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Inhibition of Both the Extrinsic and Intrinsic Death Pathways through Nonhomotypic Death-Fold Interactions

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Summary

Death-fold domains constitute an evolutionarily conserved superfamily that mediates apoptotic signaling. These motifs, including CARD (caspase recruitment domain), DD (death domain), and DED (death effector domain), are believed to exert their effects solely through homotypic interactions. Herein we demonstrate that the CARD-containing protein ARC engages in nontraditional death-fold interactions to suppress both extrinsic and intrinsic death pathways. The extrinsic pathway is disrupted by heterotypic interactions between ARC's CARD and the DDs of Fas and FADD, which inhibit Fas-FADD binding and assembly of the death-inducing signaling complex (DISC). The intrinsic pathway is antagonized by ARC-Bax binding, involving ARC's CARD and the Bax C terminus. This inhibits Bax activation and translocation to the mitochondria. Knockdown of endogenous ARC facilitates DISC assembly and triggers spontaneous Bax activation and apoptosis. Conversely, physiological levels of ARC suppress these events. These studies establish a critical role for nonhomotypic death-fold interactions in the regulation of apoptosis.

Introduction

Apoptosis is tightly regulated by the complex interplay of activating and inhibitory pathways. Death signals in vertebrates are transduced via two central pathways (Danial and Korsmeyer, 2004). In the extrinsic pathway, the activation of cell surface receptors stimulates the assembly of the death-inducing signaling complex (DISC), within which procaspase-8 is activated (Boldin et al., 1996; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). In the intrinsic pathway, the translocation of proapoptotic Bcl-2 proteins, such as Bax (Wolter et al., 1997), to the mitochondria triggers the release of cytochrome c (Kluck et al., 1997; Liu et al., 1996), which stimulates the Apaf-1-dependent activation of procaspase-9 in the apoptosome (Li et al., 1997).

Interactions between death-fold motifs are a unifying mechanism in the regulation of both of these pathways. Death-folds are an evolutionarily conserved superfamily that includes death domains (DD), death effector domains (DED), and caspase recruitment domains (CARD) (Aravind et al., 1999; Hofmann et al., 1997; Vaughn et al., 1999; Weber and Vincenz, 2001). Death-fold motifs differ markedly in primary amino acid sequence but possess similar three-dimensional structures consisting of six antiparallel α helices (Eberstadt et al., 1998; Huang et al., 1996; Vaughn et al., 1999). These motifs have been shown to engage solely in monovalent, homotypic interactions. Thus, in the DISC, the DD in the cytoplasmic tail of the death receptor Fas binds to the DD in the adaptor molecule FADD; and the DED in FADD binds a DED in procaspase-8 (Boldin et al., 1996; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). In the case of the apoptosome, the CARD in Apaf-1 binds the CARD in procaspase-9 (Li et al., 1997; Qin et al., 1999).

The pathways that activate cell death are opposed by a variety of inhibitory proteins. For example, FLIP inhibits the extrinsic pathway through its interactions with FADD and procaspase-8 (Irmler et al., 1997; Micheau and Tschopp, 2003). The intrinsic pathway is subject to negative regulation at multiple levels. Bax (Guo et al., 2003; Sawada et al., 2003) and Bak (Cheng et al., 2003; Leu et al., 2004) are suppressed by several interacting proteins. Antiapoptotic Bcl-2 proteins inhibit cytochrome c release (Kluck et al., 1997; Liu et al., 1996). Postmitochondrial antagonism of the intrinsic pathway is mediated by proteins with baculoviral inhibitor of apoptosis repeats, such as XIAP, that bind to and inhibit caspase-9 and effector caspases (Chai et al., 2001; Deveraux et al., 1998; Riedl et al., 2001; Shiozaki et al., 2003).

Most endogenous apoptosis inhibitors antagonize only one or the other of the central death pathways. An exception is ARC (apoptosis repressor with a CARD; also referred to as CARD2 or Myp), which is expressed diffusely but enriched in long-lived cells such as cardiac and skeletal myocytes and neurons (Geertman et al., 1996; Koseki et al., 1998). This death-fold protein was originally described as an inhibitor of the extrinsic pathway because it blocks apoptosis induced by a variety of death receptors (Fas, TNFR1, DR3) and adaptors (FADD, TRADD, CLARP) (Koseki et al., 1998). ARC's ability to block apoptosis and cytochrome c release induced by activators of the intrinsic pathway, such as hypoxia and oxidative stress (Ekhterae et al., 1999; Neuss et al., 2001), suggests that ARC may also modulate the intrinsic pathway. Although ARC binds to procaspases-2 and -8 (Koseki et al., 1998), the mechanism of its inhibition of either the extrinsic or intrinsic pathway is unknown.

