates. Therefore the stimulated A431 cells were responsive to EGF, though no re-localisation was observed of the GFP tagged annexin A11 proteins expressed.

Discussion

A recent genome-wide search for previously unknown genetic mutations associated with sarcoidosis, identified a single nucleotide polymorphism in annexin A11 as the most highly associated novel susceptibility locus for this disorder (Hofmann et al. 2008). Until this study the association of annexin A11 with autoimmune diseases was limited to the detection of autoantibodies in patients with a range of conditions including Raynaud's disease, rheumatoid arthritis and systemic lupus erythrematosos (Misaki et al. 1994). Annexin A11, like several other annexins, is widely expressed and it is therefore unsurprising that it, along with other annexins (Hayes et al. 2007; Rodriguez-Garcia et al. 1996; Salle et al. 2008) has been detected in sera from patients with autoimmune diseases. Therefore the finding of a mutation in annexin A11 that could potentially predispose an individual to developing sarcoidosis adds significant weight to the notion that annexin A11 has a causative role in autoimmune disorders.

We sought to further investigate this mutation (R230C) in A431 cells, which endogenously express the wild type variant of annexin A11 (R230). This was performed through the over-expression of wild type and sarcoidosis-associated variants of annexin A11 tagged to GFP. As the mutation is located in the C-terminal domain of the protein, both the full length and C-terminal domain alone tagged to GFP were investigated. No difference was seen in the localisation of GFP tagged wildtype annexin A11 or annexin $A11^{R230C}$ in resting A431 cells, suggesting that under unstimulated conditions annexin $A11^{R230C}$ is trafficked to the same domains as that of its wild type counterpart. This led us to investigate the effect of calcium on wild type annexin A11 and annexin $A11^{R230C}$.

In order to raise intracellular calcium levels the ionophore ionomycin was utilized, resulting in large, irreversible rises in intracellular calcium levels. This induced the re-localisation of GFP tagged annexin A11 first to the plasma membrane and then the nuclear envelope, consistent with our previous observations (Tomas and Moss 2003). The lag between these two events is most likely due to the strong calcium buffering capability of the nucleus (Badminton et al. 1998). This effect has also been observed in cells over-expressing GFP tagged annexin A7, which is the most closely related vertebrate annexin to annexin A11 (Clemen et al. 2003). The redistribution of endogenously expressed annexins in response to ionomycin has been investigated for several of the annexins, which show a variety of responses. Annexins A4 and A5 also re-localise to the nuclear envelope, whereas annexin A2 which is less similar to annexin A11 localises to granular structures (Barwise and Walker 1996). Therefore although all the annexins are capable of binding lipid membranes in response to rises in intracellular calcium, targeting to specific domains varies between different annexins.

In cells over-expressing GFP tagged annexin $A11^{R230C}$ the same effect was observed in response to ionomycin and with the same kinetics. A subtle difference in the time taken to re-localise to these membranous compartments between these two variants cannot be entirely ruled out, without analysing many more cells. It should be noted that the GFP tagged C-terminal domain constructs showed no significant re-localisation upon ionomycin treatment for either wildtype annexin or annexin $A11^{R230C}$. This domain contains the Ca^{2+}-binding sites of annexin A11, suggesting that although Ca^{2+} is detected within the annexin A11 core, it is incapable of sustained re-localisation to the plasma membrane or nuclear envelope in the absence of the N-terminal domain.

We also investigated the responses of these constructs to EGF but in all four cases no re-localisation of the fluorescently tagged proteins was observed, although the cells were shown to be responsive to EGF from Western blots against phosphorylated proteins. It may therefore be the case that the rise in intracellular calcium induced by EGF is too transient and/or too small to stimulate the membrane translocation of annexin A11. EGF stimulation of A431 cells is known to increase calcium levels to between 400nM and 700nM, whereas ionomycin raises levels into the low micromolar range. Furthermore, calcium levels are sustained upon treatment with ionomycin, whereas the rise in EGF-stimulated Ca^{2+} levels has been shown to return to basal levels after 30s to one minute (Moolenaar et al. 1986). The need for sustained increases in calcium for annexin relocalisation has been shown to be a requirement for endogenously expressed annexin A4, which relocalises in response to ionomycin but not to transient increases in calcium induced by weaker agonists such as bradykinin (Raynal et al. 1996).

Taken together, the failure of EGF to elicit responses from annexin A11, and the long delay in response time to ionomycin, suggest that annexin A11 is generally rather

unresponsive to Ca^{2+}, as has been reported in other studies (Lecona et al. 2003). The delay in the response to ionomycin suggests that other Ca^{2+}-dependent cellular events need to occur prior to annexin A11 translocation, such as tyrosine phosphorylation of annexin A11, which we showed is stimulated by ionomycin (Tomas and Moss 2003), or binding to an accessory protein such as ALG-2 (Satoh et al. 2002). It would be of interest in future studies to investigate other physiological agonists capable of raising intracellular calcium levels to different concentrations and different durations. Ideally, this would involve the use of different cell lines, including those of the immune system, which may show differences in response to calcium, as is the case for annexin A6 which relocalises to different compartments in fibroblasts and T cells (Barwise and Walker 1996; Podszywalow-Bartnicka et al. 2007). It would be of particular interest to investigate annexin A11 in cell types known to be directly involved in sarcoid granuloma formation such as macrophages and monocytes which form part of the granuloma core (Baughman 2006). T cells are also highly involved in sarcoidosis and it would therefore be important to investigate annexin A11 in CD4 positive T cells which are increased in sarcoid lungs (Grunewald and Eklund 2007) as well as CD8 and CD19 positive T cells, which have been shown to exhibit a striking decrease in expression of annexin A11 dependent upon activation (Hofmann et al. 2008) - over-expression in these activated T cells of wild type annexin A11 or annexin $A11^{R230C}$ may uncover differences in their function.

Although no functional differences between wildtype annexin A11 and annexin $A11^{R230C}$, were detected in this study, in terms of sensitivity to Ca^{2+}, structural analysis suggests that the R230C mutation may exert a functional effect. The tertiary structures of several of the annexins have been crystallized, though not that of annexin A11. Annexin A5 however has been crystallized and shows the highest degree of similarity with annexin A11 (of all the crystallized annexins) with 53% sequence identity and 73% sequence similarity, including conservation of the arginine at residue 230 (Fig. 5). Structural analysis shows that this residue is in close proximity to the N-terminal domain and may therefore interact with it (Fig. 5). A change from a basic arginine to a polar cysteine could alter these interactions and therefore the functionality of the protein.

Furthermore, annexin A11 contains six cysteine residues, one of which is conserved in annexin A5 (C316) (Fig. 5). This could be of importance in the context of disulphide bridge formation. Structural analysis shows that residue 230 lies near the start of an alpha helix (Fig. 5) and lies opposite the conserved cysteine residue (C316). In the wild type protein the arginine at residue 230 points away from the cysteine. Upon mutation of this arginine to a cysteine it is possible that a new conformation could arise in which the two cysteine residues are facing each other, facilitating the formation of a disulphide bridge that would alter the conformation of the protein. Disulphide bridge formation would however only be likely to occur in the extracellular environment. Interestingly, annexin A11 has been shown to be secreted by activated neutrophils (Boussac and Garin 2000), and autoantibodies against annexin A11 have been detected in several autoimmune diseases (Mizutani et al. 1995).

The conformational changes postulated here are hypothetical and would require crystallization studies to be substantiated. If structural changes do occur in annexin $A11^{R230C}$, it is not clear whether these would be sufficient to result in functional effects. It is possible that although the mutation identified in annexin A11 is strongly associated with sarcoidosis, susceptibility to the disease and cellular dysfunction requires the complete haplotype to be present. This includes five other single nucleotide polymorphisms surrounding the annexin A11 R230C mutation, which were also validated as strongly associated with sarcoidosis (Hofmann et al. 2008). Alternatively, the mutation in annexin A11 alone may be sufficient, and testing these hypotheses will be the focus of future work.

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Review

Unique S100 target protein interactions

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Abstract. Three-dimensional structures of S100B, S100A1, S100A6 and S100A11 have shown that calcium binding to these proteins results in a conformational change allowing them to interact with many biological targets. The structures of some S100 proteins in the presence of peptide targets from Ndr kinase, p53, CapZ, annexins A1 and A2 and the Siah-1 Interacting Protein indicate there are at least three modes of recognition that utilize two distinct surfaces in the S100 proteins. These surfaces have been hypothesized to simultaneously accommodate multiple binding partners. This review focuses on potential multiprotein complexes involving calcium-insensitive S100A10, annexin A2 and several other proteins including AHNAK, dysferlin, NS3, TASK-1 and TRPV5/6.

Keywords: Annexin — Multi-protein complex — Calcium-signaling — Membrane repair — Three-dimensional structure

Introduction

The S100 proteins are a group of proteins comprising at least 25 members in humans including S100B, S100A1, S100A6, S100A10 and S100A11 (Donato 2001; Heizmann et al. 2002). The proteins are dimeric having two "EF-hand" calcium-binding motifs in each subunit. *In vivo* experiments have shown that both homo- and heterodimeric S100 complexes are formed (Deloulme et al. 2000; Propper et al. 1999; Wang et al. 2005). The functions of the S100 proteins are to act as calcium-signaling molecules by converting changes in cellular calcium levels to a variety of biological responses. In this manner, many of the S100 proteins have been shown to modulate enzyme activities, oligomerization of cytoskeletal protein components (tubulin, desmin, glial fibrillary acidic protein), modulate ubiquitination, control membrane vesicle formation and participate in trafficking of proteins to the inner surface of the plasma membrane (Santamaria-Kisiel et al. 2006).

Most S100 proteins bind calcium and undergo a conformational change allowing them to interact with specific target proteins and control a cellular activity (reviewed in Santamaria-Kisiel et al. 2006; Wilder et al. 2006; Zimmer et al. 2003). In general, it has been shown that calcium binding to the first EF-hand (helix I, loop, helix II) is weaker than binding to the second EF-hand, comprised of helices III and IV. Three-dimensional structures of several S100 proteins in the calcium-free (apo) and calcium-bound states show that the major structural change involves the movement of helix III to expose previously buried residues which create a hydrophobic surface. In one S100 protein, S100A10, substitutions in both its calcium-binding sites have left this protein with the inability to coordinate calcium. Consequently, S100A10 does not undergo a calcium-induced structural change and instead adopts a structure in its calcium-free state that is very similar to the calcium-bound states of other S100 proteins (Rety et al. 1999; Rety et al. 2000). As a result S100A10 has been observed to interact and control the functions of more than a dozen proteins in a calcium-insensitive manner.

Frequent partners for the S100 proteins are members of the annexin protein family. At least 10 different S100-annexin complexes have been characterized (summarized in Santamaria-Kisiel et al. 2006). The annexins are a group of highly helical proteins having twelve members in humans. Each annexin protein has a core domain comprised of four (annexins A1-A5, A7-A11, A13) or eight (annexin A6) structurally conserved repeats, each possessing five α-helices. Although these proteins bind calcium, a large conformational change analogous to the S100 proteins does not occur. Instead, calcium binding to the annexins has been shown to promote association with phospholipid membranes. In particular, Gerke and Moss (2002) have proposed an elegant hypothesis whereby S100A10 and/or S100A11 can coordi-

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nate pairs of annexin A1 or A2 proteins allowing them to bridge two membrane surfaces. The mechanism is mediated by a high local calcium concentration that promotes the annexin-membrane interaction and/or conformational change in S100A11. This process promotes membrane fusion required for enlargeosome vesicle formation, a requirement in models for the maintenance of membrane lesions, or for vesiculation processes used in endo/exocytosis. In the last few years, several membrane-spanning proteins (TASK-1, dysferlin, NS3) have been found to interact with S100A10 together with annexin A2 as a possible means for trafficking of these proteins to the plasma membrane or assemble multiprotein complexes important in membrane repair processes. In this review we compare the three-dimensional structures of several S100-target protein complexes and use this information to rationalize some newly identified multiprotein S100-annexin complexes.

Different modes of recognition for S100 target protein complexes

The three-dimensional structures of several S100 proteins have been determined in complex with a variety of peptides derived from their parent proteins. These include calcium-bound S100B (Ca^{2+}-S100B) in complex with peptides from the C-terminal region of p53 (Rustandi et al. 2000), the N-terminal regulatory domain from Ndr kinase (Bhattacharya et al. 2003) and the actin-capping protein CapZ (TRTK12) (Inman et al. 2002; McClintock and Shaw 2003); calcium-bound S100A1 (Ca^{2+}-S100A1) in complex with peptides from CapZ (Wright et al. 2009) and the cytosolic region of the ryanodine receptor (RyR) (Wright et al. 2008); calcium-bound S100A6 (Ca^{2+}-S100A6) in complex with a C-terminal region from the Siah-1 Interacting Protein (SIP) (Lee et al. 2008) and, Ca^{2+}-S100A11 in complex with the N-terminus of the phospholipid-binding protein annexin A1 (Rety et al. 2000). In addition, the structure of S100A10, an S100 protein unable to bind calcium due to substitutions in both calcium-binding loops, is available in complex with the N-terminal region from annexin A2 (Rety et al. 1999). In general these structures show there are at least three distinct modes a target protein adopts when binding to an S100 protein (Fig. 1), all showing a symmetric relationship and 1:1 stoichiometry for the target:S100 protomer.

The first mode of binding involves the interaction between the target protein and the hydrophobic surface exposed between helices III and IV due to calcium binding to the S100 protein. In this case, all interactions between the target and the S100 protein occur at the S100 protomer level. This type of interaction is exhibited in the Ca^{2+}-S100B structures with TRTK12 (Protein Data Bank accession codes - 1MWN, 1MQ1), Ndr kinase (1PSB, Fig. 1A) and p53 (1DT7), and the Ca^{2+}-S100A1 structures with TRTK12 (2KBM, Fig. 1A) and the ryanodine receptor (2KF2). In the p53 (Rustandi et al. 2000) and Ndr kinase (Bhattacharya et al. 2003) structures, each bound peptide forms a three-turn α-helix such that its N-terminus lies near the N-terminus of helix III and its C-terminus has key interactions with the C-terminus of helix IV of Ca^{2+}-S100B. In contrast, the TRTK12 structures with Ca^{2+}-S100A1 (Wright et al. 2009) and Ca^{2+}-S100B (Inman et al. 2002) form 1.5 turn α-helices and are aligned nearly perpendicular to the orientations of the p53 and Ndr peptides such that the C-terminus of TRTK12 is closer to the middle of helix IV (Fig. 1A). Even though the TRTK12 peptide binds within the helix III-IV cleft in both structures the presentation of the peptide is different when bound to Ca^{2+}-S100A1 compared to Ca^{2+}-S100B. In Ca^{2+}-S100A1 the anchoring tryptophan (W7) of TRTK12 interacts with residues towards the C-terminus of helix IV (L81, A84, C85, F88) whereas in Ca^{2+}-S100B, this same tryptophan interacts with residues in the linker (I47) and helix III (V53, V56). These differences have the affect of rotating the entire TRTK12 peptide by about 60° and "tipping" the peptide by about 20° towards helix III in the Ca^{2+}-S100B structure. Pictorially the binding of a peptide representing the cytosolic region from the ryanodine receptor to Ca^{2+}-S100A1 (Wright et al. 2008) appears to be quite similar to that described for TRTK12. However, major differences exist as the RyR peptide forms a three-turn α-helix and is oriented nearly 180° with respect to TRTK12 such that its C-terminus is proximal to the N-terminus of helix III in Ca^{2+}-S100A1. An anchoring tryptophan (W5) in the RyR peptide interacts with residues near the middle of helix IV (L77, A80, L81) but also with I57 in helix III. The diversity of the interactions exhibited by these five S100-target peptide structures can be attributed to the binding surfaces in S100A1 and S100B that are broader and flatter than other calcium-sensor proteins (i.e. calmodulin) and have different distributions of hydrophobic and charged residues (Bhattacharya et al. 2004; Bhattacharya et al. 2003). This observation likely accounts for the broad spectrum of target proteins that interact with the S100 proteins and makes prediction of target binding based on protein sequence somewhat problematical.

The second mode of interaction utilized by the S100 proteins is displayed by structures of Ca^{2+}-S100A11 (1QLS, Fig. 1B) and S100A10 (1BT6) with N-terminal peptides from annexins A1 and A2 respectively (Rety et al. 1999; Rety et al. 2000). Unlike the diversity displayed by Ca^{2+}-S100A1 and Ca^{2+}-S100B, these structures show nearly identical locations and orientations for peptide binding such that < 1 Å rmsd exists between structures. In both structures the annexin peptide bridges the two S100 protomers such that its C-terminus interacts with the linker and helix IV of one protomer while the annexin N-terminus has contacts near the N-terminus of helix I' of the partner protomer. The

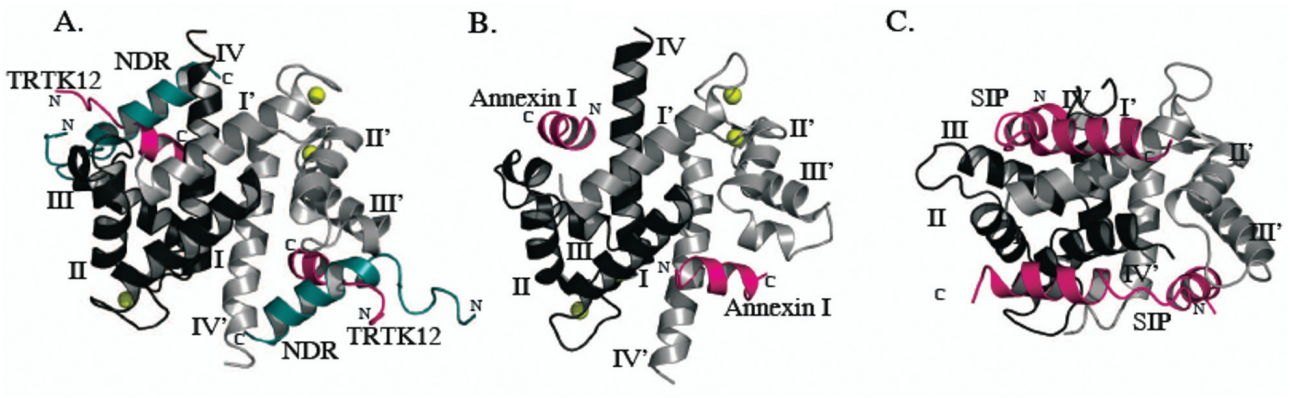

Figure 1. Different modes for target protein interaction with S100 proteins. (A) Binding to the helix III-IV hydrophobic displayed by TRTK12 (pink) bound to Ca^{2+}-S100A1 (2KBM) and NDR kinase (cyan) bound to Ca^{2+}-S100B (1PSB). (B) Binding near helix I' at the dimer interface displayed by the N-terminal region of annexin A1 (pink) and Ca^{2+}-S100A11 (1QLS). (C) Two- site surface mode displayed by the C-terminal region of SIP bound to Ca^{2+}-S100A6 (2JTT). Ribbon diagrams of the calcium-saturated S100 proteins are presented with one of the protomers shaded in black (helices labeled as I-IV) and the other protomer shown in light grey (helices labeled as I'-IV'). Calcium ions are illustrated in yellow spheres.

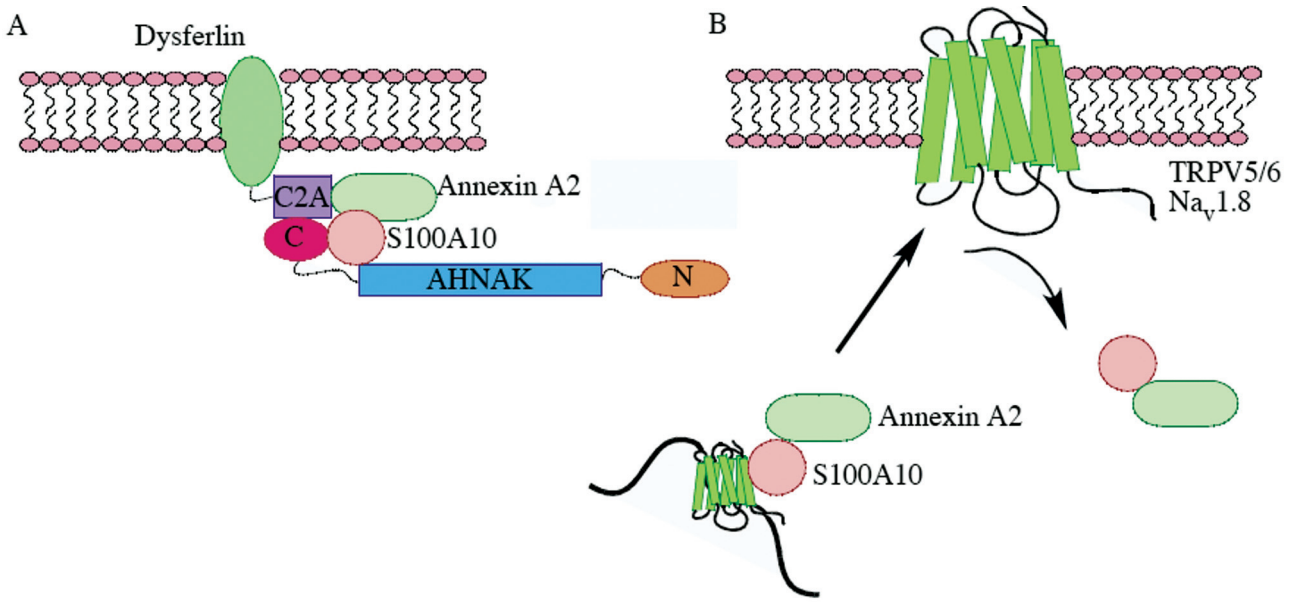

Figure 2. Possible involvement of S100A10 in multiprotein complexes. (A) Proposed interactions in the dysferlin complex based on multiple biochemical experiments. The C-terminus of AHNAK is shown to interact with S100A10 and annexin A2 and the dysferlin C2A domain is near annexin A2 (adapted from Huang et al. 2007). (B) Possible complexes formed by membrane channel proteins such as TRPV5, TRPV6 or Nav1.8 and S100A10-annexin A2 for trafficking to the plasma membrane. Once inserted into the membrane, the S100A10-annexin A2 could dissociate or become associated with phospholipids in the membrane. In all cases, dimeric S100A10 could coordinate two annexin A2 proteins although for simplicity only one is shown.

similarity of these S100-annexin structures is not surprising given the conservation of hydrophobic residues in both annexin peptides. The interacting residues correspond to V3, F6, L7 and L10 in annexin A1 and V3, I6, L7 and L10 in annexin A2 forming a XOOXXOOX interaction motif (X = hydrophobic residue, O = variable). This level of conservation does not exist between the TRTK12, RyR, p53 and Ndr sequences described earlier in complex with Ca^{2+}-S100A1 or Ca^{2+}-S100B proteins. Some similarity also exists in the annexin contacting residues in S100A10 and Ca^{2+}-S100A11,

especially in helix I' and helix IV. Although it has been shown that N-terminal peptides from annexins A1 and A2 both interact with Ca^{2+}-S100A11 (Rintala-Dempsey et al. 2006), it is interesting to note that annexin A1 does not form a tight complex with S100A10 (Streicher et al. 2009). Further, a strong level of specificity has been shown for S100 recognition of annexins A1 and A2. Other than S100A10 and Ca^{2+}-S100A11, the only other S100 protein that displays a tight calcium-dependent interaction with annexin A1 is S100A6 (Streicher et al. 2009).

Whereas both the previous modes of target peptide binding with S100 proteins utilized different albeit contiguous surfaces, the recent structure (Lee et al. 2008) of the C-terminal domain from the Siah-1 Interacting Protein (2JTT) shows a hybrid two-site surface (Fig. 1C). In this structure the SIP peptide forms two distinct three-turn α-helices oriented nearly perpendicular to each other upon binding to Ca^{2+}-S100A6. The first helix (Helix A) occupies a position and orientation similar to that observed for the RyR peptide utilizing the hydrophobic groove between helices III and IV. Helix A of SIP lies diagonally across helix III in Ca^{2+}-S100A6 with L196 and I199 anchoring the peptide through interactions with residues in helix IV and the C-terminus of helix III respectively. The second helix in SIP (helix B) lies across helix I' of the adjacent protomer, in effect bridging the two S100A6 subunits analogous to the S100-annexin complexes. Whereas the annexin structures only contact the N-terminus of helix I' in either S100A10 or Ca^{2+}-S100A11, helix B in SIP has interactions towards the more central portion of helix I' (I9', I13' and K18'). This results in helix B of SIP and helix I of Ca^{2+}-S100A6 running along opposite faces of helix I' forming a near mirror image of each other, albeit helices I and B are oriented in opposite directions. The two binding regions for SIP to Ca^{2+}-S100A6 could in part be due to the length of the SIP fragment (189-219; 30 residues) that is significantly longer than any of the other target peptides for which structures are available. Lee and co-workers show that the two helices in SIP are not equivalent in their abilities to coordinate S100A6 with helix A having a much tighter binding, while the less hydrophobic helix B enhances the affinity by about 4-fold.

The three modes of target binding to the S100 proteins utilize two distinct binding regions – the helix III-IV hydrophobic groove and the dimer interface near helix I' on the partner protomer. It has been suggested that the combination of these two regions might represent the full interaction surface for single or multiple protein interactions with the S100 proteins. Otterbein proposed this idea by superimposing the peptide binding regions for annexins A1 or A2, and p53 on the surface of Ca^{2+}-S100A6 showing these peptides occupied distinct regions of the surface with no observable steric clashes between the peptides (Otterbein et al. 2002). Subsequently, it was shown for TRTK12 binding to Ca^{2+}-S100B that a region similar to the annexin binding site was left exposed, and that a larger target protein might be accommodated (McClintock and Shaw 2003). The structure of SIP bound to Ca^{2+}-S100A6 (~1100 Å) provides the first structural evidence for a target protein that occupies both the helix III-IV groove and helix I' interface, a binding surface that is nearly twice the size of other S100 target protein surfaces (500-700 Å). Amongst the questions raised by this important structure and earlier hypotheses are – is there evidence for other S100 protein complexes that might utilize this larger target protein binding surface or can this surface possibly accommodate simultaneous binding from two distinct target proteins?

Evidence for multiprotein S100 complexes involving annexin proteins

A variety of S100 protein interactions have been shown to be more complicated than a single target interacting per S100 protomer molecule. For example multiprotein complexes between S100A10 and annexin A2 and either AHNAK (Benaud et al. 2004; De Seranno et al. 2006), TRVP5/6 (Borthwick et al. 2008; van de Graaf et al. 2003) or plasminogen (MacLeod et al. 2003) have been identified from two- and three-hybrid or direct binding experiments. The S100A10-annexin A2 complex is thought to be necessary to recruit an additional target, or allow both targets to bind simultaneously indicating that the binding sites for AHNAK, TRVP5/6 and plasminogen on S100A10 are likely distinct from those of annexin A2. The complexes described here focus on those for S100A10, largely because it functions well in yeast two-hybrid experiments. Other S100 proteins (S100B, S100A1, S100A6) do not respond well in this method due to the calcium-sensitivity of their protein interactions, although homo- and heterodimeric S100 complexes can be identified using two-hybrid experiments (Deloulme et al. 2000; Deloulme et al. 2003).

The interaction between the transient receptor potential cation channel proteins TRPV5 and TRPV6 with S100A10 has been shown using two-hybrid and co-immunoprecipitation experiments (Borthwick et al. 2008; van de Graaf et al. 2003). In this role, the S100A10-annexin A2 complex is thought mediate trafficking of the TRV5 and TRV6 proteins to the plasma membrane where they act as calcium-selective channels. The site of interaction on TRPV5 and TRPV6 for S100A10 has been localized to its intracellular C-terminal tail. Using a series of truncated and substituted proteins this has been refined to contain residues 598-603 (VATTV), a highly conserved region in both channel proteins. Although S100A10 forms a ternary complex with annexin A2 and either TRPV5 or TRPV6, there does not appear to be a direct interaction between the annexin and TRPV

proteins based on negative results from immunoprecipitation experiments. Rather, S100A10 seems to bridge the two proteins indicative of different binding sites for annexin A2 and TRPV5/TRPV6, although this has not been demonstrated yet. Since S100A10 and annexin A2 co-localize with TRPV5/TRPV6 the S100A10-annexin A2 complex has been proposed to traffic these calcium channels to the plasma membrane.

S100A10 has been shown to interact with the potassium channel TASK-1 using two-hybrid, GST pull-down and co-immunoprecipitation experiments and have a role in the trafficking of TASK-1 to the plasma membrane (Girard et al. 2002; Renigunta et al. 2006). TASK-1 is an important regulator of membrane potential that can be affected by a variety of factors including pH, hormone and neurotransmitter binding. Two studies show binding of S100A10 to the C-terminus of TASK-1 albeit to different regions of the channel protein. In one study, S100A10 was observed to interact with the extreme C-terminal sequence (SSV) of TASK-1 previously established as a site for 14-3-3 binding (Girard et al. 2002). In more recent work, the S100A10 site was localized to a more central region in the C-terminus (residues 292-331) of TASK-1 (Renigunta et al. 2006). A portion of this sequence is highly conserved in TASK-1 orthologs, but not in TASK-3, which exhibits little binding to S100A10. Further, a segment of this sequence (FRNVYAEML) bears a strong similarity to the interaction motif (XOOXXOOX) used by annexins A1 and A2 upon binding to S100A11 and S100A10, respectively (Mailliard et al. 1996; Rintala-Dempsey et al. 2008). An interaction between S100A10 and annexin A2 in the presence of TASK-1 can not be shown suggesting the S100A10 binding regions for TASK-1 and annexin A2 at least partly overlap. It has further been shown that the S100A10 interaction promotes retention of TASK-1 in the endoplasmic reticulum due to the presence of a retention signal at the extreme C-terminus of S100A10 (KQKGKK) (Renigunta et al. 2006). Alternatively, binding of S100A10 to TASK-1 has been suggested to mask an endoplasmic retention signal near the C-terminus of TASK-1 (KRR) and promote trafficking of the potassium channel to the plasma membrane (Girard et al. 2002).

S100A10 interacts with the bluetongue virus protein NS3 (Beaton et al. 2002), a membrane spanning protein thought to have a role in the export of virus particles from infected cells. Two-hybrid and affinity experiments show that residues at the N-terminus of NS3 (1-13) are most important for its interaction with S100A10. This region has the potential for α-helix formation and possesses a sequence (LSGLIQRF) corresponding to the annexin binding motif (XOOXXOOX) suggesting these two proteins likely share the same binding region on S100A10 (Fig. 1B). Using synthetic peptides corresponding to the N-terminus of NS3, it was shown that a peptide comprising residues 1-14 could compete with annexin A2 for S100A10 binding. Whereas annexin A2 could displace NS3 from an S100A10-NS3 complex, NS3 could only partially displace annexin A2 from an S100A10-annexin A2 complex indicating annexin A2 has a tighter binding to S100A10 than does NS3. It was suggested that NS3 might play a role in localizing a virus particle to the interior of the plasma membrane by bridging S100A10 and the virus particle providing an efficient route for extrusion from the cell.

The voltage-gated sodium channel $Na_V1.8$ has been observed, using two-hybrid experiments, to interact with S100A10 (Okuse et al. 2002; Poon et al. 2004). Further, this interaction facilitates trafficking of the channel protein to the plasma membrane resulting in functional Na+ currents. Other voltage-gated channels such as $Na_V1.2$, $Na_V1.5$, $Na_V1.7$ and $Na_V1.9$ have much poorer affinity for S100A10 providing evidence that some degree of specificity for the voltage-gated sodium channel $Na_V1.8$ exists. The site of interaction with S100A10 has been localized to the N-terminus of the sodium channel. In particular residues 74-103 of the voltage-gated sodium channel $Na_V1.8$ are sufficient to bind to S100A10 in GST pull-down assays. Reciprocal experiments using segments of S100A10 show that a region spanning much of the C-terminal half of the protein is able to interact with the N-terminal region of the voltage-gated sodium channel. This region does not include the extreme N- or C-termini of S100A10, known to interact with the annexin proteins so would appear to be a unique binding surface. However, it has not been shown whether annexin A2 can also bind to S100A10 in the presence of the sodium channel, an event that could facilitate association with the plasma membrane.

