The Calpain System

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I. Introduction

A great deal of information has been obtained on properties of the calpain system since purification in 1976 of the protein that was later named m-calpain (83, 84). Books dedicated to Ca^{2+}-dependent proteases and the calpains were published in 1990 (284) and 1999 (473), and a monograph on calpain methods and protocols appeared in 2000 (104). Two FASEB Summer Research Conferences devoted to the calpain system have been held: the...
first in Copper Mountain, CO in 1999 and the second in 2001 at Whitefish, MT. A web site that offers opportunities for exchange of information and discussion has been established: http://ag.arizona.edu/calpains/. A number of shorter review articles on different aspects of the calpain system have also appeared during the last 10 years (139, 196, 377, 418–420, 437, 438, 472 as examples of some of these reviews). The state of the calpain system up to 1991 was summarized in a comprehensive review on the calpains in this journal (76). A number of important advances have occurred since this 1991 review, including 1) cloning and successful expression of a proteolytically active m-calpain from E. coli (142) or baculovirus (208) expression systems; 2) crystallographic structures, first of domain VI (39, 247) and then of the entire m-calpain molecule in the absence of Ca2+ (171, 430); 3) identification of calpain-like Ca2+-activated proteases in invertebrate tissues and of a number of mRNAs encoding putative calpains, some of them evidently tissue specific (416, 417, 420, 438); and 4) generation of two knock-out mice demonstrating that the calpain system is essential for life (10) but that deletion of only one of the two “ubiquitous” calpains, μ-calpain, does not result in embryonic death (14).

Despite this wealth of new information, two of the primary questions concerning the calpain system remain unanswered: 1) How is calpain activity regulated in living cells? 2) What is the physiological function (or stated differently, what are the physiological substrates) of the calpain system in normal cells? The structural information obtained during the last 10 years has altered views as to how calpain activity may be regulated in living cells and has made it possible to propose specific structural changes that must occur to convert a catalytically inactive calpain molecule into an active proteolytic enzyme. On the other hand, although information is accumulating rapidly, the physiological function(s) of the calpain system remain poorly defined. This review summarizes the recent structural findings and some of the evolving concepts concerning regulation of calpain activity in cells and then describes some of the wide variety of physiological functions in which the calpain system has been implicated. The abundance of information on the calpain system makes it impossible to completely review all facets of this rapidly evolving area, and the review focuses on summarizing the structural information that has been firmly established and on those areas where the available information supports concrete concepts concerning regulation or function. Areas where controversy still exists, where results must be interpreted cautiously, and where new information is needed will be indicated.

The review begins with two sections describing properties of the well-characterized μ-calpain, m-calpain, and calpastatin molecules and the identification and some properties of the newest members of the calpain family, and follows with two sections on regulation and physiological function of the calpain system. The review ends with a brief overview of some of the potential pathologial involvements of the calpain system. A chapter describing the events that led to purification of m-calpain in 1976 and summarizing some of the questions regarding the calpain system at that time was published in 1990 (135), and readers should consult this chapter for information on history of the calpain system.

II. BACKGROUND INFORMATION: THE WELL-CHARACTERIZED PLAYERS μ-CALPAIN, m-CALPAIN, AND CALPASTATIN

Nomenclature of the calpain system will be discussed in section III after description of the recently identified "novel" or "atypical" calpains. The terms μ-calpain and m-calpain were first used in 1989 (62) to refer to the micromolar Ca2+-requiring (μ-calpain) and millimolar Ca2+-requiring (m-calpain) proteases, respectively, and it has been recommended that these names be used to distinguish these two calpains (434); μ- and m-calpain are sometimes referred to as the "ubiquitous," "conventional," or "typical" calpains.

A. Properties of μ- and m-Calpain

The calpains that have been isolated in a protein form are Ca2+-dependent, cysteine proteases (Table 1). Calpastatin, the third well-characterized member of the calpain system, is a protein that specifically inhibits the proteolytic activity of μ- and m-calpain but of no other proteases tested thus far. Some general properties of the three well-characterized members of the calpain system are summarized in Table 1.

1. cDNA sequences of μ- and m-calpain

cDNAs encoding μ-calpain and m-calpain have been cloned and sequenced for human (433), monkey, mouse, rat (91), bovine, porcine (411), rabbit (109, 111; partial), and chicken (324) calpains, and a great deal is known about the properties of these two proteins (Fig. 1). The large subunit has a molecular mass near 80 kDa, with the μ-calpain 80-kDa subunit usually being slightly larger than the m-calpain 80-kDa subunit (81,889 Da compared with 79,900 Da for the human calpains for example; Refs. 8, 177). Also, molecular masses for the small subunit from a number of species have ranged from 28,100 (381) to 28,235 Da (109). The amino acid sequences of calpains from vertebrate species are highly conserved with over 90% homology among the mammalian calpains sequenced thus far (91, 411, 433), and there is no evidence of any alternative splicing during expression of the calpain...
and it has been suggested that this domain binds phospholipid (180). Because Gly is near the center of the hydrophobicity scale (103), however, the amino acid composition of domain V is not characteristic of a highly hydrophobic domain, and recent studies have indicated that domain III of the 80-kDa subunit binds phospholipid (462) and has a role in interacting with cell membranes (127). Of the 101 residues in domain V, 40 are Gly, 5 are Pro, 30 are hydrophobic, and 26 are polar or charged. In addition to the long stretches of Gly residues, a region from residues 78–83 contains all five Pro residues (PEPPPP), and a region between residues 91–97 contains four Glu residues (ENESEE). Hence, the amino acid sequence of domain V suggests an unordered structure that could serve as a tether to other molecules or structures.

The COOH-terminal part of the 28-kDa subunit (residues 101–268 in Fig. 1), domain VI, is frequently termed a calmodulin-like domain, because initial analyses of the amino acid sequence of this domain suggested the presence of four EF hand Ca2+-binding sequences at residues 152–163, 182–193, 217–228, and 247–258 (325). The amino acid sequence of domain VI is only marginally homologous to that of calmodulin, however (23% identity and 30% similarity for the human molecules). Moreover, the X-ray crystallographic structures of this subunit (39, 247) have revealed the presence of a fifth Ca2+-binding site at residues 108–119, thereby identifying the calpains as members of the penta-EF-hand family of proteins (261, 484). Members of the penta-EF-hand family of proteins form dimers that involve the EF hand and associate with membranes (484), two properties that are also characteristic of the 28-kDa subunit (325). Subsequently in this review, the five EF-hand sequences in the 28-kDa subunit will be designated EF-1, EF-2, EF-3, EF-4 and EF-5, although EF-5 and probably EF-4 do not bind Ca2+ in the calpain molecule.

Recently, cloning and expression studies have identified an intronless gene that is present in both human and mice that encodes a 248-amino acid polypeptide having a predicted molecular mass of 27,659 Da and 63% amino acid sequence identity with small, 28-kDa subunit of the calpains (393). This 248-amino acid polypeptide differs from the classical 28-kDa small subunit in that it does not have the two stretches of 11 and 20 Gly residues in domain V that the 28-kDa subunit has, and it seems to bind only weakly to the large, 80-kDa subunit in in vitro assays (393). Coexpression of the 248-amino acid polypeptide with the 80-kDa subunit of m-calpain, however, produces a proteolytically active enzyme having ~70% of the activity of an expressed m-calpain. As will be described subsequently in this review, disruption of the gene encoding the 28-kDa small subunit is embryonically lethal in mice, even though the 248-amino acid polypep-
The tide is expressed in these animals. Hence, the 248-amino acid polypeptide cannot substitute for the 28-kDa subunit in cells, and the function of this polypeptide is presently unclear.

The 80-kDa subunits of μ- and m-calpain are different gene products (genes on chromosomes 11 and 1, respectively, in the human; Ref. 326), but share 55–65% sequence homology within a given species (433). The 80-kDa subunits of μ- and m-calpain were originally divided into four domains on the basis of their amino acid sequence (433; Fig. 1), but the recent crystallographic structure of m-calpain (171, 430; discussed in sect. IIA.3) has shown that the 80-kDa of this calpain has six “domains” (Fig. 1). Two of these domains contain only 18 (the NH2-terminal domain; Fig. 1) or 17 (the linker domain; Fig. 1) amino acids, and hence are not typical of domains as the term is normally used. Because the crystallographic structure of μ-calpain is not yet known, it is still unclear whether μ-calpain also contains six domains similar to those in the crystallographic structure of the Ca2+-free m-calpain molecule. In view of these uncertainties, the NH2-terminal 18 amino acids will be referred to as domain I and the 17 amino acids in the linker will be referred to as the linker domain with the realization that this nomenclature may change as additional information becomes available. This nomenclature will be limited to discussion of m-calpain and will be clearly designated as the “crystallographic domain” structure to distinguish it from the domains predicted from amino acid sequence. The domain structure based on amino acid sequence will be used in the following because it is available for both μ- and m-calpain.

A) Domain I. The NH2-terminal domain has no sequence homology with any polypeptide sequenced thus far; sequence homology of domain I among different species [human, chicken, rat, porcine, rabbit (partial)] is 72–86%.

B) Domain II. A domain with a Cys residue at position 115 (μ-calpain) or 105 (m-calpain) that is the active site Cys and with a His residue at position 272 (μ-calpain) or 262 (m-calpain) and an Asn residue at position 296 (μ-calpain) or 286 (m-calpain); these residues form a catalytic triad characteristic of cysteine proteases such as papain or cathepsins B, L, or S. Domain II, however, shares little sequence homology with these other cysteine proteases, and it is likely that it evolved from a different ancestral gene. Note that the active site Cys is in domain IIa, whereas the His and Asn that constitute the remainder of the catalytic triad are in domain IIb in the crystallographic structure of m-calpain (Fig. 1). Recent X-ray crystallographic results show that domains IIa and IIb each bind one atom of Ca2+ in a peptide loop consisting of 8 (domain IIa) or 9 (domain IIb) amino acids (298). Sequence homology of domain II among different species is high, ranging from 85 to 93%.

C) Domain III. This domain has no sequence homology with any polypeptide sequenced thus far. In addition to linking the Ca2+-binding domains of the calpain molecule to the catalytic domain (domain II), domain III may be involved in binding phospholipids (462) and in regulating calpain activity by its participation in critical electrostatic interactions (171, 430). Analysis of the amino acid sequences indicates that this domain also contains two
potential EF-hand Ca\(^{2+}\)-binding sequences, one at the domain II/III boundary (residues 329–341, for \(\mu\)-calpain; residues 318–338 for m-calpain; in domain IIb of the crystallographic structure of m-calpain), and one at the domain III/IV boundary (\(\mu\)-calpain residues 554–565; m-calpain residues 541–552; in domain IV of the crystallographic structure of m-calpain; shaded areas in Fig. 1). The sequence at the domain II/III boundary does not have an EF-hand conformation in the crystallographic structure of rat or human m-calpain, and this region does not seem to bind Ca\(^{2+}\) in m-calpain. The EF-hand sequence at the domain II/III boundary in the calpain isolated from Schistosoma mansoni, however, binds Ca\(^{2+}\) (7; see sect. iii). Because the crystallographic structures of \(\mu\)-calpain and the other calpains that have been identified in the past 10 years (see sect. iii) are not yet available, it is still unclear whether this sequence binds Ca\(^{2+}\) in these other calpains. The EF-hand sequence at the domain II/III boundary will be referred to as EF-IIb in this review with the understanding that it may not bind Ca\(^{2+}\) in some calpains:

D) Domain IV. Like the sequence of domain VI, the sequence of this domain is marginally homologous to calmodulin (24–44% identity and 51–54% similarity for \(\mu\)-or m-calpain) and contains four sets of sequences that predict EF-hand Ca\(^{2+}\)-binding sites (Fig. 1). In contrast to the domain assignment based on amino acid sequence, the crystallographic structure of m-calpain indicates that domain IV of human m-calpain (430) begins at amino acid residue 530 and thus includes the EF-hand at residues 541–552. Hence, domain IV of the 80-kDa subunit also has five EF-hand sequences, with the fifth, COOH-terminal EF-hand involved in dimerization of the 28- and 80-kDa subunits. Subsequently in this review, the six EF-hand sequences on the 80-kDa subunit will be referred to as EF-IIb, EF-1, EF-2, EF-3, EF-4, and EF-5 from the NH\(^{2+}\)-terminal to the COOH-terminal end of the 80-kDa subunit. Sequence homology among the species ranges from 65 to 93% for this domain.

2. Genomic sequences of \(\mu\)- and m-calpain

Annotation of the genomic sequences for the human, mouse, and other species whose genome has been/is being sequenced will soon make this information available for all of the calpain polypeptides, including the recently identified calpain-like molecules (see sect. iii) from a number of species. Genomic DNA sequences for the 80-kDa subunit of mouse \(\mu\)-calpain (28 exons, 21 kb; Ref. 14), the 80-kDa subunit of rat m-calpain (21 exons, >33 kb), human calpain 3a (24 exons, 40 kb; Ref. 366), human calpain 4 (11 exons, 11 kb; Ref. 296), mouse calpain 8 (23 exons, 50 kb; Ref. 157), human calpain 10 (15 exons, 31 kb; Ref. 170), and the mouse calpain 12 (21 exons, 13 kb; Ref. 88) have been determined. Studies of the promoter region of calpain genes have been done for only the chicken and human m-calpain 80-kDa subunits (112, 155, 156) and the human 28-kDa subunit (206). The human 28-kDa subunit gene is ~11 kb and contains 11 exons. Exon 1 is a non-coding sequence, and translation starts at the 16th base in exon 2. Each of the EF-2', EF-3', EF-4', and EF-5' sequences are encoded by one exon, exons 7, 8, 9, and 10, respectively, but the EF-1' sequence is split between exons 4 and 5. The positions of the intron/exon junctions are identical in domain IV of the chicken m-calpain gene and domain VI of the human 28-kDa gene. The 5'-upstream region of the human 28-kDa gene lacks a TATA or CAAT box sequence and is GC rich, containing three G-C boxes (GGGCCG). Such 5'-upstream sequences are characteristic of "housekeeping" genes.

The gene for the chicken 80-kDa m-calpain subunit is ~10 kb and contains 11 exons (112). The 5'-upstream sequence of both the chicken and the human 80-kDa m-calpain subunit (only the 5'-upstream region of the human gene was sequenced, so the size and number of introns for the human 80-kDa m-calpain subunit gene are unknown) also lack a TATA or CAAT box and are GC rich, indicating that these genes also probably belong to the "housekeeping" family of genes. CAT expression analysis identified four negative elements in the 2,500 nt upstream from the transcription start site of the human 80-kDa m-calpain gene. Removal of these elements results in a 13-fold increase in CAT expression. All four negative enhancer elements respond to the same or similar cellular trans-acting factor(s). The regions from nt −202 to −160 and from nt −130 to −80 contain two promoter elements. Both the human 80-kDa m-calpain gene and the human 28-kDa gene contain AP-1 and Sp1 binding sites upstream of the translation initiation site.

Incubation of HeLa cells with the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), results in upregulation of expression of the m-calpain 80-kDa gene but has little or no effect on expression of the genes for the \(\mu\)-calpain 80-kDa, the 28-kDa subunit, or calpastatin (156). Assay of human 80-kDa m-calpain-CAT constructs identified a cis-acting element in the −202/−80 upstream region of the m-calpain gene. This region of the human 80-kDa m-calpain gene contains a TGAATCA sequence and is GC rich, containing three G-C boxes (GGGCCG). Such 5'-upstream sequences are characteristic of "housekeeping" genes.
3. Crystallographic and solution structures

Crystallographic structural information on the calpains has become available in the past 5 years. First, the crystallographic structures of expressed domain VI polypeptides were solved to 2.3 Å (39) and 1.9 Å resolution (247). This was followed shortly afterward by the crystallographic structure of expressed rat (171) or human (430) m-calpain at 2.6 and 2.3 Å, respectively.

The expressed domain VI polypeptides contained amino acids Met-87/Ser-270 from the rat 28-kDa subunit (39) or amino acids His-84/Ser-266 from the porcine 28-kDa subunit (247; both expressed in Escherichia coli). The structures obtained in the two studies were essentially identical; the domain VI polypeptide crystallized as a homodimer with each monomer having five EF-hands. EF-1’ (residues 108–119) and EF-2’ (residues 152–163) structures were paired and were in an “open” conformation, whereas the EF-3’/EF-4’ (residues 182–193 and 217–228, respectively) pair was in a “closed” conformation.

The fifth EF-hand, EF-5’, was involved in forming the interface between the two monomers in the homodimer. Earlier studies (181, 320) indicated that the COOH-terminal ends of domains IV and VI (Fig. 1) were involved in the noncovalent association of the 28- and 80-kDa subunits of the calpain molecule, and the crystallographic structure of the m-calpain molecule (171, 430) has now shown that the fifth EF-hands of domains IV and VI are responsible for this association (at least for m-calpain). Removal of either 22 or 25 residues from the expressed domain VI, i.e., the fifth EF-hand loop and the eighth α-helix, prevents heterodimer formation (106, 294). The interactions between the two domain VI monomers in the crystal structure are principally hydrophobic and involve Ile-254, Val-256, Ile-258, and interaction of Trp-261, Leu-262, Leu-264, Met-266, and Tyr-267 on one polypeptide with Phe-243, Phe-240, Met-239, and Leu-236 on the second polypeptide. Interestingly, the same residues with only a few conservative substitutions are also present in corresponding positions of the COOH terminus of domain IV from the 80-kDa subunits of both μ- and m-calpain (Table 2). That these residues are highly conserved in the calpains that have been sequenced thus far (Table 2) indicates their importance in association of the 28- and 80-kDa subunits in both μ- and m-calpain.

The EF-1’, EF-2’, and EF-3’ sites all contained a Ca²⁺ atom when crystallization was done at “low” Ca²⁺ (1 mM; Ref. 39), whereas the EF-4’ site contained a Ca²⁺ atom only when crystallization was done at higher Ca²⁺ concentrations (20 mM; Ref. 247; or 200 mM; Ref. 39). Moreover, the Ca²⁺ atom in the EF-4’ site was not located in the loop of the EF-hand but rather at the COOH-terminal end of the loop near the NH₂ terminus of the seventh α-helix in this domain. Soaking crystals in ytterbium resulted in replacement of the Ca²⁺ atom in the EF-4’ site, supporting the suggestion that this site does not bind Ca²⁺ with an affinity as high as the EF-1’, EF-2’, and EF-3’ sites do (39). Ca²⁺-binding properties of the complete calpain molecules are discussed in section ivC2.

Comparison of structures obtained in the presence (Ca²⁺ in EF-1’, EF-2’, and EF-3’) or in the absence of Ca²⁺ indicated that the Ca²⁺-induced structural changes in domain VI crystals are very small. The largest Ca²⁺-induced structural changes occur in the EF-1’ region of the molecule (residues 98–115, Ref. 39). For reasons that presently are unclear, stability of the heterodimeric m-calpain molecule is reduced when the 28-kDa subunit of the expressed molecule has been truncated up to residue 115 (removing the EF-1’ hand); possibly, removal of this region of the domain VI polypeptide alters folding of the remainder of the polypeptide (106).

Consequently, the crystallographic structures of domain VI suggest that binding of Ca²⁺ to the EF-1’, EF-2’, and EF-3’ sites in this domain results in only a small structural change at the EF-1’ region of this domain. If Ca²⁺ binding to the EF-1, EF-2, and EF-3 sites in domain IV results in a similar pattern of conformational changes, and if Ca²⁺ binding to domains IV and VI is the trigger for Ca²⁺-induced proteolytic activity of the calpains, then the small conformational changes in the NH₂-terminal region of domain IV would need to be transmitted through domain III to the catalytic domain II (Fig. 1). This use of a “lever” to amplify a small conformational change is reminiscent of the myosin head where small structural changes associated with binding of ATP are amplified through the “stalk” of the myosin head to produce a

<table>
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<th>Amino Acids Identified <strong>in Dimerization of Domain VI Crystals</strong></th>
<th>Putative Corresponding Amino Acids on μ-Calpain 80-kDa Subunit</th>
<th>Putative Corresponding Amino Acids on m-Calpain 80-kDa Subunit</th>
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<tr>
<td>Leu-236</td>
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<td>Met-230</td>
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<td>Phe-243</td>
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<td>Met-266</td>
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<tr>
<td>Tyr-267</td>
<td>Phe-713</td>
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Except where otherwise noted, the amino acid residues for μ-calpain are conserved among eight species (human, monkey, rabbit, bovine, pig, mouse, rat, and chicken) and those for m-calpain are conserved among seven species (human, monkey, rabbit, mouse, rat, chicken, and frog). a From the crystal structures of domain VI dimers from rat (39) or porcine (247). b Conservative substitution of Tyr for Phe in the chicken. c Conservative substitution of Ala for Phe in the frog. d Conservative substitution of Leu for Val in the frog. e Conservative substitution of Val for Leu in the frog.
movement of ~10 nm relative to the actin filament (497). It is still unknown whether such an amplification occurs upon Ca\(^{2+}\) binding to the calpains. Ca\(^{2+}\) binding to domains IV and VI also weakens the affinity of these two subunits for each other, causing dissociation of the 28- and 80-kDa subunits (see sect. ivC6). An inhibitor of the calpains that does not bind to the active site and that has an inhibition constant \((K_i)\) of 0.3 \(\mu\)M (an \(\alpha\)-mercaptoacrylate derivative) was shown to bind to a hydrophobic pocket between helices B and D (2nd and 4th helices) in domain VI. Inhibitor binding caused minimal conformational changes in the crystal structure of this domain (247). It is possible that binding of the inhibitor “locks” domain VI (and domain IV) in a structure that prevents the small conformational changes in the NH\(_2\)-terminal region of this domain and thereby prevents transmission and amplification of this signal to the remainder of the molecule. Additional studies showing whether Ca\(^{2+}\) in the presence of this inhibitor can elicit the large conformational changes that have been observed in \(\mu\)- and m-calpain in solution (later in this section) would confirm or refute this possibility.

The X-ray crystallographic structure of rat (171) or human (430) m-calpain that had been expressed in an E. coli (171) or a baculovirus (430) expression system has been solved to 2.6 or 2.3 Å, respectively (Fig. 2). The two structures, which are in a Ca\(^{2+}\)-free state, are essentially identical. The 28-kDa subunit of rat m-calpain was truncated at amino acid 85 to facilitate high levels of expression in E. coli, but the first 85 amino acids of the small subunit were not represented by clear electron density in the X-ray diffraction pattern of the human m-calpain anyway. Hence, neither of the structures contains domain V. Attempts to obtain the crystallographic structure of \(\mu\)-calpain have not yet been successful, although the similarity in amino acid sequence suggests that the two structures will be similar, and the structure of \(\mu\)-calpain has been modeled on the basis of these similarities (365). m-Calpain is an elongated molecule with dimensions of \(\sim 100 \times 60 \times 50\) Å. Hydrodynamic measurements had earlier indicated that the calpain molecules were ellipsoidal (101), but the hydrodynamic estimates of \(20 \times 76\) Å were smaller than those obtained from the X-ray structure. The crystal structure suggests that the 80 kDa of m-calpain has six “domains” (or, as discussed previously, four domains, a NH\(_2\)-terminal sequence and a linker) rather than the four domains predicted from amino acid sequence (Fig. 1). The crystallographic domain I (NH\(_2\)-terminal sequence) is short, 19 amino acids, and makes contact with domain VI but not with any other parts of the calpain molecule. Domain II, the catalytic domain, is divided in the crystallographic structure into two domains, domain IIa and domain IIb, that contain the Cys and the His/Asn residues, respectively, that constitute the catalytic triad of the calpains. The crystallographic domain III is shorter than the domain III predicted from the amino acid sequence and ends with a stretch of 18 amino acids that form a “linker” with domain IV. The structure of domain IV is very similar to the structures of domain VI published previously (39, 247); indeed, the structure of domain IV in the human m-calpain resembles the Ca\(^{2+}\)-bound structure of domain VI more closely than the Ca\(^{2+}\)-free domain VI (430).

The crystallographic structure of m-calpain leads to several important conclusions (these conclusions strictly pertain only to the Ca\(^{2+}\)-free form of m-calpain).

1) The active site, which is at the top of the structure in Figure 2, is not sterically blocked by domain I (the NH\(_2\)-terminal, 18-amino acid domain) as has been widely believed. Hence, removal of domain I by autolysis (see sect. iiA4) does not activate a proenzyme by removal of a peptide that blocks access to the active site as autolytic activation of other proenzymes does.

2) The structure of the Ca\(^{2+}\)-free enzyme shows that the enzyme is catalytically inactive because the active site

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**Fig. 2.** Crystallographic structure of human m-calpain. The domains, in different colors, are labeled dI, dIIa, dIIb, dIII, dIV, dV, and dVI. I-II is the \(\alpha\)-helix linking domains I and IIa. The linker “domain” is a red line running from the gap between dIII and dIV to the bottom right of the diagram and is labeled III-IV. Locations of EF-1, EF-2, and EF-3 hands in domains IV and VI are indicated. The active site Cys, C105, is in gray as are His-262, Asn-286, and Trp-288 at the top of domain IIb. [Adapted from Reverter et al. (364).]
Cys is 10.5 Å away from the His and Asn residues, a distance too great to allow formation of a catalytically functional complex. Hence, Ca$^{2+}$ must induce a conformational change in m-calpain that reduces this distance from 10.5 to ~3.7 Å, so that Cys-105 can interact effectively with His-202 and Asn-286 to form a catalytic triad. This conformational change likely involves a rotation of Trp-288 in domain IIb (Fig. 2). Earlier studies indicated that mutation of Trp-288 to a Tyr residue reduced proteolytic activity of the mutated calpain to 5.5% of its activity before mutation (11).

3) The autolytic cleavage of domain I at amino acids 19–20 occurs at a point that is 40 Å away from the catalytic site in the Ca$^{2+}$-free enzyme, so this autolysis likely is not an intramolecular event.

4) The crystallographic structure of the m-calpain molecule could not be done in the presence of Ca$^{2+}$, but the very small conformational change resulting from binding of Ca$^{2+}$ to domain VI suggests that the “Ca$^{2+}$ switch” for initiation of proteolytic activity may involve Ca$^{2+}$ binding at some site in addition to or other than domains IV and VI, the calmodulin-like domains. A group of acidic residues, Glu-392, Glu-393, Glu-394, Asp-395, Glu-396, Asp-397, Glu-398, Glu-399, and Glu-401, are highly conserved in the calpains, and some of these acidic residues form salt bridges with Lys-226, Lys-230, Lys-234 and Lys-354, Lys-355, Lys-357 in the crystallographic structure of m-calpain (Fig. 2). Ca$^{2+}$ would disrupt these salt bridges, and this disruption could permit domain IIb to move toward domain IIa, leading to formation of the catalytic triad (430). Although this electrostatic mechanism “switch” may have an important role in the Ca$^{2+}$-induced catalytic activity of the calpains (171), recent studies (298) have shown that expressed domain IIa/IIb from μ-calpain can each bind one atom of Ca$^{2+}$ in a peptide loop and that this binding induces a conformational change that brings the catalytic residues in the expressed domain IIa/IIb within 3.7 Å of each other. Hence, at least part of the Ca$^{2+}$ switch for activating the calpains may reside in the catalytic domain itself (see sect. nC6).

In the absence of structural information on the μ- and m-calpain molecules in the presence of Ca$^{2+}$, limited proteolytic hydrolysis with trypsin or chymotrypsin has been used to compare the structures of the complete heterodimeric calpain molecules in the presence or the absence of Ca$^{2+}$ in solution (297, 459). Moldoveanu et al. (297) used an expressed m-calpain that had the active site Cys mutated to Ser to prevent autolysis in the presence of Ca$^{2+}$, whereas Thompson et al. (459) used purified preparations of both μ- and m-calpain and sodium tetrahionate oxidation (199) to reversibly inactivate calpains and prevent their autolysis in the presence of Ca$^{2+}$. The two studies produced similar results.

1) Either trypsin or chymotrypsin digestion in the absence of Ca$^{2+}$ for periods as long as 120 min produces a limited number of polypeptide fragments, suggesting that both the μ-calpain and m-calpain molecules have a compact structure that limits the number of peptide bonds accessible to proteolytic enzymes in the absence of Ca$^{2+}$. Moreover, similar proteolytic fragments were produced by these two proteases, which differ in their sub-site specificity. Both trypsin and chymotrypsin rapidly cleave either μ- or m-calpain at a region near the COOH-terminal end of domain II or IIb in the crystallographic structure (residues 245 or 266 in μ-calpain; residue 265 in m-calpain) and at several sites in domain III (residues 363 or 472 in μ-calpain; residues 383, 400, and 503 in m-calpain). Domain I is resistant to trypsin digestion for up to 120 min in the absence of Ca$^{2+}$, although chymotrypsin removes small segments (6 amino acids from μ-calpain; 9 amino acids from m-calpain) from the NH$_2$ terminus of domain I; the remainder of domain I and domain II is resistant to trypsin or chymotrypsin cleavage for 120 min, suggesting that this part of the molecule (domain IIa in the crystallographic structure) is in a compact conformation in the absence of Ca$^{2+}$.

2) The COOH-terminal regions of domain III and all of domain IV in either μ- or m-calpain also are resistant to proteolytic degradation, suggesting that these regions of the calpain molecule are also in a compact conformation.

3) Both trypsin or chymotrypsin treatment cleaved the 28-kDa subunit of either μ- or m-calpain at residues 59–61 and 85–88 within 10–20 min, leaving a 24-kDa or a 20-kDa fragment that was not degraded for 120 min.

4) The pattern of proteolytic digestion of μ- and m-calpain by trypsin or chymotrypsin changes significantly in the presence of 1 mM Ca$^{2+}$, a cation that does not directly affect trypsin or chymotrypsin activity. Both μ- and m-calpain that had been proteolytically inactivated were rapidly degraded within 5 min of incubation by either trypsin or chymotrypsin to small polypeptides. Only domains IV and VI remained after 30 min of digestion in the presence of Ca$^{2+}$. Indeed, the proteolytic fragments of the 28-kDa subunit are identical whether proteolytic digestion is done in the presence or the absence of Ca$^{2+}$. Chymotryptic digestion of either μ- or m-calpain in the presence of Ca$^{2+}$ results in only one major degradation product from the 80-kDa subunit; the NH$_2$ terminus of this fragment begins at residue 515 (μ-calpain) or 503 (m-calpain). Therefore, Ca$^{2+}$ at the concentration of 1 mM causes a substantial change in the conformation of the calpain molecules and “opens” the molecule to make it more susceptible to proteolytic degradation. Only the conformations of domains IV and VI do not seem to be significantly altered by Ca$^{2+}$, a conclusion that was also reached in the crystallographic studies of domain VI (39, 247).

In sum, the currently available evidence indicates that Ca$^{2+}$ binds to multiple sites on both the μ- and m-calpain molecules and that not all of these sites are in
domains IV and VI, the penta-EF hand ("calmodulin-like") domains. Because Ca$^{2+}$ binding to domain VI (crystallographic evidence) and to domain IV (evidence from limited proteolysis) causes only very small conformational changes in these domains, it seems likely that Ca$^{2+}$ binding to regions other than or in addition to these two domains is involved in the "switch" that initiates proteolytic activity in the calpains. It is clear that Ca$^{2+}$ binding to either $\mu$- or m-calpain causes significant conformational changes in the calpain molecules and that these changes involve a "loosening" or partial unfolding of domain II (IIa, IIb) and the NH$_2$-terminal part of domain III, making these domains that were previously resistant to proteolytic cleavage now highly susceptible. The exact nature of these changes is unknown, but they must include a moving together of critical residues in domains IIa and IIb to form a functional catalytic triad. Although the structural information that has become available during the last four to five years has changed the prevailing views as to how Ca$^{2+}$ initiates proteolytic activity of the calpains, the regions in addition to those in domains IV and VI in the calpain molecule that bind Ca$^{2+}$ and how this binding affects conformation of the calpains in a way that results in proteolytic activity remain unclear. Finally, it should be remembered that these Ca$^{2+}$ binding events must occur at physiological Ca$^{2+}$ concentrations, which are in the range of 50–300 nM (151, 192, 266). The Ca$^{2+}$ binding properties of the calpains are discussed in section IV.

4. Autolysis and the proenzyme question

It was discovered in 1981 that m-calpain autolyses rapidly in the presence of Ca$^{2+}$ (439, 440), and it is now known that both $\mu$- and m-calpain autolyze when incubated with Ca$^{2+}$ (62, 81, 101, 373, 376). Although it is not uncommon for proteolytic enzymes to autolyze, autolysis of the calpains has several unique features (autoproteolysis may be grammatically more accurate than autolysis, but the briefer autolysis will be used here with the understanding that the autolytic events are proteolytic). Brief autolysis, up to 2–3 min at 25°C and in the presence of 1 mM or greater Ca$^{2+}$, reduces the Ca$^{2+}$ concentration required for half-maximal proteolytic activity of $\mu$-calpain from 3–50 to 0.5–2.0 $\mu$M and that of m-calpain from 400–800 to 50–150 $\mu$M (137; Table 3) without affecting the specific activity of either enzyme (101). This same autolysis reduces mass of the 80-kDa subunit of $\mu$-calpain to 76 kDa, mass of the 80-kDa subunit of m-calpain to 78 kDa, and mass of the 28-kDa subunit common to $\mu$- and m-calpain to 18 kDa (SDS-PAGE; actual mass of this fragment is 20.5 kDa; Ref. 142). Amino acid sequencing studies show that the NH$_2$-terminal 27 or 18 (\$\mu$- or m-calpain) amino acids are removed from the 80-kDa subunit and that the NH$_2$-terminal 91 amino acids (hence, the entire Gly-rich domain) are removed from the 28-kDa subunit during this autolysis (433; residue numbers are for the human calpains; the autolysis sites are similar but not identical in calpains from other species).

Table 3. Estimates of Ca$^{2+}$ concentrations required for different properties of the calpains

<table>
<thead>
<tr>
<th>Calpain Property</th>
<th>Autolyzed $\mu$-Calpain</th>
<th>$\mu$-Calpain</th>
<th>Autolyzed m-Calpain</th>
<th>m-Calpain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic activity</td>
<td>0.5–2</td>
<td>3–50</td>
<td>50–150</td>
<td>400–800</td>
</tr>
<tr>
<td>Binding to calpastatin</td>
<td>0.042</td>
<td>40</td>
<td>25</td>
<td>250–500</td>
</tr>
<tr>
<td>Autolysis without PL*</td>
<td>50–150</td>
<td>550–800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autolysis with PL*</td>
<td>0.8–50</td>
<td>90</td>
<td>40–400</td>
<td></td>
</tr>
</tbody>
</table>

Numbers are Ca$^{2+}$ concentrations (in mM) required for half-maximal activity, binding, or rate of autolysis and are based largely on experiments with bovine skeletal calpains (62, 137, 190). The Ca$^{2+}$ requirement of calpains from different species may differ slightly from these for bovine skeletal muscle, but because these results are from the same species and tissue, the relative Ca$^{2+}$ requirements for different properties of the calpains can be compared directly. * PL is phospholipid; numbers are based largely on experiments using phosphatidylinositol or phosphatidylinositol 4,5-bisphosphate. Although autolysis occurs rapidly, it involves several sequential steps: 1) for the 80-kDa subunit of $\mu$-calpain, the NH$_2$-terminal 14 amino acids are removed first to produce a 78-kDa intermediate product followed by removal of an additional 12 amino acids to produce the 76-kDa autolytic fragment (504; molecular weights as estimated with SDS-PAGE); 2) for the 80-kDa subunit of m-calpain, the NH$_2$-terminal 9 amino acids are removed first followed by removal of an additional 10 amino acids to produce the 78-kDa autolytic fragment (45); and 3) for the 28-kDa subunit, the NH$_2$-terminal 26 amino acids are removed to produce a 26- to 27-kDa fragment, an additional 37 amino acids (to Gly-64) are then removed to produce a 22- to 23-kDa fragment, and finally 28 more amino acids (to Ala-92) are removed to produce the 18-kDa (20.5 kDa) autolytic fragment (272). The 28-kDa subunit of m-calpain is autolysed more rapidly than the 80-kDa subunit (45, 73), whereas autolysis of the 80-kDa subunit in the $\mu$-calpain molecule seems to proceed as rapidly as or even more rapidly than autolysis of the 28-kDa subunit in this molecule (45, 68, 504).

