REVIEW ARTICLE

Calmodulin-binding proteins as calpain substrates

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CALPAIN

Calcium-dependent neutral protease, also called calpain, has attracted increased interest in recent years. Its properties include an absolute dependence on Ca^{2+} for activity (micromolar and millimolar concentrations of Ca^{2+} for calpain I and calpain II, respectively), and a pH optimum between 7 and 8. It is isolated from the cytosolic fraction of various animal tissues or cells but it may be translocated to the cell membrane under certain conditions (for previous reviews see Murachi, 1983a; Zimmerman & Schlaepfer, 1984; Pontremoli *et al.*, 1985; Bond & Butler, 1987; Mellgren, 1987; Suzuki, 1987; Suzuki *et al.*, 1987a,b). Recently, calpain from various species has been sequenced (Ohno *et al.*, 1984; Emori *et al.*, 1986b; Sakihama *et al.*, 1985; Aoki *et al.*, 1986). Calpain I and calpain II seem to have distinct catalytic 80 kDa subunits and an identical regulatory 30 kDa subunit. Both the 80 kDa and 30 kDa subunits contain calmodulin-like 'E-F hand' Ca^{2+} -binding domains at their C-terminal ends (Ohno *et al.*, 1984). It was suggested by Suzuki and colleagues (Ohno *et al.*, 1984) that these Ca^{2+} -binding domains confer Ca^{2+} -sensitivity to calpain. The activity of calpain is also believed to be regulated by an endogenous calpain inhibitor protein, calpastatin (see Murachi, 1983a).

Despite the advances in knowledge of the structure and enzymic properties of calpain, its biological functions are still poorly understood. It is clear, however, that calpain does not have general proteolytic activity, but rather catalyses specific and limited cleavage of substrates. Interestingly, calpain I and calpain II appear to have identical substrate specificities. Endogenous protein substrates for calpains are generally classified into five subgroups: (i) enzymes (including several kinases), (ii) myofibrillar proteins, (iii) membrane proteins, (iv) cytoskeletal proteins, and (v) receptor proteins (Murachi, 1983a; Zimmerman & Schlaepfer, 1984).

CALMODULIN-BINDING PROTEINS AS CALPAIN SUBSTRATES

Calmodulin is a small, acidic Ca^{2+} -binding protein that is best known for its ability to regulate many physiological processes by interacting with various calmodulin-binding proteins (for review, see Manalan & Klee, 1984; Wang *et al.*, 1985). Vertebrate calmodulin-binding proteins can be categorized into three groups: (a) enzymes, (b) cytoskeleton/structural proteins, and (c) other proteins (Table 1). Kōsaki *et al.* (1983) reported

that several calmodulin-binding proteins (100 kDa, 90 kDa, 60 kDa and 40 kDa) were degraded by endogenous calpain I. Wallace *et al.* (1987) confirmed this study by showing that a similar set of calmodulin-binding proteins was degraded by calpain upon platelet activation. While we were studying the calpain-mediated proteolysis of the calmodulin-activated Ca^{2+} -ATPase from erythrocyte membrane (Wang *et al.* 1988a,b), we observed that another calmodulin-binding protein, adducin (103 + 97 kDa), which co-eluted as a minor component of some preparations of the Ca^{2+} -ATPase from a calmodulin-affinity column (Wang *et al.*, 1988a,b), was also fragmented by calpain treatment (Fig. 1a, lanes 2, 3 and 4). We therefore made a systematic search for other calmodulin-binding proteins which might also be calpain-sensitive (Table 1). Of the 30 calmodulin-binding proteins listed in Table 1, 16 have already been reported to be substrates for calpain *in vitro*. So far, no calmodulin-binding proteins have been reported to be resistant to calpain, although we have found that one form of calmodulin-dependent cyclic nucleotide phosphodiesterase is calpain-resistant (K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work). Nevertheless, this is a rather striking finding given the fact that calpain has a narrow range of substrate specificity. Many proteins are calpain-resistant, including bovine serum albumin, ovalbumin, sarcoplasmic reticulum Ca^{2+} -ATPase, calsequestrin, calmodulin, actin, ubiquitin, phosphorylase and cytochrome *c* (Zimmerman & Schlaepfer, 1984; Pontremoli *et al.*, 1985; K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work). Of the 16 identified calpain-sensitive calmodulin-binding proteins, five are enzymes, seven are cytoskeleton/structural proteins and the other four represent a miscellaneous group of proteins (Table 1).

Enzymes

Among the five calpain-sensitive calmodulin-dependent enzymes (phosphorylase kinase, myosin light chain kinase, plasma membrane Ca^{2+} -ATPase, phosphodiesterase and calcineurin), similarities in their proteolysis by calpain are found (Table 2): (i) the fragmentation patterns of myosin light chain kinase (Kōsaki *et al.*, 1983; Ito *et al.*, 1987), Ca^{2+} -ATPase (Wang *et al.*, 1988a,b) and calcineurin (Tallant *et al.*, 1988; K. K. W. Wang, B. D. Roufogalis & A. Villalobo, unpublished work) are different in the absence and the presence of calmodulin; (ii) proteolysis of four out of the five enzymes in the absence of calmodulin activates their respective enzymic activities (Table 2). In fact, earlier studies revealed that

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Table 1. Matching of calmodulin-binding proteins and calpain substrates

Abbreviations: CaM, calmodulin; N.D., not determined.

Protein	$10^{-3} \times$ Subunit M_r	Reference* (as calmodulin-binding protein)	Calpain-sensitivity and reference*
(a) Enzymes			
Adenylate cyclase	150	[1,2]	N.D.
Phosphorylase kinase	145 (α), 128 (β), 45 (γ), 17 (δ)	[3]	+ [34,35]
Myosin light chain kinase	150	[4]	+ [36,37]
Ca ²⁺ -ATPase (plasma membrane)	136	[5,6]	+ [38-40]
Phosphofructokinase	80	[7]	N.D.
Phosphodiesterase (cyclic nucleotide)	61, 63	[8-10]	+ [37]
Calcineurin	60 (α), 19 (β)	[11]	+ [41,42]
Inositol 1,4,5-trisphosphate kinase	53	[12]	N.D.
CaM-dependent protein kinase I	37-39	[13]	N.D.
CaM-dependent protein kinase II	50 (α), 58 (β), 60 (β')	[14]	N.D.
CaM-dependent protein kinase III	140	[15]	N.D.
Dynein ATPase	400	[16]	N.D.
(b) Cytoskeleton and structural proteins			
Nebulin	300	[17]	+ [43]
Microtubule-associated protein-2 (MAP-2)	270	[18]	+ [44,45]
Fodrin (brain spectrin)	240 (α), 235 (β)	[19]	+ [46,47]
Spectrin	240 (α), 220 (β)	[20]	+ [47,48]
Caldesmon	150, 75	[21]	+ [30]
Microvillus 110 kDa protein	110	[22]	N.D.
Tubulin	50, 55	[23]	+ [45]
Tau factor	55-62	[24]	N.D.
Adducin	97 (α), 100 (β)	[25]	+ [49]
(c) Others			
Calcium release channel	350	[26,27]	+ [26,27]
Regulatory subunit (type II cyclic AMP-dependent protein kinase)	50	[28]	N.D.
Calspermin	32	[29]	N.D.
Gap junction protein (lens)	26	[30]	N.D.
Phospholamban	5	[31]	N.D.
Neuromodulin (P-57, GAP-43)	24	[32]	+ [50]
Myelin basic protein	22	[33]	+ [51]
Histone 2B	19	[33]	+ [52]

