

Calcium-binding Proteins: Basic Concepts and Clinical Implications

C. W. HEIZMANN

Department of Pediatrics, Division of Clinical Chemistry, University of Zurich, Steinwiesstr. 75, CH-8032 Zurich, Switzerland

Abstract. Calcium ions exert their effects in part via interactions with a wide variety of intracellular calcium-binding proteins. One class of these proteins shares a common calcium-binding motif, the EF-hand. A consensus amino acid sequence for this motif has aided the identification of new members of this family of EF-hand proteins, which now has over 200 members. A few of these proteins are present in all cells, whereas the vast majority are expressed in a tissue-specific fashion. The physiological function of a few of these proteins is known to be achieved via a calcium-dependent interaction with other proteins, thereby regulating their activity. Some members, like parvalbumin, calbindin, and calretinin, proved to be useful neuronal markers for a variety of functional brain systems and their circuitries. Their major role is assumed to be buffering, transport of Ca^{2+} , and regulation of various enzyme systems. Since cellular degeneration is accompanied by impaired Ca^{2+} homeostasis, a protective role for Ca^{2+} -binding proteins in certain neuron populations has been postulated.

Another protein family are the annexins, members of which interact with phospholipids and cellular membranes in a calcium-dependent manner. In some cases members of the annexin family were even found to interact with EF-hand proteins. Certain annexins have been suggested to be involved in anti-inflammatory response, inhibition of blood coagulation, membrane trafficking or cytoskeletal organization, but several of these functions have been questioned recently. The elucidation of the interactions and functions of the majority of these proteins remains a challenging task for the coming years.

Key words: Ca^{2+} -binding proteins — EF-hand structure — Annexin — Lipocortin — Chromosomal localization

Introduction

A large number of biological processes are regulated, directly or indirectly, by the ubiquitous second messenger calcium. It is the increase of the free Ca^{2+} concentra-

tion within stimulated cells that triggers fundamental processes such as metabolism, contraction, secretion, cell division, cell growth, and memory storage. Calcium concentrations have been found to oscillate in many cell types. Additionally, different stimuli can elicit different spatial calcium concentration patterns within the same cell. This suggests that the type of physiological response that is triggered may depend on which parts of the cell are exposed to the localized calcium signal.

In normal cell homeostasis, the resting level of intracellular calcium is approximately 200 nmol/l compared to the approximately 5,000 times higher concentrations in the extracellular space. Cells thus have developed sophisticated mechanisms that precisely control influx and extrusion of calcium in the presence of this high transsarcolemmal calcium gradient. Long term maintenance of Ca^{2+} homeostasis is the concerted action of the importing systems, the Ca^{2+} channels (Neher 1992), the exporting systems (the Ca^{2+} pump, the $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger) (Carafoli 1991), and the Ca^{2+} -binding/buffering/transporting/regulating proteins (Kretsinger et al. 1991; Heizmann and Hunziker 1991).

Endogenous calcium ions – in contrast to their vital physiological role – can adopt the role of malignant 'killers' if cellular calcium metabolism is impaired and calcium levels increase severalfold above normal.

Elevated cytoplasmic calcium levels have been found in several tumor cells and it has been suggested that this might contribute to the increased motility and hence invasiveness of these cells (Durham and Walton 1982; Tsuruo et al. 1984; Banyard and Tellam 1985; Whitfield et al. 1987).

Further examples are the cardiac myocytes, which undergo severe alterations, ending up in necrosis when calcium levels increase due to ischemia (Fleckenstein et al. 1987; Barry 1991). Ca^{2+} overload in the cell results in a deficiency of high energy phosphates, due to excessive consumption of ATP caused by an overactivation of Ca^{2+} -dependent ATPases, and insufficient ATP resynthesis, due to functional and structural impairment of mitochondria. In addition, alteration of Ca^{2+} transport occurs as well as stimulation of Ca^{2+} -activated proteases (calpain), leading to a rapid degradation of cellular proteins (Fleckenstein et al. 1987; Barry 1991; Croall and Demartino 1991). It has been found that treatment with Ca^{2+} antagonists such as verapamil, nifedepine, and various derivatives thereof, has a protective effect by preventing excessive transsarcolemmal Ca^{2+} entry; these agents are used for the treatment of cardiomyopathy and arteriosclerosis (Fleckenstein et al. 1987) as well as of bipolar affective disorders (Dubovsky et al. 1986, 1989). Abnormal Ca^{2+} concentrations in cells may also be caused by an altered expression of Ca^{2+} -binding proteins, and several research groups have now started to investigate changes of these proteins in several human neurodegenerative disorders (Heizmann and Braun 1992).

