Effect of Monoclonal Antibodies Specific for the 28-kDa Subunit on Catalytic Properties of the Calpains

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Jinyang Cong, Valery F. Thompson, and Darrel E. Golli
From the Muscle Biology Group, University of Arizona, Tucson, Arizona 85721

Nine monoclonal antibodies (mAbs) specific for the 28-kDa subunit common to \( \mu \)- and m-calpains have been assayed for their effects on \( \mu \)- and m-calpains. All nine react with the COOH-terminal part (domain VI) of the 28-kDa subunit, and all nine affect the Ca\(^{2+} \) concentration required for autolysis of m-calpain, but have little effect on the Ca\(^{2+} \) concentration required for autolysis of \( \mu \)-calpain. None of the nine affect the specific proteolytic activity of \( \mu \)- or m-calpain. Two of the mAbs, 5B9 and 5B3, were selected for further study. mAb 5B9 decreased the Ca\(^{2+} \) concentration required for autolysis to one-fifth of that required in its absence; sequencing of chymotryptic fragments showed that the epitope for mAb 5B9 is between amino acid residues 92 and 104 of the 28-kDa subunit. mAb 5B3 increased the Ca\(^{2+} \) concentration required for autolysis; the epitope for mAb 5B3 is located between amino acid residues 148 and 178 of the 28-kDa subunit, which is the region that contains the first EF-hand Ca\(^{2+} \)-binding sequence in this subunit. Although it increases the Ca\(^{2+} \) concentration required for autolysis, mAb 5B3 has no effect on the Ca\(^{2+} \) concentration required for proteolytic activity of m-calpain, and unautolyzed m-calpain is not a proenzyme. That all nine mAbs react with domain VI and not with the NH\(_2\)-terminal domain V of the 28-kDa subunit suggests that domain VI (and not domain V) is involved in autolysis, contrary to the view that phosphatidylinositol lowers the Ca\(^{2+} \) concentration required for autolysis by binding to domain V.

The Ca\(^{2+} \)-dependent proteinase (calpain) system consists of at least four proteins: 1) \( \mu \)-calpain, a proteinase requiring 3–50 \( \mu \)M Ca\(^{2+} \) for half-maximal activity; 2) m-calpain, a proteinase requiring 400–800 \( \mu \)M Ca\(^{2+} \) for half-maximal activity; 3) calpastatin, a protein that specifically inhibits the two calpains; and 4) a protein identified thus far only as a cDNA having sequence homology to \( \mu \)- and m-calpains and found exclusively in skeletal muscle, skm-calpain (1, 2). In the form in which they are isolated from most cells, \( \mu \)- and m-calpains each contain two subunit polypeptides of 80 and 28 kDa (3). The 28-kDa subunit is identical in \( \mu \)- and m-calpains; the 80-kDa subunits from the two molecules differ, although they share ~50% sequence similarity (3). The calpain system has been found in every vertebrate cell that has been carefully examined for its presence (1, 4), has been isolated in a protein form from Drosophila (5, 6), and has been identified as a cDNA in the parasitic trematode Schistosoma mansoni (7). Despite its widespread presence, the physiological function of the calpain system and the mechanism by which its activity is regulated in living cells remains unknown.

It has been proposed that \( \mu \)- and m-calpains are inactive proenzymes in the state in which they are normally synthesized from cells and that in the presence of Ca\(^{2+} \), these proenzymes autolyze to produce catalytically active proteinases (8, 9). The Ca\(^{2+} \) concentration required for autolysis of the calpains, however, is slightly greater than that required for proteolytic activity (1, 10–12) and is much higher than the 100–800 nM free Ca\(^{2+} \) concentration in living cells (13–15). In \( \textit{in vitro} \) assays, certain phospholipids, with phosphatidylinositol and PIP\(_{2}\), being the most effective (16–18), lower the Ca\(^{2+} \) concentration required for autolysis of the calpains. Even in the presence of phosphatidylinositol, however, the Ca\(^{2+} \) concentration required for autolysis of \( \mu \)-calpain is reduced only from 50–150 to 1–50 \( \mu \)M and that required for autolysis of m-calpain is lowered from 550–800 to 90–400 \( \mu \)M (10, 17). The Ca\(^{2+} \) concentration required for autolysis of m-calpain in the presence of phosphatidylinositol is just above the free Ca\(^{2+} \) concentration in cells, and if \( \mu \)-calpain binds to phosphatidylinositol or PIP\(_{2}\) \( \textit{in vitro} \), there may be mechanisms that could bridge this difference and allow brief periods of \( \mu \)-calpain autolysis (e.g. transient high Ca\(^{2+} \) concentrations, etc.). The Ca\(^{2+} \) concentration required for autolysis of m-calpain, however, even in the presence of phosphatidylinositol, is much higher than would ever occur in living cells, and m-calpain activity cannot be regulated by association with phosphatidylinositol and subsequent autolysis.

Despite the uncertainty concerning the role of autolysis in calpain activity, it is clear that the calpains autolyze rapidly when incubated with sufficiently high Ca\(^{2+} \) concentrations; that during the first 1–2 min, such autolysis has little effect on the specific proteolytic activity of the calpains (19); and that autolysis lowers the Ca\(^{2+} \) concentration required for half-maximal proteolytic activity of the calpains in \( \textit{in vitro} \) assays (from 3–50 to 0.5–2.0 \( \mu \)M for \( \mu \)-calpain and from 400–800 to 50–100 \( \mu \)M for m-calpain; see Ref. 1). Moreover, cellular events associated with calpain activity, such as platelet activation, are accompanied by calpain autolysis (20–22). Therefore, it seems likely that autolysis has some role in calpain function in cells.

