Caspase-1-induced calpastatin degradation in myoblast differentiation and fusion: cross-talk between the caspase and calpain systems

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Abstract **Previously, we found that calpastatin diminished transiently prior to myoblast fusion (rat L8 myoblasts), allowing calpain-induced protein degradation, required for fusion. Here we show that the transient diminution in calpastatin is due to its degradation by caspase-1. Inhibition of caspase-1 prevents calpastatin diminution and prevents myoblast fusion. Caspase-1 activity is transiently increased during myoblast differentiation. Both calpain and caspase appear to be responsible for the fusion-associated membrane protein degradation. Caspase-1 has been implicated in the activation of proinflammatory cytokines, and in cell apoptosis. The involvement of caspase-1 in L8 myoblast fusion represents a novel function for this caspase in a non-apoptotic differentiation process, and points to cross-talk between the calpain and caspase systems in some differentiation processes.**

Key words: Calpastatin; Caspase; Calpain; Myoblast fusion; Cell differentiation

1. Introduction

Skeletal muscle formation involves myoblast differentiation and fusion to multinucleated fibers [1]. A limited membrane protein degradation is required, allowing membrane disorganization and creation of fusion-potent domains in fusing cells [1,2]. Calpain (Ca^{2+}-dependent intracellular protease) is involved in the fusion-associated protein degradation [3–5]. Most cells, in addition to having one or more of the known calpain isozymes, contain the specific inhibitor calpastatin, with ratios of calpain to calpastatin varying among different cells [6]. Using red cells as a model system, we showed that the ratio of calpain to calpastatin determines fusibility [3]. In rat L8 myoblasts, we found that calpain levels did not change significantly during myoblast differentiation, whereas calpastatin diminished prior to myoblast fusion and reappeared after fusion [4]. The transient diminution in calpastatin allows the Ca^{2+}-induced calpain activation and protein degradation in the fusing myoblasts [7].

Calpastatin has a long half-life in dividing cells [8,9]. In differentiating L8 myoblasts, calpastatin diminution is the result of inhibition of calpastatin synthesis and decrease in calpastatin stability [9]. The protease responsible for the enhanced degradation of calpastatin has not been identified. Both calpain and caspase have been shown to degrade calpastatin. Calpain degrades calpastatin in vitro and in some cells [10,11]. We have found that the calpain inhibitor calpeptin (which inhibits fusion) does not prevent the diminution in calpastatin in the inhibited myoblasts [12], indicating that calpastatin degradation in the myoblasts is not due to calpain. Caspase is known to degrade calpastatin in cell-free extracts and in several types of cells undergoing apoptosis [13–15]. Caspases have been mainly implicated in apoptosis [16–19], but may also play roles in processes not involving cell death [18–22]. Involvement of caspase-3 in skeletal muscle differentiation has recently been described [23]. This newly discovered activity of caspase-3 provides important evidence for caspase roles in non-apoptotic events.

Here we show that the transient diminution in calpastatin in differentiating L8 myoblasts is due to its degradation by caspase, specifically by caspase-1. Caspase-1 has been implicated primarily in the activation of proinflammatory cytokines [16,19,24], and in cell death [25]. The involvement of caspase-1 in myoblast fusion represents a novel function for this caspase, and points to cross-talk between the calpain and caspase systems during certain differentiation processes.

2. Materials and methods

2.1. Myoblast cultures, and treatment with caspase and calpain inhibitors

Rat myoblasts (L8 cell line) were grown in 0.1% gelatin-coated Petri dishes in Waymouth medium, supplemented with 15% fetal calf serum, and antibiotics (growth medium, GM). Myoblasts were grown in GM to about 50% confluency, then induced to differentiate as previously described [7], by changing the GM medium to Dulbecco's modified Eagle's medium, supplemented with 2% horse serum, and four units of insulin/100 ml (differentiation medium, DM). The time at which the GM is changed to DM is defined as 0 h. The DM was replaced every 48 h.

