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ARTICLES

Association of Calpain (Ca^{2+}-Dependent Thiol Protease) With Its Endogenous Inhibitor Calpastatin in Myoblasts

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Abstract Calpain isozymes (intracellular, Ca^{2+}-dependent thiol proteases) are present in the cytoplasm of many cells, along with their endogenous specific inhibitor, calpastatin. Previously, we found that the levels of μ-calpain and m-calpain (activated by μM and mM Ca^{2+}, respectively) remain about the same during myoblast differentiation and fusion. By contrast, the calpastatin level, which is high during the initial stages of differentiation, diminishes markedly before myoblast fusion, allowing the proteolysis that is required for myotube formation. In the present study, we used immunoprecipitation to investigate the molecular association between calpain and calpastatin in dividing myoblasts and in the initial stages of myoblast differentiation. Immunoprecipitation (IP) was performed in two ways: (1) IP of calpain, using an anti-calpain antibody that recognized both isozymes; and (2) IP of calpastatin (using anti-calpastatin). Calpastatin was co-precipitated when calpain was immunoprecipitated; calpain was co-precipitated when calpastatin was immunoprecipitated. The results indicate that calpastatin is associated with calpain in dividing myoblasts and in myoblasts during the initial stages of differentiation, thereby preventing calpain activation at this stage. Prior studies carried out in vitro have shown a Ca^{2+}-dependent interaction of calpain with calpastatin. The results described here suggest that an association between calpain and calpastatin could occur within cells in the presence of physiological Ca^{2+} levels. It is proposed that the status of cellular calpain-calpastatin association is modulated by cell constituents, for which some possibilities are suggested. J. Cell. Biochem. 74:522–531, 1999.

Key words: calpain-calpastatin association; myoblast differentiation; myoblast calpain; myoblast calpastatin

Calpain isozymes (intracellular, Ca^{2+}-dependent thiol proteases) are ubiquitously distributed, along with their endogenous specific inhibitor, calpastatin [Murachi et al., 1981]. Calpain and calpastatin are present mainly in the cytoplasm and the levels of each vary among different tissues and species. The two ubiquitous calpain isozymes are μ-calpain and m-calpain (activated by μM and mM Ca^{2+}, respectively). The isozymes are dimers, composed of two different subunits, a catalytic 80-kDa subunit, unique to each isozyme, and a 30-kDa subunit, common to both. In addition, some tissue-specific calpain isozymes have been described, including calpain p94, an isozyme that is specific to skeletal muscles [Saido et al., 1994]. Calpain causes a limited degradation of some membrane and cytoskeletal proteins, enzymes, and transcription factors [Mellgren, 1987; Saido et al., 1994; Kawasaki and Kawashima, 1996; Carafoli and Molinari, 1998; Ono et al., 1998]. We have shown that the calpain-calpastatin system plays a role in membrane fusion. Using red cells as an experimental model, we found that calpain-induced degradation of certain membrane proteins is a prerequisite for the red cell membrane fusion and that the fusibility depends on the ratio of the cellular calpain to calpastatin [Kosower et al., 1983; Glaser and Kosower, 1986; Kosower and Glaser, 1990]. In a study on rat L8 myoblast differentiation and fusion to myotubes, we found that the μ-calpain and m-calpain proteins remain at about the same level during myoblast differentiation and fusion. By contrast, the calpastatin level, which is high during the initial stage of differentiation, diminishes markedly before myoblast fusion, allowing the calpain-induced proteolysis that is required for myotube formation. Prevention of cal-

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pastatin diminution is accompanied by inhibition of proteolysis and of fusion [Barnoy et al., 1996, 1997, 1998]. The results indicate that the ratio of calpain/calpastatin is important in myoblast fusibility.

