Regulation of calpain and calpastatin in differentiating myoblasts: mRNA levels, protein synthesis and stability

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Calpain (Ca^{2+} -dependent intracellular protease)-induced proteolysis has been considered to play a role in myoblast fusion to myotubes. We found previously that calpastatin (the endogenous inhibitor of calpain) diminishes transiently during myoblast differentiation. To gain information about the regulation of calpain and calpastatin in differentiating myoblasts, we evaluated the stability and synthesis of calpain and calpastatin, and measured their mRNA levels in L8 myoblasts. We show here that μ -calpain and m-calpain are stable, long-lived proteins in both dividing and differentiating L8 myoblasts. Calpain is synthesized in differentiating myoblasts, and calpain mRNA levels do not change during differentiation. In contrast, calpastatin (though also a long-lived protein in myoblasts), is less stable in differentiating myoblasts than in the dividing cells, and its synthesis is inhibited upon initiation of differentiation. Inhibition of cal-

pastatin synthesis is followed by a diminution in calpastatin mRNA levels. A similar calpastatin mRNA diminution is observed upon drug-induced inhibition of protein translation. On the other hand, transforming growth factor β (which inhibits differentiation) allows calpastatin synthesis and prevents the diminution in calpastatin mRNA. The overall results suggest that at the onset of myoblast differentiation, calpastatin is regulated mainly at the level of translation and that an inhibition of calpastatin synthesis leads to the decrease in its mRNA stability. The existing calpastatin then diminishes, resulting in decreased calpastatin activity in the fusing myoblasts, allowing calpain activation and protein degradation required for fusion.

Key words: Ca^{2+} -dependent protease, cell fusion, protease inhibitor.

INTRODUCTION

The formation of skeletal muscle during embryonic development involves myoblast differentiation and fusion to multi-nucleated muscle fibres. Myoblast differentiation and fusion can be studied in myoblast cultures, employing primary myoblast cultures or myoblast cell lines [1]. Cell-membrane fusion processes, including myoblast fusion, involve destabilization of cell membrane and cytoskeletal framework, allowing the creation of membrane-fusion-potent domains [1–3]. A limited membrane-protein degradation appears to be required for cell fusion, a degradation that would allow membrane disorganization in fusing cells [1–6]. It has been proposed that calpain (Ca^{2+} -dependent intracellular protease) is involved in the fusion-associated protein degradation [4–11]. Most cells, in addition to having one or more of the known calpain isoenzymes, contain the specific inhibitor calpastatin, with ratios of calpain to calpastatin varying among different tissues and cell types [12,13]. Using red cells as an experimental model, it was found that the cell fusibility depended on the ratio of calpain to calpastatin [4,6]. In a study on rat L8 myoblast fusion, we found that the μ -calpain and m-calpain levels did not change significantly during myoblast differentiation, whereas calpastatin diminished markedly prior to myoblast fusion and reappeared after fusion [10]. A selective, limited, calpain-induced degradation of some cytoskeletal and membrane proteins was observed in the L8 fusing myoblasts [14]. The diminution of calpastatin and degradation of myoblast proteins were not observed in myoblasts prevented from fusing by transforming growth factor β (TGF- β) and EGTA [14,15]. The results indicated that the ratio of calpain to calpastatin activity determines myoblast ability to fuse. That the ratio of calpain to calpastatin may be important in controlling myoblast

fusion is also indicated by the effects of introducing calpain and calpastatin antisense oligodeoxynucleotides into myoblasts [16], and micro-injecting myoblasts with calpain and calpastatin [11].

The major calpain isoenzymes and calpastatin appear to be long-lived proteins, with metabolic half-lives estimated to be about 5 days, as shown in various dividing cell lines [17]. These data suggest stability and a low rate of synthesis of the calpain-calpastatin proteins. It is not known how the levels of the calpain isoenzymes and calpastatin are controlled in differentiating myoblasts; the reasons for the diminution of calpastatin in the differentiating myoblasts have not been clarified. In order to gain information on the regulation of calpain and calpastatin in differentiating myoblasts, we evaluated the stability and synthesis of calpain and calpastatin, and measured their mRNA levels in L8 myoblasts. We show here that μ -calpain and m-calpain are stable, long-lived proteins both in dividing and in differentiating L8 myoblasts. Calpain is synthesized in differentiating myoblasts, and calpain mRNA levels do not change during differentiation. In contrast, calpastatin is less stable in differentiating myoblasts than in the dividing cells, its synthesis is inhibited upon initiation of differentiation, and calpastatin mRNA levels diminish significantly before fusion. Temporally, the cessation of calpastatin synthesis precedes the diminution in mRNA; a similar calpastatin mRNA diminution is observed upon drug-induced inhibition of protein translation, suggesting that inhibition of calpastatin translation leads to a decrease in its mRNA. Inhibition of differentiation by TGF- β allows calpastatin synthesis and prevents the diminution in calpastatin mRNA. The overall results indicate that the decreased stability of calpastatin protein, along with inhibition of calpastatin synthesis, lead to the diminution of calpastatin in the differentiating myoblasts, prior to fusion. The