The small change in mass during autolysis of the 80-kDa subunit of m-calpain is difficult to detect in normal SDS-PAGE analysis of the calpains, and claims that this subunit has or has not undergone autolysis need to be substantiated either by direct sequencing or by use of an antibody that specifically recognizes the NH$_2$ terminus of the autolyzed molecule (78, 375). It seems likely that claims that the 80-kDa subunit of m-calpain had not undergone autolysis in some early publications were incorrect because of the difficulty in resolving the 78-kDa autolytic fragment from the 80-kDa native subunit in SDS-PAGE.

Although the biochemical changes that accompany
Autolysis have been well characterized, the physiological significance of autolysis remains controversial. The Ca$^{2+}$ concentrations required for autolysis are similar to or just slightly greater than those required for proteolytic activity (62, 218, 504; Table 3) and therefore are higher than would be encountered in living cells. Because these two Ca$^{2+}$ requirements are so similar, in vitro assays of proteolytic activity are accompanied by autolysis unless the Ca$^{2+}$ concentrations are chosen carefully to be just below those required to initiate autolysis. Proteolytic activity is less than half-maximal (usually much less) at these concentrations, and they are rarely used in in vitro assays. Many members of the cysteine protease family are proenzymes that are activated by autolytic removal of a proenzyme polypeptide that blocks access to the active site of the enzyme. That assays of proteolytic activity were always accompanied by autolysis raised the possibility that the unautolyzed calpains also were proenzymes that required autolysis for activation (274, 435).

The concept that the calpains were proenzymes was widely accepted in the 1990s, and papers have described "activation" of the calpains as being synonymous with autolysis. Although a number of studies reported that both unautolyzed $\mu$-calpain (62, 139, 299) and unautolyzed m-calpain (63, 73, 139) were active proteases, other kinetic studies (18, 68, 161, 218) found that autolysis preceded proteolytic activity of the calpains. Mutation of the residue that is cleaved during autolysis of m-calpain prevented autolysis, but the resulting unautolyzed m-calpain was an active protease (107). Furthermore, SDS-PAGE has been used to show that a cassein substrate is cleaved to small fragments by either unautolyzed $\mu$-calpain or unautolyzed m-calpain (69). Oxidation of $\mu$-calpain with 100 $\mu$M hydrogen peroxide or sodium hypochlorite does not prevent autolysis but completely inhibits proteolytic activity, suggesting that the two are separate events (147).

Finally, the crystallographic structure of m-calpain (171, 430) shows that autolysis of m-calpain removes an $\alpha$-helical NH$_2$ terminus of the 80-kDa subunit and that this NH$_2$ terminus does not block the active site in the inactive enzyme. The NH$_2$-terminal part of the 28-kDa subunit is not visible in the X-ray pattern, but it seems likely that if it were fixed in a position blocking the active site of the enzyme, it would have diffracted sufficiently to have been detected. Consequently, autolysis of m-calpain and, to the extent that structures of the two molecules are similar, of $\mu$-calpain, does not unblock an active site as autolysis of other cysteine proteases does.

Therefore, it presently seems likely that the unautolyzed calpains are capable of proteolytic activity and are not proenzymes that require autolysis for their activation. Whether the unautolyzed calpains are enzymatically active or not may be a moot question, however, because autolysis seems to occur consistently under conditions where the calpains are proteolytically active (139). Concomitant autolysis and proteolytic activity are especially well documented in activation of platelets (120, 217, 374, 378), where platelet activation is accompanied by cleavage of spectrin (118), talin, and filamin (117) in a calpain-specific manner and by autolysis of the $\mu$-calpain in platelets. Hence, autolysis can be used as an indicator that the calpains have been proteolytically active in a cell, but the absence of autolysis does not guarantee that the calpains have not been active.

It seems, therefore, that autolysis has some important role in calpain function. The nature of this role, however, remains a mystery. It has been reported that the peptides released during autolysis of the large subunit of $\mu$-calpain (225) or autolysis of the 28-kDa small subunit (226, 227) have chemotactic activities on neutrophils (225, 226) or immunocytes (227) and that peptides released by autolysis of the small subunit have spasmodogenic activity on sections of guinea pig ileum (257). There are numerous instances in biology where peptides released during limited proteolysis have important physiological functions related to the initial proteolysis, such as the vasoconstrictive effects of the peptides released during cleavage of fibrinogen to produce fibrin during blood clotting. It is possible that the peptides released during autolysis of the calpains may also have important as yet undiscovered properties. Recent studies (discussed in sect. vC6) indicate that autolysis affects stability of the calpains and may initiate dissociation of the two subunits and inactivation. The possible role(s) of autolysis in the physiological function of the calpains will remain an active area of research in the coming years.

B. Properties of Calpastatin

It was discovered during the initial studies on purification of m-calpain (83) that muscle extracts having calpain activity also contained an inhibitor of this activity (see Ref. 135). Initial studies established that this inhibitor was a heat-stable protein (to 100°C; Ref. 332), and it has subsequently been shown that it is resistant to a wide variety of denaturing agents such as urea, SDS, or trichloroacetic acid (126, 337). The name, calpastatin, was proposed for this inhibitor in 1979 by Takashi Murachi (309). The early attempts to purify this inhibitor produced inconsistent and variable results, and the inhibitor was described as a protein having molecular masses varying from 34 to 280–300 kDa (see Refs. 135, 259 for summaries). In retrospect, a number of factors likely contributed to these inconsistencies.

First, calpastatin is labile to proteolytic degradation (277, 283, 337), and it is likely that the harsh conditions used in some of the early attempts to purify calpastatin, many of which involved heating crude extracts that likely contained a number of endogenous proteolytic enzymes,
resulted in varying degrees of proteolytic degradation even though protease inhibitors were used.

Second, it is now known that the calpastatin polypeptide is nearly completely in a random coil conformation as determined by circular dichroism (212, 403) or nuclear magnetic resonance (212, 466). Because estimates of molecular weight using size exclusion chromatography are invariably based on spherical molecules as standards, size exclusion chromatography will greatly overestimate the molecular weight of a molecule having a coil conformation. The studies that reported very large molecular weights of over 200 kDa for calpastatin all used size exclusion chromatography, and it seems likely that they substantially overestimated the molecular weight of calpastatin because of this. Some of these studies also indicated that calpastatin was a dimer or tetramer in non-denaturing solutions because SDS-PAGE of their calpastatin preparations contained polypeptides that were much smaller than those estimated with size-exclusion chromatography. The available evidence indicates that calpastatin is a monomer in non-denaturing solvents (337) and functions as a monomer in cells, although de 

Calpastatin migrates anomalously in SDS-PAGE (259, 445), and it is difficult to relate a band migrating at a particular molecular weight in SDS-PAGE to a known calpastatin isoform. For example, the 46.35-kDa calpastatin in human erythrocytes, which was the first calpastatin to be purified (446), migrates at 70 kDa in SDS-PAGE. The expressed fragments of the calpastatin molecule each migrate more slowly in SDS-PAGE than would be predicted from their respective sizes (110, 445). Hence, the anomalously slow migration of calpastatin in SDS-PAGE is a property of the calpastatin polypeptide itself and is not due to posttranslational modifications. Consequently, although the multiple bands frequently observed in SDS-PAGE of calpastatin polypeptides are often ascribed to proteolytic degradation, it is not always clear without actually sequencing them whether the bands are proteolytic products, whether they are different calpastatin isoforms resulting from some combination of alternative splicing or promoter usage, or whether they are produced by some combination of proteolysis and different isoforms.

Calpastatin is the only known protein inhibitor specific for the calpains, although both L- and H-kininogen (only the central cystatin domain of the kininogen heavy chain) and α2-macroglobulin inhibit the calpains in addition to the other proteases they inhibit (72). The central domain of kininogen has a $K_i$ of 1.0 nM for m-calpain, and the rate constant for inhibition of the calpains by α2-macroglobulin is $3 \times 10^{-4} M^{-1} s^{-1}$, which is intermediate in the range of values that have been reported for the inhibition of different proteases by α2-macroglobulin (72). Calpastatin does not inhibit any other protease with which it has been tested including the cysteine proteases, papain, cathepsin B, bromelin, or ficin in addition to proteases from other classes such as trypsin, chymotrypsin, plasmin, thrombin, pepsin, cathepsin D, or thermolysin (72). Neither calpastatin nor calpastatin-like activities have been reported in invertebrate tissues, and genes

<table>
<thead>
<tr>
<th>Table 4. Summary of calpastatin isoforms</th>
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<tbody>
<tr>
<td><strong>Nature of Transcript</strong></td>
</tr>
<tr>
<td>Type I; contains 1xa, 1y, 1z, exons for domain L and all four repeats; 85-kDa polypeptide</td>
</tr>
<tr>
<td>Type II; contains 1xb, 1y, 1z, exons for domain L and all four repeats; 84-kDa polypeptide</td>
</tr>
<tr>
<td>Type III; prototypical; contains exons for domain L and all four repeats; 77–78 kDa</td>
</tr>
<tr>
<td>Deletion of exon 3 in domain L; contains all four repeats; 74–75 kDa</td>
</tr>
<tr>
<td>Deletion of exons 3 and 6 in domain L; 72–73 kDa</td>
</tr>
<tr>
<td>Lacks domains L and I; 46-kDa polypeptide</td>
</tr>
<tr>
<td>Type IV; lacks domains L and I and part of domain II; ?? kDa</td>
</tr>
<tr>
<td>Lacks domain L and domains I, II, and III; 18.7 kDa</td>
</tr>
</tbody>
</table>
having sequence homologies to calpastatin have not been detected in *Drosophila*, *Caenorhabditis elegans*, or *Saccharomyces cerevisiae*. Hence, calpastatin may be restricted to vertebrates.

1. DNA sequencing

cDNAs encoding the calpastatins for human (12), monkey, mouse (443), rat (188), pig (445), bovine (65, 206), and rabbit (108) have been cloned and sequenced (259). Although sequence homology among the calpastatins is not as high as it is among the calpains (188), there is >65% amino acid sequence identity among the calpastatins sequenced thus far. The calpastatin amino acid sequence is unique and is not homologous to any polypeptide that has been sequenced thus far, including the cystatins which inhibit many of the cysteine proteases but not the calpains (72). The initial studies on calpastatin cDNAs from rabbit lung or liver (108) or pig heart (445) indicated that the calpastatin polypeptide consisted of four repeating, marginally homologous (23 to 36%) domains of ~140 amino acids each, plus a NH₂-terminal domain, called domain L (Fig. 3), that varies in size due to alternative splicing (238, 239). The predicted mass for these calpastatins ranged from 68 to 78 kDa, depending on the size of domain L. As indicated earlier, however, the calpastatins migrate anomalously in SDS-PAGE, and relative molecular masses ranging from 107 kDa (179), to 125 kDa (93, 403), to 145 kDa (277), and to 172 kDa (241) were reported for these different calpastatins. Protein sequencing showed that the NH₂ terminus of the 46.35-kDa human erythrocyte calpastatin was Ser-Asp-Gln-Ala-Leu-Glu-Ala-Ser-Ala-Ser-Leu (178), which corresponds to the sequence of the human hepatic calpastatin beginning at Ser-287 (Fig. 3). This Ser is the first residue of domain II in human hepatic calpastatin and is located immediately after a Met residue that is encoded in a caaATGa sequence, a good Kozak consensus sequence for translation initiation. Hence, human erythrocyte calpastatin is an example of a calpastatin isoform produced from an alternative transcription/translation start site.

Sequencing the mouse calpastatin gene has shown that this gene contains 34 exons in ~60 kb of sequence (448), including 5 exons upstream from exon 2, the exon encoding the NH₂ terminus of the “prototypical” calpastatin (human hepatic calpastatin in Fig. 3). The 5 upstream exons were named 1xa, 1xb, 1y, 1z, and 1u (Fig. 4). Earlier studies (65) had identified a 5’-region of the bovine calpastatin gene containing exons 1xb, 1y, and 1z. These three exons encoded a 68-amino acid domain termed domain XL (Figs. 3 and 4) that contained three protein kinase A (PKA) phosphorylation sites. Incubation of an expressed calpastatin containing domain XL with purified PKA showed that these sites were phosphorylated in vitro. Expressed calpastatin containing the XL domain migrated as a polypeptide of ~145 kDa (molecular mass of the predicted amino acid sequence is 84 kDa), and antibodies specific for the XL domain showed that a 145-kDa calpastatin containing the XL domain is expressed in bovine liver and cardiac muscle extracts (65). These extracts also contained polypeptides of ~120 and 110 kDa that were labeled by a monoclonal antibody specific for domain IV of calpastatin (Fig. 3; Ref. 65). Hence, some bovine tissues contain at least three different calpastatins. The upstream AUG initiation site for the XL domain is in a better Kozak consensus context than the second, downstream AUG (Met-1 in the human hepatic calpastatin in Fig. 3) and was the preferred initiation site in transcription/translation assays (65).

**FIG. 3.** Schematic diagram showing the domain structure of three different calpastatin isoforms as determined from their deduced amino acid sequences. The species and tissues named under each calpastatin is the species and tissue from which the calpastatin was cloned and sequenced. The calpastatin isoforms containing a L and/or a XL domain are found in a number of tissues from a number of different species and are not unique to the species or tissue named. A, B, and C are three subdomains within each domain: the B subdomain from a number of species contains the conserved sequence indicated. ***Three sites on domain XL that are phosphorylated by protein kinase A. See text for additional details.
cDNAs encoding four different NH₂-terminal sequences have been identified in the mouse (448); these four calpastatin cDNAs were named type I, type II, type III, and type IV (Fig. 4).

A) TYPE I. Type I calpastatin started at exon 1xa; type I transcripts skip exon 1xb, continue with exons 1y and 1z, and skip exon 1u. Type I transcripts were identified in mouse brain, liver, and testis.

B) TYPE II. Type II calpastatin started at exon 1xb and continued with exons 1y and 1z before skipping exon 1u and proceeding to exon 2. Type II transcripts therefore encoded calpastatin containing the XL domain (Fig. 3). Type II calpastatin was detected in skeletal and cardiac muscle and in much lower levels in other mouse tissues.

C) TYPE III. Type III calpastatin mRNA started at exon 1u and encoded the prototypical calpastatin. Translation of type III transcripts starts in exon 2, however. Type III calpastatin was expressed ubiquitously in the mouse tissues tested.

D) TYPE IV. Type IV calpastatin expression was limited to testis. Initiation of transcription of type IV calpastatin mRNA began at a unique exon between exons 14 and 15 that was named exon 14t, and that was not expressed in type I, II, or III calpastatin mRNAs. Expression of type IV calpastatin therefore was initiated in domain II just after subdomain IIA and produced a polypeptide that migrated at ~68 kDa in SDS-PAGE.

The four mouse calpastatins are probably not unique to the mouse; the human EST database includes sequences for partial cDNAs representing types I, II, III, and IV, and calpastatin mRNA transcripts corresponding to types I, II, and III calpastatins have been identified in porcine cardiac tissue (344). Moreover, further examination of the bovine calpastatin gene (65) shows that this gene also includes a region corresponding to exon 1xa. Although type II calpastatin from the mouse and human contain the PKA phosphorylation sites described by Cong et al. (65) in the XL domain, the porcine type II calpastatin lacks these sites (344). Only type III calpastatin was detected in skeletal muscle from the pig (344).

It is unclear how many different start sites of transcription/translation may be used in expression of the calpastatin gene in different tissues or under different physiological states. In addition to the four ATGs at the 5'-ends of types I, II, III, and IV calpastatin, there is an ATG that encodes Met-189 (numbering based on Met-1 at the NH₂ terminus of type I calpastatin), which is located at the L domain/domain I junction (Figs. 3 and 4), and another that encodes Met-322, which is at the NH₂ terminus of the 46.35-kDa erythrocyte calpastatin that begins at the domain II/III boundary of calpastatin (Fig. 3). A 17.5-kDa glycoprotein with a predicted amino acid sequence identical to the COOH-terminal 186 amino acids (beginning at Gln-559 sequence number based on type I calpastatin; domain III/IV in Fig. 3) of human hepatic calpastatin has been identified in human sperm (471). This glycoprotein was not isolated, so its NH₂-terminal amino acid is unknown. Human hepatic calpastatin, however, contains a Met residue at position 542, which could be the NH₂ terminus for this “sperm calpastatin.”

The 5'-promoter region of the calpastatin gene in a number of species is GC rich and lacks a TATA box (65, 344), properties that were also observed for the 5’-promoter region of the m-calpain gene and that are characteristic of “housekeeping” genes. Both the bovine (65) and the porcine (344) calpastatin promoter contain multiple Sp-1 binding sites and putative CRE motifs. Earlier partial sequencing of the human calpastatin gene (239, 260) and
the recent, more extensive sequencing of the mouse calpastatin gene (448) now makes it possible to assign exons 2–8 to domain L and exons 9–13 to domain I (Fig. 4). The exon/intron boundaries of all four of the calpastatin inhibitory domains are highly conserved (448). With the exception of domain IV, that has an additional sixth exon at its COOH-terminal end, all inhibitory domains contain five exons. Because each domain is capable of inhibiting one calpain molecule (see sect. nB2) and because removal of either subdomain A or C within a domain does not completely ablate the inhibitory ability of that domain (see sect. nB2), the use of multiple domains and multiple exons within each domain may be a protective mechanism used to ensure that mutation within one or more exons does not completely destroy the ability of calpastatin to inhibit the calpains (448).

Sequencing of calpastatin transcripts has shown that exon 3, and less frequently, exons 4, 5, and 6, are often deleted from calpastatin mRNAs by alternative splicing. Sequences of rat calpastatin obtained in early studies of this calpastatin all lacked exon 3 (94, 238), and it was assumed initially that rodent calpastatins were unique in that they lacked this exon. Several forms of mouse type I, II, and III calpastatins, some of them containing exon 3 and some of them having exon 3 deleted (443) have been identified, however; some rodent calpastatins also contain exon 3.

The use of at least four alternative promoters and the ability to alter domain L by alternative splicing mechanisms make it possible to produce a number of different calpastatin polypeptides from a single calpastatin gene. The physiological significance of these different calpastatins remains a mystery. Deletion of exon 3 would remove a stretch of basic amino acids, Lys-Lys-Arg-His-Lys-Lys (residues 106–111; residue numbers based on Met from exon 1a as number 1), from the L domain of calpastatin. This region of calpastatin has been reported to be involved in binding calpastatin to biological membranes (275, 279, 281). Less than 2% of total cellular calpastatin, however, is estimated to be bound to the sarcolemma in cardiac muscle (279). Loss of the XL domain would remove several potential PKA sites (at least in the human, mouse, and bovine calpastatins) and could therefore alter regulation of calpastatin by this kinase. Although the three PKA sites in the XL domain are phosphorylated in in vitro assays (65), the effects of this phosphorylation on calpastatin activity have not been studied. Calpastatin is phosphorylated both in vitro by PKA (277, 384) and in vivo (1, 384) by either PKA or PKC, but this phosphorylation had only small effects on the calpastatin inhibitory properties that were measured. In vitro phosphorylation with PKA had no effect on the measured properties of calpastatin (277), whereas in vivo phosphorylation with PKC or PKA resulted in an increase in the amount of membrane-associated calpastatin from 6 to 30% (1). It is not known whether the 2% of membrane-bound calpastatin in bovine cardiac muscle (279) was phosphorylated, but membrane-bound calpastatin can inhibit the calpains as effectively as cytosolic calpastatin (280); hence, the physiological significance of membrane-associated calpastatin is unclear. In vitro phosphorylation of rat brain calpastain by either PKA or PKC (382) or by PKC (13) has been reported to decrease its efficiency (increase the concentration required for half-maximal inhibition) in inhibiting either µ- or m-calpain. The PKA phosphorylation occurred in the NHL2-terminal part of calpastatin, and the PKC phosphorylation occurred on Ser-13 of rat calpastatin (rat calpastatin lacks the XL domain, so numbering starts with Met-1 of the L-domain; Ref. 13). The S13S14 sequence for PKC is not conserved among all calpastatins, however (e.g., the mouse, Refs. 238, 443; or the human or monkey, Ref. 239; do not have this sequence), so it is unclear whether phosphorylation at this site could serve as a general mechanism of regulating calpastatin activity. Thus, although calpastatin can be phosphorylated both in vivo and in vitro by PKA or PKC, the effects of this phosphorylation on calpastatin function are still unclear.

Calpastatin levels in skeletal muscle increase 52–100% in response to β-adrenergic agonist administration (23, 215, 342, 402), and the isoform that is affected primarily is the 135-kDa polypeptide (the prototypical calpastatin without the XL domain; Fig. 3), which is also the predominant calpastatin in skeletal muscle (343, 344). β-Agonists bind to the β2-adrenergic receptor and activate signaling cascades that involve the PKA pathway. Although Cong et al. (64) identified a dibutyryl-cAMP responsive element between nt –76 to –73 upstream of the initiation of transcription of the DNA encoding the XL domain, it is unclear if this element is involved in upregulation of the 135-kDa calpastatin observed by Parr and co-workers (343, 344). Northern analysis of skeletal and cardiac muscle tissue from a number of species has typically detected two to four calpastatin transcripts in these tissues. These transcripts, however, cannot be directly related to the type I, type II, or type III calpastatins because the differences in their size evidently are due to large differences in the lengths of their 3′-untranslated regions. Again, the significance of this variability in size of the 3′-UTRs of calpastatin transcripts is unknown.

A systematic study of six different calpastatin isoforms obtained from an E. coli expression system revealed up to an eightfold difference in the ability (as measured by IC50 values) to inhibit the calpains (244). Both µ- and m-calpain and the autolyzed forms of these two enzymes were included in the study. The six expressed calpastatin isoforms were as follows: domains XL-IV, domains L-IV, domains I-IV, domains II-IV, domains III-IV, and domain IV. Domain III-IV (IC50 values of 10–34 nM) was the least effective isoform, and domain IV (IC50 values of 1.25–7.1 nM) was the most effective. Proteolytic
activity of autolyzed m-calpain was inhibited more effectively by all six calpastatin isoforms than activity of any of the other calpains (unautolyzed m-calpain, autolyzed or unautolyzed μ-calpain) was. The results thus far indicate that the different calpastatin isoforms do not differ greatly (i.e., by several orders of magnitude or more) in their ability to inhibit the calpains and do not suggest any functional reason for the different calpastatin isoforms.

2. Domain structure of calpastatin

Expression of truncated cDNAs encoding portions of the calpastatin polypeptide and assay of the expressed polypeptides has indicated that each of the domains, I, II, III, and IV (Fig. 3), can inhibit the proteolytic activity of either μ- or m-calpain (hence, theoretically, 4 calpains inhibited by I “large” calpastatin; Refs. 110, 264; 3 calpains inhibited by one “small” erythrocyte calpastatin; Ref. 178). The L domain alone has no inhibitory activity (445). Ability of the individual domains to inhibit either μ- or m-calpain has been reported to differ in the order, domain I > domain IV > domain III > domain II, from most to least effective (201). Although several studies using unpurified E. coli expression extracts found that the individually expressed domains inhibited m-calpain more effectively than they did μ-calpain (262, 263), the relative ability to inhibit μ- or m-calpain activity is not consistent among the different calpastatin domains (201, 244, 264), and the reported differences are less than an order of magnitude. Other studies have reported that rat calpastatin (110-kDa so evidently lacking the XL domain and perhaps exon 3) inhibited μ-calpain more effectively than m-calpain, but that this difference was eliminated by dephosphorylation (384). Hence, it is not clear that the different calpastatin domains differ significantly in their ability to inhibit μ- or m-calpain; additional study will be needed to clarify this question.

A conserved 12-amino acid sequence near the center of each domain (B in Fig. 3) is essential for inhibitory activity. Any combination of synthetic peptides or expressed polypeptides that lacked subdomain B had no detectable inhibitory activity (201, 258, 264). Mutation of two amino acids in this region almost completely abolishes inhibitory activity (264). Paradoxically, this 12-amino acid residue alone has no inhibitory activity, although a 27-amino acid peptide containing this domain and 11 amino acids NH2-terminal to the Gly residue at the NH2 terminus of subdomain B (legend to Fig. 3) and four amino acids added to the COOH terminus of subdomain B (i.e., lacking both subdomain A and C) inhibited both μ- and m-calpain with an IC50 of ~25 nM (m-calpain) to 800 nM (μ-calpain) (258). Inhibition by this peptide was less effective than inhibition with a complete domain. The complete calpastatin molecule with four domains (Fig. 3) is a very tightly binding inhibitor with a dissociation constant (Kd) <3 nM (72). Because traditional kinetic analysis of inhibitor mechanisms is inappropriate for very tightly binding inhibitors (233, 303), the early reports that calpastatin was a noncompetitive inhibitor of the calpains are inaccurate. The expressed domain III (residues 426–555), which completely inhibited calpain activity, bound less tightly to the calpains than the complete calpastatin molecule did (264), thereby permitting kinetic analysis of calpastatin inhibition. Such analysis indicated that domain III is a competitive inhibitor of μ-calpain with a Ki value of 3 nM. The unmutated form of domain III bound too tightly to m-calpain to permit accurate kinetic analysis, but mutated forms of domain III also inhibited m-calpain competitively with Ki values 8- to 10-fold less than those for μ-calpain (264), reflecting the greater sensitivity of m-calpain to inhibition by this domain of calpastatin.

3. Binding of calpastatin to the calpains

It was recognized early that Ca2+ is required for calpastatin to bind to and inhibit the calpains (70, 182, 337). Use of calpastatin-affinity columns (calpastatin coupled to agarose) indicated that the Ca2+ concentration required for the calpains to bind to calpastatin depended on the calpain molecule, that calpastatin inhibition was reversible, and that bound calpain could be released in an undegraded form by chelating Ca2+ with EDTA (199, 337). In all instances except for unautolyzed μ-calpain, the Ca2+ concentration required for the calpains to bind to calpastatin is significantly lower than that required to initiate their proteolytic activity (Table 3; Ref. 199). There is no evidence that calpastatin binds Ca2+, so the Ca2+ requirement for the calpastatin/calpain interaction must originate from the calpain molecule.

Binding of autolytic fragments of the calpains to calpastatin first indicated that calpastatin bound to both domains IV and VI of the calpain molecule (320). Subsequent studies using expressed segments (subdomains) of the calpastatin molecule showed that a 14-amino acid subdomain that was conserved among the four domains of the calpain molecule (subdomain A in Fig. 3) bound specifically to domain IV of calpain (444, 489) in a Ca2+-dependent manner with a Ki value of 3.1 nM. Further studies using expressed segments (256) or synthetic peptides (444) representing subdomain C of calpastatin (Fig. 3) showed that this region of the calpastatin binds specifically to domain VI of calpain with a Ki of 31 nM. The 14 amino acids in subdomain C also are conserved among the four domains in the calpastatin molecule. Neither subdomain A nor subdomain C alone has any inhibitory activity when incubated with the calpains.

Calpastatin peptides containing only subdomain B do not block binding of the calpains to cell membranes, although they reduce the rate of autolysis of the calpains (202). Expression of calpastatin fragments containing only
subdomains A and C and not subdomain B, however, block binding of the calpains to cell membranes, but do not affect the rate of autolysis. Like the Ca$^{2+}$ concentrations required for proteolytic activity of the calpains in in vitro assays, the Ca$^{2+}$ concentrations required in vitro for binding of calpastatin to the calpains (Table 3) are also much higher than 50–400 mM free Ca$^{2+}$ concentrations that normally exist in living cells (30, 32, 151, 228). Hence, it is unclear whether calpastatin is bound to domains IV and VI on calpain molecules in living cells, and thus is “poised” to prevent any inappropriate or inadvertent calpain activity. Because the Ca$^{2+}$ concentration required for calpastatin to bind to the calpains is less than that required to initiate their proteolytic activity in vitro, and because immunolocalization results suggest that the calpains and calpastatin are frequently colocalized in cells (see sect. ivB), cells must possess some mechanism to allow calpain activity in the presence of calpastatin. Otherwise, rising Ca$^{2+}$ concentrations would cause calpastatin binding before the calpains could initiate proteolytic activity. This mechanism may involve translocating the calpain binding before the calpains could initiate proteolytic activity required for proteolytic activity of the calpains in vitro or for calpastatin binding, or both these possibilities.

The available information leads to several conclusions about inhibition of the calpains by calpastatin and also raises several questions concerning this inhibition.

1) Effective inhibition of the calpains by calpastatin requires that calpastatin binds to the calpains at three sites on the calpain molecule: Ca$^{2+}$-dependent binding involving subdomain A of calpastatin to domain IV in calpain and subdomain C of calpastatin to domain VI in calpain, as well as binding of subdomain B of calpastatin to an area near the active site (domains Ib or Ib or both) of calpain. Such binding of subdomain B would account for the competitive inhibition observed in kinetic assays of inhibition by individual calpastatin domains whose binding to the calpains is sufficiently weak to allow kinetic analysis. Subdomain B binding was originally thought to be Ca$^{2+}$ independent, but there is no firm evidence to support this assumption.

2) That binding of a calpastatin fragment containing subdomains A and C to calpain prevents binding of calpain to membranes (202) suggests that binding of the calpains to membranes involves domains IV and VI, the “calmodulin-like” domains, in the calpain molecule, because calpastatin subdomains A and C bind to domains IV and VI in the calpains, respectively. The presence of Ca$^{2+}$ makes calmodulin a more hydrophobic molecule, and Ca$^{2+}$ may also increase the hydrophobicity of the “calmodulin-like” domains of the calpains. It is unclear whether binding of the calpains to cell membranes is related to binding of phospholipids to the calpains; different studies have found that phospholipids bind to a region between amino acids 39–62 in the small subunit of the calpains (9) or to domain III of the calpains (127, 462).

3) A 24-amino acid peptide synthesized to mimic the calpastatin subdomain B sequence (contained three amino acids added to the NH$_3$ terminus of the 12-amino acid subdomain B and 9 amino acids added to the COOH terminus of this subdomain) inhibited both $\mu$- and m-calpain with IC$_{50}$ values of 0.5–2 $\mu$M (77), somewhat higher than the nanomolar IC$_{50}$ values observed with the complete domain III (264). Binding of this peptide, which inhibited the calpains competitively, was greatly enhanced in the presence of Ca$^{2+}$. The region of binding was determined by covalently cross-linking the bound peptide to a Cys residue in calpain, followed by tryptic digestion. The cross-linking was not completely specific, and the cross-linked Cys was determined by inference to be between residues 300 and 510 in bovine m-calpain (COOH-terminal region of domain IIb and all of domain III) and most likely to be Cys-497 in m-calpain. $\mu$-Calpain does not have a Cys residue at this position, so no specific site of cross-linking could be determined for $\mu$-calpain, although the peptide also inhibited $\mu$-calpain activity. The residues between 300 and 510 are some distance away from the active site (Cys-105, His-262, and Asn-286) in the crystallographic structure of m-calpain, which was obtained in the absence of Ca$^{2+}$ (171, 430), and it is not known how the peptide inhibited the calpains in a competitive manner. Binding of the peptide was nearly dependent on the presence of Ca$^{2+}$ (500 $\mu$M Ca$^{2+}$); it is unclear whether Ca$^{2+}$ binding to domains IV and VI in the calpain molecule caused a conformational change that permitted binding of the inhibitory peptide, which then prevented subsequent assembly of the catalytically active conformation, or whether Ca$^{2+}$ binding at some site other than domains IV or VI was directly involved in binding of the inhibitory peptide. Recent studies have identified two Ca$^{2+}$-binding sites in an expressed calpain domain II (298; see sect. ivC6); it is unclear whether these two Ca$^{2+}$-binding sites could be involved in the Ca$^{2+}$-dependent binding of the 24-amino acid inhibitory peptide to the calpains.

4) Because the most effective inhibition of the calpains by calpastatin requires all calpastatin subdomains, A, B, and C, it seems likely that all three subdomains must bind to calpain simultaneously for maximum inhibitory efficiency. A number of calpain-like molecules have been identified in the past 12 years (see sect. ivI). Several of these recently identified calpains have been isolated and have been shown to have no small, 28-kDa subunit (for example, a Drosophila calpain and Lp82, see sects. ivA2 and ivB, respectively). Lp82 is inhibited very poorly by calpastatin, possibly because of the lack of a 28-kDa small subunit binding site. Also, the very weak proteolytic activity of the expressed domain II (298) is not inhibited by calpastatin, suggesting that calpastatin cannot bind effectively to a calpain that lacks both domain IV of the 80-kDa calpains and calpastatin are frequently colocalized in cells because immunolocalization results suggest that the calpains

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subunit and domain VI of the 28-kDa subunit. To permit
the simultaneous binding of all three calpastatin subdo-
ments to the calpains and accepting the evidence that
subdomains A and C bind to domains IV and VI in the
calpains and that subdomain B binds at or near the active
site of the calpains, the distances between the sites of
calpastatin binding of domains IV and VI and the catalytic
site in domains IIA/IIB after the Ca$^{2+}$-induced activation
must be the same as the distances between subdomains A
and B and subdomains C and B. Interestingly, the number
of amino acid residues between subdomains A and B and
B and C is remarkably conserved among the four domains
of all the calpastatins sequenced thus far (Table 5). The
length of a fully extended polypeptide chain is 7.23 Å/two
residues (102), so the maximum distance between the
calpastatin binding sites in domain IV or domain VI and
the catalytic site in domain IIA/IIB of the catalytically
active calpain is 105 and 83 Å, respectively (this assumes
that subdomain B binds at or near the active site). These
distances are nearly as large as the dimensions of the
calpain molecule in the absence of Ca$^{2+}$ (171, 430), so it
should be easily possible for all three subdomains to bind
simultaneously to the calpain molecule in the presence of
Ca$^{2+}$.

C. Purification of the Calpains and Calpastatin
Assay of Their Activity

Although expression of catalytically active proteases
(142, 268) may eventually reduce the need for conven-
tional purification of the calpains, it has not been possible
to obtain appreciable quantities of catalytically active
μ-calpain from expression systems, and catalytically ac-
tive m-calpain can be obtained in significant quantities
from E. coli expression systems only if the 28-kDa subunit
is truncated at amino acid 85 (142). Hence, it likely will
remain necessary to purify the calpains from tissues or
cells for a number of years.