* 1, Yeager *et al.* (1985); 2, Shattuck *et al.* (1987); 3, Chan & Graves (1984); 4, Klee (1977); 5, Lynch & Cheung (1979); 6, Niggli *et al.* (1979); 7, Mayr & Heilmeyer (1983); 8, Cheung (1970); 9, Kakiuchi & Yamazaki (1970); 10, Sharma *et al.* (1980); 11, Tallant & Cheung (1986); 12, Johanson *et al.* (1988); 13, Nairn *et al.* (1985a); 14, Kennedy *et al.* (1987); 15, Nairn *et al.* (1985b); 16, Blum *et al.* (1980); 17, Patel *et al.* (1988); 18, Lee & Wolff, (1984); 19, Carlin *et al.* (1983); 20, Sobue *et al.* (1981a); 21, Sobue *et al.* (1981b); 22, Glenney & Weber (1980); 23, Kumagai *et al.* (1982); 24, Sobue *et al.* (1981c); 25, Gardner & Bennett (1986); 26, Seiler *et al.* (1984); 27, K. K. W. Wang, J. S. C. Gilchrist, B. D. Roufogalis, S. Katz & A. N. Belcastro, unpublished work; 28, Hathaway *et al.* (1981); 29, Ono *et al.* (1984); 30, Welsh *et al.* (1982); 31, Molla *et al.* (1983); 32, Andreassen *et al.* (1983); 33, Grand & Perry (1980); 34, Meyer *et al.* (1964); 35, Huston & Krebs (1968); 36, Kōsaki *et al.* (1983); 37, Ito *et al.* (1987); 38, Aii (1987); 39, Wang *et al.* (1988a); 40, Wang *et al.* (1988b); 41, Tallant *et al.* (1988); 42, K. K. W. Wang, B. D. Roufogalis & A. Villalobo, unpublished work; 43, Goll *et al.* (1983); 44, Kubota *et al.* (1986); 45, Billger *et al.* (1988); 46, Siman *et al.* (1984); 47, Seubert *et al.* (1987); 48, Pant *et al.* (1983); 49, see Fig. 1(a); 50, see Fig. 1(b); 51, Banik *et al.* (1985); 51, and 52, Sakai *et al.* (1987).

phosphodiesterase (Cheung, 1971; Kincaid *et al.*, 1985), phosphorylase kinase (Depaoli-Roach *et al.*, 1979), myosin light chain kinase (Walsh *et al.*, 1982; Foyt *et al.*, 1985), calcineurin (Manalan & Klee, 1983; Tallant & Cheung, 1984), plasma membrane Ca²⁺-ATPase (Zurini *et al.*, 1984) and calmodulin-dependent protein kinase II (Levine & Sahyoun, 1987) are all activated and become calmodulin-insensitive after limited proteolysis by extracellular proteases, such as trypsin or chymotrypsin. It seems likely that these extracellular proteases mimic endogenous Ca²⁺-dependent proteolysis by calpain. The mechanism of activation of these calmodulin-dependent enzymes by limited proteolysis is envisaged as follows:

calmodulin-dependent enzymes contain a calmodulin-binding domain with an adjacent or overlapping inhibitory domain. Upon binding of calmodulin, the calmodulin-binding and inhibitory domains undergo conformational changes, which release the inhibition of enzymic activity. Limited proteolysis either removes both the calmodulin-binding and inhibitory domains or only the former. In either case, the inhibition is relieved and the enzyme expresses its full activity which is no longer calmodulin-sensitive. In the absence of calmodulin, calpain proteolysis of plasma membrane Ca²⁺-ATPase and calcineurin produces active calmodulin-insensitive fragments. On the other hand, in the presence of calmodulin,

Table 2. Effect of calpain on calmodulin-dependent enzymes

For references see Table 1. Abbreviations: CaM, calmodulin; N.D., not determined.

Enzyme	Effect of calpain on activity	Effect of calmodulin on calpain action
Phosphorylase kinase	Activated	N.D.
Myosin light chain kinase	Inactivated and loss of CaM binding	Prevents activation and loss of CaM-binding
Ca ²⁺ -ATPase (plasma membrane)	Activated and independent of CaM	Prevents activation and loss of CaM-sensitivity
Phosphodiesterase	Activated and independent of CaM	No effect
Calcineurin	Activated and independent of CaM	Slows down the loss of CaM-sensitivity

proteolysis produces active enzyme fragments which retain their calmodulin-binding capacity (Wang *et al.*, 1988a,b; Tallant *et al.*, 1988). Therefore, it seems that calmodulin can function to modulate calpain-mediated proteolysis of calmodulin-binding proteins.

The effect of calmodulin on calpain proteolysis of calmodulin-binding proteins is in some ways analogous to the effect of ubiquitin on the ATP-dependent protease system (Rechsteiner, 1987a). Thus ubiquitin, when conjugated to target proteins, signals the ATP-dependent protease to hydrolyse the conjugated protein, whereas calmodulin, when bound to the calmodulin-binding protein, modifies the pattern of proteolysis. The difference is that whereas ATP-dependent proteolysis occurs only when substrates are conjugated to ubiquitin, calpain proteolysis occurs both in the absence and in the presence of calmodulin. Intriguingly, both calmodulin and ubiquitin are small proteins (148 residues for calmodulin and 76 residues for ubiquitin) present in all eukaryotic cells and conserved to an exceptional level (Manalan & Klee, 1984; Finley & Varshavsky, 1985). Both proteins are multi-functional and are involved in various biochemical processes in cells. Furthermore, ubiquitin itself is not proteolysed by ATP-dependent protease and, likewise, calmodulin is not proteolysed by calpain.

Cytoskeletal/structural and other proteins

When we look more closely at the calpain-mediated proteolysis of calmodulin-binding proteins that are not enzymes, we find that many of these are cytoskeletal proteins, including spectrin, which forms the cytoskeleton backbone and components of microtubules (tubulin and MAP-2). The effect of calmodulin on the proteolysis of these calmodulin-binding proteins is less clear. No apparent difference in fragmentation pattern in the absence and the presence of calmodulin was found for caldesmon (Kōsaki *et al.*, 1983), erythroid spectrin (Seubert *et al.*, 1987) and adducin (K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work). However, calmodulin increases the rate of degradation of brain spectrin (fodrin) by calpain (Seubert *et al.*, 1987).

The function of the proteolysis of cytoskeletal or membrane proteins might be to induce cytoskeletal or cell membrane remodelling. For instance, cell membrane fusion requires removal of some membrane proteins to provide an enriched lipid environment at the site of fusion (Kosower *et al.*, 1983). Lynch & Baudry (1984)

hypothesized that as a result of calcium influx upon neuronal stimulation, fodrin is degraded locally by calpain and induces remodelling of the postsynaptic membrane. Consequently, initially latent glutamate receptors are exposed, which might contribute to the long-term potentiation observed in the study. Beside these passive roles on the cytoskeleton, microtubule proteins may also be involved in the repertoire of movements of the eukaryotic cell, including movements of individual cells or of organelles within the cytosol (i.e. mitosis, axonal transport or movements of pigment or secretory granules) (Bryan, 1974). Therefore, these processes may be viewed as being potentially dually controlled by calmodulin and calpain.

