Mimicking Phosphorylation of \( \alpha B \)-Crystallin on Serine-59 Is Necessary and Sufficient to Provide Maximal Protection of Cardiac Myocytes From Apoptosis

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Abstract—\( \alpha B \)-Crystallin (\( \alpha BC \)), a small heat shock protein expressed in high levels in the heart, is phosphorylated on Ser-19, 45, and 59 after stress. However, it is not known whether \( \alpha BC \) phosphorylation directly affects cell survival. In the present study, constructs were prepared that encode forms of \( \alpha BC \) harboring Ser to Ala (blocks phosphorylation) or Ser to Glu (mimics phosphorylation) mutations at positions 19, 45, and 59. The effects of each form on apoptosis of cultured cardiac myocytes after hyperosmotic or hypoxic stress were assessed. Compared with controls, cells that expressed \( \alpha BC \) with Ser to Ala substitutions at all three positions, \( \alpha BC(\text{AAA}) \), exhibited more stress-induced apoptosis. Cells expressing either \( \alpha BC(\text{AAE}) \) or (\( \text{EEE} \)) exhibited 3-fold less apoptosis than cells expressing \( \alpha BC(\text{AAA}) \), indicating that phosphorylation of Ser-59 confers protection. \( \alpha BC \) is known to bind to procaspase-3 and to decrease caspase-3 activation. Compared with cells expressing \( \alpha BC(\text{AAA}) \), the activation of caspase-3 was decreased by 3-fold in cells expressing \( \alpha BC(\text{AAE}) \). These results demonstrate that mimicking the phosphorylation of \( \alpha BC \) on Ser-59 is necessary and sufficient to confer caspase-3 inhibition and protection of cardiac myocytes against hyperosmotic or hypoxic stress. These findings provide direct evidence that \( \alpha BC(\text{S59P}) \) contributes to the cardioprotection observed after physiologically relevant stresses, such as transient hypoxia. Identifying the targets of \( \alpha BC(\text{S59P}) \) will reveal important details about the mechanism underlying the cytoprotective effects of this small heat shock protein. (Circ Res 2003;92:203-211.)

Key Words: cardiac myocytes \| \( \alpha B \)-crystallin \| apoptosis \| phosphorylation

Stress kinases, such as p38 mitogen-activated protein kinase (MAPK), are activated in the heart in response to potentially harmful stress, such as ischemia.\(^1-3\) In cardiac myocytes, when activated by the MAP kinase kinase, MKK6, p38 stimulates the MAPK-activated protein kinase-2 (MAPKAPK-2), and the cells exhibit reduced apoptosis in response to stress.\(^4,5\) Moreover, the inhibition of p38 increases cardiac myocyte apoptosis and myocardial tissue damage in response to stress.\(^5,6\) Accordingly, the p38/MAPKAPK-2 pathway serves a key role in protecting cells against stress; however, the downstream events responsible for these cytoprotective effects are uncharacterized.

The small heat shock proteins hsp25 and hsp27 and \( \alpha B \)-crystallin are targets of p38-activated MAPKAPK-2.\(^7,7-10\) Although hsp25 and hsp27 exhibit a broad tissue expression pattern, \( \alpha BC \) is expressed in a more restricted manner, being enriched in lens, neurons, skeletal muscle, and cardiac myocytes.\(^7,11-13\) In the heart, \( \alpha BC \) is thought to account for up to 3% of the total protein.\(^14\) Overexpression of \( \alpha BC \) in cultured cardiac myocytes protects them from ischemia-induced cell death and stabilizes microtubules.\(^15\) In addition, overexpression of \( \alpha BC \) in transgenic mice protects the heart from ischemic damage.\(^16\)

