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Topical Review

Calpain: A Cytosolic Proteinase Active at the Membranes

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Discovery and Initial Progress

The first report of a cytosolic Ca^{2+}-dependent proteolytic activity was that by Guroff [26] who partially purified from a rat brain homogenate a proteinase which was maximally activated by 1 mM Ca^{2+}. The first function attributed to this proteinase was the activation of muscle phosphorylase kinase [30]. The observation in the late 70s that the Ca^{2+}-dependent proteolytic activity was not detectable in crude tissue homogenates, but became evident after an anion exchange chromatography step, led to the discovery of a heat stable endogenous inhibitor which was eliminated during this purification step. The names calpain and calpastatin for the proteinase and the endogenous inhibitor, respectively, were proposed by Murachi [63]. In this review the following abbreviations will be used: CANP for the Ca^{2+} Activated Neutral Proteinase (calpain) and CALST for calpastatin. The existence of a second isozyme was established in the early 80s by the purification of a new calpain isoform which had a higher Ca^{2+} sensitivity [20, 53, 65, 91]. The nomenclature now in use refers to in vitro experiments where m-CANP shows proteolytic activity at mM Ca^{2+} concentrations, whereas μ-CANP is already active at μM Ca^{2+} concentration. Even this Ca^{2+} level, however, is 10 to 50-times higher than that in the cytosol. Nevertheless, both isoforms are likely to be able to process cellular substrates at physiological Ca^{2+} concentrations (100–300 nM) as shown for example by early experiments by Schollmeyer [95]. The problem of the requirement of excessive Ca^{2+} concentration is still open, but the presence of activating factors in the in vivo environment is an attractive possibility. The finding that m-CANP cleaves target proteins in liver nuclei in the presence of Ca^{2+} concentrations as low as 3 μM, provided that DNA is also present, is certainly of interest [57]. m- and μ-CANP (as well as their endogenous inhibitor CALST) are widely distributed in tissues, but their relative and absolute abundance varies.

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Recent Developments

The study of the involvement of CANP in physiopathological processes at the cellular level has been hampered by the lack of cell-permeable CANP-specific inhibitors able to discriminate between the Ca^{2+}-activated enzyme and other cysteine proteinases (e.g., the lysosomal cathepsins B, H and L, 115 for a review). The recent characterization of a new class of nonpeptide, cell-permeable CANP inhibitors (alpha-mercaptoacrylic acid derivatives) which can be used as neuroprotective substances in hypoxic/hypoglycemic injury [113, 114] is thus a very significant development. Another difficulty has been the uncertainty on the activation mechanism of the proteinase: while it is widely accepted that CANP activity in vitro is accompanied (or preceded) by the autolytic processing of the enzyme, recent reports indicate that autolysis may not be obligatory in vivo, i.e., under conditions in which unidentified ''factors'' or, more simply, the association with biological membranes, may be sufficient to free the active site, making it accessible to

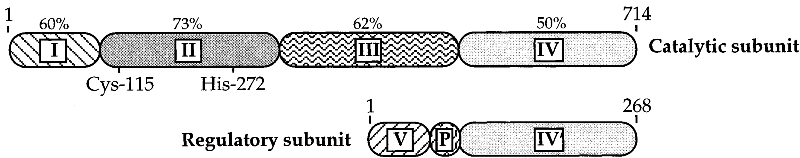

Fig. 1. Sub domain organization of the catalytic and the regulatory subunits of human μ-CANP. The percentages of identity among domains I, II, III, IV of μ- and m-CANP is given. Additional details in the text.

substrates at physiological Ca^{2+} concentration. As will be discussed in this review, the irreversible process of CANP autolysis may occur in vivo only under extreme conditions such as cell necrosis or apoptosis, whereas activation under normal cell conditions may simply reflect the reversible association of the proteinase with membrane (and/or with the underlying cytoskeleton) where preferential substrates may be cleaved in response to a limited fluctuation of the cytosolic Ca^{2+} concentration.