SUBSTRATE SPECIFICITY OF CALPAIN

Using naturally occurring and artificial peptides as substrates for calpain, Sasaki *et al.* (1984) formulated a general preference rule for calpain proteolysis at the cleavage site: a Lys, Tyr, Arg or Met residue in the P₁ position preceded by a hydrophobic amino acid residue (Leu or Val) in the P₂ position would favour cleavage at the carboxyl side of the residue in the P₁ position (nomenclature of Schechter & Berger, 1967). Very similar preference was observed using neuropeptides as substrates (Hirao & Takahashi, 1984). However, this pattern does not explain all the cases (Sasaki *et al.*, 1984). Furthermore, small peptides were known to be rather poor substrates for calpain as compared to protein substrates (Murachi, 1983b), and therefore, the preference rule derived from peptide substrates may not apply to protein substrates. Recently Sakai *et al.* (1987) studied the specificity of calpain cleavage sites in histones and found that the P₂-P₁ preference rule was not necessarily followed. Instead, they found that susceptible bonds were never located in the midst of either hydrophobic or hydrophilic amino acid clusters, but in the vicinity of the boundary between hydrophilic and hydrophobic clusters. This cleavage site specificity could account for the fact that proteolysis induced by calpain usually produces larger limit fragments which are not further susceptible to calpain cleavage. For instance, Sakai *et al.* (1987) observed that all the fragments released from histones underwent no further degradation, although many of them still contained bonds which would be susceptible to calpain if they were present in intact histones. This

finding suggests that calpain recognizes a certain higher order structure(s) in its substrates before cleavage is induced. This would explain why small peptides are in general poor substrates for calpain, since they are not large enough to contain the required recognition site(s).

When this 'recognition site' concept is applied to the calmodulin-binding proteins, which are often substrates for calpain, it follows that there may be certain common region(s) or features on these proteins that are recognized by calpain. The most obvious region would be the calmodulin-binding domain of these proteins. It has been demonstrated that the calmodulin-binding domains of many different calmodulin-binding proteins share a high degree of homology (Blumenthal *et al.*, 1985; Lukas *et al.*, 1986; Wakim *et al.*, 1987; Bennett & Kennedy, 1987; Lin *et al.*, 1987; Shull & Greeb, 1988). A typical calmodulin-binding domain is thought to be a short amphipathic α -helix peptide segment with a high percentage of basic amino acid residues (arginine, histidine and lysine) (Blumenthal & Krebs, 1988). It is believed that the resulting net positive charge favours the interaction with the strongly acidic calmodulin. As discussed earlier, calpain's large subunit (80 kDa) has its catalytic domain near the *N*-terminal region and has a calmodulin-like E-F hand Ca^{2+} -binding domain at the *C*-terminal region (Ohno *et al.*, 1984). It is conceivable that the calmodulin-like domain of calpain can bind to the calmodulin-binding domain of the substrate proteins. Once binding is achieved, the calpain catalytic domain proceeds to cleave its bound substrate. However, this seemingly attractive mechanism cannot account for the fact that some calmodulin-binding proteins, after losing their calmodulin-binding domain, are still susceptible to further proteolysis (Kōsaki *et al.*, 1983; Ito *et al.*, 1987; Wang *et al.*, 1988b). This prompted us to look for an alternative mechanism.

PEST SEQUENCES IN CALMODULIN-BINDING PROTEINS

Recently, Rogers *et al.* (1986) found that each of ten short-lived proteins examined (half-lives less than 2 h) contain one or more regions enriched in proline (P), glutamic acid (E), aspartic acid (D), serine (S) and threonine (T) (PEST regions). Similar inspection of 35 more stable proteins (half-lives between 20 and 220 h) revealed that only 15 of these contained PEST regions. The strength of a PEST sequence can be evaluated by its PEST score (Rogers *et al.*, 1986). While PEST scores theoretically can range from -45 to $+50$, two types of PEST regions exist: those with a PEST score > 0 , indicating a strong PEST region, and those with a PEST score ≤ 0 but > -5 , indicating a weak PEST region. Strikingly, in the original study nine of the ten short-lived proteins have at least one strong PEST region, whereas only three of the 35 stable proteins have a strong PEST region. These authors concluded that these PEST regions confer the property of rapid degradation to the protein containing them. They also hypothesized that since the E and D residues in the PEST sequence are both negatively charged, and S and T are potentially phosphorylatable, phosphorylation of such PEST sequences would produce very negatively charged regions which may bind calcium. In turn, this local calcium concentration may activate calpains. Therefore, in relation to our finding that many calmodulin-binding proteins are calpain substrates, we

postulate that many calmodulin-binding proteins may share sequence homology in addition to the calmodulin-binding domain, namely, PEST regions, and that it may be these PEST regions that are commonly recognized by calpain. In order to test this hypothesis, we looked for PEST sequences in calmodulin-binding proteins that have been sequenced using a PEST sequence-searching computer program 'PEST-FIND' (Rogers *et al.*, 1986). We found that all the calmodulin-binding proteins but three sequenced to date contain one or more PEST sequences (Table 3). Furthermore, the PEST scores of these regions are generally high (> 0). In fact, all of these calmodulin-binding proteins but one (phosphorylase kinase, γ -subunit) have at least one strong PEST region (with PEST score > 0) (Table 3). Of the PEST-containing calmodulin-binding proteins in Table 3, tubulin (α , β), erythrocyte Ca^{2+} -ATPase, α -spectrin and myosin light chain kinase (chicken gizzard) have been demonstrated to be fragmented by calpain (Table 1).

The three calmodulin-binding proteins that do not have PEST sequences are myelin basic protein, histone IIB and phospholamban (Table 3). Interestingly, none of the three proteins appear to have well-defined calmodulin-binding domains (Eylar *et al.*, 1971; Iwai *et al.*, 1970; Fujii *et al.*, 1987). Worth mentioning is that the plasma membrane, calmodulin-stimulated Ca^{2+} -ATPase shares a high degree of structural homology with the sarcoplasmic reticulum Ca^{2+} -ATPase which, however, lacks a typical calmodulin-binding domain (Verma *et al.*, 1988; MacLennan *et al.*, 1985). Whereas the former has five strong PEST sequences (PEST scores 2.4, 14.8, 8.3, 7.1 and 1.6) (four of them near the *C*-terminal and one near the *N*-terminal), the latter has only three weak PEST regions (PEST scores -3.4 , -4.0 and -4.9). In support of the observed correlation, the plasma membrane Ca^{2+} -ATPase is rapidly hydrolysed at four or five sites (Wang *et al.*, 1988b), whilst the sarcoplasmic reticulum Ca^{2+} -ATPase is resistant to calpain (Seiler *et al.*, 1984; K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work). Furthermore, the first few cleavage sites are also located at the *C*-terminal end of the plasma membrane Ca^{2+} -ATPase, corresponding to the region where many of the PEST sequences are found. Thus, the strong PEST regions on the plasma membrane Ca^{2+} -ATPase may be the recognition sites for calpain.