The mechanism by which \( \alpha BC \) protects cells from stress-induced damage is unclear; however, it likely involves phosphorylation. In response to various types of cellular stress, \( \alpha BC \) is phosphorylated on Ser-19, 45, and 59.\(^8\) Although the signaling pathways leading to Ser-19 phosphorylation are unknown, the extracellular signal-regulated protein kinase (ERK) MAPK pathway appears to be responsible for phosphorylation of Ser-45, and the p38 MAPK pathway is responsible for phosphorylation of Ser-59.\(^5,10,17\) In the heart, oxidative stress can lead to the activation of p38 and MAPKAPK-2\(^18,19\) and in cardiac myocytes, stress increases p38-mediated MAPKAPK-2, the latter of which phosphorylates \( \alpha BC \) on Ser-59.\(^5\) Moreover, blocking p38-mediated MAPKAPK-2 activation inhibits \( \alpha BC \) phosphorylation on Ser-59 and enhances apoptotic myocyte death.\(^5\) These results are consistent with a role for phosphorylation of \( \alpha BC \) on Ser-59 in mediating the cytoprotective actions of this small heat shock protein (hsp); however, there is no direct evidence supporting this hypothesis. Consequently, we performed the
present study to assess the role of phosphorylation of αBC on stress-induced apoptosis in a cardiac myocyte model system. Phosphorylation of αBC at Ser-19, 45, and 59 was either blocked by expressing Ser to Ala mutations or was mimicked by expressing Ser to Glu mutants in cardiac myocytes. The effects of expressing these forms of αBC on stress-induced apoptosis were then assessed. The stresses chosen for this study were sorbitol, a hyperosmotic stress known to strongly activate p38 and MAPKAPK-2 in cardiac myocytes, and hypoxia followed by reoxygenation, a stress that activates p38 in cardiac myocytes and mimics the well-studied, clinically relevant stress of ischemia/reperfusion.

**Materials and Methods**

**Cell Culture**

*Neonatal Rat Ventricular Myocytes*

Primary neonatal rat ventricular cardiac myocytes (NRVCMS) were prepared from 1- to 4-day-old Harlan Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind) as previously described. Animals were cared for according to the Guide for the Care and Use of Laboratory Animals by the NIH.

**Plasmids**

**αBC( wt)**

pcDNA3.1 HA-αBC( wt) codes for wild-type rat αBC cDNA with an N-terminal HA tag. This construct was derived from a full-length rat αBC cDNA, which was provided by A. Iwaki (Neurological Institute, Kyushu University, Fukuoka, Japan).12,20 and the N-terminal HA tag sequence (MYPYDVPDYA) was cloned into pcDNA3.1 using standard PCR protocols.

**αBC(SSE)**

pcDNA3.1 HA-αBC(SSE) codes for mutated αBC with Ser-59 replaced with Glu to mimic phosphorylation at this site. It was derived using the QuikChange site-directed mutagenesis kit (Strategene) and mutating pcDNA3.1 HA-αBC( wt) from Ser-59 to Glu using the following primers:

(Glu-59)

```plaintext
cttcctcgcgggacactGAGggtattggacactgggcttcag
gaaggacgcctcgtaggacccagCTCacctaactgtgacccgagagctc
```

The mutated nucleotides are identified in bold and underlined.

**αBC( AAA)**

pcDNA3.1 HA-αBC( AAA) codes for mutated αBC with Ser-19, -45, and -59 replaced with Ala to block phosphorylation at these sites. It was derived using the QuikChange site-directed mutagenesis kit (Strategene) and mutating pcDNA3.1 HA-αBC( wt) by mutating Ser-19, Ser-45, and Ser-59 to Ala-19, Ala-45, and Ala-59 using the following primers:

(Ala-19)

```plaintext
gggcgtcctctcttttttcagGCCccaaaggccgttcatttgaccaag
cccgaggaagaaggaaggaagtcgACgggtttccggcgaggagaactgtgc
```

(Ala-45)

```plaintext
ctcagacgcctctcctggCCGcctttctacctctcg
agtcggtgtaggagaacGCGgggaagatggaagcc
```

(Ala-59)

```plaintext
ccttctggtggglactGGCggtattggacactgggcttcag
gaaggacgcctcgtaggacccagCGCacctaactgtgacccgagagctc
```

