Apoptosis Repressor with Caspase Recruitment Domain Protects against Cell Death by Interfering with Bax Activation

Ása B. Gustafsson‡, Joseph G. Tsai‡, Susan E. Logue‡, Michael T. Crow§, and Roberta A. Gottlieb¶‡

From the ‡Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 and ¶Johns Hopkins University School of Medicine, Baltimore, Maryland 21224

Myocardial ischemia/reperfusion (I/R) is associated with an extensive loss of myocardial cells. The apoptosis repressor with caspase recruitment domain (ARC) is a protein that is highly expressed in heart and skeletal muscle and has been demonstrated to protect the heart against I/R injury (Gustafsson, A. B., Sayen, M. R., Williams, S. D., Crow, M. T., and Gottlieb, R. A. (2002) Circulation 106, 735–739). In this study, we have shown that transduction of TAT-ARCL31F, a mutant of ARC in the caspase recruitment domain, did not reduce creatine kinase release and infarct size after I/R. TAT-ARC31F also failed to protect against hydrogen peroxide-mediated cell death in H9c2 cells, suggesting that the caspase recruitment domain is important in mediating ARC’s protective effects. In addition, we report that ARC co-immunoprecipitated with the pro-apoptotic protein Bax, which causes cytochrome c release when activated. TAT-ARC, but not TAT-ARC31F, prevented Bax activation and cytochrome c release in hydrogen peroxide-treated H9c2 cells. TAT-ARC was also effective in blocking cytochrome c release after ischemia and reperfusion, whereas TAT-ARC31F had no effect on cytochrome c release. In addition, recombinant ARC protein abrogated Bax-induced cytochrome c release from isolated mitochondria. This suggests that ARC can protect against cell death by interfering with activation of the mitochondrial death pathway through the interaction with Bax, preventing mitochondrial dysfunction and release of pro-apoptotic factors.

Apoptosis plays an important role in the cardiovascular system in the process of homeostasis and development but also plays a role in the pathogenesis of certain diseases. Apoptosis is implicated in the death of myocytes in animal models of myocardial ischemia (2), in humans with acute myocardial infarction (3, 4), and in congestive heart failure (5). Even though it is clear that the extensive death of cardiac myocytes after I/R contributes to the decline of ventricular function and mortality, the pathways that initiate apoptosis during I/R in the heart are poorly understood. Studies have suggested that many factors, including Bcl-2 homologous proteins, ATP depletion, acidosis, calcium fluxes, and reactive oxygen species, cause cytochrome c release and apoptosis during I/R in cardiac myocytes (6–12).

Apoptosis repressor with CARD (ARC) is a ~22 kDa protein expressed at high levels in heart and skeletal muscle (13). ARC was initially reported to interact with caspase-2 and -8 and to inhibit apoptosis induced by caspase-8 and receptor-induced apoptosis by Fas and TNF-R1, leading to the proposal that ARC protects by interfering with the function of initiator caspases (13, 14). However, other studies have reported that ARC-mediated protection may not necessarily operate through the inhibition of caspases. For instance, ARC suppressed hypoxia-induced apoptosis by inhibiting cytochrome c release from the mitochondria in a caspase-independent manner (15). In addition, ARC overexpression inhibited oxidant stress-induced cell death in H9c2 cells by preserving mitochondrial function independently of caspase inhibition, suggesting that ARC might act at the level of the mitochondria (16). We recently reported that ARC can protect against I/R injury, where perfusion of TAT-ARC into isolated rat hearts reduced both creatine kinase release and infarct size (1). Thus, we hypothesized that ARC might interfere with the activation of the mitochondrial apoptotic pathway in ischemia. Apoptosis that occurs through the mitochondrial pathway is partly regulated by Bcl-2 family proteins. Expression of Bcl-2 family proteins has been described in both developing and adult cardiac myocytes (6, 17–20), and these proteins seem to play an important role in regulating apoptosis in the cardiovascular system. Bax is a pro-apoptotic Bcl-2 family protein that translocates to the mitochondria in response to death stimuli, causing cytochrome c release from the mitochondria (21–23). In this study, we have examined the role of ARC and ARCL31F in I/R injury and hydrogen peroxide-induced cell death and their effect on Bax. We demonstrate that the CARD domain is important in mediating the protective effects of ARC. In addition, we report that ARC interacts with Bax, which prevents its activation and subsequent cytochrome c release from the mitochondria.