A multiprotein complex has been identified between S100A10, annexin A2 and AHNAK (Benaud et al. 2004; De Seranno et al. 2006), a protein found in the lumen of enlargeosome and trafficked to the plasma membrane in response to calcium flux. AHNAK is an important protein for cell membrane differentiation and membrane repair (Kouno et al. 2004), is expressed in epithelial cells and localizes near the plasma membrane. The protein contains three main structural domains; an amino terminus (251 aa), a central region containing twenty-six 128-residue repeats and a C-terminus (1002 aa) (Shtivelman and Bishop 1993). The interaction between S100A10 and annexin A2 utilizes the N-terminus of annexin A2 similar to that observed in the S100A10-annexin A2 tetramer crystal structure (Rety et al. 1999). In the absence of annexin A2, a weak interaction between S100A10 and the C-terminal portion of AHNAK is present. However, the strength of this interaction was increased more than 150-fold in the presence of annexin A2. Further, *in vitro* binding assays showed no detectable interactions between annexin A2 and the C-terminus of AHNAK, in the absence of S100A10, or S100A10 with AHNAK in the absence of annexin A2.

These observations suggest that S100A10-annexin A2 tetramer formation is likely a prerequisite for the interaction with AHNAK. Using a series of GST pull-down assays, a 19-residue region in AHNAK corresponding to residues 5654-5673 was found to be sufficient to bind to S100A10-annexin A2 and can compete for binding to the complex with the entire AHNAK C-terminus having a Kd of about 30 nM (De Seranno et al. 2006). The AHNAK interaction appears to be specific for annexin A2 since other annexins (A4, A11) are not recovered from immunoprecipitation experiments. However, given the similarity of the binding surfaces and interacting residues for annexins A1 and A2 (Rintala-Dempsey et al. 2008), it is possible that an S100A10-annexin A1 or Ca^{2+}-S100A11-annexin A1/A2 may also participate in the AHNAK complex. Interestingly, Ca^{2+}-S100B is able to compete with S100A10-annexin A2 for binding to the AHNAK C-terminus. Further, it has been observed that Zn^{2+} binding to S100B enhances its association with AHNAK (Gentil et al. 2001). Unlike the S100A10-annexin A2 interaction that utilizes only the C-terminus of AHNAK, several regions in AHNAK are able to interact with Ca^{2+}-S100B. These include sequences in the repeat regions in the central portion of AHNAK (820-1330, 2589-3059, 3730-4188) as well as its C-terminus.

S100A10, AHNAK and the annexins have been shown to be constituent proteins in the dysferlin membrane repair complex (Huang et al. 2007). This multiprotein complex is thought to form at the site of muscle, epithelial and auditory cell damage to facilitate wound repair. The mechanism of cell membrane repair involves the aggregation of vesicles containing dysferlin, a type II membrane spanning protein, near the wound. A high calcium concentration (extracellular, via the wound) causes annexin A1 and A2 association with a dysferlin-containing vesicle and damaged plasma membranes. The central protein in this process dysferlin (Doherty and McNally 2003; Glover and Brown 2007; Han and Campbell 2007), has been shown to co-localize and co-immunoprecipitate with annexins A1 and A2 (Lennon et al. 2003). Recent three-hybrid and co-immunoprecipitation experiments have provided some details of the dysferlin repair complex. For example, S100A10 forms a ternary complex with both annexin A2 and AHNAK (Benaud et al. 2004; De Seranno et al. 2006; Huang et al. 2007) as described above. Using a series of truncated AHNAK constructs it has also been shown that the extreme C-terminal domain of AHNAK is responsible for dysferlin binding. Although dysferlin possesses six ~130-residue C2 domains, the AHNAK interaction is localized to only the N-terminal C2A domain of dysferlin, a region that typically coordinates calcium as a prerequisite for phospholipid binding. Calcium binding to the dysferlin C2A domain is not a requirement for its interaction with AHNAK. Although the exact mechanisms whereby AHNAK is recruited for plasma membrane repair are not clear, annexin A2 and S100A10 co-localize with AHNAK at the plasma membrane along with dysferlin providing some evidence this multiprotein complex functions in the repair process.

Conclusions

The multiple binding modes used by the S100 proteins to interact with a diverse array of target proteins suggests a great deal of flexibility in the manner an S100 protein recognizes a partner protein. This may prove useful to simultaneously coordinate more than a single target protein by some S100 proteins such as S100A10 that forms a tetrameric complex with annexin A2. Biochemical experiments suggest that S100A10-annexin A2 can further form multiprotein complexes with the cation channel proteins TRPV5 or TRPV6, the voltage-gated sodium channel $Na_V1.8$, or AHNAK and dysferlin. Other multiprotein complexes have been suggested between S100A10-annexin A2 and plasminogen, and Ca^{2+}-S100B with TRTK12 and annexin A6. Further, biochemical and structural experiments will be needed to ascertain the general role of S100 proteins in larger multiprotein complexes.

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Review

Calcineurin/NFAT signaling in lymphoid malignancies

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Abstract. Deregulated calcium signaling is observed at different stages of tumorigenic processes. An important signaling pathway activated in response to calcium involves the protein phosphatase calcineurin and NFAT transcriptional factors. We review here recent data that indicate an important role of the calcineurin/NFAT pathway in lymphoma/leukemogenesis and discuss the potential therapeutic implications of these findings.

Key words: Calcineurin — NFAT — Leukemia — Lymphoma

Introduction

Calcium signaling is used by all cell lineages during development, homeostatic control in adults and responses to a number of physiological and stress signals. Calcium signaling is involved in cell survival/apoptosis, cell cycle progression, differentiation, cross-talk between intracellular compartments (ER, mitochondria), general metabolism, telomerase activity and many others. Not unexpectedly therefore deregulated calcium signaling is observed at different stages of tumorigenic processes (for review see Roderick and Cook 2008), but the effectors of this altered calcium response remains to be fully characterized. We review here recent experimental evidence that support an important role for a signaling pathway involving the protein phosphatase calcineurin and NFAT factors in cell transformation and tumorigenesis.

The calcineurin/NFAT signaling pathway

Calcium (Ca^{2+})/Calcineurin/NFAT (Nuclear Factor of Activated T-cell) signaling was initially identified in mature T cells as an essential regulator of TCR-induced IL2 gene transcription (McCaffrey et al. 1992; McCaffrey et al. 1993; Shaw et al. 1988). Later studies showed that calcium/calcineurin signaling regulates the expression of a large array of genes including not only cytokines, but also genes encoding proteins involved in signal transduction, transcriptional regulation, survival/apoptosis and cell cycle control (Feske et al. 2001). This signaling pathway is by no means restricted to the immune system and plays major roles in a number of lineages (e.g. nervous system, heart and skeletal muscle, vascular system), thus regulating a wide variety of complex biological processes (Hogan et al. 2003; Wu et al. 2007). Calcineurin (PP2B; PPP3) (Klee et al. 1998) is a unique calcium-calmodulin-dependent serine/threonine protein phosphatase which is ubiquitously expressed. The calcineurin complex is composed of a catalytic subunit A (CnA) and a regulatory subunit B (CnB). In vertebrates, the catalytic subunit is encoded by three distinct genes (*CnAα/PPP3CA, CnAβ/PPP3CB and CnAγ/PPP3CC*). *CnAα* and *CnAβ* are ubiquitously expressed whereas *CnAγ* is specifically expressed in testis (Crabtree 1999; Klee et al. 1998; Rusnak and Mertz 2000). In addition to its catalytic domain (aa 70-328), CnA contains a regulatory domain including a CnB-binding domain (aa 333-390), a calmodulin-binding domain (aa 390-414) and a carboxy terminal autoinhinitory domain. Two different genes have been described in vertebrates encoding the CnB regulatory domain: the *CnB2/PPP3R2* gene is specifically expressed in testis whereas *CnB1/PPP3R1* encodes an ubiquitously expressed protein. Biochemical studies have shown that the heterodimerization of CnB and CnA is absolutely required for calcineurin activity and that both CnB and calmodulin participate in a cooperative fashion to calcineurin activation to narrow threshold of calcium ions in response to cell stimulation (Klee et al. 1998). In accordance, deletion of *CnB1* by homologous recombination results in suppression of calcineurin activity in somatic tissues and early embryonic lethality of *CnB*-deficient mouse embryos due to a failure to organize a normal vaculature (Graef et al. 2001). A major function of cal-

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modulin is to relieve the intramolecular inhibition of CnA catalytic domain by its carboxyterminal inhibitor domain in response to calcium. Thus, CnA proteolytic fragments or experimentally engineered mutants lacking the calmodulin binding and autoinhibitory domains show calcium-independent catalytic activity (Klee et al. 1998). Under physiological settings, engagement of cell surface receptors, such as antigen receptors of mature T and B cells, the Fc receptors in monocytes, natural killer cells and mast cells, a number of tyrosine kinase receptors and G protein-coupled receptors leads to the activation of phospholipase C (PLC-γ, PLCβ). Thus, activated PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to produce two second messengers, namely diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP3). Binding of InsP3 to the InsP3R calcium channel located on the endoplasmic reticulum (ER) membrane induces the release of calcium from internal stores. Store depletion triggers in turn the opening of store-operated calcium-release-activated calcium channels (CRAC, e.g. the ORAI/STIM complex, (Oh-hora and Rao 2008)) in the plasma membrane increasing levels of intracellular calcium ($Ca^{2+}{}_i$). Finally, influx of extracellular calcium leads to the calcium-calmodulin dependent activation of calcineurin and the dephosphorylation of its substrates, including NFAT transcription factors. Dephosphorylation of NFAT by activated calcineurin allows their translocation to the nucleus, where in cooperation with other transcriptional partners they regulate the expression of a number of genes (for review see Macian 2005). NFAT transcription factors form a family of 5 members: 4 calcium signaling responsive members including NFATc1 (also known as NFATc or NFAT2), NFATc2 (also known as NFATp or NFAT1), NFATc3 (also known as NFATx or NFAT4) , NFATc4 (also known as NFAT3). NFAT5, which is ubiquitously expressed and unresponsive to calcium/calcineurin activation, is activated by osmotic stress (Lopez-Rodriguez et al. 2001; Miyakawa et al. 1999). NFAT5 is the founding member of the NFAT family that emerged during evolution from the REL/NFκB gene superfamily of transcription factors since it is the only NFAT family member found to be expressed in invertebrates (Lopez-Rodriguez et al. 2001; Stroud et al. 2002). NFATc1, NFATc2 and NFATc3 are expressed in the lymphoid lineage where they are critically involved in the development, differentiation and function of multiple T-and B-cell subsets (Macian 2005). Each gene can be expressed as a number of alternatively spliced forms encoding different N-or C- terminal sequences and with distinct functions (Chuvpilo et al. 1999; Imamura et al. 1998; Luo et al. 1996; Park et al. 1996). NFATc1-c4 share a similar global modular structure, including N-terminal and C-terminal activation domains, a central Rel-homology (RHR) domain highly conserved among family members that mediates DNA binding, a moderately conserved regulatory domain (also known as the NFAT-homology region or NHR) containing 12-14 serine phosphorylation residues located in serine rich regions, a nuclear localization signal (NLS) and calcineurin docking sites centered over two critical motifs referred to as PxIxIT and LxVP respectively (Aramburu et al. 1998; Martinez-Martinez et al. 2006). In resting cells, NFATc1-c3 are located in the cytosol in a fully phosphorylated, inactive conformation that masks their NLS and inhibits their DNA binding activity. Calcium/calcineurin-induced activation leads to NFAT dephosphorylation, inducing a conformational switch that unmasks their NLS, allowing their nuclear translocation, DNA binding activity and interaction with co-regulators required for transcriptional activation (Okamura et al. 2000). To regulate transcription of their many target genes, NFAT proteins bind DNA either as monomer to specific A/TGGAA motifs, or as dimers to NFκB-like response elements, or in cooperation with other transcriptional partners activated by other signaling pathways (e.g. the AP1 complex in response to MAPK activation) on composite DNA binding sites (for review see Macian 2005). Regulation of NFAT factors activity through the regulation of their phosphorylation state has to be tightly regulated to ensure a controlled and balanced activation of the calcium/calcineurin signaling pathway (Muller et al. 2009). Several kinases are implicated in the maintenance of NFAT hyperphosphorylation in resting cells and in their nuclear re-phosphorylation after activation, including casein kinase 1, glycogen synthase kinase 3 (GSK3), JUN kinase 1 (JNK1) and DYRK protein kinases (for review see Arron et al. 2006; Gwack et al. 2006; Macian 2005). Although classic NFAT members are all phosphorylated in their NHR, several NFATs are subjected to other types of regulatory modifications including other phosphorylation events outside the regulatory domain, ubiquitination, sumoylation that modulate their activity, stability or subcellular localization (for review see Macian 2005; Nayak et al. 2009; Terui et al. 2004; Yoeli-Lerner et al. 2005). Although many mouse genetic studies have clearly demonstrated that NFATc1-c4 are critical effectors of calcineurin in several developmental processes (for review see Macian 2005), the best characterized function is their role in the immune response. Mouse genetic studies have revealed an intricate level of complexity between family members. For example in the lymphoid lineage, NFAT proteins can display either specific, redundant or antagonist functions (for review see Macian 2005). The calcineurin/NFAT signaling module is important at specific steps (preTCR signaling; TCR-mediated positive selection of antigen specific self MHC-restricted T cells) in thymocytes development as illustrated by the phenotype of the conditional deletion of mouse *CnB1* (Neilson et al. 2004) and of the single and combined knockout of *NFATc1-3* (Cante-Barrett et al. 2007; Hodge et al. 1996; Oukka et al. 1998; Peng et al. 2001; Ranger et al. 1998; Rengarajan et al. 2002; Yoshida

et al. 1998). Calcineurin/NFAT signaling plays a critical role in peripheral T cell activation following TCR engagement. Under these conditions, NFATc1 and NFATc2 play redundant roles in activating expression of several cytokine genes (Peng et al. 2001) while NFATc2 and NFATc3 function in a redundant way to negatively control peripheral T cells homeostasis (Ranger et al. 1998; Xanthoudakis et al. 1996). The crucial role of the calcineurin/NFAT signaling pathway in T cell activation downstream of TCR engagement responsible for the initiation of a productive immune response is highlighted by its sensitivity to calcineurin inhibitors such as FK506 (Tacrolimus) and Cyclosporin A (CsA). Both FK506 and CsA are extensively used in human medicine as immunosuppressive agents to improve allograft survival and to treat auto-immune diseases. These calcineurin inhibitors act through their binding to distinct intracellular receptors (Monticelli and Rao 2002) FKBP12 and cyclophilinA, respectively to inhibit access of substrates to calcineurin catalytic site (for review see Macian 2005). Although both FK506 and CsA inhibit other but distinct signaling pathways, their common inhibitory activity of calcineurin make them valuable pharmacological tools to study calcium/calcineurin/NFAT signaling pathway.

Oncogenic potential of the calcineurin/NFAT signaling pathway in hematologic disorders

Although critically involved in many aspects of normal T cells survival, proliferation and activation, the direct implication of calcineurin and/or its downstream NFAT targets in lymphomagenesis and cancer in general even if suspected for a long time has only been recently reported (Buchholz and Ellenrieder 2007; Medyouf and Ghysdael 2008). Since NFAT proteins are essential effectors of calcineurin in the control of a broad spectrum of genes in many different cell lineages which are critical for proliferation, growth, differentiation, migration and survival, processes commonly deregulated in cancer cells, it is easy to speculate an oncogenic potential for NFAT transcription factors. As some NFAT members are involved in the positive or negative regulation of cell cycle components, it is tempting to speculate that deregulation of their expression could play a role in cellular transformation. In line with this hypothesis, Clipstone and colleagues have shown that enforced expression of a constitutively nuclear and transcriptionally active NFATc1 mutant (caNFATc1, made by substitution of the serine residues of the NHR by alanine) in the 3T3L1 preadipocyte cell line impaired terminal differentiation into adipocytes and induced the acquisition of a transformed phenotype (Neal and Clipstone 2003). More recently, enforced expression of similarly mutated, constitutively active mutants of NFATc1 (short isoform) and NFATc2 (long isoform) was shown to induce distinct phenotypes in NIH 3T3 cells. Both of these caNFAT proteins are constitutively localized to the nucleus, bind DNA with high affinity and activate endogenous NFAT target genes (Monticelli and Rao 2002; Neal and Clipstone 2003; Okamura et al. 2000). Whereas enforced expression of the short caNFATc1 isoform led to increased cell proliferation and induction of cellular transformation as previously described in 3T3L1 cells (Neal and Clipstone 2003), expression of a long isoform of caNFATc2 induced cell cycle arrest and apoptosis (Robbs et al. 2008). Furthermore, enforced expression of caNFATc2 interfered with Ras-and caNFATc1-induced transformation of NIH3T3 cells, suggesting that NFATc2 long isoform may act as a tumor suppressor gene whereas the NFATc1 short isoform may function as an oncogene (Robbs et al. 2008). Genetics studies have clearly proved that in the immune system different NFAT factors can play either specific, redundant or antagonist function regarding normal T cell development (Macian 2005), these results highlight the notion that different members and splicing-dependent isoforms of the NFAT family can play different roles in tumor development (Robbs et al. 2008). The proposed tumor suppressive function of NFATc2 must however be highly context or signal dependent as NFATc2 knockout mice develop and live normally and, except for the fact that the differentiation defect observed in cartilage differentiation in these animals is associated with the emergence of chondrosarcoma (Ranger et al. 2000), these mice are not particularly tumor-prone. Interestingly, NFATc2-deficient mice are more susceptible to carcinogen-induced tumorigenesis (Robbs et al. 2008). Whether this reflects a cell-autonomous requirement for NFATc2 loss-of-function intrinsic to the tumor cells or results from impaired function in cells in the tumor environment or results from a defective tumor immune surveillance in NFATc2-/- mice remains to be addressed. Albeit involvement of calcineurin and/or NFAT proteins has been proposed in several solid tumors, in particular breast carcinoma and pancreatic cancer (for review see Buchholz and Ellenrieder 2007; Medyouf and Ghysdael 2008) we will mainly focus here on their cell-autonomous implication in hematologic malignancies. A few reports have described gain-of-function mutation in *CnA* in T or B lymphoma derived cell lines, but it is unknown whether these mutations were part of the dominant clone in the primary tumors from which these cell lines were derived. In the EL4 murine T lymphoma cells, a missense mutation changed an evolutionary conserved aspartic acid to asparagine within the autoinhibitory domain of the *CnAα* gene (Fruman et al. 1995). This substitution leads to the generation of a mutant CnA*α* hypersensitive to calcium signaling which affects normal signal transduction pathways in EL4 T-lymphoma cells (Fruman et al. 1995). A differential proteomic screen performed in a squirrel monkey-derived B-lymphoma (SML) cell line resulted in

the expression of a truncated, constitutively active form of CnA (Gross et al. 2004). In both instances, the consequences of these mutations on the cell line maintenance *in vitro* and tumorigenic activity *in vivo* have not been assesed. Moreover, large-scale retroviral insertional mutagenesis screens carried out in lymphoproliferative disorders have identified two cases of retroviral insertions in genes encoding NFAT family members, including NFATc2 and NFAT5 (Suzuki et al. 2002). In B-cell chronic lymphocytic leukemia (B-CLL), persistent nuclear localization and constitutive activation of NFATc2 (Schuh et al. 1996) were shown to be responsible, in cooperation with STAT6, to the high expression of CD23 on the surface of B-CLL cells, thus contributing to the pathogenesis of this disease (Kneitz et al. 2002). Importantly, immunohistological analysis of NFATc1 expression and subcellular localization has been performed in a large panel of Non-Hodgkin B and T-cell lymphoma (Marafioti et al. 2005). Although NFATc1 expression was not detected in classical Hodgkin's lymphoma (cHL) and plasma cell proliferations, NFATc1 was expressed in a majority of aggressive B-cell lymphomas cases, with nuclear localization of NFATc1 found in a subset of these cases (Akimzhanov et al. 2008; Marafioti et al. 2005). Suppressed expression of NFATc1 in human cHL and anaplastic large cell lymphomas (ALCLs), which both are lymphoma entities with immunoreceptor signaling, was recently explained by NFATc1 transcriptional silencing through hypermethylation of the NFATc1 P1 promoter (Akimzhanov et al. 2008). NFATc1 nuclear localization or dephosphorylation of both NFATc1 and NFATc2 were found in primary tumor samples and cell lines derived from aggressive B and T- cell lymphoma patient (Medyouf et al. 2007; Pham et al. 2005). Moreover treatment of these cell-lines with CsA triggered cell cycle inhibition and induced apoptosis. In vitro studies performed on diffuse large B cell lymphoma (DLBCL) derived cell lines have shown that NFATc1 together with NFκB are constitutively activated and cooperatively regulate the expression of target genes important for proliferation and cell survival (Fu et al. 2006; Pham et al. 2005). Of particular interest, Ford and colleagues have proposed that expression of the genes encoding CD154 and BLys, two ligands belonging to the TNFα superfamily, is synergistically regulated by activation of NFAT and NFκB (Fu et al. 2006; Pham et al. 2005), thus possibly regulating survival and proliferation of malignant B cells. A recent transcriptomic analysis of angioimmunoblastic T-cell lymphoma (AITL) also provided evidence for NFATc1 overexpression in this pathology (de Leval et al. 2007). Regarding the implication of the calcineurin/NFAT pathway in *in vivo* mouse models of human T-ALL/lymphoma, persistent activation of calcineurin/NFAT signaling was observed in primary leukemic cells and shown to be independent of preTCR/TCR signaling, but to depend upon their maintenance in their *in vivo* context (Medyouf et al. 2007). Short-term treatment of leukemic mice with calcineurin inhibitors (CsA or FK506) leads to inactivation of calcineurin/NFAT signaling (NFAT rephosphorylation), inhibition of cell cycle progression and induction of apoptosis in leukemic cells. These combined cellular effects led to severe inhibition of tumor load in treated animals and their increased survival (Medyouf et al. 2007). Pharmacological treatment of diseased mice with these compounds is likely to inhibit calcineurin in non tumor cells of the leukemic niche, possibly contributing this way to leukemia regression. For example, recent evidence shows that the calcineurin/NFAT signaling pathway is involved in tumor angiogenesis, acting downstream of the VEGFR and being negatively controlled by the DSCR1/calcipressin inhibitors of calcineurin (Ryeom et al. 2008). Ectopic expression of a constitutively activated calcineurin mutant in leukemic cells was shown to enhance leukemic cells aggressiveness *in vivo*, suggesting an intrinsic role for calcineurin in leukemic cells (Medyouf et al. 2007). Taken together these observations provide clear evidence that aberrant activation of the calcineurin/NFAT signaling module plays a critical role in the pathogenesis of hematologic disorders. It remains to be seen whether NFAT are effectors of calcineurin in leukemic cells, whether different NFAT play similar or distinct roles and whether their activity in different leukemias/lymphomas involves the deregulation of expression of cytokines, cytokine receptors, cell cycle components or components of the apoptosis machinery. In some settings, acute activation of the calcineurin/NFAT pathway has been shown to have a negative impact on tumor cell maintenance. For example, in type I Burkitt's lymphoma (BL) cells, it has been reported that B cell antigen receptor (BCR) *in vitro* cross-linking with an anti-IgM antibody leads to endogenous calcineurin activation and nuclear translocation of NFATc2 triggering lymphoma cells apoptosis probably through the induction of Nur77 (Kondo et al. 2003). Additionally, NFATc3 has been proposed to function as a tumor suppressor for the development of murine T-cell lymphomas (Glud et al. 2005). Analysis of T-cell lymphoma induced by the murine lymphomagenic SL3-3 retrovirus identified proviral integration within the *Nfatc3* locus that specifically repressed NFATc3 expression. Moreover, NFATc3-deficient mice infected with the murine SL3-3 retrovirus developed T-cell lymphoma with accelerated tumor onset as compared to wild type mice or NFATc2-deficient mice (Glud et al. 2005). It appears therefore that an appropriate balance in calcineurin/NFAT signaling can have either pro-or anti-oncogenic properties. This might be true both in signaling pathways intrinsic to tumor cells or to cells of their supportive stroma. It is thus critical to identify in specific leukemias and lymphomas the signaling cascades that function upstream of the calcineurin/NFAT signaling module and to delineate (i) how similar these pathways are in different tumor types; (ii) at what step

of disease progression they actually impinge upon the oncogenic process.

Therapeutic targeting of calcineurin/NFAT signaling

Since the NFAT activation process can be mainly divided into five steps: upstream events, calcineurin activation, NFAT dephosphorylation, NFAT nuclear translocation, NFAT target gene deregulation and cellular response, therapeutic intervention at each specific step can be envisaged to modulate the activity of this signaling module in tumorigenesis. The most potent and well characterized calcineurin inhibitors, CsA and FK506, have proved their therapeutic efficiency in pre-clinical models of T-cell leukemia/lymphoma (Medyouf et al. 2007). Importantly, therapeutic benefit of CsA has been shown in a small cohort of AITL patients (Advani et al. 2007) and larger clinical trials are ongoing to confirm these preliminary findings. Also therapeutic benefit is observed following treatment of myelodysplastic syndromes (MDS) with CsA, in particular in patients with refractory anemia and refractory anemia with excess blasts (Chen et al. 2007). However, both CsA and FK506 display off-target and severe toxic side effects ranging from neurotoxicity, nephrotoxicity, hypertension and gastrointestinal disturbances (for review see Dumont 2000). They also function as efficient immunosuppressants and their use in remission induction protocols may thus compromise tumor immunosurveillance mechanisms. Other synthetic or natural calcineurin inhibitors were identified or synthetized after the discovery of CsA and FK506. For example, a FK506 analog (designated as L-732, 531) (Dumont 2000), and a CsA analog (named $ISA_{TX}247$) (for review see Aspeslet et al. 2001; Lee and Park 2006; Stalder et al. 2003) displaying a similar or even higher efficiency with a reduced nephrotoxicity have been generated. Attempts to find novel calcineurin inhibitors through large-scale screens of chemical libraries have identified small molecules that block either NFAT activation or NFAT nuclear translocation, but turned out not to inhibit calcineurin itself but the activity of upstream calcium channels (Venkatesh et al. 2004). The interaction between calcineurin and NFAT factors and other substrates occurs at the level of two motifs, highly conserved among NFAT family members and centered over the PxIxIT and LxVP motifs in the NHR regulatory domain. Pharmacological compounds or synthetic peptide inhibitors spanning the PxIxIT motif have been identified in high throughput screens and shown to efficiently prevent NFAT dephosphorylation and nuclear translocation without affecting calcineurin activity (Aramburu et al. 1999; Kang et al. 2005; Lee and Park 2006; Noguchi et al. 2004; Roehrl et al. 2004). Designing more selective inhibitors targeting the physical interaction of calcineurin with its substrates is clearly an option to specifically target calcineurin signaling (Kang et al. 2005). Furthermore, several endogenous proteins that inhibit calcineurin activity have been identified (for review see Macian 2005) and appear to be potential targets for NFAT inhibition (for review see Lee and Park 2006). For example, therapeutic inhibition of calcipressin may prove to be an alternative or synergistic approach to existing anti-angiogenic therapies (Ryeom et al. 2008).

The most promising advance towards specificity is to bring to light the respective roles of calcineurin downstream targets that mediate its oncogenic or anti-oncogenic activity in different human malignancies, both in the tumor themselves and in supporting cells of the microenvironment. Available evidence shows that NFAT transcription factors are mediators of calcineurin in different cancers (Buchholz et al. 2006; Jauliac et al. 2002; Pham et al. 2005). It is however possible that NFAT factors are not the only targets of calcineurin in leukemogenesis as calcineurin can dephosphorylate other effectors possibly relevant to its oncogenic properties (see for example Huang et al. 2008)

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Review

Wnt up your mind – intervention strategies for S100A4-induced metastasis in colon cancer

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Abstract. Colon cancer is still a burden mainly due to metastasis formation. The latter is often associated with a constitutive activation of the Wnt/β-catenin signaling pathway and high expression of the metastasis-inducing gene S100A4. We previously identified S100A4 as a transcriptional target of β-catenin. Intervention strategies targeting Wnt/β-catenin signaling might therefore represent promising approaches to inhibit tumor growth and metastasis formation when induced by S100A4. Many inhibitors, various strategies, as well as different routes of application targeting key molecules of the Wnt signaling pathway have been reported within the last decade. Consequently, downregulation of β-catenin target genes lead to altered tumorigenic and metastastic abilities of cancer cells. This review focuses on the potential of Wnt/β-catenin signaling intervention to restrict colon cancer metastasis formation by interdicting S100A4 expression.

Keywords: Colorectal cancer — Metastasis — S100A4 — Wnt signaling — β-catenin

Abbreviations: *APC*, adenomatous polyposis coli; *CK-1α*, casein kinase-1α; *COX*, cyclooxygenase; *DKK-1*, dickkopf-1; *dn*, dominant negative; *Dvl*, dishevelled; *Fzd*, frizzled; *GSK-3β*, glycogen synthase kinase-3β; *IWP*, inhibitors of Wnt production; *LAR*, leukocyte common antigen related; *LEF-1*, lymphoid enhancer factor-1; *LRP-5/-6*, low density lipoprotein receptor-related protein-5 or -6; *MMP*, matrix metalloproteinase; *MTI*, methylation inhibitors; *NSAID*, non-steriodal anti-inflammatory drug; *PKC*, protein kinase C; *PLA2G2A*, phospholipase 2 group 2A; *PP2a*, protein phosphatase 2A; *RAGE*, receptor for advanced glycation end products; *sFRP*, secreted frizzled related protein; *tk*, transkingdom; *WIF-1*, Wnt inhibitory factor-1

Introduction

Colon carcinoma is still a major cause of cancer death worldwide. In developed countries it is the third most frequent type of cancer concerning men and second most frequent cancer occurrence in female (Garcia et al. 2007). Despite intensive health programs for early diagnosis of colon cancer, only about 40% of early stage tumors are detected. The residual 60% show already regional or even distant metastasis at the time of diagnosis.

Colon cancer in its early stages can be treated by surgery leaving patients with a good survival prognosis of about 90%. The five-year survival rate decreases to about 65% when patients are diagnosed with lymph node metastasis. However, a drastic reduction of the five-year survival rate to about 10% is observed when distant metastases have formed. In conclusion, approximately 90% of all colon cancer deaths arise from metastasis dissemination of primary tumors (Stein and Schlag 2007).