In vitro studies have shown that calpain and calpain fragments bind to calpastatin and that calpastatin fragments bind to calpain in the presence of Ca^{2+} [Kapprell and Goll, 1989; Nishimura and Goll, 1991; Crawford et al., 1993; Kawasaki et al., 1993; Ma et al., 1993; Croall and McGrody, 1994; Yang et al., 1994]. Immunocytochemical studies have shown that in some cell types, the major calpain isozymes and calpastatin are present mainly in the cytoplasm, but may also be present near the cell membrane and in the nucleus [Schollmeyer, 1986; Lane et al., 1992; Spencer and Tidball, 1996; Karlsson and Nilsson, 1997; Murray et al., 1997]; there is no information about whether a molecular association between calpain and calpastatin exists in cells. Such information is necessary in order to better understand the regulation of calpain activity within the cell. Assay of co-immunoprecipitation has been widely used as a tool to examine an association between two molecules within cells [Medvedeva et al., 1994; Burg et al., 1994; Zipser et al., 1996; Shanmugam et al., 1997; Min et al., 1998]. In the present study, we used immunocytochemistry and immunoprecipitation to study the distribution of calpain and calpastatin in myoblasts and to find out whether there is a molecular association between them at the stages when the myoblast calpastatin level is high, i.e., in dividing myoblasts and at the initial stage of myoblast differentiation. We show that myoblast calpastatin is co-precipitated when calpain is immunoprecipitated and calpain is co-precipitated when calpastatin is immunoprecipitated. The results suggest that calpain may be associated with calpastatin in certain cells and that such an association could occur in the presence of physiological Ca^{2+} levels.

MATERIALS AND METHODS

Myoblast Culture

Rat myoblasts (L8 cell line, obtained from Dr. D. Yaffe, the Weizmann Institute of Science, Rehovot, Israel) were grown in 0.1% gelatin-coated petri dishes in Waymouth medium, supplemented by 15% fetal calf serum (FCS), 1% antibiotics, and 1% glutamine (growth medium). When the number of cells reached about 50% confluency, the cells were induced to differentiate by changing the medium to Dulbecco's modified Eagle's medium (DMEM), supplemented with 2% horse serum, 1% antibiotics, 1% glutamine, and 4 U of insulin/dl medium (differentiation medium), as described previously [Barnoy et al., 1996] and cultured for additional 48 h.

Calpain, Calpastatin, and Antibodies

μ-Calpain and calpastatin were purified from human erythrocytes and m-calpain from rabbit muscle. Primary antibodies used for the detection of calpain isozymes and calpastatin in immunocytofluorescence and immunoblotting were monoclonal anti μ-calpain antibody (anti-μ-calpain) [Glaser et al., 1994], a goat polyclonal anti m-calpain antibody (anti-m-calpain) [Barnoy et al., 1997], and a rabbit polyclonal anti-calpastatin antibody (anti-calpastatin) [Schwarz-Benmeir et al., 1994]. Two antibodies were used for immunoprecipitation. A rabbit polyclonal antibody (prepared against human erythrocyte μ-calpain) that recognizes the large subunits of both μ-calpain and m-calpain (anti-calpain), was used for the immunoprecipitation of calpain; anti-calpastatin [Barnoy et al., 1996] was used for the immunoprecipitation of calpastatin.

Immunocytofluorescence

Myoblasts were grown in growth medium to about 50% confluency on glass coverslips placed in petri dishes. The coverslips were processed for immunocytofluorescence examination according to published procedures [Rapaport et al., 1993; Willingham, 1994]. The cells were washed with phosphate-buffered saline (PBS), fixed in cold methanol (precooled to −20°C) for 5 min, washed in PBS, and incubated for 15 min in the presence of 0.2% Triton X-100 in PBS. The coverslips were then placed for 30 min in a blocking buffer (PBS containing 3% bovine serum albumin [BSA], 0.2% Triton X-100) and 1.0% normal serum (goat serum for μ-calpain and calpastatin; rabbit serum for μ-calpain), then incubated for 2 h with one of the primary antibodies (anti-μ-calpain, anti-m-calpain, anti-calpastatin, described above), or

with the corresponding nonimmune serum (mouse, goat, and rabbit, respectively). The coverslips were then washed in PBS and reincubated for 1 h with the appropriate cyanine (cy3)-conjugated secondary antibodies (goat anti-mouse IgG for μ-calpain, donkey anti-goat IgG for m-calpain, and goat anti-rabbit IgG for calpastatin (Jackson ImmunoResearch, West Grove, PA) in blocking buffer, followed by washes in PBS. Mounting was done in 60% glycerol, 2% n-propyl gallate, and 0.2 M Tris-HCl, pH 8.1. The stained cells on the coverslips were visualized with a Zeiss Confocal Laser Scanning Microscope (CLSM). The microscope is equipped with a 25 mW krypton-argon laser (488 and 568 maximum lines). A 40× NA/1.2 C-apochromat water-immersion lens (Axiovert 135M, Ziess) was used for imaging. Photographs were taken from the video display.