To study effects of caspase inhibition, the following cell permeable inhibitors were used: the pan-caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) and *t*-butoxycarbonyl-Asp-(OMe)-fluoromethyl ketone (BAF) [26]; the selective caspase-1 inhibitor Boc-Asp(benzyl) chloromethylketone (BACMK) [27]; the selective caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CMK (Ac-DEVD-CMK) [28] (Calbiochem, La Jolla, CA, USA). The inhibitors were dissolved in dimethylsulfoxide (DMSO; 5.0 mM stock solutions); they were added to the DM at 0 h at a final concentration of 20 μM Z-VAD-FMK, 5–20 μM BAF, 2.5–5 μM BACMK, and 25

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Abbreviations: Ac-DEVD-CMK, Ac-Asp-Glu-Val-Asp-CMK; BACMK, Boc-Asp(benzyl) chloromethylketone; BAF, *t*-butoxycarbonyl-Asp-(OMe)-fluoromethyl ketone; DMSO, dimethylsulfoxide; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

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μM Ac-DEVD-CMK, and replenished every 24 h. The calpain inhibitor calpeptin (Calbiochem) was diluted in DM to 25 μM from 25 mM stock solution in DMSO and added to the myoblasts every 24–48 h, as described previously [12].

2.2. Measurement of caspase activity in myoblast extracts

Myoblasts, grown in GM (0 h) or cultured in DM for 48–120 h, were lysed in 10 mM Tris–HCl, pH 7.4, 130 mM NaCl, 10 mM Na-pyrophosphate, 1% Triton X-100, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4 (buffer A). Lysates were centrifuged and protein concentration in the supernatants measured (protein assay reagent kit, Pierce Biotechnology, Rockford, IL, USA). Supernatants were kept at −20°C prior to the estimation of caspase activity. Caspase activity in supernatant was estimated according to published methods [28] in 20 mM HEPES, pH 7.4, 2 mM dithiothreitol, 10% glycerol (buffer B), using the fluorogenic substrates Ac-YVAD-AMC and Ac-DEVD-AMC (Alexis Biochemicals, Lausen, Switzerland) for caspase-1 and caspase-3, respectively. Amc release was monitored at 460 nm (excitation at 380 nm), using an FL 600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA).

2.3. Preparation of cell extracts for SDS–PAGE, and immunoblotting analyses

Myoblast extracts were prepared as previously described [12], using 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, and protease inhibitors cocktail set III (Calbiochem) (buffer C). Aliquots of supernatants were mixed with Laemmli sample buffer for SDS–PAGE.

SDS–PAGE was carried out according to standard procedures, using 10% or 12% acrylamide. Samples containing 20–40 μg of myoblast proteins were electrophoresed, then transferred to nitrocellulose membranes (Schleicher&Schuell). Immunoblotting was carried out as previously described [12], using as primary antibodies monoclonal anti-μ-calpain antibody, polyclonal anti-m-calpain antibody [12]; polyclonal anti-calpastatin antibody (R19): Sc-7561 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) that recognized calpastatin of 110 kDa; polyclonal anti-caspase-1 antibody p20 (M-19): Sc-1218 (Santa Cruz); polyclonal anti-caspase-3 antibody (H-277): Sc-7148 (Santa Cruz); monoclonal anti-190 kDa talin fragment (antibody specific to calpain-induced talin degradation [29], a gift from Dr. Mitsushi Inomata, Department of Protein Biochemistry, Tokyo Metropolitan Institute of Gerontology, Japan); monoclonal anti-β-tubulin antibody (Sigma, St. Louis, MO, USA); monoclonal anti-α-fodrin antibody (Affiniti Research Products, Mamhead, UK). The appropriate peroxidase-conjugated secondary antibodies were used, and detection of bands was carried out with ECL (Pierce), as previously described [12]. Membranes were stripped off and reprobed with anti-β-tubulin antibody for estimation of loading. Bands were quantified by densitometry.

3. Results

3.1. Myoblast fusion and effects of inhibitors

Myoblasts became confluent at about 24–48 h after changing GM to DM. Alignment of myoblasts and start of fusion was observed at about 72–96 h, followed by fusion to multinucleated myotubes at 120–144 h, as previously described [4]. Previously we found that myoblast fusion was inhibited by the calpain inhibitors calpeptin and E-64 [12]. In the present study, to find out about the effects of caspase inhibition on myoblast fusion, we used two pan-caspase inhibitors. The pan-caspase inhibitor Z-VAD-FMK inhibited myoblast fusion at a concentration of 20 μM. Since Z-VAD-FMK may inhibit calpain (albeit shown only in vitro) [26], we also used the pan-caspase inhibitor BAF, which is much more specific as caspase inhibitor than as calpain inhibitor [26]. BAF inhibited myoblast fusion at a concentration of 5 μM. The results indicate that caspase activity was involved in myoblast fusion. To find out which caspase may be responsible for the inhibition, the selective caspase-1 inhibitor BACMK, and the caspase-3 inhibitor Ac-DEVD-CMK were used. BACMK partially inhibited myoblast fusion at a concentration of 2.5 μM (fusion of about 25% versus 60–80% in the control cultures). Essentially complete inhibition was achieved at a BACMK concentration of 5.0 μM (less than 4% fusing cells) (Fig. 1). Ac-DEVD-CMK did not inhibit myoblast fusion at a concentration of 25 μM (results not shown). Upon removal of BACMK (5 μM), the cells underwent differentiation and fused to myotubes 48–72 h later (Fig. 1), indicating that under the conditions used the inhibitor was not toxic to the cells.