It has been suggested that the NH\(_2\)-terminal 50 amino acids of the 28-kDa subunit are responsible for binding phospholipids to the calpains because a "trimmed" m-calpain lacking these 50 amino acids does not respond to phosphatidylinositol (23). Although the catalytic site for both \( \mu \)- and m-calpains is in the

\(^{1}\) The abbreviations used are: PIP\(_{2}\), phosphatidylinositol 4,5-biphosphate; mAbs, monoclonal antibodies; FITC-casein, fluorescein isothiocyanate-labeled casein; PAGE, polyacrylamide gel electrophoresis.

\(^{2}\) V. F. Thompson and D. E. Golli, unpublished results.
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80-kDa subunit (1, 3), removal of the 28-kDa subunit destroys all catalytic activity of the calpains, and it has been difficult to recover more than a small percentage of the original activity during reconstitution experiments (24). To obtain additional information on the role of the 28-kDa subunit in calpain autolysis and activity, we have produced mAbs specific for this subunit and have assayed the effects of these mAbs on the catalytic properties of the calpains. All mAbs we have tested thus far react with the COOH-terminal 170 amino acids of the 28-kDa subunit (domain VI), and none of them affect the specific proteolytic activity of the calpains. However, eight mAbs reduce and one mAb increases the Ca^{2+} concentration required for autolysis of m-calpain, although they have little effect on the Ca^{2+} concentration required for autolysis of μ-calpain.

EXPERIMENTAL PROCEDURES

Protein Purification and Enzyme and Autolysis Assays—The μ- and m-calpains used for assays of proteolytic activity, for autolysis, or for eliciting mAbs were prepared from bovine skeletal muscle (19, 25). The third calpain, skm-calpain, whose mRNA is present in bovine skeletal muscle, was not examined in this study. Proteolytic activity of the calpains was assayed for 30 min at 25 °C using FITC-casein as a substrate and fluorescence detection (28). Final conditions were 100 mM KC1, 100 mM Tris-HCl, pH 7.5, 2.5 mg of FITC-casein/ml, 5 mM 2-mercaptoethanol, and 0.1 mM free Ca^{2+} in the specified final volume concentration in a 125-μl final volume. FITC-casein was prepared according to Twining (27). Activity of autolyzed μ-calpain was measured specifically at 1.0 μM free Ca^{2+}, where unautolyzed μ-calpain is inactive; activity of autolyzed m-calpain was measured specifically at 150 μM free Ca^{2+}, where unautolyzed m-calpain is inactive; and total calpain activity was measured at 5.0 μM Ca^{2+}. Assays done in the presence of mAbs contained two controls: 1) a tube containing all the ingredients in the assay tubes including mAbs and Ca^{2+}, but with no calpain to ensure that the mAb preparations themselves have no proteolytic activity, and 2) tubes containing all the ingredients including mAbs with either 150 μM Ca^{2+} and unautolyzed m-calpain or 1 μM free Ca^{2+} and unautolyzed μ-calpain to ensure that the unautolyzed calpains have no proteolytic activity at these respective free Ca^{2+} concentrations. The usual controls with 1 mM EDTA and no added Ca^{2+} or with no calpain (26) were also included.

Autolysis in the presence or absence of mAbs was done at the same time as the m-calpain preparation. The selected mAb and calpain or mAb buffer and calpain were incubated for 5 h at room temperature in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA at a specific mAb/calpain molar ratio (the molecular mass of the mAbs was chosen as 150 kDa; the molecular mass of calpain was 110 kDa). Autolysis was initiated by adding enough Ca^{2+} to produce a specified free Ca^{2+} concentration and was allowed to proceed at 25 °C for 30 min at 100-μl final volume. Autolysis was stopped by adding EDTA to a final concentration that was 0.25 mM greater than the Ca^{2+} concentration used to induce autolysis; the final volume was 120 μl. Sixty μl of this mixture was used to assay for proteolytic activity, and the remaining 60 μl was used for SDS-PAGE, so autolysis patterns seen on SDS-PAGE can be compared directly with activity assays. Details of protein concentrations, etc. are given with the individual experiments.

Preparation and Characterization of Anti-28-kDa mAbs—Six-week-old female BALB/c mice were injected intraperitoneally with 50 μl murine ascites fluid in Ribi’s adjuvant 2 weeks later. Ten days before fusion, selected mice with the highest titer against m-calpain were boosted with 50 μg of purified m-calpain given intravenously. Fusion was done at a spleen/Ribi’s x 63 000 635 000 cell ratio of 10:1 using hypoxanthine/aminopterin/thymidine for selection. A total of nine cell lines selected from the 22 positive cell supernatants were used for ascites production. For reasons that are unclear to us, the fusions done in this experiment produced an unusually large percentage of mAbs that reacted specifically with the 28-kDa subunit. Subsequent use of this same protocol produced mAbs that recognized either the 28- or 80-kDa subunit.

mAbs were purified from mouse ascites fluid by precipitation at 45% ammonium sulfate saturation. The precipitated protein was dissolved; dialyzed against 10 mM Tris-HCl, pH 8.0; and eluted from a DEAE-Trisacryl M anion-exchange column with a 0–0.3 M NaCl gradient in 10 mM Tris-HCl, pH 8.0. The eluted fractions were monitored by using SDS-PAGE.

A mouse subisotyping panel (Bio-Rad) was used to determine the immunoglobulin subclass, and enzyme-linked immunosorbent assay was used to estimate the relative binding affinities for each mAb. Two-hundred ng of purified μ- or m-calpain in 100 μl of 50 mM carbonate buffer, pH 9.5, was added to each well in a 96-well microtiter plate. After incubation overnight at 4 °C, the plate was washed; 100 μl of 1.0 μM purified mAb was added to the first well of each row (12 rows); and 100 μl of 1.0 μM mAb serum in 1:100 diluted 10-fold was added successively to each of the following six wells in each row. The range of mAb concentrations tested was 10^{-6} to 10^{-12} M (wells 1–7). The last well (well 8) of each row contained 100 μl of buffer without antibody. The amount of mAb bound in each well was estimated by incubating with a secondary goat anti-mouse antibody coupled to horseradish peroxidase and measuring absorbance at 405 nm.

