Endoplasmic reticulum stress signaling transmitted by ATF6 mediates apoptosis during muscle development

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Although apoptosis occurs during myogenesis, its mechanism of initiation remains unknown. In a culture model, we demonstrate activation of caspase-12, the initiator of the endoplasmic reticulum (ER) stress-specific caspase cascade, during apoptosis associated with myoblast differentiation. Induction of ER stress-responsive proteins (BiP and CHOP) was also observed in both apoptotic and differentiating cells. ATF6, but not other ER stress sensors, was specifically activated during apoptosis in myoblasts, suggesting that partial but selective activation of ER stress signaling was sufficient for induction of apoptosis. Activation of caspase-12 was also detected in developing muscle of mouse embryos and gradually disappeared later. CHOP was also transiently induced. These results suggest that specific ER stress signaling transmitted by ATF6 leads to naturally occurring apoptosis during muscle development.

Introduction

The central executioner molecules of apoptosis are a family of cysteine proteases, the caspases, which comprise multiple cascades (Degterev et al., 2003). All caspases are synthesized as precursor proteins, procaspases, which are activated by processing to ~20- (p20) and 10-kD (p10) mature fragments. Apoptosis is often associated with differentiation during development (Glücksmann, 1951). However, the cause of apoptosis and the mechanism of initiation of caspase activation during differentiation remain largely unknown. The present study focuses on the triggering of caspase activation during myoblast differentiation. Myoblast cells begin to exhibit considerable morphological changes when cultures are altered to low concentrations of mitogens. During this process, myoblasts express muscle-specific proteins and fuse into multinucleated myotubes (Stockdale and Holtzer, 1961; Nadal-Ginard 1978). Myoblast fusion is associated with apoptosis (Fidzianska and Goebel, 1991), and activation of caspase-3, usually a downstream caspase, was detected in a cell culture model (Dee et al., 2002; Fernando et al., 2002). The caspase initiator responsible for caspase-3 activation is unknown.

Because caspase-12 is highly expressed in muscle of adult (Van de Craen et al., 1997) and embryonic mice (described later), it might play a physiological role in muscle tissue. It is an ER stress-specific caspase, functioning as the initiator caspase in response to ER stress, that involves accumulation of misfolded proteins in the ER (Nakagawa et al., 2000). Due to the nature of apoptotic stimuli, the effects of caspase-12 have been often discussed regarding pathological changes, especially in relation to neurodegenerative disorders (Nakagawa et al., 2000) and prion diseases (Hetz et al., 2003). We previously found that caspase-12 initiates the ER stress-specific cascade involving caspases-12, -9, and -3 in a myoblast cell line, C2C12 (Morishima et al., 2002), indicating that caspase-12 can function as an initiator caspase in myoblasts for implementation of apoptosis.

ER stress-induced apoptosis is preceded by activation of a cytoprotective signaling cascade termed the unfolded protein response (UPR). The UPR alters transcriptional and translational programs to cope with accumulation of unfolded or misfolded proteins. Although protein synthesis is generally down-regulated in the UPR, decreasing the load in the ER, the UPR induces a group of specific proteins, including BiP, an ER-specific molecular chaperone, and CHOP, a transcription factor known to be up-regulated by ER stress (Zhang and Kaufman, 2004). We demonstrate that both BiP and CHOP are induced in both apoptotic cells and differentiating myoblasts,

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Abbreviations used in this paper: AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride; DM, differentiation medium; E, embryonic day; p-APMSF, 4-amidinophenylmethanesulfonyl fluoride; UPR, unfolded protein response.