The calmodulin-binding protein neuromodulin (P57) was the first test of the hypothesis. From the known sequence of neuromodulin (Wakim *et al.*, 1987), we identified two strong PEST regions, with PEST scores of 8.1 and 3.5, respectively (Table 3). We predicted, therefore, that neuromodulin would be a good substrate for calpain. Subsequently, we found that neuromodulin was indeed hydrolysed by calpain into several fragments (Fig. 1b). Another test of the hypothesis was human α -fodrin (brain spectrin), whose molecular structure has recently been elucidated. Based on the partial amino acid sequence, α -fodrin, like the erythroid α -spectrin, appears to have 22 106-residue repeat structures (McMahon & Moon, 1987). Human α -fodrin possesses a highly basic calmodulin-binding region at the junction between the eleventh and twelfth repeats, which accounts for its high affinity for calmodulin (Harris & Morrow, 1988; Harris *et al.*, 1988). Calpain I and II appear to cleave α -fodrin (240 kDa) selectively in the middle of the molecule, to produce a 150 kDa calmodulin-binding fragment (Harris *et al.*, 1988). Very recently, the cleavage site was identified

Table 3. PEST sequence of calmodulin-binding proteins

Protein	Residues	PEST sequence*†	PEST score‡	Reference§
Tau factor (mouse brain)	111–121	KTPPGSGEPPK	10.6	[1]
	121–140	RSGYSSPGSPGTPGSR	5.1	
	307–338	HLSNVSTGSIDMVDSPLATL-ADEVASLAK	–4.3	
Neuromodulin (bovine brain)	112–135	KGEGAPDAATEQAAPQ-APAPSEEK	8.1	[2]
	167–209	KQADVPAAVTAAATAPAAE-DAAAMATAQPPTETAESSQAEEK	3.5	
Calmodulin-dependent protein kinase II, regulatory subunit (rat brain)	225–245	KAGAYDFPSPWDVTPEAK	2.7	[3]
α				
β	226–246	KAGAYDFPSPWDVTPEAK	2.7	[4]
Tubulin	430–451	KDYEEVGVDSEGE-GEEEGEEYend	13.7	[5]
α (rat brain)				
β (human brain)	396–443	HWYTGEGMDEMEFTEAESNMN-DLVSEYQQYQDATAEEEEEDFGE-EAEEEEAend	10.5	[6]
Ca ²⁺ -ATPase (human erythrocyte plasma membrane)	64–80	KTSPNEGLSGNPADLER	2.4	[7]
	1078–1095	KEEIPEEELAEDVEEIDH	14.8	
	1158–1174	HIPLIDDTDAEDDAPTK	8.3	
	1175–1185	RNSSPPSPNK	7.1	
	1202–1214	KSATSSSPGSPLH (no PEST)	1.6	[8]
Myelin basic protein (bovine brain)		(no PEST)	–	
Histone 2B (calf thymus)		(no PEST)	–	[9]
Regulatory subunit of cyclic AMP-dependent protein kinase II (bovine)	45–89	RASTPPAAPPSSQDFDPGAGL-VADAVADSESEDEEDLVPIGR	8.9	[10]
α -Fodrin (human brain)	93–103	RVSVCAETYNPDEEEEDTDPR	15.2	
α -spectrin	(+101)–(+111)	HPESAEDLQEK	6.8	[11]
Myosin light chain kinase (chicken gizzard)	80–94	KPDPPAGTPCASDIR	1.4	[12]
	94–127	RSSLLSWYGSSYDGGSAVQ-SYTVEIWNVDNK	–3.8	
	371–445	KVLFGTPEFVAPEVINYEPIGYETD-MWSIGVICYILVSGLSPFMGDNDNE-TLANVTSATWDFDDEAFDEISDDAK	–2.3	
	602–627	HFQIDYDEEGNCSLTISEVCGDDDAK	0.1	
(rabbit skeletal muscle)	50–59	KQDPDPSTPK	13.8	[13]
	175–201	KPLSEASELIFEGVPATPGPTEPGPAK	4.5	
	473–525	KTDMWSLGVITYMLLSGLSPFLGDDD-TETLNNVLSGNWYFDEETFEAVSDEAK	–1.9	
Phosphorylase kinase, γ subunit (rabbit skeletal muscle)	239–261	RMIMSGNYQFGSPWDYSDTVK	–5.1	[14]
Phospholamban (rabbit skeletal muscle)		(no PEST)	–	[15]
Gap junction protein (bovine lens)	241–260	RPSESNGQPEVTGEPVELK	7.4	[16]

* Partial sequences are presented with the one-letter amino acid code.

† PEST sequences are identified using the PEST-FIND computer program developed by Rogers *et al.* (1986). A PEST sequence was defined as a stretch of amino acid residues beginning and ending with positively charged residues (H, K or R) with at least eight internal residues.

‡ PEST score is calculated as described by Rogers *et al.* (1986). In brief, PEST score = $0.55 (MP_{\text{PEST}}) - 0.5 (H_0)$, where MP_{PEST} = mole percentage of P, E, D, S and T after subtracting 1 mole equivalent of P, E and S, and H_0 = average hydrophobicity of the stretch (Rogers *et al.*, 1986). To qualify as a PEST sequence, the PEST score value has to be larger than –5.0. When the stretch lacks P, E/D or S/T, then its PEST score has to be larger than 0 to qualify as a PEST sequence.

§ References of amino acid sequences are as follows: 1, Lee *et al.* (1988); 2, Wakim *et al.* (1987); 3, Bennett & Kennedy (1987); 4, Bulleit *et al.* (1988); 5, Lemischka *et al.* (1981); 6, Hall *et al.* (1983); 7, Verma *et al.* (1988); 8, Eylar *et al.* (1971); 9, Iwai *et al.* (1972); 10, Takio *et al.* (1984); 11, McMahon & Moon (1987); 12, Guerriero *et al.* (1986); 13, Kennelly *et al.* (1987); 14, Reimann *et al.* (1984); 15, Fujii *et al.* (1987); 16, Gorin *et al.* (1984).

|| Residue numbers arbitrarily assigned; for detail, see legend of Fig. 2.