SDS-PAGE and Western Blotting—SDS electrophoresis was done on 10–20% gradient polyacrylamide gels (28) following the procedure of Laemmli (28). Both 7 × 8-cm minigels (0.76 mm thick) and 14 × 16-cm gels (1.5 mm thick) were used. Gels were stained with Coomassie Brilliant Blue R-250 and destained as described previously (26). Western blotting was done according to Towbin et al. (29). After transfer, the 0.2-μm nitrocellulose membrane was blocked by incubation with Tris-buffered saline, 2.5% gelatin for 1 h at room temperature. The nitrocellulose was washed and then incubated for 2 h at room temperature with the primary antibody (diluted 1:40–100) in Tris-buffered saline, pH 7.5, 5% fetal bovine serum, 0.2% 2-mercaptoethanol, and 0.1% Tween 20, 1% gelatin. The secondary antibody (biotinylated goat anti-mouse IgG or goat anti-mouse IgG + IgM conjugated with horseradish peroxidase). The biotinylated goat anti-mouse IgG samples where incubated for 0.5 h with a streptavidin-alkaline phosphatase conjugate. Color development was done by incubation for 5 min with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (or 4-chloro-1-naphthol for the horseradish peroxidase). Details are given with descriptions of the individual experiments.

Miscellaneous—Protein concentration was measured following the Folin-Lowry procedure (30) as modified by Gell et al. (31) or the Bradford procedure (32) with bovine serum albumin calibrated by Kjeldahl nitrogen analysis to produce calibration curves. Both procedures gave similar results. Gels were scanned densitometrically either with a Krakos Model SD 3000 densitometer at 595 nm connected to an IBM 9000 digital integrator or with a Molecular Dynamics Model 300A computing densitometer with a laser light source. Half-maximal Ca^{2+} concentrations were determined using an EDTA buffer and a dissociation constant of 1.2 × 10^{-8} M.

Materials—Isove’s modified Dulbecco’s medium plus 10% fetal bovine serum, 2 mM L-glutamine, 1% antibiotic/antimycotic solution, 0.2% Fungizone was filtered through a 0.22-μm filter to produce conditioned Culture Medium 1. Interleukin-1-secreting murine macrophage cell line J774A.1 cultured in Culture Medium 1 was harvested every 2–3 days and filtered through a 0.22-μm filter to produce a conditioned medium. Isov’s modified Dulbecco’s medium plus 10% fetal bovine serum, 2 mM L-glutamine, 1% antibiotic/antimycotic solution, 0.2% Fungizone supplemented with 15% conditioned medium from the J774A.1 cell line was used as Culture Medium II. The myeloma cell line P3X63Ag8.653 (The American Type Culture Collection) was grown in Culture Medium I supplemented with 0.13 mM 8-azaguanine to maintain hypoxanthine-guanine phosphoribosyltransferase negativity.

Hamster casein was purchased from United States Biochemical Corp.; acrylamide (99.9%), bisacrylamide (99.99%), and sodium dodecyl sulfate (99%) were from Schwarz/Mann; Tria (ultrapure, 99.8%) was from Amresco (Solon, OH); and fluorescein isothiocyanate, NaN_3, EDTA, and the protease inhibitors used in the calpain preparation (25) were from Sigma. All other chemicals were reagent-grade or purer.

RESULTS

Properties of Anti-28-kDa mAbs—The mAbs from all nine cell lines selected for ascites production were of the IgG class, and all reacted specifically with the 28-kDa subunit of both μ- and
m-calpains on Western blots (Fig. 1). As determined by enzyme-linked immunosorbent assay, the relative binding affinities of all nine mAbs were similar, with apparent $K_D$ values in the 0.1–10 nM range. Although it reacted weakly on Western blots (Fig. 1), the relative binding affinity of mAb 5Al1 was not significantly lower than that of the other mAbs and was similar to the binding of mAb 5B9. In general, binding seemed to be slightly stronger to μ-calpain than to m-calpain, although this was not studied in detail. SDS-PAGE analysis showed that chromatography on DEAE-Trisacryl M purified the anti-28-kDa mAbs effectively (see Figs. 3 and 6). None of the nine purified anti-28-kDa mAb preparations had any proteolytic activity when assayed with FITC-casein in either the presence or absence of Ca$^{2+}$ (data not shown). Incubation of the mAbs with either μ- or m-calpain for 6.5 h at 25 °C under the conditions described under "Experimental Procedures" had no effect on the polypeptide subunits of the mAbs or calpains (see also Figs. 3 and 6) and did not affect the specific proteolytic activity of the calpains (FITC-casein at 5 mM Ca$^{2+}$; see Figs. 2, 4, and 7).

Preliminary experiments showed that eight of the nine mAbs (2F7, 5D8, 1B12, 5H5, 4C9, 5A11, 5B9, and 1B10) reduced and one mAb (5B3) increased the Ca$^{2+}$ concentration required for autolysis of m-calpain. The eight anti-28-kDa mAbs that lowered the Ca$^{2+}$ concentration required for autolysis of m-calpain differed slightly in the amount of reduction they elicited; depending on the antibody, the Ca$^{2+}$ concentration required for autolysis was lowered to one-third to one-fifth of that required in the absence of any mAb. All nine mAbs had little or no effect (<20% change) on the Ca$^{2+}$ concentration required for autolysis of μ-calpain, and none of the nine anti-28-kDa mAbs had any effect on the specific proteolytic activity of μ- or m-calpain assayed at 5 mM Ca$^{2+}$. Two of the anti-28-kDa mAbs, 5B9 and 5B3, were selected for further study.