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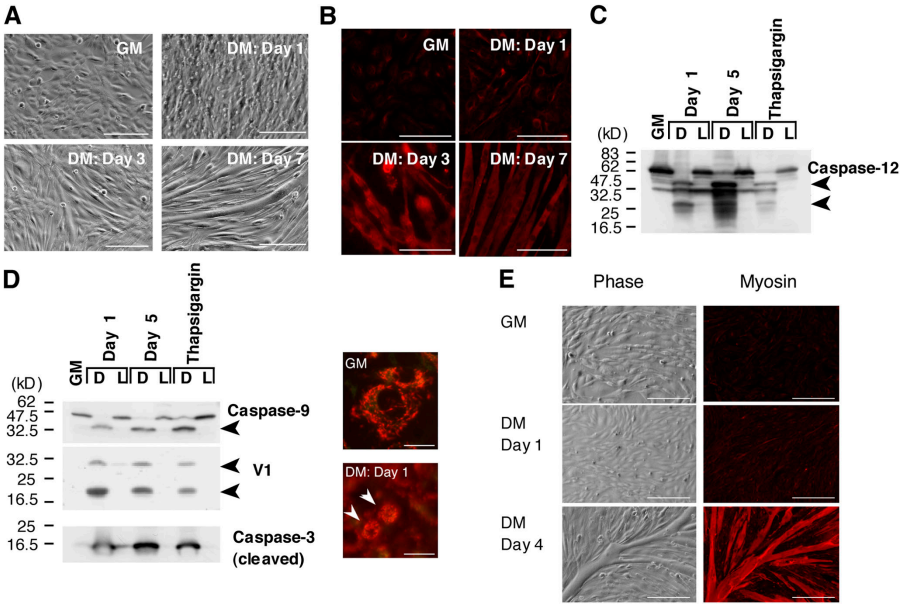

Figure 1. **The ER stress-specific caspase cascade is activated during myoblast differentiation.** (A) Induction of either apoptosis or myotube formation. Phase-contrast images of cells are shown. Note that the small round cells in growing culture (GM) are mitotic cells. Bars, 200 μm. (B) Expression of myosin in multinucleated myotubes detected by immunocytochemistry with antimyosin antibody. Bars, 100 μm. (C) Activation of caspase-12 in apoptotic cells examined by Western blot analysis. Arrowheads depict active forms of caspase-12 detected with anti–caspase-12 mAb. D, dead cells; L, live cells. As controls, cells treated with 1 μM thapsigargin (24 h) were also examined. (D) Western blot analysis of caspase-9 and -3 (left). Arrowheads depict active forms. Vimentin cleavage was detected by anti-V1 antibody (Nakanishi et al., 2001). Integrity of mitochondrial transmembrane potential was examined using the JC-1 reagent, which stains intact mitochondria orange (right). Apoptotic cells are indicated by arrowheads. Bars, 20 μm. (E) Suppression of apoptosis in MAGE-3-transfected cells cultured in DM at day 1. Left, phase-contrast; right, antimyosin immunostaining. Bars, 200 μm.

indicating that differentiation conditions elicit ER stress-specific signaling. Under such conditions, activation of caspase-12 was detected, suggesting a previously unidentified role of ER stress signaling in activating the ER-specific caspase cascade to induce naturally occurring apoptosis during development.

Results and discussion

Proliferating myoblasts undergo terminal differentiation in vitro under standard differentiation conditions (Stockdale and Holtzer, 1961; Nadal-Ginard, 1978) when cultures are changed to low concentrations of mitogens (differentiation medium [DM]). Approximately 15% of C2C12 cells died during the first 24 h of incubation in DM (Fig. 1 A); thereafter, the percentage of dead cells gradually decreased over a week, in parallel with myotube formation. Seven days after switch to DM, myotubes were abundant and apoptosis had almost ceased (Fig. 1 A). On day 3, formation of myosin was evident in multinucleated myotubes (Fig. 1 B). We collected apoptotic cells that were either floating or loosely attached to the plates by centrifugation and recovered living cells by scraping the plates. At day 1, Western blot analysis showed that procaspase-12 (48 kD) had been extensively processed to its active forms in apoptotic cells and remained largely unchanged in living cells (Fig. 1 C). The sizes of the active forms (35 and 28 kD) in these apoptotic cells were the same as observed during apoptosis in response to thapsigargin, an inhibitor of ER-specific calcium ATPase (Morishima et al., 2002). To our knowledge, this is the first example of activation of caspase-12 under nonpathological conditions.

In the ER stress-specific cascade, caspase-12 activates caspase-9, which then catalyzes cleavage of procaspase-3 (Morishima et al., 2002). Consistent with this finding, we detected considerable activation of caspase-9 and -3 as well as cleavage of vimentin by caspase-9 (Nakanishi et al., 2001) in apoptotic C2C12 cells cultured in DM (Fig. 1 D). However, mitochondrial transmembrane potential was maintained in more than 80% of apoptotic cells (Fig. 1 D), suggesting that the mitochondrial pathway is not important in apoptosis during myoblast differentiation, as in the case of apoptosis of C2C12 cells induced by ER stressors (Morishima et al., 2002).