1. Purification of the calpains

Even though purification of m-calpain was first de-
scribed over 25 years ago (83), it remains difficult to
purify the calpains from tissue sources. The initial puri-
fication of m-calpain required isoelectric precipitation at
pH 4.9 followed by five consecutive column chromatog-
raphic steps; 5 mg of purified enzyme was obtained from
12,000 g of porcine skeletal muscle. Purification of μ-cal-
pain was and still is more difficult than purification of
m-calpain. Some tissues or cells, such as human erythro-
cytes or human platelets, contain predominantly (prob-
ably entirely) μ-calpain, whereas other tissues such as
vascular or gizzard smooth muscle contain predominantly
m-calpain (136, 452). Skeletal muscle and kidney of most
mammalian species contain approximately equal amounts
of μ- and m-calpain. The reason for these differences in
μ-calpain/m-calpain ratios among different tissues/cells is
unknown, but it is much easier, for example, to purify
significant quantities of μ-calpain from erythrocytes or
human platelets than it is from liver or smooth muscle. An
outline of different procedures that can be used to purify
the calpains and calpastatin, either individually or simul-
taneously from the same tissue source if necessary, has
been described (457), and this source should be consulted
for details concerning possible difficulties or precautions.
Only a general overview of the purification process will be
described here. The presence of Ca$^{2+}$ results in rapid
autolysis of the calpains during purification, and it is
essential that the solution used to lyse/homogenize the
cells/tissue and the buffers used during purification con-
tain EDTA or some Ca$^{2+}$ chelator at a reasonably high
concentration (5–15 mM) to ensure that the free Ca$^{2+}$
concentration in the homogenate is nearly zero. Some of
the initial purification protocols did not include Ca$^{2+}$
chelators, and the 28-kDa subunit was autolyzed to the
18-kDa fragment and was not detected in SDS-PAGE of
the purified enzyme. The homogenizing/lysing solution
should also contain a cocktail of protease inhibitors; this
is especially important if calpastatin is going to be puri-
fied. An effective cocktail that uses less costly reagents
(desirable for large volumes) uses EDTA, which inhibits
metalloproteinases in addition to preventing autolysis;
phenylmethylsulfonyl fluoride (PMSF; a slow-acting but
irreversible inhibitor of serine proteases); ovomucoid,
partly purified containing ovoinhibitor (a fast acting in-
hibitor of trypsin-like serine proteases; the ovoinhibitor
also inhibits chymotrypsin-like serine proteases); and
E-64 (an irreversible inhibitor of cysteine proteases). This
cocktail contains inhibitors for each of three of the four
major classes of proteases, and homogenization at pH 8.0
inhibits aspartic proteases, the fourth class (pepstatin can

<table>
<thead>
<tr>
<th>Domains</th>
<th>Human, Monkey,</th>
<th>Porcine, and Rat</th>
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<td>A → B</td>
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<tr>
<td>B → C</td>
<td>26</td>
<td>26</td>
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<tr>
<td>III. A → B</td>
<td>29</td>
<td>29</td>
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<td>B → C</td>
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* From data in Maki et al. (259) and Lee et al. (239). † From Cong et al. (65).
be added if the homogenization has to be done at a lower pH); hence, the cocktail is more efficient than including three or four serine protease inhibitors but omitting a cysteine protease inhibitor, for example. More expensive serine protease inhibitors such as peflabloc can be used for smaller samples/homogenates. It is interesting that large quantities of E-64, an irreversible inhibitor of cysteine proteases, can be included in the homogenization buffer without adverse effects on activity of the calpains, which are cysteine proteases. Purified calpains are not inhibited by molar excess quantities of E-64 in the presence of EDTA, but are rapidly inhibited when Ca\(^{2+}\) is added to the solution (458). Evidently, conformation of the active site of the calpains in the absence of Ca\(^{2+}\) prevents or at least retards reaction of E-64 with the active site Cys. Because all other cysteine proteases except the calpains are irreversibly inhibited when E-64 is included in the homogenizing or lysing buffer, inclusion of E-64 is an effective approach to inhibiting cysteine proteases when purifying the calpains. Curiously, iodoacetate (186 Da; E-64 is 367 Da) will slowly inhibit the calpains in the absence of Ca\(^{2+}\) (481); it is not clear why these two covalent inhibitors of cysteine proteases behave differently.

The tissue homogenate can be salted out between 0 and 48–50% ammonium sulfate saturation (the calpains salt out between 30–45% ammonium sulfate; the “larger” calpastatins at slightly higher ammonium sulfate concentration 40–50% saturation), or the homogenate can be loaded directly onto a column if the volume of the extract is not too large. The calpains can also be precipitated from extracts between pH 6.2 and pH 4.9; this procedure precipitates the calpains but leaves calpastatin in the extract. The low pH seems to result in degradation of calpastatin, so isoelectric precipitation is not an effective way to purify calpastatin.

The first two column chromatographic steps in purification of the calpains are anion-exchange and hydrophobic interaction chromatography using phenyl Sepharose. Either column can be used first; anion-exchange media [either DEAE or a quaternary ion-exchange (QAE) are effective] is generally less expensive than phenyl Sepharose, and recoveries of the calpains are greater off ion-exchange than off phenyl Sepharose media (213). The existence of different calpastatin isoforms has already been described, and elution of calpastatin from anion-exchange columns is variable; in some tissues, calpastatin elution occurs just before or partly overlaps with \(\mu\)-calpain elution, and in other tissues, it almost completely overlaps \(\mu\)-calpain elution with the result that neither calpastatin nor \(\mu\)-calpain activity can be detected in the column eluant. Some of the early reports indicating that certain tissues did not contain \(\mu\)-calpain may have simply failed to detect \(\mu\)-calpain activity because it coeluted with calpastatin off the anion-exchange column used. To determine whether \(\mu\)-calpain is present, it is necessary to collect the fractions eluting in this area of the gradient off anion-exchange columns and apply them to a second column that will separate calpastatin and \(\mu\)-calpain. PKC coelutes with \(\mu\)-calpain off anion-exchange columns, and this has led to some claims that \(\mu\)-calpain is associated with PKC; the two activities can be separated, however.

The hydrophobic interaction media, phenyl Sepharose, was first used by Szpacenko et al. (441) in purification of \(\mu\)-calpain and is very effective in purification of the calpains. Both \(\mu\)- and m-calpain bind to phenyl Sepharose tightly, and \(\mu\)- and m-calpain containing fractions eluting from an anion-exchange column (120–180 and 180–300 mM salt, respectively) or in a tissue homogenate containing 100–150 mM salt can be applied directly to a phenyl Sepharose column without dialysis or salting out. The calpains are eluted with 1 mM EDTA, 2-mercaptoethanol (but see Ref. 481). Calpastatin does not bind to phenyl Sepharose under these conditions, so it is separated from any \(\mu\)-calpain that coeluted with it from the anion-exchange column. A number of additional steps (described in Ref. 457) are required to purify the calpains after anion-exchange and phenyl Sepharose chromatography.

Several studies have used Ca\(^{2+}\)-dependent affinity chromatography, either with a calpastatin peptide that contains the subdomains needed to bind calpains (5), or a calmodulin-binding motif (301), or with a substrate such as casein (74) to purify the calpains. These procedures all involve binding of the calpains to the peptide/motif/substrate in the presence of Ca\(^{2+}\) and then elution with a Ca\(^{2+}\) chelator such as EDTA. These Ca\(^{2+}\)-dependent affinity procedures are effective in purifying the calpains, but because the calpains autolyze rapidly in the presence of Ca\(^{2+}\), calpains purified by using Ca\(^{2+}\)-dependent affinity chromatography are, as pointed out by Croall (74), almost invariably autolysed to varying degrees. The small subunit is especially sensitive to such autolysis, and the 28-kDa small subunit in calpains that have been purified by using Ca\(^{2+}\)-dependent affinity columns often has been degraded.

Reactive Red dye affinity columns have been used to purify the calpains (57, 74). Reactive Red binds the calpains in high (0.5 M) salt concentrations, and the calpains are eluted at ionic strengths below 20 mM, so the binding evidently involves hydrophobic interactions. Binding of the calpains to Reactive Red also involves something in addition to hydrophobic interactions because Reactive Red chromatography provides purification that cannot be obtained with phenyl Sepharose. Reactive Red purification is most effective if the calpains have been partly purified by several chromatographic steps before loading them onto a Reactive Red column. Elution of \(\mu\)-calpain off Reactive Red columns is not always reproducible, and Reactive Red is much more efficient in purifying \(m\)-calpain than it is in purifying \(\mu\)-calpain.
Recently, it was discovered that a monoclonal antibody specific for the 28-kDa subunit common to both \( \mu \)- and m-calpain (epitope is between amino acids 92–104, the NH2-terminus of domain VI; Fig. 1) can be used in an immunoaffinity column to purify either \( \mu \)- or m-calpain (61). The antibody labels the 28-kDa subunit of both \( \mu \)- and m-calpain effectively in Western blots, but the antibody’s binding affinity for the calpains is very low when linked covalently to an agarose matrix. An immunoaffinity column containing the 5B9 monoclonal antibody will bind either \( \mu \)- or m-calpain from extracts if the extracts are loaded onto the column at neutral pH and very low ionic strength. The bound calpain can be eluted either by flushing the column with high salt or at pH 9.0, conditions that do not affect activity of the calpains. Because the binding is so weak, the column is much more effective if the extracts have been partly purified by passing them through successive phenyl Sepharose and anion-exchange columns before loading them onto the immunoaffinity column. The immunoaffinity column is more effective for purifying m-calpain than for purifying \( \mu \)-calpain; a single pass of an extract that has been partly purified with phenyl Sepharose and anion-exchange chromatography through the immunoaffinity column results in a m-calpain that is >90% pure. A single pass through the column of a partly purified \( \mu \)-calpain extract results in a \( \mu \)-calpain that is >80% pure, and two passes are required to achieve >90% purity of \( \mu \)-calpain. Even so, the immunoaffinity column is a much more efficient way to purify the calpains than the more traditional methods. The immunoaffinity column can be used to obtain partly purified calpain from small samples of tissue by loading tissue extracts directly onto the column. It is not clear why the 5B9 antibody binds the calpains so weakly. Evidently, the epitope for the antibody is shielded in the native molecules.

2. Purification of calpastatin

Calpastatin is also difficult to purify, and a number of different procedures for purifying calpastatin have been published (see Refs. 126, 285). Many of these procedures use heating to 90–100°C after one or two initial chromatographic steps (e.g., after phenyl Sepharose chromatography) to denature and precipitate most of the proteins in the fraction leaving the heat-stable calpastatin intact, or precipitation with 15% trichloroacetic acid to denature noncalpastatin polypeptides (126). Calpastatin can then be purified with anion-exchange chromatography. Heating crude extracts, however, can lead to degradation of the protease-sensitive calpastatin, whereas trichloroacetic acid precipitation decreases calpastatin activity, suggesting that 15% trichloroacetic acid may at least partly denature calpastatin. Many of the difficulties in purifying calpastatin are caused by the number of different calpastatin isoforms that may be present in a given tissue. A “purified” calpastatin may contain three to five or more different polypeptides, and it is not always clear whether these different polypeptides are due to proteolytic degradation of a native calpastatin polypeptide or whether they are different calpastatin isoforms. The “large” form of calpastatin (>100 kDa) but not the small erythrocyte calpastatin will bind to phenyl Sepharose in the presence of 1 M ammonium sulfate and is eluted between 1.0 and 0.5 M ammonium sulfate. The calpastatin can then be purified to homogeneity by using size-exclusion chromatography (e.g., Sephacryl S-300) and anion-exchange chromatography with a shallow gradient (403). This latter procedure avoids use of heating steps or denaturing solvents (457).

Calpastatin can also be purified by using an immunoaffinity column containing a monoclonal antibody specific for domain IV of calpastatin (epitope between amino acids 707–786 of bovine cardiac calpastatin; the area of this epitope contains subdomain C of domain IV but not subdomains A or B; Ref. 476). Because calpastatin is resistant to many denaturing conditions including the pH 5.6 of the immunoaffinity column that is 2.5 frequently used to elute molecules from immunoaffinity columns, it would seem straightforward to use immunoaffinity columns to purify calpastatin. For reasons that are still not clear, however, calpastatin in solution does not bind strongly to immunoaffinity columns containing specific anti-calpastatin antibodies. Even with an antibody that specifically recognizes the COOH-terminal end of calpastatin, binding is weak, and the calpastatin-containing extracts must be loaded onto the anti-calpastatin immunoaffinity column in a low-ionic-strength solution for calpastatin to bind. The calpastatin can be eluted from this column at high ionic strength or at pH 2.5, because it is stable at this pH.

3. Assays of calpain activity

Assays of proteolytic activity of the calpains usually use a protein substrate; casein and myofibrils were used in the initial purification of m-calpain (83), and casein is still widely used. The cytoskeletal proteins, MAP-2, tau, and spectrin, are also excellent calpain substrates, with MAP-2 being degraded at a rate approximately twice that of tau, and more than four times that of spectrin (148); these proteins, however, are not as readily available and are more expensive than casein. Because the calpains evidently use a large area of the substrate for binding and for subsite specificity (see sect. IV.C.3), synthetic peptides are “poor substrates” in the sense that they have high Michaelis constant \( (K_m) \) values, on the order of 0.2–5 mM (390), compared with \( K_m \) values in the low micromolar range for protein substrates (27). These peptides are also poorly soluble in aqueous solvents, so assays using synthetic peptide substrates are usually done at substrate
concentrations of ~1–2 mM or even less; the calpains are approximately half-maximally active under these conditions. Casein contains 10–15 mol Ca\(^{2+}\)/casein molecule (equivalent to 1–1.5 mM in most calpain assay conditions), but this Ca\(^{2+}\) evidently is not available to the calpains because its removal has no effect on the Ca\(^{2+}\) concentration required for half-maximal activity of the calpains (27). It has been suggested occasionally that substrate has a significant effect on the Ca\(^{2+}\) concentration required for half-maximal activity of the calpains and that use of "physiological substrate(s)" of the calpains might result in calpain activity at physiological Ca\(^{2+}\) concentrations (see sect. IV for discussion of "the Ca\(^{2+}\) requirement problem"). A systematic comparison of four protein substrates and three synthetic peptide substrates, however, found no significant difference in Ca\(^{2+}\) concentration required for half-maximal hydrolysis for any of the seven substrates (27).

Assays of calpastatin activity require calpain because calpastatin specifically inhibits calpain proteolytic activity but not the proteolytic activity of any other protease. Either pure or impure calpain can be used, but if calpastatin activity is to be measured quantitatively, the degree of inhibition must be "titered" to ensure that it does not reach 100% inhibition.

Assay temperature has unusual effects on measurement of calpain activity. Both \(\mu\)- and m-calpain rapidly lose proteolytic activity when assayed at temperatures above 25°C (84; Fig. 4). If m-calpain activity is assayed for 50 min with casein as a substrate, the activity at 0°C is as great as it is at 37°C because there is no proteolytic activity after 10 min at 37°C (84). The rapid loss in calpain activity also occurs at 30°C (Fig. 5), a temperature widely used to assay calpain activity. Loss of calpain activity occurs more rapidly at Ca\(^{2+}\) concentrations above those required for half-maximal activity and is probably associated with autolysis of the calpains. Because most assays of calpain activity use Ca\(^{2+}\) concentrations of 1–5 mM, these assays should be done at temperatures below 30°C and for periods of 20–30 min or less. Assays at temperatures of 30°C or higher and at Ca\(^{2+}\) concentrations of 1–5 mM should not be run for longer than 10–15 min or accuracy is likely to be compromised.

III. OTHER MEMBERS OF THE CALPAIN FAMILY: CALPAIN-LIKE MOLECULES

During the past 13 years, cloning and sequencing DNA has led to identification of a number of calpain-like genes in different organisms or in specific tissues. With only two exceptions, a calpain from Drosophila (351, 352) and a calpain in Schistosoma mansoni, a flatworm parasite that inhabits the portomesentric circulation of humans (409), the proteins encoded by these DNA sequences have not been isolated from tissues, so very little is known about the catalytic or other properties of the proteins encoded by these genes. Complete genomic DNA sequences have now been determined for a number of organisms including E. coli (40), S. cerevisiae (58, 132), Drosophila (3), and the human (187). Until annotation of genomic DNA sequences of the higher organisms has been completed, however, it is difficult to determine exactly how many sequences each organism has that encode molecules having significant homology to the calpains (a search of the published human genome sequence suggests that it contains 14 calpain large subunit genes; Ref. 86).

Implied in the search for calpain-like DNA sequences is that some definition exists of what a calpain-like molecule is. This is especially important for those molecules that have not been isolated in protein form and that may have only marginal amino acid sequence similarity/identity with the "ubiquitous" \(\mu\)- and m-calpains. Domain II of \(\mu\)- or m-calpain is the catalytic domain, but although it contains the Cys, His, and Asn residues characteristic of a cysteine protease, it has only marginal sequence homology to papain or other families of cysteine proteases. Consequently, the calpains have been grouped in a class of cysteine peptidases, CLAN CA, family C2, separate from the other cysteine proteases (26). Even if sequence homology with domain II of the ubiquitous calpains is used as the criteria for being a member of the calpain family of molecules, some choice must be made as to what level of sequence identity/homology with domain II of the ubiquitous calpains is needed to identify a molecule as a member of the calpain family. Recent analysis (479) has suggested that the percentage of proteins with the same class of function decreases precipitously when sequence identity falls below 35%. Using homology with domain II and > 20–25% sequence identity in this domain as criteria (an inclusive approach), 14 calpain-like genes can be identified in mammals, 4 in Drosophila, 12 in C. elegans, 2 in two species of fungi/yeast, 5 in the unicellular organism Trypanosoma brucei, and a transmembrane calpain in a variety of plants (Tables 6 and 7, Ref. 420). These 38 calpain-like genes have widely divergent properties outside the domain II homology and can be grouped into two general categories of 11 "typical" calpains, defined as those calpains having a domain structure similar to the 80-kDa subunit of \(\mu\)- or m-calpain including EF-hand sequences in domain IV (8 identified in vertebrates, 3 in Drosophila), and 27 "atypical" calpains, which can be further subdivided into six groups (Table 6). The "atypical" calpains do not have calmodulin-like EF-hand sequences in their domain IV (some are lacking a domain IV), so it is unclear whether they are Ca\(^{2+}\)-dependent (although structures in addition to the EF-hand structures in \(\mu\)- and m-calpain may be involved in the Ca\(^{2+}\)-dep-
dent proteolytic activity of these enzymes; see sect. IV C6). Six of the atypical calpains (5 genes in *T. brucei* and calpain 6) and one typical calpain in *Drosophila* are lacking one or more of the Cys, His, or Asn residues at their catalytic site (Table 6), so they probably are not proteolytic enzymes.

It is still uncertain how many “calpain-like” DNA sequences will be detected in the genomes of different organisms and what the properties of the molecules will be. The existence of polypeptides possessing domains that have 33–37% sequence identity to domain II of μ-calpain (Tables 6 and 7) but lacking one or more of the residues that constitute the catalytic triad of the calpains implies that these calpain-like polypeptides are proteolytically inactive and that the calpains have a function other than/in addition to their proteolytic activity. The nature of this function is presently a complete mystery. Finally, it should be indicated explicitly that all the sequence homologies are based on homology with the 80-kDa subunit (more specifically, domain II/IIaIIb of the 80-kDa subunit), and that with the exception of the *Schistosoma* calpain, it is often assumed that the newly identified calpains function as a single polypeptide rather than as a heterodimer as the μ- and m-calpains do. This assumption has not been proven, however, and it is possible that some “cofactor” analogous to the 28-kDa subunit of the μ- and m-calpains (for example, other members of the penta-EF-hand family of proteins such as ALG-2, peflin, sorcin, and grancalcin; calmodulin plays this role for many proteins) is required for activity of the newly identified calpains. Until the properties of at least some of the proteins encoded by these “calpain-like” DNA sequences have been deter-

**FIG. 5.** Time course of hydrolysis of a casein substrate by μ-calpain at 50 μM Ca²⁺ (top panel) or by m-calpain at 500 μM Ca²⁺ (bottom panel) at three different temperatures of 25, 30, or 37°C as indicated. Proteolytic activity was monitored continuously by using the BODIPY-FL microplate assay (460). Note that after 30 min, activity measured at 25°C is ~1.5–1.7 times greater than activity measured at 30°C and ~2–3 times greater than activity measured at 37°C.
mined, it will be difficult to develop a system for classifying the different calpains.

Proteins having properties homologous to the calpains have not yet been unequivocally detected in prokaryotes. The protease from Porphyromonas gingivalis (Table 7) illustrates some of the difficulties encountered when assigning molecules to the calpain family on the basis of cDNA-derived amino acid sequence alone. Although the predicted amino acid sequence of the P. gingivalis enzyme has 53.1% similarity (23.7% identity) to domain IIa/IIb of human \( \mu \)- or m-calpain (Table 7), the P. gingivalis enzyme has nearly the same homology (22.5% identity) with the amino acid sequence of papain; the expressed protease is not inhibited by leupeptin, an inhibitor of the calpains; is maximally active at 45°C, a temperature that results in rapid autolysis of \( \mu \)- and m-calpain; degrades azocoll, a collagen substrate that is not degraded by either \( \mu \)- or m-calpain; and the expressed protease is inhibited, not activated, by 15 mM Ca\(^{2+}\) (44). It seems unlikely that this enzyme is a member of the calpain family.

Recent genomic sequencing efforts have identified a family of transmembrane genes in Arabidopsis thaliana (455), Zea mays (246), and other plants. The polypeptides encoded by these genes all have a large transmembrane domain and an intracellular domain with significant sequence homology to domain IIa/IIb of \( \mu \)- or m-calpain (Table 7). Interestingly, the plant calpains also all have a domain III whose sequence is homologous to domain III of \( \mu \)- and m-calpain (homologies of domain III are approximately the same as the homologies of domains IIa/IIb). A search of the available data bases indicates that all plants that have significant portions of their genomes sequenced have this transmembrane "calpain" gene and have only a single copy of it (246). Because the protein form of this gene has not been isolated, the function and properties of this "plant calpain" remain unknown.

### Table 6. Subgroups within the calpain family of polypeptides

<table>
<thead>
<tr>
<th>Calpain Subgroup</th>
<th>Catalytic Triad</th>
<th>Calmodulin-like Domain</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Typical calpains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammalian calpains (8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \mu )-Calpain, m-calpain</td>
<td>C, H, N</td>
<td>Yes</td>
<td>Lp82 is alternative splicing product of calpain 3</td>
</tr>
<tr>
<td>Calpains 3, 8, 9, 11, 12, 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila calpains (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CALPA, CALPB</td>
<td>C, H, N</td>
<td>Yes</td>
<td>CALPA' is an alternative splicing product of CALPA with the calmodulin domain deleted</td>
</tr>
<tr>
<td>Drosophila CG 3692</td>
<td>R, I, D</td>
<td></td>
<td>Lacks catalytic triad</td>
</tr>
<tr>
<td><strong>Atypical calpains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calpain 6</td>
<td>K, H, N (h calpain 6)</td>
<td>No</td>
<td>Lack catalytic triad; proteolytically inactive?</td>
</tr>
<tr>
<td>Trypanosoma brucei (five similar genes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II (7)</td>
<td>C, H, N</td>
<td>No</td>
<td>Calcium dependent? ( \mu ) has two domain III-like domains in tandem at COOH terminus</td>
</tr>
<tr>
<td>Calpains 10, 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. elegans (five similar genes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (2)</td>
<td>C, H, N</td>
<td>No</td>
<td>COOH-terminal &quot;T&quot; domain in place of calmodulin-like domain</td>
</tr>
<tr>
<td>Calpain 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. elegans TRA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV (7)</td>
<td>C, H, N</td>
<td>No</td>
<td>COOH-terminal &quot;SOL&quot; domain in place of calmodulin-like domain</td>
</tr>
<tr>
<td>Calpain 15 (SOLH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila SOL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. elegans (5 similar genes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group V (4)</td>
<td>C, H, N</td>
<td>No</td>
<td>PallB-like domain followed by a domain III-like domain in COOH terminus</td>
</tr>
<tr>
<td>Calpain 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus nidulans (fungus)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. elegans CE 01070</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group VI (1 family)</td>
<td>C, H, N</td>
<td>No</td>
<td>Transmembrane NH(_2)-terminal domain</td>
</tr>
<tr>
<td>Arabidopsis thaliana (plant)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize, other plants</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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TABLE 7. Amino acid sequence similarities and identities of some purported members of the calpain family with domain II of the 80-kDa subunit of human μ-calpain

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Species</th>
<th>Predicted Number of Amino Acids/Massa</th>
<th>Sequence Similarity With μ-Calpain Domain IIb %</th>
<th>Sequence Identity With μ-Calpain Domain IIb %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain 2 (mCL)</td>
<td>Human</td>
<td>700/80,006</td>
<td>87.7</td>
<td>69.3</td>
</tr>
<tr>
<td>Calpain 3 (p94)</td>
<td>Human</td>
<td>821/94,084</td>
<td>76.6</td>
<td>54.7</td>
</tr>
<tr>
<td>Calpain 5 (htra-3)</td>
<td>Human</td>
<td>639/72,050</td>
<td>60.3</td>
<td>41.7</td>
</tr>
<tr>
<td>Calpain 6 (calpamodulin)</td>
<td>Human</td>
<td>641/74,576</td>
<td>68.7</td>
<td>37.2</td>
</tr>
<tr>
<td>Calpain 7 (PabB)</td>
<td>Human</td>
<td>813/92,652</td>
<td>59.7</td>
<td>26.7</td>
</tr>
<tr>
<td>Calpain 8 (nCL2)</td>
<td>Mouse</td>
<td>763/79,335</td>
<td>86.2</td>
<td>68.8</td>
</tr>
<tr>
<td>Calpain 9 (GC30)c</td>
<td>Human</td>
<td>664/76,168</td>
<td>79.4</td>
<td>60.8</td>
</tr>
<tr>
<td>Calpain 9 (nCL4)d</td>
<td>Human</td>
<td>690/79,096</td>
<td>79.2</td>
<td>59.9</td>
</tr>
<tr>
<td>Calpain 10 (Type A)</td>
<td>Human</td>
<td>672/74,938</td>
<td>63.4</td>
<td>36.5</td>
</tr>
<tr>
<td>Calpain 11</td>
<td>Human</td>
<td>702/80,582</td>
<td>84.0</td>
<td>60.1</td>
</tr>
<tr>
<td>Calpain 12</td>
<td>Mouse</td>
<td>720/80,588</td>
<td>75.5</td>
<td>53.4</td>
</tr>
<tr>
<td>Calpain 13</td>
<td>Human</td>
<td>557/63,600</td>
<td>68.6</td>
<td>43.7</td>
</tr>
<tr>
<td>Calpain 14</td>
<td>Mouse</td>
<td>663/76,700</td>
<td>73.1</td>
<td>46.9</td>
</tr>
<tr>
<td>Calpain 15 (SolH)</td>
<td>Human</td>
<td>1,086/177,318</td>
<td>60.0</td>
<td>33.3</td>
</tr>
<tr>
<td>CALP A</td>
<td>Drosophila</td>
<td>828/93,662</td>
<td>76.6</td>
<td>52.1</td>
</tr>
<tr>
<td>CALP B</td>
<td>Drosophila</td>
<td>791/90,132</td>
<td>78.6</td>
<td>49.7</td>
</tr>
<tr>
<td>CG3602</td>
<td>Drosophila</td>
<td>1,043/118,067</td>
<td>64.1</td>
<td>33.4</td>
</tr>
<tr>
<td>SOL</td>
<td>Drosophila</td>
<td>1,597/174,695</td>
<td>62.4</td>
<td>36.3</td>
</tr>
<tr>
<td>(S. mansoni calpain)</td>
<td>Schistosoma mansoni</td>
<td>758/86,363</td>
<td>70.2</td>
<td>45.2</td>
</tr>
<tr>
<td>(CAP5.5)c</td>
<td>Trypomysos brucei</td>
<td>853/94,772</td>
<td>50.6</td>
<td>23.2</td>
</tr>
<tr>
<td>(PabB)</td>
<td>Aspergillus nidulans</td>
<td>847/74,129</td>
<td>55.6</td>
<td>26.9</td>
</tr>
<tr>
<td>(Atig53550)</td>
<td>Arabidopsis thaliana</td>
<td>2,143/236,779</td>
<td>61.2</td>
<td>34.5</td>
</tr>
<tr>
<td>Dekl</td>
<td>Maize</td>
<td>2,159/238,999</td>
<td>66.2</td>
<td>36.0</td>
</tr>
<tr>
<td>Phytocalpain</td>
<td>Sugarcane</td>
<td>1,160 (partial)f</td>
<td>65.9</td>
<td>35.7</td>
</tr>
<tr>
<td>(Cp11p/YMR154C)</td>
<td>Saccharomyces cerevisiae</td>
<td>727/83,123</td>
<td>43.8</td>
<td>18.0</td>
</tr>
<tr>
<td>(Tpr)</td>
<td>Porphyromonas gingivalis</td>
<td>482/55,104</td>
<td>53.1</td>
<td>23.7</td>
</tr>
</tbody>
</table>

Similarities and identities were determined by using the Genstream Align program (346). Whenever possible for the vertebrate calpains, comparisons were made between the human sequences. Exceptions are noted. a Mass is in Daltons. b Domain II in μ-calpain was chosen to contain amino acids 21–352. This is consistent with the crystallographic structure of m-calpain. The protease domains of the other molecules (including calpain 2 which is included for comparison) were predicted by the Simple Modular Architecture Research Tool (SMART) (399, 400). Yoshikawa et al. (493). Lee et al. (235). Hertz-Fowler et al. (165). Full sequence not yet published.

other reports of Ca²⁺-dependent, cysteine proteolytic activity in plants such as Allomyces arbuscula (174, 327, 328, 329, 330, 331), or the algae Chara australis (302). Thus far, none of these Ca²⁺-dependent plant cysteine proteases has been cloned and sequenced, and their biochemical properties differ substantially from those of the calpains. The algae protease was assayed only in cell extracts, and its nature is unknown. Two proteases have been purified from Allomyces arbuscula (328), one eluting at 0.07 mM (CDP I) and the other at 0.2 mM NaCl (CDP II), similar to the elution pattern of μ- and m-calpain off anion-exchange columns. CDP I, however, has a molecular mass of 39 kDa, whereas CDP II is a dimer of 40- and 43-kDa subunits that degrades collagen rapidly and casein slowly (331), just the opposite of the effects of m-calpain on these two protein substrates. Also, phosphatidylinositol inhibited activity of the Allomyces protease (327), whereas it decreases the Ca²⁺ concentration required for activity of the calpains (373, 376). Consequently, it presently is uncertain whether any of these Ca²⁺-dependent plant cysteine proteolytic activities are members of the calpain family; a final conclusion will need to wait until sequence information is available for the plant proteases that have been isolated. An attempt to isolate calpain activity from a plant species rich in actin, Elodea densa, failed to find any Ca²⁺-dependent proteolytic activity similar to the calpains (482).

### A. Calpain-Like Molecules in Invertebrates

This section focuses on those molecules that have been isolated in protein form, so there is some information on their catalytic properties. There are three classes of such molecules: 1) the Schistosoma mansoni proteases, 2) the Drosophila proteases, and 3) the lobster proteases. One calpain-like molecule from Caenorhabditis elegans has been expressed and its properties studied; because this C. elegans calpain has unusual properties, and because C. elegans is a genetically tractable organism, properties of the C. elegans calpains will also be discussed. Interestingly, neither calpastatin activity nor a calpastatin-like DNA sequence has yet been detected in invertebrates; a search of the Drosophila genome failed to detect a gene homologous to calpastatin (234).

1. **Schistosoma mansoni calpains**

Screening a cDNA library from adult *Schistosoma mansoni* first indicated that these parasitic organisms...
contained a 768-amino acid, 86.9-kDa molecule having the four domains and EF-hand structures characteristic of \( \mu \)-or m-calpain (7). The \( S. \) \( m. \) calpain had 62\% amino acid sequence similarity and 42\% identity with human \( \mu \)-calpain (Table 7), clearly identifying it as a member of the calpain family, although domain IV had only three EF-hand sequences. The existence of an EF-hand sequence at the domain II/III boundary was first noticed in the \( S. \) \( m. \) calpain, and assays indicated that this EF-hand and all three EF-hands in domain IV of the \( S. \) \( m. \) calpain bound \( \text{Ca}^{2+} \). A \( \text{Ca}^{2+} \)-dependent protease activity was subsequently purified from \( S. \) \( m. \) calpain (409); the purified \( S. \) \( m. \) calpain had two subunits of 78 and 28 kDa, although the 28-kDa polypeptide seemed to be in excess of the 78-kDa polypeptide in a purified preparation (409). Two-dimensional electrophoresis indicated that there were two 78-kDa polypeptides but only one 28-kDa polypeptide, suggesting that there may be two \( Schistosoma \) calpains similar to the vertebrate \( \mu \)- and m-calpain. The purified \( Schistosoma \) calpain was labeled by antibodies elicited against either the polypeptide obtained by expression of the cDNA characterized earlier (7, 200) or the 78-kDa \( S. \) \( m. \) calpain (409). Antibodies elicited against the expressed polypeptide labeled only one of the two 78-kDa polypeptides separated by two-dimensional electrophoresis (the one with the higher isoelectric point, possibly the \( \mu \)-calpain equivalent). Neither of the \( S. \) \( m. \) antibodies labeled purified rabbit calpain, indicating that the \( Schistosoma \) calpain was immunologically distinct from mammalian calpains. The NH\(_2\)-terminal sequence in domain I of the \( S. \) \( m. \) calpain had a 28-amino acid extension that is not present in the mammalian calpains; the NH\(_2\)-terminal domain is very heterogeneous among the different members of the calpain family.

2. \( Drosophila \) calpains

A calpain-like activity was first identified in \( Drosophila \) in 1988 before any cDNA encoding a calpain-like molecule was identified (351). The following year, Müller and Spatz (306) also identified a \( \text{Ca}^{2+} \)-dependent proteolytic activity in \( Drosophila \) that they partly purified, that required 500 \( \mu \)M \( \text{Ca}^{2+} \) for half-maximal activity, and that degraded the regulatory subunit cAMP-dependent kinase. Because it was inhibited by \( p \)-chloromercuribenzoate and iodoacetate but not by various trypsin inhibitors, the proteolytic activity seemed to be due to a cysteine protease. The activity eluted off a size-exclusion column at a position corresponding to an 83-kDa molecule (306). The \( Drosophila \) \( \text{Ca}^{2+} \)-dependent proteolytic activity was subsequently purified and was shown to be a single polypeptide chain of 94-kDa that was inhibited by calpastatin and that required 600 \( \mu \)M \( \text{Ca}^{2+} \) for half-maximal proteolytic activity (352), a \( \text{Ca}^{2+} \) requirement similar to mammalian m-calpain (cf. Table 3). The \( Drosophila \) calpain was the first calpain and remains the only calpain that has been demonstrated to be proteolytically active without a 28-kDa small subunit. Yeast two-hybrid studies have suggested that calpain 3 (Table 7) does not bind a small subunit (see sect. \( \mu \)H1), and it is assumed that many of the calpains that have been identified as mRNAs also function as a single polypeptide. In the absence of purified enzymes, however, these assumptions remain unproven.

Assays of crude extracts of \( Drosophila \) heads (351) indicated that these extracts contained a second \( \text{Ca}^{2+} \)-dependent proteolytic activity (\( \mu \)-calpain?) and an inhibitor of the \( \text{Ca}^{2+} \)-dependent proteolytic activity (probably not analogous to calpastatin), but these activities have not been purified. Antibodies raised against chicken m-calpain did not label the \( Drosophila \) calpain, again indicating that these evolutionarily distant proteins are not immunologically similar.