3.2. Effects of caspase and calpain inhibitors on myoblast calpastatin and calpain

The transient diminution in calpastatin that occurred in control, differentiating myoblasts, was inhibited in BACMK-treated cells (Fig. 2A). The pan-caspase inhibitors Z-VAD-FMK and BAF also prevented the diminution in calpastatin (results not shown). Calpeptin, which inhibited myoblast fusion to the same degree as did the caspase inhibitors, did not prevent calpastatin diminution, with calpastatin level remain-

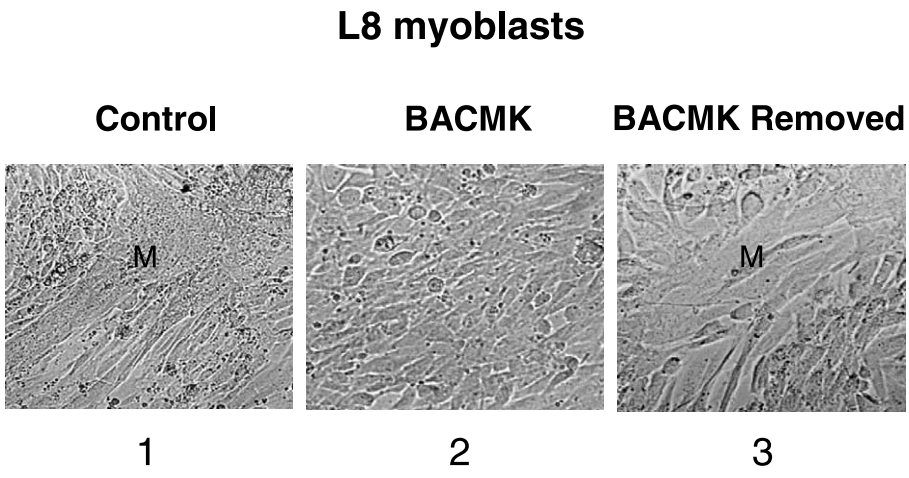

Fig. 1. Inhibition of L8 myoblast fusion by the caspase inhibitor BACMK, and reversal of the inhibition. Myoblasts were cultured in DM in the absence (control) or presence of 5 μM BACMK. 1, Control cells, and 2, BACMK-treated cells cultured in DM for 120 h; 3, BACMK was removed from treated cultures at 96 h, and cultures continued in DM for an additional 48 h (BACMK removed). M, myotubes. Representative of three experiments.