**αBC( EEE)**

pcDNA3.1 HA-αBC( EEE) codes for mutated αBC with Ser-19, 45, and 59 replaced with Glu to mimic phosphorylation at these sites. It was derived from pcDNA3.1 HA-αBC(wt) by mutating Ser-19, Ser-45, and Ser-59 to Glu-19, Glu-45, and Glu-59 using the Glu-59 primer pair (see above) and the following primer pairs:

(Glu-19)

```plaintext
ggcgtcctctcttttttcagGAGccaaaggccgttcatttgaccaag
cccgaggaagaaggaaggaagtcgACgggtttccggcgaggagaactgtgc
```

(Glu-45)

```plaintext
ctcagacgcctctcctggGAGcctttctacctctcg
agtcggtgtaggagaacGTCgggaagatggaagcc
```

**αBC(AAE)**

pcDNA3.1 HA-αBC(AAE) codes for mutated αBC with Ser-19 and 45 replaced with Ala and Ser-59 replaced with Glu to mimic phosphorylation at Ser-59 and to block phosphorylation at Ser-19 and 45. It was derived from pcDNA3.1 HA-αBC(AAA) by mutating Ala-59 to Glu using the Glu-59 primer pairs.

**pCMV6**

pCMV6 was used as an empty vector control for electroporation-mediated gene transfer TUNEL studies.

**pCMVβ-Galactosidase**

pCMVβ-gal was cotransfected with either HA-tagged αBC constructs or control pCMV6 vector to allow for identification of cardiomyocytes that were transfected.

**Recombinant Adenovirus**

**Adv-αBC( wt), Adv-αBC( AAA), Adv-αBC( EEE), and Adv-αBC( AAE)**

The AdEasy system (Stratagene) was used for preparing recombinant adenoviral strains using previously described methods.21,22 Viral titers were determined as previously described.22

**Transfection by Electroporation**

Between 5 and 12×10^6 cells were combined with the indicated amounts of plasmids in a total of 300 μL of minimal media. Cells were cotransfected with 30 μg of pCMVβ-galactosidase and 100 μg of HA-tagged αBC test constructs or the control vector pCMV6. (Preliminary experiments using various concentrations of the test constructs indicated that electroporation with 100 μg provided optimal expression levels of the relevant form of HA-tagged αBC.) Cells were electroporated at 550 V, 25 μF, and 100Ω in a 0.2-cm gap electroporation cuvette (Bio-Rad) using a Gene Pulser II (Bio-Rad). After transfection, cells were plated for ~16 hours in DMEM/F-12 supplemented with 10% FCS.

**Transfection by Adv Infection**

Cells were plated and maintained for 24 hours in DMEM/F-12 with 10% fetal bovine serum, as described in Cell Culture. Cultures were infected with Adv-GFP (control adenovirus), Adv-αBC( wt), Adv-αBC( AAA), Adv-αBC(AAE), or Adv-αBC(AEE) at a multiplicity of infection (MOI)=10 in DMEM/F-12 with 10% fetal bovine serum for 5 hours, as previously described.22 Cultures were then washed with DMEM/F-12 (1:1) and incubated in minimal medium until experimental maneuvers.

**Western Analyses**

Cultures composed of ~10^6 ventricular myocytes were lysed in Laemmli sample buffer, resolved on a 15% acrylamide gel, and transferred to a PVDF membrane in methanol transfer buffer. Membranes were probed with an HA-mouse monoclonal antibody (Stressgen Biotechnologies Corp, Victoria, British Columbia, Canada). Membranes were washed and incubated with horseradish peroxidase–conjugated anti-IgG secondary antisera (Santa Cruz Biotechnology). Visualization of immune complexes was carried out by enhanced chemilumines-
The cells visibly expressed TUNEL staining of the transfected cells could be identified; as previously observed, BC(wt)–positive cells provided a means by which transfection of the portion of the transfected cells that underwent pre-apoptosis could be assessed. Western blot using an HA antibody. TUNEL analysis of the cultured cardiac myocytes was performed with a kit (TUNEL Direct kit, MBL, Nagoya, Japan). The cell culture hypoxia/reoxygenation (H/R) model has been previously described. In experiments involving adenovirus-mediated gene transfer, cells were treated with 400 mmol/L sorbitol for 14 hours. Upon termination of the experiment, isolation of genomic DNA was performed as described previously.

