The Small Heat Shock Protein αB-crystallin Negatively Regulates Apoptosis during Myogenic Differentiation by Inhibiting Caspase-3 Activation*

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Myoblasts respond to growth factor deprivation either by differentiating into multinucleated myotubes or by undergoing apoptosis; hence, the acquisition of apoptosis resistance by myogenic precursors is essential for their development. Here we demonstrate that the expression of the small heat shock protein αB-crystallin is selectively induced in C2C12 myoblasts that are resistant to differentiation-induced apoptosis, and we show that this induction occurs at an early stage in their differentiation in vitro. In contrast, the expression of several known anti-apoptotic proteins (FLIP, XIAP, Bcl-xL) was not altered during myogenesis. We also demonstrate that ectopic expression of αB-crystallin, but not the closely related small heat shock protein Hsp27, renders C2C12 myoblasts resistant to differentiation-induced apoptosis. Furthermore, we show that the myopathy-causing R120G αB-crystallin mutant is partly impaired in its cytoprotective function, whereas a pseudophosphorylation αB-crystallin mutant that mimics stress-induced phosphorylation is completely devoid of anti-apoptotic activity. Finally, we demonstrate that αB-crystallin negatively regulates apoptosis during myogenesis by inhibiting the proteolytic activation of caspase-3, whereas the R120G and pseudophosphorylation mutants are defective in this function. Taken together, our findings indicate that αB-crystallin is a novel negative regulator of myogenic apoptosis that directly links the differentiation program to apoptosis resistance.

During skeletal muscle development, a subset of proliferating myoblasts exit the cell cycle and become resistant to apoptosis; these surviving myoblasts fuse to form multinucleated myotubes and differentiate into mature myocytes, whereas apoptosis-sensitive myoblasts are eliminated (1). Although the acquisition of apoptosis resistance by myogenic precursors is a critical event in their differentiation, only a few genes that regulate this process have been identified. One such gene is the cyclin-dependent kinase inhibitor p21 whose expression is induced at an early stage of myogenesis by the skeletal muscle-specific transcriptional regulator MyoD (2, 3). p21 expression in myoblasts promotes cell cycle withdrawal and confers resistance to apoptosis through its actions on its downstream target, the retinoblastoma (RB) protein (1, 4). The anti-apoptotic kinase Akt is also induced during skeletal muscle development and promotes the survival of differentiating myoblasts, although its anti-apoptotic mechanism(s) in muscle is unclear (5–7). Finally, Bcl-2, a protein that inhibits many of the mitochondrial events in apoptosis, is transiently expressed in myogenic precursors and promotes their clonal expansion (8). Nevertheless, given the complexity of the apoptotic cell death apparatus (9), it seems likely that other genes play important roles in myogenic apoptosis.

One particularly intriguing candidate is αB-crystallin, a member of the small heat shock protein (HSP) family that also includes αA-crystallin, Hsp27, Hsp20, Hsp22, myotonic dystrophy protein kinase-binding protein (MKBP)/HspB2, and HspB3 (10–12). With the exception of αA-crystallin, all of the small HSPs are abundantly expressed in muscle tissue where they function as molecular chaperones that facilitate refolding of non-native proteins (10, 11). Structurally, each of the small HSFs contains a highly conserved α-crystallin domain flanked by largely non-conserved amino and carboxyl termini. We postulated that αB-crystallin might be an important regulator of apoptosis during myogenesis for a number of reasons. First, the expression of αB-crystallin is induced early during skeletal myogenesis in vivo and in vitro and is regulated by MyoD (11, 13–15). Second, αB-crystallin and the related small heat shock protein Hsp27 confer resistance to apoptosis induced by a wide range of stimuli (16–18). Indeed, we have recently demonstrated that αB-crystallin negatively regulates TNF-α- and DNA damage-induced apoptosis by a novel mechanism; αB-crystallin inhibits the activation of caspase-3, a key pro-apoptotic protease (17). Third, a missense mutation of αB-crystallin (R120G) has been shown to cause an autosomal dominant myopathy characterized by the disruption of myofibrils and the accumulation of aggregates of desmin and αB-crystallin in degenerating muscle cells (19). Biochemically, the R120G mutant is severely compromised in its chaperone activity (20, 21). Fourth, mice with targeted deletion of the αB-crystallin gene, and the adjacent HspB2 gene, develop a progressive myopathy (22). These findings suggest that αB-crystallin may promote muscle survival during differentiation and in response to stress.