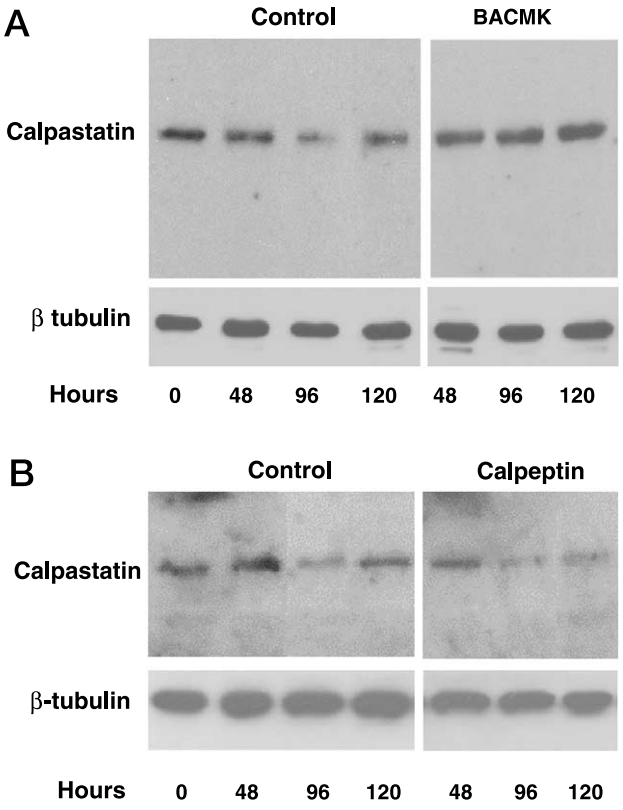

Fig. 2. Effects of caspase and calpain inhibitors on calpastatin levels in differentiating myoblasts. Myoblasts were cultured in DM in the absence or presence of 5 μM BACMK, or 25 μM calpeptin. Myoblasts were harvested at various time points, and cell extracts were analyzed by immunoblotting. Representative of two experiments. A: Effects of BACMK on calpastatin levels in myoblasts cultured in DM. Calpastatin levels (based on 100% level for 0 h, and using β-tubulin for normalizing protein loading) were 94% at 48 h, 55% at 96 h and 86% at 120 h in the control myoblasts; in the BACMK-treated cells, calpastatin levels were 95% at 48 h, 98% at 96 h, 108% at 120 h. B: Effects of calpeptin on calpastatin levels in myoblasts cultured in DM. Calpastatin levels (based on 100% level for 0 h, and using β-tubulin for normalizing protein loading) were 45% at 96 h and 82% at 120 h in the control myoblasts; in the calpeptin-treated cells, calpastatin levels were 90% at 48 h, 50% at 96 h, and 63% at 120 h.

ing low during the culture period (Fig. 2B). No change was found in μ-calpain and m-calpain (as observed by immunoblotting), neither during differentiation and fusion in the control myoblasts, nor in the myoblasts inhibited from fusing by treatment with BAF or calpeptin (results not shown). Thus, the results indicate that the transient diminution of calpastatin in differentiating myoblasts is due to caspase activity, specifically caspase-1.

3.3. *Caspase-1 and caspase-3 in differentiating and in inhibited myoblasts*

Activation of caspase-1 and caspase-3 in the myoblasts was assessed by immunoblotting and by measuring of activity of cell extracts on caspase substrates. The region of 20–22 kDa of caspase-1 (mature caspase-1, comprised of multiple species, and indicative of caspase-1 activation), increased during myoblast differentiation, then partially declined; little change was observed in myoblasts inhibited by BACMK (Fig. 3A). The activity of caspase-1 in cell extracts increased from very low level at 0 h to high levels at 72–96 h, then declined; the