**Caspase-3 Assay**

Approximately 2×10^6 cells were treated ±400 mmol/L sorbitol for 8 hours of hypoxia (12 hours)/reoxygenation (18 hours) and lysed in buffer containing 0.71% NP-40, 71 mmol/L Tris (pH 7.5), 0.71 mmol/L EDTA, and 212 mmol/L NaCl. Cellular debris was removed by centrifugation. Samples were combined with an equal volume of a reaction buffer (21 mmol/L HEPES, 105 mmol/L NaCl, 5.25 mmol/L DTT, and 50 μmol/L Ac-DEVD-AFC). The mixture was then incubated at 37°C for 1 hour and then loaded into a black 96-well titer plate. Background fluorescence resulting primarily from green fluorescent protein (GFP) contributed ~18% of the total emission. Fluorescence was read on a Spectra Max Gemini XS fluorometer (Molecular Devices) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm using Softmax Pro version 3.1.2 software.

**Statistical Analyses**

The results are presented as mean±SE or SD, as described in the figure legends. Unless indicated otherwise in the figure legends, statistical analyses included a one-way ANOVA followed by Neuman-Keuls post hoc analysis for multiple-group comparisons using SPSS software. A value of P<0.05 was considered statistically significant.

**Results**

**αB-Crystallin Is Required for Protection From Apoptosis**

To determine whether overexpression of αB-crystallin in primary cardiac myocytes could protect the cells from apoptosis after stress, cells were transfected with a construct encoding HA-tagged rat αB-crystallin/wt or with an empty vector (Figure 1A).

After treatment with sorbitol, cultures were then assessed for apoptosis by TUNEL analysis. In cultures that were transfected with the empty vector control pCMV6, treatment with sorbitol caused an ~10-fold increase in the number of

**Apoptosis Assays**

**TUNEL**

TUNEL analyses of cells plated on glass slides were carried out as previously described.

**Sorbitol Treatment**

In experiments where cells were transfected by electroporation, cells were treated with 400 mmol/L sorbitol for 6 to 7 hours. In experiments involving adenovirus-mediated gene transfer, cells were treated with 400 mmol/L of sorbitol for 14 hours. (We found that in contrast to electroporation, after AdV infection, longer treatment times with sorbitol were required to induce apoptosis.)

**Hypoxia/Reoxygenation Treatment**

The cell culture hypoxia/reoxygenation (H/R) model has been previously described. In experiments where cells were infected by AdV, the hypoxia time was 10 hours, and reoxygenation time was 24 hours.

**DNA Laddering**

Cultured NRVMs were plated at a density of either 700 cells/mm^2 (sorbitol experiments) or 100 cells/mm^2 (H/R experiments). After adenovirus infection, cells were washed with DMEM/F12 (1:1) and then maintained in minimal medium until day 4. Cells were either incubated ±400 mmol/L sorbitol for 14 hours or submitted to hypoxia (10 hours)/reoxygenation (28 hours). Upon termination of the experiment, isolation of genomic DNA was performed as described previously.

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Approximately 2×10^6 cells were treated ±400 mmol/L sorbitol for 8 hours of hypoxia (12 hours)/reoxygenation (18 hours) and lysed in buffer containing 0.71% NP-40, 71 mmol/L Tris (pH 7.5), 0.71 mmol/L EDTA, and 212 mmol/L NaCl. Cellular debris was removed by centrifugation. Samples were combined with an equal volume of a reaction buffer (21 mmol/L HEPES, 105 mmol/L NaCl, 5.25 mmol/L DTT, and 50 μmol/L Ac-DEVD-AFC). The mixture was then incubated at 37°C for 1 hour and then loaded into a black 96-well titer plate. Background fluorescence resulting primarily from green fluorescent protein (GFP) contributed ~18% of the total emission. Fluorescence was read on a Spectra Max Gemini XS fluorometer (Molecular Devices) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm using Softmax Pro version 3.1.2 software.