MATERIALS AND METHODS
Recombinant Protein Expression and Purification—Recombinant TAT-fusion proteins were purified and confirmed under denaturing conditions as described previously (1). His-tagged ARC and ARCL31F were purified under native conditions. In brief, cells from 1-liter cultures were resuspended in native buffer (50 mM NaH2PO4, pH 8.0, and 300 mM NaCl), followed by sonication. After centrifugation at 20,000 × g for 20 min, the supernatants were added to columns containing nickel-nitrilotriacetic acid (Qiagen). The resin was washed with native buffer plus 20 mM imidazole and the proteins were eluted with 250 mM nickel-nitrilotriacetic acid.
imidazole in the same buffer, followed by de-salting on PD-10 columns (Amersham Biosciences).

GST-Bax (1–171) was grown in BL21(DE) cells (Invitrogen) and expression was induced with 0.75 mM isopropyl-β-D-thiogalactoside for 4 h. The bacteria were recovered by centrifugation, and the pellet was resuspended in PBS and protease inhibitors and sonicated on ice. Triton X-100 was added to a final concentration of 1% and incubated for 30 min on a rotor at 4°C. After resuspended at the rate of 20,000 x g for 5 min at 4°C, the supernatant was mixed with glutathione-Agarose (Sigma) for 30 min at room temperature. The beads were then washed three times with PBS, and the protein was eluted with 50 mM reduced glutathione.

**Langendorff Perfusion and Ischemia/Reperfusion—Male Sprague-Dawley rats (225–250g) were anesthetized, and hearts were rapidly excised and placed in a Langendorff perfusion apparatus using a protocol adapted from Tsuchida et al. (124). The hearts were perfused with Krebs-Ringer buffer (with or without 50 nM TAT protein) at a constant pressure of 60 mm Hg. Perfused hearts were stabilized for 20 min before being subjected to 30 min of global ischemia and up to 120 min of reperfusion. The creatine kinase activity in the coronary effluent was measured using a diagnostic kit (Sigma) and infarct size was measured using triphenyl tetrazolium staining (1). All animal procedures were approved by the Animal Care and Use Committee of The Scripps Research Institute.

**Preparation of Mitochondria and Cytosol—At the end of perfusion, the ventricles were minced and briefly homogenized with the use of a Polytron homogenizer (Kinematica, Basel, Switzerland) in ice-cold PBS. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C. The pellets and supernatants were analyzed by immunoblotting for Bax and creatine kinase release.

**Immunoprecipitation and Western Blot Analysis—Rat hearts were homogenized using a Polytron homogenizer in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and complete protease inhibitor mixture (Roche). Lysates were incubated on ice for 30 min and then cleared by centrifugation at 20,000 x g for 20 min. The supernatants were incubated with anti-Bax (Santa Cruz Biotechnologies, Inc.), anti-Bax6A7 (BD Pharmingen), or anti-ARC (H-150) (Santa Cruz Biotechnologies) overnight at 4°C. The next morning, 25 μl of protein G-agarose (for monoclonal antibodies) or protein A-agarose (for polyclonal antibodies) were added to the lysates and incubated on cocker for 2 h at 4°C. The beads were washed four times in PBS and resuspended in 40 μl of 2X sample buffer. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Bax or anti-ARC (CT) (Alexis Biochemicals). The protein concentrations were determined by the Coomassie blue binding assay (Pierce Chemical) with bovine serum albumin standards.

**Immunofluorescence—Rat heart-derived H9c2 cells were grown on microscope slides for 48 h before transduction with Tat-linked proteins. Tat-fusion proteins were added to the cells for 1 h followed by treatment with hydrogen peroxide. For analysis of mitochondrial membrane potential and nuclear morphology, live cells were stained with 1 μg/ml Rhodamine 123 (Molecular Probes) for 30 min at the end of the treatment period, rinsed once with media, and co-stained with 30 μg/ml Hoechst 33342 for the last 5 min, followed by washing of cells with PBS. Images of 10 random fields totaling 500–1000 cells were captured and analyzed using MetaMorph imaging software (Universal Imaging). Mitochondrial membrane potential was measured as the ratio of total fluorescence of Rhodamine 123 to the number of nuclei in each field. Percentage nuclear condensation was measured as the number of nuclei with saturated fluorescence divided by the total number of nuclei in each field.