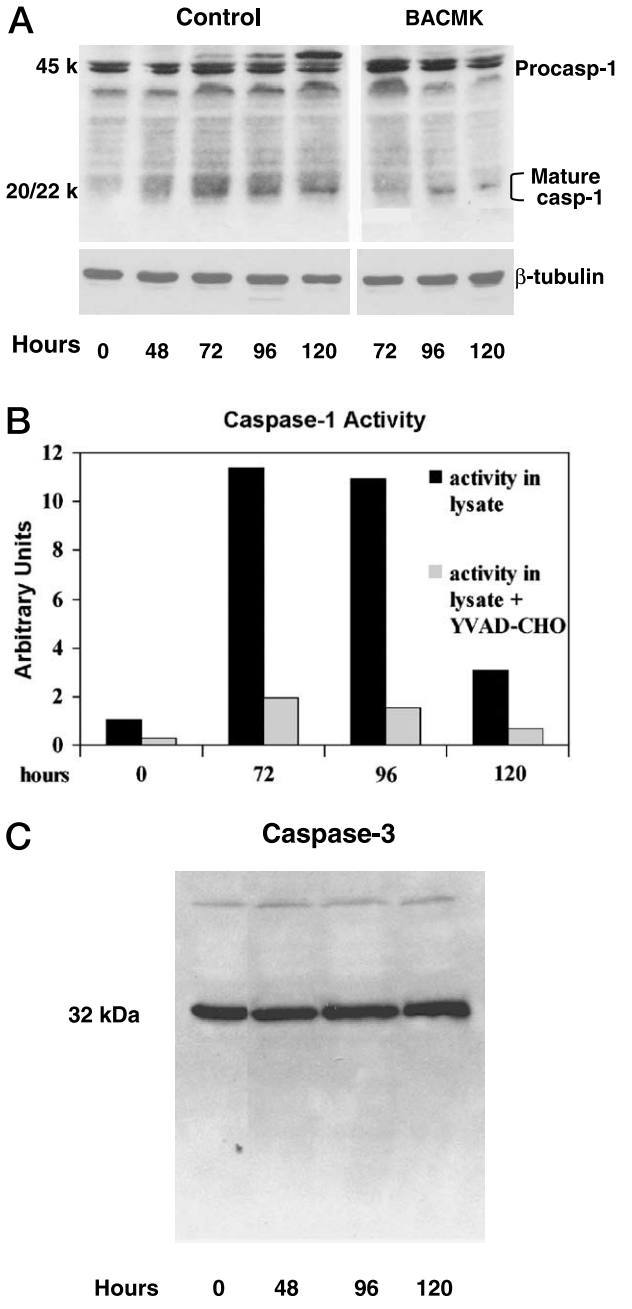

Fig. 3. Caspase-1 and caspase-3 in myoblasts during differentiation. Myoblasts, cultured in DM in the absence or presence of 5 μM BACMK, were harvested at various time points, and cell extracts were analyzed. A: Immunoblotting with anti-caspase-1 antibody. Representative of two experiments. In the control, the ratio of the mature caspase region to that of the procaspase region (considered as a ratio of 1.0 at 0 h) increased to 1.8 at 48 h, and 2.14 at 72 h, then declined to 1.75 at 96 h and 1.48 at 120 h; in the BACMK-treated cells, the ratio was 1.3–1.1 at 72–120 h. B: Caspase-1 activity in extracts of myoblasts cultured in DM. Measurements of activity were carried out by mixing 30 μl of cell extract in buffer A containing 100 μg of protein with 100 μl of buffer B, containing 40 μM of the fluorogenic substrate Ac-YVAD-AMC, with and without 40 μM of the inhibitor YVAD-CHO. Amc release (at 460 nm, with excitation at 380 nm) is shown as arbitrary units at the end of 2 h reaction. Representative of two experiments, each carried out on triplicate culture dishes. C: Immunoblotting with anti-caspase-3 antibody. Representative of three experiments.

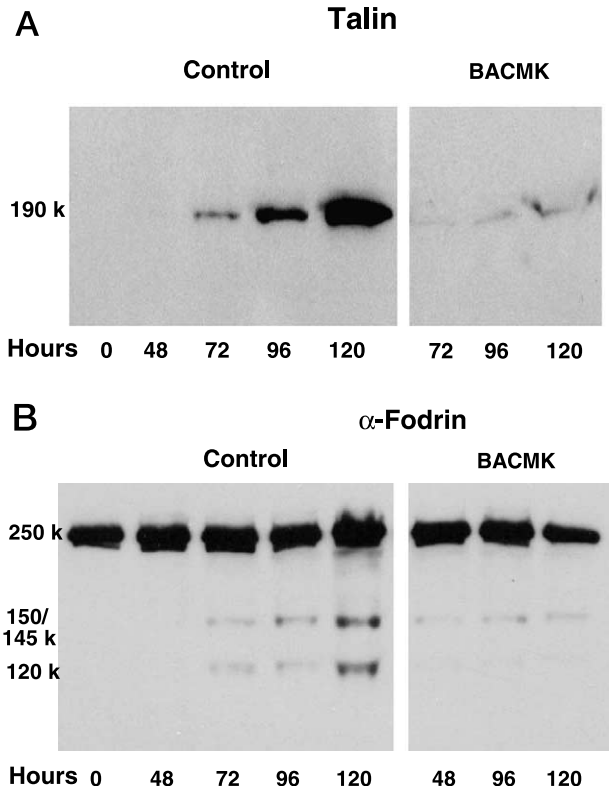

Fig. 4. Protein degradation in myoblasts. Myoblasts were cultured in DM, in the absence and presence of 5 μM BACMK. Cells were harvested at various time points and analyses by immunoblotting. A: Immunoblotting with antibody to calpain-generated talin fragment. Representative of two experiments. B: Immunoblotting with anti-α-fodrin antibody. Representative of two experiments.

addition of the caspase-1 inhibitor Ac-YVAD-CHO resulted in inhibition of a major part of the activity (Fig. 3B). Caspase-3 did not change in differentiating myoblasts, as shown by the same levels of the 32 kDa procaspase present throughout the culture period and no appearance of lower molecular weight fractions, which would be indicative of caspase-3 activation (Fig. 3C). Little activity of caspase-3 was noted in extracts of dividing myoblasts, without a significant change in the differentiating myoblasts (results not shown). These results indicate that caspase-1 is activated in the differentiating myoblasts studied here.