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In this study, we demonstrate that ARC inhibits both extrinsic and intrinsic pathways through unexpected death-fold interactions. Antagonism of the extrinsic pathway is mediated by ARC's direct binding to Fas and FADD, which occurs through heterotypic interactions between the CARD of ARC and the DDs of Fas and FADD. These heterotypic interactions inhibit the traditional homotypic interactions between the DDs of Fas and FADD that are required for Fas-FADD binding and DISC assembly. ARC inhibits the intrinsic pathway by direct binding to Bax, which requires ARC's CARD and the C-terminal regulatory domain of Bax, an interaction between a death-fold motif and a non-death-fold protein. The binding of ARC to Bax prevents Bax's conformational activation and translocation to the mitochondria in response to an apoptotic stimulus. The physiological relevance of these observations is demonstrated by the knockdown of endogenous ARC in muscle cells: DISC formation is potentiated and Bax activation and cell death occur spontaneously even in the absence of an apoptotic stimulus. Conversely, endogenous levels of ARC are sufficient to inhibit these events. These nonhomotypic death-fold interactions provide a mechanism by which a single protein inhibits both central death pathways.

Results

ARC's Inhibition of Extrinsic and Intrinsic Death Pathways Is Dependent on the CARD

Since death-fold interactions play important roles in the regulation of apoptosis, we hypothesized that ARC's antiapoptotic effects are mediated through its CARD. To test this hypothesis, the CARD was disrupted by mutation of two residues conserved in death-fold proteins back to Ced-3 (Chou et al., 1998). Leucine 31 is predicted to be a core residue in helix 2, and its mutation to phenylalanine would likely disrupt CARD structure (Chou et al., 1998). Glycine 69 is located in the linker region between helices 4 and 5, and its mutation to arginine would be expected to change the helix-turn-helix conformation (Chou et al., 1998). To assess the functional importance of ARC's CARD in inhibiting apoptosis, we cotransfected HEK293 cells, which lack detectable ARC, with wild-type ARC or ARC L31F; G69R (referred to as mutARC); and Fas (Figure 1A) or Bax (Figure 1B), direct activators of extrinsic and intrinsic pathways respectively. ARC efficiently blocked loss of the mitochondrial membrane potential and apoptotic nuclear changes due to both stimuli. In contrast, similar levels of mutARC failed to protect against Fas or Bax. Thus, ARC inhibits both extrinsic and intrinsic pathways and its CARD is critical for these effects.

Since CARDs mediate protein-protein interactions, yeast two-hybrid screening was used to identify interacting proteins with full-length ARC as bait. The most common interactor was ARC itself (data not shown). The ARC-ARC interaction was confirmed by immunoprecipitation-immunoblot experiments in HEK293 cells (see Supplemental Figure S1A at http://www.molecule.org/cgi/content/full/15/6/901/DC1). The N-terminal CARD alone was sufficient to bind full-length ARC, while the C-terminal proline-glutamic acid (PE)-rich region was not (Figure S1B). Moreover, the double point mutation

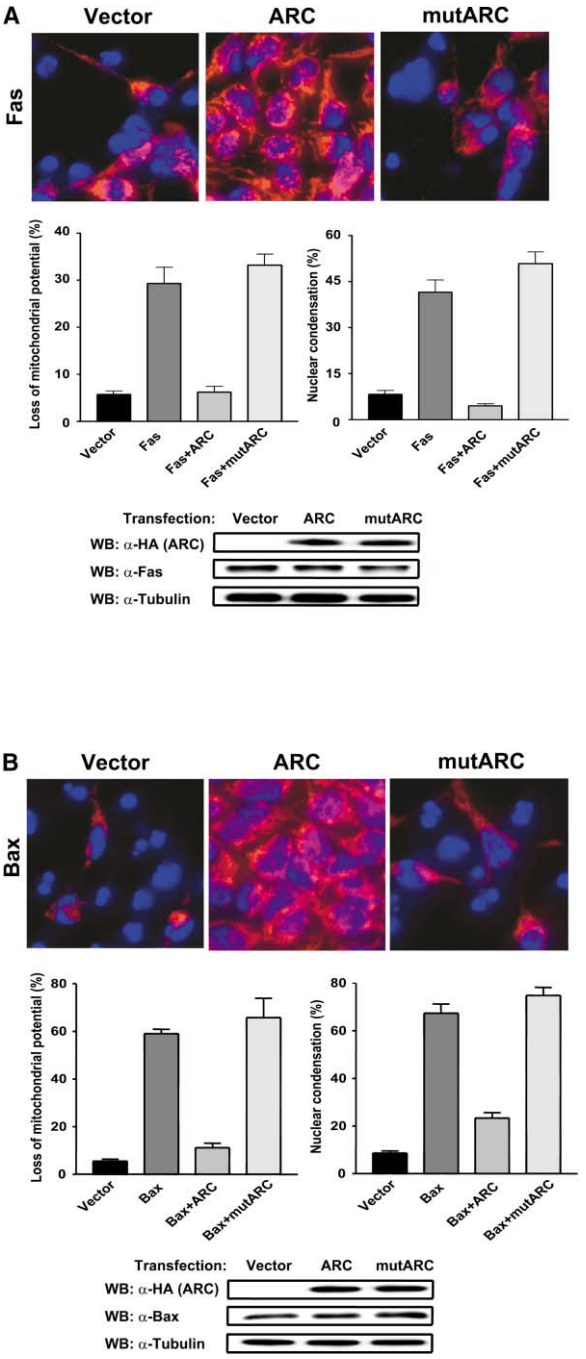

Figure 1. ARC's CARD Mediates Its Inhibition of Extrinsic and Intrinsic Death Pathways