Preparation of Cell Lysates and Immunoprecipitation

The myoblasts in the petri dishes were washed with PBS, then scraped off the plates with a rubber policeman. The cells were centrifuged, then suspended in 20 mM Hepes buffer, pH 7.6, containing 450 mM NaCl, 24% glycerol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM leupeptin, 0.02 mM pepstatin, 0.1 mM N-tosyl-L-lysylchloromethylketone (TLCK), and 10 μg of aprotonin/ml buffer (buffer A). Cells in buffer A were lysed by quick freezing (on dry ice/ethanol) and thawing at 30°C, repeated three times. Lysed samples were centrifuged at 4°C for 15 min at 14,000*g*, and protein concentration in the supernatants determined [Lowry et al., 1951].

The cell lysates were diluted to 150 mM NaCl and 8% glycerol with Hepes buffer containing the inhibitors (but without NaCl or glycerol) (buffer B). Aliquots of the lysates (100 μl containing 100–200 μg of myoblast protein) were precleared by incubation for 1 h at 4°C in the presence of 5 μl of normal rabbit serum and 15 μl of protein A Sepharose (protein A-Sepharose 4 fast flow (Pharmacia Biotech, Sweden)). The samples were centrifuged and supernatants incubated by rotation at 4°C with 5–10 μl of anti-calpain or anti-calpastatin rabbit sera. After 4 h of incubation, protein A-Sepharose (30 μl) was added and incubation continued overnight. Samples were centrifuged, the pellets washed three times, and resuspended in buffer B containing 150 mM NaCl to the original lysate volume of 100 μl.

Immunoblotting

The immunoprecipitated samples and aliquots of whole lysates were solubilized in Laemmli sample buffer and heated to 100°C for 3 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard procedures, using 10% acrylamide. Electrophoresed samples were transferred to membranes, with nitrocellulose membranes (Gelman) used for μ-calpain and calpastatin and positively charged nylon membrane (Qiagen) for m-calpain. Blocking was carried out in 20 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 2% BSA, 0.05% Tween-20 (buffer C), and 0.02% NaN_3. The membranes were then incubated overnight with the primary antibodies, as previously described [Barnoy et al., 1996]. After incubation with the primary antibodies, the membranes were washed four times with buffer C and incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies (rabbit anti-mouse IgG for μ-calpain (Amersham), rabbit anti-goat IgG for m-calpain (Sigma Chemical Co., St. Louis, MO), and goat anti-rabbit IgG for calpastatin (Sigma). Membranes were then washed in 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20. Detection of bands was carried out with the enhanced chemiluminescence (ECL) immunoblotting detection system (Amersham).

RESULTS

Immunofluorescence Localization of Calpain and Calpastatin in Myoblasts

Myoblasts cultured to 50% confluency in growth medium (0 h in differentiation medium) were immunostained for μ-calpain, m-calpain, and calpastatin, as described under Materials and Methods. Immunostaining for μ-calpain showed it to be distributed in the form of granules around and within the nuclei (Fig. 1A). Intense, mostly homogeneous cytoplasmic staining, with some in the form of granules, was observed for m-calpain, with no staining observed in the nuclei (Fig. 1B). Similar patterns of distribution were observed in myoblasts grown in differentiating medium for 48 h (not shown). Immunostaining for calpastatin showed