Very significant advances in the CANP field have been the recent discovery of the involvement of a novel CANP-isoform in a genetic disease (Limb-Girdle Muscular Dystrophy Type 2A, LGMD) [84] and the characterization of new tissue specific CANP isoforms. LGMD is characterized by the progressive symmetrical atrophy and weakness of the proximal limb muscles and by elevated blood levels of creatine kinase. In contrast with all other known muscular dystrophies, such as the Duchenne and Becker diseases [9], the severe childhood autosomal recessive dystrophy [52], the Fukuyama [51], and merosin-deficient congenital muscular dystrophies [111] and the primary adhalin deficiencies [85], this type of dystrophy is caused by mutations affecting an enzyme and not a structural component of muscle. A number of nonsense, frame-shift, splice site or missense mutations were found in the region of chromosome 15 responsible for the expression of the catalytic subunit of CANP3 (the muscle-specific CANP isoform, see next lines). The mutations described in [84] produce truncated and probably inactive forms of CANP3, which have lost their Ca^{2+} sensitivity.

The finding of DNA and RNA coding for tissue-specific CANPs (n-CANP) in skeletal and smooth muscle has opened the problem of discriminating between ubiquitous (m- and μ-CANP) and tissue-specific isoforms (novel-CANP, n-CANP) of the proteinase. Not much is known on the latter: in skeletal muscle the amount of mRNA coding for the ubiquitous CANP isoforms was found to be 10-fold lower than that of a tissue-specific isoform of the proteinase, nCL1 (p94, CANP3). Curiously, however, attempts to detect nCL1 in muscle failed, probably due to its rapid turnover, estimated at 27 min. Since mutations of the active cysteine 129 to serine stabilizes the proteinase, and since the degradation of the inactive serine 129-nCL1 was found to be significant after co-expression with wild type nCL1, its quick disappearance should be an autolytic process [101, 102]. The sequence of nCL1 includes three blocks not found in the ubiquitous CANPs [100]. Block number 2 (IS2, residues 578-653) contains a lysine-rich, nuclear translocation signal [102] and is apparently involved in the rapid turnover of the protein because its deletion stabilizes it. IS2 also mediates the interaction with a giant (3,000 kDa) protein of striated muscle, connectin, which apparently replaces the 30 kDa regulatory subunit normally associated with the catalytic subunits of m- and μ-CANPs *(see* Fig. 1). Interestingly, the sequence neighboring the nCL1 binding site of connectin is similar to that of the endogenous CANP inhibitor calpastatin (CALST) [101]. The absence of the regulatory subunit in nCL1 is typical of the tissue specific isoforms identified so far. Attempts to add the latter to the catalytic subunit of n-CANPs and binding studies using the yeast two-hybrid system [101] have confirmed that the tissue specific isoforms are unable to form the dimeric structure. When nCL1 was expressed in COS and L8 myoblast cells, it was detected mainly in the cytosol but partly also in the nucleus, in line with the presence of the nuclear localization sequence [102]. The smooth muscle (stomach)-specific CANPs have been termed nCL-2 and nCL-2′: the latter is an alternative splicing product that does not contain the putative Ca^{2+}-binding domain [102]. Since the amount of nCL-2′ mRNA in stomach muscle is comparable to that of the other CANP isoforms (μ-, m-CANP, and nCL-2), a significant amount of atypical, i.e., Ca^{2+}-*in*dependent CANP activity may be present at least in that tissue. Splicing products lacking the Ca^{2+}-binding domain have also been found in Drosophila (calpA′, [109]) and in *C. elegans* (Ce-CL-2, Ce-CL-3, [117]).

CANP Nomenclature

As can be easily deduced from the previous section, the discovery and characterization of new CANP isoforms has made the nomenclature so far used in the field inadequate and perhaps misleading. The original nomenclature proposed by Murachi [64] had distinguished Calpain 1 (later μ-CANP), the first Ca^{2+}-dependent proteolytic activity eluted with a salt gradient from a DEAE chromatography column, from Calpain 2 (later m-CANP),

eluted as a second protein peak at higher ionic strength. It did not take into account the new tissue-specific CANP isoforms known as n-calpains.