Screening \( Drosophila \) cDNA libraries has identified three cDNAs having homology to the calpain family (90, 113, 193, 456). The cDNAs encode polypeptides of 821 amino acids and a predicted molecular mass of 94 kDa (CALPA; Ref. 456); of 791 amino acids and a predicted molecular mass of 90,134 Da (CALPB; Ref. 193); and of 1,597 amino acids and a predicted molecular mass of 175 kDa (SOL protein for small optic lobes; Ref. 90). It is unclear whether either CALPA or CALPB correspond to the 94-kDa calpain that was purified in 1992 (352). The encoded polypeptides for CALPA and CALPB have the four domains characteristic of the 80-kDa subunits of \( \mu \)-and m-calpain (Fig. 2), including the four EF-hand sequences in domain IV and at the domain II/III boundary, and are therefore grouped with the “typical” calpains (Table 6). The predicted amino acid sequences of CALPA and CALPB are significantly homologous to those of the vertebrate calpains: domain II was the most highly conserved, having 50–52\% sequence identity to domain II of vertebrate calpains (Table 7); domains III and IV from CALPA and CALPB have 44–49 and 35–42\% sequence identity, respectively, with their counterparts from vertebrate calpains, whereas domain I is poorly conserved. Immunological and in situ hybridization studies showed that expression of \( Drosophila \) calpain is especially high in the central nervous system (456). A CALPA transcript that lacked the calmodulin-like EF-hand domain, \( CALPA' \), is produced from the CALPA gene by alternative splicing.

The third “calpain cDNA” in \( Drosophila \) encodes a polypeptide differing in several ways from the other calpains (90). This DNA is cytogenetically located at the \( sol \) (small optic lobe) locus of the \( Drosophila \) gene. Part of this large 1,597-amino acid (175 kDa) polypeptide between amino acids 1017 and 1320 in the COOH-terminal half of the polypeptide has 36.3\% identity to domain II of human \( \mu \)-calpain (Table 7). The sequence of the NH\(_2\)-terminal half of this molecule, however, has no homology
with the calpains but instead predicts six motifs having some similarity to zinc fingers found in steroid hormone receptors and DNA-binding proteins. The SOL protein does not have a domain IV containing EF-hand sequences nor an EF-hand sequence at the domain II/III boundary. Moreover, the sequence homology of SOL “domain II” with domain II of μ-calpain is only slightly better than with the cathepsins (21% identity with mouse cathepsin B). Hence, the Drosophila SOL calpain is an example of a calpain homolog whose predicted amino acid sequence differs considerably from the sequences of the classical μ- and m-calpains, and it will be interesting to learn about the catalytic and other properties of the SOL protein.

3. Crustacean Ca2+-dependent proteases

Ca2+-dependent protease activity that was inhibited by two inhibitors of cysteine proteases, leupeptin and antipain, but not by an inhibitor of aspartic proteases, pepstatin, was described in crustacean muscle (Bermuda land crab) in 1982 (311). This Ca2+-dependent activity was increased more than twofold during the muscle atrophy that occurs during molting and degraded the myofibrillar proteins in crab claw muscle (311).

Four different Ca2+-dependent proteolytic activities have been isolated from the claw muscle of another crustacean, the lobster (312). These four proteases have molecular masses of 310 kDa (I), 125 kDa (IIa), 195 kDa (IIb), and 59 kDa (III) as estimated by using size exclusion chromatography and require Ca2+ concentrations of 1 mM (I), 1.5 mM (IIa), 2 mM (IIb), and 0.6 mM (III) for half-maximal activity. Hence, the Ca2+ requirements for these proteases resemble the Ca2+ requirement of m-calpain more closely than they resemble that of μ-calpain. Two of these proteases, IIa and IIb (34), have been partly purified and have molecular masses of 60 and 90 kDa, respectively, in SDS-PAGE. Because these SDS-PAGE molecular masses are approximately one-half of the molecular masses obtained with size exclusion chromatography, it was assumed that the native proteases are dimers (33), although they could also be monomeric polypeptides having an asymmetric shape. All four proteases degraded the myofibrillar proteins in crustacean muscle including actin and myosin to small trichloroacetic acid-soluble peptides (270). Protease IIa resembled the vertebrate calpains in that it is not inhibited by PMSF or pepstatin, whereas protease I is inhibited by PMSF (26% inhibition) and proteases IIb and III are inhibited by pepstatin (26 and 90% inhibition, respectively). Hence, the lobster proteases differ from μ- or m-calpain in several ways: 1) they degrade the myofibrillar proteins, including myosin and actin, to small peptides, whereas μ- and m-calpain make relatively few cleavages in myofibrillar proteins leaving large fragments, do not degrade actin, and degrade myosin very slowly; 2) the lobster proteases do not possess a small subunit similar to the 28-kDa subunit and may be homodimers instead of heterodimers; 3) autolysis of the lobster proteases does not affect the Ca2+ concentration required for their proteolytic activity (33); and 4) the lobster proteases are partly inhibited by PMSF or pepstatin, which have no effect on the calpains. Despite these differences, immunological evidence suggests that the lobster proteases, at least proteases IIa and IIb, may be members of the calpain family. An antibody raised against an expressed polypeptide representing the COOH-terminal 70-kDa of Drosophila CALPA, however, labeled lobster IIb in addition to the Drosophila CALPA, but did not label the lobster IIa protease (33). Conversely, an anti-lobster IIb antibody labeled the expressed Drosophila calpain but did not label either μ- or m-calpain nor the lobster IIa protease. Hence, the lobster IIa and IIb proteases may be members of the calpain family that are evolutionarily so distant that they differ in several properties from the prototypical μ- and m-calpains. Sequencing the cDNAs for the lobster IIa and IIb proteases should eventually settle this issue.

4. Calpain-like molecules in C. elegans

Sequencing the C. elegans genome has led to identification of 12 genes, grouped in 4 classes (420), that encode calpain-like polypeptides (48). None of the putative C. elegans calpains has been isolated in protein form, so very little is known about the catalytic or other properties of these proteins.

One of the C. elegans calpain-like genes, tra-3, encodes a protein of 648 amino acids (73.6 kDa) having significant sequence homology to domains II and III of μ- and m-calpain (Table 6; Ref. 24). This homology diverges after domain III, and the COOH-terminal 142 amino acids of tra-3 constitute a domain, called domain T, that has no homology with domain IV of μ- and m-calpain. The NH2-terminal 41 amino acids of domain T, however, have 49% similarity with the COOH-terminal 42 amino acids of m-calpain, which are those amino acids involved in the 28/80 subunit interaction. Genetic analysis indicates that the tra-3 gene is involved in sex determination in C. elegans. Transgenic nematodes overexpressing TRA-3 were used to show that TRA-3 functions by selectively cleaving in a Ca2+-dependent manner the membrane protein TRA-2A, which binds FEM-3 and inhibits its ability to promote male development in C. elegans (412). The T-domain of TRA-3 does not possess the EF-hand sequences that exist in domain IV, but the TRA-3 sequence predicts an EF-hand sequence at the domain II/III boundary. Hence, the results observed in vivo with overexpressed TRA-3 sug-
gest that the Ca\(^{2+}\)-dependent proteolytic activity of this molecule may be due to an EF-hand sequence at the domain II/III boundary or to Ca\(^{2+}\) binding to domain II as described by Moldoveanu et al. (298; see sect. vC6). A homolog of TRA-3 containing the T-domain has been detected in both the mouse (87) and the human (87, 304) and has been given the name calpain 5 (Table 7). The role of calpain 5 in mammals is a mystery; is it also involved in sex determination in mammals? The highest levels of calpain 5 mRNA in human tissues were in the testis, liver, trachea, colon, and kidney (87). The ability to use a genetic approach with Drosophila and C. elegans provides a powerful approach to studying the function of the calpains.

B. Calpain-Like Molecules in Vertebrates

The first report that the calpain system contained molecules in addition to the three, by then well-characterized, proteins, \(\mu\)-calpain, m-calpain, and calpastatin, appeared in 1989 when Sorimachi et al. (414) described the presence in muscle of a mRNA that encoded a molecule having 51–54% sequence homology to the 80-kDa subunits of the calpains (414). In addition to those molecules described in the preceding section and the 80-kDa subunits of \(\mu\)- and m-calpain, 12 different mRNAs or genes encoding polypeptides with sequence homology to the calpains have been identified in vertebrates (Tables 6 and 7; Ref. 420). If the human genome contains only 14 genes that can be characterized as encoding molecules belonging to the calpain family (86), the discovery of new calpains, at least in mammals, may be nearing an end (as indicated previously, identification of new calpains will depend on what criteria are used to specify a molecule as belonging to the calpain family). It will be necessary to isolate the protein forms of these new calpains, most of which have been identified only as DNA sequences, and to characterize their catalytic and other properties before the nature of the calpain family is fully understood.

Five of the putative calpains that have been identified since 1989 seem to be tissue specific because their mRNAs are expressed principally in skeletal (calpain 3a, p94; Ref. 414) or smooth (calpain 8a, calpain 8b; Ref. 415) muscle cells, in placenta (calpain 6; Ref. 87), in the testis (calpain 11; Refs. 85, 89), in the skin during the first 16 days after birth (Capn12; Ref. 88), or in the testis and lung (calpain 13; Ref. 86). Tissue expression is more widespread for calpain 5 (269), calpain 7 (123), calpain 9 (236, 249), and calpain 10 (170). Calpain 14 mRNA could not be detected in any of 76 tissues examined (86), and the significance of the calpain 14 gene (Capn14) is unclear. Some properties of the calpains that have been identified since 1989 are summarized in Table 6. Calpain 6 is unique in that its predicted amino acid sequence has a Lys residue in place of Cys at the active site, so this calpain likely is not a proteolytic enzyme. Calpain 5 is the vertebrate analog of the TRA3 calpain in C. elegans, calpain 7 (122) is the vertebrate analog of the PaI calpain from the fungus Aspergillus nidulans, and calpain 15 is the vertebrate analog of the SOL calpain from Drosophila. Other than sequence homologies, very little is known about the properties of these putative calpains, and the remainder of this section will focus on calpain 3a (p94) and its splice variant, Lp82 (calpain 3b); on calpain 9; on calpain 10; and on an unusual \(\mu\)/m-calpain that has been identified only in the chicken.

Of the 14 calpain-like DNA sequences that have been identified in mammals, only three, \(\mu\)-calpain, m-calpain, and Lp82 (calpain 3b), have been isolated in protein form. Calpain 9 has been expressed in a proteolytically active form in a baculovirus system (236). It was necessary to coexpress the 80-kDa calpain 9 subunit and the 28-kDa small subunit of \(\mu\)- or m-calpain to obtain a proteolytically active form of calpain 9. The heterodimeric calpain 9 required 125 \(\mu\)M Ca\(^{2+}\) for half-maximal proteolytic activity; its proteolytic activity was inhibited by calpastatin and E-64, but its specific activity was <5% the specific activity of \(\mu\)- or m-calpain (236). Expression of the Capn9 gene in the stomach was suppressed in gastric cancer cells (493), and antisense-induced deficiency of calpain 9 resulted in cellular transformation and tumorigenesis of mouse NIH3T3 fibroblasts (249). Hence, calpain 9 may have antitumorigenic properties, but the inability to isolate a catalytically active calpain 9 protein from tissue makes it difficult to determine how it might function in this role. None of the other vertebrate "calpains" listed in Tables 6 and 7 has been expressed in a proteolytically active form, so little is known about their catalytic properties, although calpain 3a and to a lesser extent calpain 10 have been studied extensively.

Lp82 (calpain 3b) is a splice variant of the Capn3 gene that is produced by deletion of exons 6, 15, and 16, which encode the two unique insertion sequences in calpain 3a (Fig. 6), and by use of a different exon 1 (253),
which results in a NH₃ terminus different from calpain 3a. Lp82 (calpain 3b) is found exclusively in the eye lenses of a number of animal species but not the human (253, 316). Lp82 was partly purified by anion-exchange chromatography and shown to have Ca²⁺-dependent proteolytic activity in casein zymograms (255). Lp82 seems to be proteolytically active as a single polypeptide chain, although this has not been established conclusively because of the inability to completely purify the enzyme. Proteolytic activity of Lp82 is poorly inhibited by calpastatin (315) but is completely inhibited by cysteine protease inhibitors such as E-64, suggesting that not all of the recently identified calpains may be subject to calpastatin inhibition as μ- and m-calpain are. As indicated earlier in this review, the weak inhibition of Lp82 by calpastatin may be related to weak binding of calpastatin to a polypeptide that lacks the small subunit and that therefore does not have the third binding site of calpastatin (see sect. uB3).

1. Calpain 3a (p94) and limb girdle muscular dystrophy type 2A

Interest in calpain 3a (p94), the “skeletal muscle-specific” calpain, increased dramatically when it was demonstrated that disruption of the calpain 3a gene was the cause of limb girdle muscular dystrophy type 2A (LGMD2A; Ref. 366). Calpain 3a is by far the most extensively studied of the new calpains. Although Northern blot analysis initially suggested that this calpain was expressed only in skeletal muscle (414), more sensitive RT-PCR analyses have shown that cardiac muscle contains 40% and liver ~10% as much calpain 3a mRNA as skeletal muscle (360). The Capn3 gene consists of 15 exons spanning ~45 kb; the calpain 3a cDNA predicts a polypeptide of 821 amino acids having 54 and 51% sequence homology to the 80-kDa subunits of μ- or m-calpain, respectively (Table 7, Fig. 6; Ref. 414). Mass of the calpain 3a polypeptide is larger than mass of the 80-kDa subunits of μ- or m-calpain (414), it has been impossible to isolate the calpain 3a protein from muscle. No proteolytic activity that might originate from calpain 3a has ever been detected in muscle homogenates, although countless assays of such homogenates have been done during purifications of μ- and m-calpain from muscle. An attempt to isolate calpain 3a from rabbit skeletal muscle produced fragments of 60, 58, 55, and 31 kDa but not a 94-kDa polypeptide (207). The evidence suggested that the 60-, 58-, 55-, and 31-kDa polypeptides were produced by very rapid autolysis of the native 94-kDa polypeptide, in spite of inclusion of EDTA/EGTA, leupeptin, and E-64 in the homogenizing buffer. Earlier studies involving transfection of COS cells with a full-length cDNA encoding calpain 3a had led to the conclusion that calpain 3a could not be detected after transfection because it turned over very rapidly with a half-life of ~30 min (421). Deletion of the IS2 sequence resulted in a stable polypeptide in the transfected cells (421). The purification studies, however, showed that the degradation occurred in the IS1 sequence, with the 60-, 58-, and finally the 55-kDa polypeptide fragments being produced sequentially (207). Mutating Cys-129 (the active site Cys) to Ser resulted in a stable polypeptide that could be purified from COS cells and that eluted from a size-exclusion column at a position corresponding to 180 kDa, suggesting that the mutated calpain 3a was a homodimer and that the rapid autolysis was due to a cysteine protease that should have been inhibited by E-64. It was inferred that calpain 3a has proteolytic activity because partly purified calpain 3a degraded mutated C129S calpain 3a to produce a 55-kDa fragment similar to the 55-kDa autolytic fragment (207). The partly purified calpain 3a, however, was proteolytically inactive against an exogenous substrate (207). It seems that the two insertion sequences, IS1 and IS2, are involved in the instability of the calpain 3a molecule; removal of these sequences results in a stable protein (163), and Lp82 (calpain 3b), which lacks these sequences, is stable.

A full-length calpain 3a has been stably overexpressed in transgenic mice at mRNA levels 25–60 times greater than the levels of endogenous calpain 3a mRNA; proteolytic activity that was ascribed to calpain 3a was detected by casein zymography in muscles from the transgenic mice but not in muscles from nontransgenic mice (424). Western analysis detected polypeptides of 94, 60, 58, and 55 kDa in muscles from the transgenic mice, but only a polypeptide of 94 kDa at a much reduced level in muscles from the nontransgenic mice. Overexpression of the full-length calpain 3a had little or no effect on skeletal muscle morphology, and the mice had normal muscle strength as measured in a grip test (424). Overexpression of a calpain 3a isoform lacking the IS1 domain resulted in a muscle morphology characteristic of developmental immaturity, and overexpression of a calpain 3a lacking the
IS2 domain affected morphology of the slow soleus muscle but not the other muscles in the transgenic mice. None of the transgenic calpain 3 mice showed any signs of elevated rates of skeletal muscle wasting. No attempt was made to determine stability of the calpain 3a polypeptide in muscles of the transgenic mice.

Yeast two-hybrid analysis (208, 417) indicated that calpain 3a binds to two different sites along the large titin polypeptide: 1) at intervening sequences called M-is7 at the COOH-terminal end of the titin molecule (M-line region); this binding requires the full-length calpain 3a molecule; and 2) at the N2A region of the N2 line in skeletal muscle myofibrils; this binding involves the IS2 sequence. Binding to titin, which is part of the skeletal muscle myofibril, indicates that not all the calpain 3a in muscle cells is located in the nucleus, as was observed when calpain 3a was overexpressed in COS cells (421). The yeast two-hybrid studies did not detect any interaction of calpain 3a with calpastatin or the 28-kDa small subunit.

The experimental results leave a number of questions about the nature of the calpain 3a molecule. How can a cysteine, putatively Ca\(^{2+}\)-dependent protease, degrade itself uncontrollably in the presence of cysteine protease inhibitors and Ca\(^{2+}\) chelators? If calpain 3a is degraded so rapidly that it cannot be purified even with an immunosorbent column that requires only minutes, why can it be detected in isolated myofibrils (several hours to prepare; Ref. 417) or in biopsies of human skeletal muscle 8 h after their removal (6)? And finally, why would a cell contain such large amounts of calpain 3a mRNA if the protein is destroyed within minutes after its synthesis? If calpain 3a is stabilized by its association with titin/myofibrils in muscle; it should be possible to isolate myofibrils and show that calpain 3a is associated with them and that it has proteolytic activity. This has not yet been done. Does the function of calpain 3a require that it appear in large quantities and then be immediately destroyed? Does the lack of proteolytic activity of partly purified calpain indicate that it requires a cofactor for proteolytic activity that is present in vivo but that is removed during anion-exchange chromatography?

Because calpain 3a is degraded so rapidly and/or is proteolytically inactive after isolation, studies on calpain 3a have used antibodies elicited against synthetic peptides representing the IS1, IS2, or unique NH2-terminal sequence of calpain 3a (Fig. 6). Although these peptide antibodies recognize the expressed calpain 3a in transfected cells or in muscles from transgenic animals that are overexpressing calpain 3a, the only way at present to confirm that they label calpain 3a specifically in wild-type skeletal muscle tissue is to isolate and sequence the polypeptide(s) that are labeled in skeletal muscle extracts by these antibodies to ensure that the sequence of these polypeptides matches the sequence predicted for calpain 3a. It will be important to do this confirmation.

Over 100 pathogenic mutations have been reported for the calpain 3a gene. Analysis of nine of the point mutations that have been identified in LGMD2A patients indicated that all nine had lost the ability to degrade fodrin in transfected COS7 cells, but that only seven had lost their autolytic ability, three had lost the ability to bind to the N2A region of titin, and six had lost the ability to bind to the M-is7 region of titin (333). The results suggest that LGMD2A is associated with loss of calpain 3a’s ability to degrade a protein substrate. The crystallographic structure of calpain 3a has been modeled based on the similarity of its amino acid sequence to the amino acid sequence of m-calpain (195). These modeling studies suggest that many of the point mutations in calpain 3a that are associated with LGMD2A may affect domain/domain interactions in the calpain 3a structure and would be expected to reduce the proteolytic activity of calpain 3a.

Based on in vivo studies, Baghdiguian et al. (16) suggested that calpain 3a degrades 1β3α, which binds to NF-κB in cells and prevents its translocation to the nucleus, where it acts to induce expression of genes involved in the inflammatory response and cell survival. According to this hypothesis, the absence of proteolytically active calpain 3a prevents nuclear location of NF-κB and results in initiation of apoptosis in the affected nuclei. This mechanism would explain how loss of a putative proteolytic enzyme could result in loss of muscle mass, but it does not explain why skeletal muscle cells contain such large amounts of calpain3a mRNA.

Suppressing calpain 3a expression in primary rat myoblast cultures by antisense oligonucleotides does not block fusion but interferes with organization of the myofibrillar lattice in developing muscle. Z-disks are primarily affected and are wavy and disorganized (360). Calpain 3a is expressed relatively late during muscle cell development after fusion of myoblasts, and calpain 3a antisense oligonucleotides have little effect if administered before myoblast fusion (360). Until the protein form of calpain 3a has been isolated and its properties characterized, it will be difficult to interpret these observations. Inhibiting proteolytic activity would be expected to result in enhanced myofibrillar assembly, especially in the Z-disk area, which is a target of μ- and m-calpain degradation (and presumably, also any proteolytic activity of the homologous calpain 3a).

2. Other vertebrate calpain homologs

The human Capn10 gene maps to chromosome 2q in humans and contains 15 exons spanning 31 kb; it is expressed as eight different isoforms that are named calpain 10a, calpain 10b, and so on to calpain 10h (170). The calpain 10a isoform is the most abundant and is expressed in heart (greatest levels), brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, lens, and retina (170, 254).
Calpain 10c and calpain 10g transcripts are detected in most tissues, but calpain 10b, 10d, 10e, and 10f are less abundant. The mechanisms regulating expression of the different transcripts and the properties of the different calpain 10 isoforms are unknown. An antibody elicited against a 20-amino acid peptide from domain II of rat calpain 10 was used to show that the calpain 10 protein was expressed as a doublet of ~74.5 kDa in some tissues that contained the calpain 10 transcripts; the calpain 10 protein was located in a water-insoluble fraction of the rat tissues (254). Calpain 10 is an atypical calpain that has a T domain at its COOH-terminal end, similar to calpain 5 and 6 (Table 6).

Interest in calpain 10 increased when it was learned that a G/A polymorphism in intron 3 of the calpain 10 gene is associated with insulin resistance and type 2 diabetes (170, 490). The at-risk genotype was homozygous for the UCSNP-43 G allele within the calpain 10 gene in Mexican-American populations but not in populations of Pima Indians (17). Type 2 diabetes is a multifactorial disease, and it is unclear how polymorphisms at the UCSNP-43 locus in intron 3 are related to type 2 diabetes. Intron 3 seems to have promoter activity, with the G allele having greater promoter activity than the A allele (17). Calpain 10 may affect insulin secretion, insulin action, and hepatic glucose production, each of which is altered in patients with type 2 diabetes (170). It seems likely that the calpain 10 gene functions in some as yet unknown combination with other genes in type 2 diabetes. The positional cloning that led to identification of the association between the calpain 10 gene and type 2 diabetes is a seminal example of efforts to identify genes involved in multifactorial diseases such as diabetes, and illustrates the difficulties that will be encountered in such studies.

The first calpain that was purified from chicken skeletal muscle (189) was originally believed to be a m-calpain, but recent cloning and sequencing analysis have suggested that it is a unique calpain whose 80-kDa subunit has a sequence intermediate (in terms of homology) between the 80-kDa subunits of µ- and m-calpain in mammalian species (422). A chicken µ-calpain cDNA has been cloned and sequenced (194), and a µ-calpain protein has been purified from chicken skeletal muscle (481). It is unclear, however, whether the “high m-calpain” that was partly purified from this tissue (481) corresponds to the m-calpain cDNA sequence obtained for the chicken (422). The “intermediate” calpain, called µ/m-calpain, is expressed in a number of different chicken tissues (422) and is the predominant calpain in chicken skeletal muscle (481). µ/m-Calpain has not yet been identified at either the DNA or protein level in mammalian tissues, and it is presently unclear whether it exists only in chickens or is present in other species as well. Unlike many of the “other calpains” or calpain-like polypeptides that have been discussed in this section, the µ/m-calpain is well characterized, is a heterodimer with a 28-kDa small subunit similar to µ- and m-calpain, and has a Ca²⁺ requirement of 420 µM for half-maximal proteolytic activity (481) on the lower edge of the Ca²⁺ requirements of the m-calpains that have been studied thus far (Table 3).

C. Summary

Most of the calpains discussed in this section have been identified only as genes or mRNAs having sequence homology to the prototypal µ- and m-calpain, or more specifically to the protease domain of µ- and m-calpain. The crustacean proteases and the purified Drosophila calpain do not have a small subunit, and many of the other calpains either lack a domain IV or have an altered domain IV (e.g., domain T, PaB-like domain). Because domain IV contains the site(s) responsible for association of the 80- and 28-kDa subunits in µ- and m-calpain, it is possible, perhaps even likely, that these other, atypical calpains also will not have a small subunit when they finally are isolated in protein form. It is still unclear whether these atypical members of the calpain family associate with a cofactor different from the 28-kDa polypeptide that is essential for their proteolytic activity or whether the large subunit alone is sufficient for proteolytic activity of these calpains.

The discovery of different calpain isoforms in addition to µ- and m-calpain has opened the possibility that the calpains may participate in a wide range of hitherto unsuspected cellular functions. Does a calpain isoform that is involved in sex determination in C. elegans and whose mammalian counterpart (calpain 5) is prevalent in the testis suggest some role in sexual maturation in the human (mammals) for this calpain? The existence of calpains that lack one or more of the residues involved in the catalytic triad indicates that these calpains have some function that does not involve proteolytic activity. Is this same nonproteolytic function preserved in those calpains that have proteolytic activity? Until the identification in Arabidopsis thaliana and subsequently in Zea mays of a gene having significant sequence homology to the calpains, it had been believed that the plant kingdom did not have calpain-like molecules. Genomic sequencing now indicates that all plants may have a single “calpain” gene and that this gene encodes a polypeptide having a transmembrane domain in addition to the protease domain II and a domain III that is homologous to domain III of µ- and m-calpain. A number of studies have found that the animal calpains are translocated to the plasma membrane where they degrade membrane-associated substrates. Does the transmembrane domain in the plant calpains suggest that the plant kingdom simply tethers their calpain to the membrane rather than translocating it there? It may be anticipated that isolation and/or expression of the
protein forms of these new calpains and characterization of their properties will lead to a more completely defined role of the calpain system in general and to the physiological functions it has in cells. It is ironic in many ways that the calpains currently are better known for their roles in a variety of tissue pathologies than for what functions they play in normal cells.

The procedures described in section II C that have been used to purify \(\mu\)- and m-calpain have also been used in attempts to purify some of the recently identified calpains (calpain 3a is an example). No proteolytic activities that might be ascribed to the molecules encoded by the mRNAs for these recently discovered calpains have been identified in tissue extracts, suggesting that these calpains either have very low proteolytic activity (possibly because they are present in very small quantities) or that they require for their proteolytic activity a cofactor that is lost during tissue homogenization and fractionation. It may be more productive when attempting to obtain the protein forms of these new calpains to use expression systems and/or to use antibodies to immunoprecipitate the proteins from tissue extracts.

Information on new members of the calpain family has accumulated rapidly during the past 10 years, and it is impossible to review this information in depth here. Readers are referred to several excellent recent reviews on the “new” calpains (334, 416, 420, 438) for more detailed information.

D. Nomenclature

As indicated earlier, it has been recommended that the terms \(\mu\)-calpain and m-calpain be used to refer to the well-characterized micromolar and millimolar Ca\(^{2+}\)-requiring proteases, respectively (434). This recommendation has been widely adopted. The discoveries during the past 13 years of putative calpain-like polypeptides in a number of invertebrate species and of calpain-like genes, sometimes expressed specifically in certain tissues in vertebrates, however, has complicated nomenclature of the calpains. The situation is similar to myosin where 24 genes have been identified that encode molecules having sequences with homology to the catalytic domain of myosin but that have very little similarity to the remainder of the myosin molecule. It has been suggested (418, 438) that the calpains can be divided into two general classes: 1) "typical" calpains, those possessing a calmodulin-like domain at their COOH terminus, and 2) "atypical" calpains, those lacking a calmodulin-like domain IV at their COOH terminus, similar to the nomenclature that has been used for the PKC family (205). Because the Ca\(^{2+}\) “switch” for proteolytic activity of \(\mu\)- or m-calpain may reside in domain II or III of these enzymes (see sect. II A3), it is premature to assume that atypical calpains lacking domain IV are Ca\(^{2+}\) independent.

The recent increase in the number of calpain-like molecules has led to a corresponding increase in the number of names for the calpains. Unlike the myosin field where many of the molecules have been expressed in a catalytically active form, the lack of information on the catalytic properties of many (most) of the calpain-like molecules makes it difficult to develop a rational system of nomenclature that reflects the properties of the molecules themselves. Nevertheless, it is probably useful to discuss a systematic approach to calpain nomenclature in the hope that this discussion will provide a framework for a system that will eventually evolve. Table 8 lists most (but not all) of the molecules that have been identified as calpains or calpain-like molecules and proposes a name for the polypeptides listed. The calpain genes have been named numerically, Capn1 through Capn14, and it is proposed that the polypeptide encoded by these genes also be named numerically, calpain 1 through calpain 14 (Table 8). The polypeptides, calpain 1 and calpain 2, and probably calpain 9, do not function as monomeric polypeptides, so the names \(\mu\)-calpain and m-calpain are used for molecules containing calpain 1 plus the 28-kDa subunit and calpain 2 plus the 28-kDa subunit, respectively. To prevent confusion, the names calpain I and calpain II that have sometimes been used to refer to \(\mu\)-calpain and m-calpain, respectively, should be avoided. It is still unclear whether the other calpain polypeptides function as monomers or whether they also function in combination with some other accessory molecule. A number of small penta-EF-hand polypeptides such as ALG-2, pemlin, sorcin, and grancalin have been identified during the last several years (210). These penta-EF-hand polypeptides dimerize with their EF-5 hands involved in the dimerization, similar to dimerization of the expressed domain VI (39, 247). It has not yet been determined whether these penta-EF-hand polypeptides can form dimers with the large subunit of the typical calpains. Would such dimers stabilize calpain 3? If the calpain polypeptides function as monomers, the protein would retain the name, calpain 3, calpain 4, etc. If they function in combination with some other molecule, they can be named once this identification has been made. The human SOL calpain has been given the name calpain 15, and the Drosophila calpains have been designated CALPA, CALPB, CALPC, and dcalpain 15. It is proposed that calpains produced by alternative splicing be named calpain 10a, calpain 10b, and so on using letters to distinguish newly discovered isoforms. The term CALPA’ has been used to distinguish the alternatively spliced form of CALPA, but if a number of isoforms are identified for the invertebrate calpains, it may be more convenient to also use letters to distinguish alternatively spliced forms of invertebrate calpains: CALPAA, CALPAb, and so on. The system in Table 8 is intended as a proposal for discussion, and no attempts are made to name all the C.
TABLE 8. Some members of the calpain family, their gene names, and possible names for the polypeptides

<table>
<thead>
<tr>
<th>Species</th>
<th>Name of Gene</th>
<th>Name of Polypeptide</th>
<th>Previously Used Name</th>
<th>Typical/Atypical</th>
<th>Protein/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian</td>
<td>Capn1</td>
<td>Calpain 1</td>
<td>µCL</td>
<td>Typical</td>
<td>µ-Calpain (calpain 1 + 28 kDa)</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn2</td>
<td>Calpain 2</td>
<td>nCL</td>
<td>Typical</td>
<td>m-Calpain (calpain 2 + 28 kDa)</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn3</td>
<td>Calpain 3a</td>
<td>nCL-1, p94</td>
<td>Typical</td>
<td>Limb girdle muscular dystrophy type 2A</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn4</td>
<td>Calpain 3b</td>
<td>Lp82</td>
<td></td>
<td>Not a member of the 80-kDa calpain family</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn5</td>
<td>Calpain 5</td>
<td>nCL-3, htr3a</td>
<td>Atypical</td>
<td>Human homolog of <em>C. elegans</em> TRA3</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn6</td>
<td>Calpain 6</td>
<td>Calpamodulin</td>
<td>Atypical</td>
<td>Cys/Lys, His/Tyr, substitutions in the catalytic triad</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn7</td>
<td>Calpain 7</td>
<td>palBH</td>
<td>Atypical</td>
<td>Human homolog of <em>Aspergillus</em> Palb</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn8</td>
<td>Calpain 8</td>
<td>nCL-2</td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn9</td>
<td>Calpain 9</td>
<td>nCL-4</td>
<td>Atypical</td>
<td>Associated with gastric cancer</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn10</td>
<td>Calpain 10, 10a,</td>
<td>10b-10g</td>
<td></td>
<td>Associated with type 2 diabetes mellitus</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn11</td>
<td>Calpain 11</td>
<td></td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn12</td>
<td>Calpain 12</td>
<td></td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn13</td>
<td>Calpain 13</td>
<td></td>
<td>Atypical</td>
<td></td>
</tr>
<tr>
<td>Mammalian</td>
<td>Capn14</td>
<td>Calpain 14</td>
<td></td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>CALPA</td>
<td>CALPA</td>
<td>Calpain A</td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>CALPB</td>
<td>CALPB</td>
<td>Calpain B</td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>CALPC</td>
<td>CALPC</td>
<td>CG3002</td>
<td>Typical</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>SOL</td>
<td>Dcalpain 15</td>
<td>SOL</td>
<td>Atypical</td>
<td>SOL domain (small optic lobe) in COOH terminus</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>TRA3</td>
<td>CeCalpain 5</td>
<td>TRA-3</td>
<td>Atypical</td>
<td>Sex determination in <em>C. elegans</em></td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>CED6412.CET</td>
<td>Ce-p70</td>
<td>Atypical</td>
<td>SOL domain in COOH terminus</td>
<td></td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>T04A8.16</td>
<td>Ce-p92</td>
<td>Atypical</td>
<td>PBH domain in COOH terminus</td>
<td></td>
</tr>
<tr>
<td>Trypanosoma</td>
<td>brucei</td>
<td>Cap 5.5</td>
<td>Atypical</td>
<td>Calpastatin-like domain in NH2 terminus</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em></td>
<td>thaliana</td>
<td>FTA10.23</td>
<td>DEK 1</td>
<td>Atypical</td>
<td>Transmembrane domain in NH2 terminus</td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td>nidulans</td>
<td>palB</td>
<td>Acalpain 7</td>
<td>Atypical</td>
<td>PBH domain in COOH terminus</td>
</tr>
</tbody>
</table>

**elegans** calpains or the calpains from the lower organisms, except to suggest that those calpains having homology to a mammalian calpain be named accordingly; for example, acalpain 7 for the *Aspergillus nidulans* calpain with a palB domain similar to calpain 7, dcalpain 15 for CALPD. Hopefully, Table 8 can serve as a framework for discussion of calpain nomenclature.

**IV. BIOCHEMICAL PROPERTIES AND REGULATION OF CALPAIN ACTIVITY**

The preceding two sections have summarized the structural aspects of the calpain system, and the last three sections will discuss the enzymatic properties and physiological significance of the calpain system. The discussion in the latter three sections will be devoted almost entirely to µ- and m-calpain and calpastatin because these are the only members of the calpain system whose catalytic (and other properties) have been studied extensively thus far. The recent advances in calpain structure, the availability of amino acid sequences for the calpains and calpastatin, and the increasing information on properties of the calpains and calpastatin, all reviewed in section ii, now make it possible to propose clearly defined mechanisms for regulation of calpain activity in cells, so regulation of calpain activity will be reviewed in detail. Discussion of the physiological function(s) of the calpains will focus on those areas where there is definitive evidence implicating the calpains. There is an extensive literature suggesting that calpains are involved in a variety of physiological functions. In many instances, however, the studies have used inhibitors having proven or unsuspected effects, and it is unclear whether the findings are the result of calpain inhibition or inhibition of some other proteolytic enzyme. Hence, review of the physiological functions of the calpains will be relatively brief, considering the extensive literature in the area, and will emphasize those areas where unambiguous evidence supports a clearly defined role for the calpains. Similarly, there have been a number of reviews on the pathological consequences of unregulated calpain activity, and space restrictions (and limits of readers’ patience) will limit the discussion in this section to a listing of diseases/conditions that have been ascribed to inappropriate calpain activity.