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TUNEL-positive cells (Figure 1B). However, compared with cells transfected with the empty vector, cells expressing HA-αBC(wt) exhibited an ~40% reduction in the number of TUNEL-positive cells after sorbitol treatment (Figure 1B). To assess whether αBC displayed similar apparent cytoprotective effects in response to a different but physiologically relevant stress, a previously described cell culture model of hypoxia followed by reoxygenation (H/R) was used. This is a well-characterized cell culture model that mimics the ischemia/reperfusion that occurs in individuals with advanced coronary artery disease. Moreover, the ability of various forms of αBC to protect cardiac myocytes from H/R-induced apoptosis has never been assessed. Compared with cells transfected with the empty vector, cells expressing HA-αBC(wt) exhibited an ~50% reduction in the number of TUNEL-positive cells after H/R (Figure 1C). These findings indicated that overexpression of αBC(wt) can act in concert with endogenous αBC to protect cells from apoptosis in response to two different stresses.

**Effects of Mutations That Mimic or Block αBC Phosphorylation**

Experiments were carried out to determine the effects of recombinant forms of αBC that either mimic or block phosphorylation via Ser to Glu or Ser to Ala substitutions, respectively, at positions 19, 45, and 59 (Figure 2A). Western blot analyses confirmed that in cardiac myocytes expression levels of the HA-tagged transgenes were approximately equal (Figure 2B). (The reasons for the slightly decreased expression of αBC(AAE) and αBC(EEE) are unknown; however, preparation of multiple, independent constructs resulted in the same relative expression levels.) Compared with cells transfected with an empty vector (Con), cells expressing αBC(wt) displayed a significant decrease in the number of apoptotic cells after sorbitol treatment (Figure 2C). In contrast, compared with cells expressing similar amounts of αBC(wt), cells expressing αBC(EEE) exhibited a significant increase in sorbitol-induced apoptosis (Figure 2C). In fact, αBC(EEE)-expressing cells were more susceptible to cell death than control cells. These results were consistent with requirements for Ser-19, 45, and 59 in mediating the protective effects of αBC. To test the impact of mimicking phosphorylation at Ser-59 only, cultures were transfected with αBC(AAE). Cells expressing αBC(AAE) exhibited 3.5-fold less apoptosis than those expressing αBC(EEE) (Figure 2C). This dramatic enhancement in protection resulting from a single amino acid substitution [αBC(AAA) versus αBC(AAE)] indicates that phosphorylation of Ser-59 enhances the protective effects of αBC. Further support for the importance of phosphorylation of Ser-59, compared with Ser-19 or 45, was the finding that αBC(EEE) exhibited protective effects that were the same as αBC(AAE) (Figure 2C), as did αBC(SSE) (not shown). Cells expressing αBC(AAE) or αBC(EEE) displayed ~33% less apoptosis than those expressing αBC(wt), further supporting the importance of phosphorylation of Ser-59. Qualitatively similar results were obtained when cells were treated with H/R (Figure 2D), indicating that the various forms of αBC exhibit the same cytoprotective properties in response to two very different stressors. To examine the effects of the various

![Diagram of αBC Mutants](image)

**A** Diagram of αBC Mutants

<table>
<thead>
<tr>
<th>WT</th>
<th>AAA</th>
<th>AAE</th>
<th>EEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>19</td>
<td>A</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>45</td>
<td>A</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>59</td>
<td>A</td>
<td>E</td>
<td>E</td>
</tr>
</tbody>
</table>