We would like to propose the use of **CANP** as the acronym for **C**a^{2+} **A**ctivated **N**eutral **P**roteinase (calpain). To differentiate between ubiquitous and tissue-specific CANPs we would like to propose the use of **u-CANP1** and **u-CANP2** indicating the **u**biquitous high and low Ca^{2+}-sensitive isoforms respectively. **t-CANP** (up till now n-calpain or nCL) should indicate the tissue specific isoforms. To account for the likely discovery of new tissue-specific isoforms the acronym t-CANP could be followed by a letter code to identify the tissue of origin. Thus, nCL-1 (also indicated as p94 or calpain 3 in the literature) which is the tissue-specific isoform of the skeletal muscle would now become **t-CANPsk,** and nCL-2, the isoform of the smooth muscle would become **t-CANPsm.**

Role of CANP in Cell Physiology and Pathology

CANP has been proposed to play a role in physiological and pathological processes as diverse as differentiation and cell cycle regulation [23, 56, 95, 118], signal transduction [29, 35, 79], long-term potentiation [46] and fracture healing [68]: the latter role would be an example of the possible extracellular activity of the proteinase. CANP may be also involved in degenerative diseases of muscle and nerve [2, 6, 15, 38, 79, 84, 97, 99, 103], in the development of hypertension and in the process of cataract formation [5, 18, 19, 94]. The possibility of a role of CANP in apoptosis has recently attracted considerable attention [24, 25, 31, 50, 69, 104, 110].

Membrane permeable, active site-directed CANP inhibitors have been used as tools to investigate these suggested roles of the enzyme. As mentioned above, however, most commercially available inhibitors have insufficient specificity, i.e., conclusions on the roles of CANP based on inhibition studies must be taken with caution. Reports describing abnormal expression/activity levels of CANP or of its inhibitor CALST in various pathological conditions (e.g., blood cell syndromes [41, 42, 75] or the previously mentioned LGMD [11, 84, 101]) carry more weight.

The Ubiquitous CANP Isoforms

CANPs (EC 3.4.22.17) are widely distributed in the animal kingdom. They have been found in mammalian, avian, piscean cells [64], in crustaceans [8, 66, 67] in insects [62, 77, 78], in invertebrates (the nematode Schistosoma mansoni [98], in the octopus [27]) and in fungi [74]. As mentioned, they are members of the family of cysteine proteinases, which comprises papain, cathepsin

Table. Sequence comparison between different cysteine proteinases

Region surrounding the reactive cysteine	
104.RTDICQGALGD**C**WLLAAIASLTLND..	μ-CANP
94..RTDICQGALGD**C**WLLAAIASLTLNE..	m-CANP
14..TPVKNQGACGS**C**WAFSTIATVEGII..	Papain
130.SPVKNQGACGS**C**WTFSTTGALESAI..	Cathepsin H
Region surrounding the reactive histidine	
261.EAITFKKLVKG**H**AYSVTGAKQVNYR..	μ-CANP
251.EAITFQKLVKG**H**AYSVTGAEEVESN..	m-CANP
148.VFDGPCGTKLD**H**AVTAVGYGTSDGK..	Papain
270.TSCHKTPDKVN**H**AVLAVGYGEKNGI..	Cathepsin H
Region surrounding the reactive asparagine	
284.RGQVVSLIRMR**N**PWGEVEWTGAWSD..	μ-CANP
274.NGSLQKLIRIR**N**PWGEVEWTGRWND..	m-CANP
296.VGYGPNYILIK**N**SWGTGWGENGYIR..	Papain
287.EQNGLLYWIVK**N**SWGSNWGNNGYFL..	Cathepsin H

The catalytic amino acids are in boldface

B, H and L, and bromelain. Human μ-CANP shows sequence similarity to other cysteine proteinases in the portion surrounding the catalytic amino acids cysteine 115, histidine 272 and asparagine 295 (Table). The most remarkable difference between μ- and m-CANP (*see following sections*), in the Ca^{2+} requirement for in vitro activation. Otherwise, these two isoenzymes are rather similar in subunit and domain organization (Fig. 1) catalytic specificity and cell distribution (even though a selective nuclear transport has been reported for μ-CANP [55]). One recently reported difference, however, may be the specific inhibition of m-CANP by NO [58].