3.4. *Protein degradation in differentiating and in caspase-inhibited myoblasts*

The calpain-generated talin 190 kDa fragment [29] was observed in control, fusing myoblasts, with only traces noted in myoblasts treated with BACMK (Fig. 4A). Fodrin is a known substrate for both calpain and caspase, with a fodrin fragment of 150 kDa indicative of caspase and calpain activities, 145 kDa indicative of calpain activity and 120 kDa indicative of caspase activity [30]. Fodrin fragments of 150/145 kDa and 120 kDa were observed in the differentiating myoblasts, with little of the 150/145 kDa fractions and no 120 kDa fragment observed in the BACMK-inhibited myoblasts (Fig. 4B). Levels of myoblast β-tubulin, previously shown not to be a substrate for calpain [7], were the same in differentiating and inhibited myoblasts; therefore, β-tubulin was used to estimate loading. The results indicate that calpain-induced degradation of certain proteins in differentiating, fusing myoblasts can be inhibited via caspase inhibition.

4. Discussion

The conserved regions in calpastatin are rich in aspartic acid residues [31] with cleavage motives for caspases [32], and thus calpastatin is a suitable substrate for caspases. It has been shown to be a substrate for caspase-1 and caspase-3, both in vitro and in cells in several cases of apoptosis [13–15]. Since calpastatin is a substrate for calpain [10,11,30], the diminution in calpastatin during L8 myoblast differentiation may have been due to calpain or caspase activity. Calpeptin, which inhibits calpain, does not prevent calpastatin diminution in the differentiating myoblasts (for reasons which are not clear at present, calpeptin also appears to delay or prevent calpastatin recovery). Thus, calpeptin appears to inhibit fusion by inhibiting calpain activity directly [12]. The results presented here point to caspase, specifically caspase-1, as the protease responsible for calpastatin degradation during L8 myoblast differentiation. This conclusion is based on effects of caspase inhibitors, on immunoblotting analysis and on activity estimation in cell extracts. The caspase-1-induced calpastatin degradation would allow calpain activation. In turn, activated calpain promotes protein degradation, as illustrated here by the calpain-induced talin degradation. Fodrin, which is a substrate for both calpain and caspase, appears to be degraded in fusing myoblasts by caspase and possibly also by calpain. Calpastatin subdomains A and C have recently been shown to be calpain activators [33], and thus some caspase-induced calpastatin fragments may enhance calpain activation. The presence of such calpastatin fragments may be responsible for the continuation of calpain-induced protein degradation at a time when calpastatin has partially recovered.

While this manuscript was being prepared for publication, a study was published on caspase-3 involvement in mouse skeletal muscle differentiation [23]. The caspase-3 substrate mammalian sterile twenty-like kinase (MST1) was shown to mediate the caspase-3 effect [23]. Caspase-1 was not evaluated in that study. The fact that caspase-3 activity is not necessary for rat L8 myoblast fusion may be due to differences in the levels and regulation of caspases in different species and cell types [13] and/or differences in target substrates (e.g. MST1 [23]). It is also possible that the rat L8 myoblasts studied here are beyond the stage of myoblast differentiation at which caspase-3 is involved. The triggers for caspase activation in the myoblasts are not known. It is of interest to note that the anti-apoptotic protein bcl-2 is expressed in the early stages of myogenesis in proliferating myoblasts but not in differentiating myoblasts [18,34]. It is also of interest to note that the transcription factor FKHR, that usually induces cell cycle arrest and apoptosis, is required for mouse myoblast fusion [35]. The relation of FKHR activity to the loss of bcl-2 and to caspase activation in differentiating myoblasts remains to be studied.

Overall, the published results [23] and those presented here indicate that one or more of the caspases are involved in myoblast differentiation and fusion. The participation of caspases in non-apoptotic processes may be ascribed to partial activation of caspase cascades, so that some initial steps occur, but the reactions do not proceed to apoptosis [18–23]. In

the case of myoblast differentiation, such partial caspase-mediated events might play a role in the membrane and cytoskeleton disorganization required for myoblast fusion to viable myotubes. Most studies have concentrated on caspase-3 as the downstream effector caspase in non-apoptotic events [19–23]. Information is lacking on caspase-1 in such processes, including differentiation processes, and it would be of interest to carry out such studies on other myoblasts and other cell types. We have recently found that neuronal cell differentiation is inhibited by caspase-1 inhibitor (S. Barnoy, T. Vaisid, and N.S. Kosower, unpublished results). Thus, caspase-1 involvement in cell differentiation may not be unique to L8 myoblasts. The involvement of caspase-1 in L8 myoblast fusion represents a novel function for this caspase and points to cross-talk between the calpain and caspase systems in some cases of non-apoptotic, differentiation processes.

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