Fig. 1. Proteolysis of human erythrocyte adducin and bovine brain neuromodulin by calpain I

(a) Adducin (97 kDa and 103 kDa) was solubilized and co-purified with the Ca^{2+} pump from human erythrocyte as described by Wang *et al.* (1988b). Adducin preparation (100 μ g of protein) was then subjected to either no treatment (lane 2) or calpain treatment for 15 min (lane 3) or 30 min (lane 4) at 25 $^{\circ}\text{C}$ in 50 mM-Hepes (pH 7.4), containing 10 mM-dithiothreitol, 0.5 mM-EDTA, 0.7 mM- CaCl_2 , 65 mM-KCl, 0.06% (w/v) sonicated asolectin, 0.03% (w/v) Triton X-100 and 2 μ g of calpain I purified from human erythrocyte (Wang *et al.*, 1988b). Proteolysis was terminated with 20% (w/v) ice-cold trichloroacetic acid before being subjected to gel electrophoresis (Laemmli, 1970). The open arrow indicates the bands of adducin and solid arrows indicate fragments of adducin. The Ca^{2+} pump (136 kDa) and its fragment (124 kDa) are indicated by the solid triangles, whilst calpain's large subunit (76 kDa) is indicated by the open triangle. Molecular mass standards used in lane 1 (Bio-Rad) are: myosin, 200 kDa (a); β -galactosidase, 116 kDa (b); phosphorylase b, 97 kDa (c); bovine serum albumin, 66 kDa (d); ovalbumin, 43 kDa (e); carbonic anhydrase, 31 kDa (f); soybean trypsin inhibitor, 21.5 kDa (g). (b) Neuromodulin purified from bovine brain was a gift from Dr. Yuechung Liu, Dr. Edwin R. Chapman and Dr. Daniel R. Storm, University of Washington, U.S.A. Neuromodulin (4 μ g) was subjected to either no treatment (lane 2) or calpain treatment for 15 min (lane 3) or 30 min (lane 4) at 25 $^{\circ}\text{C}$ in 50 mM-Hepes (pH 7.4), containing 10 mM-dithiothreitol, 0.5 mM-EDTA, 0.7 mM- CaCl_2 and 0.5 μ g of calpain I. Proteolysis was terminated as in (a). The open arrow indicates the band of neuromodulin (appearing as a 57 kDa band) while solid arrows indicate fragments of neuromodulin. Purified calpain I (0.5 μ g) from human erythrocyte was applied to lane 5. Open triangles indicate the large subunits of calpain (80 kDa) and its active fragment (76 kDa). Molecular mass standards applied in lane 1 are as described in (a).

to be 10 residues upstream from the calmodulin-binding domain (see Fig. 2) (Harris *et al.*, 1988), which is consistent with the finding that calmodulin stimulates the degradation of brain spectrin by calpain (Seubert *et al.*, 1987). A search for PEST sequences in α -fodrin based on the partial amino acid sequence covering the seventh to fifteenth repeats (McMahon & Moon, 1987), revealed that the only PEST sequence (PEST score 6.8) was located in close proximity (128 residues downstream) to the calpain cleavage site (Fig. 2).

RECOGNITION OF CALMODULIN-BINDING PROTEINS BY CALPAIN

A schematic drawing of how calmodulin-binding proteins may be recognized by calpain is presented in Fig. 3. Since PEST regions are highly hydrophilic and therefore are very likely to form surface loops (Rechsteiner, 1987b), they would be easily accessible to calpain molecules. In the model, the negative charge of Glu/Asp residues inside the PEST regions of calmodulin-binding proteins localize calcium ions at the negatively-charged calcium-binding domain of calpain (C-terminal end of the 80 kDa subunit). Upon interaction with substrate-associated Ca^{2+} , calpain's catalytic domain is activated (at the N-terminal end of the 80 kDa subunit) and attacks the

adjacent cleavage site(s), which are of course distinct from the PEST regions. Also, because of the complex tertiary structure of proteins, the PEST regions and cleavage sites need not be close in the primary amino acid sequence in order to be in spatial proximity. In some calmodulin-binding proteins the binding of calmodulin might induce conformational changes which protect certain cleavage sites from calpain attack (Fig. 3b), as seen with the erythrocyte Ca^{2+} -ATPase (Wang *et al.*, 1988b). Alternatively, such conformational changes may expose new cleavage sites that are otherwise inaccessible to calpain. Consequently, fragmentation of calmodulin-binding proteins by calpain could be quite different in the absence versus the presence of calmodulin (Wang *et al.*, 1988b; Ito *et al.*, 1987; Tallant *et al.*, 1988). For some calmodulin-binding proteins (e.g. α -fodrin), the binding of calmodulin can simply modify the rate of proteolysis of the molecule by calpain (Seubert *et al.*, 1987), probably by controlling the accessibility of certain cleavage sites via conformational changes.

It is possible that the PEST regions in calmodulin-binding proteins function solely in calpain recognition. Alternatively, the PEST region might have other functions as yet unknown. In this case, it will be important to define such functions, for it would most certainly help in the further understanding of the function or regulation of calmodulin-binding proteins in general.

Fig. 2. Localization of PEST regions, calmodulin-binding domain and calpain cleavage site within human brain α -fodrin

Of the proposed 22 repeats, only the tenth, eleventh, twelfth and thirteenth (represented by Roman numerals) are illustrated (McMahon & Moon, 1987; Harris *et al.*, 1988). Since the complete amino acid sequence of α -fodrin is not available, the first residue of the XII repeat is arbitrarily assigned residue +1 and is used as the reference residue. Residues moving downstream (towards the C-terminal), and away from residue 1, have increasing positive residue numbers, whereas residues moving upstream and away from residue 1 have increasing negative residue numbers (starts from -1 and on). The PEST region (residue numbers +101 to +111) is illustrated by the stippled area. The calmodulin-binding domain (residue numbers -18 to +5) is shown by the hatched area. A calpain cleavage site (between residue -28 and -27) is illustrated by the arrow and the broken line.

Fig. 3. Model of calpain-mediated proteolysis of calmodulin-binding protein

A schematic drawing illustrating that calpain recognizes calmodulin-binding protein PEST regions with its Ca^{2+} -binding domain (a) and subsequently attacks cleavage sites 1 and 2. (b) Upon calmodulin (CaM) interaction with the calmodulin-binding domain (hatched region), there is a conformational change that prevents the adjacent cleavage site (site 2) from attack by calpain. Note that the size of calpain, its substrate and calmodulin are not drawn to the same proportions.

PEST SEQUENCES IN OTHER CALPAIN SUBSTRATES

After we formulated the hypothesis that calmodulin-binding proteins are recognized by calpain through their PEST sequences, we asked the question whether other calpain substrates also contain PEST sequences. To answer this question, we looked at the known amino acid

sequences of 41 protein substrates for calpain. Of these 41 proteins, 29 have one or more PEST sequences (Table 4), whilst 12 do not (Table 5). Of the 29 PEST-containing substrates, 26 contain one or more strong PEST sequences. The other three substrates which contain only a weak PEST sequence are vimentin, pyruvate kinase (L-type) and α -actinin. Among the PEST-containing proteins of known amino acid sequence are five calmodulin-binding enzymes, as described above, the large subunit of calpain I and calpain II, calpain inhibitor protein (calpastatin), the protein kinase C family and three receptor proteins (EGF receptor, progesterone receptor and oestrogen receptor). The number of PEST regions of these substrate proteins varies from 1 to 12 (for Factor V). The PEST scores are as high as 34.6 (for the progesterone receptor). Of the four cloned isoenzymes of protein kinase C, three (α , β I and β II) have a single highly conserved PEST region (residues 276–302) located at the end of the C2 region of the regulatory domain (Kikkawa *et al.*, 1987). The γ -isoenzyme has a higher degree of variation in that region, which causes the PEST score to fall below -5. However, about 15 residues downstream there is a PEST sequence (residues 316–334) unique to this isoenzyme, with a PEST score of 14.0 (Table 4). Furthermore, we have identified the cleavage site of protein kinase C by calpain (Ohno *et al.*, 1987) to be in the same region in which the PEST sequence is found. Interestingly, HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, has its two PEST-sequences and two calpain cleavage sites in adjacent regions [the PEST sequences being about 40 kDa and 47 kDa, respectively, from the N-terminus (Chin *et al.*, 1984), whilst the calpain cleavage sites are about 35 kDa and 44 kDa from the N-terminus (Liscum *et al.*, 1985)]. The high conservation of PEST sequences among isoenzymes and the proximity of PEST sequences to calpain cleavage sites suggest that PEST regions are recognition sites on calmodulin-binding proteins and some other calpain substrates.