As mentioned, the tissue-specific CANPs are monomeric enzymes, whereas the ubiquitous CANPs (μ- and m-CANP) are heterodimeric proteins consisting of a specific catalytic subunit of 80 kDa and a regulatory subunit of 30 kDa. The primary structure of both subunits has been deduced from the cDNA from different sources [1, 21, 22, 33, 72, 73, 90]. The two subunits have been divided in domains; the catalytic subunit in domains I, II, III and IV and the regulatory subunit in domains IV′ and V.

Domain I is the NH_2-terminal portion of the catalytic subunit and has no significant homology to other proteins. It becomes autoproteolyzed when the proteinase is exposed to Ca^{2+}. The autoproteolytic processing also involves domain V of the regulatory subunit and requires 10 to 50 μM Ca^{2+} for μ-CANP and 300 to 500 μM Ca^{2+} for m-CANP. Autolysis has been found to be essential for the irreversible activation of CANP in vitro [54, 105]. However, as will be discussed later, the activation of CANP in vivo must occur at physiological, i.e., much lower Ca^{2+} concentration. Since it should be reversible, it may thus not involve autolysis. The autopro-

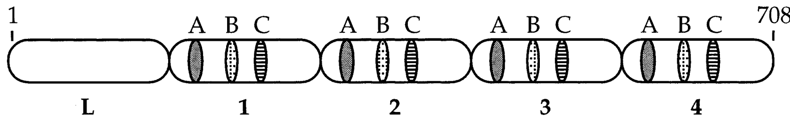

Fig. 2. A scheme of the domain organization of human CALST. Details in the text.

teolytic step, i.e., its mechanistic properties and its necessity for the activation of the proteinase are one of the obscure points in the CANP field.

Domain II has homology to the catlytic domain of other cysteine proteinases (*see* Table) and its active site contains the essential residues cysteine, histidine, and asparagine.

Domain III is similar to the first domain and does not have significant homology to other proteins. Its function remains unknown, but the proposal has been made that a conformational change of this domain is responsible for the transduction of the Ca^{2+} signal from subdomain IV (Ca^{2+}-binding) to subdomain II (catalytic). A role of domain III in the interaction of CANP with its endogenous inhibitor CALST has also been proposed [17].

Domains IV and *IV′* (calmodulin-like domains, CaMLDs) are assumed to confer Ca^{2+} sensitivity to the enzyme. They show evident homology with Ca^{2+}-binding proteins of the EF-hand family, particularly in the Ca^{2+}-binding loops.

Domain V represents the NH_2-terminal portion of the regulatory subunit. Of 66 residues, 57 are hydrophobic (the sequence contain 40 glycines). The suggestion has been made that this region could be the anchor responsible for the insertion of CANP into membranes which is likely to be a step of the activation pathway in vivo. However, a recent study has shown that the association of the proteinase with the membrane was inhibited by CALST fragments that bind to the CaMLDs of CANP. The finding suggests that the association of the proteinase with the membrane is mediated by the interaction of domains IV and IV′ with membrane proteins and/or phospholipids, rather than by the insertion of domain V into the phospholipids bilayer [40].