The calcium signal is transmitted into the intracellular response, in part by Ca^{2+} -binding proteins that are involved in the regulation of many cellular activities.

In this review I would like to summarize their protein and gene structures, localization, tissue-specific expression, physiological functions, and their possible roles in health and disease.

According to their structural features Ca^{2+} -binding proteins can be subdivided into the following families:

(a) Proteins belonging to the same evolutionary family (Moncrief et al. 1990) and sharing a type of Ca^{2+} -binding domain known as the EF-hand (Kretsinger et al. 1991; Heizmann and Hunziker 1991; Heizmann, 1991)

(b) Hemolysin-type putative Ca^{2+} -binding proteins (Bairoch and Cox 1990; Economou et al. 1990; Chauvaux et al. 1990). Hemolysins were isolated from various bacteria. Bacterial hemolysins are exotoxins that attack blood cell membranes and cause cell rupture. These proteins bind Ca^{2+} and contain a variable number of tandem repeats of a nine amino acid motif that is rich in glycine, aspartic acid, and asparagine. It has been suggested that a domain consisting of four of these repeats could bind one calcium ion. Members of this family include hemolysins, cyclolysin, extracellular zinc proteases, and a nodulation protein (nodo).

(c) The annexin protein family, members of which interact with phospholipids and cellular membranes in a calcium-dependent manner (Klee 1988; Moss et al. 1991; Cirino and Flower 1991).

The EF-hand protein family

Calmodulin, troponin-C, parvalbumin, calbindin, S-100 proteins, calpain, and altogether more than 200 proteins now belong to this family (Heizmann and Hunziker 1991; Kretsinger et al. 1991). All of these proteins exhibit a common structural motif, the EF-hand, which is present in multiple copies and binds calcium selectively and with high affinity. Each of these domains consists of a loop of 12 amino acids (a variant loop with 14 amino acids is present in the S-100 protein subfamily) that is flanked by two α -helices. This structural principle was first identified with the crystal structure of the calcium-binding carp parvalbumin and has been termed EF-hand after the E- and F-helices of parvalbumin (Kretsinger and Nockolds 1973; Kumar et al. 1990; Roquet et al. 1992).

From this structural information, the putative EF-hand-dependent calcium-binding ability of a protein can be predicted or confirmed on the basis of the amino acid or cDNA-sequence, leading to the discovery of many new EF-hand proteins. The model even allows us to predict whether an EF-hand domain is still functional or whether it has lost its calcium-binding ability due to mutations.

EF-hand proteins are thought to have evolved from a single ancestral EF-hand motif. During evolution, multiple reiterations of this primordial gene gave rise to genes coding for proteins with multiple EF-hands. Subsequent gene duplications led to a large family of proteins (Moncrief et al. 1990; Lee et al. 1991). Together

with the growth in their numbers, their functions and expression patterns also diverged. Through mutations in the EF-hand loop, some domains have lost the ability to bind calcium. Acquisition of different promoters has allowed some to be ubiquitously expressed (e.g. calmodulin), whereas most of the others show a tissue-specific pattern of expression, some even in a hormonally regulated fashion (e.g. calbindins).

So far the genes of only a few members of the EF-hand Ca^{2+} -binding proteins have been analyzed, but already a picture of a family tree is emerging from comparisons of the numbers and positions of the introns (Heizmann and Hunziker 1991).

One branch of the family is the S100-like proteins. All genes of this group that have been analyzed contain an intron in the linker region that separates the two EF-hand domains.

The second family branch contains proteins with four EF-hands (e.g. calmodulin, troponin C) and a related protein with three EF hands (parvalbumin), related on the basis of intron positions and numbers.

The third family branch contains the six EF-hand domain proteins, calbindin D-28K, and calretinin.

Interestingly, in the S-100 branch the introns separate functional domains whereas in the other two branches the introns are preferentially located within the EF-hand domains (Heizmann and Hunziker 1991).

Adjacent localization of two related genes on the same chromosome can be indicative of a recent gene duplication event and therefore suggests a close kinship. Several members of the S-100 protein family are located on chromosome 1 but their distances are not yet known. As an exception, the human S-100 β , a member of the same subfamily, is located on chromosome 21. Chromosome localization of a gene in conjunction with the chromosome localization of certain inherited diseases could yield some clues as to the physiological function of the protein. It has been speculated that some of the impaired brain functions observed in trisomy 21 might be a consequence of an additional copy of the S-100 β gene. An elevated S-100 β level in the blood and lymphocyte fraction of patients with Down syndrome have been reported. The known chromosomal assignments of other EF-hand calcium-binding proteins are listed in Table 1.