**A. Expression of Proteolytically Active Molecules**

The calpains have been difficult to study, partly because it has been difficult to purify quantities of them in an undegraded form and partly because addition of Ca2+ to study their catalytic properties or their structural response to Ca2+ results almost immediately in self-destruction through autolysis. Therefore, the ability to express catalytically active calpains would significantly enhance study of the calpains by making quantities of these proteins available in purified form and making it possible to
use mutagenesis to determine the effects of modifying specific residues/domains on the catalytic properties of the expressed molecules. Catalytically active rat m-calpain was expressed in 1994 in an *E. coli* expression system with yields of 0.6 mg/l (142); much higher yields are now obtained from this expression system (104). Although a m-calpain containing the full-length 28-kDa subunit could be expressed in a catalytically active form, the 28-kDa subunit was truncated at amino acid 85 to obtain reasonably high yields of expressed m-calpain. Catalytically active human m-calpain was subsequently expressed in a soluble form in a baculovirus expression system with yields of 20 mg/l Sf9 cell culture (268). The Ca\(^{2+}\) concentrations required for a half-maximal rate of proteolytic activity for the two expressed calpains were the same as those required by the calpains that had been purified from tissues (400 \(\mu\)M for the baculovirus calpain; 350 \(\mu\)M for the *E. coli* calpain; cf. Table 3).

Attempts to express catalytically active \(\mu\)-calpain have not been as successful as expression of m-calpain. It has been difficult to repeat the early studies reporting that catalytically active \(\mu\)-calpain could be obtained from either *E. coli* (469) or baculovirus (292, 469) expression systems. Both reports indicated that the 80-kDa subunit of \(\mu\)-calpain was proteolytically active when expressed alone, and Vilei et al. (469) were able to express proteolytically active \(\mu\)-calpain mutants lacking domains III or IV and a mutant \(\mu\)-calpain containing domains I, II, and III from \(\mu\)-calpain and domain IV from m-calpain. When \(\mu\)-calpain is expressed in *E. coli*, the \(\mu\)-calpain is deposited in inclusion bodies and only very low yields of catalytically active \(\mu\)-calpain are obtained (104). Similar low yields of proteolytically active \(\mu\)-calpain have been obtained when the baculovirus system is used (104). Expression of a catalytically inactive form of \(\mu\)-calpain with the active site Cys-115 mutated to Ser has been reported using a baculovirus system (167), but the yields of this expression were not specified. Wild-type and mutant expressed \(\mu\)-calpain have already been used to study the effects of site-directed mutagenesis and amino acid deletions on properties of m-calpain (discussed in sect. v, C2 and C6), and the ability to express catalytically active \(\mu\)-calpain would make such studies possible also with \(\mu\)-calpain.

**B. Tissue Localization**

1. **Presence in different tissues**

One or more of the three well-characterized members of the calpain system, \(\mu\)-calpain, m-calpain, and calpastatin, has been detected in every vertebrate cell that has been carefully examined for their presence (139, 196, 308). Different tissues/cells, however, differ widely in their ratios of the three proteins. For example, human platelets and erythrocytes have no detectable m-calpain (308, 452), whereas bovine platelets (452), turkey gizzard smooth muscle (136), and vascular smooth muscle (271) have no detectable \(\mu\)-calpain. In general, skeletal muscle from domestic animals contains approximately equal amounts of \(\mu\)-calpain and m-calpain and contains calpastatin activity in excess of the activity of the two calpains together. Calpastatin activity exceeds activity of the two calpains together in most but not all cells/tissues. The approximate relative proportions of \(\mu\)-calpain, m-calpain, and calpastatin in 20 different mammalian tissues are summarized in Thompson and Goll (457). As indicated earlier in this review, calpastatin has not been detected in invertebrate tissues, although *Drosophila, Schistosoma mansoni*, and *C. elegans* contain calpain.

2. **Immunolocalization**

Immunolocalization studies have shown that both \(\mu\)- and m-calpain and calpastatin are located exclusively intracellularly; the few reports of extracellular localization have been attributed to poor fixation procedures or to diseased tissues where the calpains/calpastatin had “leaked” from cells (222, 430). Many of the immunolocalization studies have been done on skeletal or cardiac muscle. In normal skeletal muscle, most of the calpain and calpastatin is located on or next to the Z-disk with smaller amounts in the I-band and very little in the A-band area (134, 139, 222, 425). Immunogold electron microscope studies using an antibody specific to \(\mu\)-calpain also found that \(\mu\)-calpain was localized primarily in the Z-disk/I-band region of sections of rat skeletal muscle (494).

Most immunolocalization studies have found that the calpains and calpastatin are colocalized in cells (134, 139, 222), and Barnoy et al. (25) found that they coprecipitate from extracts of rat L9 myoblasts. Immunofluorescence studies indicated that calpastatin was localized at the Z-disk in sections of cardiac muscle (232), the same location as the calpains (222). Several studies reported that m-calpain is localized at the plasma membrane in dividing (397) or fusing (396) L9 or L6 myoblasts and during metaphase or interphase in cultured PtK1 cells (398). These and similar results in other tissues (378) have led to the suggestion that the calpains relocate from a widely dispersed distribution to a preferential location near the cell periphery in response to some cellular signal, possibly a Ca\(^{2+}\) flux. A recent study using COS 7 and LCLC 103H cells supports this suggestion. Both the 28- and 80-kDa calpain subunits and calpastatin were widely distributed in the cytoplasm of unstimulated cells with a slight predominance around the nucleus (127). After stimulation with a Ca\(^{2+}\) ionophore, ionomycin, the calpain subunits redistributed to a predominate plasma membrane location, but the calpastatin localization did not change. No such redistribution of the calpains has ever been observed, however, in sections of postnatal rat soleus mus-
cle (134) or in neuronal tissue (20), although calpastatin was localized at or near the sarcotubular in cultured cardiocytes (232). It is unclear whether the calpains and calpastatin can be translocated to the plasma membrane in cultured, mitotically active cells, but remain stationary in mitotically inactive tissues such as striated muscle fibers or neurons. Immunoelectron localization found that 93% of the calpain in human erythrocytes was cytosolic and the remaining 7% was associated with the plasma membrane (386). If the calpains are activated by association with the plasma membrane, as has been suggested (184, 217, 218; see sect. nCI), these results would suggest that only 7% of the calpain in human erythrocytes was enzymatically active. In dystrophic skeletal muscle, the calpains seem to be distributed more uniformly throughout the muscle fiber with less concentration at the Z-disk than in normal muscle (223, 425).

Several studies have suggested that a substantial proportion of the calpains are not free in the cell cytoplasm, as has been widely assumed, but that they are associated with subcellular structures (222). In skeletal muscle, these structures are myofibrils (222), whereas in other cells they may be cytoskeletal (actin) filaments (231). The immunolocalization studies thus far have not provided much insight into function of the calpain system because most studies find a widespread cytosolic distribution, rather than an association with a particular organelle.

C. Regulation of Calpain Activity

It can be estimated that all 2 disks in a skeletal muscle cell/fiber would be destroyed in ≤5 min if all the calpain, which as indicated in the preceding section is largely located directly on the Z-disk, in that fiber were proteolytically active. Hence, most of the calpain in a cell must be proteolytically inactive most of the time. Based on the current knowledge of the catalytic properties of the calpains, it seems that calpain activity in cells is regulated by altering the Ca2+ concentration required for its proteolytic activity, by calpastatin, and probably also by the intracellular location (access to substrate) and the seemingly fastidious subsite specificity of the calpains.

1. The Ca2+ requirement problem

Ever since the first partial characterization of m-calpain (84), it has been clear that the Ca2+ concentrations required for proteolytic and other activities of the calpains were much higher than the 50–300 nM Ca2+ concentrations that exist in living cells (192, 266; cf. Table 3). Only the Ca2+ concentrations required by autolyzed μ-calpain are in the physiological range, but Ca2+ concentrations in the 800–15,000 nM range are required to initiate autolysis of μ-calpain (Table 3). Consequently, there have been numerous attempts to identify a mechanism for reducing the Ca2+ requirements of the two ubiquitous calpains. So far, these efforts have not produced any conclusive answers.

Efforts at identifying a mechanism for reducing the Ca2+ requirements of μ- and m-calpain (an “activation” mechanism) have focused on two areas: 1) finding a mechanism(s) for reducing the Ca2+ concentration needed to induce autolysis, with the assumption that the autolyzed enzymes could be active at physiological Ca2+ concentrations, and 2) attempts to find molecules in cells that would interact with/alter the calpains and reduce their Ca2+ requirements.

It was discovered in 1984 that certain phospholipids, with phosphatidylinositol (PI) being the most effective, would lower the Ca2+ concentration required for autolysis of m-calpain by nearly eightfold (66). Subsequent studies have shown that several phospholipids, with phosphatidylinositol, 4,5-bisphosphate (PIP2) being the most effective (373, 376), lower the Ca2+ concentration required for autolysis of either μ-calpain (62, 373, 376) or m-calpain (62) by three- to sixfold (Table 3). Very high molar ratios of 375–4,100 phospholipid (PI or PIP2) to one calpain, however, were necessary to reduce the Ca2+ concentration required for initiation of autolysis in vitro assays, and neither PIP2 nor PI had any effect below these concentrations. The PIP2 and PI were micellar at these concentrations, and it is uncertain whether PIP2 has to be in a micellar form to induce a reduction in Ca2+ requirement. It seems unlikely that the calpains would ever encounter PIP2 molar ratios of 375–4,100 to one calpain or PIP2 micelles in vivo. Finally, even in the presence of phospholipid, the Ca2+ concentrations required for autolysis of m-calpain are far above those that exist in living cells, and the Ca2+ concentrations required for autolysis of μ-calpain are at the upper limits of in vivo Ca2+ concentrations (Table 3). Hence, μ-calpain would autoamylase only in the presence of Ca2+ “spikes” of 1–10 μM that might occur in localized areas. It has been suggested that the calpains interact with a phospholipid at the plasma membrane (or other cellular membranes), perhaps in the area of a Ca2+ channel, and that this interaction is of a nature that would reduce the Ca2+ requirement for autolysis. Several studies, however, have reported that the calpains bind to proteins and not phospholipids in the plasma membrane (184, 186, 219), and other studies have shown that when the calpains are incubated with inside-out erythrocyte membranes, the Ca2+ concentration required for their autolysis is not affected (T. Zalewska, V. Thompson, and D. Goll, unpublished data). If these latter results are indeed accurate, it is unclear whether interaction of the calpains with the plasma membrane would have any effect on autolysis. Studies using two irreversible inhibitors to label μ-calpain in human platelets indicated that the calpain in these platelets was autolyzed in the cytosol before being translocated to the membrane.
(4). In sum, there are a number of uncertainties as to whether phospholipids/autolysis could be the “activation” mechanism sought for the calpains, although as indicated earlier, autolysis and possibly also interaction with phospholipids likely has some as yet unknown role in calpain function.

A number of studies have described molecules that seemed to reduce the Ca$^{2+}$ requirements of the calpains in vitro assays. It was reported that isovalerylcarnitine reduced the Ca$^{2+}$ concentration required for maximal proteolytic activity of m-calpain to <10 μM and increased its specific activity by 1.5-fold, but did not affect either the Ca$^{2+}$ requirement or the specific activity of μ-calpain (354, 356). Other studies described a heat-stable polypeptide of ~40–45 kDa isolated from human neutrophils (353) or rat skeletal muscle (357) that reduced the Ca$^{2+}$ concentration required for maximal activity of m-calpain to 20 μM or less without affecting its specific activity. The 40- to 45-kDa “activator” also had no effect on the catalytic properties of μ-calpain. A similar 40-kDa “activator” protein that was purified from erythrocytes (383) reduced the Ca$^{2+}$ concentration required for half-maximal activity of erythrocyte calpain, which is a μ-calpain, from 50 to ~0.2 μM; this activator also reduced the Ca$^{2+}$ requirements of m-calpain similar to the previously isolated ones. More recently, a protein having a monomeric mass of 15 kDa but functioning as a dimer of 30 kDa reduced the Ca$^{2+}$ requirement of μ-calpain from a variety of sources to 0.4–0.5 μM (289), but had no effect on m-calpain. Sequence analysis indicated that this protein was very similar to a goat liver protein, UK114. The UK114-like protein was active at a 1:1 molar ratio of UK114:calpain. A subsequent study identified a 10-kDa polypeptide that also functioned as a dimer of 20 kDa and that reduced the Ca$^{2+}$ requirement of m-calpain from several sources to 6–7 μM (290). Sequencing followed by cloning and expression identified this protein as acyl-CoA binding protein. The acyl-CoA binding protein was maximally active at a 1:1 molar ratio, acyl-CoA binding protein:m-calpain, and had only a minimal effect on the Ca$^{2+}$ requirement of μ-calpain.

The significance of these activator proteins is still unclear. The isovalerylcarnitine studies required molar ratios of nearly 20,000 isovalerylcarnitine:1 calpain for a reduction in Ca$^{2+}$ requirement, raising the possibility that some contaminant was the active agent or that nonspecific effects were involved. It is difficult to determine the molar ratios used in the studies describing the 40- to 45-kDa activators, but if 4 μg activator was required to “activate” 3 μg m-calpain (353), a molar ratio of ~4 activator:1 m-calpain was required for activity of this activator. The 40- to 45-kDa activator bound to m-calpain in a 1:1 molar ratio (293), and it is unclear whether the 40- to 45-kDa activator reduced the Ca$^{2+}$ requirement of m-calpain at this ratio. One to one molar ratios of either the UK114-like or the acyl-CoA binding protein were required to reduce the Ca$^{2+}$ requirement of the calpains. Hence, these two activators did not act catalytically (for example, via phosphorylation or dephosphorylation) to change the Ca$^{2+}$ requirement of the calpains, but would need to bind to the calpains in a manner similar to the calmodulin binding that activates a number of different enzymes. The UK114-like activator was reported to enhance dissociation of the two subunits of μ-calpain (289), but recent results have shown that dissociation of the two subunits results in aggregation of the 80-kDa subunit and loss of proteolytic activity, not a reduction in Ca$^{2+}$ requirement (245).

Several other calpain activator proteins have been described. These proteins increased the catalytic activity (Vₘₐₓ) of the calpains by 25- (92), 10- (449), or 2-fold (406) without affecting the Ca$^{2+}$ concentration required for proteolytic activity. There have been no subsequent studies describing these activators, and their nature is unknown.

2. Ca$^{2+}$-binding properties of μ- and m-calpain

Attempts to determine the number and binding affinities of the Ca$^{2+}$ bound to the calpains have encountered several technical difficulties. First, as described in section μA4, the calpains autolyze rapidly in the presence of Ca$^{2+}$, so ways must be found to prevent autolysis. Second, the calpains aggregate/precipitate at Ca$^{2+}$ concentrations above 1 mM (11, 98), and this precipitation is likely to impact Ca$^{2+}$ binding measurements.

To prevent difficulties due to autolysis, domain IV (Fig. 1) from chicken μ-m-calpain, rabbit μ-calpain, or rabbit m-calpain and domain VI of rabbit calpain were expressed and were assayed for their Ca$^{2+}$-binding properties (295). Chicken μ-m-domain IV bound 2.1 Ca$^{2+}$ with a Kᵦ of 61 μM; rabbit μ-calpain domain IV bound 1.6 Ca$^{2+}$ with a Kᵦ of 35 μM; rabbit m-calpain domain IV bound 4.0 Ca$^{2+}$ with a Kᵦ of 130 μM; and the rabbit small subunit bound 2.1 Ca$^{2+}$ with a Kᵦ of 150 μM. Site-directed mutagenesis studies of domain VI suggested that the EF-2’ and EF-5’ sites bound the two Ca$^{2+}$ that were bound by this domain and that the EF-4’ site did not bind Ca$^{2+}$ (294). Binding to proteins enhances the fluorescence of terbium, and this property was used to show that bovine erythrocyte μ-calpain bound 4 mol Tb$^{3+}$/mol with Kᵦ values of 10⁻⁵ to 10⁻⁷ M and that bovine brain m-calpain bound 6 mol Tb$^{3+}$/mol with Kᵦ values of 10⁻⁶ to 10⁻⁴ M (503).

The studies using expressed domains IV and VI were done before identification of the two Ca$^{2+}$-binding sites in domains IIA and IIB in the 80-kDa subunit (see sects. μA1 and νC6), and because the isolated domains IV and VI were used, the EF-5 hands, which now are known to be involved in association of the two subunits and do not
bind Ca\(^{2+}\) in the intact calpain molecule, bound Ca\(^{2+}\).

Critical, bidentate Ca\(^{2+}\)-ligating residues in individual
EF-hands were mutated in expressed rat m-calpain to reduce or eliminate Ca\(^{2+}\) binding to the mutated EF-hand (98). The mutations were done either in single EF-hands or in various combinations of EF-hands. X-ray crystallography was used to confirm that the mutations abolished Ca\(^{2+}\) binding at the mutated site, and proteolytic activity was used to infer Ca\(^{2+}\) binding; direct measurements of Ca\(^{2+}\) binding were not attempted. The results showed that 1) mutations in EF-1 in either subunit had only small effects on the Ca\(^{2+}\) concentration required for half-maximal activity (\(K_{0.5}\)); changes in \(K_{0.5}\) ranged from -115 to +66 \(\mu\)M from the 367 \(\mu\)M Ca\(^{2+}\) \(K_{0.5}\) for the wild-type calpain; 2) mutations in EF-2 in either subunit increased the \(K_{0.5}\) slightly, from 14 to 232 \(\mu\)M above the 367 \(\mu\)M; 3) mutations in EF-3 had the largest effects on \(K_{0.5}\); with mutations in EF-3' of the small subunit increasing \(K_{0.5}\) by -330 \(\mu\)M and mutations in EF-3 of the large subunit increasing \(K_{0.5}\) by 466 to 590 \(\mu\)M; 4) mutations in EF-4 of either subunit had little effect on the \(K_{0.5}\) of the enzyme, in agreement with the crystallographic results showing very weak and abnormal Ca\(^{2+}\)-binding to this site (39, 247); 5) when three EF-hands (omitting EF-4) in either subunit (but not both simultaneously) were mutated, the \(K_{0.5}\) rose to 940 \(\mu\)M, indicating that Ca\(^{2+}\) binding to either subunit was sufficient for proteolytic activity; and 6) mutation of EF-3 in both the large and small subunits increased the \(K_{0.5}\) from 367 to 1,689 \(\mu\)M, and mutation of EF-1, EF-2, and EF-3 in both subunits simultaneously increased the \(K_{0.5}\) from 367 to 7,410 \(\mu\)M, illustrating the importance of EF-3 in either subunit (98). The results of this study indicate that Ca\(^{2+}\) binding to EF-hands in the calpain molecule is highly cooperative, with Ca\(^{2+}\) binding at any one site (possibly excluding EF-4) being sufficient to initiate proteolytic activity of the enzyme (98). The fifth EF-hand in each domain (EF-5 and EF-5') was not studied because it is involved in subunit association and does not bind Ca\(^{2+}\). That mutation of EF-4 in either the 28- or the 80-kDa subunit has almost no effect on the Ca\(^{2+}\) requirement of expressed m-calpain makes it unlikely that the two EF-4 sites in the calpain molecule are involved in functional Ca\(^{2+}\) binding. Similarly, the EF-hand-like sequence identified at the domain II/III boundary does not have a EF-hand-like structure in the crystallographic structures of m-calpain (171, 430), and it is uncertain whether this site binds Ca\(^{2+}\), at least in the m-calpain molecule (it does bind Ca\(^{2+}\), however, in the calpain isoleted from Schistosoma mansoni). Recent studies have identified two additional Ca\(^{2+}\)-binding sites in domains IIa and IIb; these Ca\(^{2+}\)-binding sites are not EF-hand sequences but are in peptide loops (see sect. vC6; Ref. 298).

Equilibrium dialysis and a membrane filtration assay were used in an attempt to measure the Ca\(^{2+}\)-binding properties of intact, unmutated, unautolyzed calpins (M. Matsuishi, V. Thompson, and D. Goll, unpublished data). The membrane filtration assay was rapid, so autolysis was minimized in these experiments, and all studies were done at 4°C, also to minimize autolysis. In addition, a complete set of experiments was done using calpins that had been reversibly inactivated by using tetrathionate oxidation (199) to prevent autolysis altogether. Oxidative inactivation had little effect on the Ca\(^{2+}\)-binding properties of the calpins. Scatchard analysis was used to determine \(K_d\) values and the number of Ca\(^{2+}\) bound per molecule. For \(\mu\)-calpain: membrane filter assay-YM-10 membranes, 7.3 Ca\(^{2+}\)/molecule and \(K_d = 61.1 \mu\)M; membrane filter assay-PVDF membranes, 8.3 Ca\(^{2+}\)/molecule and \(K_d = 20.6 \mu\)M; equilibrium dialysis, 4.6 Ca\(^{2+}\)/molecule and \(K_d = 32 \mu\)M. For m-calpain: membrane filter assay- YM-10 membranes, 15.9 Ca\(^{2+}\)/molecule and \(K_d = 502 \mu\)M; membrane assay-PVDF membranes, 20.3 Ca\(^{2+}\)/molecule and \(K_d = 1,300 \mu\)M; equilibrium dialysis, 11.0 Ca\(^{2+}\)/molecule and \(K_d = 322 \mu\)M. Hence, based on this study, \(\mu\)-calpain binds \(\sim 5-8\) Ca\(^{2+}\)/molecule with a \(K_d\) of \(\sim 21-61 \mu\)M, whereas m-calpain binds many more Ca\(^{2+}\), \(\sim 11-20/molecule with a much higher \(K_d\) 322-1,300 \(\mu\)M. The variability in the results attests to the inherent difficulty in measuring the Ca\(^{2+}\)-binding properties of the calpins. The data, however, indicate that m-calpain has more Ca\(^{2+}\)-binding sites than would be accounted for by the six EF-hand structures in the molecule, although these sites have low affinity for Ca\(^{2+}\), whereas the Ca\(^{2+}\) bound by \(\mu\)-calpain can be accounted for by the six EF-hand Ca\(^{2+}\)-binding sites and the two Ca\(^{2+}\)-binding sites in domains IIa and IIb. Also, the \(K_d\) values for the two calpins reflect their respective Ca\(^{2+}\) requirements for proteolytic activity. Hopefully, it will be possible to find new approaches to accurately measure the Ca\(^{2+}\)-binding properties of the calpins with precision; such measurements are needed to show whether Ca\(^{2+}\) in addition to those binding to the EF-hand structures in domains IV and VI are involved in the catalytic activity of the calpins.

3. Subsite specificity

The initial studies showing that m-calpain degraded tropomyosin, troponins T and I, and C-protein but did not degrade myosin and actin, the two major proteins in skeletal muscle myofibrils, implied that the calpains had a limited and very specific subsite specificity (82). In general, the calpains cleave proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides or amino acids (138, 139, 310). Early studies suggested that the calpains preferentially cleaved peptide bonds having a Leu or a Val residue in the P2 position (reviewed in Ref. 310), although this was not an absolute
requirement. More detailed analysis, however, has indicated that subsite specificity of the calpains is not governed by amino acid sequence, but rather by conformation of the polypeptide chain (75, 116, 152, 428). A summary of 23 sites cleaved by m-calpain in 5 different proteins indicates that only 3 of them have a Leu or Val in the P₂ position (Table 9; the cleavage sites listed are intended only as an example; see Ref. 76 for a more complete list). Indeed, the variety of amino acids in the P₂ position ranging from negatively charged to positively charged to polar suggests that the P₂ amino acid has only a marginal effect on the specificity of the calpains.

Brain α-spectrin (α-fodrin) is cleaved at a site in repeat 11 by μ-calpain: Q-Q-E-V-Y(1176)—G(1177)-M-M-P-R, to produce fragments of ~150 and 145 kDa in SDS-PAGE. This site-directed mutagenesis was used to substitute 20 different amino acids into the P₂ position (428). The substitutions significantly affected the rate of digestion of the expressed polypeptide by μ-calpain, with Arg, Val, and Ala favoring cleavage and Gly, Asp, Pro, and His protecting against cleavage (428). Those amino acid substitutions that increased rate of calpain cleavage also disrupted the structure of the expressed polypeptide, as indicated by molecular modeling studies and additional proteolytic degradation of the expressed polypeptide, whereas those amino substitutions that decreased rate of calpain cleavage stabilized the expressed polypeptide; the results suggested that rate of calpain cleavage is directly linked to polypeptide conformation. Nine of the 11 calpain cleavages in the α-tropomyosin polypeptide are in the COOH-terminal half of the molecule (Table 9). Although the tropomyosin polypeptide is 100% α-helical, the COOH-terminal half of the helix is significantly less stable than the NH₂-terminal half, again suggesting that subsite specificity of the calpains depends on the conformation of the polypeptide, with a more open structure favoring cleavage. An early study on the subsite specificity of m-calpain found that m-calpain cleaved vimentin at 10 sites and that all 10 were in the nonhelical, unordered part of the molecule (116). The very limited degradation of most polypeptides by the calpains, however, indicates that specificity is regulated by more than just an "open" structure, but the nature of these additional regulatory interactions is unknown. Small peptides (3–5 amino acids) are very poor substrates for the calpains with Kₗ values in the millimolar range (390), again indicating that the subsite recognition by the calpains uses a fairly large area of the polypeptide substrate.

The specificities of μ- and m-calpain are very similar if not identical (75; Thompson and Goll, unpublished data). Both μ- and m-calpain seem to cleave the same peptide bonds in α-tropomyosin, although they may cleave them at slightly different rates (Thompson and Goll, unpublished data).

Rates of calpain digestion of some proteins are affected significantly by phosphorylation of the protein substrate, and in some instances, the sites that are cleaved seem to be affected. PKA phosphorylation of troponin I (TN I) reduced its susceptibility to degradation by μ-calpain, whereas those amino substitutions that decreased rate of calpain cleavage stabilized the expressed polypeptide; the results suggested that rate of calpain cleavage is directly linked to polypeptide conformation. Nine of the 11 calpain cleavages in the α-tropomyosin polypeptide are in the COOH-terminal half of the molecule (Table 9).

### Table 9: Amino acid residues on either side of the peptide bonds cleaved by m-calpain

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cleavage</th>
<th>P₁</th>
<th>P₂</th>
<th>P₃</th>
<th>P₄</th>
<th>P₁</th>
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<tbody>
<tr>
<td>Rabbit skeletal α-tropomyosin*</td>
<td>Cleavage 1</td>
<td>Gln-Met-Leu-Lys (12)</td>
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<td>Cleavage 2</td>
<td>Gln-Ala-Glu-Ala (27)</td>
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<td></td>
<td>Cleavage 3</td>
<td>Gln-Glu-Arg-Ala (183)</td>
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<td>Cleavage 4</td>
<td>Thr-Asn-Asn-Leu (204)</td>
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<td>Cleavage 5</td>
<td>Asn-Asn-Leu-Lys (205)</td>
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<td>Cleavage 6</td>
<td>Lys-Ser-Glu-Glu (208)</td>
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<td>Cleavage 7</td>
<td>Ala-Glu-Ala-Glu (212)</td>
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<td>Cleavage 8</td>
<td>Gln-Lys-Glu-Asp (219)</td>
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<td>Cleavage 9</td>
<td>Lys-Tyr-Glu-Glu (223)</td>
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<td>Cleavage 10</td>
<td>Arg-Ala-Glu-Phe (241)</td>
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<td>Cleavage 11</td>
<td>Ile-Asp-Arg-Leu (256)</td>
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<td>EGF receptor kinase†</td>
<td>Cleavage 1</td>
<td>Arg-Leu-Leu (659)</td>
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<td>Cleavage 2</td>
<td>Thr-Ile-Pro (709)</td>
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<td>Cleavage 3</td>
<td>Ser-Thr-Ser (1006)</td>
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<td>Cleavage 4</td>
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<td>Cleavage 5</td>
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<td>Cleavage 6</td>
<td>Ser-Thr-Phe (1127)</td>
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<td>Cleavage 7</td>
<td>Pro-Ala-Gly (1161)</td>
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<td>Clupeine Y11‡</td>
<td>Cleavage 1</td>
<td>Pro-Val-Arg-Arg (14)</td>
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<td></td>
<td>Cleavage 2</td>
<td>Arg-Val-Ser-Arg (22)</td>
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<td></td>
<td>Cleavage 1</td>
<td>Pro-Val-Arg-Arg (15)</td>
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<td></td>
<td>Cleavage 2</td>
<td>Arg-Val-Ser-Arg (23)</td>
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<td></td>
<td>Cleavage 3</td>
<td>Ser-Thr-Phe (98)</td>
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<td>p55§</td>
<td>Cleavage 1</td>
<td>Ile-Asp-Asp-Leu (256)</td>
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* V. F. Thompson and D. E. Goll, unpublished results. Digestion at 25°C was allowed to go to completion, and the fragments were separated and analyzed. † From Gregoriou et al. (144). ‡ From Hayashi et al. (160). § From Lee et al. (237).
pain by approximately fivefold, whereas PKC phosphorylation increased susceptibility of TN I to \( \mu \)-calpain degradation by approximately twofold (95). In situ phosphorylation of platelet filamin (actin-binding protein) by PKA decreased its susceptibility to degradation by \( \mu \)-calpain by nearly twofold (53). The effect was specific for \( \mu \)-calpain because phosphorylation in vitro by PKA did not affect filamin degradation by trypsin, papain, or thermolysin (500). Cleavage of phosphorylated and dephosphorylated filamin occurred at similar sites (500). PKA phosphorylation of the microtubule-associated protein tau decreased its susceptibility to m-calpain and also altered the pattern of peptide fragments produced during digestion (248). Binding of calmodulin may also affect rate of cleavage by the calpains (191, 450).

That binding of calmodulin or phosphorylation can alter the rate and in some instances the sites that calpains cleave in proteins substantiates the conclusion that subsite specificity of the calpains is regulated by polypeptide conformation and raises the possibility that degradation of physiological substrates by the calpains may be regulated not only by modifying the calpains but also by altering the substrates. It has been suggested that PEST sequences favor calpain cleavage, but subsequent studies have indicated that PEST sequences do not influence susceptibility to the calpains (300).

4. Phosphorylation of the calpains

Although an early study found that the calpains were not phosphorylated in vivo (2), reexamination of calpain phosphorylation by using antibodies specific for phospho-Ser, phospho-Thr, and phospho-Tyr and MALD-TOF mass spectrometry found that both \( \mu \)- and m-calpain are phosphorylated at multiple sites in situ (60). Calpains prepared from a variety of tissues including bovine skeletal muscle, bovine cardiac muscle, human placenta, and human platelets were used. The calpains were isolated from these tissues with no attempt to inhibit intracellular phosphatases, and the results, therefore, are representative of the calpains that have been used in in vitro studies during the past 35 years. Both types of assay indicated that \( \mu \)-calpain is phosphorylated at nine sites and that m-calpain is phosphorylated at eight sites (Fig. 7; Ref. 60). All sites that have been identified by MALDI are on the 80-kDa subunit; in several instances (4 of 19), the antiphospho-antibodies have detected a phospho-Ser in domain V of the 28-kDa subunit. This phospho-Ser would be either S65 or S68. Phosphorylated residues have never been detected in domains IV or VI. There are two phospho-Tyr, three phospho-Thr, and four phospho-Ser in \( \mu \)-calpain and one less phospho-Ser in m-calpain (Fig. 7). The phosphorylated residues are clustered in two areas of the calpain molecule: 1) at the domain I/II junction (IIa) where there are four phosphorylated residues in residues 77–81 (\( \mu \)-calpain) or residues 66–70 (m-calpain) and 2) at the NH\(_2\) terminus of domain II (IIb) where there are four (\( \mu \)-calpain) or three (m-calpain) phosphorylated residues (Fig. 7). The phosphorylated residues are in equivalent positions in the \( \mu \)- and m-calpain polypeptides, except for S366 and T362 on \( \mu \)-calpain and T316 on m-calpain (Fig. 7).

![Diagram of calpains phosphorylation sites](https://physiol.org/images/calpain_diagram.png)
The sequences surrounding the phosphorylated residues suggest that there are two PKA sites, two (μ-calpain) or one (m-calpain) PKC sites, two calmodulin kinase II sites, a casein kinase I site, and a protein kinase G site.

Not all sites are phosphorylated in all molecules; the sites at residues 379/380 (μ-calpain) or 369/370 (m-calpain) are phosphorylated in most calpains, but the other sites are phosphorylated less frequently. Direct assays indicated that, on average, the calpains contained 2–4 phosphates/molecule. The phosphates cannot be removed by various phosphatases unless the calpain is in a solution containing 500 μM Ca2+ or more. Removal of all the phospho-Ser and phospho-Thr residues with calcineurin results in a calpain that is nearly inactive proteolytically (<15% of its original activity).

At this point it is difficult to determine what the physiological significance of calpain phosphorylation might be. It evidently has some effect on catalysis, and based on the demonstrated importance of phosphorylation in regulating activity of countless other enzymes, some combination of phosphorylation might be the long-sought activator. On the other hand, phosphorylation may have a primarily structural role, and the phospho-Tyr residues may target the calpains to some signal transduction function.

5. Calpastatin

The calpain/calpastatin interaction has been well-characterized (see sect. II3), and phosphorylation of calpastatin has been discussed in section II1. It is clear that calpastatin has an important role in regulating activity of μ- and m-calpain, but how calpastatin regulates calpain activity in living cells is not well understood. Also, the role of calpastatin in regulation of the “other calpains” that have been identified as DNA sequences is unknown. Calpastatin does not inhibit the weak proteolytic activity of expressed domain IIa/Iib and only weakly inhibits the activity of LP82. Both the expressed domain IIa/Iib and LP82 do not have a small subunit, and hence, one of the three sites that calpastatin uses to bind to the μ- or m-calpain molecule is absent. These results suggest that calpastatin requires all three binding sites to be an effective inhibitor and that calpastatin may not inhibit single-chain calpains. Any regulation of calpain activity by calpastatin is likely limited to the vertebrate calpains because calpastatin has not been identified in invertebrate cells (the invertebrate calpains may be all single-chain calpains anyway). There have been reports that the calpains can autolyze in the presence of levels of calpastatin that completely inhibit the proteolytic activity of the calpains. It is possible to envision combinations of calpastatin phosphorylation/calpain phosphorylation that completely ablate the ability of calpastatin to bind to and inhibit the calpains at Ca2+ concentrations sufficient for maximal proteolytic activity of the calpains. Because there is so little information on regulation of the calpain/calpastatin interaction, a number of different possible mechanisms can be proposed without knowing if any of them is close to the truth. Regulation of the calpain/calpastatin interaction will likely be an area that will receive considerable attention in the coming years.