Effect of mAb 5B9 on Autolysis and Proteolytic Activity of m-Calpain—Incubation of m-calpain with a 1:1 molar ratio of mAb 5B9 lowered the Ca$^{2+}$ concentration required for a half-maximal rate of autolysis from 635 to 120 μM (Figs. 2 and 3). Reducing the mAb/calpain molar ratio to 1 mAb/3 m-calpain lessoned the effect (the Ca$^{2+}$ concentration required for a half-maximal rate of autolysis decreases from 635 to 410 μM), whereas increasing the mAb/m-calpain molar ratio to 2:1 did not elicit a further reduction in Ca$^{2+}$ concentration required for a half-maximal rate of autolysis (Fig. 2). Therefore, it seems that one mAb molecule reacts with one m-calpain molecule to cause the reduction in Ca$^{2+}$ requirement. SDS-PAGE indicated that the polypeptides resulting from autolysis were the same in the presence or absence of mAb 5B9; the 28-kDa subunit disappeared and its 18-kDa fragment appeared, whereas the 80-kDa subunit diminished slightly at higher Ca$^{2+}$ concentrations, with the appearance of small amounts of 34- and 22-kDa fragments (Fig. 3) that originated from the 80-kDa subunit (34).

When proteolytic activity of m-calpain was assayed after 5 h of incubation in the presence or absence of mAb 5B9 without the calpain first being autolyzed for 30 s, the Ca$^{2+}$ concentration required for half-maximal activity decreased slightly, from 550 to 375 μM (Fig. 4). This decrease was probably due to the mAb 5B9-induced increase in the rate of autolysis at Ca$^{2+}$ concentrations below 500 μM, so that during the assay for proteolytic activity, m-calpain was changed from an unautolyzed molecule with little activity below 300 μM to an autolyzed molecule nearly fully active at 300 μM Ca$^{2+}$. As discussed below, it is
significant that simply assaying proteolytic activity detects this effect of the mAb on autolysis.

Effect of mAb 5B3 on Autolysis and Proteolytic Activity of m-Calpain—Incubation of m-calpain with a 1:1 molar ratio of anti-28-kDa mAb 5B3 significantly increased the Ca\(^{2+}\) concentration required for a half-maximal rate of autolysis (Figs. 5 and 6). In the presence of mAb 5B3, a maximal rate of autolysis was not yet reached at 1000 \(\mu\)M Ca\(^{2+}\) under the conditions used in our experiments (Fig. 5), and much of the 28-kDa subunit remained unautolyzed after 30 s at 1000 \(\mu\)M Ca\(^{2+}\) (Fig. 6). In contrast to the results obtained with mAb 5B3, increasing the mAb/m-calpain molar ratio to 2:1 further decreased the rate of autolysis of m-calpain (Fig. 5). m-Calpain caused no detectable degradation of the IgG mAb molecule during the 30-s autolysis incubation period (Figs. 3 and 6), although calpain completely degrades casein at a 2:1 molar ratio of casein to calpain in 30 s.\(^2\)

When proteolytic activity of m-calpain was assayed after 5 h of incubation in the presence or absence of mAb 5B3 without Fig. 4. Effect of mAb 5B9 on Ca\(^{2+}\) concentration required for proteolytic activity of m-calpain. After incubation for 5 h in the absence or presence of mAb 5B9, m-calpain or mAb 5B9/m-calpain (1:1 molar ratio) was added to FITC-casein, and proteolytic activity was measured at the free Ca\(^{2+}\) concentrations indicated (the calpain was not autolyzed for 30 s before assaying for proteolytic activity). Assays for proteolytic activity contained 13.5 \(\mu\)g of m-calpain and 18.3 \(\mu\)g of mAb 5B9 and were done under the conditions described under "Experimental Procedures."

the calpain first being subjected to autolysis for 30 s, the Ca\(^{2+}\) concentration required for a half-maximal rate of proteolytic activity remained unchanged at \(~530\ \mu\)M (534 and 527 \(\mu\)M in the absence and presence of mAb 5B3, respectively) (Fig. 7). SDS-PAGE, however, showed that the calpain had autolyzed only slightly at 500 \(\mu\)M Ca\(^{2+}\), even though it was nearly half-maximally active proteolytically at this Ca\(^{2+}\) concentration (Fig. 7). The SDS-PAGE shown in Fig. 7 was done on samples
that had been incubated for 30 min at 25 °C, the same conditions used in the assay for proteolytic activity. Therefore, the detectable in the presence of mAb 5B3, even after 30 min at 25 °C in the presence of mAb 5B3 (Fig. 7, mAbHC and mAbLC, heavy and light chains of mAb 5B3, respectively.

![Graph showing Effect of Monoclonal Antibodies on the Calpains](attachment:image.png)

Fig. 6. SDS-polyacrylamide gels showing effect of mAb 5B3 on Ca²⁺ concentration required for autolysis of m-calpain. Autolysis was done for 30 s in the absence (upper) or presence (lower) of mAb 5B3 as described under "Experimental Procedures." A molar ratio of 2 mAb to 1 m-calpain was used in this experiment. Each lane contains 15 μg of m-calpain (upper) or 15 μg of m-calpain and 43 μg of mAb 5B3 (lower). mAbHC and mAbLC, heavy and light chains of mAb 5B3, respectively.

that had been incubated for 30 min at 25 °C, the same conditions used in the assay for proteolytic activity. Therefore, the extent of autolysis observed in these samples was the amount of autolysis that had occurred by the end of the 30-min assay period and was greater than that observed after 30 s at 25 °C (compare gels in Figs. 6 and 7). Densitometric analysis showed that autolysis (disappearance of the 28-kDa subunit) was not detectable in the presence of mAb 5B3, even after 30 min at 25 °C, until the Ca²⁺ concentration exceeded 400 μM. At 500 μM free Ca²⁺, nearly 60% of the 28-kDa subunit remained after 30 min at 25 °C in the presence of mAb 5B3 (Fig. 7, right gel), whereas only 20% of the 28-kDa subunit remained under these same conditions in the absence of mAb 5B3 (left gel). Hence, mAb 5B3 did not inhibit autolysis, but rather increased the Ca²⁺ concentration required to attain a given rate of autolysis. Incubation with m-calpain for 30 min at 25 °C resulted in slight degradation of the mAb light chain (Fig. 7).