Efforts to identify main molecular players in metastasis formation revealed the metastasis progressor protein S100A4 (Ebralidze et al. 1989). We demonstrated that S100A4 was useful as a prognostic biomarker for metachronous colon cancer metastasis formation. Thus, S100A4 contributes to the early identification of patients with high risk to develop distant metastasis. Moreover, we also identified S100A4 as one of the target genes of the canonical Wnt/β-catenin pathway (Stein et al. 2006). Furthermore, S100A4 was found to be

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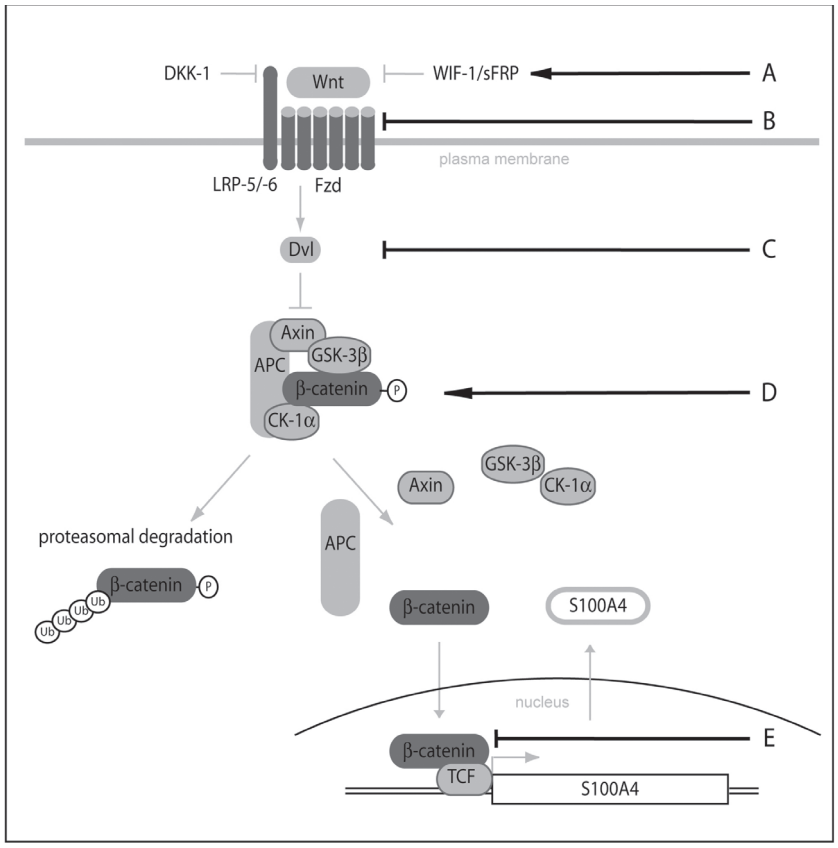

Figure 1. Potential target sites within the canonical Wnt/β-catenin signaling pathway for inhibition of S100A4 induced metastasis formation (adapted from Barker and Clevers 2006; Barker 2008; Klaus and Birchmeier 2008).
A. Activation of naturally occurring Wnt antagonists: DKK-1, WIF-1, sFRP.
B. Inhibition of Wnt receptor signaling initiation complex: Wnt; Fzd, LRP-5/-6.
C. Inhibition of Wnt signal transduction *via* inhibiting Dvl.
D. Re-activation of the β-catenin destruction complex: APC, Axin; GSK-3β, CK-1α.
E. Prevention/Disruption of the β-catenin/TCF transcription activating complex.

specifically expressed at the invasion front of colorectal cancer together with nuclear β-catenin (Hlubek et al. 2007). The link of S100A4 expression to the Wnt/β-catenin pathway was also shown for gastric cancer (Yoon et al. 2008; Li et al. 2008). In osteosarcoma, metastasis formation was also mediated by the regulation of S100A4 through the Wnt signaling pathway (Guo et al. 2009). Many inhibitors and intervention strategies targeting the Wnt signaling pathway have been reported within the last decade. This review focuses on their potential to restrict S100A4 induced metastasis formation (Fig. 1).

I. Wnt signaling in metastasis formation of colorectal cancer

Active Wnt signaling is crucial for cell differentiation along the crypt-villus axis and hence plays a central role in maintaining the homeostasis of colon tissue (Pinto et al. 2003). Consequently, deregulation of this important signaling pathway is a major event in tumorigenesis of colon carcinoma (Segditsas and Tomlinson 2006). Although further pathways such as K-ras, Notch/bHLH, BMP-4 and hedgehog signaling contribute to colorectal cancer, the Wnt signaling pathway plays a decisive role for this cancer entity (Klaus and Birchmeier 2008; Fre et al. 2008).

The Wnt signaling pathway can be distinguished between the non-canonical and the canonical signaling pathway. The non-canonical Wnt signaling pathway regulates cell adhesion, cell polarity and coordinated tissue movement (Vincan and Barker 2008) *via* binding of β-catenin to the cytoplasmic region of cadherin adhesion receptors (Jeanes et al. 2008). The canonical Wnt signaling pathway regulates cell differentiation, proliferation, migration and invasion by strictly controlling the amount of cytoplasmic β-catenin (Barker

2008). In the absence of active Wnt signaling, β-catenin levels are reduced by the destruction complex. In this complex β-catenin is anchored by APC and Axin which builds a platform for the protein kinases CK-1α and GSK-3β and the protein phosphatase 2A (PP2A) to enter the complex and sequentially phosphorylate β-catenin (Komiya and Habas 2008). Phosphorylation of β-catenin generates a binding site for E3 ubiquitin ligase, which leads to polyubiquitinylation and rapid proteasomal degradation. Suppressed levels of β-catenin enable Groucho proteins to interact with T-cell factor (TCF)/lymphoid enhancer factor-1 (LEF-1) transcription factors and Wnt signaling enhancers to inhibit transcription of β-catenin target genes (Behrens et al. 1996; Brabletz et al. 1998; Barker 2008).

Activation of the Wnt/β-catenin signaling pathway is initiated by binding of secreted Wnt proteins to their receptor from the Frizzled (Fzd) family and their co-receptors LRP-5/-6 at the plasma membrane. The formed Wnt-Fzd-LRP-5/-6 initiation complex triggers the association of dishevelled (Dvl) with the cytoplasmic tail of Fzd receptor. That activates phosphorylation of LRP-5/-6 and further sequesters Axin from the destruction complex to the plasma membrane. Disruption of the destruction complex leads to accumulation of cytoplasmic β-catenin which then translocates into the nucleus (Barker 2008). Nuclear β-catenin displaces Groucho proteins from their complex with TCF/LEF-1 transcription factors and initiates transcription of β-catenin target genes (Daniels and Weis 2005). This target gene transcription is further amplified by the recruitment of transcriptional enhancers such as CBT, TBP, BRG-1, legless, Bcl-9, mediator or hyrax (Barker 2008).

In non-cancer cells Wnt signaling is tightly controlled by its antagonists. They can be divided into two functional classes: the secreted frizzled related protein (sFRP) class and the dickkopf (DKK) class. Proteins of the sFRP class such as sFRP family members, Wnt inhibitor factor-1 (WIF-1) and Cerberus bind directly to secreted Wnts and thereby diminish non-canonical and canonical Wnt signaling. In contrast, proteins of the DKK class specifically inhibit canonical Wnt signaling by binding to LRP-5/-6 and interfering with the formation of the Wnt receptor signaling complex (Kawano and Kypta 2003).

In a major part of colon cancers the canonical Wnt/β-catenin pathway is constitutively active. About 90% of colorectal cancers bear mutations in the APC gene, which lead to a disruption of the β-catenin destruction complex and consequently to a nuclear accumulation of β-catenin. Another set of colorectal cancers displays a loss of conserved β-catenin phosphorylation sites which results in abolished proteasomal degradation. Constitutive Wnt signaling can further be potentiated by deregulated and thus overexpressed proteins of the Wnt and Fzd family (Vincan and Barker 2008). Deregulation of Wnt signaling in colon cancer leads to aggressive tumor growth and poor prognosis. One of the Wnt target genes that directly promotes this increased metastatic potential is S100A4 (Stein et al. 2006).

II. S100A4 promotes metastasis formation in many types of cancer

S100A4 was firstly discovered in 1989 by Ebralizde *et al.* who found it overexpressed in metastatic cells (Ebralidze et al. 1989). S100A4 is a 11 kDa small Ca^{2+}-binding protein which is encoded in the epidermal differentiation complex located on human chromosome 1 (1q21). This region is frequently rearranged in human cancers (Fernandez-Fernandez et al. 2008). Consistently, S100A4 is overexpressed in many different types of cancer such as gallbladder, bladder, breast, oesophageal, gastric, pancreatic, hepatocellular, non-small lung, and colorectal cancer, and its presence highly correlates with the aggressiveness of a tumor (Helfman et al. 2005). Furthermore, it was found that S100A4 expression levels positively correlate with the chance of a tumor to metastasize. S100A4 itself is not necessarily tumorigenic since transgenic mice overexpressing S100A4 do not develop tumors *per se* (Ambartsumian et al. 1996). However, when crossed with MMTV-neu or GRS/A mice which are characterized by forming mammary tumors that rarely metastasize, S100A4 overexpression leads to highly aggressive primary tumors and formation of metastasis (Davies et al. 1996; Ambartsumian et al. 1996). On the other hand, S100A4 null mice injected with highly metastatic mouse mammary carcinoma cells displayed no metastases (Grum-Schwensen et al. 2005). These observations suggest that S100A4 is not simply a marker for metastatic disease but rather has a causal role in mediating this process. Large efforts have been made in the last years to investigate the mechanisms by which S100A4 promotes metastasis formation.

S100A4 protein activity is dependent on intracellular Ca^{2+}

S100A4 is one of the currently known 25 members of the S100 protein family that share high sequential and structural homology. Their structure consists of two EF hands connected by a hinge region (Gingras et al. 2008). EF hands are defined by a helix-loop-helix motif and the loop creates a binding pocket for one Ca^{2+}-ion. Additionally to the canonical EF hand at the C-terminus S100 proteins are characterized by their N-terminal pseudo EF hand that comprises a slightly elongated loop region. This elongation results in a lower affinity to Ca^{2+} which allows ion binding to occur sequentially (Dutta et al. 2002). Ca^{2+}-binding triggers a conformational shift which generates two major hydrophobic binding sites partly formed by the hinge region (Pathuri et al. 2008). Residues of this hinge region as well as the lengthy C-terminal tail

of S100A4 share almost no sequence homology within the S100 protein family. Hence those regions confer specificity towards S100A4 protein interaction partners (Vallely et al. 2002). Several protein binding partners were identified, such as proteins of the cytoskeleton, the tumor suppressor protein p53, the receptor for advanced glycation end products (RAGE), and annexin II.

Intracellular S100A4 increases cell motility

S100A4 directly increases cell motility by its interaction with proteins of the cytoskeleton. It was found to coaggregate with actin filaments in sedimentation assays (Watanabe et al. 1993) and to bind non-muscle tropomyosin (Takenaga et al. 1994). However, the most prominent binding partner of S100A4 is non-muscle myosin II (Chen et al. 2001). Myosin II is a chemomechanical protein that participates in cell division, cell motility and secretion. S100A4 binding to myosin II inhibits myosin polymerization and even promotes the disassembly of myosin filaments at the leading edge of migrating cells. This local enrichment of myosin II monomers is needed for the formation of flexible directed protrusions as a first step in migration (Li et al. 2003; Li and Bresnick 2006).

Besides increasing cell motility and cell polarization S100A4 affects cell adhesion by binding to liprin β1. Liprin β1 itself interacts with a transmembrane protein tyrosine phosphatase called leukocyte common antigen related (LAR) which is localized at the end of focal adhesions. It is suggested that S100A4 affects LAR function by binding to liprin β1 and thus modulates cell adhesion causing a migratory phenotype (Kriajevska et al. 2002).

Grigorian *et al.* firstly discovered the binding of S100A4 to the tumor suppressor p53 and the modulation of its transcriptional activity. Additionally, binding of S100A4 masks several C-terminal PKC phosphorylation sites of p53 (Grigorian et al. 2001). Although the meaning of the phosphorylation status of p53 is still controversially debated, inhibition of PKC phosphorylation can stabilize p53 (Chernov et al. 1998). Furthermore, increased p53 levels lead to increased migration and invasion rates in colon cancer cell lines (Sablina et al. 2003). However, the exact role of S100A4 in p53 mediated migration still needs to be further elucidated.

Extracellular S100A4 induces metastasis formation and angiogenesis

Beside its intracellular interactions, S100A4 is released to the extracellular space and initiates metastasis formation and angiogenesis. For instance, exogenously added oligomeric S100A4 increased cell motility of endothelial cells *in vitro* (Ambartsumian et al. 2001). Injection of recombinant S100A4 protein increased metastasis formation of a mouse mammary adenocarcinoma cell line *in vivo* when tumor cells were in contact with stroma cells. This also implies a role for S100A4 to enhance intercellular communication. S100A4, when secreted by stroma cells, further increased vascularization of the tumor (Schmidt-Hansen et al. 2004a). Extracellular S100A4 activates expression of matrix metalloproteinases (MMPs) such as MMP-1, -3, -9 and 13 (Schmidt-Hansen et al. 2004b; Senolt et al. 2006). MMPs cleave proteins of the extracellular matrix and thereby enable cell invasion into adjacent tissues. In chondrocytes S100A4 induces MMP-13 expression via binding to RAGE (Yammani et al. 2006). However, extracellular S100A4 can also activate expression of MMP-13 independently of RAGE via NF-κB (Schmidt-Hansen et al. 2004b).

Furthermore, S100A4 also promotes angiogenesis. Extracellular S100A4 dimers form heterotetramers with annexin II dimers. This activates the plasminogen activated system which stimulates MMPs and together facilitates angiogenesis (Semov et al. 2005).

III. Targeting the Wnt/β-catenin pathway to suppress S100A4 expression

Concentrating on the regulation of S100A4 expression, we identified S100A4 as a target gene of the canonical Wnt/β-catenin pathway. We compared gene expression profiles of the human colon carcinoma cell line HCT116, which carries one allele with a gain-of-function mutation of β-catenin and a wildtype β-catenin allele, with the derivative cell line HAB-92^{wt}, where the mutant β-catenin allele was ablated by homologous recombination (Kim et al. 2002). In this experiment we found a massive upregulation of S100A4 in cells with mutant β-catenin due to an increased level of nuclear β-catenin. The promoter analysis of S100A4 showed a direct regulation by the β-catenin/TCF complex. Effects on both cell migration and invasion induced by constitutive nuclear β-catenin are mediated by S100A4 and were significantly reduced upon S100A4 siRNA application. The identification of S100A4 as a target gene of the canonical Wnt/β-catenin pathway provides the link between two previously unconnected molecular pathways which play important roles in tumor progression and metastasis formation in colorectal cancer (Stein et al. 2006). This finding constitutes a new basis for improved anti-metastatic treatment in colon cancer since the Wnt/β-catenin pathway contains several potential sites to interfere with active signaling.

Blockage of the Wnt-Fzd-LRP-5/-6 initiation complex

The effect of active β-catenin to transform a tumor towards a more aggressive state by activating S100A4 can be blocked by interfering with the interaction of Wnt and its receptors. Successful inhibition of Wnt-1 signaling was achieved by

application of siRNA in human cancer cells *in vitro*. Treatment of Wnt-1 overexpressing cell lines with Wnt-1 siRNA resulted in increased apoptosis. *In vivo* tumor growth was inhibited by application of a specific Wnt-1 antibody (He et al. 2004). Wnt-2b is a mediator of the mesenchymal-epithelial transition needed for secondary tumor formation. Treatment of xenograft models with a specific Wnt-2b antibody also resulted in decreased tumor growth (You et al. 2004). These data present the possibility to abort the interaction of secreted Wnt proteins with their Fzd receptors in a highly specific manner. Therefore, it is promising to investigate whether targeting other proteins of the Wnt family could prevent S100A4 induced migration despite of inducing apoptosis.

Besides targeting the interaction between Wnt and Fzd receptors, inhibiting secretion of Wnts interferes at the very beginning of Wnt signaling. Chen *et al.* recently reported on the identification of a group of small molecules they defined as inhibitors of Wnt production (IWP). Those IWPs all blocked porcupine, an acetyltransferase needed for palmitoylation and thus the secretion of Wnts. Interestingly, the blockage of Wnt secretion was effective to abort Wnt signaling despite the presence of mutated APC and thus constitutive activity of the pathway (Chen et al. 2009). The ability of those inhibitors to prevent S100A4 expression in human colon cancer and thus S100A4 induced metastasis would be a worthwhile investigation.

Disruption of the Wnt-Fzd-LRP-5/-6 signaling initiation complex can also occur by targeting the co-receptors LRP-5/-6. For instance, knockdown of LRP-5 expression by siRNA in human mesenchymal stem cells resulted in a decreased ability of the cells to invade (Neth et al. 2006). Colony formation, cell migration and invasion rates of osteosarcoma cells were reduced when dominant negative LRP-5 (dnLRP-5) was stably expressed. DnLRP-5 competes with endogenous LRP-5 in the Wnt signaling initiation complex and thus disrupts Wnt signaling. *In vivo* dnLRP-5 expressing cells reduced the number and size of lung metastasis in a spontaneous pulmonary metastasis model (Guo et al. 2008). Therefore, dnLRP-5 treatment also bears great potential to be applied against S100A4 induced metastasis.

Abortion of Wnt-Fzd downstream signaling

Wnt signaling is initiated by formation of the Wnts-Fzd-LRP-5/6 receptor complex. Intracelullar downstream transmission of the signal is dependent on Dvl proteins. Upon Wnt signaling Dvl activates recruitment of Axin to the plasma membrane and thereby disrupts the β-catenin destruction complex. Dvl proteins are overexpressed in many cancers leading to constitutive β-catenin signaling (Mizutani et al. 2005).

Dvl proteins consist of three functional domains of which the central PDZ domain is essential for protein-protein interaction. Via this domain Dvl binds to the intracellular part of Fzd receptors. This binding is essential for transmission of Wnt signals. Accordingly, deletion of the PDZ domain in Dvl protein resulted in a drastic reduction of β-catenin target gene expression (Uematsu et al. 2003b). Since the structure of the PDZ domain of Dvl is known (Khlebtsova et al. 2000), Shan *et al.* applied computational structure-based ligand screening to identify potential Dvl inhibitors (Shan et al. 2005). Virtual 3D screening revealed the small molecule NSC668036 which was validated to bind to the PDZ domain of Dvl and further to inhibit Wnt-3a induced secondary axis formation.

By creating a chemical library which was applied in a high throughput screening, You *et al.* identified a new small molecule called FJ9. FJ9 inhibited Dvl induced Wnt activation in HEK293 cells which resulted in decreased TCF-dependent transcription. Furthermore, FJ9 suppressed tumor growth *in vivo* (You et al. 2008). By large scale screening of peptides that can bind to the PDZ domain of Dvl-2, Zhang *et al.* recently published the peptide pen-N3 to inhibit Wnt signaling. Pen-N3 inhibited Dvl induced β-catenin/TCF reporter signaling to comparable extents as FJ9. Pen-N3 was nontoxic at Wnt inhibiting concentrations (Zhang et al. 2009).

Targeting Dvl protein with siRNA resulted in growth inhibition and reduced colony formation in lung cancer cells. However, in colon cancer cells that bear mutated APC Dvl siRNA had no effect on cellular growth (Uematsu et al. 2003a).

Those promising data on blocking Wnt signaling by acting on Dvl offer alternative strategies to reduce S100A4 expression and might thus be applied in anti-metastatic treatment.

Stimulation of the destruction complex

Stabilization or reactivation of the β-catenin destruction complex bears great potential to restrict constitutive Wnt/β-catenin signaling. Calcimycin is a Ca^{2+}-ionophor that increases intracellular Ca^{2+} by binding extracellular Ca^{2+} and shuffling it into the cell. Calcimycin was found to stimulate β-catenin destruction by activating protein kinase C (PKC). Upon increased intracellular Ca^{2+}-levels, PKC phosphorylates β-catenin and stimulates proteasomal degradation of the latter (Gwak et al. 2006). Calcimycin treatment of hematopoetic cells resulted in a decrease of S100A4 mRNA level (Grigorian et al. 1994). Combining those two findings, calcimycin could be applied against S100A4 induced metastasis.

Another strategy to stimulate the β-catenin destruction complex is the treatment with dexamethasone. In osteoblasts dexamethasone stimulated the upregulation of Axin-2 which

is the scaffold protein of the destruction complex (Hayashi et al. 2009). Moreover, induced Axin stabilization in colorectal cancer cells was recently reported. A panel of small molecules was able to block accumulation of free β-catenin that was not in complex with E-cadherin by elevating Axin levels in the cell. Strikingly, increased Axin levels compensated the loss of APC tumor suppressor function (Chen et al. 2009). The potential of those compounds to reduce S100A4 induced metastasis formation is a promising approach for future research.

RNA interference technology can also be applied to target pathway key molecules such as β-catenin. Very recently, an interesting *in vivo* approach using transkingdom(tk)RNAi against β-catenin was reported. The application of tkRNAi led to reduced β-catenin expression in the mucosa, when delivered to the gastrointestinal tract after oral feeding (Silva et al. 2009).

Prevention/Disruption of the β-catenin/TCF complex

Non-steriodal anti-inflammatory drugs (NSAIDs) have been repeatedly evaluated as potential Wnt/β-catenin pathway therapeutics. Prominent Wnt signaling inhibition by NSAIDs was recently investigated in clinical studies (e.g. Meyskens et al. 2008; Sporn and Hong 2008; Tuma 2008). Traditional NSAIDs include aspirin, sulindac or indomethacin that all inhibit cyclooxygenase (COX) activation. Elevated COX expression levels in cancer lead to increased prostaglandin levels which subsequently activate Wnt signaling. Therefore reduction of COX signaling via NSAIDs can reduce Wnt-signaling and induce β-catenin degradation (Tuynman et al. 2008).

For instance, sulindac has been well established as a colon cancer chemopreventive agent for many years. It has pleiotropic activities as a COX inhibitor and as an inhibitor of polyamine biosynthesis. However, it is less known, that sulindac also acts *via* its inhibition of the nuclear accumulation and expression of β-catenin. Sulindac inhibits the nuclear accumulation of β-catenin in colon carcinoma cell lines, but also in adenomas of patients with familial adenomatous polyposis leading to reduced downstream signaling (Orner et al. 2003; Rice et al. 2003; Boon et al. 2004). Moreover, sulindac inhibits β-catenin expression in colorectal cancer cells and also in patients with hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis (Gardner et al. 2004; Koornstra et al. 2005; Han et al. 2008). As a consequence, upregulated target genes of β-catenin, like Met and cyclin D1, are downregulated following sulindac treatment (Boon et al. 2004; Dihlmann and von Knebel Doeberitz 2005).

For other molecules such as silibinin, a flavonolignan extracted from milk thisthle (*Silybum marianum*) plant, the modulation of the Wnt/β-catenin cascade has been reported. The caused decrease of β-catenin expression resulted in downregulation of target genes such as cyclin D1 and c-myc and inhibited the spontaneous intestinal tumorigenesis in $APC^{min/+}$ mice (Rajamanickam et al. 2009).

Since the artificial disruption of the β-catenin/TCF complex consequently results in target gene inactivation, many efforts have been made to identify small molecule inhibitors that are able to disrupt the β-catenin/TCF interaction. By high throughput screening, natural compounds were found, e.g. PKF115-584, PKF222-815, and CPG049090, that target the binding of β-catenin to TCF. Their cancer therapeutic potential has been shown by blocking growth of colon cancer cell lines (Lepourcelet et al. 2004; Barker and Clevers 2006).

Thus, inhibitors of the β-catenin/TCF complex may also offer potential as anti-metastatic agents by interdicting S100A4 expression. It might serve as the basis for chemoprevention of metastasis formation in individual colon cancer patients at high metastatic risk.

Activation of naturally occurring Wnt antagonists

Wnt antagonist expression is repressed in many types of cancer mainly due to promoter hypermethylation. Thereby, constitutively active Wnt signaling is provoked. For instance, the DKK-1 promoter was found to be hypermethylated in colon cancer (Aguilera et al. 2006). Furthermore, WIF-1 promoter hypermethylation frequently occurs in gastrointestinal tumors resulting in loss of WIF-1 expression and its negative regulation of the Wnt/β-catenin pathway (Taniguchi et al. 2005). Decreased expression of phospholipase 2G2A (PLA2G2A) was also found in late tumor stages of gastric cancer. PLA2G2A is a direct target of the Wnt/β-catenin signaling pathway which was recently identified as Wnt antagonist. Its expression correlated with the inhibition of S100A4 expression and the reduced ability of a tumor to metastasize (Ganesan et al. 2008).

Transcriptional reactivation of Wnt antagonist expression showed promising anti-cancerous results *in vitro*. For transcriptional reactivation methylation inhibitors (MTI) are useful drugs and many of them have already shown promising results in clinical trials. However, MTIs act on DNA methyltransferases and thus mainly activate transcription in general. To act more specific on the Wnt/β-catenin pathway, overexpression of naturally occurring Wnt antagonist represent an alternative opportunity to reduce β-catenin target gene expression and thereby S100A4 induced metastasis formation.

Conclusion and future perspectives

Colon cancer is still an unsolved burden mainly due to metastasis formation. Therefore, successful therapies need to target right at these cellular and molecular processes.

The Wnt/β-catenin pathway is constitutively active in almost all colon cancers which leads to highly metastasizing phenotypes. S100A4 as a target gene of this pathway is one of the main promoters of metastasis formation. Strikingly, S100A4 was shown to function as a precious biomarker for the identification of patients with high risk to develop metastases metachronously or who already have developed metastases. For those patients the demonstrated intervention strategies provide high potential to improve their survival prognosis.

Many approaches were made to identify S100A4 inhibitors that target the interactions of S100A4 with its target proteins. However, the role of S100A4 in metastasis formation is manifold. It acts on cellular adhesion to further increase cell motility. Furthermore, secreted S100A4 initiates cell invasion and angiogenesis. With all those various actions it still needs to be shown that specific inhibition of S100A4 protein is sufficient to restrict metastasis formation. Targeting the Wnt/β-catenin pathway to inhibit S100A4 gene expression bears the huge advantage to generally inhibit all S100A4 functions at once.

We certainly know of the complexity of this pathway, of the various described β-catenin target genes, as well as of the lack of strict specificity of the compounds mentioned here. The specificity of each Wnt signaling pathway-based intervention approach towards downregulation of S100A4 expression should be evaluated to prevent unwanted effects in the tumors as well as in the non-tumor tissues. Thus, the evaluation of potential side effects should be balanced together with the therapeutic potential with respect to metastasis reduction in preclinical studies.

Moreover, targeting exclusively the Wnt/β-catenin pathway can be insufficient in some cases, since other signaling cascades can also impinge on the expression of S100A4. Beside Wnt signaling, for instance, ErbB2 signals can activate S100A4 expression in medulloblastoma cell lines during EMT via the Ras/Raf/Mek/Erk1/2 signaling pathways (Hernan et al. 2003). Additionally, more recent studies by O'Connor and colleagues identified α6β4 integrin to activate S100A4 expression in breast cancer cell lines *via* NFAT5 transcription factor (Chen et al. 2009).

Each pathway might be active to a different extent in distinct types of tumors, and should be therefore differentially used as therapeutic target. The role of each transcriptional regulation in colon cancer needs to be further evaluated, before specific intervention strategies can be designed. The decisive impact of the Wnt/β-catenin signaling pathway, however, is without any doubt. A major aim of future therapies is the S100A4-based early identification of high risk patients for metastasis intervention strategies. Targeting the Wnt signaling pathway offers promising approaches for the ultimate goal – the reduction or even prevention of the S100A4-induced colon cancer metastasis.

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RAGE and S100 protein transcription levels are highly variable in human melanoma tumors and cells

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Abstract. The Receptor for Advanced Glycation Endproducts (RAGE) has been suggested to play an important role in melanoma. Animal studies with anti-RAGE antibodies have shown that RAGE blockade leads to reduced melanoma tumor growth and metastasis formation. RAGE is a multiligand receptor and among its ligands are the Ca-binding S100 proteins. Certain S100 proteins are differentially expressed in melanoma. For example, S100B is currently used as a reliable prognostic biomarker in patients with malignant melanoma. We have surveyed 40 human melanoma tumor samples for the transcription of RAGE and five of its known S100 protein ligands. Compared to normal skin tissue, we found highly significant ($p < 0.0001$) over-expression of S100B and under-expression of S100A2, whereas no significant difference in transcription of S100A6 and S100A10 was observed. RAGE showed slightly increased transcription in stage IV. Between individual tumor samples tremendous differences in transcription of the S100 proteins were observed, whereas RAGE expression showed relatively little variance. We also analyzed three well-characterized melanoma cell lines for S100 and RAGE expression. The S100 protein transcription profile showed clear differences between cultured melanoma cells and melanoma tumor tissue. Detailed profiling of S100 and RAGE transcription in melanoma tumors in combination with imunohisto-chemical and clinical data may lead to improved molecular diagnostic of melanoma and subsequently may facilitate improved treatment in the future.

Key words: RAGE — sRAGE — S100 protein — Melanoma

Introduction

Metastatic melanoma is characterized by poor (<20%) five-year survival rates. Many melanomas respond poorly to chemotherapy and aggressively form metastases. A better understanding of melanoma tumor biology may allow personalized chemotherapeutic treatment strategies. One of the most reliable biomarkers for melanoma is S100B. It is used as a marker for overall tumor load as well as for tumor response and survival prognosis during the therapeutic process: high serum levels of S100B predict poor treatment outcome (Egberts et al. 2008; Hamberg et al. 2003; Hauschild et al. 1999; Heizmann 2004; Schultz et al. 1998; Bolander et al. 2008; Oberholzer et al. 2008; Andres et al. 2008).

S100B is a member of the S100 protein family, which encompasses 21 members of sequence and structure related proteins (Marenholz et al. 2006). S100 proteins are small EF-hand calcium binding proteins that possess various intra- and extra-cellular functions, including cell proliferation, migration, muscle contraction, dynamics of the cytoskeleton and metabolic regulation (Donato 2003; Heizmann 2002). Members of the S100 protein family have been demonstrated to play important roles in tumor progression and tumorigenesis in several cancers, including melanoma, through their interaction with a number of intracellular target proteins (Emberley et al. 2004; Harpio and Einarsson 2004; Van Ginkel et al. 1998; Donato 2003; Zimmer et al. 2003). Extracellular S100 proteins have been described as signals of tissue damage and to function as alarmins (Bianchi 2007;

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Foell et al. 2007). Recently, extracellular S100 proteins have been implicated with cancer through their interaction with RAGE (Donato 2003; Heizmann et al. 2007). We are focusing in this study on five S100 proteins (S100B, S100A2, S100A4, S100A6 and S100A10) that have been associated in cancer progression and development.

S100B is highly expressed in melanocytes and in the brain. Intracellular S100B interacts with the p53 tumor suppressor (Baudier et al. 1992) and is an active target for anti-cancer drug development(Markowitz et al. 2005).

S100A2 was first described as a tumor-suppressor in mammary carcinoma cells (Lee et al. 1992) but recent studies have found that it is over-expressed in ovarian and gastric cancers, suggesting a more complex role for S100A2 in cancer (El-Rifai et al. 2002; Hough et al. 2001). In cultured epithelial cells, S100A2 has been shown to inhibit cell motility, probably through modification of actin polymerization/depolymerization mechanisms (Nagy et al. 2001).

The gene of S100A4 was first identified in metastatic tumor cell lines and is thought to play an important role in tumor progression and metastasis (Ambartsumian et al. 2005; Ambartsumian et al. 2001; Ebralidze et al. 1989). The role of S100A4 in melanoma tumor progression is not yet clear (Maelandsmo et al. 1997; Nonaka et al. 2008).

S100A6 is mainly expressed in fibroblasts and epithelial tissue but can also be found in neurons and smooth muscle cells (Kuznicki et al. 1989; Kuznicki et al. 1989). S100A6 expression is up-regulated in several cancers such as acute myeloid leukemia (Calabretta et al. 1985), pancreatic cancer (Vimalachandran et al. 2005), neuroblastomas and human melanomas (Boni et al. 1997; Shrestha et al. 1998; Weterman et al. 1992; Weterman et al. 1993)(Nonaka et al. 2008).

S100A10 was first identified in complex with annexin 2 (Gerke and Weber 1984; Glenney and Tack 1985) and is insensitive to calcium due to crucial mutations in its calcium binding domains resulting in a locked active conformation (Rety et al. 1999). S100A10 is thought to play an important role in the trafficking of plasma membrane proteins (Rescher and Gerke 2008). S100A10 has been shown to play a role in invasiveness of colorectal cancer cells through the interaction with plasminogen (Zhang et al. 2004). High expression of S100A10 has also been shown in anaplastic large cell lymphoma (Rust et al. 2005) and in renal cell carcinoma (Domoto et al. 2007). Down-regulation of S100A10, both at the transcript and at the protein levels has also been observed in three melanoma cell lines in culture suggesting a putative role of S100A10 in melanoma progression (Petersson et al. 2009).