We examined stable transfectants of C2C12 overexpressing a caspase-12–specific suppressor, MAGE-3, which are resistant to ER stress inducers but not to other apoptotic stimuli (Morishima et al., 2002). The transfectant cells underwent myotube formation with a much lower rate of apoptosis (<1%) than did the parental C2C12 cells (Fig. 1 E). Activation of caspases (-12, -9, and -3) was not detected in the transfectants (unpublished data). These results suggest that execution of apoptosis during myotube formation is mainly dependent on the ER-specific cascade. Myoblast differentiation may proceed, to some extent, without caspase activation because the transfectants expressed myosin, which is in parallel with morphological changes (Fig. 1 E).

Western blot analysis showed that BiP was induced at comparable levels in dying and living cells (Fig. 2 A), indicating that the UPR is activated under differentiating conditions, irrespective of cell fate. At day 5, myosin was expressed in apoptotic cells in DM, indicating that apoptosis takes place even after partial cell differentiation (Fig. 2 B).

The evidence for ER stress signals provided by the induction of BiP is supported by the immunocytochemistry of the C2C12 cells. One day after the cells were transferred to DM, CHOP was induced and accumulated in the nuclei of over 80% of differentiating myoblasts (Fig. 2 C). CHOP induction was also detected in MAGE-3 transfectants (Fig. 2 D), suggesting that generation of ER stress is an upstream event of caspase activation.

Nuclear accumulation of CHOP was still evident at day 2 (~40%), when the differentiating cells began to fuse into myotubes (Fig. 2 C). However, only a fraction of them underwent

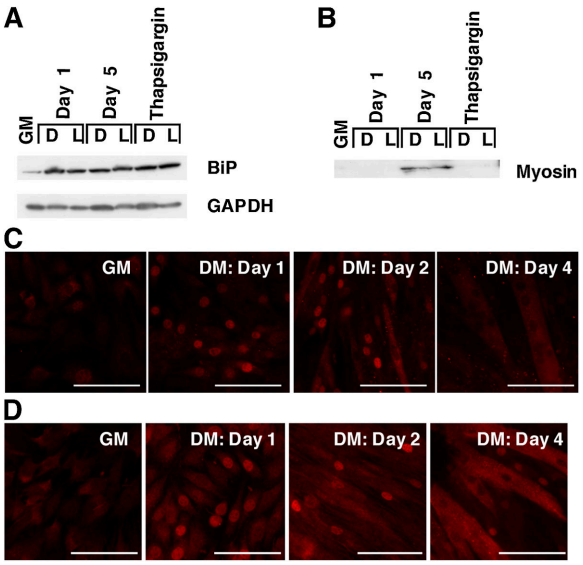

Figure 2. **ER stress signals are generated in C2C12 cells in DM.** (A) Induction of BiP in dying (D) and living (L) cells determined by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as loading control. Cells treated with 1 μM thapsigargin (24 h) were also examined as positive controls. (B) Myosin was expressed in both apoptotic cells and differentiating cells. (C) Detection of induction of CHOP by immunocytochemical analysis of differentiating C2C12 cells. Bars, 100 μm. (D) CHOP induction in MAGE-3 transfectants. Bars, 100 μm.