Interestingly, the non-PEST-containing substrates are mostly small proteins (Table 5). Why these non-PEST-containing proteins also serve as calpain substrates has two possible explanations. (a) The PEST-containing proteins and non-PEST-containing proteins may represent two separate groups of calpain substrates. The non-PEST proteins may be recognized at other sequence(s), perhaps analogous to PEST. (b) The definition of PEST regions and the PEST score calculation may have minor flaws which might affect identification of some potential PEST sequences and also their PEST scores. Therefore, it is possible that the non-PEST containing calpain substrates might in fact contain 'PEST' or related regions. The first possibility seems to be supported by the fact that many small peptides that definitely do not have PEST sequences still serve as calpain substrates. Among these are insulin β -chain, protamine, glucagon and dynorphin (Sasaki *et al.*, 1984). On the other hand, in support of the second possibility, we observed that many non-PEST-containing substrates had a high number of basic amino acid residues such as histidine (H), lysine (K) and arginine (R), (e.g. myelin basic protein), which, by definition precludes the existence of 'PEST' regions. Also, the strict requirement for starting and ending a PEST sequence with H, K or R sometimes significantly reduces the PEST scores of a highly concentrated PEST-enriched region which lacks a

Table 4. PEST sequences of PEST-containing calpain substrates

Protein	Species used as calpain substrate and reference*	Species for amino acid sequence and reference*	Residues	PEST sequence	PEST score
Calpain I (large subunit)	Human [1 ^R] and others	Human [27]	295–322	RNPWGVEVEWTGAWSDSSE-WNNVDPYER	4.2
			517–548	KSAGTVELDDQIQANLPDEQ-VLSEEEIDENFK	3.3
Calpain II (large subunit)	Chicken [1 ^R] and others	Chicken [28]	288–315	RNPWGQVEWTGAWSDGSSSE-WDNIDPSDR	4.1
			508–539	KQSDTAELDEEISADLADEEE-ITEDDIEDGFK	14.8
Calpastatin	Rabbit [1 ^R] and others	Rabbit [29]	150–197	KSGMDAALDDLITLGEPSSE-TQEDSTAYTGPEISDPMSSSTY-IEELGK	9.5
			212–249	KTGVAGPPPDSVTPLGPDDAI-DALSSDFTCSSPVASGK	4.4
			496–533	KPLLPSEPTAQLPALSEDLLL-DALSEDFSGPSSASSLK	2.7
Myosin light chain kinase (chicken gizzard)	Chicken [2,3]	Chicken [30]		See Table 3	
Ca ²⁺ -ATPase (plasma membrane)	Human [4–6]	Human [31]		See Table 3	
Neuromodulin	Bovine [7]	Bovine [32]		See Table 3	
Tubulin					
α	Cattle [8]	Rat [33]		See Table 3	
β	Cattle [8]	Human [34]		See Table 3	
α -Fodrin	Human [9,10] and rat	Human [35]		See Table 3	
Protein kinase C	Rat [11]	Rat [36]			
α isoenzyme			276–302	KLLNQEEGEYYNVIPEGDE-EGNVELR	0.7
β I isoenzyme			276–302	KLLSQEEGEYFNVPVPEGSE-GNEELR	3.5
β II isoenzyme			276–302	KLLSQEEGEYFNVPVPEGSE-GNEELR	3.5
γ isoenzyme			316–334	RMGPSSSPIPSPSPSPTDSK	15.8
3-Hydroxy-3-methylglutaryl-CoA reductase	Chinese hamster [12]	Chinese hamster [37]	381–395	KLSSVEEPEGVSQDR	4.2
			429–442	RTQELEIELPSEPR	5.5
			442–456	RPNEECLQILESAEK	–4.4
Casein					
α_{s1}	Cattle [13 ^R]	Cattle [38]	58–79	KEMEAESISSSEEIVPNSVEQK	6.6
			151–193	RQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIG-SENSEK	–1.9
α_{s2}	Cattle [13 ^R]	Cattle [39]	6–16	HVSSEESIISQETYK	2.2
			45–70	RNANEEYISIGSSSEESA-EVA-TEEVK	11.0
β	Cattle [13 ^R]	Cattle [40]	1–25	RELEELNVPGEIVESLSSEE-SITR	6.6
			113–134	KYPVQPFTESQSLTLTDVENLH	–2.8
Vimentin	Human [14]	Hamster [41]	406–419	RETNLESPLVDTH	–2.8
Band 3 (anion channel)	Rat [15]	Mouse [42]	72–105	RDLTIPVTEMQDPEALPTEQT-ATDYVPSSTSTPH	11.1
			245–272	HQPSLETQLYCGQAEGGSEG-PSTSGTLK	1.1
			362–383	RSLESFLDCSLVLPPTDAPSEK	0.4
			910–929	KVTFDEENGLDEYDEVPM-PVend	2.4
von Willebrand factor	Human [16]	Human [43]	463–505	HCDVVNLTCEACQEPGGLVV-PPTDAPVSPTTLYVEDISEPPLH	0.6
			687–728	RDEIVSYLCDLAPEAPPPTLP-PDMAQVTVGPGLLVSTLGP	–3.9
			905–948	RCCSGEGLQIPTLSPAPDCSQPL-DVILLLDGSSSFASYFDEM	–3.7
			1622–1666	KTQCCDEYECACNCVNSTVS-CPLGYLASTATNDCGCTTTT-CLPDK	–2.9
			1947–1967	KIPGTCCDTCEEPECNDITAR	1.5

Table 4. (cont.)