The Endogenous Protein Inhibitor of CANP: Calpastatin (CALST)

Calpastatin is the ubiquitous intracellular reversible inhibitor of CANP. It does not inhibit other cysteine proteinases such as cathepsin B or papain. It is a heat stable, acidic (pI 4.7), protein rich in hydrophobic residues and in prolines, and very poor in aromatic residues. Two isoforms of the protein are known: the tissue-type (110 kDa) has the molecular organization illustrated in Fig. 2. The erythrocyte-type (70 kDa) lacks domains L and 1 [108]. The gene of CALST is located on the long arm of chromosome 5; a deletion in this chromosomal region was detected in myeloid disorders known as 5q-syndromes [48]. CALST has 4 repeated inhibitory domains of about 140 amino acids (1 to 4 in Fig. 2). The conserved residues among the four domains are clustered in three restricted regions (A, B and C) encoded by separate exons in the human gene. The analysis of recombinant CALSTs with various deletions in the inhibitory domains has revealed that the conserved **regions B** are essential for the (Ca^{2+}-dependent) inhibition of CANP [39, 49]. Interestingly, however, the inhibitory region of CALST does not interact directly with the active site cysteine of CANP, since the latter can be masked by covalent inhibitors without impairing the binding of CALST [M. Molinari, J. Anagli, E. Carafoli, *unpublished results*].

The conserved **regions A** and **C** do not display inhibitory activity, but potentiate that of region B since they promote the tight binding of CALST to CANP. Recent reports [107, 117] have shown that a 19-mer peptide derived from region A interacted with the CaMLD of the catalytic subunit of CANP, and that the corresponding 19-mer peptide derived from region C interacted preferentially with the CaMLD of the regulatory subunit; both interactions were Ca^{2+}-dependent. Moreover, a portion of CALST covering the conserved regions C of domain 1 through regions A of domain 2 was found to inhibit CANP-binding to membranes. This peptide bound to the CaMLDs which are likely to be involved in the interaction of the protease with membrane protiens and/or phospholipids [40]. Ca^{2+} can be replaced in the binding of the proteinase to its inhibitor by higher concentrations of other divalent cations (such as Ba^{2+}, Sr^{2+} and Mn^{2+}). However, no binding was observed in the presence of Mg^{2+} [47].

CANP Is Active at the Membranes

The problem of the activation of CANP under physiological conditions in vivo is controversial. In vitro, the cleavage of substrates by CANP is always preceded by its autolysis. However, the autoproteolytic cleavage of the catalytic and regulatory subunits requires Ca^{2+} concentrations which are at least one order of magnitude higher than the normal cytosolic level (*see above*), even with the more sensitive μ-CANP.

This discrepancy has led to two hypotheses: (i) The *proenzyme hypothesis* [54, 105] proposes that the acti-

vation of CANP requires an autolytic process that ''frees'' the active site allowing the cleavage of substrates. The hypothesis is mainly based on in vitro experiments, in which the kinetic of the autolytic reaction is so fast that it always precedes the processing of the substrates. SDS gel electrophoresis techniques have documented the rapid autolysis of the regulatory subunit from 30 to 18 kDa and the simultaneous autolysis of the catalytic subunit from 80 to 76 kDa via a short-lived 78 kDa intermediate product. Upon longer incubations in the presence of high concentrations of Ca^{2+}, autolysis progresses to the complete degradation of the proteinase into smaller inactive fragments [70]. Since these reactions, which require unphysiological Ca^{2+} concentrations, invariably precede the processing of substrates, the 80/30 kDa dimer would be a *proenzyme* and the 76/18 kDa the ''active CANP species.'' The conundrum of the necessity of high Ca^{2+} concentrations was circumvented by proposing cytosolic activating factors (proteins, phospholipids, but *see above* p. 1 for the role of DNA) that would lower the Ca^{2+} requirement for autolysis, or by suggesting that particular sequences on substrates, rich in acidic amino acids (PEST sequences, for a discussion *see* [60]) could increase the local Ca^{2+} concentration to levels adequate to promote autolysis and then the cleavage of substrates [83, 86]. A number of recent observations, however [10, 12, 59, 60, 71, 87, 88, 118], have made the proenzyme hypothesis less appealing. Among the more recent, there is the finding that the unproteolyzed form of CANP can cleave substrates [59]. Another is the demonstration that the active site of the nonautolyzed enzyme is fully accessible to substratelike peptide inhibitors [M. Molinari, J. Anagli and E. Carafoli, *unpublished data*], an observation that suggests that the enzyme should also be able to process substrates. Furthermore, it has been reported that deletions or mutations lowering the PEST score of PEST-rich sequences of various CANP substrates (the plasma membrane Ca^{2+}-ATPase [60], the D1 polypeptide of photosystem 2 [71], and the transcription factor c-fos [10]) do not prevent the degradation by CANP. Moreover, no cytosolic CANP activator has been so far identified and characterized unambiguously. Lastly, the very recent finding of the long half-life of CANPs (and their inhibitors CALST) in ^{35}S-methionine-pulse and chase experiments is difficult to reconcile with the proenzyme theory, since autolysis would imply relatively short half-life of the enzyme in vivo [118].