Abbreviations used: TGF- β , transforming growth factor β ; GM, growth medium; DM, differentiation medium; IP, immunoprecipitation; 2,5A synthetase, 2',5'-oligoadenylate synthetase; IGFBP-3, insulin growth factor-binding protein 3.

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transient diminution of calpastatin protein [10] and of its mRNA may be part of the myogenic programme responsible for myoblast differentiation [18].

MATERIALS AND METHODS

Myoblast cultures and inhibition of myoblast fusion and protein synthesis

Rat myoblast L8 cell line was obtained from Dr D. Yaffe (The Weizmann Institute of Science, Rehovot, Israel). The cells were grown in 0.1 % gelatin-coated 90-mm Petri dishes in Waymouth medium, supplemented by 15 % fetal calf serum, 1 % antibiotics and 1 % glutamine (growth medium, GM), and induced to differentiate, according to the procedures described previously [10]. When the cells reached 50 % confluency, the medium was changed to Dulbecco's modified Eagle's medium, supplemented by 2 % horse serum, 1 % antibiotics, 1 % glutamine and 4 units of insulin/100 ml of medium (differentiation medium, DM). The time at which the GM was changed to DM is defined as 0 h. Under these conditions, the cultured myoblasts reach confluency at 24–48 h (i.e. the cells withdraw from the cell cycle after completing an additional division). The DM was replaced every 48 h. In some experiments, the cells were grown to 70–80 % confluency in GM, then medium changed to DM, as specified below.

For inhibition of fusion, cells were grown in GM to about 50 % confluency, then the medium was changed to DM. TGF- β 1 (TGF- β ; Sigma, St. Louis, MO, U.S.A.) was added to the DM at 0 h, at a final concentration of 0.6 nM (diluted from an aqueous stock solution of 1.0 μ M), and replenished every 48 h, along with the replacement of the DM [14]. For inhibition of protein synthesis by cycloheximide, cells were grown in GM to about 70–80 % confluency. In some dishes, the medium was changed to DM, and others replenished with GM. Cycloheximide was added at a final concentration of 100 μ M (diluted from a stock solution of 100 mM in ethanol), and cells cultured for an additional 24 or 48 h. For inhibition of protein synthesis by puromycin, myoblasts were grown in GM to about 70–80 % confluency, then the medium changed to DM, with or without 0.6 nM TGF- β . Puromycin was added at a final concentration of 1.0 μ g/ml (diluted from an aqueous stock solution of 1.0 mg/ml) and replenished at 48 h.

Preparation of cell extracts and immunoblotting procedures

The cells in the Petri dishes were washed with PBS and then detached from the dishes with PBS containing 5 mM EDTA. Cells were centrifuged, then lysed in a solution containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, 0.01 mM *p*-aminoethylbenzenesulphonyl fluoride (AEBSF) and 10 μ g of aprotinin/ml of solution (lysis buffer). Samples were kept on ice for 30 min, then centrifuged at 8000 *g*. Protein concentration in the supernatants was determined by the method of Lowry et al. [19]. Aliquots of supernatants were mixed with Laemmli sample buffer for SDS/PAGE and immunoblotting analysis.

SDS/PAGE was carried out according to standard procedures, using 10 % acrylamide. Samples containing 40 μ g of myoblast proteins were electrophoresed, then transferred to membranes, with nitrocellulose membranes (Gelman) used for μ -calpain and calpastatin, and positively-charged nylon membrane (Qiagen) used for m-calpain. Immunoblotting was carried out as described previously [14], using monoclonal anti- μ -calpain antibody, polyclonal anti-m-calpain antibody, polyclonal anti-calpastatin antibody and the appropriate peroxidase-conjugated secondary

antibodies [14]. Detection of bands was carried out with the ECL immunoblotting detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). Bands were quantified by densitometric scanning.