It could be argued that the small amount of autoxylized m-calpain in 500 μM Ca²⁺ is nevertheless sufficient for a half-maximal rate of proteolytic activity. That mAb 5B3 increased the Ca²⁺ concentration required for autolysis but had no effect on the Ca²⁺ concentration required for proteolytic activity, however, indicates that unauxolized m-calpain is an active proteinase with a specific activity similar or identical to autoxylized m-calpain. The results in Figs. 3 and 4 with mAb 5B9 show that this assay is sufficiently sensitive to detect the effects of autolysis on proteolytic activity.

Mapping the Epitopes of mAbs 5B9 and 5B3—When the nine anti-28-kDa mAbs were incubated with autoxylized μ- or m-calpain after separation by SDS-PAGE and transfer to a nitrocellulose membrane, all nine reacted with the 18-kDa fragment produced by autolysis of the 28-kDa subunit (see Fig. 8 for blots with mAbs 5B9 and 5B3). Hence, the epitopes for these mAbs, which alter the Ca²⁺ concentration required for autolysis of m-calpain, are on the COOH-terminal part of the 28-kDa subunit and not in the NH₂-terminal 50 amino acids of this subunit, where phosphatidylinositol/phospholipids have been proposed to react with the calpains. Additional degradation of the 18-kDa fragment using chymotrypsin indicates that mAbs 5B9 and 5B3 reacted with different regions within the 18-kDa fragment (Fig. 8). The epitope for mAb 5B9, which decreases the Ca²⁺ concentration required for autolysis, is in residues 92–104 of the amino acid sequence of the 28-kDa subunit (Fig. 8 and Table 1), at the very NH₂-terminal part of the 18-kDa autolytic fragment and at the boundary between domains V and VI (this boundary has been assigned to amino acids 100–101). The epitope for mAb 5B3, which increases the Ca²⁺ concentration required for autolysis, is in residues 148–178 of the amino acid sequence of the 28-kDa subunit (Fig. 8 and Table 1). The amino acid sequence for the first predicted EF-hand Ca²⁺-binding region in domain VI occurs at amino acids 152–163, so the epitope for mAb 5B3 is at or very close to the first predicted EF-hand Ca²⁺-binding region. Site-directed mutagenesis studies (35) on the 28-kDa subunit of rabbit calpain have indicated that this first EF-hand Ca²⁺-binding site does indeed bind Ca²⁺.

**DISCUSSION**

The results of this study support three conclusions about the calpains. First, the calpains are not proenzymes that require autolysis to become proteolytically active. We have shown that by careful selection of Ca²⁺ concentration (between 10 and 40 μM free Ca²⁺ for bovine skeletal muscle μ-calpain), it is possible to detect significant proteolytic activity of unauxolized μ-calpain (10). Although this activity is <50% of maximal activity attained at higher Ca²⁺ concentrations, it originates entirely from unauxolized μ-calpain and occurs because the Ca²⁺ concentration required for autolysis is slightly higher than that required for proteolytic activity (1, 11, 12). For m-calpain, however, the Ca²⁺ concentrations required for autolysis and for proteolytic activity are so close that it is difficult to find a free Ca²⁺ concentration that supports significant proteolytic activity without also resulting in significant autolysis (10). mAb 5B3 significantly inhibits autolysis of m-calpain without affecting the Ca²⁺ requirement for its proteolytic activity and allows these two properties of m-calpain to be distinguished.

Although the calpains are not proenzymes in the sense that they require autolysis before being proteolytically active, several studies using incubation with a Ca²⁺ ionophore, such as A23187, in the presence of millimolar extracellular Ca²⁺ concentrations to stimulate calpain activity have observed autolysis of intracellular calpain under these conditions (see Ref. 36 for a study on erythrocytes). These experiments are difficult to interpret because the intracellular Ca²⁺ concentration in the presence of a Ca²⁺ ionophore probably increases to a level much higher than the 500–800 nM normally observed during stimulation of cells (13–15). A clearer example of the relationship
Assays contained 9 pg of m-calpain and, when present, 12.2 pg of mAb 5B3 and were done under the conditions described under "Experimental Procedures." The remaining 5.0 pl of the 5-h incubation mixture was incubated at 25 °C for 30 min (same conditions as for the proteolytic assay) on SDS-PAGE were done and the activity was measured at the free Ca2+ concentrations indicated (the m-calpain was not autolyzed for 30 s before assaying for proteolytic activity). Assays contained 9 pg of m-calpain and, when present, 12.2 pg of mAb 5B3 and were done under the conditions described under "Experimental Procedures." The remaining 5.0 pl of the 5-h incubation mixture was incubated at 25 °C for 30 min (same conditions as for the proteolytic assay) in the absence of casein and was then loaded onto an SDS-polyacrylamide gel. Consequently, the assays for proteolytic activity (graph) and SDS-PAGE were done on the same samples. Protein concentrations on the gel were 9 pg of m-calpain, 12.2 pg of mAb 5B3 (when present), and 20 pg of myofibril standard. MF, bovine skeletal muscle myofibril standard; mAbHC and mAbLC, heavy and light chains of mAb 5B3, respectively.

between autolysis and calpain activity occurs in platelets, where activation by thrombin or other agonists that are involved in platelet activation in vivo results in cleavage of talin and filamin, two calpain-susceptible proteins, and autolysis of platelet calpain (20–22). Calpain autolysis in platelets, however, occurs only when thrombin-activated platelets are stirred to promote aggregation and does not occur in unstirred platelets, even though other calpain-associated events such as prothrombinase release occur in the absence of stirring (37–39). Moreover, it has been reported that neutrophil calpain does not require autolysis for activation (40).