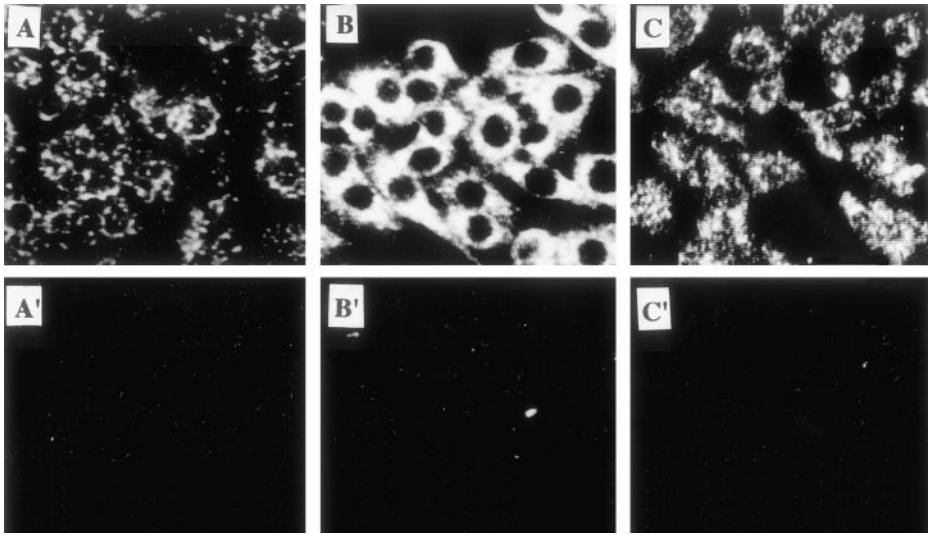

Fig. 1. Immunofluorescence labeling of calpain and calpastatin in rat L8 myoblasts. Myoblasts, obtained at the time of change from growth medium to differentiation medium, were fixed and stained, as described under Materials and Methods. Immunolabeling with: anti-μ-calpain **(A)**, anti-m-calpain **(B)**, and anti-calpastatin **(C)**. **A′**,**B′**,**C′**, cultures treated with the corresponding nonimmune serum.

it to be present in the cytoplasm in the form of granules, mainly around the nuclei, with some staining noted in the nuclei as well (Fig. 1C).

Specificity of Antibodies Used for Immunoprecipitation

Purified μ-calpain, m-calpain, and calpastatin were electrophoresed and samples immunoblotted, as described under Materials and Methods. As shown in Figure 2, the anti-calpastatin recognized several bands of calpastatin (Fig. 2, lane 2), in agreement with published information on the appearance of calpastatin bands of various molecular weights [Takano et al., 1993; Schwarz-Benmeir et al., 1994]; this antibody did not react with m-calpain (Fig. 2, lane 1) or with μ-calpain (Fig. 2, lane 3). The anti-calpain recognized the large subunit of both calpain isozymes (Fig. 2, lanes 6 and 7); it did not react with calpastatin (Fig. 2, lane 5).

Immunoprecipitation of Myoblast Calpain: Calpastatin Is Co-precipitated

Immunoprecipitation was carried out on myoblast lysates, using the anti-calpain, followed by immunoblotting for the identification of calpastatin, as described under Materials and Methods. As shown in Figure 3, a 110-kDa calpastatin band was co-precipitated with the immunoprecipitated calpain in the myoblasts

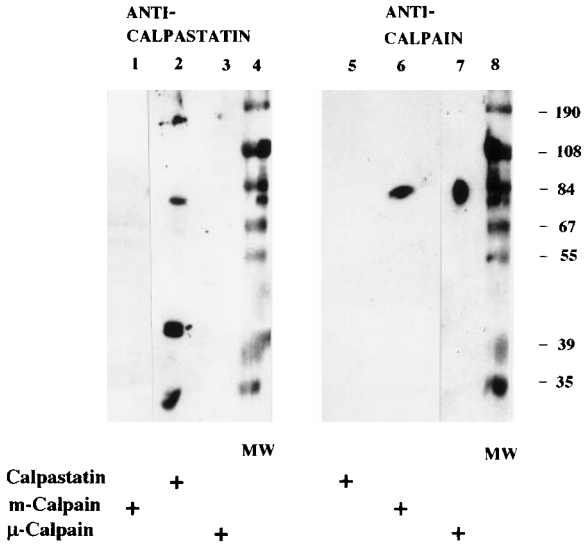

Fig. 2. Immunoblotting of purified μ-calpain, m-calpain, and calpastatin. The calpain isozymes and calpastatin were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to blotting membranes and immunoblotted with the corresponding antibodies. Electrophoresis: **lanes 2**, **5**, calpastatin (1.5 μg loaded per lane); **lanes 1**, **6**, m-calpain (1.0 μg loaded per lane); **lanes 3**, **7**, μ-calpain (1.0 μg loaded per lane); **lanes 4**, **8**, MW standards. Primary antibodies used for immunoblotting: lanes 1–3, anti-calpastatin; lanes 5–7, anti-calpain. For details, see under Materials and Methods.