Metabolic labelling, and immunoprecipitation (IP) of calpain and calpastatin

Myoblasts were grown in GM to about 80 % confluency, then the Petri dishes were washed three times with PBS. Methionine-free Dulbecco's modified Eagle's medium, containing 2 % dialysed fetal calf serum, was added with or without 0.6 nM TGF- β , and Petri dishes cultured for 1 h, followed by the addition of 100 μ Ci of [35 S]Met (NEN, Boston, MA, U.S.A.). The cells were labelled for 12 h, then the dishes were placed on ice and washed three times with cold PBS. Cells were detached from the Petri dishes with a rubber policeman, centrifuged and rewashed with PBS. Lysis buffer containing 0.5 % deoxycholate was added to the packed cells (20–40 μ l/1 $\times$ 10⁶ cells), and cell lysates were kept on ice for 30 min and then centrifuged at 8000 *g* at 4 °C for 20 min.

IP of calpain and calpastatin from the supernatants was carried out according to published procedures [17]. For IP of calpain, SDS was added to the supernatant to a concentration of 0.5 %, the sample was heated to 100 °C for 3 min, diluted by the addition of 4 vols of lysis buffer lacking Triton X-100 (IP buffer), and divided into aliquots of 1.0 ml. The aliquots were precleared by rotation at 4 °C for 1 h with 20 μ l of Protein A–Sepharose (Amersham Pharmacia Biotech) and 5 μ l of normal rabbit serum, then centrifuged. Polyclonal anti-calpain antibody that recognizes the large subunits of both μ -calpain and m-calpain [20] was added to two aliquots, and normal rabbit serum was added to the third. The aliquots were incubated by rotation at 4 °C for 4 h, then mixed with 30 μ l of Protein A–Sepharose and samples were incubated for additional 16 h. The samples were then centrifuged, the pellets washed three times with IP buffer, solubilized in Laemmli sample buffer, and separated on SDS/PAGE (7.5 % acrylamide). Gels were treated with 2,5-diphenyloxazole (PPO) and subjected to autoradiography to visualize the 35 S-labelled bands. Other gels were used for immunoblotting, to verify IP of μ -calpain and m-calpain.

For calpastatin IP, myoblast extracts were prepared as described above, then heated to 100 °C for 5 min without the addition of SDS, and centrifuged to remove denatured proteins. Under these conditions, calpastatin is present in the supernatant, usually as a single band of 110 kDa [17]. The supernatants were immunoprecipitated by incubation at 4 °C for 4 h with anti-calpastatin antibody, addition of 30 μ l of Protein A–Sepharose, and continued incubation for 16 h. Following the incubation, the immunoprecipitates were processed as described above for calpain.

Isolation of cellular RNA, preparation of calpain and calpastatin 32 P-labelled cDNA probes and Northern-blot analysis

Myoblasts from the various stages of differentiation (0, 48, 72, 96 and 120 h) were washed twice with PBS and detached from the Petri dishes with 5 mM EDTA in PBS. Total RNA was extracted from the cells according to established methods [21], using the TRI-Reagent kit (Molecular Research Center, Cincinnati, OH, U.S.A.). The pellet, obtained after propan-2-ol precipitation and washing with ethanol, was suspended in diethyl pyrocarbonate (DEPC)-treated water. Aliquots of the extracted RNA samples were electrophoresed on agarose gels and stained with ethidium bromide to verify the quality and intensity of the 28 and 18 S bands.

Plasmids pUC8 and pTV119N, carrying the cDNAs of the large subunits of μ -calpain and m-calpain, respectively [22,23], were provided by Dr K. Suzuki (University of Tokyo, Tokyo, Japan). Plasmids were amplified, and plasmid DNA recovered and purified, using standard techniques [24]. The cDNA inserts were recovered by restriction-enzyme digestion, using *EcoRI* for μ -calpain and *BamHI* and *NcoI* for m-calpain.