The *second hypothesis* is supported by observations made both in vitro and in vivo. It proposes that the activation of CANP is independent of its autolysis [59, 88], requires the association of the proteinase to the cell membrane [59], and possibly results in the dissociation of the regulatory from the catalytic subunit [106].

Under physiological conditions, CANP is not membrane bound. Fractionation experiments on erythrocytes as cell models showed only a minor amount of CANP in the membrane fraction. However, when the cytosolic Ca^{2+} concentration was increased by treating the cells with Ca^{2+} and Ca^{2+} ionophores, a significant amount of nonautolyzed proteinase was detected in the plasma membrane. In experiments of this type, no autolyzed form of CANP was ever found associated with the erythrocyte membrane. This is at variance with a newly proposed activation model [106], where a fraction of active CANP is associated to the membrane in the autolyzed form. On the other hand, CANP was only detected in the cytosolic fraction of A23187/Ca^{2+}-treated erythrocytes in the 80 and in the 78 kDa forms: apparently, the rapid autolysis to the 76 kDa form observed in in vitro experiments, does not occur or is greatly slowed down in vivo. It was thus proposed that CALST blocks the irreversibly activated 78 kDa form of the enzyme which leaves the membrane after autolysis and which, if converted to the extremely active 76 kDa form, would be dangerous to the cell. It was further suggested that the physiologically active CANP is the 80 kDa form which becomes *reversibly* activated by membrane association: in agreement with this, the nonautolyzed form of membrane-bound CANP was found to be active on its preferred substrates. The proposal that autolysis could be a post-activation process, irrelevant to the cleavage of substrates, is now gradually gaining acceptance [12, 13, 36, 59, 76, 80, 82, 88, 89, 106]. Since both the binding of CANP to CALST, and CANP activation, require Ca^{2+}, and since both the proteinase and its inhibitor are cytosolic proteins, CANP must escape CALST control to be active in the cell. Although the association of CALST with membranes has been reported in a single case [96], one could speculate that the translocation of CANP to the membrane during the activation process could be a way to segregate the enzyme away from the inhibitor.

The CANP/CALST balance may become impaired in pathology: e.g., in erythrocytes of aging individuals, CALST is largely degraded, resulting in the decrease of its inhibitory activity [96]. The balance could be genetically disturbed, e.g., in essential hypertension, in which the cytosolic CALST level has been found to be particularly low [93]. In these conditions, the permanently activated CANP dissociating from the membrane would not be completely buffered by the cytosolic CALST, thus escaping its control [59]. Recent reports indicate that CANP and CALST are substrates of different protein kinases, e.g., PKA [45]. The modulation of their activity by phosphorylation/dephosphorylation cycles is thus a likely possibility. Two forms of cytosolic CALST differing in phosphorylation state and specificity towards μ- and m-CANP have been described [92]. An additional control mechanism for the proteolytic activity of

CANP could thus be the phosphorylation/dephosphorylation level of its endogenous inhibitor.