S100 proteins have specific and common target proteins: the tumor suppressor p53 protein appears to be a common intracellular target for S100 proteins. p53 is a transcription factor that directs cell cycle arrest and apoptosis under stress conditions, probably through its tetramerization (Natan et al. 2009). S100B, S100A2, S100A4, S100A6 and S100A11 have been shown to interact with p53 but in distinct ways that could result in different effects of p53 dependent cell-cycle regulation (Baudier et al. 1992; Rustandi et al. 2000; Rustandi et al. 1998; Mueller et al. 2005; Fernandez-Fernandez et al. 2008; Fernandez-Fernandez et al. 2005).

The Receptor for Advanced Glycation Endproducts, RAGE, has been suggested as a common extracellular receptor for S100 proteins (Donato 2007; Heizmann et al. 2007; Leclerc et al. 2008; Schmidt et al. 2000). RAGE itself is important for tumor development, progression and metastasis in several cancers including melanoma (Fuentes et al. 2007; Heizmann et al. 2007; Logsdon et al. 2007; Turovskaya et al. 2008; Sparvero et al. 2009; Abe et al. 2004). RAGE is a cell surface receptor of the large immunoglobulin like receptor family and can be activated by structurally unrelated ligands that include members of the S100 protein family, the advanced glycation endproducts (AGE), amphoterin, and amyloid forming peptides or proteins. In cells, RAGE is present in multiple splice- and proteolytically truncated isoforms, the most important being soluble RAGE (sRAGE) that plays the role of decoy to antagonize ligand induced RAGE signaling (Hudson et al. 2008; Hudson et al. 2005; Ramasamy et al. 2009).

Melanomas express RAGE and S100 proteins and the stimulation of RAGE by S100 proteins may be a critical factor for tumor development, progression and metastasis. The splice isoforms of RAGE might also play an important role in melanoma. In order to explore this hypothesis we have undertaken a quantitative analysis of mRNA transcript levels of S100 proteins (S100B, S100A2, S100A4, S100A6 and S100A10) and of RAGE (full-length and sRAGE) of tissue samples of advanced melanoma (stage III and IV). We have also analyzed S100 and RAGE transcription levels in three cultured melanoma cell lines to complement the data obtained from the melanoma tumor samples.

Materials and methods

Quantitative PCR

A human melanoma tissue cDNA array (TissueScan MERT501) was purchased from OriGene (Rockville, MD) and consisted of normalized cDNA (against β-actin) from three non-tumor skin samples, 21 stage III malignant melanoma tumor samples and 19 stage IV malignant melanoma tumor samples. The age and gender distribution of tumor samples was as followed: stage III, 11 females and 10 males ranging from age 34 to 81 (median 56); stage IV, 5 females and 14 males ranging from age 42 to 81 (median 56). The average tumor content was 82 % (Supplemental Table 1). The cDNA of the non-tumor and tumor samples

Supplemental Table 1. Description of the melanoma tumor tissue cDNA array: Metastatic melanomas can be classified as stages III to IV depending of the spreading of the tumor cells. In stage III, the melanoma has spread to 1 to 3 lymph nodes near the primary tumors. In IIIA, the melanoma is not ulcerated and has not spread to other parts of the body. In stages IIIB and C, the melanoma has not spread to other parts of the body, it may or may not be ulcerated and may have spread to nearby small areas of skin or lymphatic channels. Stage IV melanomas have spread to other part of the body or to distant lymph nodes.

Sample	Gender	Age	Tissue	Diagnosis	Stage	% Tumor	% Necrosis	% Other tissue
1	Male	36	Skin	Fracture of bone	0	0	0	100
2	Not Sp	69	Skin	Skin Carcinoma, Merkel cell	0	0	0	100
3	Male	51	Skin	Obesity	0	0	0	100
4	Female	42	Lymph node	Malig. melanoma, metastatic	III	80	15	5
5	Female	59	Lymph node	Malig. melanoma, metastatic	III	95	5	0
6	Female	66	Lymph node	Malig. melanoma, metastatic	III	80	0	20
7	Male	56	Lymph node	Malig. melanoma, metastatic	III	85	5	10
8	Male	46	Lymph node	Malig. melanoma, metastatic	III	96	2	2
9	Female	66	Lymph node	Malig. melanoma, metastatic	III	80	20	0
10	Female	34	Lymph node	Malig. melanoma, metastatic	III	90	0	10
11	Female	56	Lymph node	Malig. melanoma, metastatic	III	80	20	0
12	Male	59	Lymph node	Malig. melanoma, metastatic	III	90	5	5
13	Female	49	Lymph node	Malig. melanoma, metastatic	III	95	5	0
14	Female	67	Lymph node	Malig. melanoma, metastatic	IIIA	80	0	20
15	Male	61	Lymph node	Malig. melanoma, metastatic	IIIB	90	10	0
16	Male	43	Lymph node	Malig. melanoma, metastatic	IIIB	85	7	8
17	Male	42	Lymph node	Malig. melanoma, metastatic	IIIB	90	0	10
18	Female	49	Lymph node	Malig. melanoma, metastatic	IIIB	90	0	10
19	Female	72	Lymph node	Malig. melanoma, metastatic	IIIB	75	0	25
20	Male	50	Groin	Malig. melanoma, metastatic	IIIB	60	0	40
21	Male	56	Lymph node	Malig. melanoma, recurrent	IIIC	95	0	5
22	Male	81	Lymph node	Malig. melanoma, metastatic	IIIC	90	5	5
23	Male	66	Lymph node	Malig. melanoma, metastatic	IIIC	70	5	25
24	Female	52	Lymph node	Malig. melanoma, metastatic	IIIC	55	0	45
25	Male	66	Lymph node	Malig. melanoma, metastatic	IV	75	5	20
26	Female	42	Lung	Malig. melanoma, metastatic	IV	75	20	5
27	Female	45	Lymph node	Malig. melanoma, metastatic	IV	80	15	5
28	Female	74	Lung	Malig. melanoma, metastatic	IV	98	0	2
29	Male	54	Lymph node	Malig. melanoma, metastatic	IV	70	20	10
30	Male	48	Mesentery	Malig. melanoma, metastatic	IV	95	0	5
31	Male	60	Jejunum	Malig. melanoma, metastatic	IV	80	0	20
32	Male	58	Lung	Malig. melanoma, metastatic	IV	80	0	20
33	Male	45	Lung	Malig. melanoma, metastatic	IV	90	5	5
34	Male	75	Small intest.	Malig. melanoma, metastatic	IV	80	15	5
35	Male	56	Liver	Malig. melanoma, metastatic	IV	75	1	24
36	Male	46	Jejunum	Malig. melanoma, metastatic	IV	90	10	0
37	Male	56	Lung	Malig. melanoma, metastatic	IV	90	10	0
38	Female	66	Omentum	Malig. melanoma, metastatic	IV	90	0	10
39	Male	56	Small intest.	Malig. melanoma, metastatic	IV	90	5	5
40	Male	81	Lymph node	Malig. melanoma, metastatic	IV	70	0	30
41	Male	56	Lung	Malig. melanoma, metastatic	IV	60	40	0
42	Male	73	Lung	Malig. melanoma, metastatic	IV	65	30	5
43	Female	42	Skin	Malig. melanoma, metastatic	IV	90	5	5

of the plate were provided already normalized against β-actin. Description of the tumor samples can be found in the Supplemental Table 1. PCR primers were designed with the help of PrimerBank (Wang and Seed 2003; Spandidos et al. 2008). Primers used are summarized in Table 1. Brilliant II SYBR Green QPCR master mix was from Stratagene (La Jolla CA). Real-time PCR measurements were performed on a Stratagene Mx3005p instrument, in duplicate for the tissue sample array and in triplicate for the cell samples. At the end of each RT-PCR run, the melting curves were obtained. In addition, the correct size of the amplicons was controlled by agarose gel electrophoresis.

Cell lines and tissue culture

Melanoma cell lines WM-115 (passage P80), WM-266-4 (P42) and SK-MEL2 (P40) were generously provided by Dr. G.B. Fields (University of Texas Health Science Center, San Antonio, Texas). Cells were maintained in opti-MeM (Invitrogen, Carlsbad, CA) supplemented with 4% FBS 100 U/ml penicillin, 100 μg/ml streptomycin. Cells at 70-80% confluence were used for mRNA preparation using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The mRNA was immediately reverse transcribed into cDNA using a reverse transcription kit (Promega, Madison, WI). The cDNA was used in quantitative PCR using a standard protocol. The cDNAs were normalized to β-actin.

Statistical analysis

Statistical analysis was performed on the Ct values using the Student t test for 2 groups of unpaired data with unequal variance.

Results

RAGE transcription levels in human melanoma.

We have determined the transcription levels of RAGE in a panel of 40 human melanoma tumor tissue samples. 33 out of 43 tumor samples contained 75% or more tumor tissue according to the manufacturer's documentation (Origene). The tumor samples provided were classified as tumor stage III, IIIA-C, tumor stage IV, or lesion stage IV. Three samples of normal skin tissue stage 0 were also provided and originated from non-melanoma patients (Supplemental Table 1). We measured the transcript levels of full length RAGE (Fig. 1A) and sRAGE (Fig. 1B) using the isoform specific primer pairs (Table 1). Transcript levels were normalized relative to healthy skin and data for individual tumor samples are shown in Figure 1. Immediately notable are large differences between tumor samples. Some tumors showed close to ten-fold over-expression of RAGE whereas other samples showed over five-fold under-expression relative to normal skin. We averaged and compared RAGE transcription in stage III and stage IV tumors and analyzed them for significant differences. We found statistically significant differences ($p < 0.05$) between stage III and stage IV melanoma tumors, with stage IV tumors over-expressing RAGE relative to stage III (Fig. 1A).

The analysis of the transcription levels of the sRAGE isoform (Fig. 1B) also showed great differences between samples, with up to 22 fold difference between the lowest and the highest transcription levels in either stage III or stage IV melanoma tumors. 90% of stage III and stage IV tumors were significantly ($p < 0.05$) under-expressed compared to non-melanoma tissue sample. The over-expression of full-length RAGE between stage III and stage IV tumors and the under-expression of sRAGE in both stage III and stage IV tumors support the hypothesis of a protective role of sRAGE against the detrimental effects of RAGE ligands in normal conditions. In pathological conditions, such as melanoma, reduced sRAGE expression could enhance the amount of free ligand available to full-length RAGE,up-regulated, resulting in increased RAGE dependent signaling.

S100 transcription levels in melanoma tumors.

We next analyzed the transcription levels of four S100 genes in our tumor samples. Tremendous differences in transcription levels for S100B of over 2,000 fold were observed between tumor samples (Fig. 1C). On average transcription levels for S100B exceeded normal skin by 100 fold in stage III and 200 fold in stage IV tumors. We found a strong statistical correlation between S100B expression and tumor stage ($p < 0.0001$). Higher stage tumors showed higher S100B transcript

Table 1. Primer pairs used in real-time PCR experiments

Primer	5'-3' sequence	Amplicon size
RAGE fwd RAGE rev	TGTGTGGCCACCCATTCCAG GCCCTCCAGTACTACTCTCG	309
sRAGE fwd sRAGE rev	AGCCCTCTCCTCAAATCCACT CTTTATCAAACCCCTCACCTGC	304
S100B fwd S100B rev	CCGAACTGAAGGAGCTCATC AGAACTCGTGGCAGGCAGTA	155
S100A2 fwd S100A2 rev	CACTACCTTCCACAAGTACT GAAGTCATTGCACATGAC	229
S100A6 fwd S100A6 rev	GTGGCCATCTTCCACAAGTA TTCACCTCCTGGTCCTTGTT	285
S100A10 fwd S100A10 rev	AAAGACCCTCTGGCTGTGG AATCCTTCTATGGGGGAAGC	247
β-actin fwd β-actin rev	CATGTACGTTGCTATCCAGGC CTCCTTAATGTCACGCACGAT	250

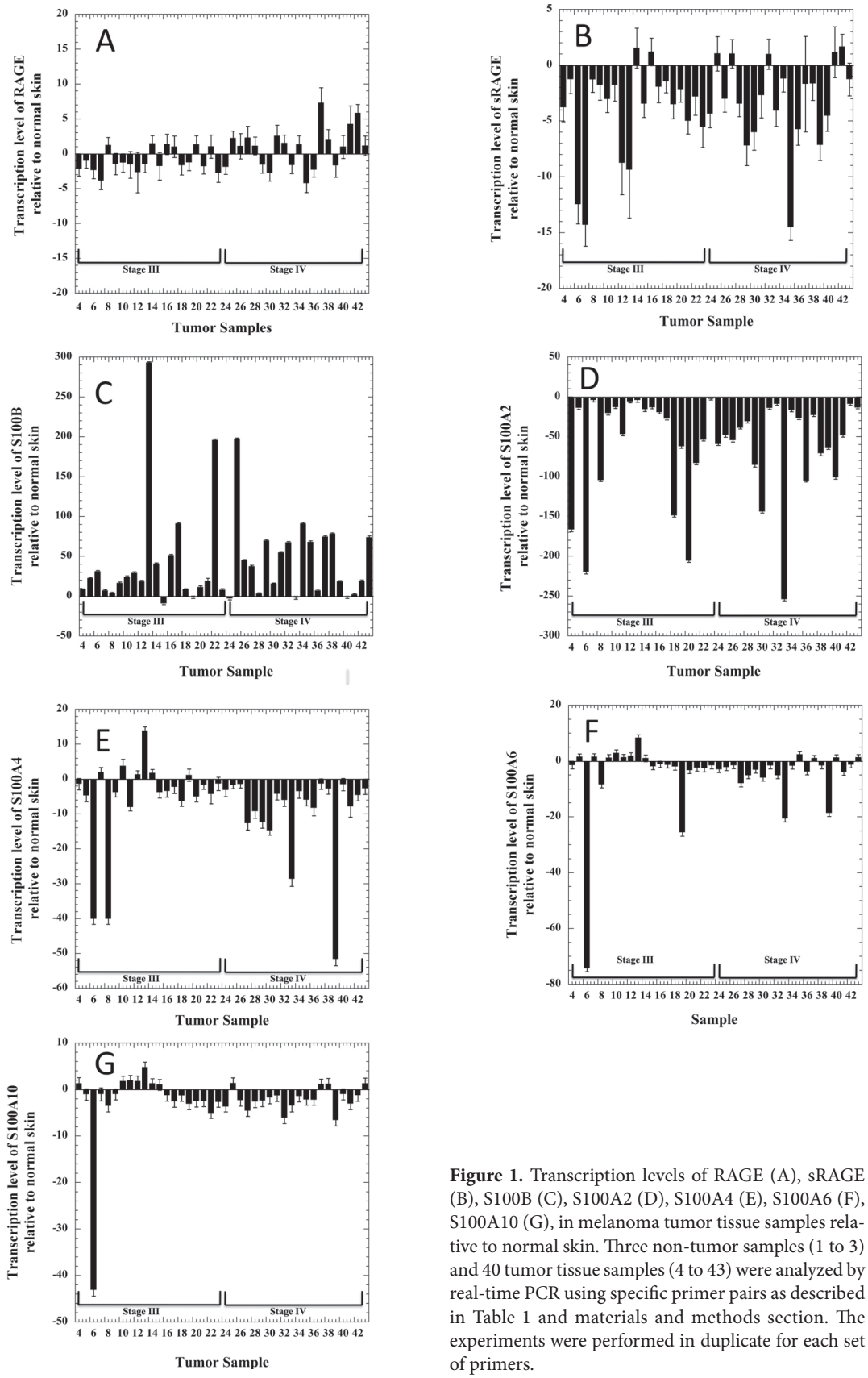

Figure 1. Transcription levels of RAGE (A), sRAGE (B), S100B (C), S100A2 (D), S100A4 (E), S100A6 (F), S100A10 (G), in melanoma tumor tissue samples relative to normal skin. Three non-tumor samples (1 to 3) and 40 tumor tissue samples (4 to 43) were analyzed by real-time PCR using specific primer pairs as described in Table 1 and materials and methods section. The experiments were performed in duplicate for each set of primers.

levels. Interestingly, five out of 40 melanoma tumor samples presented under-expression of S100B of 3.2 and 9.5 fold compared to non tumor samples, in agreement with previous studies showing the absence of S100B over-expression in 4% of all melanoma tumors (Aisner et al. 2005).

We also measured the transcription levels of the tumor suppressor S100A2, which we found to be generally in lower abundance in tumor samples (Fig. 1D). S100A2 was statistically significantly down-regulated ($p < 0.0001$) in both stage III and stage IV tumors when compared to normal skin. S100A2 was down-regulated on average 40- to 50-fold but showed tremendous differences between individual tumors. We did not detect up-regulation of S100A2 transcripts in any of the tumor samples (Fig. 1D).

S100A4 was found in higher abundance than S100B in normal skin ($Ct_{S100A4} = 20.8$; $Ct_{S100B} = 27.8$) and its transcript levels were significantly decreased ($p < 0.05$) in stage IV tumors (Fig. 1E). We also observed a significant ($p < 0.05$) under-expression of S100A4 transcripts in 76% of stage III tumors. Again, large differences in transcripts levels (>500 fold) were observed between melanoma tumors (Fig. 1E).

S100A6 did not show any significant difference in transcription levels between either stage III or stage IV tumors and normal skin even so individual tumors show great differences in relative expression: S100A6 transcription levels varied by more than a factor of 600 fold among stage III tumors and by 50 fold among stage IV tumors (Fig. 1F). A detailed analysis showed that 43% and 38% stage III tumors showed significant ($p < 0.05$) over-expression and under-expression, respectively, of S100A6 transcripts relative to non-tumor samples. Similarly 58% stage IV melanoma tumors showed significant ($p < 0.05$) under-expression of S100A6 transcripts compared to reference skin. Similar to S100A4 (Ct = 20.8), S100A6 (Ct = 22.2) was highly expressed in normal skin. Our results support an earlier study where 33% of melanomas, characterized by dermato-pathologists were found to express S100A6 at the protein level (Ribe and McNutt 2003).

S100A10 transcription levels in stage III and stage IV tumor samples did not show significant differences when compared to reference skin (Fig. 1G). Here again large variations between samples were observed with more than 200 fold differences between the lowest and the highest transcription levels in melanoma tumors. Detailed analysis showed that 58% stage IV melanoma tumors showed significant ($p < 0.05$) under-expression of S100A10.

The analysis of tumor samples for transcription of S100 proteins and RAGE revealed that S100B was generally strongly over-expressed and that the transcription level increased with the tumor stage. S100A2 on the other side was strongly under-expressed compared to normal skin. In average, the transcription ratio between S100A2 and S100B was approximately 6:1 in normal skin but reached 1:600 in stage III and 1:1500 in stage IV melanomas. Also, the ratio between full length RAGE and sRAGE changed between normal skin and melanoma tumor tissues from 6:1 to 12,1 for stage III and to 38,1 for stage IV melanomas.

RAGE and S100 transcription in melanoma cell lines

Although our panel of tumor tissue samples contained a high percentile of tumor tissue (mean = 82%), they also contained non-tumor tissue that could influence the evaluation of the transcription levels of the S100 proteins and RAGE. Furthermore "tumor tissue" is intrinsically heterogeneous due to non-melanoma cells that invade and constitute to tumor mass. We therefore decided to evaluate the transcription levels of S100B, S100A2, S100A4, S100A6, S100A10, RAGE and sRAGE in three well characterized melanoma cell lines: WM-115, WM-266 and SK-Mel2. These cell lines are free of non-melanocyte derived cell types. WM-115 and WM-266 originate from the same patient, WM-115 derives from the primary tumor whereas WM-266 comes from a secondary tumor. The transcription levels of RAGE and S100 proteins in these cell lines were normalized to the transcription level of β-actin in order to be comparable to the results from tissue culture arrays. As in the case of tumor tissue, we observed large variations in the transcription levels of RAGE and the S100 proteins between the three cell lines (Fig. 2).

As a general trend gene transcription levels for RAGE and the S100s relative to β-actin were always highest in the primary tumor cell line WM-115. RAGE was transcribed at lower levels than the S100s, by about two to three orders of magnitudes and varied by a factor of approximately 2.5 between the primary (WM-115) and the metastatic cell line WM-266 (Fig. 2A). sRAGE was found in ten times lower amount than full-length RAGE in all three cell lines. Interestingly, the ratio between RAGE and sRAGE was generally constant between the three melanoma cell lines although it varied significantly between the normal skin and melanoma stage III ($p < 0.0001$) or stage IV ($p < 0.0001$) tumor samples (Fig. 2A).

S100B was most highly expressed and exceeded RAGE transcription levels by more than three orders of magnitude (Fig. 2B). Differences between cell lines did not exceed four-fold. S100A2 transcripts were found at low levels and in the same order of magnitude as RAGE transcripts (Fig. 2C). Eight fold variation in transcription levels were observed between the three cell lines.

The S100B:S100A2 ratios were approximately 625:1, 2800: 1 and 560:1 for WM-115, WM-226 and Sk-Mel2, respectively.

S100A4 was strongly transcribed and showed greater differences between cell lines than the other genes. An almost 20-fold difference between WM-115 and WM-266 and a six-fold difference between WM-266 and SK-Mel2 were detected (Fig. 2D).

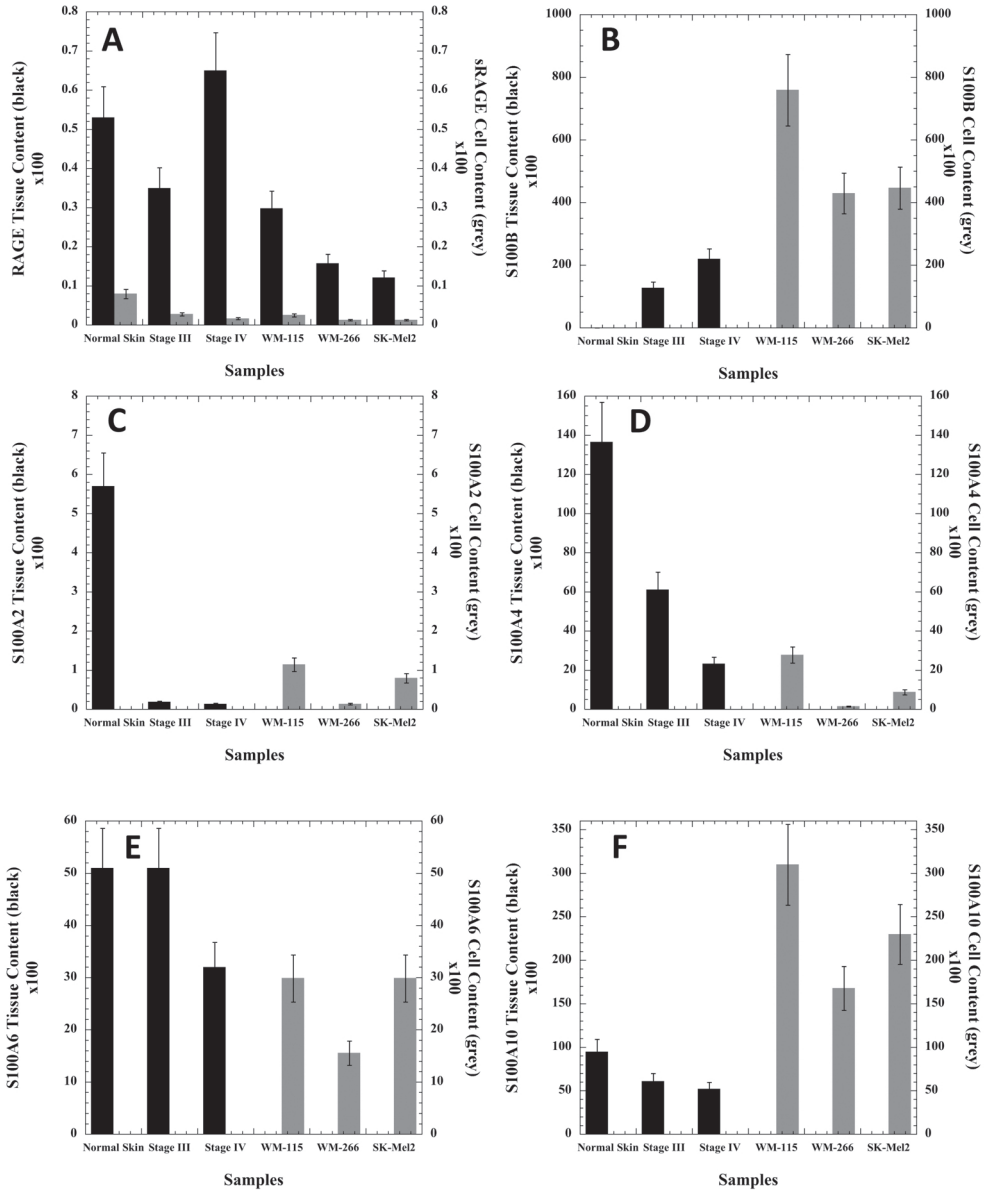

Figure 2. Transcription levels of RAGE and sRAGE (A), S100B (B), S100A2 (C), S100A4 (D), S100A6 (E), S100A10 (F), in melanoma cell lines WM-115, WM-266, sk-Mel2. The expression levels of the transcripts were normalized against β-actin. The experiments were performed in triplicate. The values of the transcript levels were also normalized by an additional factor of 100 for better representation.

S100A6 differed from the emerging pattern between the cell lines in that WM-115 and SK-Mel2 had approximately equal transcription levels (Fig. 2E).

S100A10 transcription level in the three cell lines was the closest to the one of S100B and was similar in the three cell lines (Fig. 2F).

The increase in the S100B:S100A2 and RAGE:sRAGE ratios observed by the comparison of normal skin with melanoma tumor tissues were also observed for cultured melanoma cells.

Discussion

RAGE has been suggested to be involved in melanoma progression and development (Abe et al. 2004). Animal studies using anti-RAGE antibodies have demonstrated that RAGE blockade can reduce melanoma tumor growth and metastasis formation. Among the RAGE ligands are several S100 proteins including S100B, S100A2 and S100A6 that have also been suggested to be potential biomarkers in melanoma: S100B is currently used by clinicians as prognostic marker

and high serum levels of S100B correlate with poor survival rates (Bolander et al. 2008; Oberholzer et al. 2008; Andres et al. 2008). The exact role of S100B in melanoma progression is still unknown. S100A2, generally described as tumor suppressor, was found to be strongly expressed in benign nevi and absent in metastatic melanoma, suggesting that loss of S100A2 expression could play a role in melanoma (Maelandsmo et al. 1997). A recent report also showed that over-expression of S100A2 could synergize with interferon α resulting in growth inhibition of cutaneous melanoma (Foser et al. 2006). In uveal melanoma, up-regulation of S100A2 was observed following treatment with decibatine and resulted in cell death (Gollob and Sciambi 2007). S100A6 gene expression was also found in melanocytic lesions (Weterman et al. 1993) and was shown to correlate with melanoma survival rate, a higher S100A6 expression rate being less favorable (Maelandsmo et al. 1997).

In our present study, we used a panel of cDNA prepared from 40 melanoma tissue samples and from three normal skin samples. The objective of our study was to evaluate differences in transcription levels between individual tumors and between tumor stages relative to normal skin.

Our study detected RAGE, sRAGE, S100B, S100A2, S100A4, S100A6 and S100A10 transcripts in melanoma tumor cDNA. Averaging stage III and stage IV tumor samples and comparing them to normal skin we found 100 to 200 fold increased transcript levels for S100B and 50 fold lower transcript levels for S100A2. These differences were statistically highly significant ($p < 0.0001$) and in the case of S100B differences between stage III and stage IV were also significant ($p < 0.001$). Differences between normal skin and melanoma for RAGE, S100A6 and S100A10 were not statistically significant on average. However, differences were significant for RAGE and S100A4 between tumor stage III and IV, for sRAGE between stage III tumors and normal skin and for S100A4 between tumors stage IV in comparison to normal skin. However, although no significant difference in sRAGE transcription levels was observed when comparing all stage IV tumors with normal skin, a significant decrease in expression level ($p < 0.05$) was observed for 89% of these tumors. Moreover, although no significant difference could be observe, on average, in the transcript levels of S100A6 and S100A10 between normal skin and melanoma tumors, the finding that 58% of stage IV tumors showed significant ($p < 0.05$) decrease in transcript levels of S100A10 and that 43% of stage III tumors showed a significant ($p < 0.05$) increase in transcript levels of S100A6 supports previous studies performed on both cell lines and tumor tissue samples (Petersson et al. 2009; Ribe and McNutt 2003).

More important than the average values are the differences in transcription levels observed between individual tumor samples: 500 fold for S100B, S100A4, S100A6, 200 fold for S100A10, 100 fold for S100A2. These variations between individual tumors are too large to be the result of tumor tissue content in the biopsied sample. We suggest instead that these differences are intrinsic properties of the tumor itself and that melanoma tumors develop a molecular signature, which can differ between tumors.

Molecular signatures in tumors are an emerging concept. Cluster classification of melanoma tumors according to the expression of several S100 proteins could be useful at several levels: it could help to understand the role of these S100 proteins in the development and progression of the tumor and also help in the diagnosis and prognosis of the cancer. S100B is already an established biomarker in melanoma. S100A2 shows promising properties as well. The presence of large variations in RAGE transcription among melanoma tumors may also indicate a possible role of RAGE in a subset population of melanoma tumors.

Tumor profiling based on gene transcription levels does not necessarily mirror protein levels in the tumor. However, transcript levels can be determined with very high specificity for a given gene and at very low cost in a very fast manner. PCR based diagnostic methods are significantly faster and more economical when compared to traditional immuno-histochemical methods. This is an important consideration when it comes to diagnostic and preventive screening efforts offered to an expanded spectrum of patients. The integration of the genetic profiling of RAGE and S100 transcription in combination with immuno-histological analysis of tumors could result in earlier diagnosis and treatment of melanoma in the future (Kashani-Sabet et al. 2009; Viros et al. 2008). For example, suspicious skin moles are routinely removed by family doctors and send in for immuno-histopathological characterization. A routine PCR based ratio determination of S100B:S100A2 and RAGE:sRAGE may provide an additional and quantitative measure for potential malignancy.

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Review

S100B in schizophrenia: an update

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Abstract. Recent research has supported a potential role of immune pathology in the etiopathogenesis of schizophrenic. In the CNS various viruses were identified in the brains of schizophrenic patients. Pro-inflammatory cytokines were found to be associated with the stage of disease. Microglial cells were reported to be activated in a subgroup of schizophrenic patients in post mortem as well as imaging studies.

New research has demonstrated that astrocytes together with microglial cells are the major immunocompetent cells of the brain and play an important role in the regulation of neuronal proliferation and differentiation. S100B, a calcium binding astrocyte-specific cytokine, presents a marker of astrocytic activation.

Scientific evidence for increased S100B in acute schizophrenia is very consistent. The picture is not as clear regarding schizophrenia subtypes in acute states but patients with persistent negative symptoms or deficit syndrome show constant high S100B concentrations. There is an association between high S100B and poor therapeutic response. The increased S100B concentrations appear to be functionally relevant since they are reflected by poor cognitive performance and cross validation with other methods make it unlikely that the findings are merely an epiphenomenon. These findings suggest that the activation of astrocytes might be an important pathogenic factor for the development of schizophrenia.