The S-100 protein family

S-100 proteins are a group of small acidic Ca^{2+} -binding proteins expressed in a cell type-dependent fashion (Table 2) (for reviews see Hilt and Kligman 1991; Longbottom and van Heyningen 1991). The amino acid sequences of a number of S-100 proteins have been conserved in a wide variety of organisms ranging from protozoa to man. This strong conservation argues for an important (and preserved) biological role for S-100 proteins.

Table 1. Chromosomal assignment of the genes encoding calcium-binding proteins

Protein	Human chromosome	Reference
S-100 proteins		
– S-100 β	21q22	Duncan et al. 1989
– S-100 α	1	Morii et al. 1991
– calcyclin	1q21-q25	Ferrari et al. 1987
– MRP-8 and MRP-14 (also termed CF-antigen, CAGA or CAGB)	1q12-q21	Brüggen et al. 1988 Dorin et al. 1990
– placental calcium-binding protein (CAPL)	1q12-q21	Dorin et al. 1990
α -Parvalbumin	22q12-q13.1	Berchtold et al. 1987 Ritzler et al. 1992
β -Parvalbumin (oncomodulin)	7p11-p13	Berchtold et al. 1987 Ritzler et al. 1992
Calbindin D-28K	8q21.3-q22.1	Parmentier et al. 1989
Calretinin	16q22-q23	Parmentier et al. 1989
Myosin alkali light chains (MLC-1 and MLC-3)	2q32.1-9ter	Seidel et al. 1988
Myosin light chain (MLC-1emb/A)	17q	Seharaseyon et al. 1990
Sorcin	7	Van der Blik et al. 1988
Sorcin-related gene	4	Van der Blik et al. 1988
Calmodulin pseudogene	17	Sen-Gupta et al. 1989
Calpains (L ₁ , L ₂ , L ₃ , S)	1,11,15,19	Ohno et al. 1990
Calcineurin (catalytic subunits)	4,10	Giri et al. 1991

Table 2. Properties of the S-100 protein family

S-100 protein ¹ family	Synonyms and species homologs	Cell type/source	Context described
S-100 α		neurons, cardiac and skeletal (type I) muscle fibres	transduction of Ca ²⁺ in these cells
S-100 β		glial cells other cells	regulation of cell morphology, cytoskeletal organization and cell proliferation; extracellular function: neurite extension
S-100L		lung and other cells	expressed in lung cells
Ca[1]	p10 p11 42C	many cells PC12 cells	regulators subunit of tyrosine kinase substrate NGF induced

CAPL	p9Ka	breast myoepi- thelial cells	induced during differen- tiation of cuboidal cells to myoepithelial cells
	18A2	fibroblasts and other cells	serum-stimulated mRNA
	pEL98	mammary carci- nomas and many other cells	S-phase cell-cycle specif. induced in transformed cell lines
	42A Metastasin (mtS1)	PC12 cells tumor cells	NGF induced mRNA spe- cies increased expression in tumor cells with metastatic potential
CACY	2A9	fibroblasts, other cells	serum-stimulated mRNA
	PRA	mammary carcino- ma and other cells	G1 phase cell-cycle specific co-purifies with ¹ prolactin receptor
CaBP9K	IaBP Calbindin-D9K	intestinal cells	vitamin D ₃ -induced Ca ²⁺ -binding protein; Ca ²⁺ transport
CAGA	CFAg MIF MRP8 p8 L1 Ag light chain MAC387 Ag α chain 60B8Ag	myeloid cells	expressed in cystic fibrosis and rheumatoid arthritis
CAGB	MIF MRP14 p14 L1 Ag light chain MAC387 Ag β chain 60B8Ag CFP	myeloid cells	expressed in rheumatoid arthritis

¹ Nomenclature according to: Longbottom and van Heyningen (1991).

Ca[1], calpactin-1 light chain; CAPL, placental Ca²⁺-binding protein; CACY, calcyclin; CaBP9K, 9 kDa vitamin D₃-induced intestinal Ca²⁺-binding protein; CAGA, calgranulin A; CAGB, calgranulin B.

The individual members of this family are described in detail in Heizmann (1991).

The number of S-100 proteins are steadily increasing. We cloned S-100 α CAPL and other S-100 proteins from human tissue (Engelkamp, et al. 1992) and detected two novel S-100 proteins (Engelkamp et al., in preparation).