**B** Western Blot of αBC Mutants

**C** Sorbitol: TUNEL

**D** H/R: TUNEL

**Figure 2.** Effects of various forms of αB-crystallin on sorbitol or H/R-induced apoptosis: TUNEL analysis. A, Diagram of the mutant forms of αBC used in this study. B, Western blot analysis of αBC expression. Cells were transfected by electroporation or with empty vector (con) or with the αBC constructs shown in panel A. Culture extracts were then analyzed by SDS-PAGE followed by Western blotting using an anti-αBC antiserum, as previously described. C and D, TUNEL analysis. Cells were cotransfected with pCMV-β-gal and αBC constructs or empty vector, as described in panel B, plated on glass slides in serum-containing medium for 24 hours, and then maintained in serum-free medium for 48 hours. Cultures were then treated with sorbitol (panel C) or H/R (panel D) and then fixed and assessed for apoptosis by β-gal immunocytofluorescence coupled with TUNEL analysis, as described in Figure 1 and in Materials and Methods. The data obtained with each construct were normalized to the treatment that resulted in the maximum number of TUNEL-positive cells, which was set to 100%; 17.6% or 16.1% of the cells transfected with αBC(AAA) were TUNEL-positive after sorbitol or H/R treatment, respectively. Shown are the mean±SE of at least 3 experiments, each of which included at least 3 cultures for each treatment. *P<0.05 different from Con; †P<0.05 different from wt.
adenoviral (AdV) strains encoding HA-\(\alpha\)BC would provide higher transfection efficiency, recombinant in nearly 100% transfection efficiency of cardiac myocytes.\(^{22}\) The data were normalized to at least 3 experiments, each of which included at least 2 cultures for each treatment. \(\ast P<0.05\) different from wt; \(\dagger P<0.05\) different from \(\alpha\)BC(LLL) and H/R treatment, respectively, which was set at 100%, as described in Figure 2. Shown are the mean\(\pm\)SE of at least 3 experiments, each of which included at least 2 cultures for each treatment. \(\ast P<0.05\) different from wt; \(\dagger P<0.05\) different from \(\alpha\)BC(LLL).

forms of \(\alpha\)BC using a different means of DNA transfer that would provide higher transfection efficiency, recombinant adenoviral (AdV) strains encoding HA-\(\alpha\)BC(LLL) AAA, AAE, and EEE were prepared. (AdV-mediated DNA transfer results in nearly 100% transfection efficiency of cardiac myocytes\(^{22}\) compared with the \(\approx 5\%\) efficiency afforded by electroporation.) Western blot analyses demonstrated that the recombinant AdV strains encoded the expression of similar levels of each form of HA-\(\alpha\)BC without affecting the levels of endogenous \(\alpha\)BC (Figure 3A). TUNEL analyses after treatment with either sorbitol or H/R showed that using AdV-mediated gene transfer, the various forms of \(\alpha\)BC exhibited effects that were similar to those observed using electroporation (Figures 3B and 3C). These results indicated that in response to either sorbitol or H/R, the various strains of AdV-\(\alpha\)BC exhibited cytoprotection for the various forms of \(\alpha\)BC against apoptosis with a rank order of

\[
\text{AAE} = \text{EEE} > \text{wt} > \text{Con} = \text{AAA},
\]

such that (AAE) displayed the greatest protection and AAA the least. Thus, either electroporation or AdV-mediated gene transfer resulted in similar profiles of cytoprotection for the various forms of \(\alpha\)BC, as assessed by TUNEL analysis.

The cytoprotective effects of the various forms of \(\alpha\)BC were further analyzed using DNA fragmentation, or laddering, as a measure of the nucleosomal DNA cleavage, a hallmark of apoptosis.\(^{4}\) Compared with cells infected with AdV-Con, those infected with AdV-\(\alpha\)BC(LLL) displayed less DNA fragmentation on treatment with sorbitol, indicating protection from apoptosis (Figure 4A). AdV-\(\alpha\)BC(LLL) exhibited no apparent protective effects and actually fostered somewhat more DNA fragmentation than infecting cells with AdV-Con. Infection of cells with either AdV-\(\alpha\)BC(AAE) or (EEE) resulted in levels of DNA fragmentation that were reduced significantly below levels observed in all other cultures. Similar results were obtained when these AdV strains were tested in cultures treated with H/R (Figure 5). Thus, in comparison to the TUNEL analyses, the DNA fragmentation analyses showed that in response to either sorbitol or H/R, the various strains of AdV-\(\alpha\)BC exhibited cytoprotective effectiveness against apoptosis with a rank order of

\[
\text{AAE} = \text{EEE} > \text{wt} > \text{Con} = \text{AAA},
\]

where (AAE) displayed the greatest protection and AAA the least. Thus, two different methods of \(\alpha\)BC transgene delivery and two different methods of assessing apoptosis supported the hypothesis that when phosphorylated on serine-59, \(\alpha\)BC protects cells from apoptosis in response to either hyperosmotic or hypoxic stress.