Key words: S100B — Schizophrenia — Inflammation — Glia cells

Background

Recent research has supported a potential role of immune pathology in the etiopathogenesis of schizophrenia. Epidemiological studies reported an increased risk to develop schizophrenia for individuals who were in the second trimester of fetal development during an influenza epidemic (Mednick et al. 1988; Sham et al. 1992; McGrath et al. 1994). This finding is supported by a report demonstrating a 7-fold increased risk for schizophrenia in the offspring of mothers in whose blood influenza antibodies were discovered during the first trimester of pregnancy (Brown et al. 2004). It has been hypothesized that there might be a cross reaction between maternal antibodies against influenza and the fetal brain representing a pathogenic process for the development of schizophrenia later in life (Wright et al. 1993). Human endogenous retroviruses (HERVs) and Borna disease virus (BDV) are also suspected to contribute to the development of schizophrenia since higher rates of antibodies and viral sequences were discovered in patients with schizophrenic psychosis (Yolken et al. 2000; Karlsson et al. 2001; Huang et al. 2006; Bechter et al. 1997; Iwahashi et al. 1997). Elevated concentrations of proinflammatory cytokines and phagozyting macrophages in the CSF of patients point towards an inflammatory reaction in the brains of schizophrenics (Licinio et al. 1993; McAllister et al. 1995; Nikkilä et al. 1999; review: Rothermundt et al. 2001a). Also, in a subgroup of schizophrenics microglial activation has been observed with various methods (Bayer et al. 1999; Radewicz et al. 2000; Wierzba-Bobrowicz et al. 2005; van Berckel et al. 2005, 2008) although this finding is not consistent in all studies (Togo et al. 2000; Falke et al. 2000; Arnold et al. 1998; Kurumaji et al. 1997; Steiner et al. 2006b).

Considering these findings astrocytes need to be focused since apart from regulating the extracellular ionic and chemical environment they serve as immunocompetent cells within the brain. They are able to express class II major histocompatibility complex (MHC) antigens and costimula-

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tory molecules (B7 and CD40) that are critical for antigen presentation and T-cell activation. As immune effector cells they influence aspects of inflammation and immune reactivity within the brain e.g. by promoting Th2 responses. In addition, astrocytes produce a wide array of chemokines and cytokines (Dong et al. 2001).

Early studies focusing on astrocytes in schizophrenia were initiated to search for astrogliosis as a sign of neurodegeneration supporting the neurodegenerative hypothesis (Arnold et al. 1998; Arnold 1999; Falkai et al. 1999). None of these studies could demonstrate an increase of astrocyte numbers. On the contrary, Webster et al. (2001) reported a reduction of glial fibrillary acidic protein (GFAP)-immunoreactive astroglia adjacent to blood vessels of the prefrontal cortex. Rajkovska et al. (2002) also found decreased GFAP-positive astrocytes, but in layer V of the dorsolateral prefrontal cortex. In the serum of schizophrenic patients antibodies against astrocyte muscarinic cholinoceptors were reported (Borda et al. 2002).

Microarray studies revealed alterations in astrocyte- and oligodendrocyte-related genes in the brains of schizophrenic patients (Tkachev et al. 2003; Sugai et al. 2004). Matute et al. (2005) discovered a 2.5-fold increase in astrocytic glutamate transporter (GLT-1) mRNA in the prefrontal cortex of schizophrenics. Protein concentration and function of the transporter were also elevated.

The progressive brain volume reduction leading to poor clinical outcome in a subgroup of schizophrenia patients (Gur et al. 1998; DeLisi 1999; Pearlson and Marsh 1999; Lieberman et al. 2001; Shenton et al. 2001) is not caused by a loss of neurons but rather by neuronal pruning and a reduction of neuropil (Harrison 1999; Powers 1999; Selemon and Goldman-Rakic 1999; McGlashan and Hoffman 2000; Jones et al. 2002; Danos et al. 2005). Here the potential influence of glia cells has to be considered as well.

A clinical approach to assess the functionality of astrocytes in patients suffering from schizophrenia is to measure astrocytic markers that can be detected in CSF and serum. This offers the opportunity to investigate patients in various stages of disease including drug-naïve first episode patients. S100B, a protein of the EF-hand type (helix–loop–helix) with 4 Ca^{2+}-binding sites mainly produced by astrocytes, can serve as such a marker. S100B is involved in the regulation of energy metabolism in brain cells. It modulates the proliferation and differentiation of neurons and glia. Furthermore, it interacts with many immunological functions of the brain (for a review see Donato 2003; Heizmann et al. 2007; Rothermundt et al. 2004).

CSF studies

In 2004 Rothermundt et al. demonstrated increased concentrations of S100B in CSF of schizophrenia patients in an acute psychotic episode compared to matched healthy controls. Serum levels concomitantly measured were also elevated correlating closely with the CSF concentrations. Apart from the finding of increased levels in schizophrenia this study revealed that serum concentrations reliably reflect CSF concentrations not only in healthy individuals as has been shown by Nygaard et al. (1998) but also in patients suffering from schizophrenia. This finding is supported by the study of Steiner et al. (2006a) who reported elevated S100B concentrations in the CSF and serum of acute schizophrenia patients compared to healthy controls but no differences regarding GFAP, MBP or NSE. Their conclusion was that there are no hints towards a destruction of astrocytes (GFAP), oligodendrocytes (MBP) or neurons NSE) in schizophrenia. The increase in S100B therefore appears to be caused by an active secretion of S100B from astrocytes.

Serum studies

In the first published study measuring S100B in the serum of schizophrenic patients 20 patients at various stages of disease were investigated (Wiesmann et al. 1999). All patients were on neuroleptic medication. A significantly increased serum concentration of S100B was found in schizophrenic subjects compared to matched healthy controls. No correlation between S100B and age at onset or duration of illness was seen. However, S100B levels tended to be higher in patients with residual symptomatology and with long-term continuous psychotic symptoms without reaching statistical significance potentially due to a lack of statistical power.

A study on 23 schizophrenic outpatients all medicated with antipsychotic drugs (16 on clozapine) reporting significantly lower S100B concentration compared to healthy controls has to be considered with caution (Gattaz et al. 2000). Citrate plasma was used as substrate to measure S100B. However, the assay used is not designed for plasma and the S100B concentrations measured in healthy controls were almost ten times higher than the usual S100B concentrations measured in serum.

In 2001 the first data on 20 medication free schizophrenic patients were published (Lara et al. 2001). The serum of 6 outpatients and 14 patients who had just been admitted for inpatient care contained significantly higher concentrations of S100B than that of the matched healthy controls. No correlations between S100B levels and PANSS total, positive subscale or negative subscale scores could be detected. The total group showed a significant negative correlation with illness duration. However, when one outlier with an extremely high serum level (0.603 μg/l, the only drug naïve patient) was excluded from analysis the negative correlation with illness duration was not significant any more.

Rothermundt et al. (2001b) published a longitudinal study on 26 initially unmedicated or even drug naïve patients suffering from an acute episode of paranoid type schizophrenia. All patients were examined in the acute psychotic stage and after 6 weeks of neuroleptic treatment. Upon admission, the S100B serum level in schizophrenic patients was significantly higher compared to the matched healthy controls. After 6 weeks of treatment the level of significance was no longer reached. However, there was a significant positive correlation between negative symptoms (blunted affect, emotional withdrawal, poor rapport, passive social withdrawal, difficulty in abstract thinking, lack of spontaneity and flow of conversation, and stereotyped thinking) and the S100B concentration after 6 weeks indicating that little change or even deterioration of the negative symptomatology was associated with high S100B levels. Continuously increased S100B levels were associated with the persistence of negative symptoms.

Schroeter and colleagues (2003) reported increased S100B serum concentrations in schizophrenic patients treated with antipsychotic drugs for 3 weeks while untreated patients showed normal values. Patients with deficit schizophrenia had higher S100B concentrations than non-deficit subtypes. The authors concluded that treatment with antipsychotic agents might increase S100B levels. However, since medicated and unmedicated patients belonged to different samples and were not longitudinally studied this conclusion needs to be verified.

The question whether a persistence of negative or deficit symptoms might be associated with increased S100B concentrations was focused in a study including 98 schizophrenic patients with predominant negative symptoms (Rothermundt et al. 2004). The patients were monitored for 24 weeks under standardized pharmacological treatment (risperidone or flupenthixol). S100B serum concentrations were increased throughout the whole study period in comparison to healthy controls. Those patients who showed S100B concentrations that were above the mean plus two standard deviations of the healthy controls showed decelerated therapeutic response compared to patients with lower S100B levels. It was hypothesized that an increased release of S100B might indicate an activated state of glia cells as a response to an unknown inflammatory or degenerative process.

Ryoun Kim and colleagues (2007) observed significantly higher S100B serum concentrations in long-term medicated schizophrenics compared to medication-free recent onset patients. Recent onset patients did not differ from healthy individuals with respect to S100B levels. The study by Schmitt et al. (2005) in 41 elderly chronic schizophrenic patients on stabile antipsychotic medication reproduced the increased S100B serum concentrations but saw a negative correlation with deficit symptoms.

In a very recent study Schroeter and colleagues (2009) observed increased S100B serum concentrations on admission and upon discharge in a sample of chronic schizophrenic patients containing various subtypes and medicated as well as unmedicated patients. Neuron specific enolase (NSE) was unchanged in this sample at all time points. A meta-analysis including 12 studies with n = 380 schizophrenia patients and n = 358 healthy controls revealed a high mean effect size of 2.07 ± 1.85 confirming higher S100B serum levels in schizophrenic patients (Schroeter et al. 2009).

In a treatment study administering Erythropoetin (EPO) to 39 chronic schizophrenic patients with stable disease state and medication suffering from cognitive impairment it was shown that cognitive performance significantly improved in the EPO group compared to the placebo group. Treatment with EPO and cognitive improvement were associated with a decline in S100B serum concentration.

Since negative symptoms and deficit syndrome are often accompanied by cognitive disturbance in schizophrenia a study in 75 patients was performed investigating global verbal memory (AVLT), figural memory (DCS) and abstract rule learning (LPS-3, Pedersen et al. 2008). 40 patients experienced their first episode while 35 patients suffered from chronic schizophrenia. While first episode patients and chronic schizophrenics with normal S100B serum concentration showed no cognitive deficits the chronic patients with significantly increased S100B levels were impaired in memory performance.

In a study by Rothermundt and colleagues (2007) MR-Spectroscopy was carried out in addition to S100B measurements. The rationale behind this study was to include an independent method to evaluate astrocytic activation status. Myo-Inositol is considered the glial marker in MR-Spectroscopy since it indicates energy metabolism which predominantly takes place in glia cells. In this study it could be shown that patients with increased Myo-Inositol show elevated S100B levels and vice versa. It was concluded from this study that increased S100B concentrations indeed indicate glial activation and do not have to be considered an epiphenomenon.

A new aspect regarding S100B metabolism has recently been focused by Steiner and colleagues (2008b) looking at the soluble receptor for advanced glycation products (sRAGE). RAGE, a major receptor for S100B, is located on neurons, glia and many other cell types. The actions of S100B are mainly transduced via RAGE. Soluble RAGE (sRAGE), lacking the transmembrane and signaling domains, is generated by alternative splicing (endogenous secretory RAGE/esRAGE) or by matrix-metalloproteinase induced ectodomain shedding (Geroldi et al. 2006). sRAGE is hypothesized to counteract the detrimental action of RAGE as a competitive inhibitor of the signaling pathway and as ligand scavenger, leading to renal clearance of soluble receptor-ligand complexes. In this study 26 schizophrenic patients were included. In the acute phase S100B and sRAGE were elevated in comparison

to healthy controls. After 6 weeks of treatment and psychopathological improvement S100B returned to normal while sRAGE even further increased. The authors concluded that successful treatment might lead to an increase of sRAGE to serve as a scavenger for increased S100B.

Genetic studies

In humans the gene encoding S100B is located on chromosome 21q22.3. Liu and colleagues (2005) analyzed four relevant SNPs in a sample of 384 patients with schizophrenia and 401 healthy subjects (all Han Chinese). Only rs1051169 showed a marginal association with schizophrenia. However, the haplotype V3(rs1051169)-V4(rs9722) (G-C) was significantly associated with schizophrenia in this study. Hohoff et al. (2009) investigated SNPs of the S100B gene with respect to their relevance regarding S100B mRNA and protein expression. Two SNPs (rs9722, rs11542311) of the S100B-gene were associated with S100B mRNA expression and S100B serum concentration. At least one of the SNPs included in the schizophrenia-relevant haplotype as shown by Liu et al. (rs9722) proved to be relevant regarding mRNA and protein expression in the Hohoff study.

Post mortem studies

In a post mortem study including brain tissue of 18 patients with schizophrenia Steiner and colleagues (2008a) reported more S100B-immunopositive glia in cortical brain regions of schizophrenia patients. This was the case only in the paranoid subtype while residual schizophrenics showed no significant findings. Dean et al. (2006) had reported no differences between brain homogenates (DLPF-C and OF-C) of schizophrenic and healthy subjects but did not specifically look at subtypes of schizophrenia.

Summary and conclusion

Scientific evidence for increased S100B in acute schizophrenia is very consistent. The picture is not as clear regarding schizophrenia subtypes in acute states but patients with persistent negative symptoms or deficit syndrome show constant high S100B concentrations. There is an association between high S100B and poor therapeutic response. The increased S100B concentrations appear to be functionally relevant since they are reflected by poor cognitive performance and cross validation with other methods make it unlikely that the findings are merely an epiphenomenon. Further research is needed to verify the interesting, but preliminary finding regarding sRAGE as scavenger for increased S100B.

Increased S100B concentrations in schizophrenic psychosis are currently considered to reflect glial activation. The question remains whether this activation in fact promotes pathological processes in the brain of patients with schizophrenia. It could well be possible that the activation of glia cells represents an effort of the brain to fight against an unknown pathogenic mechanism such as inflammation of unidentified origin. A persistence of astrocyte activation indicated by increased S100B concentration would then direct towards an ongoing pathogenic process not successfully limited by glial activation.

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Structural diversity of calcium binding sites

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Abstract. Calcium Binding Proteins (CBPs) play a major role in many biological processes. The three dimensional (3D) structure of several CBPs has been resolved by means of X-ray crystallography and nuclear magnetic resonance. We consulted several databases to compile a collection of CBPs of known 3D structure. The analysis of these data shows, the CBP structures are distributed over many different functional families and fold types. The binding site itself is less frequently formed by a continuous sequence segment. In the majority of the cases Ca^{2+} ion coordination is spread over different secondary structure elements with considerable distance on the amino acid sequence. The sidechain of amino acids Asp and Glu are the major interaction partner for the ion. Less frequently it is the side chain of Asn, Gln, Ser and Thr. Often main chain oxygen contributes to the Ca^{2+} coordination. In addition, water molecules are frequently involved.

Key words: Calcium binding — Structural motif

Introduction

Calcium is a key regulator in many biological processes. Binding and release of Ca^{2+} ions changes structural properties of the involved calcium binding proteins (CBPs) such that they switch their state regarding interaction with other protein or performing enzymatic activities (Ikura 1996). By means of X-ray crystallography or nuclear magnetic resonance the 3D structure of a considerable number of CBPs has been resolved. For a subset, Ca^{2+} bound and Ca^{2+} free forms have been determined to investigate the molecular impact of the Ca^{2+} ion.

Here we focus on the three dimensional structure of Ca^{2+} binding sites. We have been interested in the different types and topologies of the secondary structural elements which host the residues involved in Ca^{2+} binding. Three databases, Pfam (Finn et al. 2008), PDB (Berman et al. 2000) and SCOP (Murzin et al. 1995) have been consulted for this purpose. Pfam-A is a manually curated collection of protein families which are represented by multiple alignments and annotated with biological information. PDB is the central public repository for structurally resolved proteins. SCOP, the Structural Classification of Proteins is a hierarchical, expert knowledge supported system, which groups protein domains with respect to their tertiary structure and evolutionary and functional relationships.

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Results

We consulted the databases Pfam (version 23.0), PDB and SCOP (version 1.73) to retrieve structurally resolved proteins associated with Ca^{2+} binding. Pfam has been used to generate a list of families with assigned keywords 'calcium binding' and a link to a PDB entry. PDB and SCOP was used to determine the respective Ca^{2+} binding domain and their type of fold. This way, redundancies and false crosslinks arising from multidomain proteins have been removed. A representative structure was selected for each CBP family and its Ca^{2+} binding site was investigated in detail. Loosely following the SCOP hierarchy, we classify the sites into alpha-helix, beta-sheet and mixed alpha/beta based motifs. In addition we consulted the PROSITE database (Hulo et al. 2008) for relevant sequence pattern.

Table 1 provides a summary of the different types of binding sites including a representative structure and its classification in Pfam and SCOP. It is evident that Ca^{2+} binding sites are found in all SCOP secondary structural classes (a,b,c,d, and g), and in different fold types therein. Indicated by the number of Pfam domain architectures, about half of the CBP motifs are found within single domain proteins representing an integral component of the protein domain

Table 1. Classification of structurally known CBPs

Motif	SCOP code	Scop domain	Pfam ID	Pfam Arch.	Figure
Phospholipase A2	a.133.1.2	d1bp2a_	PF00068	3	2C
EF-hand	a.39.1.5	d1up5b1	PF00036	277	1
Phospholipase C	a.39.1.7	d1djwa1	PF09279	217	4A
Annexin	a.65.1.1	d2rana_	PF00191	18	2A/B
Peroxidase	a.93.1.1	d1hsra_	PF00141	17	4D/E
Carbohydrate binding module	b.18.1.10	d1gmma_	PF03422	135	3D
Collagen-binding domain	b.23.2.1	d1nqda_	PF04151	131	3F
Legume lectin	b.29.1.1	d2bqpa_	PF00139	45	3E
C2-domain	b.7.1.1	d1djwa2	PF00168	217	3H
Pectate lyase	b.80.1.1	d1o88a_	PF00544	18	3A
Hemolysin-type Ca-binding repeat	b.80.7.1	d1kapp1	PF00353	246	3B/C
CutC	c.1.30.1	d1x7ib1	PF03932	2	5B
Leucin rich repeat	c.10.2.6	d1jl5a_	PF00560	539	
Pancreatic lipase	c.69.1.19	d1lpbb2	PF01477	48	4B
Severin	d.109.1.1	d1svya_	PF00626	34	4C
C-type lectin domain	d.169.1.1	d2msba_	PF00059	144	3G
Lactalbumin	d.2.1.2	d1b9oa_	PF00062	4	
Transglutaminase	d.3.1.4	d1ggub4	PF01841	55	5A
LDL repeat	g.12.1.1	d1ajja_	PF00057	203	4F
GLA domain	g.32.1.1	d1j34c_	PF00594	25	5C

Motifs are named according to the Pfam family or by the protein name. Pfam ID: Pfam database accession code. Pfam Arch.: Number of different domain architectures found for the CBP domain. A high numbers indicates that a domain is found in many different multi-domain proteins. SCOP code: indicates class, fold, superfamily and family. SCOP domain: domain identifier; character 2-5 correspond to the four letter PDB code, character 6 is the PDB chain identifier, character 7 the domain number. An underscore means the protein appears as single domain respective 3D structure.

function. The other half are part of multidomain proteins and combined with many other functional domains such as kinases, peptidases or receptors.

Below we describe the different motifs as well as possible repetitive arrangements in the respective CBP domain. Detailed molecular information was derived from PDBsum (Laskowski 2009). USCF Chimera (Pettersen et al. 2004) has been used to visualize and investigate the respective representative molecules and to prepare the corresponding figures.

Alpha-Helix Based Motifs

The EF-hand, a (E)helix-loop-(F)helix supersecondary structure motif, is presumably the best known Ca^{2+} binding site conformation (Finn and Forsen 1995). The helices are arranged in an orthogonal manner. The loop has usually a size of 12 residues (Fig. 1A). The Ca^{2+} ion is predominantly coordinated by residues located in the loop region and one additional residue in the F-Helix. PROSITE defines a sequence pattern which covers a single EF-hand (pattern PS00018). Numerous EF-hand containing proteins have been structurally resolved and could be associated with a typical sequence pattern. EF-hand motifs predominantly appear in an even number of copies. An EF-hand subdomain consists of two of such motifs (Fig. 1B), as found for example in calbindin, S100 proteins or polcalcin. Osteonectin contains one additional helix, parvalbumin contains two additional helices. Calmodulin-like domains represent a duplicated EF-hand subdomain and thus host four EF-hand motifs (Fig. 1C). The SCOP classification currently lists 23 functionally diverse calmodulin-like domains such as calmodulin, troponin C or calcium-dependent protein kinase sk5. SCOP also describes a penta-EF-hand protein family including the proteins calpain and sorcin.

Several single domain EF-hand proteins appear as multimeres. Dimers are found for S100 proteins or polcalcin Phl p 7. Polcalcin Che a 3 is a tetramer. The symmetry and repetitive nature of EF-hand subdomains occasionally leads to domain swapping (Carey et al. 2007). An EF-hand subdomain is built by a continuous region of the two different chains. This has been observed for example in polcalcins or S100 proteins (Fig. 1D/E) (Wopfner et al. 2007).

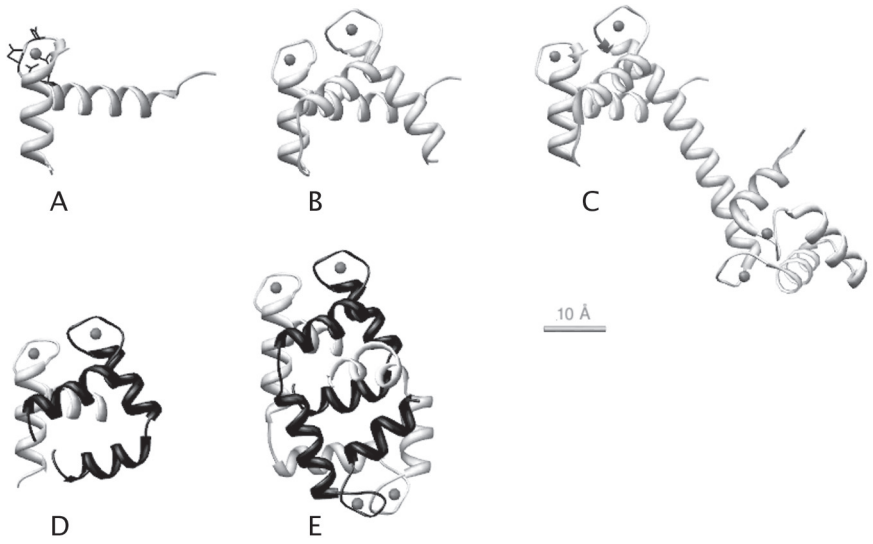

Figure 1. EF-hands and their arrangements in domains. (A) shows a single EF-hand. The Ca^{2+} interacting residues are highlighted in dark grey and the corresponding side chains are drawn sticks. The Ca^{2+} ion is the large grey sphere. Small spheres represent the oxygen of water molecules. (B) Two EF-hands build an EF-hand subdomain. (C) Calmodulin combines two EF-hand subdomains. (D) Domain swapping in Phl p 7: The two differnet chains are in light grey and dark grey respectively. Phl p 7 has two EF-hand subdomains, each composed of EF-hands which come from a different chain. In (E) the complete Phl p 7 is shown.

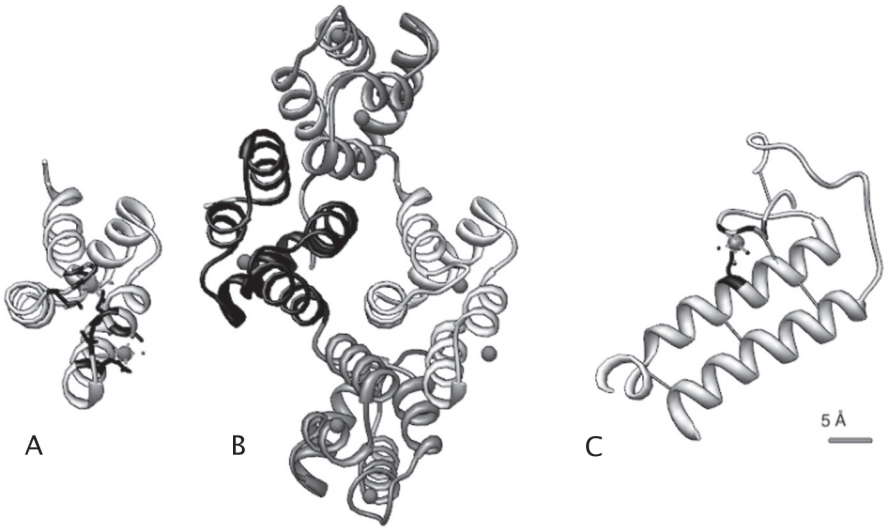

Figure 2. Other alpha helix based motifs. (B) Annexin is composed of four annexin repeats show in different shades of grey. (A) A single annexin repeat binds two Ca^{2+} ions. (C) Ca^{2+} ion binding in phospholipase A2. The complex loop and the helices are fixed by several disulphide bonds (indicated by solid lines).

A similar secondary structure pattern is the basis for the annexin repeat, which consists of a five helix folded leaf (Fig. 2A). The Ca^{2+} ions are bound in the loop region between the first and the second helix and the fourth and fifth helix. The Ca^{2+} binding site loop has about seven residues and therefore is shorter compared to the one in EF-hands, the helices are almost parallel. An annexin repeat is covered by the PROSITE pattern PS00223. The annexin core domain is made up of four repeats (Fig. 2B). In addition to the core domain animal and fungal annexins also have variable amino-terminal domains (Moss and Morgan 2004). Finally lactalbumin has a helix-loop-helix motif for Ca^{2+} binding

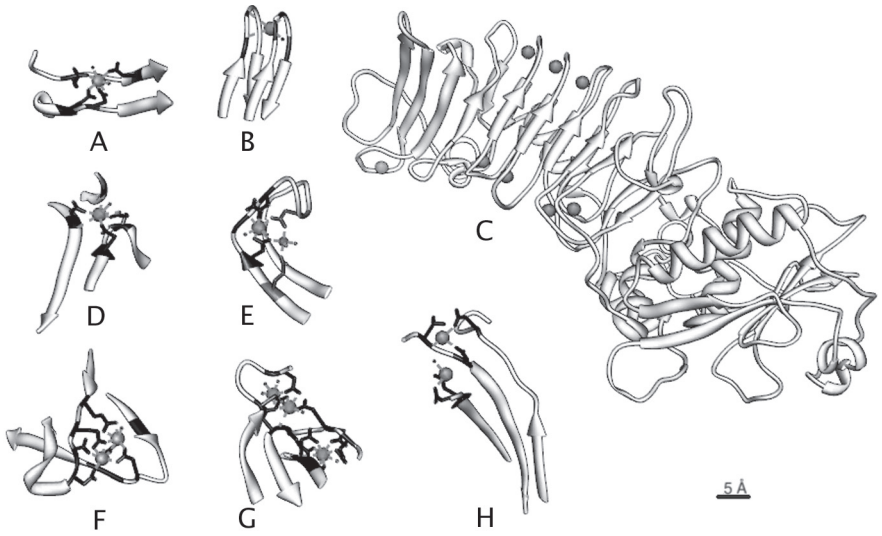

Figure 3. Beta sheet based motifs. (A) Motif from Pectate lyase C. (B) Hemolysin-type repeat of a metalloprotease. (C) Complete metalloprotease with several Ca^{2+} ions bound between consecutive loops. (D) Ca^{2+} binding site in Carbohydrate Binding Module CBM6. (E) In legume lectins there is Ca^{2+} site (larger sphere) near a Mn^{2+} site (middle sized spheres). Other motifs are from the collagen binding domain (F) with two ions bound, from C-lectin (G) which binds three Ca^{2+} ions and from the C2 domain (H).

(Acharya et al. 1989) with the associated PROSITE pattern PS00128.

In contrast to the continuous helix-loop-helix motif, phospholipase A2 and related proteins form a motif where a helix and a large complex loop is involved (Fig. 2C). A disulphide bond connects loop and helix, a second disulphide bond is found within the loop. The loop is rich of glycine residues such that the main chain can form tight turns. Several main chain oxygen atoms establish close contacts to the Ca^{2+} ion. The ion is required for activity in phospholipase A2 (Dennis 1994).

Beta-sheet based motifs

Beta-helix folds are formed by the association of beta strands into a helical topology. In SCOP, two Ca^{2+} binding protein families are found in this fold category. The pectate lyase C protein has a single binding site composed of four Asp/Glu residues located in neighbouring beta strands (Fig. 3A), and resembles the most simple beta-sheet based motif (Herron et al. 2003). In the metalloproteases the binding sites are mainly located between two loops of hemolysin-type calcium-binding repeats (Baumann et al. 1993). The loops themselves are glycine rich and thus rather tight, exposing main chain oxygen atoms (Fig. 3B). Due to highly repetitive nature of sequence and structure up to eight Ca^{2+} ions are bound by a single protein (Fig. 3C). A third class of highly repetitive beta domains is composed of leucin rich repeats (Bella et al. 2008). Several Ca^{2+} ions are bound in the loop regions by Asp and Asn residues.

More complex architecture of the Ca^{2+} binding site can be observed in other beta proteins. The Carbohydrate Binding Module CBM6 from xylanase (Czjzek et al. 2001), which belongs to galactose-binding domain-like fold class, comprise a binding motif, where three different strands and a loop are involved in Ca^{2+} coordination (Fig. 3D). Legume lectins (Sharon and Lis 1990) own a binding site, where two strands and a considerably large loop is involved (Fig. 3E). In addition a manganese ion is bound close to the Ca^{2+}.

In several other beta-sheet domains the Ca^{2+} binding site is located in a loop region. Two Ca^{2+} ions are coordinated by the collagen binding domain of class 1 collagenase (Wilson et al. 2003). The domain is described as CUB-like fold (Fig. 3F). The C2 domain is found in many proteins involved in signal transduction or membrane trafficking (Brose et al. 1995). It binds two Ca^{2+} ions in the loop region, although the topology is different compared to CUB-like folds (Fig. 3H). The C-lectin domain from the mannose-binding protein (Weis et al. 1992) binds three Ca^{2+} ions close to the carbonhydrate binding site (Fig. 3G).

Alpha/Beta based motifs

Phospholipase C (Rhee and Choi 1992) is composed of three domains. We already described the C2 domain in the previous section. The catalytic domain has a TIM-Barrel fold, which is found in many enzymes. In addition to the Ca^{2+} ions in the C2 domain there is also one Ca^{2+} ion located in the catalytic domain (Fig. 4A). It is in close proximity to the

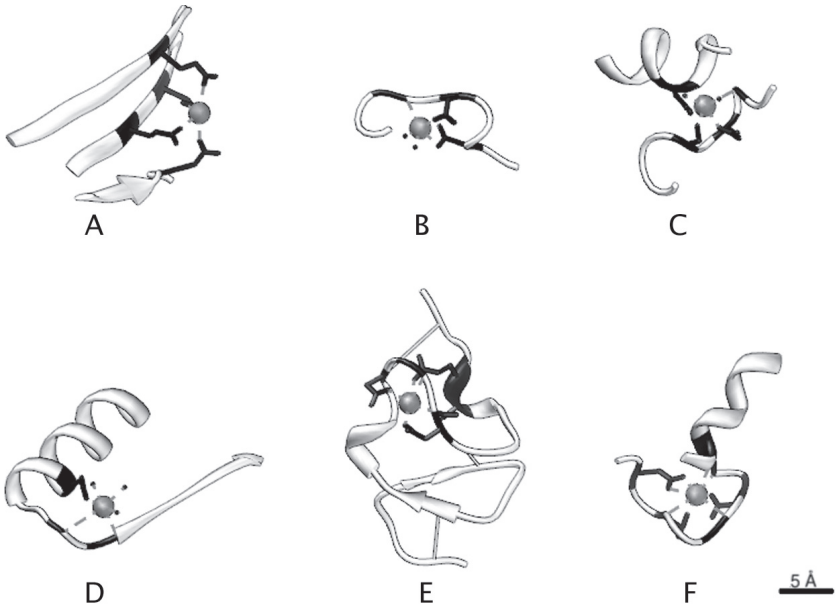

Figure 4. Alpha/beta based motifs. Ca^{2+} binding pattern in phospholipase C catalytic domain (A), N-terminal domain of pancreatic lipase (B), severin (C), heme dependent peroxidase (D and E). In (F) a complete LDL receptor repeat is shown. Disulphide bonds are indicated by solid lines.

catalytic site known to be involved in the reaction (Essen et al. 1997). Interestingly, also the third domain contains potential Ca^{2+} binding sites in form of four EF-hand motifs, although there are no Ca^{2+} ions bound in the X-ray structure. Both, the C2 and EF-hand domain possibly act as feedback regulator for the enzymatic domain.