Ca^{2+}-Independent CANP Activation

In a very recent report [3] an activation model of CANP in vivo has been proposed which appears not to depend on variations of intracellular Ca^{2+} concentration. Exploiting the findings that hepatocellular carcinoma cells are more resistent to anoxia than normal hepatocytes, and that CANP is likely to be involved in this kind of cell death, the report has examined the differences in CANP and CALST expression level and CANP activity in these two cell types: increased CANP activity by unchanged enzyme level was observed in normal hepatocytes. At variance with the Ca^{2+} level, which did not change in both cell cultures during the 60 minutes of induced anoxia, increased amounts of phospholipid degradation products were detected in normal hepatocytes. The finding that necrosis (and CANP activity) in hepatocellular carcinoma cells is increased by mellitin, an agonist of phospholipase A_2, is in line with the concept of a phospholipase-mediated, Ca^{2+}-independent CANP activation [3].

CANP and Membrane Fusion

Erythrocyte membrane fusion can be promoted by Ca^{2+} in vitro [43], limited degradation of membrane proteins by CANP being a prerequisite for it [28, 44]. More recent studies have extended the findings to myoblast cell lines in culture, confirming the involvement of the CANP/CALST system in the process of myoblast differentiation and fusion: a marked decrease of the CALST level was detected during differentiation, leading to the increase in the activity of CANP required for myoblast fusion [7].

Phospholipids as CANP Activators

CANP migrates from the cytosol to the membrane in response to the increase of the cytosolic-free Ca^{2+} concentration. Membrane bound cytoskeletal (e.g., spectrin) and membrane-intrinsic proteins (e.g., the Ca^{2+}-ATPase) as well as cytosolic proteins targeted to the cell membrane under specific conditions (e.g., PKC), are usually considered as preferential CANP substrates, (*see* [88, 112] for a detailed discussion of the CANP substrates). The physical vicinity (membrane or cytoskeleton) and/or the presence in the candidate substrates of structural motifs (e.g., PEST sequences, CaM-binding domains) favor the attack by membrane-associated CANP.

Another important point is the concentration of Ca^{2+} and/or the sensitivity of CANP to it. Membrane phospholipids may be key factors, since they have been variously reported to increase the Ca^{2+}-sensitivity of μ-CANP in in vitro tests. Polyphosphoinositides may be particularly important, as underlined by the inhibition of their stimulatory effect by the polyphosphoinositide-binding antibiotic neomycin [88]. The matter of phospholipids in CANP activation however, still has obscure aspects: activation, i.e., lowering of the calcium requirement for CANP autolysis by phosphatidyl inositol and no effect for phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidic acid has been reported [14], whereas others have found that phosphatidyl inositol, phosphatidyl choline, phosphatidyl ethanolamine and to a lesser extent phosphatidyl serine, could all reduce the calcium requirement for autolysis in m-CANP [81]. More recently, [4] activation has been reported for all the phospholipids tested (phosphatidyl inositol, phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidic acid and sphingomyelin). The sequence $[G]_{17}$TAMRILGG in the V domain of the regulatory subunit has been suggested to be essential for the role of phosphatidylinositol in the decrease of the optimal Ca^{2+} concentration for CANP-autolysis [4, 16, 32]: possibly, the sequence is the phosphatidylinositol-binding site of CANP.

Alternatively to the phospholipids, however, a number of studies have indicated that proteins could be the binding sites for CANP in the membrane. Thus it was demonstrated that the trypsinization of inside-out plasma membrane vesicles, but not the treatment with phospholipase, decreased the amount of CANP associated with the membrane after Ca^{2+} exposure [34, 37]. Further support for the proteinaceous nature of the CANP-receptor(s) in the membrane has been provided by recent experiments [40] in which a portion of CALST spanning the conserved regions C of domain 1 through region A of domain 2 (*see* Fig. 2) was found to inhibit CANP-binding to membranes. As already mentioned above, this finding suggests that domain IV, and not the Gly-rich domain V is principally responsible for the interaction. The role of domain IV in the interaction of CANP with membrane-inserted substrates is also supported by recent independent work [61]. A conciliatory proposal would be that the migration of CANP to the membrane following the increase in cytosolic Ca^{2+} is based on both the direct interaction of the protease with membrane substrates or with the underlying cytoskeleton and on the insertion of the hydrophobic domain V of the regulatory subunit into the phospholipid bilayer.

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