Because \(\alpha\)BC is a chaperone, one possible mechanism by which it might confer protection against apoptosis is by binding to and altering the function of proteins involved in apoptosis. In support of this possibility is a recent study demonstrating that \(\alpha\)BC binds to and inhibits the proteolytic conversion of the inactive caspase-3 intermediate p24 to active caspase-3.\(^{23}\) However, that study did not assess whether \(\alpha\)BC phosphorylation might be required for maximal inhibition of caspase-3 maturation. Accordingly, the effects of the various recombinant AdV strains on caspase-3 activation were assessed. In cells infected with AdV-Con, sorbitol or H/R increased caspase-3 activation (Figure 6A). Cultures were then infected with the test AdV-\(\alpha\)BC strains, and the effects of the various forms of \(\alpha\)BC on sorbitol or H/R-acti-
vated caspase-3 were assessed. Whereas overexpression of αBC(wt) reduced caspase-3 activation by 15% to 30%, expression of αBC(AAA) resulted in slightly more caspase-3 activation than αBC(wt), although this increase did not reach statistical significance (Figure 6B). And, cells infected with AdV-αBC(AAE) or (EEE) displayed 20% to 40% reductions in caspase-3 activation, compared with cells infected with AdV-αBC(wt) (Figure 6B). In comparison to the apoptosis results, αBC(AAE) displayed an ~2-fold greater ability to inhibit caspase-3 activation compared with αBC(AAA).

To determine whether the effects of the AdV-encoded forms of αBC might be due to their abilities to alter either the expression level or phosphorylation status of endogenous αBC, Western blot analyses were carried out. None

Figure 4. Effects of various recombinant strains of adenovirus αB-crystallin on sorbitol-induced apoptosis: DNA ladder analysis. A, Cultured cardiac myocytes were infected with the recombinant AdV-αBC strains shown, treated with sorbitol, the DNA was extracted, fractionated on an agarose gel, stained with ethidium bromide, and photographed. A 1-kb DNA ladder run in parallel was used for estimating fragment sizes (not shown); the fastest migrating band in each sample is the 200-bp band. B, The intensity of the 200-bp band (lowest) from each sample shown in panel A was assessed using Image Quant software (Molecular Dynamics). Each intensity was normalized to the maximum intensity, which was obtained with AdV-αBC(AAA); shown are the mean intensities ± SE from triplicate cultures. *P<0.05 different from wt; †P<0.05 different from αBC(AAA).

Figure 5. Effects of various recombinant strains of adenovirus αB-crystallin on H/R-induced apoptosis: DNA ladder analysis. A, Cultured cardiac myocytes were infected with the recombinant AdV-αBC strains shown, treated with H/R, and the DNA was extracted and fractionated as described in Figure 4A. B, The intensities of the 200-bp bands obtained from each sample shown in panel A were quantified and are plotted as described in Figure 4B. *P<0.05 different from wt; †P<0.05 different from αBC(AAA).
of the AdV-encoded forms of αBC affected the expression levels of endogenous αBC (Figure 6C, Total αBC). When these same blots were probed using an antiserum that detects αBC S59P, the only AdV-encoded form of αBC that was phosphorylated was HA-αBC(wt), as expected (Figure 6C, Ser-59P wt). None of the AdV-encoded forms of αBC had any effect on the level of endogenous αBC phosphorylation on serine-59. These results indicate that the various forms of AdV-encoded αBC affect sorbitol- or H/R-induced apoptosis by virtue of their abilities to inhibit (AAA) or mimic (AAE or EEE) αBC S59P interaction with appropriate cellular targets.

