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.

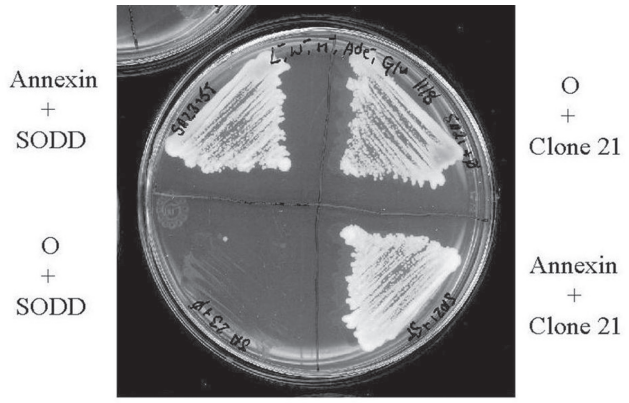

Figure 4. Yeast two hybrid evidence for an interaction between SODD (Suppressor Of Death Domains) and the N-terminal region of annexin A7. AH109 cells have been streaked on a leu$^-$, trp$^-$, his$^-$, ura$^-$ plate. Strains harboring the following plasmids are streaked in each quadrant: Upper left, 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.

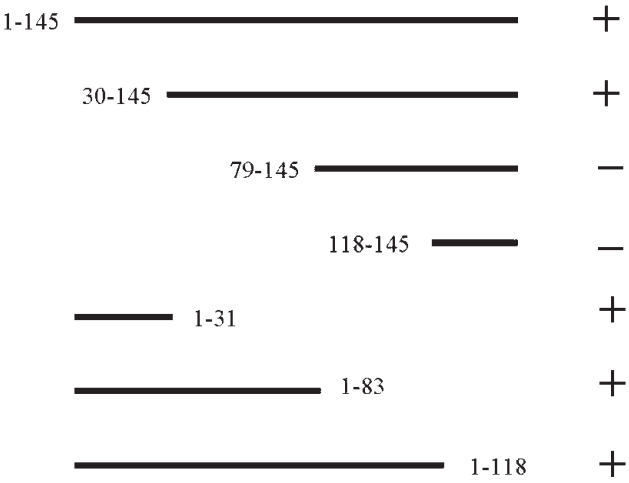

Figure 6. Deletion mapping of the binding site for SODD on the annexin A7 N-terminal region. cDNAs encoding the residues of the annexin (indicated on the left), from the full N-terminal sequence (1-145) to the fragments as labeled were cloned into the yeast two hybrid bait vector. Cells were co-transformed 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.

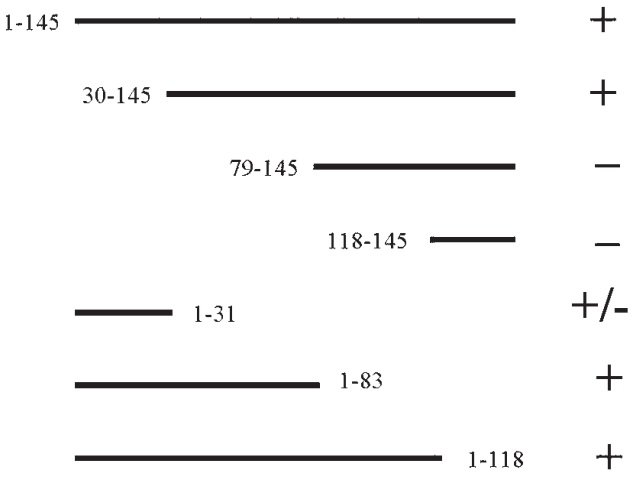

Figure 7. Deletion mapping of the interaction between the N-terminal region of annexin A7 with itself. The full N-terminal sequence (residues 1 to 145) was cloned into the prey vector and then tested in the two hybrid system with the same fragments of the N-terminal region in the bait vector as used 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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