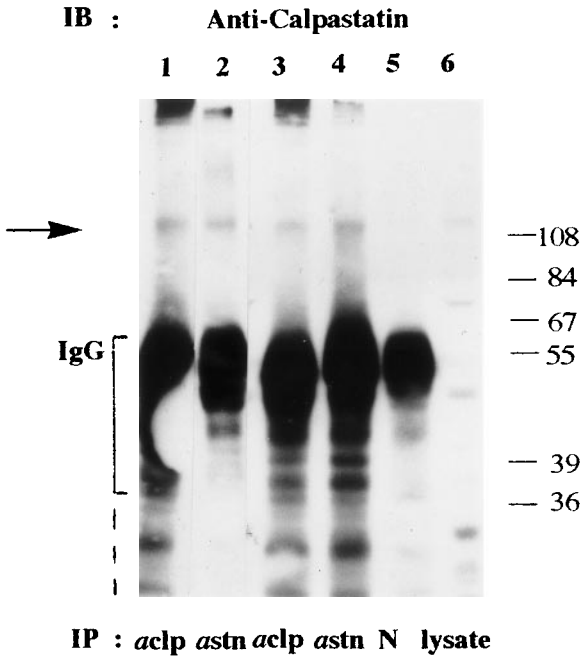

Fig. 3. Immunoprecipitation of myoblast calpain and calpastatin followed by immunoblotting with anti-calpastatin. Immunoprecipitation (IP) was carried out using anti-calpain (aclp) or anti-calpastatin (astn). Aliquots of the immunoprecipitates (corresponding to about 30 µg of the proteins in the original lysates) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to blotting membranes, and immunoblotted (IB), using anti-calpastatin. **Lanes 1**, **3**, IP with aclp, using myoblasts at 0 h and 48 h, respectively; **lanes 2**, **4**, IP with astn, using myoblasts at 0 h and 48 h, respectively; **lane 5**, IP with preimmune serum; **lane 6**, myoblast lysate used for the IP. MW standards are indicated at the right side. Arrow, calpastatin band of 110 kDa. For details, see under Materials and Methods.

cultured in growth medium (0 h) and in differentiating myoblasts, grown in differentiating medium for 48 h (Fig. 3, lanes 1 and 3). The 110-kDa band was similar in molecular weight to the calpastatin band observed in the immunoprecipitates when anti-calpastatin was used (Fig. 3, lanes 2 and 4). No calpastatin was observed in precipitates obtained using nonimmune rabbit serum (Fig. 3, lane 5). Multiple calpastatin bands are present in myoblast lysates prepared at 0 h (Fig. 3, lane 6), with a similar pattern observed at 48 h of differentiation (not shown). It should be noted that the presence of IgG in the immunoprecipitates precludes the identification of calpastatin bands of molecular weights lower than 110 kDa, which are present in the original lysates, so that only the calpastatin band of 110 kDa could be identified in the immunoprecipitates.

Immunoprecipitation of Myoblast Calpastatin: Calpain Is Co-precipitated

Immunoprecipitation was carried out on myoblast lysates, using anti-calpastatin, followed by immunoblotting for the identification of m-calpain and µ-calpain, as described under Materials and Methods. As shown in Figure 4, m-calpain was immunoprecipitated by anti-calpain (aclp) (Fig. 4, lanes 1 and 3), and co-precipitated from lysates of dividing and differentiating myoblasts (myoblasts at 0 and 48 h after changing from growth medium to differentiating medium), when anti-calpastatin was used as the precipitating antibody (Fig. 4, lanes 2 and 4). No m-calpain was precipitated from lysates treated with nonimmune serum (Fig. 4, lane 5).