For the preparation of calpastatin probe, reverse transcriptase-PCR was carried out, using 5 μ g of total RNA incubated with oligo-dT primer (Promega, Madison, WI, U.S.A.) at 70 °C for 10 min and cooled on ice; 25 μ M of each deoxyribonucleotide, 0.5 units of RNasin (Promega), 200 units of Moloney-murine-leukaemia virus reverse transcriptase and first-strand buffer (Gibco, Gaithersburg, MD, U.S.A.), containing 1 mM dithiothreitol, were added to a final volume of 35 μ l and reaction was carried out at 42 °C for 1 h, followed by incubation at 95 °C for 5 min to inactivate the enzyme. A fragment of calpastatin corresponding to nucleotides 650–1889 was amplified using the forward primer 5'-CCCTGCAGGCTCTGTCAGAT-3' and the reverse primer 5'-CATCAAAGCCTTTGATCTTC-3' [25]. PCR was carried out using 5 μ l of the reverse transcriptase product, 1.0 unit of *Taq* polymerase (Perkin Elmer-Roche Molecular Systems, Branchburg, NJ, U.S.A.), 25 μ M of each deoxyribonucleotide, 10 μ M of each primer and reaction buffer in a total volume of 20 μ l. PCR was carried out for 32 cycles; each cycle included denaturation at 94 °C for 1 min, annealing at 56 °C for 45 s and extension at 72 °C for 1 min; the last cycle was followed by extension at 72 °C for 5 min. The PCR product was electrophoresed on 1 % agarose gel and appeared as a single band of the expected size [25]. The fragment was cloned in the pGEM-vector system (Promega), as specified by the manufacturer. The plasmid was amplified, plasmid DNA recovered and purified [24], and the cDNA insert recovered by the restriction enzymes *PstI* and *NcoI*. Identification of the probe as calpastatin was confirmed by sequencing. The probes for μ -calpain, m-calpain and calpastatin were labelled with [³²P]dCTP (NEN), using a random-priming DNA-labelling kit (Biological Industries, Beit Haemek, Israel).

Aliquots of 20 μ g of total RNA were separated on 1.2 % agarose gel containing 0.22 mM formaldehyde and 1 $\times$ Mops. RNA was transferred from gel to Hybond-N nylon membranes (Amersham Pharmacia Biotech). After the transfer, the mRNA was immobilized by baking for 2 h, the membranes stained with 0.1 % Methylene Blue and photographed. Membranes were incubated at 42 °C for 2 h in prehybridization solution [24]. The labelled probes (2 $\times$ 10⁶ c.p.m./ml) were added to the membranes in the same solution, and hybridization was carried out by rotation at 42 °C for 14–16 h. Membranes were then washed three times at room temperature with 0.2 % SDS in 2 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), and once more with 0.1 % SDS in 0.1 $\times$ SSC at 50 °C. Membranes were exposed to X-ray film (Fuji, Tokyo, Japan) for between 1 day and 1 week. Intensities of mRNA bands were quantified by densitometric scanning.

RESULTS

Stability of calpain and calpastatin in dividing and differentiating myoblasts

Cycloheximide was used to inhibit *de novo* protein synthesis and thus allowed us to estimate the stability of existing calpain and calpastatin. Myoblasts in GM and in DM were treated with cycloheximide, and analysed by immunoblotting for μ -calpain, m-calpain and calpastatin, as described in the Materials and methods section. Following the addition of 100 μ M cycloheximide, the levels of μ -calpain and m-calpain changed little

Figure 1 Effects of cycloheximide on myoblast μ -calpain, m-calpain and calpastatin levels

Myoblasts were grown in GM to about 70–80 % confluency. Some Petri dishes were replenished with GM and in others the medium was changed to DM; cycloheximide was added (final concentration 100 μ M), and the cells were cultured for an additional 48 h. Extracts were analysed by immunoblotting.

over the course of 48 h in myoblasts grown in either GM or in DM (Figure 1, top and middle panels). Densitometric analysis showed that 95 and 93 % of the initial μ -calpain were present at 48 h in cycloheximide-treated myoblasts cultured in GM and DM, respectively (means of three experiments), with similar values obtained for m-calpain. Treatment of the myoblasts with higher concentrations of cycloheximide or for over 48 h caused morphological changes, followed by cell detachment from the Petri dishes.