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In this report, we demonstrate that the expression of αB-crystallin is selectively induced in surviving C2C12 myoblasts at an early stage in their differentiation in vitro. Moreover, we show that ectopic expression of αB-crystallin, but not Hsp27, is sufficient to inhibit differentiation-induced myoblast apoptosis. We also demonstrate that the myopathy-causing R120G mutation is partly impaired in its cytoprotective function, whereas a pseudophosphorylation αB-crystallin mutant that mimics stress-induced phosphorylation is completely defective in its anti-apoptotic function. Finally, we show that αB-crystallin negatively regulates differentiation-induced myoblast apoptosis by inhibiting the proteolytic activation of caspase-3. Overall, our findings demonstrate for the first time that the small HSP αB-crystallin is a novel negative regulator of myogenic apoptosis that directly links the differentiation program to apoptosis resistance.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Murine C2C12 cells were maintained in growth medium (GM): Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). To induce differentiation, cells were washed twice in phosphate-buffered saline (PBS) and transferred to differentiation medium (DM): Dulbecco’s modified Eagle’s medium supplemented with 0.5% FBS.

Western Blot Analyses—Whole cell lysates were prepared and analyzed by immunoblotting as described (23) using the following Abs: αB-crystallin (StressGen), Hsp27 (StressGen), tubulin (Sigma), desmin (Sigma), p21 (Oncogene Science), M2 FLAG (Sigma), FLIP (kindly provided by Dr. H. Perlman), XIAP (BD PharMingen), and Bel-xL (BD PharMingen).

Construction of FLAG Epitope-tagged cDNAs—The FLAG-tagged, wild-type human αB-crystallin and Hsp27 cDNAs have been described previously (17). The R120G mutant αB-crystallin cDNA was made using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with the following oligonucleotide primers: 5′-CTCCAGGGATTTCCAGGGAATATCCGATCC-3′ and 5′-GGGATCCGGATTTCCCCGCTGGAACCTTCTACCTTCGGCC-3′. The triple pseudophosphorylation mutant αB-crystallin cDNA (S19E,S45E,S59E, abbreviated 3XSE) was made using this same site-directed mutagenesis kit by sequentially altering each of these serine residues using the following primers: 5′-CCCTCTCTTCTTCTTCTCCAGGAAAGTCTGGCTGGTCTC-3′ and 5′-GCATAAACTTCCACCTGCCC-3′ and 5′-GGCCGAGAGTTAGAAGGTTTGCAAGAAACCGG-3′ (S19E), 5′-CCAGGCTGTCATTCCCTGGACCC-3′ and 5′-GGCCGAGAGTTAGAAGGTTTGCAAGAAACCGG-3′ (S45E), 5′-CCCTCTCTTCTTCTTCTCCAGGAAAGTCTGGCTGGTCTC-3′ and 5′-GGCCGAGAGTTAGAAGGTTTGCAAGAAACCGG-3′ (S59E). The αB-crystallin cDNA encoding amino acids 1-161 and lacking its carboxyl-terminal 14 amino acids (ΔC) was PCR-amplified using the following primers: 5′-GGCGAATTCTAGGATCTCGGAGGAGCTCTCACCC-3′ and 5′-GGCCGCGCGAGGAGGTGGGAGTGGGAGGAGCTCTCACCC-3′. The PCR product was then digested with EcoRI and XhoI and cloned into a modified pcDNA3 vector in which a sequence encoding FLAG was inserted upstream of the multiple cloning site. All sequences were confirmed by automated DNA sequencing.

Transfection of C2C12 Cells—C2C12 cells were grown on glass coverslips to 40% confluence in GM and transiently transfected with 1 μg of plasmid DNA using the FuGENE 6 transfection reagent (Roche). To obtain transfection efficiencies, 2 μg of a mixture of the following plasmids were transfected: pCI-neo (Novagen), pCMV-myc (Clontech), pCMV-HA (Clontech), pCMV-FLAG (Clontech), pCMV-B (Clontech), pCMV-X (Clontech), and pCMV-Bcl-xL (Clontech). Transfections were performed using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. After a 24-h incubation, C2C12 cells were washed and transferred to DM for an additional 24 h prior to their analysis by immunofluorescence.