Discussion

In cardiac myocytes, p38 activation can result in protection from apoptosis and in MAPKAPK-2–mediated phosphorylation of αBC on serine-59. However, it is not known whether such phosphorylation is required for αBC-mediated cytoprotection. In the present study, αBC(AAE), which mimics phosphorylation only on Ser-59, protected cardiac myocytes against apoptosis, whereas αBC(AAA) conferred increased susceptibility to apoptosis. These results indicate that phosphorylation of αBC on Ser-59 alone is necessary and sufficient for this hsp to confer maximal cytoprotection. The molecular mechanisms underlying the cytoprotection mediated by αBC(S59P) or increased apoptosis conferred by αBC(AAA) are unknown. The finding in the present study that αBC(AAE) reduced caspase-3 activation suggests that the antiapoptotic effects of αBC(S59P) may involve inhibition of the conversion of p24 caspase-3 to active caspase-3. Although it has been shown that αBC can bind to p24 and decrease the rate of activated caspase-3 generation, the role, if any, of αBC serine-59 phosphorylation has not been established.

It is most likely that αBC effects cytoprotection by interacting with many targets in addition to caspase-3. αBC monomers, which are ≈25 kDa, associate into oligomers made of ≈32 subunits, reaching a mass of 645 kDa. The mass of the oligomers decreases in response to cellular stresses that also increase the phosphorylation of αBC(S59P) or mimic Ser-59P) or increased apoptosis conferred by αBC(DDD), where serines-19, 45, and 59 have been replaced by aspartic acid, which mimics phosphorylation at these sites, resulted in oligomers that are smaller and display reduced chaperone-like activity. However, a naturally occurring form of
αBC carrying an R120G mutation, which is known to cause desmin-related cardiomyopathy, forms oligomers that exceed 645 kDa; yet, this form is a poorer chaperone. Thus, the relationships between αBC oligomer size, phosphorylation status, and chaperone and cytoprotective activities remain unclear and will require additional study.

The finding in the present study that αBC(AAA) leads to less protection from apoptosis than the other forms of αBC tested is supported by several previous reports. In one study, it was shown that compared with αBC(wt), αBC(AAA) exhibited reduced chaperone-like activity when tested in an in vitro assay system. In another report, when expressed in Escherichia coli, αBC(AAA) displayed a nearly 10-fold reduction in the ability to protect cells from heat shock–induced death compared with αBC(wt). These findings support the notion that in the present study, αBC(AAA) may confer enhanced apoptosis because of its reduced chaperone activity. Indeed, our findings that expression of αBC(AAA) did not alter the phosphorylation of endogenous αBC on S59 indicate that the ability of αBC(AAA) to increase apoptosis is not because it acts in a dominant-negative manner on MAPKAPK-2. Instead, αBC(AAA) may compete with αBC(wt) for binding to downstream targets, such as p24 and procaspase-3; however, because αBC(AAA) possesses reduced chaperone activity compared with αBC, susceptibility to apoptosis increases.

In summary, this is the first study to report that mimicking Ser-59 phosphorylation of αBC is necessary and sufficient to confer protection during hyperosmotic and hypoxic stress of cardiac myocytes. Future studies directed toward identifying targets of αBC(S59P) will be required to better understand the molecular events underlying the potent cytoprotective roles of this small hsp in the stressed myocardium.

Acknowledgments

This work was supported by grants from the NIH (HL-63975 and NS/HL-25037) to C.C.G. This work was supported by an award from the American Heart Association to H.E.H. The authors wish to thank Jason Wall for developing the caspase-3 assay. The authors wish to thank Dr. Kanefusa Kato, Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Japan, for the S59P-specific αBC antisera.

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Lisa E. Morrison, Holly E. Hoover, Donna J. Thuerauf and Christopher C. Glembocki

Circ Res. 2003;92:203-211; originally published online December 19, 2002;
doi: 10.1161/01.RES.0000052989.83995.A5

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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