The N-terminal domain of pancreatic lipase, a alpha/beta-hydrolase fold, contains one Ca^{2+} binding site in a loop region (Egloff et al. 1995) (Fig. 4B). In the severin domain 2 (Fig. 4C), classified in SCOP as gelsolin-like (three layer alpha/beta/alpha) fold, the Ca^{2+} binding site is located between a helix and strand, close to the N-terminal end of the strand (Puius et al. 2000). Heme dependent peroxidase from *Arthromyces ramosus* contains two Ca^{2+} binding sites in different buried regions of the protein (Fig. 4D/E). The structure of the two sites is rather dissimilar(Itakura et al. 1997).

The LDL receptor hosts six Ca^{2+} binding modules at the N-terminus (Fig. 4F). Each module has approximately 40 residues and forms three disulphide bonds. Several of these repeats have been investigated by X-ray(Fass et al. 1997) and NMR (Daly et al. 1995).

Out of the ordinary

In transglutaminase, only a single main chain oxygen from an alanine residues is involved in the Ca^{2+} coordination (Fig. 5A). Apart from that water molecules perform that task (Fox et al. 1999). Solely by main chain O atoms a Ca^{2+} ion is coordinated in the copper homeostasis protein CutC (Gupta et al. 1995) (Fig. 5B). Note that CutC has been structurally determined in a structural genomics project. Although annotated as copper ion binding, such a ion is not found in the different crystal structures.

Several coagulation factors are composed of two domains. The Gla domain is named after the modified amino acid gamma-carboxy-glutamat. The additional carboxy group enables high-affinity binding of calcium ions (Fig. 5C). The Gla fold has low secondary structure content. It undergoes an considerable conformational change on Ca^{2+} binding to form a compact fold, as show by NMR determination of the metal free form (Freedman et al. 1995). The C-type lectin-like domain also binds Ca^{2+}. The involded Glu residues are unmodified.

A different type of Ca^{2+} binding is found in the moderate affinity but high capacity Ca^{2+} binding of Calsequestrin. The protein consists of three identical domains which contain numerous acidic side chains. This yields to a highly negatively charged surface which can accommodate 40-50 Ca^{2+} ions. Binding of Ca^{2+} ions increases the secondary structure content of Calsequestrin (Wang et al. 1998).

Prediction of Ca^{2+} binding sites

It would be desirable to predict potential Ca^{2+} binding sites in proteins. As seen from the data above, the Ca^{2+} binding

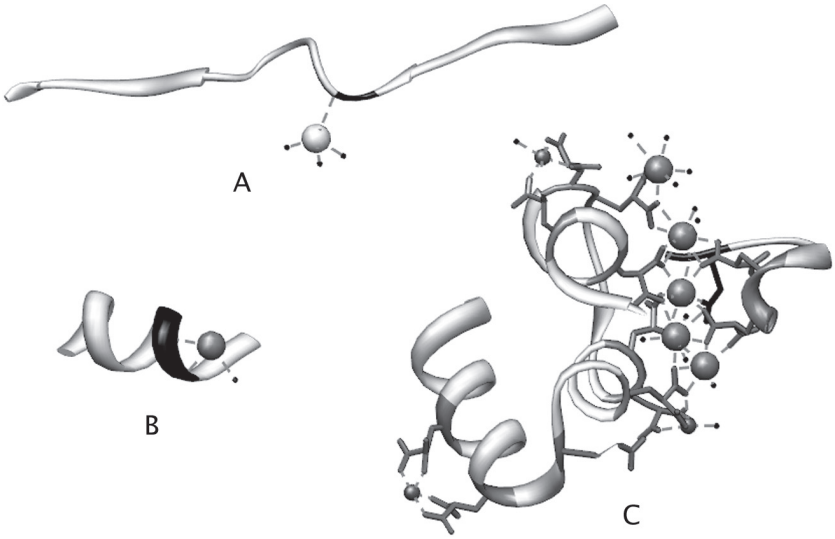

Figure 5. Unusual binding. Only coordinated by main chain oxygen is the Ca^{2+} in transglutaminase (A) and CutC (B). Modified Glu residues (gamma-carboxy-glutamate) bind the Ca^{2+} ion in Gla domains (C), the middle sized spheres are magnesium ion.

is rarely local in sequence. Furthermore, main chain oxygen atoms of different amino acids are involved which impedes to deduce strong sequence signals. Therefore sequence based methods hardly succeed. PROSITE defines at least a few pattern which match the Ca^{2+} binding region, e.g. for EF-hand and lactalbumin.

Therefore the 3D structure seems to be the better target for prediction methods. A few methods to predict potential Ca^{2+} binding sites in known 3D structures have been implemented. They are based on geometrical rules combined with atom types or charge distributions which describe potential binding cavities (Nayal and Di Cera 1994; Deng et al. 2006). These approaches should be applicable regardless the Ca^{2+} ion is bound by side chain or main chain oxygen. But however, this will also run into troubles if water molecules are involved.

Summary

Ca^{2+} ion binding sites are structurally diverse. It does not appear that a certain type or arrangement of secondary structure elements is preferred by nature. In several cases, a loop is involved. Especially if Ca^{2+} coordination is accomplished by the main chain oxygen atoms, glycine rich tight loops are incorporated. Water molecules may mediate Ca^{2+} coordination via H-bonds. Most of the binding sites are discontinuous regarding the involved residues in the proteins sequence. This complexity results in a challenge for the development of bioinformatics methods for binding site detection and prediction.

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Review

Does any relationship exist between P-glycoprotein-mediated multidrug resistance and intracellular calcium homeostasis

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Abstract. Multidrug resistance (MDR) of neoplastic tissue represents a real obstacle to the effective chemotherapy of cancer. Several mechanisms of MDR were identified, from which the over-expression and efflux activity of P-glycoprotein (P-gp) – a plasma membrane ATPase (ABCB1 member of ABC transporter family) – represents the most commonly observed reason for neoplastic disease chemotherapy malfunction. The process of P-gp-mediated MDR seems to be related to intracellular calcium homeostasis, at least indirectly, for the following reasons: i. substances blocking calcium influx through L-type of calcium channels like verapamil were often found to antagonize P-gp-mediated MDR; ii. calcium signal abnormalities were observed in cells over-expressing P-gp; iii. cells with P-gp-mediated MDR were often resistant to thapsigargin; iv. several differences in intracellular calcium localization were observed when P-gp-negative and P-gp-positive cells were compared; and v. differences in the contents of several proteins of the endoplasmic reticulum involved in calcium homeostasis were observed to be associated with P-gp over-expression. This current study represents an attempt to summarize the knowledge about the possible relationship between P-gp-mediated MRD and intracellular calcium homeostasis.

Keywords: P-gp-mediated MDR — Calcium homeostasis — Ca^{2+}-antagonists — Calcium binding proteins — Endoplasmic reticulum

Introduction

P-glycoprotein (P-gp) represents a transport ATPase of the plasma membrane that is responsible for the efflux of diverse substances (including cancer chemotherapeutic agents) out of animal cells (Breier et al. 2005). This protein is a member of the ABC (**A**TB-**B**inding **C**assette) transporters family and is characterized by broad substrate specificity for hydrophobic molecules with high membrane affinity (Pawagi et al. 1994). We have proposed that for recognition at P-gp binding sites, a substrate should possess the following characteristic features: (i) flexible structure giving rise to different structural conformers; (ii) molecular weight lower than 1,300 g/mol; and (iii) existence of a non-charged tertiary amino group at neutral pH (Breier et al. 2000). Over-expression of P-gp in neoplastic cells confers a lack of cell sensitivity to P-glycoprotein substrates that represents the most often observed mechanism of multidrug resistance as a real obstacle to effective chemotherapy of cancer (Zhou 2008).

Several authors have focused on whether a relationship exists between processes controlled by intracellular calcium homeostasis and P-gp-mediated multidrug resistance. These investigators were inspired by following data: i. Substances blocking calcium influx through L-type calcium channels like verapamil were often found to antagonize P-gp-mediated MDR (Barancik et al. 1994; Kimura et al. 2004); ii. Calcium signal abnormalities were observed in cells over-expressing P-gp (Witkowski and Miller 1999); iii. Cells with P-gp-mediated MDR were often resistant to thapsigargin (Gutheil et al. 1994; O'Neill et al. 2006; Seres et al. 2008; Wagner-Souza et al. 2003); and iv. Differences in the contents of several proteins of the endoplasmic reticulum involved in calcium homeostasis were detected in P-gp expressing cells (Seres et al. 2008).

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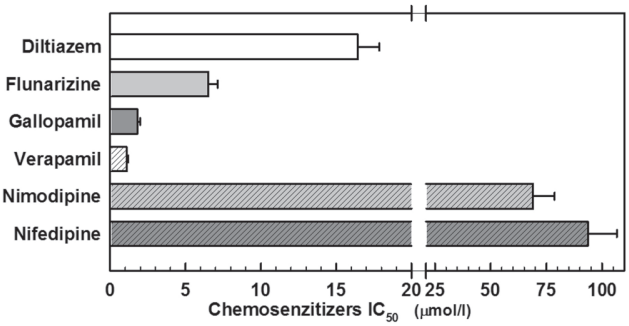

Fig. 1. Values of IC50 characteristic of calcium-channel blockers effects on the P-gp-mediated vincristine resistance of L1210/VCR cells. Drugs were added directly to the cultivation medium for cell cultivation (Barancik et al. 1994). The effects of drugs were measured in the presence of 0.2 mg/l of vincristine that exerts a total cell death effect on parental P-gp-negative cells and did not influence the proliferation activity of P-gp-positive L1210/VCR cells. Calcium blockers in the applied concentration did not significantly influence the proliferation activity of L1210/VCR cells. Data are expressed as mean ± S.E.M. for nine independent values.

The current paper represents a state of the art review of the available knowledge on the multidrug resistance of neoplastic cells (mediated by P-gp) associated with alterations in intracellular calcium homeostasis.

Calcium entry blockers as P-gp antagonizing agents

Substances that are able to antagonize P-glycoprotein-mediated multidrug resistance represent a diverse group of chemicals with different structures and mechanisms of primary pharmacological action, including calcium channel blockers, calmodulin antagonists and many others (Ford 1996). The effectiveness of calcium channel blockers indicated that their MDR-antagonizing effect may be directly related to their action on calcium entry and, consequently, to intracellular calcium concentration regulation. We described the effects of verapamil, galopamil, diltiazem, flunarizine, nimodipine and nifedipine on the reduction of P-glycoprotein-mediated vincristine resistance of L1210/VCR cells (Barancik et al. 1994). All these drugs altered the resistance of cells to vincristine in a concentration-dependent manner with different effectiveness (IC50 values for these actions are summarized in Fig. 1). However, no correlation between the effectiveness of these substances in the reduction of calcium channel activity and P-gp antagonizing activity was observed. Therefore, the idea that the P-gp antagonizing activity of calcium channel blockers is directly related to their effect on calcium entry is improbable. Consistent with the latter idea, stereoisomers of calcium antagonists that differ markedly in their potencies as calcium blockers were equally effective in modulating the drug transport by P-glycoprotein (Hollt et al. 1992). Interestingly, both verapamil and nifedipine stimulated the ATPase activity of P-gp, i.e., they may act also as substrates of P-gp (Shapiro and Ling 1994). The dual function of several substances as both substrates and inhibitors of P-gp was described by Didziapetris et al. (2003) and represents relevant confusion in his attempt to classify the P-gp substrates. Moreover, verapamil was proved to interact with P-gp directly, and P-gp may be specifically labeled by its fluorescent derivatives (Safa 1988). A direct interaction with P-glycoprotein could not be postulated as a common feature of all calcium blockers. The newly synthesized calcium entry blocker, SR33557, with several structural similarities to verapamil failed to label P-gp (i.e., protein band with molecular weigh about 170 kDa) but labeled another protein with a molecular weight of about 65 kDa (Jaffrezou et al. 1991). Labeling of this protein was found to be antagonized by diltiazem and nifedipine but not by verapamil. While verapamil was proved to inhibit P-gp transport activity by direct interaction with a P-gp molecule effect of verapamil on P-gp-mediated MDR could also include alterations in P-gp expression because this drug was proved to significantly reduce the P-gp protein levels (Sulova et al. 2008; Takara et al. 2002). All of the above facts indicated that calcium antagonists are overcoming P-gp-mediated MDR by mechanisms distinct from their inhibitory effect on voltage-dependent calcium channels. These mechanisms include not only direct interaction with the P-gp protein but also interactions with several other proteins that may indirectly alter P-gp transport/activity.

Role of calmodulin and other calcium binding proteins in P-gp mediated MDR

The fact that calmodulin inhibitors like neuroleptics potently reduce P-gp-mediated MDR indicates that calmodulin may also play a role in this phenotype. Consistent with this, calmodulin was proved to interact directly with P-gp (Schlemmer et al. 1995). A more potent interaction was observed in the absence than in presence of calcium, which was associated with calcium-dependent restoration of the P-gp ATPase activity inhibited by calmodulin. We found a statistically significant correlation between the MDR reversal effects of several substances (neuroleptics, local anesthetics and calcium entry blockers) and their affinity to calmodulin using P-gp-positive L1210/VCR cells (Barancik et al. 1994). Moreover, the applied substances were effective against P-gp-mediated MDR via an interaction with something located in the internal space of the cells. Trifluoperazine improved the doxorubicin sensitivity in P-gp-positive L1210/DOXO cells and, moreover, induced an increase of P-glycoprotein

phosphorylation (Ganapathi et al. 1991). In the similar cell model, i.e., L1210/adr, trifluoperazine induced a lower expression of P-gp (Shin et al. 2006). The mechanism of calmodulin inhibitory action on P-gp-mediated MDR is unknown but may be related to the effect of substances on the activity of calmodulin kinase II. Activation of this kinase by substances that induce the increase of the intracellular calcium concentration, like the sodium pump inhibitors (oubain and digoxin) or SERCA inhibitors (artemisinin and parthenolide) potentiated HIF-1α phosphorylation and the induction of P-gp expression (Riganti et al. 2009a; Riganti et al. 2009b). Thus, the interaction of calmodulin inhibitors with calmodulin may oppositely reduce the activity of calmodulin kinase II and consequently cause a decrease in P-gp expression.

Another calcium-binding protein that was described to be related to P-gp-mediated MDR is sorcin (Sugawara et al. 1989). Sorcin was found to be over-expressed in cells expressing P-gp and was closely associated with free ribosomes, rough endoplasmic reticulum, mitochondria, microfilament bundles and perinuclear membranes. Sorcin is a 22 kDa protein that: i. Modulates the cardiac L-type Ca^{2+} current (Fowler et al. 2009), probably by a functional interaction with the alpha1C subunit (Fowler et al. 2008) and ii. Interacts with the calcium/calmodulin-dependent protein kinase and indirectly modulates the ryanodine receptor function (Anthony et al. 2007). A significant correlation between the contents of mRNA encoding sorcin and P-glycoprotein was described in leukemic blast cells of 65 acute myeloid leukemia patients (Tan et al. 2003). The same author described that sorcin over-expression was associated with poor clinical outcomes. However, the over-expression of sorcin alone induces a low level of paclitaxel resistance in human ovarian and breast cancer cells by P-gp-independent mechanisms (Parekh et al. 2002). On the other hand, the knock-down of sorcin induces the up-regulation of MDR1 in HeLa cells (Kawakami et al. 2007). Thus, it could be concluded that the over-expression of sorcin may cause the reduction of cell sensitivity to diverse drugs by mechanisms independent from P-gp. P-gp-mediated MDR and resistance associated with over-expression of sorcin may exist together in the same cells.

Calcium signal abnormalities were observed in cells over-expressing P-gp

P-gp-positive T cells exert smaller elevations of the intracellular calcium ion concentration induced by Con A, anti-CD3 antibodies or ionomycin than P-gp-negative cells (Witkowski and Miller 1999). The latter authors discussed the differences in intracellular calcium signaling by the higher expression of sorcin that may influence intracellular calcium homeostasis. For the measurement of intracellular calcium content, they used indo-1/AM as an intracellular calcium indicator. However, intracellular calcium indicators are known as P-gp substrates (Breier et al. 2005). Low levels of accumulation of calcium indicators in P-gp-positive cells that could be antagonized by P-gp inhibitors were described for: i. Indo-1/AM (Brezden et al. 1994); ii. Fura-2/AM (Fu et al. 1997; Fu et al. 2002); iii. Calcein/AM (Eneroth et al. 2001; Karaszi et al. 2001); iv. Fluo-3/AM (Orlicky et al. 2004; Van Acker et al. 1995). For this reason, the measurement of the intracellular calcium concentration by calcium indicators in P-gp-positive cells may produce incorrect results.

Therefore, to resolve the question if any differences in calcium entry exist between P-gp-negative and -positive cells, we had to measure differences in $^{45}Ca^{2+}$ uptake between sensitive L1210 and resistant L1210/VCR cells (Sulova et al. 2005). The $^{45}Ca^{2+}$ uptake was more pronounced in resistant cells and could not be antagonized by verapamil (Fig. 2). This indicates the fact that calcium entry into P-gp-positive cells is more effective than in P-gp-negative cells. Consistent with this, a higher intracellular calcium concentration in P-gp-positive MCF-7 cells as compared with its P-gp-negative counterparts was described (Mestdagh et al. 1994). The lack of the ability of verapamil to alter calcium uptake to L1210 and L1210/VCR cells indicate that there are other systems responsible for calcium entry besides voltage-dependent calcium channels. Consistent with this, Tarabova and Lacinova (from the Institute of Molecular Physiology and Genetics SAS) measured calcium currents in L1210 and L1210/VCR cells by the whole-cell patch-clamp technique, and they did not find any calcium channel that fulfills the criteria characteristic for a voltage-dependent one. Sensitive L1210 and resistant L1210/VCR cells differ not only in the level of calcium uptake but also in intracellular calcium localization detected by cytochemical precipitation methods using electron microscopy (Sulova et al. 2005). In sensitive cells, calcium precipitates were found to be localized predominantly extracellularly along the surface of cells and within mitochondria, frequently delineating the cristae. In resistant cells, precipitates were also found inside of nuclei, predominantly at the border of heterochromatin clumps and scattered within the cytoplasm, probably corresponding to elements of the endoplasmic reticulum and masking to some extent the image of the mitochondria. Thus, P-gp-positive and P-gp-negative cells differ in the effectiveness of calcium uptake and its intracellular localization. Interestingly, application of high calcium concentrations in extracellular space, which magnified differences in calcium uptake when L1210 and L1210/VCR cells were compared (Sulova et al. 2005), were also improving P-gp expression in clonal parathyroid epithelial cells (Axiotis et al. 1995).

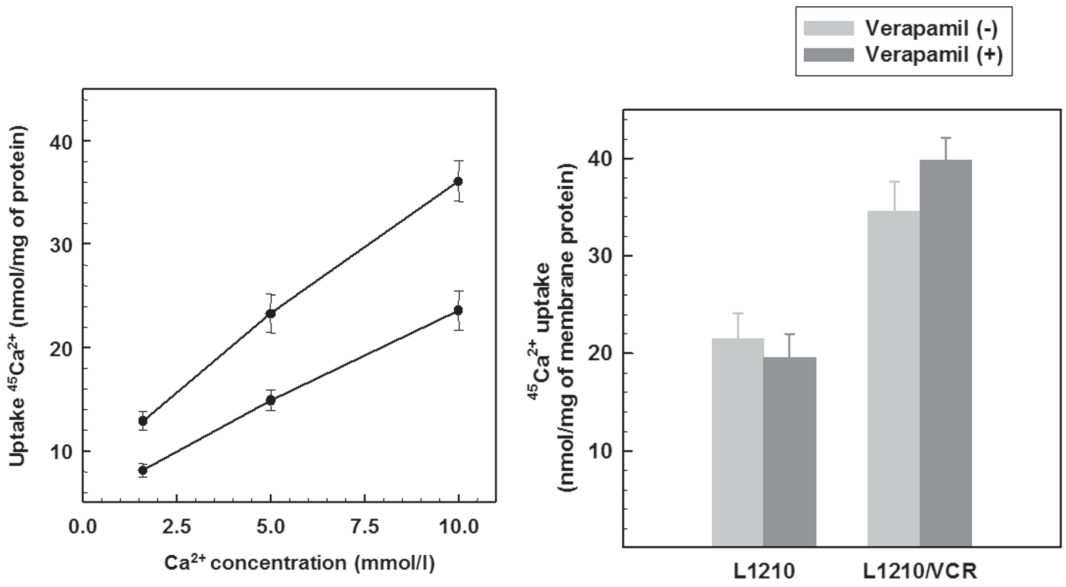

Fig. 2. Differences of $^{45}Ca^{2+}$-uptake by P-gp-negative L1210 and P-gp-positive L1210/VCR cells. Cells were incubated in 20 mmol/l Mops with 10 mmol/l glucose, 117 mmol/l NaCl, 3 mmol/l KCl, and 1 mmol/l $MgSO_4$ supplemented with 1.6, 5.0 and 10.0 mmol/l $CaCl_2$ labeled by [$^{45}Ca^{2+}$] (Amersham, UK) to a specific radioactivity of 24 GBq/mol (final cell density of 3×10^6 cells/ml in 2.5 ml) in 24-well tissue cultivation plates in a humidified atmosphere with 5% CO_2 at 37 °C for 20 min. Then, 200 µl aliquots were removed, filtered on Whatman GF/A glass fiber filters (Whatman) and washed three times with 5 ml ice-cold medium supplemented with 1 mmol/l $LaCl_3$ and 5 mmol/l EGTA. The filters were dried, and the radioactivity was measured in SLT scintillation cocktail on a liquid scintillation counter Beckman LS 6500. The effects of verapamil (10 µmol/l) on Ca^{2+} uptake were also measured. Verapamil at these concentrations induced a total inhibition of P-gp transport activity in L1210/VCR cells measured by calcein/AM as a substrate (Orlicky et al. 2004). Left panel: Concentration dependency of $^{45}Ca^{2+}$ uptake. Symbols: circles – L1210 cells; diamonds – L1210/VCR cells. Right panel: Effect of verapamil on $^{45}Ca^{2+}$ uptake. Data are expressed as mean ± S.E.M. for nine independent values.

Resistance of P-gp positive cells to thapsigargin and changes in protein expressions involved in intracellular calcium homeostasis

Thapsigargin is a well-known inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-pump, SERCA. The inhibitory action of thapsigargin on this transport ATPase will induce intracellular calcium mobilization and calcium depletion from sarco/endoplasmic reticulum as a major intracellular calcium store. Thapsigargin was found to be a P-gp substrate, i.e., it may be eliminated from the cytoplasm in P-gp-positive cells (Gutheil et al. 1994). Thus, the expression of P-gp in the cells may also cause a resistance to thapsigargin. In P-gp-positive cells, thapsigargin did not induce the calcium mobilization effect that was observed in P-gp-negative cells (Wagner-Souza et al. 2003). However, resistance to thapsigargin can involve not only alterations in P-gp expression but also in SERCA isoform expression (Gutheil et al. 1994). L1210/VCR cells were also found to be resistant to thapsigargin (Seres et al. 2008). However, this resistance could not be explained only by the thapsigargin efflux by P-gp for the following reason. The efflux activity of P-gp in L1210/VCR cells may be fully inhibited by the presence of 10 µmol/l of verapamil (Orlicky et al. 2004). However, a total blockade of P-gp activity by verapamil (at this concentration) in L1210/VCR cells did not restore their sensitivity to thapsigargin to the level observed in L1210 cells (Seres et al. 2008). Consistent with this, thapsigargin-induced calcium mobilization could not be achieved even if P-gp was inhibited by verapamil or cyclosporine A (Wagner-Souza et al. 2003). The latter authors showed in the model of the human leukemic cell line K562, selected for its resistance to vincristine, that cross-resistance to thapsigargin is not associated with an elevation of SERCA expression or with the prevalence of thapsigargin less-sensitive SERCA isoforms.

In L1210/VCR cells, the over-expression of P-gp was associated with the depression of SERCA 2 expression (Seres et al. 2008). Ryanodyne receptor expression was found to be unchanged, and IP3 receptor expression was depressed only when L1210/VCR cells were cultivated prior to estimation in the presence of vincristine. The obtained data indicated that L1210 and L1210/VCR cells did not differ from the point of view of expression of calcium-induced calcium release channels. Thus, release of calcium ions from the endoplasmic reticulum should be of similar extent in both cell counterparts. On the other hand, a decrease in SERCA2 expression

in L1210/VCR cells as compared with L1210 cells indicated slower calcium ion uptake to the luminal space of the endoplasmic reticulum. Thus, the predominance of calcium release over calcium uptake should take place in L1210/VCR cells when compared with L1210 cells. This indicated that in P-gp-positive L1210/VCR cells, the calcium intracellular concentration has to be higher than in P-gp-negative L1210 cells. A higher intracellular calcium concentration in P-gp positive MCF-7 cells as compared with its P-gp negative counterparts was also described (Mestdagh et al. 1994). In lymphoid leukemia cells, expression of the SERCA3 isoform may also take place (Papp et al. 2004). Whether depression of SERCA2 content in L1210 cells is partially compensated by an increase in SERCA3 expression remains to be elucidated. However, SERCA3 is known to have a lower affinity for calcium than other isoforms (Wuytack et al. 1995). Thus, the prevalence of SERCA3 over SERCA2 expression has to induce an increase in intracellular calcium concentration (Papp et al. 2004).

Calnexin – a Ca^{2+}-dependent molecular chaperone of the endoplasmic reticulum – was described to be involved in the quality control of newly synthesized P-gp molecules in the endoplasmic reticulum (Loo and Clarke 1994). Only a P-gp molecule with an accurate structure was able to escape association with calnexin in the endoplasmic reticulum and be targeted to the plasma membrane. The interaction of calnexin and the non-mature protein molecule depends on the calcium content in the endoplasmic reticulum. Therefore, substances like thapsigargin that inhibit endoplasmic reticulum calcium pumps consequently induced a lowering in the amount of stored calcium ions in the endoplasmic reticulum and may have influenced the interaction between calnexin and non-matured protein. Consistent with this, thapsigargin induced an escape of the mutant ΔF508-CFTR protein (Cystic Fibrosis Transmembrane conductance Regulator – ABCC7 member of ABC protein family) and its functional cell surface localization (Egan et al. 2002). This phenomenon, however, was not fully proved because other authors were not able to induced CFTR cell surface localization with the SERCA inhibitors curcumin and thapsigargin (Grubb et al. 2006; Loo et al. 2004). Loo and colleagues (2004) also described the inability of both SERCA inhibitors to induce the escape of mutant P-gp from the interaction with calnexin and its functional cell surface localization. All of the above reasons motivated us to measure if there were any differences in the calnexin expression between P-gp-positive L1210 and P-gp-negative L1210/VCR cells. The expression of calnexin was considerably decreased in our resistant cell variant that expressed P-gp (Seres et al. 2008). However, the P-gp protein was present in the material obtained by immuno-precipitation with anti-calnexin antibody of the cell homogenate originating from L1210/VCR cells. Thus, calnexin acts as a P-gp chaperone also in L1210/VCR cells, even if its amount in this cell variant is considerably reduced. These facts indicated that P-gp-positive cell variants may differ from parental cells also in protein maturation, i.e., in protein glycosylation reactions. Interestingly, we described real alterations between the compositions of sugar parts of glycoproteins located on the surface of L1210 and L1210/VCR cells (Fiala et al. 2003; Sulova et al. 2009).

Conclusions

Several facts indicated that some relationship between intracellular calcium homeostasis and P-gp-mediated MDR exists. However, straight connections between intracellular calcium-dependent processes and the regulation of P-gp expression or efflux activity are still unknown. They may involve intracellular calcium concentration regulation, calcium-dependent protein phosphorylation, quality control of newly synthesized protein via calcium dependent molecular chaperons and others. In the end, it could be stressed that P-gp-mediated MDR in neoplastic cells is linked to intracellular calcium homeostasis, but the mechanisms and meaning of this interplay are still unclear.

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Review

Calcium binding chaperones of the endoplasmic reticulum

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Abstract. The endoplasmic reticulum is a major Ca^{2+} store of the cell that impacts many cellular processes within the cell. The endoplasmic reticulum has roles in lipid and sterol synthesis, protein folding, post-translational modification and secretion and these functions are affected by intraluminal endoplasmic reticulum Ca^{2+}. In the endoplasmic reticulum there are several Ca^{2+} buffering chaperones including calreticulin, Grp94, BiP and protein disulfide isomerase. Calreticulin is one of the major Ca^{2+} binding/buffering chaperones in the endoplasmic reticulum. It has a critical role in Ca^{2+} signalling in the endoplasmic reticulum lumen and this has significant impacts on many Ca^{2+}-dependent pathways including control of transcription during embryonic development. In addition to Ca^{2+} buffering, calreticulin plays important role in the correct folding and quality control of newly synthesized glycoproteins.

Key words: Calcium binding — Chaperones — Endoplasmic reticulum

Introduction

The endoplasmic reticulum (ER) of eukaryotic cells is an extensive, continuous network of membrane tubules and is a separate metabolic compartment that houses many functions critical to the survival of a cell (Baumann and Walz 2001; Schroder 2008). The ER lumen provides a unique environment with a high concentration of Ca^{2+} binding proteins which directly influences the functioning of the ER such as its roles in Ca^{2+} storage and release, membrane and secretory protein synthesis and folding including post-translational modifications such as N-linked glycosylation and the formation of disulfide bonds, lipid and sterol synthesis and metabolism and signal transduction (Michalak et al. 2002; Schroder 2008). Many ER Ca^{2+} binding proteins have dual functions and are also molecular chaperones involved in protein folding and quality control (Ashby and Tepikin 2001). The functions of these chaperones and formation of folding complexes is dependent on Ca^{2+} concentrations (Ashby and Tepikin 2001). ER luminal Ca^{2+} impacts all downstream functions of the ER including apoptosis, stress response, organogenesis and transcriptional activity (Michalak et al. 2002). The protein folding machinery of the ER is highly sensitive to ER luminal Ca^{2+} fluctuations and the ER has evolved a sophisticated system of quality control as well as an unfolded protein response (UPR) pathway in order to deal with mis-folded proteins. Impaired function of the ER leads to many severe diseases (Michalak et al. 2002). This review will focus on ER luminal Ca^{2+} and the ER luminal Ca^{2+} buffering chaperones, their role in ER-dependent Ca^{2+} homeostasis and how they impact quality control in the secretory pathway.

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ER, a calcium storage organelle

Cytoplasmic Ca^{2+} is a versatile signalling molecule affecting many cellular functions including exocytosis, contraction, metabolism, transcription, fertilization and proliferation (Berridge et al. 2003). The ER is the major intracellular Ca^{2+} store in the cell. The total ER Ca^{2+} concentration is estimated to be 2 mmol/l while the free ER Ca^{2+} concentration varies from 50 to 500 μmol/l (Groenendyk 2006; Meldolesi and Pozzan 1998). This is magnitudes higher than the free cytoplasmic Ca^{2+} level which is approximately 100 nmol/l (Michalak et al. 2009). The ER Ca^{2+} stores play an essential role in Ca^{2+} signalling. The buffering of ER luminal Ca^{2+} is critical to the many diverse ER functions. Overload and depletion of ER Ca^{2+} stores have detrimental effects on the entire cell. Ca^{2+} release from ER stores is controlled by the inositol 1,4,5-triphosphate ($InsP_3$) receptor and the ryanodine receptor (RyR) (Taylor and Laude 2002). ER

Ca^{2+} store refilling is controlled by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Lipskaia et al. 2009) while the Na^{+}/Ca^{2+} exchanger and the plasma membrane Ca^{2+} ATPase actively remove Ca^{2+} from the cells (Rhodes and Sanderson 2009). Taken together, both the ER Ca^{2+} buffering proteins and the ER pumps and exchangers exert powerful effects via ER Ca^{2+} fluctuations on the varied functions of the ER (Frischauf et al. 2008). Store-operated calcium influx occurs when there is a depletion of ER Ca^{2+} stores which activates channels in the plasma membrane to refill the internal stores (Putney 1986). High throughput RNAi screens led to the identification of stromal interaction molecule 1 (STIM1) that functions as an ER Ca^{2+}-sensor that accumulates in punctuate close to the plasma membrane upon store-depletion (Frischauf et al. 2008). It clusters at the plasma membrane with the Ca^{2+} channel, Orai1, to activate Ca^{2+} influx (Frischauf et al. 2008).