Indirect Immunofluorescence and Apoptosis Assays—To detect ectopically expressed cDNAs, C2C12 cells were prepared as above. To detect endogenous αB-crystallin, C2C12 cells were grown up to 40% confluence on glass coverslips in GM, washed twice in PBS, and transferred to DM for 0–72 h. Cells were then fixed in 100% methanol for 2 min at −20 °C and incubated for 2 h at 37 °C with αB-crystallin mAb (1:500 dilution in PBS) to detect the endogenous protein or with FLAG M2 mAb (5 μg/ml) to detect the ectopically expressed proteins. After incubation with the primary Ab, cells were washed in PBS and incubated with fluorescein-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, 1:20) and rhodamine-conjugated antibody to rabbit IgG (1:200). The percentage of apoptotic cells was determined by scoring fragmented/condensed nuclei by fluorescence microscopy as described (17, 24). In each experiment, at least 200 cells were counted, and experiments were performed in triplicate. Statistical significance was assessed by a two-tailed, paired Student’s t test.

RESULTS

Induction of αB-crystallin Expression During an Early Stage of Myogenic Differentiation—To begin to determine whether αB-crystallin might play a role in regulating apoptosis during myogenesis, we examined its expression in C2C12 myoblasts that were induced to undergo differentiation by mitogen withdrawal, a well characterized in vitro model (1). C2C12 myoblasts were grown in GM containing 0.5% FBS for 0–72 h, and the expression of αB-crystallin was analyzed by immunoblotting. As shown in Fig. 1A, the expression of αB-crystallin was rapidly induced in myoblasts within 4 h of transfer to DM and increased in a time-dependent manner throughout the entire 72-h interval, a critical period during which myoblasts differentiate into multinucleated myotubes and become resistant to apoptosis (see Fig. 2 and Ref. 1). Indeed, the myogenic induction of αB-crystallin expression preceded that of desmin, a muscle-specific intermediate filament protein that is one of the earliest markers of myogenic commitment (25, 26), and p21, a mediator of cell cycle exit and apoptosis resistance (1). In contrast, the expression of Hsp27, a closely related small HSP, was transiently increased at a later time point (18–24 h), its expression falling below baseline levels at 48–72 h in DM. Fur-

![Fig. 1. Induction of αB-crystallin expression during an early stage of myogenic differentiation in C2C12 myoblasts. A and B, murine C2C12 myoblasts were cultured in differentiation medium (DM) containing 0.5% FBS for 0–72 h, and whole cell lysates were then analyzed by immunoblotting as detailed under "Experimental Procedures." C, C2C12 myoblasts were heat-shocked at 44°C for 1 h, transferred to 37°C for the indicated time, and the expression of αB-crystallin was determined by immunoblotting as described under "Experimental Procedures."](http://www.jbc.org/content/25/8/A350/F1.large.jpg)
thermore, the expression levels of other anti-apoptotic proteins (XIAP, FLIP<sub>L</sub>, and Bcl-x<sub>L</sub>) were not significantly altered during myogenic differentiation (Fig. 1B). Interestingly, the induction of αB-crystallin expression observed in C2C12 myoblasts during differentiation was similar to that observed in these cells in response to heat shock at 44 °C for 1 h (Fig. 1C).

αB-crystallin Is Preferentially Expressed in Surviving Myoblasts during Myogenesis—If the expression of αB-crystallin confers resistance to myogenic apoptosis, αB-crystallin should be selectively expressed in surviving myoblasts. To examine this hypothesis, C2C12 cells were transferred to DM for 0–72 h, and cells were then simultaneously examined for αB-crystallin expression by indirect immunofluorescence and scored for apoptotic (condensed/fragmented) nuclei as described under “Experimental Procedures.” A, representative photomicrographs showing αB-crystallin expression (upper panels) and nuclear morphology (lower panels), the nuclei corresponding to αB-crystallin-positive myoblasts are indicated by arrows. B, the data represent the mean ± S.E. of three independent experiments (*, p < 0.05 for αB-negative versus αB-positive cells at each time point).

αB-crystallin Inhibits Myogenesis-induced Caspase-3 Activation—Because we have previously demonstrated that αB-crystallin negatively regulates TNF-α- and DNA damage-induced apoptosis by inhibiting the activation of caspase-3 (17), we wanted to determine whether αB-crystallin inhibited myogenic apoptosis by the same mechanism. To this end, we transfected C2C12 myoblasts to DM for 72 h and examined cells for expression of αB-crystallin and active caspase-3; the latter was detected using an antibody that specifically recognizes the large subunit of activated caspase-3 (and does not detect pro-caspase-3). As shown in Fig. 2A, the vast majority of C2C12 cells expressing αB-crystallin had intact nuclei (the nuclei corresponding to αB-crystallin-positive myoblasts are indicated by arrows in the lower panels) even after 72 h in DM, whereas virtually all of the apoptotic myoblasts with fragmented/condensed nuclei lacked αB-crystallin. These results are presented quantitatively in Fig. 2B. Indeed, αB-crystallin-negative myoblasts had dramatically higher rates of differentiation-induced apoptosis than αB-crystallin-positive cells at 24, 48, and 72 h after transfer to DM. This striking inverse correlation between the induction of apoptosis and the expression of αB-crystallin suggests that αB-crystallin may promote myoblast survival during differentiation.