Anti-µ-calpain was used for the identification of µ-calpain in the immunoprecipitates. No µ-calpain was observed when anti-calpain (aclp) was used as the immunoprecipitating antibody (Fig. 5, lane 1); µ-calpain was identified in the immunoblot after immunoprecipitation by anti-

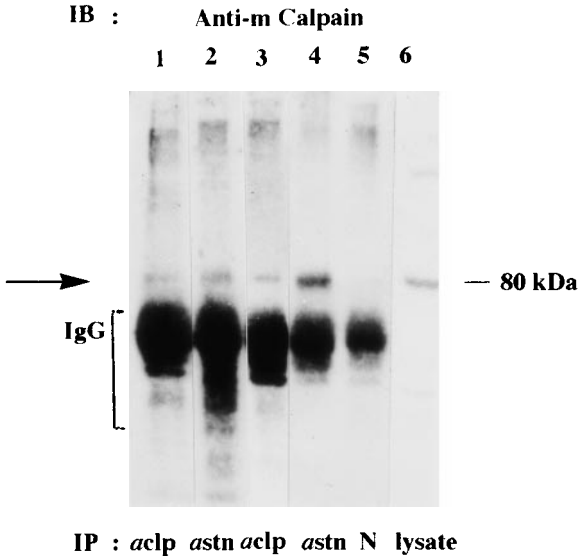

Fig. 4. Immunoprecipitation of myoblast calpastatin and calpain, followed by immunoblotting with anti-m calpain. Immunoprecipitation (IP) was carried out with anti-calpastatin (astn) or anti-calpain (aclp). Aliquots of the immunoprecipitates (corresponding to about 30 µg of the proteins in the original lysates) were electrophoresed, transferred to membranes, and immunoblotted (IB), using anti-m calpain. **Lanes 1**, **3**, IP with aclp, using myoblasts at 0 h and 48 h, respectively; **lanes 2**, **4**, IP with astn, using myoblasts at 0 h and 48 h, respectively; **lane 5**, IP with preimmune serum; **lane 6**, myoblast lysate used for the IP. Arrow, m-calpain 80-kDa large subunit. For details, see under Materials and Methods.

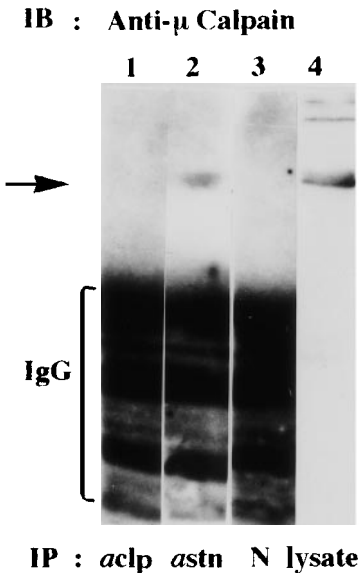

Fig. 5. Immunoprecipitation of myoblast calpastatin and calpain, followed by immunoblotting with anti-μ-calpain. Immunoprecipitation (IP) was carried out with anti-calpastatin (astn) or anti-calpain (aclp), using myoblasts grown in differentiation medium for 48 h. Immunoprecipitates (corresponding to about 70 μg of proteins in the original lysates) were electrophoresed, transferred to membranes, and immunoblotted (IB), using the monoclonal anti-μ-calpain antibody. **Lane 1**, IP with aclp; **lane 2**, IP with astn; **lane 3**, IP with preimmune serum; **lane 4**, myoblast lysate used for the IP. Arrow, 80-kDa large μ-calpain subunit. For details, see under Materials and Methods.

calpastatin (astn) (Fig. 5, lane 2), and no μ-calpain was observed when nonimmune serum was used for the precipitation (Fig. 5, lane 3). It should be pointed out that very little μ-calpain was observed in the astn-precipitate, necessitating the use of a large fraction of the precipitate for immunoblotting.