Calpastatin levels changed little over the course of 48 h in myoblasts grown in GM, but diminished significantly in myoblasts

Figure 2 IP of calpains from [^{35}S]Met-labelled myoblasts

Myoblasts were cultured in GM to about 80% confluency, then cells were cultured in methionine-free DM for 1 h, followed by labelling with [^{35}S]Met for 12 h. Myoblasts were then lysed, immunoprecipitated with anti-calpain antibody, or with normal serum, and analysed by autoradiography (AR) and immunoblotting (IB) with the anti-m-calpain antibody [14] (for details see the Materials and methods section). Lane 1 (N), IP with normal rabbit serum; lane 2 (+), IP with anti-calpain antibody, in the presence of excess unlabelled μ -calpain (6 units), added prior to the addition of anti-calpain antibody; lane 3 (—), IP with anti-calpain antibody, in the absence of unlabelled calpain; lane 4 (N), immunoblotting of precipitate of lane 1; lane 5 (IP), immunoblotting of precipitate of lane 3; lane 6 (sup), immunoblotting of supernatant obtained by centrifugation of the precipitate of lane 3. IgG, Ig heavy chains present in the immunoprecipitates.

cultured in DM (Figure 1, bottom panel). Densitometric analysis showed 95 ± 4.45 and 64.6 ± 4.76 % (means $\pm$ S.E.M., $n = 5$) of initial calpastatin in the myoblasts after 48 h of treatment with cycloheximide in GM and DM, respectively. The results indicate that μ -calpain and m-calpain are stable, long-lived proteins in both dividing and differentiating myoblasts. Calpastatin also appears to be a stable, long-lived protein in dividing myoblasts, but is less stable in differentiating myoblasts.

Synthesis of calpain and calpastatin in differentiating myoblasts

[^{35}S]Met was added to myoblasts in methionine-free DM, and cells were labelled for 12 h, as described in the Materials and methods section, then washed and lysed. Aliquots were used for IP of calpain and calpastatin, and immunoprecipitates were electrophoresed and analysed by autoradiography and immunoblotting. Under the experimental conditions used, about 70–80 % of the m-calpain large subunit present in the extract was immunoprecipitated (Figure 2, IB); 30–40 % of the μ -calpain was immunoprecipitated (results not shown). As shown in the autoradiograph in Figure 2, the calpain in the immunoprecipitate obtained with anti-calpain antibody was labelled (Figure 2, lane 3), with the identity of the labelled band ascertained by competitive co-precipitation with excess, non-labelled calpain (Figure 2, lane 2) and by immunoblotting of the precipitate of lane 3 (Figure 2, lane 5). No labelled and no immunoprecipitated calpain was observed in control samples, in which normal serum was used for IP (Figure 2, lanes 1 and 4). The results indicate that calpain is synthesized in the differentiating myoblasts.

Calpastatin was immunoprecipitated from myoblasts cultured in DM, in the absence and presence of TGF- β , which blocks differentiation and fusion of myoblasts [26–28]. The calpastatin was not labelled in the control, differentiating myoblasts, but was

Figure 3 IP of calpastatin from [^{35}S]Met-labelled myoblasts

Myoblasts were cultured as described in Figure 2, in the presence and absence of 0.6 nM TGF- β . Myoblast lysates were prepared and subjected to IP with anti-calpastatin antibody [14], and analysed by autoradiography (AR) and immunoblotting (IB) with the anti-calpastatin antibody (for details, see the Materials and methods section). Lanes 1 and 2, autoradiography of immunoprecipitates of myoblasts cultured in the absence (C) and presence of TGF- β ; lane 3 (IP), immunoblotting of precipitate of lane 1; lane 4 (N), immunoblotting of precipitate obtained with normal rabbit serum; lane 5 (lys), immunoblotting of the heated lysate (the calpastatin high-molecular-mass band is present in heated lysates [17]); lane 6 (sup), supernatant obtained by centrifugation of the precipitate of lane 1. IgG, Ig heavy chains present in the immunoprecipitates. Arrows indicate the calpastatin band of 110 kDa.