Ectopic Expression of αB-crystallin Protects Myoblasts from Differentiation-induced Apoptosis—We next wanted to determine whether ectopic expression of αB-crystallin was sufficient to protect myoblasts from differentiation-induced apoptosis. C2C12 cells were transiently transfected with empty vector or FLAG-tagged cDNAs encoding wild-type αB-crystallin or Hsp27. After overnight incubation, cells were transferred to DM for 24 h. Transfected cells were then identified by immunofluorescence with an anti-FLAG mAb, and the percentage of transfected cells with apoptotic nuclei was determined. As shown in Fig. 3A, ectopic expression of wild-type αB-crystallin potently inhibited differentiation-induced myoblast apoptosis. In contrast, Hsp27 did not significantly inhibit differentiation-induced myoblast apoptosis, thereby underscoring the specificity of the role of αB-crystallin in regulating this process.

To delineate the domains of αB-crystallin that mediate its anti-apoptotic actions, we examined the ability of several well defined αB-crystallin mutants to inhibit differentiation-induced myoblast apoptosis. Specifically, we used the following mutant constructs: (i) the myopathy-causing R120G missense mutation, which severely impairs its chaperone activity (19–21); (ii) a pseudophosphorylation triple mutant (S19E,S45E,S59E, abbreviated 3XSE) in which each of the three amino-terminal Ser residues that are phosphorylated in response to stress has been altered to a Glu residue to mimic phosphorylation (27, 28); and (iii) a truncated αB-crystallin, which lacks its carboxyl-terminal tail (ΔC), a region which mediates substrate binding and stabilizes the protein (10, 29). These mutant proteins were expressed at levels comparable with those of wild-type αB-crystallin and Hsp27 in transiently transfected C2C12 myoblasts (see Fig. 3C). As shown in Fig. 3B, the R120G and ΔC mutants were partly impaired in their ability to protect myoblasts from differentiation-induced apoptosis compared with wild-type αB-crystallin. In contrast, the 3XSE triple pseudophosphorylation mutant was completely devoid of anti-apoptotic activity. Because stress-induced phosphorylation of these Ser residues triggers the dissociation of the large oligomeric complexes of αB-crystallin (28), our findings strongly suggest that the anti-apoptotic function of αB-crystallin is tightly linked to its oligomerization state. Importantly, as demonstrated in Fig. 3D, the degree of apoptosis inhibition conferred by αB-crystallin was similar to that of p21, a previously described regulator of myocyte survival (1), whereas neither Bcl-2 nor wild-type Akt significantly inhibited myogenic apoptosis under these conditions.

**Fig. 2. αB-crystallin is preferentially expressed in surviving myoblasts during myogenesis.** C2C12 myoblasts were grown in DM for 0–72 h, and then cells were examined for αB-crystallin expression by indirect immunofluorescence and scored for apoptotic (condensed/fragmented) nuclei as described under “Experimental Procedures.” A, representative photomicrographs showing αB-crystallin expression (upper panels) and nuclear morphology (lower panels), the nuclei corresponding to αB-crystallin-positive myoblasts are indicated by arrows. B, the data represent the mean ± S.E. of three independent experiments (*, p < 0.05 for αB-negative versus αB-positive cells at each time point).
**αB-crystallin Inhibits Myogenic Apoptosis**