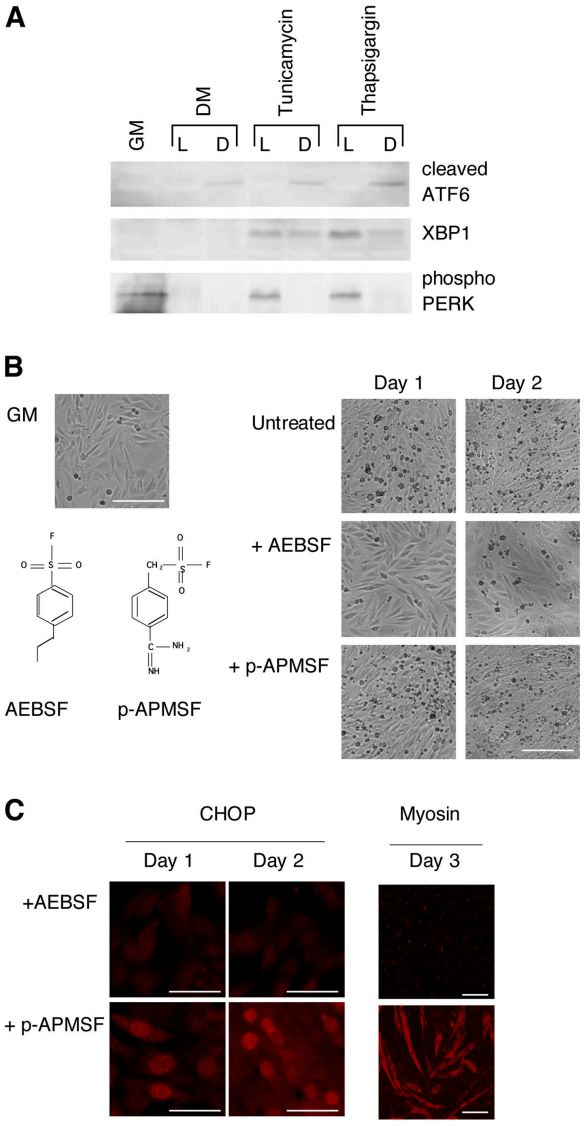

Figure 3. **ATF6 is important in caspase-12 activation during myotube formation.** (A) Activation states of ER stress sensors. Western blot analysis of myoblast cells cultured in DM for 24 h. L, live cells; D, dying cells. As positive controls, cells treated with either 2 μg/ml tunicamycin or 1 μM thapsigargin for 24 h were included in the analysis. (B) AEBSF inhibits apoptosis in C2C12 cells in DM. Proliferating cells were pretreated with 400 μM AEBSF or 100 μM p-APMSF for 1 h, and then transferred to DM in the presence of inhibitor for 24 or 48 h. Bars, 200 μm. (C) AEBSF treatment prevents induction of CHOP and myosin. Cells were immunostained by anti-CHOP antibody and antimyosin antibody. Bars: (CHOP) 50 μm; (Myosin) 100 μm.

apoptosis on day 2 or thereafter. Thus, nuclear localization of CHOP did not necessarily induce apoptosis. CHOP immunostaining was detected in the cytoplasm at a later stage of differentiation (Fig. 2 C), suggesting that CHOP translocates into the nucleus at an early stage and later moves back out, being no longer functional as a transcription factor. Stress is probably reduced during differentiation, allowing most cells to recover and differentiate into myotubes.

To gain insight into the mechanism of induction of caspase-12 activation in C2C12 cells in DM, we examined activation states of upstream factors that sense ER stress. Three ER-resident transmembrane proteins have been identified as proximal sensors of ER stress: the kinase and endoribonuclease IRE1, PERK kinase, and the transcription factor ATF6 (Rutkowski and Kaufman, 2004). In the cases of IRE1 and PERK, ER stress induces autophosphorylation and activation. In contrast, ER stress leads to ATF6 transit to the Golgi complex, where it is cleaved for activation by the protease S1P, and then by S2P. These sensors comprise parallel pathways that are connected by signal cross talk through gene expression. Activation of these pathways can be assessed by posttranslational modification of these sensors themselves or by their downstream targets; e.g., generation of a splicing variant of XBP1 by active IRE1.

Culturing C2C12 cells under differentiation conditions resulted in activation of the ATF6 pathway in dying cells but not of IRE1 and PERK (Fig. 3 A). Although PERK seemed to be constitutively active in live cells in growth medium, active PERK disappeared in DM. In control experiments, the ER stressors tunicamycin (inhibitor of *N*-glycosylation) and thapsigargin also activated ATF6 specifically in dying cells and IRE1/XBP in both living and dying cells (Fig. 3 A). Active PERK was detected in surviving cells, whereas it again disappeared in dying cells treated with ER stressors. The reason for disappearance of active PERK from these dying cells is unknown. These results suggest that active ATF6 plays a decisive role in induction of apoptosis in C2C12 cells, whereas activation of IRE1 or the PERK pathway is not required for apoptosis. ER stress during myoblast differentiation may be weaker than that generated by ER stressors, so that it activates only the ATF6 pathway.

Next, we treated C2C12 cells with a serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), which specifically inhibits S1P (Okada et al., 2003). At days 1 and 2,

AEBSF in DM conferred resistance to differentiation-associated apoptosis, resulting in little cell death (Fig. 3 B), with neither activation of ATF6 and caspase-12 (not depicted) nor induction of CHOP and myosin (Fig. 3 C). Control experiments using another serine protease inhibitor, 4-amidinophenylmethanesulfonyl fluoride (p-APMSF), revealed neither a protective effect against apoptosis (Fig. 3 B) nor prevention of induction of CHOP and myosin (Fig. 3 C). Although this result suggests the importance of the ATF6 pathway in apoptosis, transient transfection of the cleaved form of ATF6 did not enhance apoptosis in DM (unpublished data). It seems likely that an additional signal pathway is also involved in apoptosis induction.