Consequently, it seems likely that calpain autolysis has some role other than activation. For example, the peptides released during calpain autolysis may have a physiological function analogous to other situations such as the proteolytic conversion of fibrinogen to fibrin, where the peptides released act as vasoconstrictors. Indeed, it has been reported that the peptides released during calpain autolysis have weak chemotactic effects on neutrophils (41, 42). Hence, there may be instances when calpain activity occurs without autolysis and other situations in which calpain activity is accompanied by autolysis depending on the physiological requirements of the cell.

Second, this study and earlier studies (10, 11, 16–18, 23) indicate that the 28-kDa subunit of the calpains has a role in calpain autolysis, although the exact nature of this role remains unknown. None of the anti-80-kDa mAbs that we have obtained thus far have any effect on the Ca2+ concentration required for autolysis of the calpains. Also, a report describing characterization of 15 mAbs that reacted with the 80-kDa subunit of m-calpain did not mention any effects on autolysis (43). Mellgren and Lane (44) found that two anti-28-kDa mAbs they obtained both reduced the specific activity of μ- or m-calpain and reduced the Ca2+ concentration required for half-maximal proteolytic activity of m-calpain from 807 to 477 μM. Although Mellgren and Lane did not report any effect of their anti-28-kDa mAbs on the Ca2+ concentration required for autolysis, it seems possible that the reduction they observed in Ca2+ concentration required for proteolytic activity was due to a reduction in the Ca2+ concentration required for autolysis, similar to the situation we observed in the experiments summarized in Fig. 4.

Our anti-28-kDa mAbs all recognize the 18-kDa fragment produced by autolysis of the 28-kDa subunit of the calpains. The anti-28-kDa mAbs of Mellgren and Lane (44) recognized a tryptic fragment of the 28-kDa subunit, and we have found that tryptic digestion of either μ- or m-calpain removes the NH2-terminal 60 amino acids from the 28-kDa subunit and leaves a stable 24-kDa polypeptide.2 Hence, it is probable that Mellgren and Lane’s anti-28-kDa mAbs also recognize the COOH-terminal domain VI of the 28-kDa subunit. The epitopes for mAbs 5B9 and 5B3, which affected the Ca2+ concentration required for autolysis in opposite ways, are near the NH2 terminus of the 18-kDa subunit of autolyzed m-calpain. The first predicted EF-hand Ca2+-binding site in the 28-kDa subunit is also near the NH2 terminus of the 18-kDa fragment (the NH2 terminus is amino acid residue 92, and the first predicted EF-hand Ca2+-binding site is residues 152–163; see Ref. 3), suggesting that this particular Ca2+-binding site may be involved in the Ca2+ binding event(s) associated with inducing autolysis (1).

Although removal of the NH2-terminal 50 amino acids from the 28-kDa subunit of m-calpain (the effect of removal of the NH2-terminal 50 amino acids from the 28-kDa subunit of μ-calpain has not been reported) eliminates the ability of phosphatidylinositol to reduce the Ca2+ concentration required for autolysis (23), this result does not necessarily mean that phosphatidylinositol/phospholipid reacts with this part of the

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**Fig. 7. Effect of mAb 5B3 on Ca2+ concentration required for proteolytic activity of m-calpain.** Ten μl of m-calpain was incubated for 5 h in the absence or presence of mAb 5B3. After incubation, 5.0 μl of m-calpain or mAb 5B3/m-calpain was added to FITC-casein, and proteolytic activity was measured at the free Ca2+ concentrations indicated (the m-calpain was not autolyzed for 30 s before assaying for proteolytic activity). Assays contained 9 pg of m-calpain and, when present, 12.2 pg of mAb 5B3 and were done under the conditions described under "Experimental Procedures." The remaining 5.0 μl of the 5-h incubation mixture was incubated at 25 °C for 30 min (same conditions as for the proteolytic assay) in the absence of casein and was then loaded onto an SDS-polyacrylamide gel. Consequently, the assays for proteolytic activity (graph) and SDS-PAGE were done on the same samples. Protein concentrations on the gel were 9 pg of m-calpain, 12.2 pg of mAb 5B3 (when present), and 20 pg of myofibril standard. MF, bovine skeletal muscle myofibril standard; mAbHC and mAbLC, heavy and light chains of mAb 5B3, respectively.
Effect of Monoclonal Antibodies on the Calpains

FIG. 8. Western blot analysis of mAbs 5B9 and 5B3 against fragments of 18-kDa polypeptide from autolyzed m-calpain. m-Calpain was autolyzed in 2.0 mM CaCl₂, 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.1% 2-mercaptoethanol for 2 min at 25 °C. The 18- and 78-kDa polypeptides produced by this autolysis were separated using a Superose 12 size-exclusion column in 1.0 mM KCl, 50 mM Tris-HCl, pH 7.5, at a ratio of 1:25 (w/w) chymotrypsin/18-kDa polypeptide for 10 min at 25 °C. The resulting chymotryptic fragments were separated on a 20% (acylamide) SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose for Western blot analysis. Lanes 1–4 were stained with naphthol blue black. MW Std 1 is Sigma SDS-7 molecular weight standard mixture containing polypeptides with Mr values of 66,000, 45,000, 36,000, 29,000, 24,000, 20,100, and 14,200. MW Std 2 is Sigma SDS-17S molecular weight standard mixture containing polypeptides with Mr values of 16,950, 14,440, 10,600, 8,160, 6,210, 3,460, and 2,510 (the smallest two fragments are not seen on this gel). 18kDa and 18kDa Digest are 8 µg each of the 18-kDa polypeptide before and after 10 min of chymotrypsin treatment, respectively. 5B3 and 5B9 lanes contain 9 µg of 10-min chymotrypsin-treated 18-kDa polypeptide incubated with mAbs 5B3 and 5B9, respectively. Arrowheads indicate the positions of Fragments 1–3 in the 18-kDa digest. See Table I for amino acid sequences of Fragments 1–3.