**DISCUSSION**

We have demonstrated that αB-crystallin is a novel negative regulator of myogenic apoptosis; its expression is selectively induced in surviving myoblasts during an early stage of their differentiation in vitro. Indeed, the αB-crystallin promoter contains a canonical skeletal muscle-specific E-box element that binds MyoD family members and accounts for the early expression of αB-crystallin during skeletal myogenesis (11, 13–15). In this way, αB-crystallin provides a direct and previously unrecognized link between the myogenic differentiation program and the acquisition of apoptosis resistance, the latter event being a critical step in myogenesis because apoptosis-sensitive myogenic precursors are eliminated (1). Although a few other regulators of myogenic apoptosis have been described (1, 5–8), αB-crystallin is the first HSP to be implicated in this process. Interestingly, we observed that Hsp27, a protein ~40% identical to αB-crystallin (10, 11), was only transiently induced during myogenic differentiation and did not confer resistance to differentiation-induced apoptosis, thereby indicating that there is considerable specificity among HSPs with respect to their role in myogenic cell death. Moreover, our finding that several other anti-apoptotic proteins (XIAP, FLIP, and Bcl-xL) were not induced during skeletal myogenesis indicates that the apoptosis resistance acquired by myogenic precursors represents the activation of a specific subset of anti-apoptotic proteins. However, the long term survival of a subset of αB-crystallin-negative myoblasts indicates that other anti-apoptotic proteins must also contribute to the apoptosis resistance of mature myocytes.

We have also demonstrated that αB-crystallin antagonizes myogenic apoptosis by inhibiting the activation of caspase-3. This observation is consistent with our previous findings in cancer cells that αB-crystallin inhibits caspase-3 activation by disrupting its proteolytic maturation (17). In contrast, Hsp27 did not inhibit myogenic differentiation-induced caspase-3 activation or apoptosis. This latter finding provides additional evidence that the anti-apoptotic mechanisms of these closely related small HSPs are likely to be quite distinct. Indeed, we have demonstrated previously that Hsp27 does not directly inhibit apoptosis by binding to cytosolic cytochrome c and preventing the recruitment of procaspase-9 to the apoptosome (30), a cytosolic caspase-9 activator.

![Fig. 3. Ectopic expression of αB-crystallin protects myoblasts from differentiation-induced apoptosis.](http://www.jbc.org/). In both A and B, after overnight incubation, cells were transferred to DM for 24. Transfected cells were identified by indirect immunofluorescence using an anti-FLAG mAb, and the percentage of transfected cells with apoptotic nuclei was scored as detailed under “Experimental Procedures.” In A, B, and D, the data represent the mean ± S.E. of three independent experiments (*, p < 0.02). C, immunoblot of C2C12 cells transiently transfected with empty vector or FLAG-tagged wild-type or mutant (R120G, ΔC, or 3XSE) αB-crystallin or Hsp27 cDNAs. The ectopically expressed proteins were detected with a FLAG mAb as described under “Experimental Procedures.” D, αB-crystallin confers a similar degree of protection against differentiation-induced apoptosis as p21. C2C12 cells were transiently co-transfected with pEGFPN1 or excess plasmid containing αB-crystallin, p21, Bcl-2, wild-type Akt, or Hsp27, and apoptosis was scored in GFP-positive cells 24 h after transfer to DM as described under “Experimental Procedures.”
We have also reported for the first time structure-function analyses of the anti-apoptotic domains of αB-crystallin. We observed that the myopathy-causing R120G mutation and deletion of its carboxyl-terminal tail partly suppressed the ability of αB-crystallin to inhibit differentiation-induced caspase-3 activation and apoptosis, whereas a pseudophosphorylation triple mutant that mimics stress-induced phosphorylation was severely impaired in these functions. Because each of these mutations likely diminishes the chaperone activity of αB-crystallin (20, 21, 28, 29), but only the triple pseudophosphorylation mutant is defective in its ability to form large 500-kDa oligomers (28), these findings strongly suggest that oligomerization of αB-crystallin is essential for its cytoprotective actions, as has been demonstrated for Hsp27 in other systems (32).

Furthermore, the novel anti-apoptotic function of αB-crystallin reported here and the impaired ability of the R120G mutant to inhibit caspase-3 activation may provide new insights into the etiology of the progressive myopathy caused by this mutation (19). Although the R120G mutation is also compromised in its ability to stabilize desmin intermediate filaments, thereby resulting in the accumulation of aggregates of desmin and αB-crystallin and the disruption of myofibrils (19–21, 33), the defect in its ability to inhibit caspase-3 activation would likely sensitize muscle cells to stress-induced apoptosis. Indeed, the observation that the R120G mutation in patients or in transgenic mice causes muscle degeneration in early adulthood (19, 33) suggests that the cumulative oxidative stress of years of contractile activity is required to unmask the deleterious consequences of this mutation in vivo. Finally, given the central role that caspase-3 plays in the execution of apoptosis (9), our results suggest that αB-crystallin may participate broadly in the regulation of muscle cell death.

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