We could not detect activation of ATF6 in live cells (Fig. 3 A), in which both BiP and CHOP were induced. BiP and CHOP are induced by either active ATF6 or IRE1, the former being upstream of the latter (Rutkowski and Kaufman, 2004). It is possible that activation of these sensors is below the level of detection in Western blot analysis.

To confirm involvement of caspase-12 in myogenesis in vivo, we examined caspase-12 activation in developing muscle tissues. We generated specific antibodies (Nakanishi et al., 2001) to active caspase-12, which recognized the COOH terminus of the p20 subunit as well as to the NH_2 terminus of the p10 subunit (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200412024/DC1).

Immunostaining of embryonic muscle tissues with anti–caspase-12 monoclonal antibody was negative in embryonic day (E) 12.5 sections (not depicted); however, at E13.5, E15.5, and E18.5, caspase-12 was abundant in filamentous cells of muscle tissues around the neck, tongue, and back (Fig. 4 A). At E13.5, about half of the filamentous muscle cells in this region reacted with the antibody to active caspase-12, and intensity of staining decreased at E15.5 and E18.5 (Fig. 4 B), suggesting the presence of transient ER stress. Staining with caspase-12 monoclonal antibody was detected throughout filamentous cells, with more intense signals around the nucleus (Fig. 4 C), whereas the signals obtained with the two anti-active caspase-12 antibodies were generally restricted to the region around the nucleus (Fig. 4 C). Evidently, only a fraction of caspase-12 molecules are activated in these tissues. Double-staining of these cells with active caspase-12 antibody and antimyosin antibody showed that activated caspase-12 was indeed present in cells undergoing differentiation, and thus does not necessarily induce apoptosis (Fig. 4 C).

Only tissue sections at E13.5 appeared to express CHOP, indicating that CHOP induction is transient (Fig. 4 D). This finding coincides with the onset of caspase-12 expression (Fig. 4 A), as well as muscle-tissue formation (Ontell and Kozeka, 1984), in the developing mouse. CHOP exhibited either nuclear or cytoplasmic location (Fig. 4 E). This pattern is similar to that observed in the culture model (Fig. 2 C), perhaps reflecting the production and subsequent extinction of stress signals. It is possible that CHOP leaves the nucleus as the result of decrease in ER stress. Double-staining of muscle tissues showed that caspase-12 is activated in cells that express CHOP (Fig. 4 F). These findings suggest that activation of caspase-12 by ER stress occurs in these myoblasts.