DISCUSSION

The results presented indicate that calpain is associated with calpastatin in dividing and in differentiating myoblasts at the initial stages of differentiation. An association between the protease and the inhibitor was shown by carrying out immunoprecipitation in two ways: one of calpain, using anti-calpain, which recognized the large subunits of μ-calpain and m-calpain, and a second one, immunoprecipitation of calpastatin, using anti-calpastatin. We found that calpastatin was co-precipitated when calpain was immunoprecipitated and calpain was co-precipitated when calpastatin was immunoprecipitated. The lack of cross-reactivity of the anti-calpain with calpastatin and anti-calpastatin with calpain ensured that the antibodies used immunoprecipitated directly only calpain or calpastatin, respectively. Thus, any calpastatin observed in the calpain-immunoprecipitate is associated with the precipitated calpain and conversely, any calpain observed in the calpastatin-immunoprecipitate would be associated with the precipitated calpastatin. As to which isozyme was associated with calpastatin, the results indicate that both are associated with calpastatin in the myoblasts. The results are clear with respect to m-calpain, which was identified in the anti-calpain immunoprecipitates and in the anti-calpastatin immunoprecipitates, consistent with the association of this isozyme with calpastatin in these cells. The results with respect to μ-calpain are less clear, but they do indicate that μ-calpain is associated with calpastatin, since μ-calpain was identified in the calpastatin immunoprecipitate. Purified unautolysed μ-calpain has been shown to require slightly more Ca^{2+} for in vitro half-maximal binding to calpastatin than for half-maximal activity, whereas the opposite is true for m-calpain [Kapprell and Goll, 1989]. The intracellular association between μ-calpain and calpastatin may therefore be weaker than the association of m-calpain with calpastatin. The failure to identify μ-calpain in the anti-calpain immunoprecipitate may be due to an inability of the anti-calpain used here to immunoprecipitate native μ-calpain (the anti-calpain precipitated SDS-solubilized μ-calpain (Barnoy et al., unpublished results)). It may also be due to an instability of μ-calpain during the immunoprecipitation procedure, as we found that myoblast μ-calpain is unstable under a variety of conditions, such as column chromatography and casein gel zymography (Barnoy et al., unpublished results). Such instability would also account for the minute amounts of μ-calpain observed in the calpastatin immunoprecipitates.

The major calpains are present mainly in the cytoplasm of mammalian cells, with some variations noted among different cell types (the p94 protein has not been identified by immunohistochemistry, apparently because of an extremely short half-life time [Saido et al., 1994]); μ-calpain is present in the cytoplasm, with peripheral presence in some cells, and with nuclear

presence in other cases; m-calpain appears to be distributed uniformly throughout the cytoplasm of various cells in tissue cultures [Lane et al., 1992; Karlsson and Nilsson, 1997; Murray et al., 1997], similar to its appearance in the myoblasts studied here. After elevations in cellular Ca^{2+}, calpain can undergo relocalization to the membrane or nucleus [Molinari et al., 1994; Mellgren and Lu, 1994]. Previously, it was shown that fusing and nonfusing myoblasts exhibit cytoplasmic and probably nuclear staining for μ-calpain; m-calpain is seen near the plasma membrane in fusing cell, whereas it is present predominantly in the cytoplasm in nonfusing myoblasts [Schollmeyer, 1986]. Degenerating muscle fibers in dystrophin-deficient mice showed a homogeneous, cytosolic distribution of calpain, with similar location in regenerating fibers, whereas in fully differentiated muscle fibers, calpain is present throughout the cytosol, but more concentrated near the plasma membrane [Spencer and Tidball, 1996]. The distribution of calpain may thus be different for each isozyme and depend on the type of cell and on the physiological state and differentiation stage of the cell. For calpastatin to exert its inhibitory role in the cell, it should be present in adequate amounts relative to calpain, located in the same cellular compartment, and associated with calpain. Calpastatin is present in a granular form, mainly around the nucleus [Lane et al., 1992; Karlsson and Nilsson, 1997; present results] or throughout the cytoplasm [Murray et al., 1997] and in some cases also in the nucleus, as observed here and in osteoblasts [Murray et al., 1997]. Though there may be differential distributions of calpain and calpastatin to certain cellular sites, in the areas in which both calpain and calpastatin are present and calpastatin is abundant, an interaction is expected between calpain and calpastatin.

Calpastatin interaction with calpain has been studied in vitro, using intact molecules and fragments of calpain and calpastatin. Using calpastatin affinity chromatography, it was shown that intact μ-calpain, m-calpain, and their autolytic fragments that contain the calmodulin (CaM)-like domains bind to calpastatin-affinity column in a Ca^{2+}-dependent manner, whereas the large subunit fragment containing the calpain active site does not bind or binds poorly to calpastatin [Kapprell and Goll, 1989; Nishimura and Goll, 1991]. In another study, using calpastatin-affinity matrix, it was shown that m-calpain binds to calpastatin in the presence of Ca^{2+}, that the isolated large subunit does not bind to calpastatin, and that when the Ca^{2+}-binding domains in the small and large subunits are separated from each other, they also do not bind to calpastatin. These results indicate that an association between the Ca^{2+}-binding domains in the two subunits is necessary for efficient binding to calpastatin [Crawford et al., 1993]. A required association between the two calpain subunits for binding to calpastatin is consistent with the resistance of p94 (a monomer isozyme) to inhibition by calpastatin [Ono et al., 1998]. In addition, calpastatin has been shown to bind with higher efficiency to the Ca^{2+}-induced autocatalytic μ-calpain 78-kDa intermediate than to the intact calpain [Melloni et al., 1996), indicating that Ca^{2+} may in part be indirectly involved in calpain-calpastatin interaction, via formation of high-affinity fragments produced by Ca^{2+}-induced calpain autocatalysis.