Calreticulin, a major calcium buffering chaperone of the ER

Calreticulin is a 46-kDa ER resident Ca^{2+} binding and buffering protein and molecular chaperone (Michalak et al. 2009). Calreticulin contains ER targeting signal sequence and it terminates with ER retrieval signal, Lys-Asp-Glu-Leu-COOH (KDEL) (Fliegel et al. 1989). The protein has been implicated to play a role in many diverse cellular processes (Michalak et al. 2009). Many of these functions are due to calreticulin's role as an ER Ca^{2+} buffering protein (Michalak et al. 2009). The protein can be divided into three major structural and functional domains (Fliegel et al. 1989; Nakamura et al. 2001b; Ostwald and MacLennan 1974). The N-domain (residues 1-180) of calreticulin contains both polypeptide- and carbohydrate binding sites and, together with the P-domain, it is critical to the chaperone function of the protein (Kapoor et al. 2004; Leach et al. 2002). Within the N-domain, there are specific amino acid residues that contribute to oligosaccharide binding and conformational stability of the protein (Kapoor et al. 2004; Leach and Williams 2003; Martin et al. 2006; Thomson and Williams 2005). The P-domain (residues 181-290) immediately follows the N-domain and forms a flexible arm domain (Ellgaard et al. 2001a; Ellgaard et al. 2001b). This central proline-rich core is characterized by three copies of two repeat amino acid sequences (denoted type 1 and 2) and are arranged in a "111222" pattern (Ellgaard et al. 2001a). These repeats may play a role in oligosaccharide binding contributing to the lectin-like function of calreticulin. They may also be involved in forming complexes between calreticulin and ERp57, an oxidoreductase folding enzyme of the ER (Vassilakos et al. 1998). Indeed, NMR studies revealed that ERp57 docks on the tip of the P-domain of calreticulin (Frickel et al. 2002). This may involve specific amino acid residues including Glu^{239}, Asp^{241}, Glu^{243}, Trp^{244} (Frickel et al. 2002; Leach et al. 2002; Martin et al. 2006). Interestingly, *in vitro* studies indicate that the P-domain binds Ca^{2+} with a high affinity (K_d = 1 μmol/l) and a low capacity (1 mol of Ca^{2+} per mol of protein) (Baksh and Michalak 1991). The third domain of calreticulin is the low affinity (K_d = 2 mol/l), high capacity (25 mol of Ca^{2+} per mol of protein) Ca^{2+} binding C-domain (residues 291-400) (Baksh and Michalak 1991; Nakamura et al. 2001b). This domain is responsible for binding over 50% of Ca^{2+} in the ER (Nakamura et al. 2001b). The Ca^{2+} binding capacity of the C-domain is derived from large clusters of acidic amino acid residues consisting of aspartic and glutamic acid interrupted with basic residues of lysine and arginine (Breier and Michalak 1994). Interruption of the basic residues, interestingly, results in a reduction in the Ca^{2+} binding capacity of calreticulin (Breier and Michalak 1994).

Mutational analysis revealed that specific amino acid residues are required for the chaperone function of calreticulin (Guo et al. 2003; Martin et al. 2006). Trp^{302} in the carbohydrate binding N-domain as well as Trp^{244} at the tip of the P-domain are both critical to the chaperone function of calreticulin (Martin et al. 2006). The N-domain amino acid His^{153} was found to not only be essential for chaperone function but also important in the structure of calreticulin (Guo et al. 2003). Surprisingly, when the disulfide bridge in the N-domain was disrupted (Cys^{88} and Cys^{120}) there was only partial disruption of the chaperone function of calreticulin (Martin et al. 2006). Overall, conformational changes of calreticulin resulting from mutations in specific amino acids residues of the protein or due to fluctuations in Ca^{2+} concentrations of the ER lumen impact the functionality of the chaperone function of the protein (Corbett et al. 2000; Guo et al. 2003; Martin et al. 2006).

Calreticulin, loss-of-function and gain-of-function

The direct link of calreticulin to Ca^{2+} signalling is highlighted by data that shows that changes in calreticulin expression level directly correlates to changes in Ca^{2+} signalling (Nakamura et al. 2001b). It is not surprising, therefore, that the regulation of expression of calreticulin impacts on ER Ca^{2+} release and storage capacity and ultimately results in abnormal embryonic development (Li et al. 2002; Mesaeli et al. 1999; Nakamura et al. 2001a). Key to understanding the chaperoning functions and Ca^{2+} binding properties of calreticulin comes from studies involving animal and cellular models of calreticulin-deficient systems and over-expressing calreticulin (Michalak et al. 2009). These studies further demonstrate the role of calreticulin as a buffering protein and a regulator of Ca^{2+} homeostasis might be far

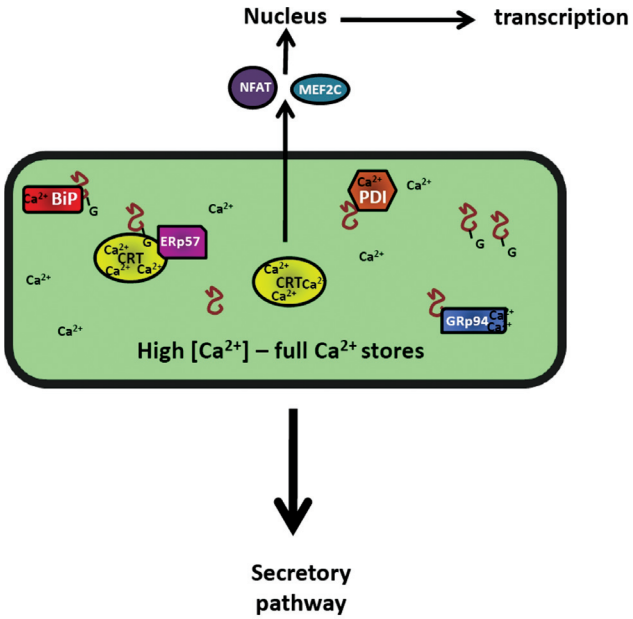

Figure 1. Calcium binding chaperones in the endoplasmic reticulum. Under conditions of high [Ca^{2+}] or full Ca^{2+} stores, the chaperones, calreticulin (CRT), glucose-regulated protein 78 (Grp78/BiP), glucose-regulated protein 94 (Grp94), protein disulfide isomerase (PDI) and the oxidoreductase ERp57 are fully active to bind mis-folded proteins. Both the secretory pathway and Ca^{2+} dependent transcriptional processes are also fully active.

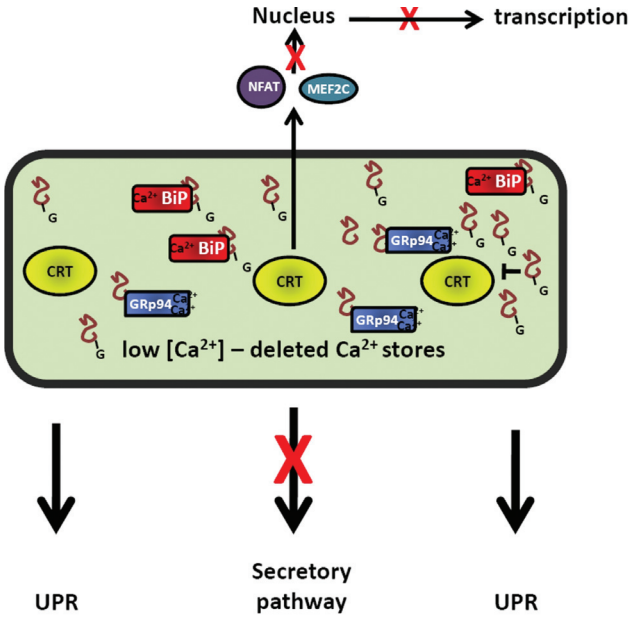

Figure 2. Calcium binding chaperones in the endoplasmic reticulum. When there is low [Ca^{2+}] or empty Ca^{2+} stores, there is an increase in accumulation of mis-folded proteins, activation of the unfolded protein response (UPR) and subsequent increase in expression of BiP, Grp94 and calreticulin (CRT). There is also significant inhibition of the secretory pathway and Ca^{2+} dependent transcriptional processes. NFAT, nuclear factor of activated T-cells; MEF, myocyte-specific enhancer factor; G, glucose.

more critical than its role as molecular chaperone (Figs. 1 and 2) (Michalak et al. 2009).

Loss-of-Function

Studies with calreticulin-deficient mice and embryonic stem cells show a critical role for calreticulin in maintenance of ER Ca^{2+} (Li et al. 2002; Mesaeli et al. 1999; Nakamura et al. 2001a). Calreticulin-deficient mice are embryonic lethal at 14.5 post-coitum due to impaired cardiac development (Mesaeli et al. 1999). Specifically, there is a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls, however, development of all other tissues is normal (Mesaeli et al. 1999; Rauch et al. 2000). Interestingly, fibroblast cells derived from calreticulin-deficient embryos show significantly reduced ER Ca^{2+} capacity although free ER Ca^{2+}, as measured by the ER-targeted "cameleon" reporter, remains unchanged (Nakamura et al. 2001b). Also, in *crt*$^{-/-}$ derived fibroblasts, there is inhibition of bradykinin-induced Ca^{2+} release. This is likely due to the impairment of bradykinin binding to its cell surface receptor suggesting that calreticulin plays a role in the correct folding of the bradykinin receptor (Mesaeli et al. 1999; Nakamura et al. 2001b). This inability for bradykinin Ca^{2+} release results in aberrant nuclear translocation of a number of factors such as NF-AT (nuclear translocation of nuclear factor of activated T-cells) and MEF2C (myocyte-enhancer factor 2C) (Guo et al. 2001; Lynch et al. 2005; Mesaeli et al. 1999). These transcription factors are all essential during vertebrate cardiac morphogenesis and hypertrophy (Chien and Olson 2002; Lynch et al. 2005; Qiu and Michalak 2009; Srivastava and Olson 2000). These studies suggest that the lethality observed in the calreticulin-deficient mice is due to the Ca^{2+} buffering role of calreticulin but not its chaperone function (Michalak et al. 2009; Nakamura et al. 2001b). Interestingly, the embryonic lethality of the calreticulin-deficient mice is rescued by over-expression of the serine/threonine phosphatase, calcineurin (Guo et al. 2002). These mice have rescued cardiac development however they show impeded growth, hypoglycaemia, increased levels of serum triacylglycerols and cholesterol indicating an important role of calreticulin in postnatal energy metabolism (Guo et al. 2002). Most importantly, over-expression of activated calcineurin rescues the nuclear localization of NF-AT and MEF2C that is aberrant in the absence of calreticulin (Guo et al. 2002; Lynch et al. 2005). The rescue of these calreticulin-deficient mice with activated calcineurin underscores the importance of the calreticulin and calcineurin relationship in the Ca^{2+}

signalling cascade for normal cardiac development (Guo et al. 2002).

Studies using calreticulin-deficient embryonic stem cells further support the role of the ER, calreticulin and Ca^{2+} in cardiogenesis (Li et al. 2002). *crt*$^{-/-}$ ES-derived cardiomyocytes have a severe disruption of myofibrillogenesis due to insufficient expression and Ca^{2+} -dependent phosphorylation of ventricular myosin light chain 2 (MLC2v) (Li et al. 2002). Myofibrillogenesis is rescued in *crt*$^{-/-}$ ES-derived cardiomyocytes when they are supplied with a Ca^{2+} ionophore highlighting the importance of calreticulin and ER Ca^{2+} signalling in cardiac development (Li et al. 2002).

Gain-of-Function

Increased expression of calreticulin results in significant increase in Ca^{2+} capacity of the ER (Arnaudeau et al. 2002). Transgenic mice over-expressing calreticulin in the heart display bradycardia, complete heart block and sudden death, cardiac edema and abnormal sarcomere structure of the heart, dilated ventricular chamber and atria, thinner ventricular walls and disarrayed cardiomyocytes (Nakamura et al. 2001a; Hattori et al. 2007). Over-expressing mice also had reduced HCN1 (hyperpolerization-activated cyclic nucleotide-gated channel1) activation, which regulates cardiac pacemaker activity (Hattori et al. 2007). A decrease in the protein level of connexin40 (Cx40) and connexin43 (Cx43), components of gap junction, and MEF2C were also observed (Hattori et al. 2007). Together, decreased levels of HCN1, Cx40 and MEF2C results in impaired structure and function of the heart in calreticulin over-expressing mice (Hattori et al. 2007). Interestingly, calreticulin autoantibody has been detected in patients suffering from congenital heart block and transgenic mice over-expressing calreticulin in the heart have a similar phenotype to children suffering from congenital heart block (Nakamura et al. 2001a; Hattori et al. 2007; Orth et al. 1996; Moak et al. 2001). This suggests a role for calreticulin in the pathogenesis of adult and paediatric congenital heart block (Orth et al. 1996; Moak et al. 2001).

Calcium binding chaperones and folding enzymes of the ER

In addition to calreticulin there are a number of other Ca^{2+} buffering chaperones and folding enzymes that affect ER-dependent Ca^{2+} homeostasis. These include calnexin, BiP/Grp78, glucose-regulated protein 94 (Grp94), protein disulfide isomerase (PDI)/Calcistorin, and ERp72. BiP/Grp78 binds Ca^{2+} at relatively low capacity (1-2 mol of Ca^{2+} per mol of protein) but is responsible for as much as 25% of the Ca^{2+} binding capacity of the ER (Lievremont et al. 1997). Grp94 is one of the most abundant Ca^{2+} buffering proteins of the ER (Argon and Simen 1999). It is a low-affinity, high-capacity Ca^{2+} binding protein with 15 moderate-affinity sites (K_d =~ 2 μM) with low capacity (1 mol Ca^{2+} per mol of protein) and 11 low-affinity sites (K_d ~ 600 μM) with high capacity (10 mol of Ca^{2+} per mol of protein) (Argon and Simen 1999). Grp94 is highly expressed in the early stages of embryonic hearts and suggests that it may have a role in the process of myocardial cell differentiation and heart development (Barnes and Smoak 1997). It has been found that the selective increase in Grp94 in response to Ca^{2+} levels protects cardiomyocytes in ischemia (Vitadello et al. 2003).

There are several ER oxidoreducatases in the ER lumen that also have roles buffering ER Ca^{2+}. PDI is a 58-kDa protein that binds Ca^{2+} with a high capacity (19 mol Ca^{2+} per mol of protein) and weak affinity (K_d =2-5mM) (Lebeche et al. 1994). ERcalcistorin/PDI is an ER luminal calsequestrin-like protein that binds Ca^{2+} with a high capacity (23 mol of Ca^{2+} per mol of protein) and low affinity (K_d = ~1mM) (Lucero and Kaminer 1999). ERp72, a 72-kDa member of the PDI family, is known to bind with a high capacity (12 mol of Ca^{2+} per mol of protein) and low affinity (Lucero et al. 1998).

ER Ca^{2+} and quality control in the secretory pathway

The ER is a multifunctional organelle and aside from its role in Ca^{2+} storage it is also well-known for its role in the synthesis, folding and post-translational modification of all secreted and integral membrane proteins (Groenendyk 2006). The critical importance of the ER as a site for protein storage machinery is underscored by the numerous diseases that result from impaired protein folding or post-translational machinery (Groenendyk 2006). Many of the ER Ca^{2+} buffering proteins also have dual functions as folding chaperones for newly synthesized proteins, so it is not surprising that their structure, complex formation with other foldases as well as substrates is dependent on fluctuations within the ER lumen (Corbett et al. 1999; Corbett and Michalak 2000). In addition, exit of properly folded proteins from the ER and trafficking to the Golgi apparatus is also an ER Ca^{2+} dependent process (Lodish and Kong 1990).

Calreticulin, calnexin and other ER Ca^{2+} binding chaperons and folding enzymes are important component of protein folding and quality control. A lectin-like chaperone function of calreticulin and calnexin is especially important in this process. Both proteins assist in folding of glycosylated proteins via their interaction with mono-glucosylated protein (Oliver et al. 1999). The protein is released from the folding machinery when the glucose residue is removed by glucosidase II (Hebert and Molinari 2007). Monoglucosylated carbohydrate binding to calreticulin or calnexin is dependent on the presence of Ca^{2+} (Williams 2006). In the

absence of Ca^{2+}, these interactions are broken resulting in accumulation of mis-folded proteins, activation of UPR and frequently cell death (Michalak et al. 2009). The interaction of the nascent polypeptide to calnexin and calreticulin is a Ca^{2+} -dependent process (Corbett et al. 2000). Under conditions of low ER luminal Ca^{2+} (<100 μmol/l), calreticulin is rapidly degraded by trypsin while under high luminal Ca^{2+} concentrations (500 μmol/l to 1 mmol/l) calreticulin formed an N-domain protease resistant core (Corbett et al. 2000). This suggests that fluctuations in Ca^{2+} within the ER lumen can affect the conformation of calreticulin and this will inevitably impact on the function of this protein (Corbett et al. 2000). Correctly folded proteins are transported to their biological destinations. However, if the protein is still mis-folded, it is recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT1) which specifically re-glucosylates allowing the protein to re-enter the calreticulin/calnexin/ERp57 cycle for another round of protein folding (Hebert and Molinari 2007). If the protein is terminally mis-folded it is removed from the ER by ER associated degradation (ERAD) which involves translocation of the mis-folded proteins to the cytoplasm where they are degraded by ubiquitine-dependent pathway (Vembar and Brodsky 2008). Native proteins, however, are transported from the ER through the Golgi to their correct location within the cell (Malhotra and Kaufman, 2007).

ER Ca^{2+} and the unfolded protein response

Disruption of any of the protein folding machinery results in an increase in mis-folded proteins in the ER lumen and this is termed ER stress (Schroder and Kaufman 2005a). In order to deal with this increased load of proteins in the ER, the cell has evolved a sophisticated system called the unfolded protein response (UPR) (Kozutsumi et al. 1988; Schroder and Kaufman 2005a). UPR is modulated by three ER transmembrane proteins called activating transcription factor-6 (ATF6), inositol-requiring kinase 1 (IRE) and double-stranded RNA-activated protein kinase-like ER kinase (PERK) (Schroder and Kaufman 2005b). These proteins work to alleviate stress on the ER by decreasing protein load through three simple adaptive mechanisms. First, there is an up-regulation of chaperone proteins and foldases as well as an increase in the size of the ER. Secondly, there is an inhibition of translation of newly synthesized proteins in the ER and, thirdly, there is an increase in ERAD machinery to rapidly clear mis-folded proteins from the ER lumen (Schroder and Kaufman 2005b). Activation of the three sensors is maintained by the regulatory protein, glucose regulated protein-78 (Grp78) or BiP (Hendershot 2004). Therefore, BiP has been referred to the as the master regulator of the ER because this role in the ER that prevents aggregation of newly synthesized proteins and associates with UPR sensors to prevent their activation (Hendershot 2004; Groenendyk and Michalak 2005; Hebert and Molinari 2007). Interestingly, drugs that interfere with intracellular Ca^{2+} stores are known to activate UPR (Tombal et al. 2002). For example, thapsigargin, a Ca^{2+}-ATPase inhibitor, decreases the intracellular Ca^{2+} concentration resulting in activation of all branches of the UPR pathway (Lytton et al. 1991; Li et al. 2000). Additionally, the ionophore, ionomycin, which increases intracellular Ca^{2+} levels, is also known to induce the UPR pathway and its treatment of cells is marked with an increase in expression of BiP/Grp78 (Miyake et al. 2000).

PERK, an ER transmembrane kinase, is the first sensor activated in mammalian UPR and its function is to transiently attenuate mRNA translation decreasing the load of newly synthesized proteins into the already stressed ER (Malhotra and Kaufman 2007; Lin et al. 2009). When there is an accumulation of mis-folded proteins in the ER lumen, BiP is sequestered by mis-folded proteins, released from PERK which results in the dimerization of PERK and eventual *trans*-autophosphorylation (Malhotra and Kaufman 2007). This autophosphorylation event causes the activation of its alpha subunit of eukaryotic initiation factor 2 (eIF2α) phosphorylation activities where PERK goes on to phosphorylate eIF2α at Ser^{51} (Malhotra and Kaufman 2007; Lin et al. 2009). Phosphorylated eIF2α inhibits translation of mRNA and consequently reduces protein synthesis (Malhotra and Kaufman 2007). Phosphorylated eIF2α also plays a selective role in the transcription by inducing the translation of activating transcription factor 4 (ATF4) mRNA which results in the transcription of genes involved in mechanisms such as apoptosis and anti-oxidative stress response (Ameri and Harris 2008).

In mammalian cells, IRE1 is a bi-functional protein with not only a cytosolic carboxy-terminal kinase domain but also an endoribonuclease domain (Back et al. 2005). Under conditions of no stress, IRE1, like PERK, is maintained as an inactive homodimer by the protein chaperone Grp78/BiP. However, when mis-folded proteins accumulate in the ER lumen, IRE1 is released from the Grp78/BiP protein and it homodimerizes and *trans*-autophosphorylates to activate its endoribonuclease (RNase) activity (Malhotra and Kaufman 2007). The RNase domain of IRE1 targets the mRNA of the basic leucine zipper domain (bZIP) containing transcription factor, X-box binding factor-1 (Xbp1) (Malhotra and Kaufman 2007). There is splicing of a 26-nucleotide intron from the mRNA of Xbp1 causing a translational frameshift to an active and stable transcription factor (Back et al. 2005; Malhotra and Kaufman 2007). The splicing of the mRNA results in the activation of a potent transcription factor whose protein, *XBP1*, translocates to the nucleus where it activates the transcription of UPR element (UPRE) containing genes to alleviate ER stress (Malhotra and Kaufman 2007). There is activation in the transcription of genes involved in ER associ-

ated degradation (ERAD) in order to alleviate the build up of mis-folded proteins in the ER lumen (Back et al. 2005).

Upon ER stress and UPR, ATF6 is released from Grp78/BiP, cleaved and activated in response to ER stress and their bZip domains allow them to bind ERSEs as homodimers or as a heterodimer and this modulates the ER stress response (Kondo et al. 2005; Murakami et al. 2006; Thuerauf et al. 2004). Under normal conditions, the luminal domain of ATF6, like PERK and IRE1, is bound by Grp78/BiP however, it is also bound by calreticulin (through its three glycosylation sites) and upon ER stress, the luminal domain of ATF6 is released from Grp78/BiP and from calreticulin (due to underglycosylation of the three luminal glycosylation sites), revealing non-consensus Golgi localization sites (GLSs) (Shen et al. 2002; Hong et al. 2004; Shen et al. 2005). Since carbohydrate binding to calreticulin is Ca^{2+}-dependent it is conceivable that calreticulin-ATF6 interactions may be regulated by ER luminal Ca^{2+}. Once free of calreticulin and Grp78/BiP, ATF6 then translocates to the Golgi apparatus where it is subject to proteolytic processing (Shen et al. 2002; Hong et al. 2004) to release N-ATF6 portion of the ATF6. N-ATF6 translocates to the nucleus to activate promoters containing ERSE's (Thuerauf et al. 2007). The involvement of players such as Grp78/BiP and calreticulin in UPR suggest that Ca^{2+} may have role in the mediation of this stress response. It is of interest that these sensors are maintained in their "off" states by binding Grp78/BiP (PERK, IRE, ATF6) and calreticulin (ATF6) (Hendershot 2004; Hong et al. 2004).

Conclusions

The ER is a continuous, dynamic and multifunctional organelle and is the major Ca^{2+} store of the cell. It plays a vital role in many cellular processes of the cell including protein folding and secretion, post-translational modification, lipid and sterol biosynthesis and Ca^{2+} buffering and homeostasis. Calreticulin, an ER resident protein, is the major Ca^{2+} binding protein of the ER. Modulation of this protein is tightly controlled to impact Ca^{2+} stores and signalling. Ca^{2+} fluctuations as a result of calreticulin modulation impact the cells at the molecular level, as seen with impacts on protein folding machinery, as well as at the whole tissue level, specifically cardiac development. Normal cardiac development is ultimately controlled by calreticulin and its mis-regulation (loss-of-function or gain-of-function) leads to lethal cardiac pathologies. It is exceedingly important to continue to study the role calreticulin plays in the ER as it is a valuable player in normal development.

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Review

Cross-talk of intracellular calcium stores in the response to neuronal ischemia and ischemic tolerance

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Abstract. Ischemic/reperfusion brain injury (IRI) is a very severe event with the multiple etiopathogenesis. Ischemic preconditioning (IPC) is an important phenomenon of adaptation of CNS to subsequent ischemia. An altered cross-talk between intracellular calcium stores is presumed in the mechanisms of ischemic damage/protection. We show here that IRI leads to the inhibition of mitochondrial respiratory complexes I and IV, however due to the excess of their capacities, the mitochondrial Ca^{2+} uptake rate is not significantly depressed. IPC acts at the level of both initiation and execution of IRI-induced mitochondrial apoptosis and protects from IRI-associated changes in integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria. Inhibition of the mitochondrial p53 pathway might thus provide a potentially important mechanism of neuronal survival after ischemic brain damage.

In addition, IRI initiates a time dependent differences in endoplasmic reticular (ER) gene expression of the key UPR proteins at both the mRNA and protein levels. Moreover, gene expression of the UPR proteins is affected by preischemic treatment by the increased expression of Ca^{2+} binding protein: GRP 78 and transcriptional factor ATF6 in reperfusion times. Thus, IPC exerts a role in the attenuation of ER stress response, which might be involved in the neuroprotective phenomenon of ischemic tolerance.

Hippocampal cells respond to the IRI by the specific expression pattern of the secretory pathways Ca^{2+} pump (SPCA1) and this pattern is affected by preischemic challenge. IPC also incompletely suppresses lipo- and protein oxidation of hippocampal membranes and leads to partiall recovery of the ischemic-induced depression of SPCA activity. The data suggests the correlation of SPCA function with the role of secretory pathways (Golgi apparatus) in response to preischemic challenge.

Documented functional alterations of mitochondria, ER and Golgi apparatus put light into the understanding of cross-talk between intracellular Ca^{2+} stores in cerebral ischemia and ischemic tolerance and might suggest for possible targets of future therapeutic interventions to enhance recovery after stroke.

Key words: Cerebral ischemia — Ischemic tolerance — Intracellular calcium stores

Introduction

Ischemic/reperfusion brain injury (IRI) is a very severe event with the multiple, parallel and sequentional pathogenesis (Endres 2008). Its etiology includes dysregulation of the energetic metabolism with intracellular derangement of ion homeostasis. Altered Ca^{2+} dysregulation, triggering Ca^{2+}-dependent bio-polymer degradation and mitochondrial and bioenergetic failure, ultimately culminates in the activation of reactions leading to necrotic/apoptotic cell death. It is not yet clear which sources of Ca^{2+} and which pathways are involved, however the defective cross-talk between intracellular stores is presumed in the etiopathogenesis of the injury (Bano and Nicotera 2007).

Ischemic preconditioning (IPC) represents an important phenomenon of adaptation of CNS to sub-lethal short-term

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ischemia, which results in increased tolerance of CNS to the lethal ischemia (Kirino 2002; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008). The mechanisms underlying ischemic tolerance are rather complex and not yet fully understood. In all multiple paradigms for IPC, two windows have been identified. One window that represents very rapid and short-lasting post-translational changes and second, which develops slowly (over days) after initial insult as a robust and long lasting transcriptional changes which culminate to prolonged neuroprotection (Gidday 2006; Obrenovitch 2008; Yenari et al. 2008; Dirnagl et al. 2009).

Mitochondria are important regulators of neuronal cell life and death through their role in metabolic energy production and involvement in apoptosis (Yuan and Yanker 2000). Remarkably, mitochondrial dysfunction is considered to be one of the key event linking ischemic/recirculation insult with neuronal cell death (Berridge et al. 2003). In addition, mitochondria play a dual role in intracellular calcium. They are involved in the normal control of neuronal Ca^{2+} homeostasis (Berridge et al. 2003), such as Ca^{2+} signalling, Ca^{2+} -dependent exocytosis and stimulation of oxidative metabolism and ATP production (Rizzuto 2001; Gunter et al. 2004). On the other side, mitochondrial Ca^{2+} overload and dysfunction, due to excitotoxic activation of glutamate receptors, is a crucial early event which follows ischemic or traumatic brain injury (Nicholls et al. 2007). Evidence for mitochondrial Ca^{2+} accumulation after excitotoxic stimulation comes from the experimental studies which support the idea that mitochondrial depolarization during glutamate exposure is neuroprotective (Pivovarova et al. 2004), while its reduction correlates with excitotoxicity (Ward et al. 2007).

In addition, activation of apoptosis has been documented after brain ischemia in several studies (Cao et al. 2003; Endo et al. 2006), and that this phenomenon might be closely linked to mitochondrial dysfunction. In fact, mitochondrial dysfunction provoked activation of apoptotic machinery by direct triggering of cytochrome c release (Clayton et al. 2005), or induction of Bax-dependent neuronal apoptosis through mitochondrial oxidative damage (Endo et al. 2006).

The endoplasmic reticulum (ER) of neural cells responds to the interruption of blood flow by the unfolded protein response (UPR), which can be highly variable, depending on dosage and duration of ischemic treatment (Imaizumi et al. 2001), and intensity of UPR signals (Yoshida et al. 2003). However, when ER stress is too severe and prolonged, apoptosis is induced. Various enzymes and transcription factors, such as ATF4 and ATF6 (activating transcription factor 6) and the inositol-requiring enzyme IRE1 (Shen et al. 2001) are involved in the UPR. In the physiological state, activities of these factors are suppressed by binding of the ER chaperone: Ca^{2+} binding, glucose regulated protein 78 (GRP78). Remarkably, induction of GRP78 prevents neuronal damage and its increased expression may correlate with the degree of neuroprotection (Morimoto et al. 2007). Under ER dysfunction, GRP78 dissociates and subsequently induces expression of the ER stress genes. ATF6 is a key transcription factor in the resolution of the mammalian UPR, and unlike IRE1 and PERK, there is no evidence that ATF6 is involved in proapoptotic pathways (Yoshida et al. 2001). Activated IRE1 specifically cuts out the coding region of X-box protein 1 (XBP1) mRNA (Calfon et al. 2002) which after translation functions as a transcription factor specific for ER stress genes including GRP78 and GRP94. Previous studies shown that changes of the UPR gene expression induced by IRI occur during the first 24 h (Paschen 2003) or the first few days after the insult (Qi et al. 2004).

The secretory pathways (SP) in neural cells represents a dynamic Ca^{2+} store where high luminal Ca^{2+} concentration, and also Mn^{2+} are required for optimal activity of many enzymes and for processes such as secretion of neurotransmitters and secretory proteins (Michelangeli et al. 2005). In addition, SP are involved in the stress sensing, neuronal aging and transduction of apoptotic signals (Maag et al. 2003; Sepulveda et al. 2008). The SP derived Ca^{2+}-ATPases (SPCAs) represent a subfamily of P-type ATPases related to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+}-ATPase (PMCA) (Van Baelen et al. 2004; Murín et al. 2006). The SPCA1 isoform is considered as a house-keeping isoform with pronounced expression in neural cells (Wootton et al. 2004; Murín et al. 2006; Sepulveda et al. 2008). The higher expression levels of SPCA1 in the brain coincide with a relatively high ratio of SPCA activity (thapsigargin insensitive) to the total activity of Ca^{2+}-dependent ATPases, implying a significant role of SPCA-facilitated transport of Ca^{2+} for calcium storage within the brain (Wootton et al. 2004).