Protein	Species used as calpain substrate and reference*	Species for amino acid sequence and reference*	Residues	PEST sequence	PEST score
Pyruvate kinase (L-type) Factor V	Rat [17, 18] Human [19]	Rat [44] Human [45]	104–118	REATESFATSPLSYR	–0.9
			18–34	RPEPTNSSLNLSVTSFK	–2.3
			420–439	HGVTFSPTYEDEVNSSFTSGR	0.0
			712–757	RNSSLNQEEEEFNLTALALEN- GTEFVSSNTDIIVGSNYSSPS- NISK	0.7
			799–820	HSSPYSEDPIEDPLQPDVTGIR	8.2
			877–892	RPWFDLPSQDTGSPSR	10.2
			894–907	RPWEDPPSLLLLK	–5.0
			1044–1072	KSNETSLPTDLNQTLPSM- DFGWIASLPDH	–0.1
			1072–1098	HNQNSSNDTGQASCPGLY- QTVPEEH	3.8
			1136–1151	KSFTDISQMSPSEH	4.4
			1151–1182	HEVWQTVISP- DLSQVTLSPELSQTNLSPDLSH	1.9
			1299–1335	HTTSLDFSQTNLSPELSQ- TNLSPALGQMPLSPDPSH	2.3
			1335–1477	HTTSLDLSQTNLSPELSQTNL- PDLSEMPFLFADLSQIPLTP- DLQMTLSPDLGETDLSPN- FGQMSLSPDLSQVTLSPDISD- TTLLPDLSQISPPDLDQIFY- PSESSQSLLLQEFNESFPYPD- IGQMPPSPSPTLNDTFLSK	6.4
			1572–1588	RETDIEDSDDIPEDTTYK	19.3
Fibrinogen α	Human [20]	Human [46]	29–45	KDSDWPFCSDDEDWNYK	1.7
			252–268	RGGSTSYGTGSETESPR	11.0
β	Human [20]	Human [47]	461–491	KEVVTSEDGSDCPEAMDLG- TLSGIGTLDGFR	0.0
			217–234	KGGETSEMYLIQPDSSVK	–3.5
Kininogen	Human [21]	Human [48]	380–391	RDNDGWLTS DPR	0.3
			129–143	HPISTQSPDLEPIR	–2.9
			251–266	RDIPTNSPELEETLTH	9.2
			379–388	KEETTVSPPH	10.7
			505–523	HLASSSEDSTTPSAQTQEK	12.3
			523–535	KTEGPTPIPSLAK	–2.3
			535–595	KPGVTVTFSDFQSDLIATM- MPPISPAPIQSDDDWPDIQT- DPNGLSFNPISDFPDTTSPK	5.0
Epidermal growth factor (EGF) receptor	Human and rat [22, 23]	Human [49]	237–260	KDTCPLMLYNPTTYQM- DVNPEGK	–2.9
			359–374	HTPPLDPQELDILK	–3.2
			889–905	KPYDGIPASEISSILEK	–4.9
			975–1008	RALMDEEDMDDVVDAD EY- LIPQQGFFSSPSTSR	2.6
			1044–1075	RYSSDPTGALTEDSIDD TFL- PVPEYINQSVPK	4.4
Progesterone receptor	Chicken [24]	Chicken [50]	45–69	RSSDEEEEEEEQEEEEEE- EEPQQR	34.4
			169–179	RPGPEDASENR	8.7
			257–278	RSSPSVPAADLA EYGYPPPDGK	3.1
			335–374	KAEPPLLP GAYGPPAAPDS- LPSTSAAPPGLYSPLGLNGH	–0.8
			400–419	RPDTETSQSSQYSFESLPQK	8.7
Oestrogen receptor	Cattle [25]	Human [51]	560–573	KPETPSSLLTSLNH	–1.0
			303–335	KNSLALSLTADQMVSALL- DAEPPILYSEYDPTR	–3.8
			555–567	RGGASVEETDQSH	1.1
α -Actinin Neurofilament Light	Chicken [26]	Chicken [52]	137–153	KDDPLTNLNTAFDVAEK	–4.7
			53–82	RSYSSSSGSLMPSLENLDLSQ- VAAISNDLK	–4.9
	Human [54]	Cattle [53]	456–472	HVQEEQTEVEETIEASK	8.2
			477–491	KDEPPSEGEAEEEEK	28.4
			516–531	KEEEEGGEGEGEETK	26.2

Table 4. (cont.)

Protein	Species used as calpain substrate and reference*	Species for amino acid sequence and reference*	Residues	PEST sequence	PEST score
Medium	Human [54]	Cattle [55]	495–509	KEEEPEAEVEEVAAK	18.4
			544–555	KSDQAEEGGSEK	8.4
			561–583	KEEGEQEEGETEAEAEAGEE-AEAK	22.4
Heavy	Human [54]	Cattle [56]	776–790	KAGGEGGSEEEGSDK	10.3
			66–95	RGAGAASSTDSDLTSLNG-PEGCMVAVATSR	–4.4
			452–476	KETVIVVEEQTEETQVTE-EVTEEEEEK	17.7
			502–514	KSPPAEAAASPEK	12.1

* 1, Suzuki *et al.* (1987b); 2, Kōsaki *et al.* (1983); 3, Ito *et al.* (1987); 4, Au (1987); 5, Wang *et al.* (1988a); 6, Wang *et al.* (1988b); 7, see Fig. 1(b); 8, Billger *et al.* (1988); 9, Seubert *et al.* (1987); 10, Siman *et al.* (1984); 11, Kishimoto *et al.* (1983); 12, Liscum *et al.* (1983); 13, Murachi (1983a); 14, Nelson & Traub (1981); 15, Croall *et al.* (1986); 16, Kunicki *et al.* (1986); 17, Ekman & Eriksson (1980); 18, Dahlqvist-Edberg & Ekman (1981); 19, McGowan *et al.* (1983); 20, Kunicki *et al.* (1984); 21, Schmaier *et al.* (1986); 22, Cassel & Glaser (1982); 23, Gates & King (1983); 24, Vedeckis *et al.* (1980); 25, Puca *et al.* (1977); 26, Ishiura *et al.* (1980); 27, Aoki *et al.* (1986); 28, Ohno *et al.* (1984); 29, Emori *et al.* (1987); 30, Guerriero *et al.* (1986); 31, Verma *et al.* (1988); 32, Wakim *et al.* (1987); 33, Lemischka *et al.* (1981); 34, Hall *et al.* (1983); 35, McMahon & Moon (1987); 36, Kikkawa *et al.* (1987); 37, Chin *et al.* (1984); 38, Mercier *et al.* (1971); 39, Brignon *et al.* (1977); 40, Ribadeau-Dumas *et al.* (1972); 41, Quax-Jeuken *et al.* (1983); 42, Kopito & Lodish (1986); 43, Titani *et al.* (1986); 44, Inoue *et al.* (1986); 45, Jenny *et al.* (1987); 46, Rixon *et al.* (1983); 47, Chung *et al.* (1983); 48, Kellermann *et al.* (1986); 49, Ullrich *et al.* (1984); 50, Gronemeyer *et al.* (1987); 51, Green *et al.* (1986); 52, Baron *et al.* (1987); 53, Julien *et al.* (1987); 54, Malik *et al.* (1983); 55, Myers *et al.* (1987); 56, Lees *et al.* (1988).

^R Review article.