The biological functions of the S-100 proteins are less clear. They likely exert their biological effects by interacting with secondary effector proteins in a Ca^{2+} -dependent fashion. This mode of protein-protein interaction and modulation of the activity of the secondary effector protein is similar to that seen with calmodulin S-100 proteins, however, they interact with a different set of binding proteins than does calmodulin. One member of the S-100 protein family, the p11, is associated with the tyrosine kinase substrate p36 (or annexin II) (Gerke 1991). It has been suggested that the $\text{p36}_2\text{p11}_2$ complex might be involved in the membrane-cytoskeletal linkage and/or the control of membrane fusion events during exocytosis (Burgoyne and Geisow 1989). The most difficult and interesting question to date remains that of the exact biological function of these proteins. Toward this goal experiments of over- and underexpression of S-100 proteins in cells or transgenic animals have been started in several laboratories.

For example, antisense inhibition of S-100 β production in cultured glial cells resulted in the alteration in cell morphology, cytoskeletal organization, and cell proliferation (Selinfreund et al. 1990). It will now be possible to study the molecular mechanism of S-100 β function, its action and interaction, in more detail. There is increasing evidence that S-100 proteins may also be secreted and may act extracellularly (Kligman and Marshak 1985; Van Eldik and Zimmer 1987; Selinfreund et al. 1991). There are also data indicating that intracellular and secreted levels of S-100 β are differently regulated and that ACTH and isoproterenol stimulate the release of S-100 β (Suzuki et al. 1987).

Calcium-binding proteins in health and disease

Calcium-binding proteins are involved in a wide variety of activities, such as cytoskeletal organization, cell motility and differentiation, cell cycle regulation, calcium buffering and transport. One might therefore suppose that altered levels of some calcium-binding proteins (e.g. due to a deletion or mutation of the corresponding genes) should lead to an impaired calcium homeostasis in cells and to pathological conditions.

Several research groups have now started to search for an altered expression of the Ca^{2+} -binding proteins, parvalbumin, calbindin D-28K, and S-100 proteins, in affected brain regions of patients. Cases examined include those suffering from acute insults such as stroke and epileptic seizures, and from chronic neurodegenerative disorders, such as Alzheimer's, Huntington's, Parkinson's, and Pick's diseases. In addition, altered calcium levels have been found in platelets of patients with bipolar affective disorders (Dubovsky et al. 1986, 1989), and calcium antagonists have been suggested for treatment of psychotic depression.

Calcium overload as a result of seizures or ischemia is supposed to activate biochemical processes leading to enzymatic breakdown of proteins and lipids, to malfunctioning of mitochondria, energy failure and ultimately to cell death. There

is reason to assume that neurons that contain certain intracellular calcium-binding proteins, and therefore have a greater capacity to buffer calcium, would be more resistant to degeneration. Investigations of the vulnerability of such neurons in human brain as well as in experimental animal models have revealed contradictory results concerning the postulated protective role of calcium-binding proteins (for review see Heizmann and Braun 1992).

The increase in intracellular calcium that occurs as a result of excitatory amino acid receptor activation has been suggested to be the initiating factor in seizure associated degeneration and neuronal death. Since only certain subsets of neurons are susceptible to irreversible damage the positive correlation between parvalbumin- or calbindin D-28K-content and relative resistance to seizure-induced neuronal damage in certain hippocampal neuron populations is in support of the neuroprotective hypothesis of calcium-binding proteins. However, in human epileptic brain tissue some authors have reported that the parvalbumin- and calbindin D-28K immunoreactive neurons in the hippocampus were relatively spared from degeneration and only some calbindin D-28K positive granule cells were lost, while other authors have found a clear loss of parvalbumin- and calbindin D-28K immunoreactive neurons.

The levels of Ca^{2+} -binding proteins have also been investigated in chronic neurodegenerative diseases, e.g., Alzheimer's disease and Parkinson's disease, however, results from those studies on human pathological material are inconsistent.

Ca^{2+} -binding proteins have also been suggested as diagnostic tools, e.g., S-100 protein has been measured in cerebrospinal fluid and blood of patients with cerebral infarction, transient ischemic attack, hemorrhage, and head injury (Persson et al 1987). An enhancement of S-100 β was also found in the blood of patients with Down syndrome (Kato et al 1990). These and other Ca^{2+} -binding proteins might be selective markers in the future to estimate the extent of brain damage in the various neurological disorders and also to classify various brain tumors in children (Ishiguro et al 1983) and in adults (Fagnart et al 1988).