For Bax immunofluorescence, H9c2 cells were fixed in 4% paraformaldehyde after treatment with hydrogen peroxide, permeabilized with 0.2% Triton X-100 in PBS, and then blocked in 5% normal goat serum in PBS. The cells were incubated for 1 h with anti-Bax NT (1:100; Upstate Biotechnology) and anti-COX IV (1:100; Molecular Probes) in blocking solution, washed three times in PBS, and then incubated with goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody coupled to Alexa 488 or Alexa 594 (Molecular Probes). After a 1-h incubation, the cells were washed three times with PBS; in the last wash, 30 μg/ml of Hoechst 33342 was added to the cells. All images were captured with a Nikon TE300 fluorescence microscope (Nikon) and digitally collected with a Spot2 digital camera (Diagnostic Instruments).

**Cytochrome c Release Assays—H9c2 cells seeded on 150-mm dishes were transduced with 500 nM of Tat-ARC or Tat-ARCL31F for 24 h in low-glucose Dulbecco’s modified Eagle’s medium for 1 h and then treated with 600 or 800 μM H2O2 for 4 h. At the end of the treatment, cells were collected by trypsinization, washed once with PBS, and resuspended in 500 μl of buffer B (100 mM sucrose, 1 mM EGTA, pH 7.4, 20 mM MOPS, pH 7.4, 10 mM triethanolamine, and 5% Percoll). The cells were disrupted by nitrogen cavitation at 420 psi for 5 min, and supernatants were incubated with the fractionated cells to a final concentration of 300 μM. Unbroken cells and nuclei were removed by centrifugation at 1,000 x g for 5 min. The supernatants were centrifuged at 10,000 x g for 10 min to pellet mitochondria. The pellets were resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, 10 mM triethanolamine, and 5% Percoll). The cells were disrupted by nitrogen cavitation at 420 psi for 5 min, and supernatants were incubated with the fractionated cells to a final concentration of 300 μM. Unbroken cells and nuclei were removed by centrifugation at 1,000 x g for 5 min. The supernatants were centrifuged at 10,000 x g for 10 min to pellet mitochondria. The pellets were resuspended in 200 μl of buffer D (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, 10 mM triethanolamine, and 5% Percoll). The cells were disrupted by nitrogen cavitation at 420 psi for 5 min, and supernatants were incubated with the fractionated cells to a final concentration of 300 μM. Unbroken cells and nuclei were removed by centrifugation at 1,000 x g for 5 min. The supernatants were centrifuged at 10,000 x g for 10 min to pellet mitochondria. The pellets were resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4), washed twice, and finally resuspended in 40 μl of buffer C. Equal amounts of mitochondrial protein were loaded onto 10–20% Tris glycine gels (Invitrogen) and transferred to nitrocellulose membranes for Western blot analysis. Cytochrome c was identified by anti-cytochrome c antibody (BD Biosciences) and voltage-dependent anion channel by anti-Porin antibody (Calbiochem).

For Bax-mediated cytochrome c release studies, mitochondria were isolated from rat hearts as described above. Purified recombinant Bax (1 μg) was pre-incubated with or without recombinant ARC or ARCL31F for 10 min at 4°C. The samples were then mixed with 40 μg of mitochondrial protein at 30°C for 3 h in a final volume of 60 μl. The pellets and supernatants were analyzed by immunoblotting for cytochrome c.

![FIG.1](http://www.jbc.org/)

**Fig. 1. Effect of TAT-ARC and TAT-ARCL31F on infarct size and creatine kinase release.** Rat hearts were perfused with or without 50 nM Tat-ARC or Tat-ARCL31F for 15 min and then washed out before 30 min of global ischemia and 2 h of reperfusion. A, creatine kinase (CK) activity in the coronary effluent was measured for 15 min before ischemia (pre) and for the first 15 min of reperfusion (post). Values represent mean ± S.E., n = 6–8, *, p < 0.01; **, p is nonsignificant compared with I/R. B, infarct size was determined by TTC staining. Data mean ± S.E., n = 6–8, *, p < 0.01; **, p is nonsignificant compared with I/R. C, representative TTC-stained sections from perfused rat hearts.
For in vitro Bax binding, 400 ng of recombinant Bax was combined with 1 μg ARC or ARCL31F in Incubation Buffer. After a 1-h incubation at 30 °C, 500 μl of lysis buffer was added to each binding mixture. Samples were agitated overnight at 4 °C in the presence of 5 μg of anti-Bax6A7. Protein G-agarose (25 μl) was added to each sample, incubated for 2 h, recovered by centrifugation, and washed four times in ice-cold PBS. Proteins bound to the beads were eluted by addition of 50 μl of 2× sample buffer and boiled for 5 min. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting.