calpain molecule. We have found that although phosphatidylinositol has no effect on the Ca²⁺ concentration required for proteolytic activity of either autolyzed μ- or m-calpain, it inhibits the specific proteolytic activity of autolyzed p-calpain by 90% (10). Therefore, phosphatidylinositol reacts with autolyzed μ-calpain that has the NH₂-terminal 91 amino acids removed from the 28-kDa subunit. Approximately 75 µM phosphatidylinositol is required for maximal stimulation of autolysis in an assay containing 0.2 µM calpain, and phosphatidylinositol concentrations below 5 µM have little effect on the Ca²⁺ concentration required for autolysis in assays containing 0.2 µM calpain (17). Studies involving the effects of PIP₂ on the Ca²⁺ concentration required for autolysis of μ-calpain have used 25 µM PIP₂ in assays containing 0.006–0.010 µM calpain (16, 45). The critical micellar concentration for phosphatidylinositol or PIP₂ under the conditions used in calpain assays is not known, but we have found that 50 µM phosphatidylinositol is in the form of micelles under the conditions usually used to assay the effect of phosphatidylinositol on calpain autolysis. Consequently, it seems likely that the phosphatidylinositol/PIP₂ must be in a micellar form to reduce the Ca²⁺ concentration required for autolysis, and the stoichiometry of the phosphatidylinositol/calpain interaction is not known.

It has been proposed that the calpains are activated intracellularly by migrating to the cell membrane and by binding through the NH₂-terminal region of the 28-kDa subunit to a phosphatidylinositol/phospholipid moiety (8, 9). Our results involving mAbs specific for the 28-kDa subunit, however, suggest that domain VI and not the NH₂-terminal domain V region of the 28-kDa subunit affects the Ca²⁺ concentration required for autolysis and that the calpains do not require autolysis for activation anyway. Also, it is not clear how an in vitro phospholipid micelle/calpain interaction is related to a membrane/calpain interaction. Angli et al. (46) found that autolysis of calpain upon platelet activation occurs largely in the platelet cytosol and not in the particulate fraction of platelets. The autolyzed calpain is then translocated to the membrane or cytoskeletal sites. Therefore, the physiological significance of phosphatidylinositol/phospholipids in the regulation of calpain activity in living cells remains unclear, although the 28-kDa subunit is evidently involved in autolysis of m-calpain.

Third, that the nine anti-28-kDa mAbs we tested affected the Ca²⁺ concentration required for autolysis of m-calpain but had little effect on the Ca²⁺ concentration required for autolysis of μ-calpain, even though they bound to the 28-kDa subunits of both calpains, suggests that interaction of the 28-kDa subunit with the catalytic 80-kDa subunit in μ-calpain differs from its interaction with the 80-kDa subunit in m-calpain. The nature of this difference is unclear. If phosphatidylinositol binds to the COOH-terminal domain VI of the 28-kDa subunit in both μ- and m-calpains, the different effects that phosphatidylinositol has on the specific proteolytic activities of autolyzed μ- and m-calpains (10) may also be due to this difference in the nature of the 28-kDa/80-kDa subunit interaction in the μ- and m-calpain molecules. That we and others (44) have been able to obtain mAbs that are specific for the calmodulin-like domain of the 28-kDa subunit and that do not recognize the calmodulin-like domain (domain IV) of the 80-kDa subunit in either μ- or m-calpain indicates that these domains differ, although they share amino acid sequence homology with calmodulin. None of our nine anti-28-kDa mAbs reacted with purified calmodulin in an enzyme-linked immunosorbent assay.

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TABLE I

| Polypeptide | Amino acid sequence |
|-------------|---------------------|
| Fragment 1  | A-Q-L-A-G-D-D-M-E-V |
| Human 28-kDa subunit | A-Q-L-A-G-D-D-M-E-V |
| Fragment 2  | V-A-V-M-D-S-D-T-T-G |
| Human 28-kDa subunit | V-A-V-M-D-S-D-T-T-G |
| Fragment 3  | K-Q-F-D-V-D-F-I-G-T |
| Human 28-kDa subunit | K-Q-F-D-T-D-R-S-G-T |

Numbers above the sequence of the human 28-kDa subunit refer to the position of that amino acid in the human calpain subunit. Residues that differ from the sequence of the 28-kDa subunit of human calpain are underlined. Position 183 in the pig and rabbit 28-kDa subunits also contains a V residue, the same as our bovine 28-kDa subunit does.