6. Mechanisms involved in catalysis

The 28-kDa small subunit of the μ- and m-calpain molecules has been referred to as the “regulatory subunit,” as having chaperone activity needed for proper folding of the 80-kDa subunit (495), or as being essential for proteolytic activity of μ- or m-calpain, without any clear indication of what its function(s) actually is. It was first proposed in 1984 that activation of rat liver μ- or m-calpain occurred in the presence of substrate and Ca2+ and that it correlated with dissociation of the two calpain subunits (291, 355). Eleven years later, Yoshizawa et al. (496) reported that the subunits of m-calpain dissociated reversibly in the presence of 1 mM Ca2+ and that the 80-kDa monomer had full catalytic activity and a reduced Ca2+ requirement (495, 496). Other laboratories, however, found that the two subunits of calpain communoprecipitated under conditions where they were proteolytically active (499); that an inactive mutant m-calpain, C-105 → S-105 did not dissociate during anion-exchange chromatography in the presence of 3 mM Ca2+ (106) nor during affinity chromatography in the presence of 5 mM Ca2+ (97); and that expressed m-calpain was catalytically inactive when the COOH-terminal 25 amino acids were removed from the small subunit, thereby preventing association of the two subunits, whereas a fully active enzyme was obtained when the subunits were expressed in a manner that allowed their association (106). Furthermore, mutation of the EF-1, EF-2, and EF-3′ sites in a domain VI increased the Ca2+ concentration required for proteolytic activity of the expressed m-calpain in the presence of Ca2+, which would be impossible if the small subunit did not remain associated with the large subunit during catalysis (98). Finally, fluorescence resonance energy transfer (FRET) fluorescence is not diminished during hydrolysis in vivo of a synthetic calpain substrate, Suc-Leu-Leu-Val-Tyr-AMC, in either COS 7 or LCLC 103H cells (127). Moreover, immunofluorescence and confocal microscopy showed that the two subunits of μ-calpain that had been transfected into the cells remained colocalized following activation of the calpain by a Ca2+ ionophore (127). Hence, it seems very likely that the subunits of the calpains remain associated during proteolysis in cells.

Although the two subunits of the calpains evidently remain associated during proteolytic activity in cells, a number of studies have indicated that the two calpain
subunits may indeed dissociate under relatively mild conditions in vitro (243, 245, 313, 338). The physiological significance of this dissociation remains unclear. Size-exclusion chromatography showed that the expressed domains IV from either \( \mu \)- or m-calpain and domain VI associated as homodimers in 20 mM Tris \( \cdot \) HCl, pH 7.5, and 1 mM dithiothreitol (431). The \( \mu \)-domain IV homodimers dissociated to monomers in the presence of 500 \( \mu \)M Ca\(^{2+} \), whereas 5 mM Ca\(^{2+} \) was required to even partly dissociate the m-domain IV and domain VI homodimers (431). Because these studies involved homodimers and not the domain IV/domain VI association that exists in \( \mu \)- and m-calpain, it is unclear whether they relate to dissociation of the calpain molecules. Also, it should be noted that all Ca\(^{2+} \) concentrations used in these studies were far higher than the 50–300 nM that exists in vivo.

Other studies have used complete 80/28-kDa or 80/21-kDa m-calpain molecules and catalytically inactive mutants (C-105 \( \rightarrow \) S-105) of these molecules expressed in *E. coli* (338) or in a SF9 baculovirus system (313) to study dissociation of the two calpain subunits. Subunit dissociation was determined by measuring exchange of a truncated 28-kDa subunit (beginning at residue 86) in 5 mM Ca\(^{2+} \) (338) or by separation of the small and large subunits during chromatography of a His-tagged 28-kDa subunit/large subunit heterodimer in 1 mM Ca\(^{2+} \) on a Ni\(^{2+} \)-immobilized ion affinity column (313). Use of the catalytically inactive mutants prevented autolysis in the presence of Ca\(^{2+} \). Dissociation of the 80- and 21-kDa subunits occurs in the presence of 5 mM Ca\(^{2+} \), but the extent of this dissociation, as measured by exchange of the truncated 21-kDa subunits, is incomplete, with only \( \sim 30\% \) of the subunits exchanging (338). Sedimentation equilibrium and Rayleigh light scattering showed that the expressed m-calpain aggregated in the presence of 200–2,000 \( \mu \)M Ca\(^{2+} \), which significantly complicates interpretation of studies using 1,000–5,000 \( \mu \)M Ca\(^{2+} \) to dissociate the calpain subunits (338). Pal et al. (338) suggested that interaction of the NH\(_2\)-terminal 19 amino acids on the large subunit with domain VI in the small calpain subunit stabilized the calpain molecule and limited the extent of subunit dissociation. This suggestion was supported by the results of Nakagawa et al. (313) who found that the subunits of m-calpain dissociated in the presence of Ca\(^{2+} \) only if the interaction of the first 19 amino acids in domain I with domain VI was disrupted. The crystallographic structure of m-calpain in the absence of Ca\(^{2+} \) shows that K\(_7\) of the 80-kDa subunit forms an electrostatic interaction with D\(_{154}\) in the EF-2’ sequence of the 28-kDa subunit and that R\(_{12}\) of the large subunit forms an electrostatic interaction with E\(_{200}\) in the EF-5’ sequence of the 28-kDa subunit. Disruption of either one of these interactions alone does not result in dissociation in the presence of 1 mM Ca\(^{2+} \), but disruption of both results in \( \sim 50\% \) dissociation of the two subunits in 1 mM Ca\(^{2+} \). Autolysis removes the NH\(_2\)-terminal 19 amino acids of the 80-kDa subunit of m-calpain, and Nakagawa et al. (313) found that \( \sim 50\% \) of the autolyzed form of m-calpain also dissociated in the presence of 1 mM Ca\(^{2+} \). It was proposed that Ca\(^{2+} \) binding to EF-2’ disrupted the salt bridge between K\(_7\) and D\(_{154}\) in the EF-2’ sequence, that this disruption allowed domains Ia and Ib to move together to form a catalytically active molecule, and therefore that Ca\(^{2+} \) binding to D\(_{154}\) in EF-2’ was a “calpain activation” step (313). It is not clear, however, how such an activation process can be reconciled with the Ca\(^{2+} \) binding studies showing that mutation of EF-1’, EF-2’, and EF-4’ in the small subunit to prevent Ca\(^{2+} \) binding to these sites produced a mutant m-calpain that was proteolytically active and that required 542 \( \mu \)M Ca\(^{2+} \) for half-maximal proteolytic activity, only 1.5-fold more than the wild-type m-calpain (98).

Both Pal et al. (338) and Nakagawa et al. (313) reported that the dissociated calpains were “unstable” and lost proteolytic activity rapidly. Several earlier reports (124, 125) had indicated that autolyzed \( \mu \)-calpain lost its proteolytic activity rapidly at ionic strengths above 100 mM but in the absence of the high Ca\(^{2+} \) concentrations used in the subsequent studies (313, 338). A more detailed examination has shown that the subunits of both autolyzed \( \mu \)- and autolyzed m-calpain but not the unautolyzed forms of these molecules dissociate rapidly at ionic strengths above 100 mM in the absence of Ca\(^{2+} \) and that the dissociated 80-kDa subunits aggregate to form a larger species, possibly a mixture of trimers and tetramers (243, 245). The aggregated forms of these 80-kDa subunits have no proteolytic activity (243, 245). The salt-induced aggregation is not reversible by dialysis, occurs within 2–5 min after exposure of the autolyzed calpain to salt, and the aggregates seem stable after formation (i.e., larger aggregates do not form with increasing time). The extent and rate of dissociation/subsequent aggregation as estimated by loss of proteolytic activity depends on ionic strength; after 45 min in 100 mM KCl, \( \sim 40–50\% \) of the proteolytic activity of either autolyzed \( \mu \)- or autolyzed m-calpain is lost; after 45 min in 300 mM KCl, \( \sim 55–65\% \) of the activity is lost, and after 45 min in 500 mM KCl, \( \sim 75–80\% \) of the proteolytic activity is lost (243, 245). It seems likely that the loss of proteolytic activity observed by Pal et al. (338) and Nakagawa et al. (313) also resulted from aggregation of the dissociated 80-kDa subunit that was induced by high concentrations of Ca\(^{2+} \) in the latter studies (313, 338). Evidently, removal of the NH\(_2\)-terminal 27 (m-calpain) or 18 (m-calpain) amino acids from the 80-kDa subunit and possibly also the NH\(_2\)-terminal 91 amino acids from the 28-kDa subunit has a substantial effect on stability of the calpain molecules, such that salt-induced disruption of ionic bonds present after autolytic removal of the NH\(_2\)-terminal amino acids from domains I and V results in subunit dissociation and loss of proteolytic activity.
activity. Thus the $K_I$ (80 subunit)-D$_{154}$ (28 subunit) and $R_{12}$ (80 subunit)-E$_{260}$ (28 subunit) interactions are not involved in this dissociation. Because interaction of domains IV and VI in the 80- and 28-kDa subunits, respectively, involves hydrophobic forces (see sect. 1A3 and Table 2), it seems likely that dissociation of the two subunits exposes a hydrophobic surface in domains IV and VI and that the dissociated subunits then self-aggregate. Ionic strengths of 100 mM are similar to (slightly less than) those in living cells, but the 1–5 mM Ca$^{2+}$ concentrations (313, 338) are several orders of magnitude higher than those that exist in living cells. Hence, the effect/role of autolysis in functioning of the calpains continues to mystify (if autolysis results in dissociation, subunit aggregation, and loss of proteolytic activity, what do assays of autolysis in functioning of the calpains mean?; C 2+-dependent proteolytic activity, what do assays of autolytic calpains measure?)?), although it is becoming clear that autolysis results in a considerably less stable molecule.

Several recent studies have shown that a polypeptide corresponding to domain IIa/IIb has very weak, Ca$^{2+}$-dependent proteolytic activity (158, 298). Degradation of casein by an expressed human m-calpain domain IIa/IIb (amino acids 19–342; see Fig. 1) was Ca$^{2+}$-dependent but was incomplete even after 14 h at 30°C in 5 mM Ca$^{2+}$ (158). Activity of the expressed domain IIa/IIb was estimated to be less than 1/100 that of the complete m-calpain molecule, and the Ca$^{2+}$ concentration required for half-maximal activity was estimated to be 900 μM. The activity was inhibited by cysteine protease inhibitors such as E-64c but only weakly by calpastatin. Nishimura and Goll (320) reported that the 34–35-kDa (μ-μ-calpain) polypeptides obtained by extensive autolysis of μ- or m-calpain and that contained amino acids 28–330 (μ-calpain) or amino acids 20–330 (m-calpain) had no proteolytic activity in either the absence or the presence of Ca$^{2+}$, although they did not run the assays for sufficiently long periods of time to detect the small activity recorded by Hata et al. (158). An expressed rat μ-calpain domain IIa/IIb (amino acids 29–356) degraded a catalytically inactive mutant m-calpain and a synthetic substrate in a Ca$^{2+}$-dependent manner with a Ca$^{2+}$ concentration of 42 μM required for half-maximal activity (298). Activity of the μ-calpain domain IIa/IIb also was low: ~1/40 that of intact m-calpain. The expressed domain IIa/IIb was not inhibited by calpastatin domain I but was inhibited by leupeptin, E-64, and calpain inhibitor I (Table 10). Crystallographic analysis showed that the expressed μ-domain IIa/IIb bound two Ca$^{2+}$, one in domain IIa and the other in domain IIb (298). The two Ca$^{2+}$ were not bound in EF-hand structures, but in two peptide loops, one providing eight coordinations to the bound Ca$^{2+}$ and the other providing seven coordinations (domain IIb). Ca$^{2+}$ binding

<table>
<thead>
<tr>
<th>Type and Name</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Calpastatin</td>
<td>Specific for the calpains; reversible; tight binding with IC$_{50}$ in pM range, requires Ca$^{2+}$ to bind to the calpains</td>
</tr>
<tr>
<td>Kininogen</td>
<td>Calpains are inhibited by both L- and H-kininogen (but only the central domain of H-kininogen); kininogens are extracellular, but calpains are intracellular; $K_I = 1.0$ nM</td>
</tr>
<tr>
<td>α-Macroglobulin</td>
<td>Partially inhibits the calpains; rate constant of 30,000 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td><strong>Reversible inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Ac-Leu-Leu-arginal; $K_I = 3.2$ (μ-calpain) to 4.3 (m-calpain) $\times 10^{-7}$ M; inhibits a number of cysteine and some serine proteases</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>[(S)-1-carboxy-2-phenylethyl]-carbamoyl-α-[2-iminohexahydro-4(S)-pyrimidyl]-((S)-glycyl-X-phenylalaninal with X as L-Leu, L-Val, or L-Ile; inhibits the calpains weakly; chymotrypsin, cathepsins A and B more strongly</td>
</tr>
<tr>
<td>Antipain</td>
<td>[(S)-1-carboxy-2-phenylethyl]-carbamoyl-L-Arg-L-Val-arginal; for calpains, $K_I = 1.4 \times 10^{-6}$ M; inhibits cathepsins A, B, papain, and trypsin</td>
</tr>
<tr>
<td>Calpain inhibitor I</td>
<td>N-Ac-Leu-Leu-norleucinal; $K_I = 0.12-0.23 \times 10^{-6}$ M for μ-calpain; $K_I \sim 2$-fold higher for m-calpain; inhibits cathepsins B ($K_I = 0.5$ nM) and L, papain and the proteosome; membrane permeable</td>
</tr>
<tr>
<td>Calpain inhibitor II</td>
<td>N-Ac-Leu-Leu-methioninal; $K_I = 0.12-0.23 \times 10^{-6}$ M for m-calpain; $K_I \sim 2$-fold higher for μ-calpain; inhibits cathepsins B and L, weak inhibition of the proteosome; membrane permeable</td>
</tr>
<tr>
<td>Calpeptin</td>
<td>Carbobenzyloxy-Leu-norleucinal; IC$_{50} = 0.0029$ μM for μ-calpain; 0.0028 μM for m-calpain; membrane permeable</td>
</tr>
<tr>
<td><strong>Irreversible inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>General inhibitor of all cysteine proteases</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>General inhibitor of all cysteine proteases</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>General inhibitor of all cysteine proteases</td>
</tr>
<tr>
<td>E-64</td>
<td>L-3-Carboxy-trns,2,3-epoxypropionyl-L-leucylamido-(4-guanidino)butane; one of a class of epoxy inhibitors of cysteine proteases; poor membrane permeability; second-order rate constant for inhibition 7,500 M$^{-1}$ s$^{-1}$; E-64d a derivative of E-64c (with 3-methylbutane in place of the 4-guanidino group) is a membrane permeable but less effective calpain inhibitor</td>
</tr>
<tr>
<td>Diazomethylketones</td>
<td>Carbobenzyloxy-Leu-Leu-Tyr diazomethyl ketone, second-order rate constant of inhibition 230,000 M$^{-1}$ s$^{-1}$; poor inhibitory of the proteosome; potent inhibition of cathepsin L as are all compounds of this class</td>
</tr>
</tbody>
</table>

There are over 50 reported inhibitors of the calpains, and this list includes only those most widely used. See Wang and Yuen (472) and Wells and Bihovsky (477) for more complete lists.
induced conformational changes that brought the catalytic residues to within 3.7 Å from each other compared with the 10.5 Å separating them in the Ca$^{2+}$-free structures of the intact m-calpain molecule (171, 430).

The ability to express a catalytically active calpain was used in an attempt to learn what parts of the calpain molecule were responsible for differences in the Ca$^{2+}$ requirements of μ- and m-calpain (99). Mutating the residues in each EF-hand individually to exchange residues present in μ-calpain for the corresponding ones in m-calpain had little effect on the Ca$^{2+}$ concentration required for half-maximal proteolytic activity of the expressed m-calpain. Substituting residues 2–50 from the μ-calpain 80-kDa subunit for residues 2–49 from m-calpain but leaving the remainder of the 80-kDa subunit unchanged in a recombinant m-calpain reduced the Ca$^{2+}$ concentration required for half-maximal activity for the expressed calpain by 23%, from 325 to 250 μM even though this part of the calpain molecule ostensibly does not bind Ca$^{2+}$. Substituting residues 2–498 from μ-calpain for residues 2–487 from m-calpain (residue 487 is almost to the COOH-terminal end of domain III of m-calpain; Fig. 1) had little additional effect on the Ca$^{2+}$ concentration required for half-maximal activity, reducing it from 250 to 230 μM. This substitution includes the structure in domain III of the calpains that is marginally homologous to a C2 domain and that has been suggested to be involved in binding phospholipid and reduction of the Ca$^{2+}$ concentration required for autolysis. Substituting residues 2–581 from μ-calpain for residues 2–569 from m-calpain (this extends the substitution past the linker and EF-1 hand parts in the crystallographic structure of m-calpain; Fig. 1) had a marked effect on the Ca$^{2+}$ concentration required for half-maximal activity, reducing it from 230 to 110 μM even though mutating the EF-1 hand alone actually increased the Ca$^{2+}$ concentration required for half-maximal activity from 325 to 398 μM. Additional substitutions involving residues 2–649 from μ-calpain for residues 2–637 from m-calpain (this substitution includes EF-2 and EF-3 but not EF-4 and EF-5 of the 80-kDa subunit of m-calpain) reduced the Ca$^{2+}$ concentration required for half-maximal activity from 110 to 80 μM (wild-type μ-calpain required 25 μM Ca$^{2+}$ for half-maximal activity). The results of this study indicate that the Ca$^{2+}$ concentration required for proteolytic activity of the calpains is regulated by the entire calpain molecule and not by a specific set of Ca$^{2+}$-binding sites.

Consequently, the mechanisms regulating calpain activity in cells remain unknown despite the rapid accumulation of information on properties of the calpains and the recent demonstration of weak Ca$^{2+}$-dependent proteolytic activity of expressed domains IIA/IIB. The Ca$^{2+}$ concentrations required by the expressed domains IIA/IIB are similar to those required by the intact molecules and are therefore much higher than those that exist in cells. It seems clear that reducing the Ca$^{2+}$ concentration required for calpain activity and perhaps also modifying the Ca$^{2+}$ concentration needed for its inhibition by calpastatin are crucial regulators of calpain activity. It is unclear how many Ca$^{2+}$ atoms are bound by the calpains, and until some way is found to circumvent the autolysis and aggregation/precipitation that occurs in the presence of Ca$^{2+}$, it will be difficult to measure Ca$^{2+}$ binding carefully. Two recent studies have advanced the understanding of calpain activity. The FRET studies indicate that μ- and m-calpains function as heterodimers during proteolytic activity in cells, and the substitutions of μ-calpain domains for m-calpain domains suggest that regulation and Ca$^{2+}$ activation are likely to involve interactions that include the entire calpain molecule rather than only one or two domains (365). If so, this adds to the complexity of the regulation and the difficulty in eventually understanding it.

V. PHYSIOLOGICAL FUNCTIONS OF THE CALPAIN SYSTEM

The physiological functions of the calpain system remain unclear despite the efforts of many laboratories. The calpain system almost certainly has a number of different roles in cells, including but not limited to “remodeling” of cytoskeletal attachments to the plasma membrane during cell fusion and cell motility, proteolytic modification of molecules in signal transduction pathways, degradation of enzymes controlling progression through the cell cycle, regulation of gene expression, substrate degradation in some apoptotic pathways, and an involvement in long-term potentiation. The discovery of members of the calpain family that lack some residues necessary for catalysis suggests that the calpains may in some instances have functions that do not require proteolytic activity; the nature of these functions is completely unknown. That the calpains cleave polypeptides at a limited number of sites leaving large, often catalytically active fragments indicates that the calpains have a regulatory or signaling function in cells rather than a digestive function such as the lysosomal proteases or the proteosome. It is unclear why different cells have such widely differing ratios of μ-calpain to m-calpain. It is reasonable to suggest that the two calpains can perform identical physiological functions but respond to different cellular signals: 1) they have very similar if not identical subsite specificities, and 2) human platelets contain only μ-calpain (at least within the ability to detect any m-calpain) but bovine platelets, on the other hand, contain only m-calpain (452); it seems likely that the calpains perform identical functions in the platelets from these two species.

As indicated in the introduction to section IV, many of the studies attempting to determine the physiological...
functions of the calpain system have used protease inhibitors with the aim of identifying which properties of cells are affected and then using this information to infer how the calpains are involved. There is, however, only one specific inhibitor of the calpains, calpastatin, and the other inhibitors that have been used have effects in addition to inhibition of the calpains. In some instances, these other effects are known (for example, calpain inhibitor I is also a good inhibitor of cathepsins B and L and the proteasome), but in other instances, they are not. Hence, the results of experiments that rely totally on the effects of a "calpain inhibitor" must be interpreted cautiously. Table 10 lists some of the compounds that inhibit proteolytic activity of the calpains. Many of the reversible inhibitors of the calpains are peptidyl aldehydes. Two recent reviews summarize the properties and structures of 59 calpain inhibitors (477) and the properties and therapeutic values of some of these inhibitors (472); these reviews should be consulted for a more complete list and information on the properties of these inhibitors.

The two best approaches currently available for identifying the actions of the calpains in cellular functions are introduction of calpastatin or a fragment of calpastatin into cells by transfection or some other procedure, or the use of selective antibodies such as the one that specifically labels the calpain-cleaved fragment of spectrin (379). Hopefully, additional antibodies that specifically label the calpain-cleaved fragments from other putative calpain substrates, such as talin, PKC, ppFAK125, and some of the transcription factors that are cleaved by the calpains in vitro, will become available in the future. The availability of such antibodies will significantly enhance the ability to define the roles of the calpains in cells. Until then, studies will need to rely on a combination of approaches involving different inhibitors having different known selectivities, sensitivity to changes in Ca2+ concentration, cleavage of polypeptides that are known calpain substrates in vitro, and autolysis of the calpains as an indication that the calpains have been active. As will be discussed in the following sections, it seems that not all calpain-sensitive substrates are cleaved in a given cellular event; in some instances, spectrin is cleaved and talin is spared, and in other instances, talin is cleaved and another calpain-sensitive polypeptide is spared. Degradation of talin and filamin in platelets from Capn4-/- mice was normal, although these platelets lacked μ-calpain. Consequently, clearly defining the role of the calpains in cellular functions will likely continue to be difficult without the availability of more specific assays.

A. What Do The Transgenic Mice Show?

The short answer to the question posed is: 1) knocking out both μ- and m-calpain by interfering with expression of the small subunit is embryonically lethal (10, 502), 2) knocking out only μ-calpain results in platelet dysfunction but otherwise the mice survive (14), and 3) disrupting the gene for calpain 3a has minimal effect on the mice (367, 442). The two studies involving disruption of expression of the small subunit had identical results: 1) Capn4-/- embryos died by 11.5 days of gestation and had no detectable calpain activity (10), but Capn4+/+ mice were viable, fertile, had normal calpain activity and were phenotypically normal (10, 502); 2) the Capn4-/- embryos appeared normal up to day 10.5 but began to show cardiovascular defects at that stage (10); 3) RNA levels for the 80-kDa subunits of μ- and m-calpain were normal in Capn4-/- embryonic stem (ES) cells, but the 80-kDa μ- and m-calpain polypeptides are barely detectable in these cells; and 4) growth rates of Capn4-/- ES cells were normal and fibroblasts from the Capn4-/- embryos had no calpain activity but grew and proliferated normally (10). The results raise a number of questions. That lack of a 28-kDa small subunit is embryonically lethal implies that μ- and m-calpain function as heterodimers in cells, at least to a considerable extent (see sect. IV), or that the 28-kDa subunit has some presently unknown function that is vital to cell survival. That the embryos survived to day 11.5 and that the Capn4-/- fibroblasts divide mitotically but have no detectable calpain activity is counter to a number of studies indicating that μ- and/or m-calpain have an essential role in the mitotic cycle and cell growth. Can one or more of the “other calpains” described in section iiB compensate for the absence of μ- and m-calpain activity, at least for the first 10 days of development and in fibroblast function? If so, this implies that these calpains can function without the 28-kDa subunit, a conclusion consistent with the predicted amino acid sequences of the atypical calpains. Or, can the results be explained by the presence of small residual amounts of μ- and/or m-calpain activity or to the 80-kDa subunits having a small amount of residual activity acting alone? The Zimmerman et al. (502) knockout experiment eliminated exons 4–8 (EF-1’, EF-2’, EF-3’; amino acids 82–202) of the 28-kDa subunit and resulted in lethality before day 9, whereas the Arthur et al. (10) knockout experiment disrupted the COOH-terminal 25 amino acids of the small subunit and was lethal at day 10–11. It is unclear whether the differences in survival are due to a “more complete” disruption of the Capn4 transcript in the Zimmerman study (502).

The Capn1-/- mice are viable and fertile, but they have a significant reduction in platelet aggregation and clot retraction; surprisingly, bleeding times were normal (14). Although it has been widely accepted that one of the physiological roles of the calpains is degradation of filamin, talin, and spectrin during platelet activation, the cleavage patterns and kinetics of degradation of both talin...
and filamin were normal in platelets from the \textit{Capn1}^+/− mice. Levels of tyrosine phosphorylation were decreased in \textit{Capn1}^+/− platelets. Western and Northern analysis confirmed that \(\mu\)-calpain expression was ablated in \textit{Capn1}^+/− mice. Again, the results raise several questions. Evidently, the presence of \(m\)-calpain, which was normal in the \textit{Capn1}^+/− mice, compensates for the absence of \(\mu\)-calpain in these animals. This finding is consistent with but does not prove the hypothesis that \(\mu\)- and \(m\)-calpain can cleave the same substrates in cells and are capable of performing the same physiological functions. Because platelets contain only (or in mice, largely) \(\mu\)-calpain, is the \(m\)-calpain present in these platelets sufficient to produce normal bleeding times and cleavage of talin and filamin in the absence of \(\mu\)-calpain? Or, does the seemingly normal pattern of filamin and talin cleavage in the \textit{Capn1}^+/− platelets suggest that one of the “accepted” roles of the calpains requires reexamination?

Recent studies have examined the effects of disruption of the 80-kDa subunit of \(m\)-calpain on mouse physiology. Although still preliminary, there is strong evidence that disruption of the \textit{Capn2} gene in mice is embryonically lethal at a very early stage. If this is correct, it would suggest, when considering the lethality of the \textit{Capn4}^+/− mice (10, 502) and the very mild effects in the \textit{Capn1}^+/− mice, that \(m\)-calpain is absolutely essential for embryonic development (P. Dutt, J. S. Elce, and P. A. Greer, personal communication) and that \(m\)-calpain performs some function during development that \(\mu\)-calpain does not or cannot. The lack of effect of \textit{Capn1}^+/− on embryonic development may be due to late expression of \(\mu\)-calpain during normal development so that it is not involved in calpain cleavages required for development or to the ability of \(m\)-calpain to perform certain cleavages that \(\mu\)-calpain cannot.

Two types of calpain 3a transgenic mice have been studied: one in which expression of the \textit{Capn3} gene has been disrupted (367) and one expressing an inactive mutant of calpain 3a (the active site Cys mutated to Ser) (442). The phenotypes of both mice were nearly normal; the \textit{Capn3}^−/− mice had a postnatal appearance similar to their wild-type littermates, exhibited normal behavior, were fertile, and were kept alive for 1.5 years with no increase in death rate (367). The \textit{Capn3}^−/− mice had some loss of sarcolemmal integrity as indicated by increased membrane permeability. Expression of the inactive/mutant calpain 3a was low in the transgenic Cys-129/Ser-129 mice, but examination of old (2 years) mice indicated that they had slightly lower “grip strengths” and greater levels of centrally placed nuclei, more tubular aggregate-like structures, and more lobulated fibers than their wild-type littermates (442); these differences were evident only at advanced ages. As in the \textit{Capn3}^−/− studies, the Cys-129/Ser-129 mice appeared normal and were actually heavier than the wild-type littermates at 20 and 32 wk of age.

In sum, the transgenic mice studies have raised a number of questions concerning the physiological functions of the calpains; roles that various studies had suggested for the calpains such as mitosis and cleavage of some cytoskeletal proteins during platelet activation seem to proceed normally in the absence of \(\mu\)- and \(m\)-calpain. Although it has been demonstrated that disruption of the \textit{Capn3} gene is responsible for LGMD2A, disrupting expression of this gene in mice has minimal consequences. Clearly, a great deal remains to be learned about how the calpains function in different cells and the signals that trigger calpain activity.

**B. In Vitro Substrates**

A large number of proteins (over 100 have been reported) are cleaved by the calpains in in vitro assays (see Refs. 76, 138 for proteins in muscle/cytoskeleton; see Ref. 474 for others). Many but not all of these in vitro substrates can be placed in one of four general categories: 1) cytoskeletal proteins, especially those involved in cytoskeletal/plasma membrane interactions; 2) kinases and phosphatases; 3) membrane-associated proteins, including some receptors and ion-channel proteins; and 4) some transcription factors (Table 11 lists some of the substrates in the first two of these categories). It is important when evaluating reports of proteins cleaved by the calpains to ensure that the proteins were not denatured and to consider the rates of cleavage. For example, undenatured sarcomeric myosin heavy chain is degraded very slowly and undenatured sarcomeric actin is not degraded at all, although denatured actin is degraded rapidly. Similarly, undenatured \(\alpha\)-actinin is degraded very slowly and in a limited manner (133). In vitro degradation of many kinases or phosphatases by the calpains leaves large catalytically active fragments that lack the physiological controls of the undegraded molecule (Table 11). In addition to the proteins listed in Table 11, \(m\)-calpain cleavage of brain calmodulin-dependent, cyclic nucleotide phosphodiesterase leaves an enzyme that is active in the absence of calmodulin (197), and calpain cleavage of p53 ablates its ability to regulate gene expression (141, 216, 340). Many of the cytoskeletal proteins involved in linking the cytoskeleton to the plasma membrane are cleaved rapidly by the calpains; among these proteins are talin, vinculin, spectrin, filamin, band 4.1a, band 4.1b, ezrin, but not \(\alpha\)-actinin, which is cleaved slowly, or the ERM proteins, moesin and radixin, which are not cleaved (404). Similarly, most of the intermediate filament proteins such as desmin and vimentin are cleaved rapidly by the calpains. In most instances, calpain cleavage of the cytoskeletal proteins severs their cross-linking ability.
TABLE 11. Some known substrates of the calpains

<table>
<thead>
<tr>
<th>Polypeptide Class and Name</th>
<th>Effects of Calpain Cleavage</th>
</tr>
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<tbody>
<tr>
<td><strong>Cytoskeletal proteins</strong></td>
<td></td>
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<tr>
<td>Adducin 4.1a, 4.1b</td>
<td>Both the 103-kDa α-subunit and the 97-kDa β-subunit are degraded within 60 min to produce stable 57- and 49-kDa fragments, respectively; the 49-kDa β-subunit fragment does not bind calmodulin as its parent 97-kDa does (392).</td>
</tr>
<tr>
<td>Ankyrin IIE1, IIE2</td>
<td>The 97-kDa fragment is cleaved to the 57-kDa fragment, which is further degraded to 49- and 31-kDa fragments (392).</td>
</tr>
<tr>
<td>Desmin</td>
<td>Both the brain (212 kDa) and erythrocyte (239 kDa) isoforms are rapidly degraded to a 160-kDa polypeptide (150).</td>
</tr>
<tr>
<td>Calpain cleavage at the C-protein</td>
<td>The C-protein is bound to the myofilament (82).</td>
</tr>
<tr>
<td>Desmin</td>
<td>Very rapidly degraded to polypeptide fragments of 125, 115, 100, 105, and 88-90 kDa; the latter fragment is stable (75).</td>
</tr>
<tr>
<td>Cadherin E-cadherin</td>
<td>E-cadherin is not degraded by calpain (47), whereas the intracellular domain of N-cadherin is degraded by μ-calpain (391).</td>
</tr>
<tr>
<td>Calponin</td>
<td>Very rapidly degraded at approximately the same rate as caldesmon to 30-, 27-, and 19.5-kDa fragments; the latter fragment is stable (75).</td>
</tr>
<tr>
<td>Filamin/actin-binding protein MAP1, MAP2</td>
<td>MAP2 is more susceptible than MAP1 and is one of the most sensitive proteins known; rate of MAP2 degradation is decreased by phosphorylation of MAP2.</td>
</tr>
<tr>
<td>Myosin</td>
<td>Very slowly degraded of undenatured myosin; the 210-kDa large subunit is degraded to fragments of 150, 105, and 180 kDa; slow degradation of LC2 light chain (347).</td>
</tr>
<tr>
<td>Nebulin</td>
<td>Very rapidly degraded to a series of smaller polypeptides ranging from 30 to several hundred kDa; fragments produced by calpain may remain bound to actin (453); calpain degradation severs nebulin connection to Z-disk (75).</td>
</tr>
<tr>
<td>Neurofilament proteins</td>
<td>The three neurofilament proteins, NFH (−200 kDa), NFM (−150 kDa), and NFL (−68 kDa), are all cleaved by the calpains, in the order of NFM, NFL, and NFH (198). Phosphorylation increases rate and extent of NFH degradation, has little effect on rate of NFM degradation, and does not affect NFL degradation (143). NFH is degraded to ~190 kDa, NFM to ~68 kDa, 55 kDa, and 30 kDa, and NFL to ~57 kDa.</td>
</tr>
<tr>
<td>Protein 4.1a, 4.1b</td>
<td>Both isoforms are degraded very rapidly to 43-, 40-, 38-, and 31-kDa fragments; the 31-kDa fragment is stable and accumulates (75).</td>
</tr>
<tr>
<td>αI-Spectrin</td>
<td>Rigidly degraded to 145- and 150-kDa fragments; the 150-kDa fragment may be detected by a specific antibody; the 145- and 150-kDa fragments are then more slowly degraded to smaller fragments (401); the initial calibration-sensitive site is at Tyr^{176}Gly^{177} (428).</td>
</tr>
<tr>
<td>β-Spectrin</td>
<td>All three isoforms are cleaved at a single site in helix B of repeat 17 at Trp-Ala (βE1), Thr-Ala (βE2), or Ala-Ala (βE1) to produce fragments of 125- and 165-kDa; calpain cleavage seems to be modulated by binding of calmodulin (250).</td>
</tr>
<tr>
<td>Synemin</td>
<td>Very rapidly degraded to a large number of polypeptide products ranging from 40- to 220-kDa with major fragments of 40 (stable), 45, 50, 66, and 200 kDa (36).</td>
</tr>
<tr>
<td>Talin</td>
<td>Very rapidly cleaved between Q^{121} and Q^{141} (chicken talin) to produce a 190-kDa COOH-terminal actin binding fragment and a 474-kDa NH2-terminal fragment; after cleavage, can no longer cross-link integrin to cytoskeletal elements (162, 305).</td>
</tr>
<tr>
<td>Tau</td>
<td>Very rapidly cleaved to major, stable fragments of 35 and 45 kDa and a minor fragment of 25 kDa; rate of digestion is decreased by phosphorylation of tau (248).</td>
</tr>
<tr>
<td>Titin</td>
<td>The ~3000-kDa titin polypeptide is very rapidly cleaved to a large ~2000-kDa fragment by removal of a 1200-kDa NH2-terminal fragment; this cleavage severs the connection of titin to the Z-disk; the 1200-kDa fragment is degraded to smaller fragments of 100–500 kDa, with the 500-kDa fragment being stable (452); chicken titin is quickly degraded to a T2 (2000 kDa) fragment which is then degraded to a 1700- and a 400-kDa fragment; the 1700-kDa fragment is further degraded to a 1400-kDa fragment (432).</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>Rapidly cleaved to a 14-kDa fragment and several smaller polypeptides (see Table 9); rate of degradation is decreased greatly if tropomyosin is in the groove of the actin helix in myofilaments (82).</td>
</tr>
<tr>
<td>Troponin I</td>
<td>Degraded to smaller polypeptides (the 32-kDa cardiac troponin I is degraded to a 26-kDa fragment as measured by SDS-PAGE) at a moderate rate; not protected while in the myofilament structure (82).</td>
</tr>
<tr>
<td>Troponin T</td>
<td>Very rapidly degraded to smaller fragments of ~35, 30, and 28 kDa as measured by SDS-PAGE; the 30-kDa fragment is stable; a 15-kDa fragment is produced by longer digestion (168); not protected when in the myofilament structure (82).</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Very slowly degraded from a 55- to a 50- to 52-kDa fragment (37); or not degraded at all (388).</td>
</tr>
</tbody>
</table>
Doublecortin-like kinase

Calpain cleaves the 85-kDa kinase at two sites to produce a 35-kDa NH₂-terminal fragment that retains the Calcineurin

Calcium/calmodulin-dependent protein kinase

The 52- to 54-kDa CaM kinase II is degraded to a major 35- to 40-kDa fragment which contains the entire catalytic core and is active in the absence of calmodulin; degradation of the kinase occurs in situ in cultured neurons and in vitro (140).