**RESULTS**

**Effect of TAT-ARC and TAT-ARCL31F on Myocardial Injury**

The CARD is a sequence that is present in many proteins in-
involved in the regulation of cell death pathways and is believed to mediate signaling through protein-protein interaction (25). To determine whether the CARD is important in mediating the protective effects of ARC in I/R injury, we perfused hearts with TAT-ARC or TAT-ARCL31F, a point mutant of ARC with a single amino acid change in the conserved region of the CARD. Mutation of this conserved leucine residue in other CARD-containing proteins has previously been shown to lead to a disruption of conformation and protein binding (26, 27). As reported previously (1) and as shown in Fig. 1, perfusion with TAT-ARC significantly reduced both infarct size and creatine kinase release after I/R. In contrast, perfusion of hearts with TAT-ARCL31F did not reduce creatine kinase release or infarct size after I/R, suggesting that the CARD domain is important for ARC function in mediating a protective effect in the heart.

**Effect of TAT-ARC and TAT-ARCL31F on Mitochondria**—ARC has been shown to inhibit hydrogen peroxide-induced cell death in H9c2 cells (1, 16). To determine whether the CARD is important in protecting against oxidative stress, we investigated whether TAT-ARC or TAT-ARCL31F could protect against H2O2-induced cell death. Fig. 2 shows that treatment of H9c2 cells with hydrogen peroxide caused loss of mitochondrial membrane potential and nuclear condensation and that TAT-ARCL31F did not protect H9c2 cells against hydrogen peroxide-mediated cell death and, in some experiments, exacerbated the effects, raising the possibility that it interferes with the action of endogenous ARC. These results demonstrate that the CARD domain of ARC is important in protecting against oxidative stress.

**Heterodimerization of ARC with Bax**—Mitochondria are the primary sites of action of pro-apoptotic Bel-2 proteins that respond to cellular stress by causing the release of apoptosis-promoting factors, including cytochrome c, from the mitochondria. Because overexpression of ARC can protect the mitochondria and prevent cytochrome c release in response to oxidative stress and hypoxia (15, 16), we hypothesized that ARC might have an effect on the pro-apoptotic protein Bax in the heart. To determine whether ARC directly associates with Bax, we examined the interaction between ARC and Bax in the heart by immunoprecipitation of Bax from rat heart lysate and then analysis of the precipitate by immunoblotting for coprecipitation of ARC. In a reciprocal experiment, ARC immunoprecipitate was analyzed for co-IP of Bax. As shown in Fig. 3A, ARC was present in the precipitate obtained by using anti-Bax, and Bax was seen in the anti-ARC immunoprecipitates.
Effects of ARC on Bax Activation and Cytochrome c Release—To examine whether Bax activation can be inhibited by ARC, we treated H9c2 cells with TAT-ARC or TAT-ARCL31F followed by 2 h of hydrogen peroxide. We assessed activation of Bax using an antibody that preferentially recognizes the active conformation of Bax (28). Immunostaining of cells subjected to hydrogen peroxide treatment demonstrated an increase in immunoreactivity for activated Bax (Fig. 4A). TAT-ARC treatment before hydrogen peroxide prevented the increase in Bax immunoreactivity, suggesting that ARC inhibits hydrogen peroxide-induced Bax activation. In contrast, TAT-ARCL31F failed to prevent the increase in Bax immunoreactivity hydrogen peroxide-treated cells.

The appearance of Bax immunoreactivity in intact cells correlates with the release of cytochrome c from mitochondria (28–30). We assessed cytochrome c release in H9c2 cells treated with hydrogen peroxide and determined whether the presence of ARC or ARCL31F would inhibit hydrogen peroxide-induced cytochrome c release. Hydrogen peroxide treatment resulted in the loss of cytochrome c from the mitochondria in H9c2 cells, whereas cells transduced with TAT-ARC did not release cytochrome c from the mitochondria after hydrogen peroxide treatment (Fig. 4B). Hydrogen peroxide treatment was still able to elicit cytochrome c release in the presence of TAT-ARCL31F. Likewise, we found that the amount of cytochrome c increased in the cytosol in rat hearts subjected to I/R (Fig. 5A). Hearts perfused with TAT-ARC, but not TAT-ARCL31F, exhibited reduced cytochrome c release after I/R. These data suggest that ARC is blocking cytochrome c release by inhibiting Bax activation in the ischemic heart.