Calcium-dependent and phospholipid-binding proteins, the annexins

Members of the annexin protein family interact with phospholipids and cellular membranes in a calcium-dependent manner (Klee 1988, Moss et al 1991, Cirino and Flower 1991). A common nomenclature has recently been proposed for these proteins, which have previously been known as lipocortins, calcimedins, calpactins, or chromobindins (Table 3). These proteins show a high degree of sequence homology and share a repetitive conserved sequence. This segment of 70 amino acid residues might include the site for calcium binding and for association with phospholipids. Although these proteins lack sequences conforming to the typical EF-

Table 3. Nomenclature of the annexin family

Annexin	Synonyms
I	Lipocortin I Calpactin II p35 Chromobindin 9
II	Calpactin I heavy chain Lipocortin II p36 Chromobindin 8 Protein I heavy chain Placental anticoagulant protein IV
III	Lipocortin III Placental anticoagulant protein III 35- α Calcimedlin
IV	Endonexin I Protein LL 32.5 kDa-Calelectrin Lipocortin IV Chromobindin 4 Placental anticoagulant protein II Placental protein 4-X 35- β Calcimedlin
V	Placental anticoagulant protein I Inhibitor of blood coagulation Lipocortin V 35 kDa-Calelectrin Endonexin II Placental protein 4 Vascular anticoagulant-a 35- γ Calcimedlin Calphobindin I Anchorin CII
VI	p68, p70, 73k 67 kDa-calelectrin Lipocortin VI Protein III Chromobindin 20 67 kDa-calcimedlin Calphobindin II

VII	Synexin
VIII	Vascular anticoagulant- β
IX and X	from <i>Drosophila</i>

For details and references see Crumpton and Dedman (1990); Moss et al. (1991); Huber et al. (1992); Moss (1992).

hand structure, they do contain sequences resembling mutated EF-hands. In some cases members of the annexin family were even found to interact with EF-hand proteins. The chromosomal assignment of the genes encoding annexins is shown in Table 4. Certain annexins have been suggested to be involved in anti-inflammatory

Table 4. Chromosomal assignment of the genes encoding annexins

Protein	Human chromosome	Reference
Annexin I	9q11-greater than q22	Huebner et al. 1988
Annexin II	4q21-q31.1; 9pter-q34 proximal to c-abl; 10q proximal to 10q24; 15q21-q22 proximal to the 15q22 translocation breakpoint	Huebner et al. 1988
Annexin III	4q21	Tait et al. 1991a
Annexin IV	2p13	Tait et al. 1992
Annexin V	4q28-q32	Modi et al. 1989
	4q26-q28	Tait et al. 1991b

response, inhibition of blood coagulation, membrane trafficking, or cytoskeletal organization. Interestingly, some of these integral membrane proteins were reported to exhibit voltage-dependent calcium channel activities (Burns et al. 1989; Brisson et al. 1991). Annexin 1 was found to inhibit eicosanoid production, replication of A 549 cells, carrageenin oedema, cytokine-induced neutrophil migration, chemotaxis, and fever (Rothwell and Flower 1992). In addition, anti-annexin-1 antibodies were found to reverse anti-inflammatory and antipyretic actions of glucocorticoids *in vivo*, suggesting that endogenous annexin-1 mediates these effects. In the brain, annexin-1 is expressed in neurons and glia. A biologically active recombinant fragment of annexin-1 inhibits the central actions of cytokines on fever and thermogenesis, and may protect against ischaemic and excitotoxic damage. Lipocortin-1 expression in brain is increased in response to many forms of cell damage (Johnson et al. 1989). Administration to rats of anti-lipocortin-1 antibody exacerbates brain damage, indicating that annexin acts as an endogenous neuroprotective agent. The first evidence of a cellular 'receptor' for annexin was reported

recently. A Ca^{2+} -dependent binding site on human peripheral blood leukocytes was identified. A marked decrease in the density of binding sites was observed in patients with rheumatoid arthritis.

Preliminary data also indicate the presence of two specific 15 kDa and 18 kDa binding proteins, and further characterization may reveal the structure of a putative receptor for annexin-1.

In conclusion

At present, information on new calcium binding proteins, their primary and X-ray structures, their distribution, localization, and regulation of expression is accumulating at a rapid pace. The most difficult and challenging question to date remains that of their exact biological roles and their mechanism of action and interaction. In the future, gene transfer or gene disruption experiments could prove to be useful especially for those Ca^{2+} -binding proteins that are expressed in a tissue-specific fashion. Overexpression or disruption of these genes could yield interesting information on some of the distinct functions and targets of these large families of proteins.

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