Doublecortin-like kinase

Calpain cleaves the 85-kDa kinase at two sites to produce a 35-kDa NH₂-terminal fragment that retains the microtubule-binding properties of the parent kinase and a 50-kDa COOH-terminal fragment that retains the kinase activity. A similar cleavage occurs in vitro in embryonic mouse brain (46).

EGF receptor kinase

Degraded at seven sites, three of them in a "calpain-sensitive" hinge region between residues 996 and 1059 to produce a 150-kDa fragment from the 170-kDa EGF receptor kinase (144). The 150-kDa fragment retains EGF binding and EGF-stimulated kinase activity, but has reduced autophosphorylation activity.

Myosin light-chain kinase (MLCK)

In the presence of calmodulin, the 130-kDa MLCK is cleaved to a 62-kDa fragment that retains complete kinase activity and that binds calmodulin; in the absence of calmodulin, MLCK is cleaved to a 60-kDa fragment that has no kinase activity and that does not bind calmodulin (191).

ppFAK125

Rapidly degraded to 90 kDa and the 90 kDa then to 45- and 40-kDa fragments with a concomitant loss of autokinase activity; similar degradation occurs in platelets (67). Calpain degradation of ppFAK125 is associated with its loss from the platelet cytoskeleton.

pp60src

Cleaved to 52- and 47-Da fragments by removal of the NH₂-terminal region of the kinase, which is the region that binds the kinase to the membrane; pp60src calpain fragments are exclusively in the platelet cytoplasm; calpain cleavage occurs in situ in platelets and results in decreased tyrosine kinase activity (323); cleaved 50% as rapidly as ppFAK125 and protein tyrosine phosphatase 1B and less rapidly than talin or filament in platelets.

Protein kinase C (PKC)

The PKC-α, PKC-βI, PKC-βII, and PKC-γ isoforms are all rapidly cleaved from a 82-kDa polypeptide to a 45- to 49-kDa fragment that is fully active as a kinase but that does not require Ca²⁺ or phospholipids for activity and to a 36-kDa regulatory fragment (200); rate of cleavage is increased in the presence of phospholipid and diacylglycerol, suggesting that the active form of the kinase is preferentially cleaved; cleavage occurs at one or two sites in the third variable region of the PKC polypeptide. At least some of the novel PKC isoforms (PKC-δ; Ref. 486; PKC-ε; Ref. 372) are also cleaved to a constitutively active form by the calpains.

Calcineurin

The 60-kDa subunit is cleaved sequentially in the presence of calmodulin to 58-, 55-, and 48-kDa fragments; in the absence of calmodulin, the 60-kDa subunit is cleaved sequentially to a 58- and then to a 45-kDa fragment; the calpain-degraded phosphatase is 1.4–1.6 times more active than the native enzyme and does not require calmodulin or Ca²⁺ for activity; cleavage is more rapid in the presence of calmodulin than in its absence; the 19-kDa Ca²⁺-binding subunit is not cleaved by the calpains (450).

Insoluble polyphosphate

The NH₂-terminus of the 105-kDa phosphatase is clipped to produce a 104-kDa fragment that is then degraded to a 70-kDa fragment; degradation occurs in vivo in platelets, and appearance of the 70-kDa fragment is accompanied by loss of phosphatase activity (322).

Protein tyrosine phosphatase 1B (PTP-1B)

The 50-kDa phosphatase is cleaved to a stable 42-kDa fragment, removing the COOH-terminal 60–70 amino acids which are involved in binding PTP-1B to the plasma membrane; the 42-kDa fragment is located entirely in the cytoplasm of platelets and has approximately twofold higher phosphatase activity than the native 50-kDa polypeptide (368); PTP-1B cleavage occurs in situ in platelets (121); integrin signaling is necessary and sufficient to induce calpain cleavage of PTP-1B in platelets; PTP-1B is cleaved at approximately the same rate as ppFAK125.

Most of the masses listed are from SDS-PAGE and are known in some instances (for example, troponin T, troponin I, and synemin) to be inaccurate. This table is a sampling of the more than 100 reported substrates for the calpains and is intended to provide examples of the limited degradation resulting from calpain proteolysis; only two (cytoskeletal proteins; kinases and phosphatases) of the several classes of calpain substrates are included.

C. In Vivo Substrates and Some Proposed Functions

Calpain cleavage of a protein in vitro does not indicate that it is a substrate for the calpains in vivo. One obvious example is the reports indicating that the calpains cleave certain extracellular proteins such as fibrinogen or factor V. The immunolocalization studies all show that the calpains are located exclusively intracellularly except in diseased or injured tissues. Indeed, it seems possible that one of the physiological reasons that kininogen can inhibit the calpains (Table 10 and sect. II) is to ensure that proteolytically active calpain is not present in the circulatory system. On the other hand,
there is no conclusive evidence that the polypeptides cleaved by the calpains in in vitro assays cannot also be cleaved in vivo, although it is clear that not all potential calpain substrates in a cell will be cleaved during a single cellular event.

An antibody raised against a pentapeptide mimicking the NH₂ terminus produced by calpain cleavage of spectrin (G-M-M-P-R; Ref. 379) has been used to demonstrate that the calpains cleave spectrin in postischemic gerbil hippocampus (379) and during apoptosis in a murine T-cell lymphoma (267). A number of studies have used synthetic inhibitors of the calpains to conclude that the calpains are responsible for degradation of various cytoskeletal proteins, p53, and different proteins. Although several of these inhibitors have some selectivity for the calpains, there presently are no synthetic inhibitors that are absolutely specific for the calpains (see Table 10), and results of these studies should be evaluated cautiously. If polypeptides that are known to be highly susceptible to calpain cleavage are degraded in response to a Ca²⁺ flux and this degradation is inhibited by synthetic calpain inhibitors, it is reasonable to suggest that the cleavage is calpain-mediated, but it does not prove that the calpains are involved. In a novel attempt to determine calpain activity in living cells, a fusion protein containing enhanced yellow and enhanced cyan fluorescent protein was linked by a peptide containing the α-spectrin cleavage sequence specific for calpain (468). The fusion protein exhibited FRET when expressed in cells. FRET was lost when the peptide linking the two fluorescent proteins was cleaved, so calpain activity could be measured in vivo by loss of FRET. FRET decreased in dendritic spines of cultured neurons when the neurons were treated with glutamatergic agonists to increase intracellular Ca²⁺ concentrations (468). The decrease in FRET in the dendritic spines was paralleled by an increase in the 150-kDa calpain fragment of α-spectrin, implying a relationship between the 150-kDa calpain fragment of α-spectrin and calpain activity. Thus far, calpastatin is the only inhibitor known to be specific for the calpains. Several studies have used expressed or microinjected single domains (e.g., domain I) of calpastatin in cells to learn whether the intracellular proteolytic degradation observed was due to the calpains (see Refs. 51, 345 as examples). Production of antibodies specific for other calpain-cleaved substrates, development of the long-sought synthetic inhibitor specific for the calpains, and overexpression of calpastatin will be needed, either singly or in combination, in future studies to identify calpain cleavages in vivo with certainty.

1. Cytoskeletal/membrane attachments: cell motility

Cleavage of cytoskeletal/membrane attachments is likely the most thoroughly documented function of the calpains. Some of the strongest evidence for calpain involvement in cleavage of cytoskeletal/membrane attachments originates from three systems: 1) fusion of skeletal muscle myoblasts during muscle development, 2) fibroblasts from calpain knock-out mice (10), and 3) cell spreading/motility and platelet activation. During skeletal muscle development, embryonic myoblasts withdraw from the mitotic cycle, align, and then fuse to form multinucleated myotubes that accumulate skeletal muscle proteins such as actin, myosin, and titin to form skeletal muscle fibers. Fusion of myoblasts requires extensive remodeling of the cytoskeletal/plasma membrane attachments at the point of fusion. Microinjection of calpastatin into myoblasts just before fusion stops fusion completely but does not kill the myoblasts, which can go on to fuse after turning over the excess calpastatin (454). An antisense oligodeoxyribonucleotide to m-calpain decreased the number of myoblasts that fused by ~50% (19), and the membrane-permeable inhibitors calpeptin, Z-Leu-Met-H (230), and E-64d (224) also nearly completely inhibited fusion, depending on the dosage applied. Although the latter two inhibitors affect enzymes in addition to the calpains, calpastatin and antisense oligos to m-calpain are specific for the calpains. Hence, it seems likely that calpain activity is necessary to cut the cytoskeletal membrane attachments that must be severed to allow myoblast fusion.

Fibroblasts from Capn4⁻/⁻ embryos have no calpain activity, are viable, and proliferate (10). These fibroblasts, however, do not generate the calpain-specific degradation products of spectrin that fibroblasts from wild-type animals do; do not degrade talin, a protein that is involved in cytoskeletal/membrane attachments and that is degraded rapidly in in vitro systems by the calpains; have decreased migration rates and an abnormal cytoskeleton with loss of central stress fibers; display delayed retraction of membrane protrusions and filopodia; and have a decreased number of focal adhesions (96). These properties are characteristic of a loss of ability of the cells to undergo normal remodeling of cytoskeletal/membrane attachments. Expression of the rat small calpain subunit partially rescues the aberrant phenotype of the Capn4⁻/⁻ fibroblasts, indicating again that the 28-kDa subunit is required for calpain activity in vivo (96).

Spreading and cell motility require calpain degradation of focal adhesions at attachment sites at both the leading and the rear edges of cells (129, 348). Overexpression of calpastatin in NIH-3T3-derived clonal cells impairs the ability of these cells to extend lamellipodia, reduces by 90% the ability of the cells to spread, and results in an
increase in ezin content (suggesting that calpastatin over-expression prevents normal degradation of this calpain-sensitive substrate; Ref. 359). Interestingly, levels of PKC-α/β, ppFAK125, and talin, all calpain substrates in in vitro assays and also in other in vivo situations, are not affected in the calpastatin overexpressing cells, suggesting that degradation of calpain-sensitive proteins in vivo may depend in part on the physiological signals that the cell has received and possibly also the subcellular location of calpain relative to the substrate proteins. Introduction of a 24-amino acid sequence of calpastatin into platelets inhibited thrombin-induced α-granule secretion, platelet aggregation, and spreading of platelets on glass (79). The 24-amino acid calpastatin also inhibited Ca2+-ionophore (A23187)-induced degradation of talin and filamin, which have been shown to be degraded during platelet aggregation (117, 120). The results indicate that calpain activity is involved in early events (the first 30 s) after platelet activation, such as α-granule release, and also in aggregation. Earlier studies indicating that calpain activity was not involved in platelet aggregation may have been the result of poor membrane permeability of the ZLLY-CHN2 (benzyloxy carbonyl-Leu-Leu-Try-diazomethyl ketone) inhibitor used.

In sum, the calpains have a role in severing cytoskeletal/membrane attachments, although the exact nature of this role remains unclear. Some functions that clearly require extensive cytoskeletal/membrane remodeling, such as cytokinesis for example, proceed in the Capn4−/− fibroblasts that have no or very little calpain activity, and some calpain-sensitive cytoskeletal proteins are degraded in the presence of levels of calpastatin that are sufficient to prevent cell spreading.

2. Signal transduction pathways

Calpain cleavage of PKC to produce a constitutively active enzyme (209) led to suggestions that the calpains were involved in signal transduction. In vitro studies have shown that many of the kinases (e.g., ppFAK125, pp60src), phosphatases (e.g., tyrosine phosphatase PTP-1B), and cytoskeletal proteins (e.g., talin, filamin, paxillin, vinculin) involved in signal transduction pathways are rapidly cleaved by the calpains, but it remains difficult to identify specific calpain cleavages in vivo that are related to specific signal transduction events. Recent studies indicate that the calpains are involved in integrin-mediated signal transduction pathways (119) and that only the β-integrin family of integrins is cleaved by the calpains (349). Calpain cleavage occurs at several sites in the COOH-terminal half of the cytoplasmic domains of the β-integrins, specifically in regions flanking the conserved NPYX/NXXY motifs; these motifs are involved in binding of integrins to the cytoplasmic proteins, talin and filamin. The cleavage sites are not influenced by the linear sequence of amino acids on either side of the scissile bonds, but rather by the structural framework provided by the NXXY motifs.

Part of the role of the calpains in signal transduction is related to their role in cytoskeletal/membrane interactions. Integrin clustering or binding of ligands to certain receptors leads via some unknown mechanism to calpain activation. Calpain cleavage of integrin/cytoskeletal proteins in focal adhesions severs the interactions among these molecules, resulting in disassembly of the focal adhesions, cell rounding, loss of submembranous actin filament networks, increased rates of cell spreading and cell motility (129, 348), and, in platelets, to retraction of fibrin clots (395). Calpain cleavage of proteins in focal adhesions may be necessary for detachment at the trailing edges of motile cells, and the breakdown and formation of new focal contacts in extending lamellipodia allows cells to spread (129, 175). A study using cultured smooth muscle cells showed that aggregation of integrin receptors induced by collagen fragments that bind to the αβ1 integrin in these cells leads to a rapid cleavage of pp125FAK, talin, and paxillin; this cleavage blocks downstream signal transduction signals and results in focal adhesion disassembly, cell rounding, and increased motility (50). Cleavage was prevented by a synthetic calpain inhibitor; neither α-actinin nor actin, which are poor calpain substrates, was cleaved.

The calpains also seem to have a role in signal transduction pathways in addition to cleaving integrin/cytoskeletal protein interactions. Synthetic inhibitors of the calpains abrogate ability of bovine aortic endothelial cells (BAEC) to spread and to form focal adhesions, actin filament networks, and stress fibers. Cells expressing a constitutively active form of RhoA, however, can still spread and form focal adhesions when calpain inhibitors are present; cells expressing active Rac1 can extend lamellipodia, form focal complexes and networks of subcortaneous actin fibers, but cannot form focal adhesions or stress fibers (35, 221). The use of different inhibitors and overexpression of a catalytically inactive form of μ-calpain indicated that only μ-calpain and not m-calpain was involved in these changes in BAEC. The results suggest that the calpains (μ-calpain in this instance) act in signal transduction at a site upstream of both Rac1 and RhoA. Western analysis showed that both talin and PKC were cleaved in adherent (plated on fibronectin; α5β1-integrin) BAEC (221). Immunofluorescence studies using BAEC have shown that integrin, calpain-induced clusters, assemble before formation of Rac1-containing focal complexes and the larger, RhoA-containing focal adhesions (35). These calpain-induced clusters contain calpain, calpain-cleaved β-integrin subunit, and Rac1; require catalytically active calpain for their formation; and seem to be
involved in activation of Rac1. In contrast to the earlier studies that found m-calpain in adhesion plaques in several cell types (BS-C-1; EBTr; MDBK; Ref. 31), calpain was not detected immunologically in either focal complexes or focal adhesions in BAEC. Do different focal adhesions/focal complexes in different cells contain either μ- or m-calpain but not both? Transfection with constitutively active Rac1 or RhoA showed that focal complexes and focal adhesions can form in these cells via a mechanism that does not require calpain. The results indicate that calpain is involved, by some as yet unknown means, in pathways that lead to activation of Rac1 and RhoA but that focal complexes and focal adhesions can form independent of calpain if active Rac1 or RhoA is present.

RhoA activity is dynamic during transmembrane signaling. During the early stages of integrin-mediated cell spreading, activity of RhoA is downregulated, but at a later stage, RhoA activity is increased and focal adhesions and stress fibers are formed. Downregulation of RhoA is caused by specific cleavage of RhoA between Q180 and A191 to produce a 20-kDa fragment that inhibits integrin-induced stress fiber formation and focal adhesions (220). The RhoA cleavage is inhibited by the synthetic calpain inhibitors calpeptin and calpain inhibitor I, but not by proteasome or caspase inhibitors (220). Neither Rac1 nor cdc42 was cleaved when incubated with purified μ-calpain, although RhoA was cleaved to a 20-kDa fragment. Hence, the calpains (μ-calpain) can modulate RhoA activity directly.

The studies with BAEC suggest that several types of integrin complexes exist: 1) calpain-induced clusters that contain active calpain, calpain-cleaved β-integrin fragment, vinculin, phosphotyrosine, Rac1-binding protein, α-actinin, and skelemin but not talin; 2) Rac-induced focal complexes that contain intact integrin, vinculin, and phosphotyrosine but not calpain and probably not skelemin; and 3) RhoA-induced focal adhesions that contain intact integrin, vinculin, phosphotyrosine, and talin, but not α-actinin, skelemin, or calpain (35, 362). These different integrin complexes are formed by activation of different signaling molecules and at different stages of spreading and involve different integrin/cytoskeletal interactions. Only α-actinin and skelemin can bind to the calpain-cleaved, membrane-associated part of β-integrin. A skelemin-based peptide that blocks the interaction of skelemin with β-integrin also completely blocks formation of focal complexes and focal adhesions, which form downstream from the calpain-induced clusters (362).

In sum, the studies thus far suggest a complex and dynamic pattern of integrin signaling that may involve the calpains in several ways ranging from cleavage of integrin/cytoskeletal proteins that ablates the interaction between them to activation of Rac1 and to direct inactivation of RhoA. Other studies have indicated that epidermal growth factor (EGF) receptor-mediated fibroblast motility requires activation of m-calpain downstream of ERK/mitogen-activated protein kinase signaling (128, 131). Calpain activation in this system is triggered only by membrane-restricted EGF receptor, and not by the internalized form of the receptor (131). EGF-induced activation in fibroblasts (α5β1- and α6β1-integrins) was specific for m-calpain and did not involve μ-calpain (128). Immunological studies have shown that m-calpain and not μ-calpain is present in focal adhesions of several cell types (31) and that m-calpain but not μ-calpain is involved in adhesion and spreading of T-cell lymphocytes (368).

Transformation of cells by v-src results in deregulated cell growth, cytoskeletal disassembly, and loss of integrin-linked focal adhesions, contributing to a highly mitogenic and motile phenotype. Synthesis of m-calpain was increased in chick embryo fibroblasts transformed by v-src, and this increase was accompanied by degradation of ppFAK125 and calpastatin, which is a calpain substrate when calpains are in excess. Overexpressing calpastatin in the v-src cells suppressed ppFAK125 degradation, morphological transformation, anchorage-independent growth, and rate of progression through the G1 stage of the mitotic cycle (51). The decreased rate of progression through the cell cycle was accompanied by a decrease in hyperphosphorylation of the tumor suppressor protein pRb and attenuation of the increases in cyclin A, cyclin D, and cdk2 that normally accompany v-src transformation. The v-src transformation, however, had little effect on the phenotype of fibroblasts from Capn4−/− mice, suggesting that calpain activity is needed for v-src transformation of cells (51).

Although it is becoming clear that the calpains are activated in a number of signal transduction processes, it will likely require a number of years before the precise role of the calpains in signal transduction is clarified. It presently seems that different cells and different signaling pathways involve either μ- or m-calpain but not both and may involve different roles for the calpains in the same cell depending on the signal the cell receives.

An unanswered question in the role of the calpains in signal transduction is, how are the calpains activated by a clustering of integrins or by binding of EGF to the EGF receptor? The Ca2+ influx in response to ligand binding is insufficient to activate μ-calpain let alone m-calpain. Glading et al. (130) reported that ERK kinase phosphorylated m-calpain on a Ser residue, probably Ser-50. The phosphorylated m-calpain was fully active catalytically at Ca2+ concentrations below 1 μM. It is tempting to speculate that the results suggest that some combination of phosphorylation is the calpain “activator” that enables calpain activity at physiological Ca2+ concentrations.
3. The cell cycle

A number of studies using synthetic inhibitors have suggested that calpain activity is required for progression through the cell cycle, specifically through the G1 to S transition. As indicated previously, however, synthetic inhibitors may have unexpected effects, such as inhibition of protein tyrosine phosphatase (PTP) 1-B by calpeptin (394), a calpain inhibitor that has been used widely. Also, that fibroblasts from the Capn4−/− mice proliferate normally but have no detectable calpain activity raises uncertainties as to the role of the calpains in the mitotic cycle.

Nevertheless, several studies using combinations of different inhibitors to minimize contributions of the proteasome or using the specific calpain inhibitor, calpastatin, have indicated that the calpains have a role in the cell cycle. Cyclin D1 is rate limiting and is involved in progression through G1. Serum starvation of NIH 3T3 cells results in a rapid loss of cyclin D1 that is prevented by synthetic calpain inhibitors or by overexpressing calpastatin (55). Hyperphosphorylation of the retinoblastoma gene product pRb is associated with increased rate of progression of cells through the G1 stage of mitosis; overexpression of calpastatin represses progression of v-src transformed chick embryo fibroblast through G1; decreases the levels of cyclin A, cyclin D, and cdk2; and is correlated with decreased levels of pRb phosphorylation (51). Although cyclin D1 is also degraded by the proteasome, synthetic proteasome inhibitors did not prevent the rapid loss of cyclin D1 following serum starvation. Progression of serum-starved WI-38 fibroblasts through G1 was partially inhibited by E-64d, a cell-permeant inhibitor of calpain, whereas the proteasome inhibitor lactacystin caused only modest inhibition of this degradation (276). Addition of 20 μM benzoyloxycarbonyl-Leu-Leu-Tyr-diazomethyl ketone (ZLLY-CHN2), a synthetic calpain inhibitor, to cultures of WI-38 fibroblasts completely blocked their proliferation at the late G1 stage and resulted in a twofold increase in p53 levels (498). Immunofluorescence studies showed that μ-calpain appeared transiently in the nucleus at late G1. A proteasome inhibitor also resulted in an increase in p53 levels, so it seems that inhibition of either protease alone can retard p53 degradation. In contrast to the results of Choi et al. (55), ZLLY-CHN2 did not affect the levels of cyclin D in WI-38 fibroblasts and also did not affect the levels of c-Jun or c-Fos (498). Selection of a line of Chinese hamster ovary (CHO) cells that were resistant to ZLLY-CHN2 resulted in cells that had decreased levels of μ-calpain, unaltered levels of m-calpain and calpastatin, and a 50% increase in doubling time compared with CHO cells before selection (282). The increased doubling time was due to a prolonged G1 phase. Addition of ZLLY-CHN2 to cultures of TE2 cells (simian virus 40-transformed human esophageal epithelial cells) or C-33A cells (derived from human cervical carcinoma) inhibited growth of both cell lines and lowered the Ca2+-dependent proteolytic activity in both cell lines to 20% of the original activity (286). Addition of ZLVG-CHN2, a synthetic inhibitor that has little effect on activity of the calpains, but inhibits cathepsin L, however, had no effect on either the growth or the calpain activity of these two cell lines, suggesting that the effects of ZLLY-CHN2 were due to the calpains.

A recent study found that both EcR-CHO cells overexpressing calpastatin and fibroblasts from Capn−/− mice proliferated at the same rate as mock-infected cells or fibroblasts from Capn+/+ mice if the cells were plated at densities of 1,500 cells/cm² (485). If the EcR-CHO cells overexpressing calpastatin or the Capn−/− fibroblasts were plated at clonal densities (2–4 cells/cm²), however, their rates of proliferation were only 20–50% as great as the rates of mock-infected cells or fibroblasts from Capn+/+ mice (485). That both the calpastatin-overexpressing cells and the Capn−/− fibroblasts proliferated, albeit at a reduced rate, even at clonal densities indicates that cells can proliferate in the absence of calpain activity. Interestingly, the levels of ezrin, talin, and spectrin, all purported in vivo substrates of the calpains and presumably proteins that would be cleaved as cytoskeletal/membrane attachments are broken during cytokinesis, were the same in the calpastatin-overexpressing and the mock-infected cells (485). As noted previously, it seems that, for reasons that are not yet clear, some calpain-susceptible polypeptides in cells are spared from degradation in vivo in circumstances where they would be expected to be cleaved.

Several studies have implicated the calpains in meiosis. Calpain is present and undergoes autolysis and re-localization in concert with an increase in intracellular Ca2+ concentration in rat eggs (265). Reinitiation of meiosis in starfish oocytes is accompanied by a Ca2+ transient, disassembly of the nuclear envelope, and degradation of the lamins, which are calpain substrates in vitro (389). Microinjection of calpain into the nuclei of prophase-arrested oocytes induced meiosis. Finally, Watanabe et al. (475) have shown that m-calpain degrades the protein kinase pp39mos in Xenopus eggs, thereby allowing progression through metaphase.

In sum, the role of the calpains in mitosis/meiosis is still poorly understood. The results thus far suggest that calpain activity may not be essential for mitosis to occur. The calpains may serve as an alternative to or have a function supplemental to the proteasome in the proteolytic pathways that regulate cell proliferation. Although it will likely be difficult to sort out what role the calpains have in the mitotic cycle, recent advances in understanding the cell cycle and its regulation indicate that interest-
ing new information of the role of the calpains in the cell cycle may be expected in the future.

4. Regulation of gene expression

The exact nature of the role that the calpains may have in regulation of gene expression also is presently unclear. The concept that the calpains are involved in regulation of gene expression seems to be based to a considerable extent on ability of the calpains to cleave several transcription factors such as c-Jun, c-Fos (166, 341), and p53 (141, 216, 340) in vitro assays. These same transcription factors are also degraded by the proteasome, however, and it seems likely that the proteasome is the principal route of degradation of these transcription factors in cells. Gonen et al. (141), for example, indicate that, although they cannot absolutely rule out a role for the calpains in degradation of p53, a rigorous proof for their involvement is still missing. If the calpains have a role in degradation of transcription factors, it is likely to occur in the cell cytoplasm because immunolocalization studies indicate that calpains are not generally located in the nucleus.

On the other hand, overexpression of calpastatin in NIH3T3 cells decreases and introduction of an anti-sense calpastatin increases the rate of degradation of c-Jun in these cells (161). Hence, the evidence suggests that c-Fos, c-Jun, and p53 can be degraded via several different proteolytic pathways in vivo and that one of these pathways may involve the calpains (385), although the proteasome pathway is likely the predominant pathway in most instances. The factors regulating participation of the calpains in degradation of transcription factors are unknown. For example, addition of a calpain inhibitor to cells expressing wild-type p53 resulted in stabilization of the p53, whereas neither in vivo nor in vitro degradation of p53 mediated by human papillomavirus E6 protein was affected by the presence of the same calpain inhibitor (216). The mutated transduced v-FosFBR but not the mutated transduced v-FosFBD (transduced by Finkel-Biskis-Reilly murine sarcoma virus-FBR-MSV or by Finkel-Biskis-Jenkins murine sarcoma virus-FBJ-MSV) was resistant to calpain cleavage, raising the possibility that decreased sensitivity to the calpains might contribute to the tumorigenic potential of FBR-MSV (429). Additional work using specific calpain inhibitors such as calpastatin will be needed to clarify the role of the calpains in gene regulation.

5. Apoptosis

The term *apoptosis* was first used in 1972 (203) to describe the process of programmed cell death. Reports in 1993 first suggested that the calpains were involved in apoptosis, but the exact role of the calpains in this process has been controversial. It presently seems that involvement of the calpains in apoptosis is limited to certain cell types and to specific stimuli (204). There are several difficulties in clearly defining the role of the calpains in apoptosis. A number of proteolytic enzymes are involved in apoptosis. It is clear that cysteine (but not Ca\(^{2+}\)-dependent) proteases, the caspases, have a major role in apoptosis in most cell types, but lysosomal cathepsins and the proteasome also seem to be involved in some apoptotic pathways. Some of the currently available “calpain inhibitors” inhibit cathepsins B and L in addition to inhibiting the calpains, and calpain inhibitor I inhibits the proteasome (Table 10). Hence, use of synthetic inhibitors does not distinguish clearly among calpain, cathepsin, and proteasome activity in apoptosis, and results of studies using calpain inhibitors should be interpreted cautiously. Some studies have used combinations of proteasome inhibitors, cathepsin inhibitors, and caspase inhibitors and differences in the abilities of the different inhibitors to affect apoptosis in attempts to distinguish the roles of the different proteolytic pathways in apoptosis. Although these studies provide suggestive evidence for the role of the calpains in apoptosis, it remains difficult to be certain that the inhibitors used completely inhibited their putative targets and that they did not have unexpected effects.

Recent studies using injection or overexpression of the calpain-specific inhibitor calpastatin or domains of calpastatin have now shown that the calpains are clearly involved in some types of apoptosis in specific cell types and in response to certain apoptotic signals (54, 251, 369, 427). Other evidence supporting a role of the calpains in apoptosis has relied on the ability to detect the calpain-specific 150/145-kDa degradation products of α-spectrin (see sect. II.C3) in apoptotic cells by using the antibody specific for the NH\(_2\) terminus of the 150-kDa calpain fragment (379). Caspase degradation of α-spectrin produces 120- and 150-kDa fragments (267), so spectrin degradation by the calpains can be clearly distinguished from spectrin degradation by the caspases.

Understanding the role of the calpains in apoptosis is complicated by the ostensible ability of the calpains to cleave the caspases themselves and to cleave several proteins that regulate progression of apoptosis. Several studies have found that the calpains cleave caspase-7, -8, and -9 and that calpain cleavage of caspase-7 and -9 inactivates them (56). On the other hand, m-calpain has been reported to cleave procaspase-12 to generate an active caspase and to cleave the loop region of Bcl-xl to change an antiapoptotic molecule into a proapoptotic molecule (314). Therefore, it seems that in some instances, the calpains may act as negative regulators of apoptosis by inactivating “upstream” caspases, whereas in other instances, they may act as positive regulators of apoptosis; these activities would be in addition to the presumed direct role of the calpains in degradation of
cytoskeletal proteins in some types of apoptosis. Studies involving Chinese hamster cells overexpressing μ-calpain or calpastatin found that calpain actually protected against tumor necrosis factor-α-induced apoptosis, whereas calpastatin protected against apoptosis induced by thapsigargin, A23187, or serum deprivation in these cells (251).

There are several other examples of “indirect” effects of the calpains on apoptosis. The chaperone glycoprotein GRP94 possesses antiapoptotic properties. During apoptosis induced in three different cell types by etoposide, a topoisomerase II inhibitor, a fraction of total cellular GRP94 that is associated with the endoplasmic reticulum (~40% of total GRP94) is degraded by a protease that is completely inhibited by a synthetic calpain inhibitor but that is only partly inhibited by synthetic caspase inhibitors and is not affected by a proteasome inhibitor (363). Immunolocalization studies using confocal microscopy showed that calpain and GRP94 were colocalized in the perinuclear region of Chinese hamster fibroblast K12 cells after treatment with etoposide. The use of antisense GRP94 to reduce cellular levels of GRP94 resulted in initiation of apoptosis in Jurkat cells (363). Hence, calpain cleavage of GRP94 may assist in progression of the apoptotic pathway independently of any direct effect of the calpains on cleavage of cytoskeletal proteins. The synthetic calpain inhibitor calpeptin prevents cleavage of the pro-apoptotic protein Bax in a human hematopoietic cell line, HL-60 (483). Both the caspases and the calpains can cleave Bax, and cleavage enhances the proapoptotic properties of Bax. In HL-60 cells induced to undergo apoptosis by the topoisomerase I inhibitor 9-amino-20(α)-camptothecin (9-AC), cleavage of α-spectrin to a 150-kDa polypeptide (caspase fragment) and activation of caspases occurred early in apoptosis, whereas activation of the calpains seemed to be related to caspase activity and occurred later, simultaneously with cleavage of Bax. The authors speculate that calpain activation, as estimated by the autolytic cleavage of its 28-kDa subunit, occurred downstream of caspase activity and only after the caspases had degraded endogenous calpastatin. Pretreatment with calpeptin, which would inhibit the calpains, did not prevent apoptosis in the HL-60 cells in this study, indicating that apoptosis in HL-60 cells can proceed in the absence of calpain activity.

Examination of apoptosis of senescent neutrophils indicated that the caspases, calpains, and proteasome function synergistically to complete apoptosis in these cells and that both calpains and the proteasome act downstream from the caspases (211). On the other hand, apoptosis in human platelets, which contain caspase-9 and caspase-3 and the caspase activators APAF-1 and cytochrome c, seems to be initiated by calpain (μ-calpain in human platelets), and not by the caspases (480). Anti-IgM-induced apoptosis in an immature B-cell line was blocked completely by overexpression of calpastatin, but apoptosis induced by actinomycin D in these same cells was not impeded by calpastatin overexpression (369), indicating that the role of the calpains in apoptosis is cell and apoptotic-signal specific.

Finally, studies on primary cultures of v-src-transformed and nontransformed chick embryo fibroblasts indicate that the calpains may regulate focal adhesion turnover but may not be involved in apoptosis in these cells. Levels of m-calpain increased substantially during the first 18 h after temperature-sensitive induction of v-src expression, whereas the levels of μ-calpain and calpastatin were unaffected (49). Induction of v-src resulted in disassembly of focal adhesions, cell rounding, disorganization of the actin cytoskeleton, and increased cell motility. These changes were prevented by synthetic calpain inhibitors and a calpastatin peptide, but not by a lysosomal inhibitor or by synthetic caspase or proteasome inhibitors. ppFAK<sup>125</sup>, the focal adhesion kinase, was degraded during v-src transformation, but other calpain-sensitive cytoskeletal proteins such as talin, paxillin, and pp60<sup>src</sup> were not degraded. Rat-1 fibroblasts expressing v-src undergo apoptosis when cultured in low serum; this apoptosis is also accompanied by ppFAK<sup>125</sup> degradation, but in this instance, ppFAK<sup>125</sup> degradation is prevented by synthetic caspase inhibitors and is not affected by synthetic calpain inhibitors (49). Hence, the calpains seem to be involved in disassembly of focal adhesions and loss of substrate anchorage in v-Src-transformed fibroblasts, but not in apoptosis of these cells.

The latter studies all used synthetic inhibitors of the calpains and the proteasome and therefore are limited by the uncertain specificity of these inhibitors. Nevertheless, the results indicate that the role of the calpains in apoptosis is highly cell and apoptotic signal dependent. It is unclear how much calpain degradation of the caspases themselves and of some of the regulatory proteins of apoptosis contribute to the progression of apoptosis and whether this degradation is also cell and apoptotic signal dependent. Apoptosis is a rapidly evolving area, and it can be anticipated that a great deal of additional information on the role of the calpains in apoptosis will become available in the coming years.