Table 5. Non-PEST-containing calpain substrates

Protein	Species used as calpain substrate and reference*	Species for amino acid sequence and reference*
Calpain (small subunit)	Rabbit, human and others [1 ^R]	Pig and human [7, 8]
Troponin I	Rabbit [2]	Rabbit [9]
Troponin T	Rabbit [2]	Rabbit [10, 11]
Tropomyosin		
α	Rabbit [2]	Rabbit [12]
β	Rabbit [2]	Rabbit [13]
Haemoglobin		
α	Human [3]	Human [14]
β	Human [3]	Human [15]
Myelin basic protein	Cattle [4] and others	Cattle [16]
Histone IIA	Cattle [5]	Cattle [17]
Histone IIB	Cattle [5]	Cattle [18]
Histone III	Cattle [5]	Cattle [19]
Desmin	Human [6]	Chicken [20]

* 1, Suzuki *et al.* (1987b); 2, Dayton *et al.* (1975); 3, Melloni *et al.* (1984); 4, Banik *et al.* (1985); 5, Sakai *et al.* (1987); 6, Nelson & Traub (1981); 7, Sakihama *et al.* (1985); 8, Emori *et al.* (1986a); 9, Wilkinson & Grand (1975); 10, Pearlstone *et al.* (1977a); 11, Pearlstone *et al.* (1977b); 12, Stone & Smillie (1978); 13, Mak *et al.* (1980); 14, Konigsberg & Hill (1962); 15, Braunitzer *et al.* (1961); 16, Eylar *et al.* (1971); 17, Yeoman *et al.* (1972); 18, Iwai *et al.* (1970); 19, DeLange *et al.* (1972); 20, Geisler & Weber (1982).

^R Review article.

positively charged residue to mark its boundary. The 30 kDa subunit of calpain is a good example of this argument. It has regions which are quite rich in P, D/E and S/T but which are either disrupted by H, K or R or are lacking these to start or end the potential PEST region properly. Taken together, this suggests to us that the restriction of the requirement of positively charged amino acid residues to start or end a PEST sequence may not always be justified.

Another important point is that not all PEST-containing proteins are calpain substrates. For instance, calsequestrin, which is insensitive to calpain (Seiler *et al.*, 1984; K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work), is a PEST-containing protein according to its amino acid sequence (Reithmeier *et al.*, 1987). Such cases could be explained by a dual requirement for proteolysis by calpain: (i) the requirement of the PEST-region as a recognition site and (ii) the

Fig. 4. Resistance of the troponin C superfamily of Ca^{2+} -binding proteins to calpain I

Troponin I and C mixture (2 μg , rabbit skeletal muscle; Sigma), calmodulin (1 μg , bovine brain; Calbiochem.), S-100A (1 μg , bovine brain; Sigma), S-100B (1 μg , bovine brain; Sigma), oncomodulin (1 μg , rat hepatoma; a gift from Dr. B. Mutus, University of Windsor, Canada), parvalbumin (1 μg , rat muscle; Calbiochem) and calcineurin (3 μg , bovine brain; purified essentially according to Tallant & Cheung, 1986) were subjected separately to either no treatment (–) or calpain treatment (+) at 25 °C for 60 min in 50 mM-Tris/HCl containing 10 mM-dithiothreitol, 0.3 mM-EGTA, 0.5 mM- CaCl_2 and 0.2 μg of purified calpain I (see Fig. 1). Termination of proteolysis and gel electrophoresis are as described in Fig. 1. The letters I and C on the left indicate the bands of troponin I and C, respectively, whilst the letters A, A' and B on the right indicate the subunit A (60 kDa), major fragment of subunit A (43 kDa) and subunit B (17 kDa) of calcineurin, respectively. The 80 kDa subunit of calpain and its major active fragment (76 kDa) are indicated with an open triangle. Molecular mass standards: 200 kDa (a), 116 kDa (b), 97 kDa (c), 66 kDa (d), 43 kDa (e), 31 kDa (f) and 21.5 kDa (g) were as described in Fig. 1 with the addition of egg white lysozyme (14.4 kDa; h), also from Bio-Rad.

existence of a susceptible cleavage site (e.g. fulfilling the $\text{P}_2\text{-P}_1$ requirement or another as yet undefined restriction) which is in close proximity to the PEST region. In other words, PEST-containing proteins which do not have suitable cleavage sites will not be attacked by calpain.

RESISTANCE OF THE TROPONIN C SUPERFAMILY TO CALPAIN

During our study of the effect of calpain on the plasma membrane Ca^{2+} -ATPase and calcineurin, we came to the interesting finding that both calmodulin and the calmodulin-like small subunit of calcineurin (calcineurin B) were resistant to calpain (Wang *et al.*, 1988a; K. K. W. Wang, A. Villalobo & B. D. Roufogalis, unpublished work). Therefore, we looked at the calpain-susceptibility of other members of the troponin C superfamily of

calcium binding proteins (Fig. 4). There has been some controversy about the susceptibility of troponin C to calpain (Dayton *et al.*, 1975; Ishiura *et al.*, 1979; Murakami & Uchida, 1979). In order to clarify this discrepancy, we treated a mixture of troponin I and troponin C with calpain I from human erythrocytes. We found that calpain I rapidly cleaved troponin I while leaving troponin C intact (Fig. 4). To expand this study, we also looked at the calpain-sensitivity of other members of the troponin C superfamily, including oncomodulin, S-100 α protein, S-100 β protein, and parvalbumin (Fig. 4). Consistent with our prediction, none of these proteins is cleaved by calpain I (Fig. 4). This leads us to speculate that the members of the troponin C superfamily are in general calpain-resistant.

To take this hypothesis one step further, we speculate that the various binding proteins (target proteins) of the troponin C superfamily of Ca^{2+} -binding proteins are calpain substrates. The evidence comes from the following observations: (i) many calmodulin-binding proteins are calpain substrates; (ii) calcineurin A, which binds calcineurin B, is hydrolysed by calpain; (iii) troponin I, which binds troponin C, is calpain-sensitive; (iv) protein kinase C, a well-established calpain substrate, appears to bind to an 21 kDa inhibitor protein which belongs to the troponin C superfamily (McDonald & Walsh, 1985; McDonald *et al.*, 1987). If this hypothesis is correct, the target proteins of these Ca^{2+} -binding proteins may be considered to belong to a large subgroup made up of substrates of calpain. In addition, the troponin C-like Ca^{2+} -binding proteins might control the hydrolysis of these proteins by calpain at the substrate level.

CONCLUSION

In this review, we have discussed evidence which strongly suggests that calmodulin-binding proteins are substrates for calpain. We point out that calmodulin might have a controlling role in such proteolysis. We have clearly demonstrated that most of the calmodulin-binding proteins contain strong PEST sequences. We hypothesize that the PEST sequences of calmodulin-binding proteins and many other substrates may be the recognition sites for calpain. Lastly, we present an expanded theory proposing that the target proteins of the troponin C superfamily of calcium-binding proteins belong to a large subgroup of calpain substrates. If these hypotheses are correct, they would form the first consistent molecular basis for substrate specificity of calpain and eventually might help in the further understanding of protein metabolism in the cell.

Future directions

In order to test the hypotheses presented in the review, at least two directions can be taken: (a) obtain amino acid sequences of calmodulin-binding proteins and other potential substrates for calpain and identify their PEST regions; (b) determine whether the remaining calmodulin-binding proteins are hydrolysed by calpain. Subsequently, efforts can be directed to determine whether the calpain cleavage sites and the PEST regions are in proximity. We also predict that the loss of PEST region(s) by initial cleavage with calpain or other proteases will render the remaining fragment insensitive to further cleavage by calpain. Site-directed mutagenesis of 'important' residue(s) in the PEST sequence of a

protein should predictably affect its proteolysis by calpain. It would also be of interest to find out whether calmodulin-binding proteins and other PEST-containing substrates for calpain have short half-lives *in vivo*.

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