Figure 4 Levels of μ -calpain mRNA in differentiating myoblasts

Myoblasts were grown in GM to about 50% confluency, then medium was changed to DM (0 h). RNA was isolated at 0, 48, 96 and 120 h of differentiation, and subjected to Northern blotting (for details, see the Materials and methods section). Top panel, Northern-blot autoradiogram of hybridization with μ -calpain probe; bottom panel, Methylene Blue staining to visualize the 28 and 18 S bands. Representative of two experiments.

labelled in the TGF- β -treated myoblasts (Figure 3). Under the experimental conditions used, the calpastatin band present in the original heated lysate was immunoprecipitated quantitatively

Figure 5 Levels of calpastatin mRNA in differentiating myoblasts and in TGF- β -treated, differentiation-arrested myoblasts

Myoblasts were grown in GM to about 50% confluency, then medium changed to DM (0 h), and myoblasts cultured in the presence or absence of TGF- β . RNA was isolated at 0, 48, 72, 96 and 120 h of differentiation, and subjected to Northern blotting (for details, see the Materials and methods section). Top panel: Northern-blot autoradiogram of control (differentiating) myoblasts and TGF- β -inhibited myoblasts. Upper section, hybridization with calpastatin probe; lower section, Methylene Blue staining to visualize the 28 and 18 S bands. Representative of three experiments. Bottom panel: densitometric quantification of the Northern-blot autoradiogram shown in the top panel.

(Figure 3), so that the observed lack of labelling in the differentiating myoblasts was not caused by absence of calpastatin in the precipitate, but rather due to inhibition of synthesis. Thus the results indicate that calpastatin was not synthesized in the differentiating myoblasts, whereas it was synthesized in the myoblasts inhibited by TGF- β from differentiating.

Calpain and calpastatin mRNA levels in differentiating and inhibited myoblasts

RNA was extracted from myoblasts during differentiation and fusion, and analysed by Northern blotting. A Northern blot for μ -calpain mRNA is shown in Figure 4 (representative of two experiments). Densitometric analysis of the blot (mRNA bands

Figure 6 Effects of puromycin on the levels of calpastatin mRNA in differentiating myoblasts and in TGF- β -treated, differentiation-arrested myoblasts

Myoblasts were grown in GM to about 70–80% confluency, the medium changed to DM containing puromycin (0 h), and myoblasts cultured in the absence (control) or presence of TGF- β . RNA was isolated at 0, 24, 48 and 72 h of treatment, and subjected to Northern blotting (for details, see the Materials and methods section). Top panel: Northern-blot autoradiogram of control myoblasts and TGF- β -inhibited myoblasts. Upper section, hybridization with calpastatin probe; lower section, Methylene Blue staining to visualize the 28 and 18 S bands. Lower panel: densitometric quantification of the Northern-blot autoradiogram shown in the top panel. The results are representative of two experiments.

relative to the 28 or 18 S RNA) showed mRNA levels at 95–115% of the level obtained at 0 h, indicating that the μ -calpain mRNA levels did not change significantly during differentiation and increased slightly in the myotubes. Similarly, m-calpain mRNA did not change significantly during differentiation (results not shown).

A Northern blot for calpastatin mRNA and densitometric analysis is shown in Figure 5 (representative of three experiments). Calpastatin mRNA appeared as two species of mRNA, one of 5.1 kb and the second of 3.8 kb (Figure 5, top panel). The calpastatin mRNA diminished significantly in differentiating myoblasts before the beginning of fusion. Subsequently, at the time of myotube formation, the mRNA regained its predifferentiation levels. In contrast, the calpastatin mRNA levels did not

diminish in the non-fusing, TGF- β -treated myoblasts (Figure 5). Densitometric analysis of the blots showed levels of calpastatin mRNA in the differentiating myoblasts to be 55 % at 72 h and 36 % at 96 h of culture in DM (means of three experiments), as compared with levels present at 0 h, with similar behaviour for both fractions; in the TGF- β -treated myoblasts, the levels of mRNA were 94 and 106 % at 72 and 96 h, respectively, with a further increase at 120 h to 120 % of the initial level.

To find out about the relation of calpastatin mRNA steady-state levels to the presence or absence of calpastatin synthesis, we investigated the effects of puromycin (which inhibits translation and causes dissociation of polyribosome complexes and release of mRNA [29]) on the myoblast calpastatin mRNA levels. Puromycin addition to TGF- β -treated myoblasts led to diminution in calpastatin mRNA (Figure 6). Calpastatin mRNA levels in these myoblasts were 49 % at 48 h and 45 % at 72 h (means of two experiments). When puromycin was added to differentiating myoblasts, calpastatin mRNA levels were 53 and 48 % at 48 and 72 h, respectively. Thus in TGF- β -treated myoblasts that maintain their calpastatin mRNA levels (as shown in Figure 5), the addition of puromycin induced a diminution in the calpastatin mRNA. In the case of differentiating myoblasts, puromycin did not cause any further loss of calpastatin mRNA beyond the loss that occurred in the differentiating cells (as shown in Figure 5). The results indicate that calpastatin mRNA is less stable in myoblasts that do not synthesize calpastatin (i.e. in differentiating myoblasts, and in TGF- β -treated myoblasts, which are inhibited by puromycin) than in myoblasts that continue to synthesize calpastatin (TGF- β -treated myoblasts).