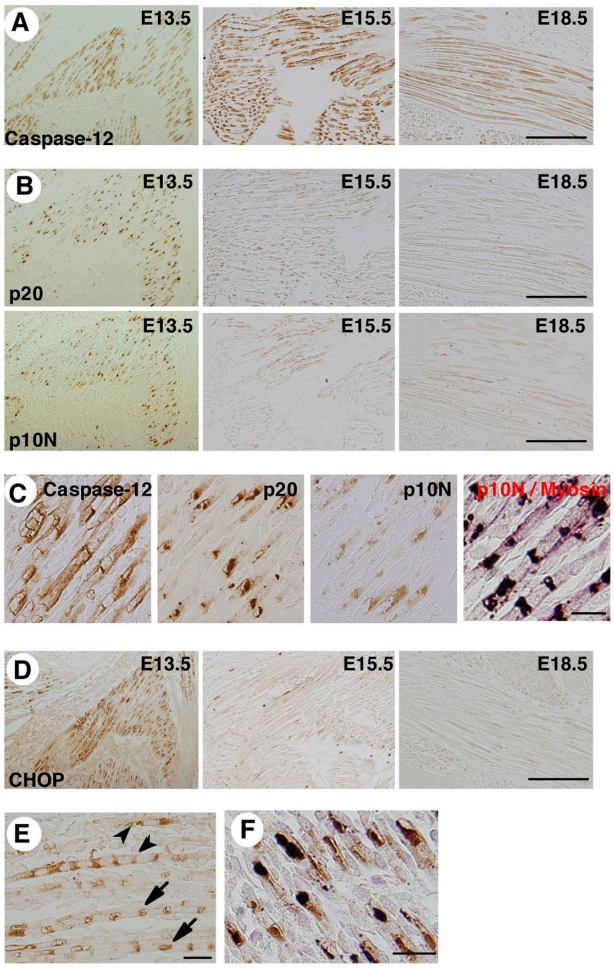

Figure 4. **Activation of caspase-12 and induction of CHOP in developing muscle tissue.** (A) Caspase-12 was induced at the onset of muscle formation at E13.5 in embryo back muscle tissue. (B) Back muscle at E13.5 was specifically stained with antibody to active caspase-12. (C) Higher magnification of images in A and B. The muscle tissue at E13.5 was stained with antibody to caspase-12. Also shown is double-labeling of cells with anti-active caspase-12 antibody (brown) and antimyosin antibody (purple). (D and E) CHOP induction in back muscle tissues at E13.5. Arrows indicate nuclear accumulation of CHOP, whereas arrowheads point to cells with cytoplasmic CHOP. (F) Activation of caspase-12 in the presence of CHOP induction. Anti-p10N antibody, purple; CHOP, brown. Bars: (A, B, and D) 100 μm; (C, E, and F) 10 μm.

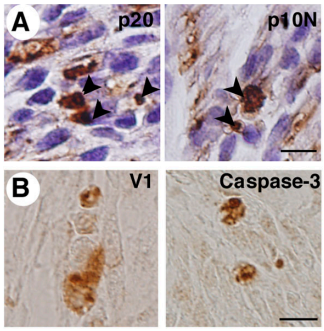

Figure 5. **Activation of the ER stress-specific caspase cascade in apoptotic cells in developing muscle tissue at E13.5.** (A) Small round cells stained by anti-active caspase-12 antibodies contained condensed nuclei (arrowheads). Nuclei were stained by Gill's modified hematoxylin solution (Merck). (B) Small round cells stained with anti-V1 and anti-active caspase-3 antibodies. Bars, 10 μm.

A few of the immunopositive cells were apoptotic (round cells with condensed nuclei; Fig. 5 A). However, other positive cells were filamentous (Fig. 4 C), indicating that most of the stained cells were not apoptotic but differentiating. Activation of caspase-9 and -3 was also detected in the round cells (Fig. 5 B). A single section might contain a few cells undergoing apoptosis because of caspase-9 and -3 activation. It seems likely that activation of caspase-12 does not always result in full activation of downstream caspases and that antiapoptotic proteins such as XIAPs might inhibit downstream caspases (Deveraux and Reed, 1999). Most of the cells probably experience stress when they start to differentiate, but only a small minority die, whereas the majority differentiate toward the terminal phenotype.

In summary, we propose that ER stress signaling, especially that mediated by ATF6, plays a role in the induction of developmental apoptosis in muscle tissues through activation of caspase-12. Identification of ER stress as a trigger was made possible by detection of caspase-12 activation because its activation is nearly synonymous with generation of ER stress. Because the major cause of apoptosis appears to be ER stress, we speculate that this type of apoptosis contributes to selecting out of a subpopulation of cells that are vulnerable to cellular stress. Our findings also imply that differentiating myoblasts suffer cellular stress, and thus provide clues to how myotube formation is regulated in vivo. ER stress and its downstream signaling might play roles in myogenesis, in addition to apoptosis. One remaining issue is how ATF6 signaling is activated at the beginning of differentiation. Changes in the ER, other than accumulation of misfolded proteins, may cause specific activation of ATF6. The suggestion that caspase activation is involved in myoblast differentiation by itself (Fernando et al., 2002) must also be considered. Caspase-12 knockout mice develop normally (Nakagawa et al., 2000), indicating that caspase-12–dependent apoptosis is not essential for muscle development. Although caspase-12 plays a major role in ER stress-induced apoptosis, caspase-12−/− cells are still sensitive to ER stress (Nakagawa et al., 2000), suggesting that a mechanism other than that involving caspase-12 also participates in execution of apoptosis in response to ER stress. Recent studies have shown that ER stress can induce caspase-9 by a mitochondria-dependent and probably caspase-12–independent route in several cell lines (Häcki et al., 2000; Ito et al., 2001). It is thus possible that other caspases, such as caspase-9, compensate for the absence of caspase-12, as such compensatory effects are sometimes observed in caspase knockout mice (Zheng et al., 2000). Nevertheless, our study strongly suggests that ER stress plays an important role in triggering apoptosis associated with muscle development because activation of caspase-12 was detected in all the apoptotic cells in differentiating muscle tissues.