6. Long-term potentiation

It was reported in 1981 that micromolar Ca<sup>2+</sup> concentrations markedly enhanced glutamate binding to receptors in hippocampal synaptic membranes and that this effect was partly irreversible (29). The increased binding was associated with selective degradation of a 180-kDa doublet by a Ca<sup>2+</sup>-dependent protease that was inhibited by leupeptin and that had many of the properties of m-calpain, whose purification had just been described (29). It was proposed that Ca<sup>2+</sup>-dependent cleavage may
be a mechanism for the long-term potentiation of synaptic transmission observed in the rat hippocampus. Since this early report, evidence has continued to accumulate suggesting that calpains cleave cytoskeletal and other proteins that anchor the N-methyl-D-aspartate (NMDA) receptors in structures named postsynaptic densities (PSDs) that underlie the postsynaptic membranes. In vitro studies have shown that μ-calpain cleaves the NR2 subunits of the NMDA receptor in their COOH-terminal regions (146) and that m-calpain cleaves a protein, PSD-95, in the PSD that binds to the NR2 subunits to anchor the receptor (470). The current hypothesis suggests that proteolytically induced changes in the composition and the anatomy of NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors lead to long-term potentiation (252). These changes may involve changes in the membrane environment of the receptors, a greater access to modulatory proteins, a clustering of the receptors, a modification of the space available for receptor insertion, or any combination of these possibilities. The interest in long-term potentiation is its purported role as a molecular mechanism that underlies learning and memory formation. Theta pattern stimulation results in an influx of Ca$^{2+}$ and activates calpain in the PSDs as determined by the appearance of the calpain-specific 150-kDa fragment of α-spectrin (252). Many of the studies implicating the calpain system in long-term potentiation have used Ca$^{2+}$-dependent proteolysis in crude preparations and synthetic inhibitors of the calpains to infer a role for calpain in glutamate receptor function, and the conclusions therefore should be interpreted cautiously. Nevertheless, it seems likely that the calpains are responsible for degradation of key polypeptides in NMDA-induced potentiation, although the complexity of the system makes it difficult to determine the exact nature of the role that the calpains have.

VI. PATHOLOGICAL IMPLICATIONS OF THE CALPAIN SYSTEM

The calpains have been implicated in a wide range of pathological states in cells or tissues. Some but not all of these “calpain-associated” pathologies are listed in Table 12. The putative role of the calpains in various pathologies has been the subject of several recent reviews (173, 461, 472, 473), and the evidence linking the calpains to these pathologies will not be reviewed in depth here. With three exceptions, the role of calpain 3 in limb girdle muscular dystrophy, the possible role of calpain 9 in tumorigenesis, and the role of calpain 10 in type II diabetes (Table 12) that have all been discussed earlier in this review, the calpains are not the genetic cause of these diseases; rather, conditions leading to loss of Ca$^{2+}$ homeostasis in cells are in many instances accompanied by degradation of proteins/polypeptides that are known substrates of the calpains in in vitro assays. When synthetic inhibitors of the calpains prevent or reduce this proteolytic degradation, it is frequently assumed that the increased intracellular Ca$^{2+}$ concentrations have “activated” the calpains and that the proteolytic degradation in these situations is the result of increased calpain activity. Hence, the calpains become involved in a “guilt by association” process. The vexing Ca$^{2+}$ problem discussed earlier, however, appears again. The increases in intracellular Ca$^{2+}$ concentration that occur in ischemic or other areas where Ca$^{2+}$ homeostasis is impaired are not sufficiently great to activate the calpains directly (this is certainly true for m-calpain; in some instances, Ca$^{2+}$ concentrations may increase to levels that could elicit minimal μ-calpain activity, and it can be argued that such minimal and unregulated μ-calpain activity could over time result in substantial tissue damage). A great deal of circumstantial evidence implicating the calpains in the pathologies listed in Table 12 has accumulated during the past 10–15 years, however, and it currently seems likely that the calpains have some role in many of the conditions listed in Table 12. Rather than activating the calpains directly, however, it seems probable that the increased Ca$^{2+}$ concentrations that occur during disruption of Ca$^{2+}$ homeostasis in cells alter regulation of calpain activity. For example, the increased Ca$^{2+}$ concentration may affect activity of the “activator” discussed in section IV C1, or it may alter regulation of calpain activity by calpastatin, phosphorylation, or other mechanisms that are presently unknown.

The muscular dystrophies were the first disease to be associated with inappropriate calpain activity (190). Nearly all of the more than 20 different kinds of muscular dystrophies involve some loss of Ca$^{2+}$ homeostasis. The Duchenne and Beckers muscular dystrophies are among the most thoroughly studied of the muscular dystrophies; both are caused by disruption of the gene encoding the protein dystrophin. This protein is associated with the skeletal muscle cell membrane and links the subsarcolemmal actin cytoskeleton to the extracellular matrix. Loss of dystrophin leads to weakening of the plasma membrane of skeletal muscle cells and “leakage” of extracellular Ca$^{2+}$ into the muscle cell. Intracellular free Ca$^{2+}$ concentrations rise nearly two- to threefold up to 200–500 nM in skeletal muscles of the mdx mouse (169), an animal model of Duchenne muscular dystrophy that also lacks dystrophin. There are several features of the putative role of the calpains in muscle wasting that apply also to many of the other “calpain pathologies.” These features are summarized in the following model for turnover of myofibrillar proteins that was proposed a number of years ago (82).

First, the specific subsite specificity of the calpains indicates that inappropriate calpain activity is restricted to a limited number of cleavages in a few proteins. Muscle
tissue contains three classes of proteins: 1) the sarcomplasmic or cytoplasmic proteins that constitute ~30–35% of total muscle protein; 2) the myofibrillar or contractile proteins that constitute ~55–60% of total muscle proteins; and 3) the stroma proteins that constitute 10–15% of total muscle protein and that include the plasma membranes and membrane receptors but that otherwise are largely extracellular and are insoluble in most aqueous solvents. Although the calpains can cleave many of the kinases and phosphatases in the sarcoplasmic fraction and some of the signal transduction molecules in the stroma protein fraction, they do not catalyze bulk degradation of either the sarcoplasmic proteins (451) or the stroma proteins, many of which would be inaccessible to the intracellular calpains. Hence, any role of the calpains in muscle protein degradation is likely to involve principally the myofibrillar or the cytoskeletal proteins. Of the known myofibrillar proteins, the calpains rapidly cleave titin and nebulin at sites near the Z-disk in striated muscle (Table 11), thereby severing their attachment to the proteins in the Z-disk. In addition, the calpains cleave the intermediate filament protein desmin that attaches the Z-disk to the sarcolemma; hence, the proteins constituting the Z-disk, including α-actinin, are released, and the Z-disk disappears leaving a space in the myofibril (82, 84). The released α-actinin can rebind to actin, indicating that

<table>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Evidence/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limb girdle muscular dystrophy type 2A</td>
<td>Ca2+ influx of 1,900 µM or higher activates m-calpain, the predominant calpain in lens, and α- and β- but not γ-crystallins are cleaved. The crystallin fragments aggregate to form cataracts. No Lp82 in human lens; see section VI.</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Cataract formation</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Muscular dystrophies</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Myocardial infarcts</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Multiple sclerosis (MS) (demyelination)</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Neuronal ischemia (stroke)</td>
<td>Ca2+ homeostasis-linked pathologies</td>
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<tr>
<td>Obsessive-compulsive disorders</td>
<td>Ca2+ homeostasis-linked pathologies</td>
</tr>
<tr>
<td>Traumatic spinal cord (brain) injury</td>
<td>Ca2+ homeostasis-linked pathologies</td>
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The calpain system 783

**Table 12.** Some pathological conditions that have been linked to the calpains or to inappropriate calpain activity

<table>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Evidence/Comments</th>
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<tbody>
<tr>
<td><strong>Genetic diseases</strong></td>
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</tr>
<tr>
<td>Limb girdle muscular dystrophy type 2A</td>
<td>Caused by disruptions in the gene for calpain 3a (366); disease-related disruptions are linked to loss of calpain 3a proteolytic activity (333); see section VI.</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Downregulation of Capn9 gene is associated with human gastric cancer (493); antisense induced deficiency of calpain 9 is associated with tumorigenesis in NIH3T3 fibroblasts (240).</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>Mutations in intron 3 of Capn10 gene associated with increased incidence of type 2 diabetes in some populations; see section VI.</td>
</tr>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>Amount of m-calpain in the cytosolic but not the membranous fractions (464) and in the neurofibillary tangles (145) of brain from Alzheimer’s patients is increased. Ratio of autolyzed to unautolyzed μ-calpain is threefold higher and amount of calpastatin is lower in AD prefrontal cortex than in normal brain (321). Calpain evidently is not involved in cleavage of APP to produce amyloid β-peptides; see section VI.</td>
</tr>
<tr>
<td>Cataract formation</td>
<td>Ca2+ influx of 1,900 µM or higher activates m-calpain, the predominant calpain in lens, and α- and β- but not γ-crystallins are cleaved. The crystallin fragments aggregate to form cataracts. No Lp82 in human lens; see section VI.</td>
</tr>
<tr>
<td>Muscular dystrophies</td>
<td>Many muscular dystrophies are accompanied by loss of Ca2+ homeostasis, either locally or involving entire fibers; Duchenne and Beckers muscular dystrophy are caused by loss of a membrane-associated protein, dystrophin, which results in increased Ca2+ concentrations in muscle, loss of Ca2+ homeostasis, and inappropriate calpain activity (461); see section VI.</td>
</tr>
<tr>
<td>Myocardial infarcts</td>
<td>Ca2+ homeostasis is lost in ischemic areas, triggering inappropriate calpain activity. Desmin and α-spectrin are degraded in ischemic hearts (339, 463, 491); this degradation occurs after an increase in [Ca2+]i (463) and is inhibited by synthetic calpain inhibitors. Protein and mRNA levels of first m-calpain and then μ-calpain increase after a myocardial infarction (387).</td>
</tr>
<tr>
<td>Multiple sclerosis (MS) (demyelination)</td>
<td>All major myelin proteins, the 68- and 200-kDa neurofilament proteins, myelin basic protein, and myelin-associated glycoprotein, are calpain substrates (407); m-calpain is the major calpain in myelin sheaths. Levels of both calpain protein and calpain activity are increased in animal models of MS (408); in the demyelinating disease, experimental allergic encephalomyelitis; and as measured immunologically, in astrocytes, activated microglia, and activated T cells and macrophages that infiltrate sites of inflammation. The 150-kDa calpain-specific degradation product of α-spectrin increases 50% in human MS plaques (408). Degradation of the 68-kDa neurofilament protein is inhibited by a synthetic calpain inhibitor (22).</td>
</tr>
<tr>
<td>Neuronal ischemia (stroke)</td>
<td>Intracellular [Ca2+]i increases in ischemic areas (487), partly due to overactivation of NMDA receptors; MAP2 and α-spectrin ( fodrin) are degraded in animal models of cerebral ischemia with the calpain-specific 150-kDa α-spectrin fragment appearing 5 (28) or 60 min (319) after ischemia in rat brains; rate of expansion of the area of spectrin degradation is reduced by synthetic calpain inhibitors, A275 and A295 (28). Calpastatin is degraded by calpain to a membrane-bound 50-kDa polypeptide in ischemic brain (41). Tissue damage in ischemic areas involves both apoptosis and necrosis and the calpains participate in both processes (42).</td>
</tr>
<tr>
<td>Obsessive-compulsive disorders</td>
<td>Erythrocytes from patients with obsessive-compulsive disorder have significantly higher calpain activities than erythrocytes from normal patients; this difference could not be attributed to differences in memory function (307). Calpastatin activities do not differ between obsessive-compulsive and normal patients.</td>
</tr>
<tr>
<td>Traumatic spinal cord (brain) injury</td>
<td>Most studies done in the rat; injury results in increased intracellular [Ca2+]i (370). Levels of calpain-specific 145-kDa α-spectrin fragment increased up to 660% within 72 h after traumatic injury to rat brain (350). Loss of neurofilament protein 68 (35% decrease), and NF200 (65% decrease) in ipsilateral rat cortex is prevented by calpain inhibitor 2 (358); treatment with synthetic calpain inhibitor, AK295, improves motor scores and performance on cognitive tests for rats after fluid percussion injury (370). Both μ- and m-calpain activities increase globally following brain injury (501). Spinal cord injury in traumatized rats increases ryanodine receptors and m-calpain activity (331).</td>
</tr>
</tbody>
</table>

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it has been released from the Z-disk in a substantially undegraded form (133). Light and electron microscope examination of muscle tissue in many conditions of muscle wasting, including the muscular dystrophies, show that such muscle often has abnormal Z-disks, and in some instances of severe muscle wasting, the Z-disks are missing altogether. The calpains also rapidly cleave troponins T and I and tropomosin, and C-protein, which contribute to stability of the thin and thick filaments, respectively, but they cleave myosin and actin, the two major proteins in striated muscle, very slowly if at all. Consequently, the net effect of the calpains on striated muscle myofibrils is progressive disruption of the Z-disk leading eventually to its complete loss; the subsequent release of myosin thick filaments and actin thin filaments from the surface of the myofibril; and production of fragments of titin (some as large as 500 kDa), nebulin, desmin, and other calpain-susceptible proteins (Fig. 8). Because of the limited specificity of the calpains, further degradation of the titin, nebulin, etc. fragments and of actin and myosin to amino acids requires participation of other proteases. It seems likely that the proteasome has a major role in degradation of the released actin and myosin molecules and the other myofibrillar protein fragments (100, 413, 478). The proteasome, on the other hand, cannot degrade intact myofibrils (413) or cytoskeletal complexes, likely because the entrance to the central cavity of the proteasome containing the active sites is only 10–13 Å in diameter and is much too narrow to allow entry of myofibrils that range from 10 to 100 μm in diameter. Hence, loss of myofibrillar proteins in muscle wasting requires the concerted action of at least two proteolytic systems: 1) the release of filaments from

![Fig. 8. Schematic diagram of a model for turnover/degradation of proteins assembled in myofibrillar structures in striated muscle. The angled lines on the thick filaments represent bands of C-protein that are spaced at regular intervals along the length of the thick filament. The myosin cross bridges and C-protein bands are not drawn to scale and represent only a few of the cross bridges and C-protein bands in the thick filament. The lines distributed longitudinally on the surface of the thin filaments represent troponin molecules, again not drawn to scale. Nebulin and titin filaments are not shown to prevent the diagram from becoming overly complex. In this model, either μ- or m-calpain degrade nebulin and titin filaments on the surface of the myofibril at the point where they enter the Z-disk. This degradation severs the attachments of Z-disk proteins to the remainder of the myofibril, releasing Z-disk proteins such as α-actinin and also releasing thick and thin filaments from the surface of the myofibril. After the release of one "layer" of filaments by the calpains, the remaining myofibril is narrower by two thick filaments and two thin filaments but is otherwise unchanged and remains functionally capable of contraction. The released filaments can reassemble back onto the surface of the myofibril, or additional degradation of troponin and tropomyosin on the thin filament and of C-protein on the thick filament (these proteins are all rapidly cleaved by the calpains) will result in dissociation of the thick and thin filaments to myosin and actin molecules, respectively. The actin and myosin molecules and the polypeptide fragments produced by calpain degradation of titin, nebulin, desmin, troponin, tropomyosin, and C-protein can be ubiquitinated and degraded to amino acids by the proteasome and cellular peptidases or perhaps also directly degraded by lysosomal cathepsins.](http://physrev.physiology.org/)

![Diagram](http://physrev.physiology.org/)
the myofibril, a process likely mediated by the calpains (82, 138, 153, 478), and 2) degradation of the released molecules and polypeptide fragments to amino acids/small peptides, a process likely mediated by the proteasome (Fig. 8; Refs. 82, 100, 138). Rates of muscle wasting are often estimated by measuring release of free amino acids such as tyrosine or 3-methylhistidine; the latter amino acid originates entirely from actin and myosin in muscle (there are other sources of 3-methylhistidine in animals, but actin and myosin are the only sources in muscle). Such measurements estimate the rate of degradation of cytoskeletal (and other) polypeptides to amino acids (i.e., the proteasome contribution) and do not include the effects of the calpains, because the calpains do not degrade proteins to amino acids. Moreover, free amino acids are also produced by degradation of the sarcoplasmic proteins, which is probably due largely to the proteasome or lysosomal cathepsins. It seems likely that concerted action of the calpains and the proteasome is also involved in other calpain pathologies such as neuronal ischemia and traumatic injury (Table 12) where proteins that are known calpain substrates in vitro are degraded first to large fragments and then to amino acids. Several studies have used the antibody (379) that specifically labels the 150-kDa calpain degradation product of α-spectrin (fodrin) to detect calpain cleavage of spectrin in rat heart that has been reperfused after ischemia (491) or in postischemic gerbil hippocampus (379).

Second, although disruption of Ca²⁺ homeostasis in dystrophic muscle, ischemic areas, or in other calpain-related pathologies results in an increase in intracellular Ca²⁺ concentration, this increase is not sufficient to support calpain activity directly; intracellular free Ca²⁺ concentrations of 10–50 μM (μ-calpain; Table 3) or 400–800 μM (m-calpain; Table 3) would lead to immediate cell death regardless of any effect of the calpains. Hence, it seems likely that the increase in free Ca²⁺ concentration results in deregulation of one or more of the control mechanisms that normally regulate calpain activity in cells. Because the mechanisms that regulate calpain activity are still unknown, it is also unclear what the target(s) of this increase in free Ca²⁺ concentration are.

Third, measurements of dystrophic muscle, either from the mdx mouse (59, 423, 425) or human dystrophic muscle (223, 461), often indicate that calpain activity has increased (423, 425, 467), that calpastatin has been degraded (317), that expression of mRNAs for the calpains has increased (59, 467), or that the amount of calpain protein has increased (223, 423, 425) in this muscle. These measurements suggest that net calpain activity is increased (loss of calpastatin has the potential to effectively increase net calpain activity) in dystrophic muscle and in other “calpain pathologies.” Because the mechanisms used to regulate calpain activity in situ are still unknown, however, it is possible that calpain activity in diseased cells could increase substantially with no increase in amount of calpain protein (or decrease in amount of calpastatin protein). Indeed, based on estimates that <10% of the calpain in a muscle cell is active at any one time, it is unclear why additional calpain would have any effect on rate of substrate degradation unless the “extra calpain” disrupts the normal regulation of calpain activity.

Fourth, intramuscular injection of the synthetic calpain inhibitor leupeptin to mdx mice prevents the decrease in muscle fiber diameter characteristic in such mice; fiber diameters in the soleus and anterior tibialis muscles from the injected mice were even larger than those from the same muscles in a control line of mice. Hence, a protease inhibitor that inhibits the calpains can prevent loss of muscle mass in a dystrophic mouse (15). Calpain activities were 44–84% lower in the injected muscles than in untreated muscles from mdx mice. Because leupeptin does not inhibit proteasome activity, these results illustrate the importance of the calpain system as the initiating step in myofibrillar protein turnover. Inhibiting the first step in release of myofilaments would prevent loss of myofibrils but would not necessarily affect the release of amino acids from the sarcoplasmic proteins. Although a large number of studies have shown that calpain inhibitors prevent or retard proteolytic degradation in the pathologies listed in Table 12, these studies have usually used synthetic inhibitors that are not completely specific for the calpains. Calpeptin, a synthetic calpain inhibitor, prevents degradation of troponin I in hypoxic neonatal cardiomyocytes (214). Intracellular protease activity increased by 80% after hypoxia in cultured rat myocytes, and this protease activity and hypoxic cell death were inhibited by E-64 and calpain inhibitor I (173).

The relative roles of the calpains and the proteasome in muscle protein turnover have been debated for a number of years. A recent study using microarray assays found that expression of two genes, MuRF1 (muscle ring finger 1) and MAFbx (muscle atrophy F-box), was upregulated in three models of muscle wasting: denervation, immobilization, and unweighting (43). A similar microarray assay independently found that expression of MuRF1 was upregulated in fasting, uremic, and diabetic mice (140). The proteins encoded by MuRF1 and MAFbx are both skeletal muscle-specific ubiquitin ligases, implicating the proteasome in these models of muscle wasting. Expression of neither of the calpains nor of any of the subunits of the proteasome itself was notably upregulated as measured in these microarray assays. As indicated previously, however, the calpains are present in large amounts in skeletal muscle, as is the proteasome (335). Hence, the amounts of the calpains and the proteasome do not need to change to increase the rate of muscle protein degradation; regulation of the activity of these two proteolytic systems needs to change. The rate at which polypeptides are ubiquitinated has been consid-
erated to be the rate-limiting step for proteasome degradation (164, 335). Hence, it would be expected that components of the ubiquitinating system would be upregulated in atrophying muscle with little or no change in expression of the proteasome or calpain subunits. It seems unlikely that the myofibrillar proteins are ubiquitinated while still assembled in the myofibrillar structure, and the proteasome clearly cannot degrade myofibrillar proteins until they have been disassembled from the myofibril (100, 413); hence, it is necessary that something dissociate these proteins from the myofibril before they can be degraded by either the proteasome or lysosomal proteases. The available evidence indicates that this “something” is the calpains (153, 154, 478). Because the calpains do not degrade proteins to amino acids, estimating rate of muscle protein turnover by measuring release of amino acids such as tyrosine or 3-methylhistidine does not relate directly to calpain activity.

The purported role of the calpains in Alzheimer’s disease (AD) is more complex than simple unregulated/excessive calpain activity in response to loss of Ca²⁺ homeostasis. AD is diagnosed by the presence of two brain lesions: 1) the extracellular amyloid plaques that contain deposits of the amyloid β-peptides, Aβ40 and Aβ42; and 2) intracellular neurofibrillary tangles that are composed principally of hyperphosphorylated tau, a microtubule-associated protein. The Aβ40 and Aβ42 peptides are produced by proteolytic cleavage of an amyloid precursor protein, β-APP. These proteolytic cleavages are mediated by a class of proteases called secretases: 1) α-secretase, which removes the large extracellular NH₂ terminus of APP; 2) β-secretase, which also removes the extracellular domain of APP; and 3) γ-secretase, which removes the COOH-terminal 60 or 58 amino acids of APP and together with the β-secretase cleavage, produces the Aβ40 or Aβ42 amyloid peptides. The α, β, and γ-secretases are not related to the calpains, so the calpains are not involved in Aβ peptide production (115). On the other hand, Ca²⁺ concentrations are elevated in AD brains (240), immunohistochemical studies indicate that m-calpain levels are increased in brains from AD patients (145, 464), and autolysis of μ-calpain to its 76- and 78-kDa forms is enhanced in brains from AD patients (380). It has been suggested that abnormalities in calpain activity may affect secretion of the β-amyloid peptides, possibly through the conversion of PKC to a constitutively active form by the calpains (321). The nature of such possible indirect effects of the calpains on β-amyloid secretion, however, is very poorly defined at present. The clearest involvement of the calpains in AD is calpain cleavage of p35, which activates the cyclin-dependent kinase 5 (CDK5), to a 25-kDa form (p25); the activated 25-kDa form of the kinase then causes prolonged activation of CDK5 (229). Tau is a substrate of CDK5, and p25 accumulates in brains of Alzheimer’s patients (237), suggesting that calpain-cleaved p25 is responsible for the hyperphosphorylation of tau in the intracellular neurofibrillary tangles observed in AD brains (401). Hyperphosphorylation makes tau, which normally is rapidly cleaved by the calpains (148), highly resistant to calpain degradation (248), so the neurofibrillary tangles in AD are resistant to calpain degradation. Finally, because the neurofilament proteins are excellent substrates of the calpains (Table 11), the calpains may have an important role in the necrotic neuronal death that accompanies AD. Diagnosis of AD involves structural analysis of brain tissue, and it is possible (even likely) that not all neurofibrillary tangles or β-amyloid deposits originate from the same pathway, adding an extra complicating factor in determining the role of the calpains in AD.

More than 75% of human cataract cases involve elevated Ca²⁺ concentrations, and cataract formation in young rat lens is probably one of the most compelling instances of a relationship between inappropriate calpain activity and tissue pathology (405). A wide variety of insults can cause a large influx of Ca²⁺ into the lens, with Ca²⁺ concentrations sometimes reaching 1,000 μM or more (316), a concentration sufficient to directly initiate proteolytic activity of m-calpain, which is the principal ubiquitous calpain in lens. Hence, in cataract formation, the increase in intracellular Ca²⁺ concentration is sufficient for direct activation of calpain activity, and it is not necessary to propose some deregulation of a regulatory mechanism. The m-calpain in lens cleaves the NH₂-terminal region of α-crystallin to remove 4–49 amino acids and cleaves 9 sites in the NH₂ terminus of β-crystallin, but does not degrade γ-crystallin. The truncated crystallins aggregate, are resistant to additional proteolysis, and form cataracts that scatter light. Crystallin fragments produced in vivo during cataract formation in young rats are identical in two-dimensional electrophoresis to the crystallin fragments produced by m-calpain in vitro. Lp82, a splice variant of calpain 3 described in section III, is present in young rat lens at approximately the same levels as m-calpain and degrades both α- and β-crystallins (316). Expression of Lp82 decreases with advancing age, however, so its role in cataract formation in older animals is uncertain. Although human lens also contain m-calpain, they do not contain Lp82 because of a stop codon inserted in exon 1 (316). The role of the calpains in human cataracts is not as well documented as it is in rat lens, although human lenses contain m-calpain and human crystallins are substrates for m-calpain in vitro.

The other “calpain pathologies” listed in Table 12 are more directly linked to alterations in Ca²⁺ homeostasis than AD or cataract formation, and the points made previously in the discussion of the muscular dystrophies apply more directly to these pathologies.
VII. SUMMARY AND CONCLUSIONS

A great deal of information has been obtained on the calpain system since the last comprehensive review in 1991 (76). The crystallographic structure of m-calpain shows that m-calpain at least, and probably also μ-calpain, is catalytically inactive in the absence of Ca$^{2+}$ because the three residues constituting the calpain catalytic triad, Cys, His, and Asn, are not sufficiently close in the Ca$^{2+}$-free state to assemble a functional active site. Hence, the calpains are not proenzymes in the sense that homolog, calpain 9, has been associated with gastric cancer. Suppression of expression of another calpain homolog, calpain 10, is associated with type 2 diabetes LGMD2A, and a mutation in the gene of another calpain one of these calpain homologs, calpain 3a, is the cause of Ca$^{2+}$-regulated in cells, many questions still remain. The high deal of new information on how activity of the calpains is does not have an EF-hand structure in the crystallographic structure of m-calpain, and it seems unlikely that this sequence binds Ca$^{2+}$ in the catalytic triad, Cys, His, and Asn, are not sufficient conditions for activity of the calpains that lack one or more of the catalytic residues indicates that the calpains have a function in addition to their proteolytic activity. In almost all instances, these calpain homologs are expressed in specific tissues, and in several instances, the calpain homolog does not have all three residues necessary for a catalytic triad. It is still unclear whether existence of calpains that lack one or more of the catalytic residues indicates that the calpains have a function in addition to their proteolytic activity. In almost all instances, however, these calpain homologs have been identified only as mRNAs, and the proteins encoded by these mRNAs have not been isolated. Hence, almost nothing is known about the catalytic properties of these calpain homologs, whether they function as monomers or require a cofactor for proteolytic or other activity, or whether they inhibit activity of calpastatin, the specific inhibitor of μ- and m-calpain. Disruption of the gene for one of these calpain homologs, calpain 3a, is the cause of LGMD2A, and a mutation in the gene of another calpain homolog, calpain 10, is associated with type 2 diabetes mellitus. Suppression of expression of another calpain homolog, calpain 9, has been associated with gastric cancer. The absence of information on properties of the proteins encoded by these mRNAs makes it difficult to infer how these calpain homologs are involved in these diseases.

Although the past 11–12 years have produced a great deal of new information on how activity of the calpains is regulated in cells, many questions still remain. The high Ca$^{2+}$ concentrations required for in vitro proteolytic ac-

tivity of the calpains have been a central problem in determining how calpain activity is regulated in cells, and it remains a problem today, 26 years after the first characterization of purified m-calpain. Technical difficulties have plagued attempts to determine how many Ca$^{2+}$ atoms are bound by the calpains, and which Ca$^{2+}$ atoms are involved in inducing catalytic activity. The “EF-hand” predicted from the amino acid sequence at the boundary of domains II/III in the 80-kDa calpain subunit does not have an EF-hand structure in the crystallographic structure of m-calpain. The fifth EF-hand in domains IV and VI is involved in association of the 28- and 80-kDa subunits and does not bind Ca$^{2+}$ in the μ- and m-calpain molecules. Ca$^{2+}$-binding and crystallographic studies have shown that the fourth EF-hand in domains IV and VI also binds Ca$^{2+}$ very weakly and has little influence on the Ca$^{2+}$-dependent proteolytic activity of the calpains, leaving 6 (or 7) of the 11 predicted (from amino acid sequence) EF-hands as Ca$^{2+}$-binding sites. Crystallographic and limited proteolytic digestion studies, however, indicate that the conformation of domains IV and VI changes very little when Ca$^{2+}$ is added, raising questions as to whether Ca$^{2+}$ binding at some site in addition to or other than the EF-hands is involved in inducing proteolytic activity of the calpains. Recent studies have indicated that domain IIa/IIb (in the crystallographic structure) of the 80-kDa subunit has very weak, Ca$^{2+}$-dependent proteolytic activity by itself and that this domain binds one Ca$^{2+}$ atom each in two peptide loops that provide seven or eight coordinations to the Ca$^{2+}$ atom. The Ca$^{2+}$ concentrations required for the weak activity of domain IIa/IIb, like the Ca$^{2+}$ concentrations required for the proteolytic activity of the entire calpain molecules, are much higher than the Ca$^{2+}$ concentrations that exist in living cells. The recent finding that μ- and m-calpain are phosphorylated at multiple sites on the calpain molecule may provide a new approach to studying the Ca$^{2+}$-requirement problem. It is now clear that calpastatin binds to the μ- and m-calpain molecules at three sites and that binding to at least two of these sites is Ca$^{2+}$ dependent. Two single-chain calpains that have been recently isolated are not inhibited by calpastatin, suggesting that removal of one of the three calpastatin-binding sites may weaken calpastatin binding to a point where it can no longer inhibit calpain activity effectively. The physiological significance of the eight or more calpastatin isoforms that are produced from the single calpastatin gene is still unclear. That knocking out expression of the 28-kDa subunit is embryonically lethal in mice implies that catalytic activity of μ- and m-calpain requires the 28-kDa subunit, although recent studies have indicated that the 28-kDa subunit can be dissociated from the 80-kDa subunit and that this dissociation occurs much
more readily after the calpains have undergone autolysis. Additional studies are needed to understand the physiological significance of autolysis and the role of the 28-kDa subunit in calpain function.

The physiological functions of the calpain system remain poorly defined, despite a great deal of work showing that the calpains are involved in functions as diverse as cytoskeletal/plasma membrane attachments, cell motility, signal transduction pathways including activation of some signaling molecules and assembly of focal adhesions, some aspects of the cell cycle and regulation of gene expression, and some but not all apoptotic pathways, in addition to roles in other processes such as long-term potentiation. Some additional reported functions of the calpain were not discussed in this review simply because of space and time limitations, and because the evidence supporting these functions is not yet conclusive. It seems likely that the calpains are capable of cleaving a large number of different “physiological substrates” but that not all calpain-susceptible molecules may be cleaved in a given cellular event; the molecules that are degraded may depend on the signals that the cell has received, the location of the calpains (and possibly calpastatin), and possibly other factors. This conclusion is supported by the finding that overexpressing calpastatin can prevent extension of lamellipodia and degradation of ezrin, but has no effect on degradation of talin, PKC, ppFAK25, and other calpain-sensitive polypeptides in NIH3T3 cells. Additional information on the properties of the recently identified calpain homologs will be needed before the physiological functions of the calpain system are fully understood. Finally, it is not clear why cells would use proteolytic cleavage to regulate cytoskeletal/plasma membrane interactions or signal transduction pathways. It would seem much more efficient for cells to use some easily reversible procedure such as phosphorylation rather than destroying an entire polypeptide and then having to resynthesize it. Perhaps the polypeptide fragments produced by limited calpain cleavage have some physiological function essential to the cytoskeletal/plasma membrane remodeling or to the particular signal transduction event that cannot be performed by simple phosphorylation/dephosphorylation.

The calpain system has been implicated in a number of diseases or pathological conditions. In three instances, abnormalities in expression of the genes for the newly identified calpain homologs, calpain 3a, calpain 9, and calpain 10, are related to LGMD2A, gastric cancer, and type 2 diabetes, respectively. The other “calpain pathologies” involve loss of Ca\(^{2+}\) homeostasis by cells/tissues and inappropriate/excessive degradation of proteins that are known calpain substrates. It is widely assumed that loss of Ca\(^{2+}\) homeostasis leads to increases in intracellular Ca\(^{2+}\) concentrations that “activate” the calpains. Based on the Ca\(^{2+}\) requirements of the calpains in in vitro assays, however, the increases in intracellular Ca\(^{2+}\) concentrations are not sufficient to initiate proteolytic activity of the calpains directly. It seems more likely that loss of Ca\(^{2+}\) homeostasis results in disruption of some physiological process that regulates calpain activity in cells and that disruption of this process results in unregulated calpain activity. Most of the studies implicating the calpains in situations involving loss of Ca\(^{2+}\) homeostasis and degradation of calpain-susceptible molecules have used synthetic inhibitors of the calpains. Because none of the presently available synthetic calpain inhibitors is completely specific for the calpains, the ability of these inhibitors to prevent inappropriate protein degradation does not provide conclusive proof that the calpains are the cause of the degradation. The use of calpastatin or calpastatin peptides, which are specific for inhibiting the calpains, will provide more definitive evidence on the role of the calpain system in these pathologies related to loss of Ca\(^{2+}\) homeostasis.

We are very grateful to a number of individuals who read sections of the draft of this review: to Dr. Zongchao Jia, Queens University, Kingston, Canada, for his comments on the part on structure of the calpains; to Dr. Masatoshi Maki, Nagoya University, Japan, for his comments on the section on calpastatin; to Dr. Hiroyuki Sorimachi, University of Tokyo, Japan, for reviewing some of his preliminary results on the other members of the calpain family; to Drs. Joan Fox and Sucheta Kulkani from the Cleveland Clinic Foundation, Cleveland, OH, for reviewing the section of calpains in signal transduction pathways; to Dr. Ronald L. Mellgren, Medical College of Ohio, Toledo, OH, for reading the entire section on physiological functions of the calpains; and to Dr. John S. Elce, Queens University, who read nearly the entire draft of the manuscript and began his comments with “phew!”, our first indication that someone might actually be able to read the entire thing. Any omissions or errors are, of course, the responsibility of the authors. We also thank Janet Christner for assistance in assembling the manuscript into a readable form. We apologize to those individuals whose work was not cited; we have emphasized references that have appeared since 1990 when the last major review of the calpains was published. Medline lists 2,534 references for the calpains since 1990, and it was necessary to restrict the number of citations severely.

The work described in this review that originated from the authors’ laboratory was supported by grants from the National Institutes of Health; the Muscular Dystrophy Association; the American Heart Association, Arizona Affiliate; and National Research Initiative Competitive Grants 2001–35503–10776 and 2002–35206–11630.

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10.8475/technical-v9.1/issue-ix.1.1.1.21.1.21

11. THE CALPAIN SYSTEM

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The present document contains text that is not easily readable due to the formatting issues. It appears to be a collection of scientific references, possibly from a research paper or a review article in the field of biochemistry. The text seems to be discussing various aspects of calpains, including their expression patterns, functions, and interactions with other proteins.

The text is rich with scientific terms and references to various studies, indicating a comprehensive discussion of the topic. However, due to the formatting issues, it is challenging to extract specific details or key findings from the text. The references cited in the text are from reputable journals and conferences, suggesting a well-supported discussion on the subject.

In summary, this document provides a detailed exploration of calpains, their roles, and interactions, which is typical in scientific literature on proteinases and their functions in cellular processes. The references are essential for anyone looking to delve deeper into the subject or verify the information presented.


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