DISCUSSION

Myoblasts proliferate when cultured in GM in the presence of serum-containing growth factors. When placed in DM medium with reduced concentrations of growth factors, the myoblasts withdraw from the cell cycle, differentiate and fuse to myotubes. Differentiation involves expression of muscle-specific genes, as shown by the appearance of certain mRNAs and proteins, such as α -actin, myosin, troponin-T and creatine kinase. Expression of other genes is suppressed in differentiating myoblasts, as indicated by the diminished levels of some mRNAs and proteins, such as junB, β - and γ -actin, fibronectin and the neurofilament protein NF-140K [26–28,30]. Still other proteins seem to be unchanged during myoblast differentiation, such as α - and β -tubulin. For some proteins, there is a transient increase during differentiation, followed by diminution in the myotubes, as observed for 2',5'-oligoadenylate synthetase (2,5A synthetase) [31] and cathepsin B [32]. Other proteins are transiently diminished before myoblast fusion, as observed for calpastatin [10] and insulin growth factor-binding protein 3 (IGFBP-3) [33].

Muscle-specific gene expression is known to be regulated at the transcriptional and post-transcriptional levels, as well as at the translational level [26–28], leading to the appearance and accumulation of the muscle-specific proteins. Little is known, however, about the regulation responsible for the diminution of proteins in differentiating myoblasts; such protein diminution might be due to repressed transcription, altered mRNA processing, and/or inhibition of translation, and/or decreased stability. For proteins such as junB and possibly NF-140K, β -actin and γ -actin, down-regulated transcription or diminished mRNA stability may be the primary event involved, since their mRNA levels appear to decrease soon after shifting the myoblasts to DM [27,28,30]. No information is available on the step(s) at which calpastatin gene expression is regulated

and the reasons for its diminution in the differentiating myoblasts studied here have not been elucidated.

In order to probe possible mechanisms involved and to gain information as to the step(s) at which calpastatin expression is regulated, we measured calpastatin mRNA levels during L8 myoblast differentiation and fusion to myotubes, and also estimated calpastatin synthesis and stability. We show that calpastatin mRNA diminishes in the differentiating myoblasts before their fusion, and regains initial levels after fusion. Calpastatin synthesis is arrested in differentiating myoblasts at a time when calpastatin mRNA is still abundant. Therefore, the results suggest that the initial event is inhibition of translation, followed by a diminution in calpastatin mRNA. The results also indicate that calpastatin is a stable protein in dividing myoblasts, consistent with the long half life of a few days estimated for calpastatin in human fibroblast cell lines [17]. Calpastatin stability appears to be diminished in differentiating myoblasts, but it is still quite stable in these cells, with a half life estimated to be about 48–72 h from the time at which cells cease dividing and withdraw from the cell cycle. The overall results suggest that the primary step concerning calpastatin regulation in the differentiating myoblasts is at the level of translation. The existing calpastatin protein then diminishes, resulting in decreased calpastatin activity in the fusing myoblasts, as shown previously [10].

As for the observed fall in calpastatin mRNA, it may be due to diminished transcription and/or post-transcription processing of the mRNA during myoblast differentiation, occurring as a separate event, independent of the inhibition of translation. However, the decrease in mRNA may be due to instability as a result of arrested translation [34–36]. In order to probe this possibility, we studied the effects of puromycin on control, differentiating myoblasts and on TGF- β -treated, differentiation-arrested myoblasts. TGF- β inhibits myoblast differentiation without promoting myoblast proliferation, and prevents the appearance of muscle-specific mRNAs and proteins. On the other hand, TGF- β prevents the loss of some myoblast mRNAs and proteins, such as β - and γ -actin, that are high in proliferating myoblasts and which are lost during differentiation, so that their levels remain high in the TGF- β -treated, non-fusing myoblasts [26–28]. We found previously that TGF- β prevented the decrease in calpastatin at the protein level [14]. In the present study we show that, in contrast to the inhibition of calpastatin synthesis in differentiating myoblasts, calpastatin is synthesized in TGF- β -treated, differentiation-arrested myoblasts. We also show that calpastatin mRNA remains at the initial levels in the TGF- β -treated myoblasts. The calpastatin mRNA did diminish in the TGF- β -treated myoblasts when protein synthesis was inhibited by puromycin, which causes the dissociation of polysomes to monosomes and the release of mRNA [29]. The fact that puromycin had no effect on the loss of calpastatin mRNA in differentiating myoblasts is consistent with the finding that calpastatin synthesis is already inhibited in the differentiating myoblasts.