Materials and methods

Creation of antibodies to caspase-12 fragments
Cleavage-site–directed antibodies were prepared as described previously (Nakanishi et al., 2001). Immunizing rabbits with the synthetic peptides CRYNGTINVSTNKGIATAD and TDEERC generated an anti-p20 antibody and an anti-p10N antibody, respectively.

Cell culture
C2C12 cells were grown in medium with 10% FCS as described previously (Morishima et al., 2002). Differentiation was induced in medium with 2% horse serum (Invitrogen) and 1 μg/ml insulin (Sigma-Aldrich). Mitochondrial transmembrane potential was examined by staining cells with JC-1 reagent (Molecular Probes), according to the manufacturer's protocol. For inhibition studies, either 400 μM AEBSF or 100 μM p-APMSF (Sigma-Aldrich) was included in the medium. Effective concentrations of these inhibitors are 100–1,000 μM (AEBSF) and 10–100 μM (p-APMSF), as suggested by the manufacturer. Transfection was performed as described previously (Morishima et al., 2002). The active ATF6 cDNA was provided by K. Mori (Kyoto University, Kyoto, Japan).

Immunocytochemistry
C2C12 cells grown in 8-chamber slides (Nunc) were fixed in 4% PFA/PBS for 10 min and permeabilized in 0.1% Triton X-100 for 20 min. In some cases, they were fixed and permeabilized in methanol at −20°C for 2 min. The fixed and permeabilized cells were blocked in PBS containing 3% BSA (Jackson ImmunoResearch Laboratories) and incubated overnight at 4°C with primary antibodies in blocking solution. Immunoreactivity was detected with either Alexa-conjugated secondary antibody (Molecular Probes) or a combination of biotin-conjugated secondary antibody (Vector Laboratories) and Alexa-conjugated streptavidin (Molecular Probes).

Western blot analysis
Cells were lysed in SDS-PAGE sample buffer and homogenized. Western blot analysis was performed as described previously (Morishima et al., 2002).

Immunohistochemistry
Paraffin-embedded sections were prepared as described previously (Nakanishi et al., 2001). For immunohistochemistry, sections were rehydrated through a graded series of alcohols, rinsed in PBS, and blocked in PBS containing 2% normal serum. After overnight incubation with primary antibody at 4°C, immunoreactivity was detected using a TSA Fluorescein System (NEN Life Science Products) followed by incubation with 3,3′-DAB (Wako Chemicals). For double-staining, DAB and VIP substrate (Vector Laboratories) were used for discrimination of different primary antibodies. TUNEL staining was performed as described previously (Nakanishi et al., 2001).

Microscopy
Immunostained images were captured with an ORCA-ER cooled charge-coupled camera (Hamamatsu Photonics) mounted on a microscope (model BX51; Olympus). All images were captured at either 10-, 20-, or 40-fold magnification using Plan-Apochromat objective lenses (10×, 0.40 NA; 20×, 0.70 NA; 40×, 0.85 NA). Images were acquired and processed using IPLab software (Scanalytics). Selected images were pseudocolored for presentation using Adobe Photoshop. Live cells during culture were observed under a microscope (model IX-70; Olympus) using a 4× Plan-Semi-Apochromat lens (0.13 NA).

Antibodies
Primary antibodies used for immunostaining were anti–caspase-12 p20 (this study), anti–caspase-12 p10N (this study), anti–caspase-12 (Nakagawa et al., 2000; provided by J. Yuan, Harvard Medical School, Boston, MA), anti–caspase-9 (Medical and Biological Laboratories), anti–vimentin V1 (Nakanishi et al., 2001), anti-active caspase-3 and anti-phospho PERK (Cell Signaling Technology), anti-CHOP, anti-ATF6, and anti-XBP1 (Santa Cruz Biotechnology, Inc.), anti-BiP (Transduction Laboratories), antimyosin (Progen; Sigma-Aldrich), anti-GAPDH (Chemicon), and anti-hexahistidine (CLONTECH Laboratories, Inc.).

Online supplemental material
Fig. S1 shows the efficacy of anti-active caspase-12, which was confirmed by specifically stained, paraffin-embedded sections of adult mouse kidney treated with tunicamycin. Also shown are TUNEL-positive staining, induction of CHOP, cleavage of vimentin by caspase-9, and active caspase-3 in the damaged kidney. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200412024/DC1.

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