Remarkably, the SPCA plays a pivotal role in normal neural development, neural migration and morphogenesis (Sepulveda et al. 2007, 2008). Likewise, deficiency of SPCA in knock-out mice caused alteration in neural tube development and Golgi stress with its dilatation and reduction in the number of stacked leaflets (Okunade et al. 2007). Such morphological changes in the Golgi complex, like its fragmentation, represents an early causative step rather than a secondary event, and it is very commonly found associated with several neurodegenerative diseases, such as amyotrophic lateral sclerosis, corticobasal degeneration, Alzheimer's and Creutzfeldt-Jacob diseases, and spinocerebelar ataxia type 2 (Gonatas et al. 2006).

Impact of IRI and IPC on mitochondrial calcium transport, p53 translocation and neuronal apoptosis

Mitochondria are involved in the control of neuronal Ca^{2+} homestasis and neuronal Ca^{2+} signalling. In a series of recent

papers (Racay et al. 2007, 2009a,b,c), we have studied the effect of global cerebral ischemia/reperfusion injury (IRI) and ischemic preconditioning (IP) on mitochondrial Ca^{2+} homeostasis and mitochondrial way of apoptosis.

At first, we have analyzed effect of global brain ischemia-reperfusion on mitochondrial Ca^{2+} uptake in relation to ischemia-induced inhibition of complex I and IV. Although in previous experiments was detected decrease of Ca^{2+} uptake capacity in isolated hippocampal mitochondria (Friberg et al. 2002), in our experimental setting we did not observe any significant changes on the rate of active Ca^{2+} uptake in isolated mitochondria from hippocampi of rats submitted to ischemia-reperfusion. Neither ischemia nor reperfusion had significant impact on the rate of active Ca^{2+} uptake, statistically significant reduction of mitochondrial capacity to accumulate Ca^{2+} was observed only after 15 min of global ischemia (80.8% of control). The capacity of mitochondria to retain Ca^{2+} was completely restored during reperfusion (Racay et al. 2009a,b).

It is generally accepted that the rate of mitochondrial Ca^{2+} uptake is proportional to changes of membrane potential which is generated by respiratory chain complexes (Duchen, 2004). Our results have shown that rate of mitochondrial Ca^{2+} uptake decreased proportionally with decrease of membrane potential (Racay et al. 2009a). Inhibition of key mitochondrial enzyme complexes is thought to be a cause of ischemia-induced mitochondrial dysfunction. It seems that mitochondrial complex I, the initial complex of respiratory chain, plays crucial role in mitochondrial functioning since complex I inhibition has been implicated in a number of brain pathologies (Duchen 2004). Indeed, ischemia induced progressive inhibition of complex I with the minimal activity expressed at 24 h after ischemia (63% of control). In addition, an inhibition of complex IV activity to 80.6% of control was observed 1 h after ischemia. This discrepancy between unaltered rate of Ca^{2+} uptake and activities of both complexes was explained by titration experiments (Racay et al. 2009a). As shown from relationship between inhibition of respiratory complexes and generation of mitochondrial transmembrane potential, complex I and IV activities must be decreased by approximately 40, and 60%, respectively, before significant decline of the transmebrane potential. Thus, mitochondrial Ca^{2+} uptake was not significantly affected by IRI, apparently due to excess capacity of the complexes I and IV. Inhibition of complex I is favourable of reactive oxygen species (ROS) generation. Maximal oxidative modification of membrane proteins was documented 1 h after ischemia. Although enhanced formation of ROS might contribute to neuronal injury, depressed activities of complex I and IV together with unaltered rate of Ca^{2+} uptake are conditions favourable of initiation of other cell degenerative pathways like opening of mitochondrial permeability transition pore or apoptosis initiation, and might represent important mechanism of ischemic damage to neurones.

Ischemic preconditioning (IPC) represents an important phenomenon of adaptation of CNS to sub-lethal short-term ischemia, which results in increased tolerance of CNS to the lethal ischemia (Kirino 2002; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008). As documented by Racay et al. (2007, 2009a), global ischemia led to progressive decrease of complex I activity after IRI to 65.7% of control at 24 h after reperfusion. In preconditioned animals, the activity of complex I was also significantly inhibited after ischemia (to 65.4% of control) and ischemia/reperfusion for 1, 3, and 24 h (62-78% of control). Although the values in preconditioned animals were significantly smaller compared to naive ischemia, IPC did not protect complex I from ischemia induced inhibition. On the other hand, activity of the terminal enzyme complex of respiratory chain, complex IV were slightly pretected by IPC and the net effect of IPC was the shift of its mininal actvity from 1 h to 3 h after reperfusion (Racay et al. 2009c).

As shown earlier by several studies, 10 min of global cerebral ischemia initiates decreased capacity for active Ca^{2+} sequestration by isolated forebrain mitochondria, while a 5-h period of reperfusion after 30 min of forebrain ischemia in the rat also inhibited the ability of isolated mitochondria both to actively accumulate and retain Ca^{2+} (Sciamanna et al. 1992). Decreased Ca^{2+} uptake capacity was observed in isolated hippocampal mitochondria during reperfusion (Friberg et al. 2002). The discrepancy between our and previous studies might be attributable to different models of ischemia and different anaesthetics. As shown in our laboratory, the effect of ischemia on active Ca^{2+} accumulation by endoplasmic reticulum depends on ischemic model and used anaesthetics (Racay et al. 2000). In addition, the ischemia/reperfusion-induced inhibition of mitochondrial Ca^{2+} transport was parallel with decline of mitochondrial respiration (Sciamanna et al. 1992). In our study, the ischemia-induced inhibition of complex I and IV was not accompanied by significant changes in the rate of mitochondrial Ca^{2+} uptake. It is generally accepted and was confirmed in our studies (Racay et al. 2007, 2009a,b,c) that the rate of mitochondrial Ca^{2+} uptake is proportional to transmebrane potential which is generated by respiratory chain complexes. However, based on our titration experiments, we suppose that the initial rate of mitochondrial Ca^{2+} uptake was not altered apparently due to excess capacity of the complex I and complex IV documented by energy thresholds (Racay et al. 2009c).

Mitochondrial dysfunction and oxidative stress were often implicated in pathophysiology of neurodegenerative diseases, including cerebral ischemia (Lin and Beal 2006). Inhibition of complex I itself or in combination with elevated Ca^{2+} led to enhanced ROS production in different *in vitro*

(Panov et al. 2005) and *in vivo* systems (Yadava and Nicholls 2007). Importantly, an enhanced production of ROS and consequent induction of p53-dependent apoptosis due to damage to neuronal DNA has also been documented after inhibition of complex I. Recent study showed that spare respiratory capacity rather then oxidative stress is involved in excitotoxic cell death (Yadava and Nicholls 2007).

As shown by experimental and clinical studies, IRI -induced mitochondrial pathway of apoptosis is an important event leading to neuronal cell death after blood flow arrest. Impact of IRI and ischemic preconditioning on the level of apoptotic and anti-apoptotic proteins was assessed in both cortical and hippocampal mitochondria by Western blot analysis of p53, bax, and bcl-x (Racay et al. 2007, 2009b). Remarkably, IRI led to increase of p53 level in hippocampal mitochondria, with significant differences after 3 h (217.1 ± 42.2% of control), 24 h (286.8 ± 65% of control), and 72 h (232.9 ± 37.3% of control) of reperfusion. Interestingly, translocation of p53 to mitochondria was observed in hippocampus but not in cerebral cortex. However, level of both, the apoptotic proteins bax and the anti-apoptotic bcl-xl were unchanged in both hippocampal and cortical mitochondria. Ischemia-induced translocation of p53 to mitochondria was completely abolished by IPC since no significant changes in mitochondrial p53 level were observed after preconditioned ischemia. Similar to naive ischemia, the levels of both bax and bcl-xl were not affected by IPC. In addition, IPC had significant protective effect on ischemia-induced DNA fragmentation, as well as on number of positive Fluoro-Jade C staining cells. Thus, it indicates that IPC abolished almost completely both initiation and execution of mitochondrial apoptosis induced by global brain ischemia in vulnerable CA1 layer of rat hippocampus (Racay et al. 2007, 2009b).

Interestingly, Bcl-xl can prevent mitochondrial membrane permeabilization by competing with Bax (Billen et al. 2008), and it seems that Bax is already inserted in outer membrane of hippocampal mitochondria but the pore forming properties of bax are neutralized by high mitochondrial level of bcl-xl. Thus, our results are consistent with the recent view, that p53 protein can directly induce permeabilization of the outer mitochondrial membrane by forming a complex with protective Bcl-xl protein, resulting in oligomerization of Bax, cytochrome c release, and initiation of neuronal apoptosis after cerebral ischemia (Endo et al. 2006).

A considerable delay from the preconditioning stimulus until onset of ischemic tolerance is consistent with a role for transcriptional changes in adaptation (Kirino 2001; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008; Otani 2008). Tanaka and co-workers (2004) have shown that IPC acts downstream of caspase-3 activation and upstream of its target caspase-activated DNase to prevent the onset of apoptotic cell death (Tanaka et al. 2004). The IPC-induced inhibition of caspase-activated DNase was consistent with observations that IPC induces over-expression of heat shock protein 70 kDa, in which protective effect from cerebral ischemia via inhibition of caspase death cascade and mitochondrial apoptosis is well documented. Recently, it has also been shown that hsp70 inhibits apoptosis upstream of mitochondria by preventing bax translocation (Gidday 2006; Obrenovitch 2008; Otani 2008). The molecular mechanisms driving translocation of p53 to mitochondria after brain ischemia are not yet known. Thus, we can only speculate about the possible mechanism involved in inhibition of mitochondrial p53 translocation observed after IPC. Since several different mechanisms, like IPC-induced over expression of heat shock protein 70 kDa (Tanaka et al. 2004) or activation of Akt pathway (Gidday 2006; Obrenovitch 2008; Pignataro et al. 2009), might be considered, the exact mechanism of IPC-induced prevention of p53 translocation to mitochondria has to be clarified by further experiments.

Collectively, our studies showed that ischemia induced inhibition of mitochondrial complexes I and IV, however inhibition is not accompanied by decrease of mitochondrial Ca^{2+} uptake rate apparently due to the excess capacity of the complex I and complex IV. On the other hand, depressed activities of complex I and IV are conditions favourable of initiation of cell degenerative pathways, e.g. opening of mitochondrial permeability transition pore, ROS generation and apoptosis initiation, and might represent important mechanism of ischemic damage to neurons. In line of this, ischemic preconditioning acts at the level of both initiation and execution of ischemia-induced mitochondrial apoptosis and protects from ischemia associated changes in integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria. Inhibition of the mitochondrial p53 pathway thus might provide a potentially important mechanism of neuronal survival in the face of ischemic brain damage (Otani 2008).

Stress reaction of neuronal endoplasmic reticulum after IRI and IPC

Ischemic tolerance can be developed by prior ischemic non-injurious stimulus - preconditioning. The molecular mechanisms underlying ischemic tolerance are not yet fully understood, therefore in a series of papers (Urban et al. 2009; Lehotsky et al. 2009; Pavlikova et al.2009) we focused our attention on both the mRNA and the protein levels of ER stress genes after ischemic/reperfusion damage (I/R) in naive and preconditioned groups of rats.

In the UPR response, an activated IRE1 specifically cuts out the coding region of X-box protein 1 (*XBP1*) mRNA (Calfon et al. 2002) which after translation functions as a transcription factor specific for ER stress genes including GRP78 and GRP94. As we have observed in our experiments,

the hippocampal mRNA for XBP1 has shown elevated level in the naive IRI group of animals in ischemic phase (about 43%) and persisted non-significantly changed in all analyzed periods (Lehotsky et al. 2009; Urban et al. 2009). Preischemic treatment (IPC) induces on the level of hippocampal mRNA in ischemic phase only slight, not significant differences compared to controls, followed by significant decrease at 24 hours of reperfusion (by about 12.8 ± 1.4% compared to controls). When we analyzed translational product, the hippocampal XBP1 protein level in naive IRI animal group showed significant differences in ischemic phase (39.2 ± 1.6% compared to controls) and the levels were significantly elevated at later reperfusion periods (3 and 24 h) (82 ± 2.4% and 24.1± 1.6% respectively compared to controls). The influence of preischemia (IPC) on protein level was significant mainly in later ischemic times. The protein level reached maximum at 3 h of reperfusion (about 230% of controls) and persisted elevated in the later reperfusion (40.3 ± 4.9% compared to controls) (Lehotsky et al. 2009; Urban et al. 2009).

Endoplasmic reticular chaperone, the Ca^{2+} binding, glucose regulated protein 78 (GRP78) was shown to prevent neuronal damage (Morimoto et al. 2007). Under ER dysfunction and GRP78 dissociation it subsequently induces expression of ER stress genes. On the level of mRNA for *GRP78* in hippocampus from naive IRI group of animals we have observed that maximal differences are seen in later reperfusion phases. Preischemic pretreatment (IPC) led to the elevated mRNA hippocampal levels in reperfusion period by about 11.7 ± 3.6 at the first hour and by about 8.7± 1.8% at 24 hours of reperfusion in comparison to mRNA levels in corresponding ischemic/reperfusion times. Remarkably, the level of GRP78 protein in naive IRI showed rapid increase in ischemic time (by about 217% of controls) and remained elevated also at 3 and 24 hours of reperfusion (about 213% and 43%, respectively, compared to controls). Increased mRNA values in preconditioned animals also corresponded with the significant increase of the levels of GRP78 protein. The changes are documented in the ischemic phase and also in all reperfusion times (by about 250% of controls and about 50% of corresponding ischemic/reperfusion times) (Lehotsky et al. 2009; Urban et al. 2009).

ATF6 works as a key transcription factor in the resolution of the mammalian UPR (Yoshida et al. 2001). As shown in our experiments, the mRNA level for *ATF6* in naive IRI animals showed gradual elevation reached to significant increase at 24 hours of reperfusion (9.2 ± 4 % higher than control) and preconditioning (IPC) did not change significantly mRNA levels in all analyzed periods. Similarly to mRNA levels, the hippocamapal ATF6 protein level in naive IRI animals followed the mRNA levels. Interestingly, IPC induced remarkable changes in the protein levels at ischemic phase reaching significantly (about 170%) increased levels in comparison to controls and remained elevated in earlier reperfusion times (about 37 and 62 % higher than in controls) and later reperfusion time (about 15% of controls).

In general, IRI initiates suppression of global proteosynthesis, which is practically recovered in the reperfusion period with the exception in the most vulnerable neurons, such as pyramidal cells of CA1 hippocampal region (de la Vega et al. 2001). On the other hand, ischemia is one of the strongest stimuli of gene induction in the brain. Different gene systems related to reperfusion processes of brain injury, repair and recovery are modulated (Gidday 2006). In fact, IRI induces transient inhibition of translation, which prevents the expression of UPR proteins and hindered recovery from ischemia-induced ER dysfunction (Kumar et al. 2001; Paschen et al. 2003) which possibly leads to a pro-apoptotic phenotype (DeGracia and Montie 2004). Similarly to our data, Thuerauf et al. (2006) found that myocardial ischemia activates UPR with the increased expression of XBP1 protein and XBP1-inducible protein. They contribute to protection of the myocardium during hypoxia. Also the results of Paschen et al. (2003) by semi-quantitative RT-PCR showed a marked increase in *XBP1* mRNA levels after focal ischemia in the cerebral cortex.

Preischemia induced elevation of mRNA and protein GRP78 levels in reperfusion periods. GRP78 is a member of the 70-kDa heat shock protein family that acts as a molecular chaperone in the folding and assembly of newly synthesized proteins within the ER. As shown by Yu et al. (1999) the suppression of GRP78 expression enhances apoptosis and disruption of cellular calcium homeostasis in hippocampal neurons that are exposed to excitotoxic and oxidative insults. This indicates that a raised level of GRP78 makes cells more resistant to the stressful conditions (Aoki et al. 2001). Similar results were obtained by Morimoto et al. (2007) in the focal ichemia model. Our results are similar to the findings of Hayashi et al. (2003) and Garcia et al. (2004), who documented an increase in GRP78 expression after 2 days of preconditioning. Authors proposed that the development of tolerance includes changes in PERK/GRP78 association, which were responsible for the decrease in eIF2a phosphorylation induced by preconditioning. On the other hand, Burda et al. (2003), failed to find any differences in the level of GRP78 protein in rats with or without acquired ischemic tolerance. This was probably due to exposure to very short reperfusion times.

ATF6 is an ER-membrane-bound transcription factor activated by ER stress, which is specialized in the regulation of ER quality control proteins (Adachi et al. 2008). Interestingly, Haze et al. (1999) found that the overexpression of full-length ATF6 activates transcription of the GRP78 gene. Explanation of generally higher levels of protein p90ATF6 in preischemic group is probably connected with an increased promotor activity of GADD153 to UPR genes (Oyadomari et al. 2004).

The data from these experiments (Lehotsky et al. 2009; Urban et al. 2009) suggest that IRI initiates time dependent differences in endoplasmic reticular gene expression at both the mRNA and protein levels and that endoplasmic gene expression is affected by preischemic treatment. These data and recent experiments of Bickler et al. (2009) also suggest that preconditioning paradigm (preischemia) may exert a role in the attenuation of ER stress response and that $InsP_3$ receptor mediated Ca^{2+} signaling is an important mediator in the neuroprotective phenomenon of acquired ischemic tolerance. Changes in gene expression of the key proteins provide an insight into ER stress pathways. It also might suggest possible targets of future therapeutic interventions to enhance recovery after stroke (Yenari et al. 2008; Pignatato et al. 2009).

Secretory pathways Ca^{2+} ATPase (SPCA1) gene expression is altered following ischemic precondtioning

The secretory pathways (SP) in neural cells represents a dynamic Ca^{2+} store required for optimal activity of enzymes and for secretion of neurotransmitters and secretory proteins (Michelangeli et al. 2005). In addition, SP are involved in the stress sensing, neuronal aging and transduction of apoptotic signals (Maag et al. 2003; Sepulveda et al. 2008). The SPCA Ca^{2+}-ATPase has a significant role for calcium storage within the brain (Wootton et al. 2004) and was shown to play a pivotal role in normal neural development, neural migration and morphogenesis (Sepulveda et al. 2007, 2008).

Collective studies confirm, that reactive oxygen species (ROS) contribute to neuronal cell injuries secondary to ischemia and reperfusion (Lehotsky et al. 2004; Burda et al. 2005; Danielisova et al. 2005; Shi and Liu 2007) and might initiate cell death signaling pathways after cerebral ischemia and parallels with selective post-ischemic vulnerability of the brain (Valko et al. 2007; Shi and Liu 2007; Otani 2008; Dirnagl et al. 2009).

As shown in our laboratory by measurement of steady state fluorescence of ANS in hippocampal mitochondria (Racay et al. 2007, 2009a), naive IRI induced significant increase in ANS flurescence (it binds to hydrophobic part of membrane lipids and proteins) in both ischemic and reperfusion periods. These results support data from our previous experiments (Lehotsky et al. 2004; Babusikova et al. 2008), which showed that IRI induced structural changes on hippocampal membrane lipids and both, the lipoperoxidation dependent and the direct oxidative modifications of membrane proteins. Remarkably, preconditioning (IPC) induces significant decrease of ANS fluorescence, which indicates protective effect of IPC on mitochondrial membranes.

In addition, as shown by recent paper of Pavlikova et al. (2009), microsomes prepared from injured hippocampus after IRI also manifested considerable lipoperoxidation and protein oxidation as analyzed from the level of TBARS and fluorescence intensities of tryptophan (Trp) and bityrosine (biTyr). The experiments also indicated an IRI-induced depression of Ca^{2+} ATPase activity which is attributable to SPCA-associated thapsigargin insensitive activity. Interestingly, preconditionig (IPC) partially protects hippocampal membranes from oxidative damage, as shown by recovery of both the Trp and biTyr fluorescence intensities and lower levels of TBARS. However, the oxidation of membrane proteins still persists (to 94 and 105%, respectively). It is important from functional aspects, that IPC had also partial protective effect on the SPCA-associated Ca^{2+}-ATPase activity. Thus, results from these experiments showed that IPC incompletely suppresses lipo- and protein oxidation in hippocampal membranes and led to partiall recovery of the ischemia-induced depression of SPCA activity.

As shown by earlier studies, SP are involved in the stress sensing, neuronal aging and transduction of apoptotic signals (Maag et al. 2003; Sepulveda et al. 2008). In order to evaluate whether the severe metabolic stress induced by IRI and/or IPC affects transcription of SPCA1 gene, we analyzed the mRNA and protein levels of SPCA1. RT-PCR clearly detected, that hippocampal cells responded to the IRI by induction of mRNA level in reperfusion period with maximum at 3 h reperfusion (to 171% of control). Preconditioning (IPC) initiates earlier tissue response to the injury by the significant elevation of mRNA expression already at 1 h of reperfusion and the level of mRNA expression reached 142% comparing to 1 h ischemia, and to 164% comparing to control.

Western blot analysis demonstrated that immunosignal for SPCA1 showed similar profile to that of the mRNA and it increased in the later reperfusion period. Although IPC did not induce any statistically significant changes at 3 and 24 h of corresponding ischemic levels, in the line of results of mRNA expression, the IPC induced a 149% rise in the level of SPCA1 protein corresponding to a 1 h reperfusion level. Thus, these results showed that the SPCA expression and the post-translational changes induced by ischemia are modulated by the IPC. These data might also serve to understand the molecular mechanisms involved in the structural integrity and function of the secretory pathways after ischemic challenge. Data also suggests that there is a correlation between SPCA function and the role of SP in the response to pre-ischemic challenge.

Neuronal microsomes are vulnerable to physical and functional oxidative damage (Lehotsky et al.1999, 2002a, 2004; Urikova et al. 2006). We have shown here that SPCA activity, similarly to other P-type ATPases, is also subject of ischemic damage likely due to free radicals action (Lehotsky et al. 2002b). In addition, oxidative alterations detected in mitochondria and microsomes after IRI in our experiments,

may at least partially explain functional postischemic disturbances of neuronal ion transport mechanisms (Lipton 1999; Lehotský et al. 2002a; Obrenovitch 2008) and inhibition of global proteosynthesis (Burda et al. 2003), which both are implicated in neuronal cell damage and/or recovery from ischemic insult. Ischemic preconditioning induced reductions of lipoperoxidation products and protein oxidative changes (Racay et al. 2009b; Pavlíková et al. 2009), probably due to upregulation of defence mechanisms (antioxidant enzymes) against oxidative stress in the preconditionig challenge (Danielisova et al. 2005; Gidday 2006; Obrenovitch 2008).

In addition, IRI causes significant drops in the SPCA associated Ca^{2+}-ATPase activity and ischemic preconditioning had a partial protective effect on this activity. As shown in earlier studies, preconditioning upregulates defence mechanisms against oxidative stress (Danielisova et al. 2005; Pignataro et al. 2009) which might partialy restore the depression of enzyme activity. Additionally, as shown in this study by Western blot analysis, ICP induced an elevation of SPCA protein level in comparison to corresponding naive ischemic control.

Golgi apparatus is strategically located which predict its common Ca^{2+} signaling communication and contribution to the spatial and temporal complexity of Ca^{2+} signals (Michelangeli et al. 2005). However, the Golgi Ca^{2+} stores also reflect a number of different functions. The cis-Golgi appears to express SERCA and $InsP_3$ receptors, while the trans-Golgi contains SPCA1 and lacks $InsP_3$ receptors. In fact, we (Murin et al. 2006) and others (Sepulveda et al. 2008), have demonstrated the presence of SPCA1 protein in hippocampal neurons either in neuronal cell cultures or in rat hippocampus. Interestingly, particularly these cells are highly vulnerable to ischemic challenge.

One of the most pronounced morphological features following IRI is the mitochondrial and Golgi swelling and activation, which could be suppressed by neuroprotective treatment (Hicks and Machaner 2005; Strosznajder et al. 2005; Gonatas et al. 2006). The secretory pathways are apparently involved in sensing stress and transducing signals during the execution phase of apoptosis (Maag et al. 2003; Hicks and Machamer 2005). Our results showed a partial recovery of Ca^{2+}-ATPase activity and earlier hippocampal response to later ischemia by the induction of mRNA and protein expression. In fact, the mechanism of transcriptional regulation of SPCA1 gene is not yet fully understood. The transcription factors Sp1 and YY1 were shown to be involved in the gene regulation by the cis-enhancing elements in 5-untranslated regions (Kawada et al. 2005), or the expression of the putative endogenous activator of SPCA or the changes in local membrane environment are suggested as a cause for the increase in SPCA activity (Sepulveda et al. 2008).

In conclusion, results of these series of experiments indicate for the specific SPCA1 expression pattern in injured ischemic hippocampus and might serve to understand the molecular mechanisms involved in the structural integrity and function of the Golgi complex after ischemic challenge. They also suggest the correlation of SPCA function with the role of secretory pathways in response to preischemic challenge.

Conclusions

Collectively, our studies showed that ischemia induced inhibition of mitochondrial complexes I and IV without depression of mitochondrial Ca^{2+} uptake rate, apparently due to the excess capacity of the complex I and complex IV. Depressed activities of complex I and IV are conditions favourable for initiation of cell degenerative pathways, e.g., opening of mitochondrial permeability transition pore, ROS generation and apoptosis initiation. Ischemic preconditioning acts at the level of both initiation and execution of ischemia-induced mitochondrial apoptosis and protects from ischemia associated changes in the integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria and thus IPC affects downstream processes connnecting mitochondrial dysfunction. Inhibition of the mitochondrial p53 pathway might provide a potentially important mechanism of neuronal survival in the face of ischemic brain damage (Fig. 1 and Fig 2).

Our data also suggests, that IRI initiates time dependent differences in endoplasmic reticular gene expression at both the mRNA and protein levels and that endoplasmic gene expression is affected by preischemic treatment. Preconditioning paradigm (preischemia) exerts a role in the attenuation of ER stress response in the neuroprotective phenomenon of acquired ischemic tolerance.

Results of experiments also indicate a specific SPCA1 expression pattern in injured ischemic hippocampus and might serve to understand the molecular mechanisms involved in the structural integrity and function of the Golgi complex after ischemic challenge. They also suggest a correlation of SPCA function with the role of secretory pathways in response to preischemic challenge.

Ischemic induced alterations of mitochondria, endoplasmic reticulum and Golgi apparatus (Fig.1 and Fig. 2) shed light on the understanding of cross-talk between intracellular Ca^{2+} stores in cerebral ischemia/reperfusion and in the phenomenon of ischemic tolerance. Documented neuroprotective response of intracellular organelles in the phenomenon of ischemic tolerance might suggest possible targets for future therapeutic interventions to enhance recovery after stroke (Dirnagl et al. 2009; Pignataro et al. 2009).

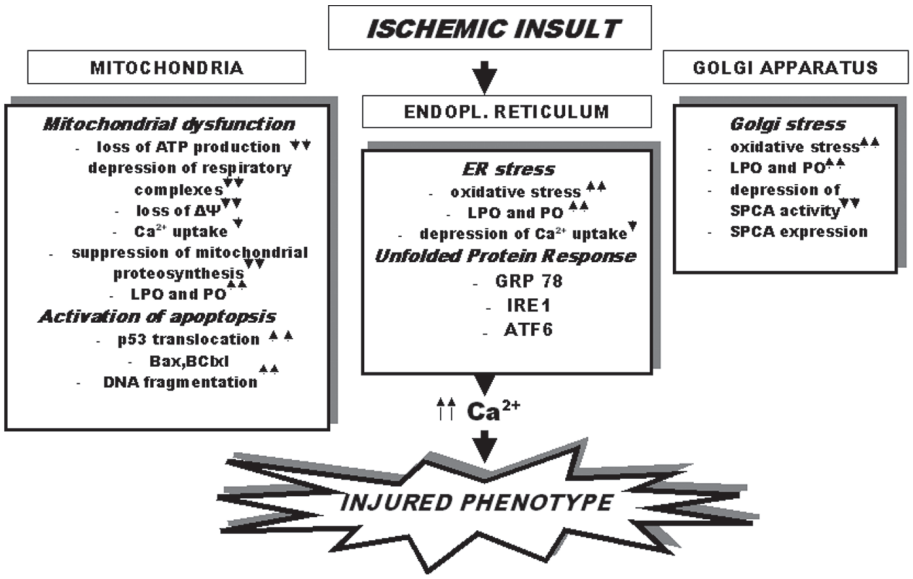

Figure 1. Schematic drawing of the documented mechanism for intracellular organelles dysregulation leading to ischemic injurious phenotype (Lehotsky et al. 2009; Pavlikova et al. 2009; Racay et al. 2007, 2009a,b,c; Urban et al. 2009). ↑↑ = significant alterations, ΔΨ = membrane potential, LPO = lipoperoxidation, PO = protein oxidation.

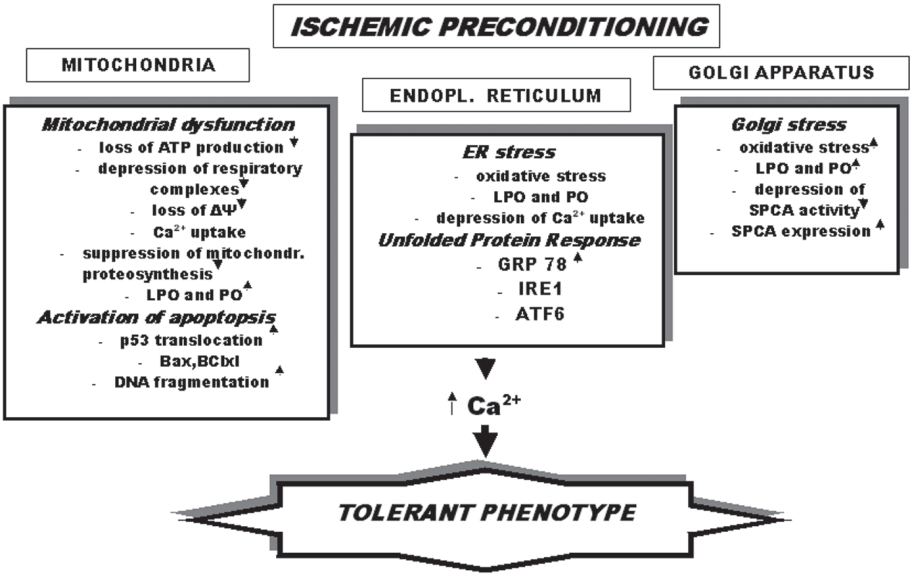

Figure 2. Schematic drawing of the mechanism for documented intracellular events activating after preconditioning which eventualy lead to ischemic tolerant phenotype (Lehotsky et al. 2009; Pavlikova et al. 2009; Racay et al. 2007, 2009a,b,c; Urban et al. 2009). ↑ = medium alterations, ΔΨ = membrane potential, LPO = lipoperoxidation, PO = protein oxidation.

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      Hodgkin A. L., Huxley A. F., Katz B. (1952): Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. J. Physiol. (London) **116**, 424–448

   b) *Monographs:*

      Zachar J. (1971): Electrogenesis and Contractility in Skeletal Muscle Cells. University Park Press, Baltimore and London

c) *Chapters from monographs:*

Haggis G. H. (1964): The structure and function of membranes. In: Introduction to Molecular Biology. (Eds. G. H. Haggis, D. Michie, A. R. Muir, K. B. Roberts and P. M. B. Walker), pp. 151–192, Longmans, London

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