The results suggest that when calpastatin synthesis is inhibited (as would normally occur in differentiating myoblasts, or when puromycin is added to TGF- β -treated myoblasts), the mRNA becomes unstable. Various calpastatin mRNA species have been described [37–42]. The 3'-untranslated regions of some calpastatin mRNA species have several polyadenylation sites and contain sequences reported to modulate mRNA stability [37–41]. Thus calpastatin mRNA may belong to a class of mRNAs that can be degraded under certain conditions, i.e. when translation is inhibited and the appropriate factors and RNase are present. It is of interest to note that 2,5A synthetase, which is transiently

increased to high levels in differentiating myoblasts, remains at a low level in TGF- β -treated myoblasts [31]. The induction of 2,5A synthetase in differentiating L8 myoblasts [31] appears to be a mirror image of the diminution in calpastatin mRNA shown in the present study. The synthetase polymerizes ATP to 2',5'-oligoadenylates, which in turn activate latent RNase. Thus 2,5A synthetase could provide a pathway for degrading calpastatin mRNA, and such a possibility should be studied further.

The behaviour of calpastatin mRNA in the differentiating myoblasts studied here seems to be similar to the behaviour of IGFBP-3 mRNA studied in porcine myogenic cells, i.e. a transient decrease, followed by reappearance of the mRNA in the myotubes, and prevention of mRNA decrease by TGF- β [33]. Since no data on the synthesis and stability of the IGFBP-3 protein are available, it is not clear whether the overall mechanism of diminution in IGFBP-3 mRNA and protein levels [33] is similar to that proposed here for calpastatin. In addition, the factors responsible for the reappearance in myotubes of calpastatin mRNA (and of IGFBP-3 mRNA [33]) are not known.

As for the regulation of calpain expression, the results indicate that μ - and m-calpain mRNAs do not change, and that calpain is synthesized and is stable in the differentiating myoblasts. The results thus suggest that the regulation of calpastatin is independent of that of calpain, and that in some cell types calpain expression at the mRNA and protein levels may be constant and similar to those of genes such as glyceraldehyde phosphate dehydrogenase [31,33]. In some other types of myoblast, m-calpain protein appears to increase during myoblast differentiation with no change or increase in calpastatin [7,11]; in yet other myoblasts, m-calpain increases and calpastatin mRNA and protein decrease [9,16]. The reasons for the differences observed are not clear at present. The overall results, however, are consistent with the notion that the myoblast calpain-to-calpastatin ratio determines fusibility. Thus in the myoblasts studied here, the diminution in calpastatin protein and activity allows the activation of the existing calpain [10], resulting in calpain-induced protein degradation that is required for myoblast fusion [15]. That the calpain-calpastatin system plays a role in muscle-protein degradation is also shown by the inhibition of protein degradation in myoblasts obtained when m-calpain is inhibited, either by transfection with a dominant-negative m-calpain gene or by overexpression of the calpastatin inhibitory domain [39].

The calpain-calpastatin system in muscle is also regulated under conditions of muscle atrophy or hypertrophy, associated with enhancement or suppression of protein degradation respectively [40,41]. Fasting, which causes an increased degradation of skeletal-muscle myofibrillar proteins, induces increased levels of calpain and calpastatin mRNA, and an increase in polysome-associated calpain mRNA [40]. β -Adrenergic stimulation, which causes muscle hypertrophy and suppresses protein degradation, has been shown to induce a very significant increase in calpastatin mRNA and activity, with some increase in calpain mRNA and activity [41]. These results indicate that an increased calpastatin-to-calpain ratio may play a role in the β -adrenergic-induced diminution of muscle-protein degradation. Additional work is necessary to identify the factors participating in calpain and calpastatin regulation in myoblasts and muscle tissues.

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