Calpastatin contains four internal repeating domains, each having about 140 amino acid residues. Each calpastatin domain exhibits inhibitory activity and has been shown to have two sites for interaction with calpain [Kawasaki et al., 1993; Ma et al., 1993]. It has been suggested that, in the presence of Ca^{2+}, one calpastatin sequence in each domain inhibits calpain activity by binding to the protease active site and a second calpastatin sequence inhibits the binding of calpain to the membrane by interacting with another site of calpain, presumably in the CaM-like domain [Kawasaki et al., 1993]. A synthetic calpastatin-like peptide, which mimics the inhibitory regions of calpastatin, has been used to map the binding site in intact calpain; the peptide inhibits calpain activity, and cross-linking to calpain occurs preferentially in the presence of Ca^{2+}, with cysteine residue in the calpain catalytic domain identified as the cross-linked site [Croall and McGrody, 1994]. Further studies on calpastatin-calpain association, using recombinant and synthetic peptides, have identified the sequences (subdomains) in the calpastatin domains involved in calpain-calpastatin interaction; two subdomains exhibit Ca^{2+}-dependent binding to the CaM-like sequences in calpain,

but do not inhibit calpain activity, whereas another subdomain inhibits calpain activity but does not bind to the CaM-like calpain domains. It was suggested that the binding of calpastatin subdomains to calpain CaM-like domains is similar to the interaction of CaM-binding peptides and CaM [Yang et al., 1994].

Thus, an efficient in vitro interaction of calpastatin with calpain appears to require Ca^{2+} and is achieved at Ca^{2+} concentrations higher than the usual cellular Ca^{2+} levels [Mooren and Kinne, 1998]. The relevance of the mode and sites involved in calpain-calpastatin interaction in vitro to that in vivo is unclear. We found a Ca^{2+} concentration of 10–20 nM in the myoblast extracts used here for immunoprecipitation, representing about 10-fold dilution of packed myoblasts (Barnoy et al., unpublished results). It is thus consistent with the Ca^{2+} level in myoblasts, reported to be about 120 nM [Constantin et al., 1995]. The finding of low Ca^{2+} in these extracts lends further support to the conclusion that the calpain-calpastatin association shown here represents an endogenous association and was not due to Ca^{2+} contamination in the extract. Conformational changes in calpain, which in vitro are dependent on Ca^{2+} [Blanchard et al., 1997; Lin et al., 1997], may be promoted in the cell by other molecules (proteins, peptides, and small molecules). An association between calpain and calpastatin within the cell may then occur under physiological conditions, i.e., in the presence of physiological Ca^{2+} levels. This would explain the results obtained here. However, we must also consider how calpain can be activated in the cell. In some cases, a diminution in calpastatin is found, allowing the activation of calpain [Schwarz-Benmeir et al., 1994; Nixon et al., 1994; Barnoy et al., 1996]. In most cases, it has not been resolved as to how calpain is activated in the presence of cellular high calpastatin activity. One explanation offered is that calpastatin and calpain may be located in different cellular compartments [Lane et al., 1992]. However, it is possible that, under some conditions, certain modulating factors inhibit calpain-calpastatin association or cause their dissociation. Modulating factors (some of which are known to promote calpain activity in the presence of low Ca^{2+}) may include membrane phospholipids, DNA [Mellgren et al., 1993], phosphorylated calpastatin [Salamino et al., 1997], calpain activator protein [Melloni et al., 1998], and CaM. In view of the possible involvement of calpain in various processes [Ilian and Foresberg, 1992; Nixon et al., 1994; Morimoto et al., 1997; Shields et al., 1998], it would be of interest to study whether calpain-calpastatin association occurs within various types of cells and whether there are modulating factors that may promote or inhibit their association under physiological conditions and in pathological disorders.

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