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REFERENCES

1. Goll, D. E., Thompson, V. F., Taylor, R. G., and Zalewski, T. (1992) BioEssays 14, 549-556
2. Ji, S. Q., Hancock, D. L., Bidwell, C. A., and Anderson, D. B. (1992) J. Anim. Sci. 70, Suppl. 1, 208
3. Suzuki, K. (1990) in Intracellular Calcium Dependent Proteolysis (Mellgren, R. L., and Mursuchi, T., eds) pp. 25-25, CRC Press, Inc., Boca Raton, FL
4. Wolfe, F. H., Srpacenko, A., McGee, K., and Goll, D. E. (1989) Life Sci. 45, 2093-2101
5. Pittér, M., and Friedreich, P. (1988) Biochem. J. 253, 467-473
6. Pittér, M., Stierandova, A., and Friedrich, P. (1992) Biochemistry 31, 8201-8206
7. Andreasen, K., Thom, T. D., and Strand, M. (1991) J. Biol. Chem. 266, 15085-15090
8. Mellgren, R. L. (1987) FASEB J. 1, 110-115
9. Suzuki, K., Inagaki, S., Emori, Y., Kawasaki, H., Minami, Y., and Ohno, S. (1987) FEBS Lett. 220, 271-277
10. Cong, J., Goll, D. E., Peterson, A. M., and Kapprell, H. P. (1989) J. Biol. Chem. 264, 10996-11003
11. Kuboki, M., Iashi, H., and Kazama, M. (1987) Biochim. Biophys. Acta 929, 164-172
12. Zimmerman, U.-J. P., and Schlepfer, W. W. (1991) Biochim. Biophys. Acta 1078, 192-198
13. Mark, P. W., and Maxfield, F. R. (1990) J. Cell Biol. 116, 43-52
14. Corp, A. N., Check, T. B., Moreton, B. B., Berriço, M. J., and Brown, K. D. (1989) Cell Regul. 1, 75-86
15. Kurobayashi, N., Harkins, A. B., and Baylor, S. M. (1993) Biophys. J. 64, 1934-1960
16. Saito, T. C., Miura, K., and Suzuki, K. (1991) Biomed. Biochim. Acta 50, 485-489
17. Ohta, S. A., and Hathaway, D. R. (1984) J. Biol. Chem. 259, 11627-11630
18. Pontremoli, S., Mioni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O., and Horecker, B. L. (1985) Biochim. Biophys. Res. Commun. 129, 388-395
19. Edmunds, T., Naganiz, P. A., Emori, Y., Thompson, V. F., and Goll, D. E. (1991) Biochim. Biophys. Acta 1079, 197-208
20. Iashi, H., Suzuki, Y., Horie, S., Nakagawa, M., and Kozama, M. (1992) Biochim. Biophys. Acta 1175, 37-43
21. Kuboki, M., Iashi, H., Horie, S., and Kozama, M. (1992) Biochem. Biophys. Res. Commun. 185, 1122-1127
22. Els, J. S., Sigmund, L., and Fox, M. J. (1989) Biochem. J. 261, 1039-1042
23. Imajoh, S., Kawasaki, H., and Suzuki, K. (1986) J. Biochem. (Tokyo) 99, 1281-1284
24. Kikuchi, T., Umeda, N., Sasaki, T., and Murachi, T. (1984) Arch. Biochem. Biophys. 234, 629-645
25. Goll, D. E., Kleese, W. C., Sloan, D. A., Shannon, J. D., and Edmunds, T. (1986) Cien. Biol. 11, 75-83
26. Wolfe, F. H., Sato, S. K., Goll, D. E., Kleese, W. C., Edmunds, T., and Dup-erret, S. M. (1989) Biochim. Biophys. Acta 998, 236-250
27. Twining, S. S. (1984) Anal. Biochem. 143, 30-34
28. Kosem, U. K. (1970) Nature 227, 680-685
29. Towbin, H., Staeelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 186, 265-275
31. Goll, D. E., Horecker, B. L., and Bray, R. W. (1964) J. Food Sci. 29, 608-614
32. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
33. LeGendre, N., and Matsudaira, P. (1989) in A Practical Guide to Protein and Peptide Purification and Microsequencing (Matsudaira, P. T., ed) pp. 49-69, Academic Press, Inc., San Diego, CA
34. Nishimura, T., and Goll, D. E. (1991) J. Biol. Chem. 266, 11842-11850
35. Minami, Y., Emori, Y., Imajoh-Okami, S., Kawasaki, H., and Suzuki, K. (1988) J. Biochem. (Tokyo) 104, 927-933
36. Hayaishi, M., Saito, Y., and Kawasaki, S. (1992) Biochem. Biophys. Res. Commun. 182, 939-946
37. Fox, J. E. B., Austin, C. D., Reynolds, C. C., and Steffen, F. K. (1991) J. Biol. Chem. 266, 13289-13295
38. Fox, J. E. B., Reynolds, C. C., and Austin, C. D. (1990) Blood 76, 2510-2519
39. Verhallen, P. F. J., Bevers, E. M., Comfurius, P., and Zwaal, R. F. A. (1987) Biochim. Biophys. Acta 905, 206-217
40. Pontremoli, S., Meloni, E., Damiani, G., Salamino, P., Sparatore, B., Michetti, M., and Horecker, B. L. (1988) J. Biol. Chem. 263, 1915-1919
41. Kunimata, N., Hishigamiya, S., Sato, K., Ohkubo, I., and Sasaki, M. (1989) Biochim. Biophys. Res. Commun. 164, 875-882
42. Kunimata, M., Ma, X. J., Nishimura, J., Baba, S., Hamada, Y., Shiotri, T., and Sasaki, M. (1990) Biochem. Biophys. Res. Commun. 168, 1242-1247
43. Kazai, Y., Inomata, M., Hayashi, M., Imahori, K., and Kawasaki, S. (1986) J. Biochem. (Tokyo) 100, 183-190
44. Mellgren, R. L., and Lane, R. D. (1988) Biochim. Biophys. Acta 954, 154-160
45. Seide, T. C., Shibata, M., Takewara, T., Murufushi, H., and Suzuki, K. (1992) J. Biol. Chem. 267, 24595-24590
46. Anagli, J., Hagmann, J., and Shaw, E. (1993) Biochem. J. 289, 93-99