To determine whether ARC could directly inhibit Bax-induced cytochrome c release, we investigated the ability of ARC to inhibit cytochrome c release from mitochondria isolated from the rat heart. Addition of recombinant Bax directly to mitochondria in vitro results in the release of cytochrome c (23). The results in Fig. 5B show that Bax induced cytochrome c release from isolated heart mitochondria. Pre-incubation of Bax with recombinant purified ARC reduced cytochrome c release from isolated mitochondria, confirming that the major effect of ARC in these mitochondria is to interfere with Bax function (Fig. 5B). In contrast, ARCL31F did not interfere with the effects of Bax on isolated mitochondria. We compared the ability of ARC and ARCL31F to bind to recombinant Bax. Consistent with the results obtained with the endogenous proteins immunoprecipitated from ischemic and reperfused heart (Fig. 3B), we found that both forms of ARC bound to Bax in a reconstituted system consisting of only the recombinant proteins (Fig. 5C). These results suggest that the interaction between ARC and Bax does not depend upon the intact CARD domain, but the ability of ARC to block Bax function is CARD dependent. In addition, it reveals that accessory or bridging proteins are not required for the interaction.

DISCUSSION

ARC is expressed at high levels in the heart, consistent with the need to prevent apoptosis in the terminally differentiated cardiomyocytes. We have previously shown that increasing the levels of ARC through TAT-mediated protein transduction can reduce the incidence of cell death after myocardial ischemia and reperfusion. In the present study, we have begun to evaluate the basis for cardioprotection. We show that the CARD domain of ARC is essential for its protective effect; mutation of Leu to Phe at position 31 is sufficient to disrupt CARD func-

![FIG. 5. Effect of TAT-ARC and TAT-ARCL31F on cytochrome c release from rat heart mitochondria. A, rat hearts were perfused with or without 50 nM TAT-ARC or TAT-ARCL31F were subjected to 30 min of ischemia followed by 15 min of reperfusion. Cytosol and mitochondrial fractions were analyzed by immunoblotting (IB) using an antibody to cytochrome c or voltage-dependent anion channel (VDAC). Western blots are representative of three replicate experiments. B, isolated rat heart mitochondria were incubated with recombinant Bax with or without ARC or ARCL31F proteins for 3 h at 30 °C. Mitochondria were then subjected to centrifugation, and the resulting pellets and supernatants were analyzed by Western blotting using anti-cytochrome c (n = 4). C, recombinant GST-Bax was incubated with His-tagged ARC or ARC L31F, then Bax was immunoprecipitated and analyzed for coprecipitation of ARC.](http://www.jbc.org/)

preservation of mitochondrial integrity is essential for maintaining energy production and preventing apoptosis. Consistent with this, ARC was able to preserve mitochondrial membrane potential and prevent cytochrome c release in H9c2 cells exposed to hydrogen peroxide and in the isolated perfused heart subjected to ischemia and reperfusion. Bax has been shown to be a key determinant of the mitochondrial pathway of apoptosis. Using hydrogen peroxide to simulate the oxidative stress of ischemia/reperfusion, we showed that Bax undergoes the conformational change of activation in H9c2 cells. ARC interacts with Bax, as demonstrated by co-immunoprecipitation of endogenous ARC and Bax. We speculated that there could be a bridging protein, such as Aven or Bar, that would form an anti-apoptotic scaffold containing ARC and Bax (31, 32). However, our experiments with recombinant proteins demonstrated that ARC and Bax interact directly, without a requirement for additional proteins to bridge the two molecules. There are precedents for interactions between CARD proteins and Bcl-2 family members, such as that between Bcl-XL and...
Activation. Likewise, the intact CARD domain is required to interact with Bax to prevent its activation. ARC is not disrupted by the L31F mutation within the CARD domain; however, the intact CARD domain is required to prevent Bax activation. Likewise, the intact CARD domain is required to prevent cytochrome c release from isolated mitochondria incubated with Bax.

Our results demonstrate that ARC preserves mitochondrial integrity through an interaction with Bax to prevent its activation. Previous work had shown that ARC interacts with caspase 8 to interfere with receptor-mediated death signaling (13) and suppresses potassium efflux through voltage-gated Kv channels (38). ARC has also been shown to block necrosis by preserving mitochondrial integrity (39). Taken together, these results suggest that ARC prevents cell death by interfering with multiple pathways.

Acknowledgments—We thank Dr. Richard N. Kitis for stimulating discussions.

REFERENCES
Apoptosis Repressor with Caspase Recruitment Domain Protects against Cell Death by Interfering with Bax Activation

Åsa B. Gustafsson, Joseph G. Tsai, Susan E. Logue, Michael T. Crow and Roberta A. Gottlieb

doi: 10.1074/jbc.M400695200 originally published online March 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400695200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 23 of which can be accessed free at http://www.jbc.org/content/279/20/21233.full.html#ref-list-1