(A and B) Cell death was assessed in HEK293 cells transfected with Fas (A) or Bax (B) and the indicated ARC constructs. MutARC denotes the L31F; G69R mutant. Red (tetramethyl rhodamine ethyl ester) and blue (Hoechst 33342) denote intact mitochondrial membrane potential and nuclear morphology respectively. Immunoblots show expression levels of proteins.

in mutARC ablated the interaction (Figure S1C). These data demonstrate that ARC interacts with itself and that the CARD is both necessary and sufficient for this binding.

The preceding experiments show that ARC-ARC bind-

ing and ARC's inhibition of apoptosis both require the CARD. The simplest model suggested by these data is that CARD-dependent homodimerization of ARC is required for cytoprotection. To test this model, we constructed FK506 binding protein (FKBP)-ARC fusion proteins, with which we could induce ARC-ARC binding using the divalent FKBP ligand AP20187 (Spencer et al., 1993). Addition of "dimerizer" increased ARC-ARC binding from basal levels in the case of FKBP-ARC (Figure S2A, upper two blots). Binding of FKBP-mutARC was absent at baseline (as expected) and augmented by dimerizer (Figure S2A, lower two blots). FKBP-ARC potently inhibited Bax-induced apoptosis in the absence of dimerizer (Figure S2B). In contrast, this protection was significantly decreased, rather than enhanced, in the presence of dimerizer. As expected, FKBP-mutARC was unable to protect in the absence of dimerizer, and this lack of protection persisted in the presence of dimerizer (Figure S2B). Similar results were obtained using procaspase-8 as the apoptotic stimulus (data not shown). Thus, forced dimerization of ARC decreases, rather than increases, ARC's ability to inhibit apoptosis.

Consistent with this observation, most ARC is not homodimeric during apoptosis. Immunoblotting of native gels showed that the percentage of dimeric ARC decreases from 71% to 6% during apoptosis induced by procaspase-8 (Figure S3A) or Bax (data not shown). To confirm these findings, we performed immunoprecipitation-immunoblot experiments on HEK293 cells transfected with Myc- and HA-tagged ARC along with either vector or procaspase-8 (Figure S3B). While Myc- and HA-tagged ARC coimmunoprecipitated under control conditions, their interaction decreased markedly during apoptosis. Thus, only a small percentage of ARC is homodimeric during apoptosis. Taken together with the data demonstrating that homodimeric ARC is poorly protective (Figure S2B), these experiments indicate that ARC's inhibition of apoptosis is not mediated by homodimerization.

Given that ARC's inhibition of apoptosis is dependent on the CARD but cannot be attributed to homodimerization, we considered an alternative model in which ARC's cytoprotection is mediated by interactions of its CARD with other proteins. To assess this possibility, we screened for ARC-interacting proteins in heart lysates using an antibody array approach (Wang et al., 2000). We found that ARC binds Fas and FADD, and procaspase-8, the latter previously shown (Koseki et al., 1998), as well as Bax.

Interactions between ARC's CARD and the DDs of Fas and FADD Inhibit DISC Assembly

We first confirmed ARC's interactions with Fas, FADD, and procaspase-8, which are components of the DISC. Immunoprecipitation of endogenous ARC demonstrated interactions with endogenous Fas, FADD, and procaspase-8 in lysates from mouse heart (Figure 2A). Similar results were obtained from the reciprocal immunoprecipitations (data not shown). The interactions of ARC with Fas and FADD were further investigated using size exclusion chromatography of heart lysates. A significant portion of endogenous ARC coeluted with endogenous Fas or FADD (Figure 2B). In addition, ARC coimmunoprecipitated with these proteins in only a subset of coeluting fractions reinforcing the specificity of these interactions. These data demonstrate that ARC interacts with Fas and FADD in a cell that endogenously expresses each of these proteins.

To further test the specificity of ARC's interactions with Fas (containing a DD), FADD (containing a DD and a DED), and procaspase-8 (containing two DEDs), we assessed whether ARC also interacts with FLIP (containing two DEDs), RIP (containing a DD), RICK (containing a CARD), and TRADD (containing a DD) (Figure 2C). While ARC interacted with procaspase-8, it did not bind FLIP, RIP, RICK, or TRADD even in this overexpression system. Thus, ARC's interactions with Fas, FADD, and procaspase-8 are specific.

To determine whether ARC's CARD, which is critical for its antiapoptotic function (Figure 1A), mediates its interactions with Fas, FADD, and procaspase-8, we performed immunoprecipitation-immunoblot experiments on lysates from HEK293 cells transfected with wild-type ARC or mutARC. While wild-type ARC interacted with endogenous Fas, FADD, and procaspase-8, mutARC did not (Figure 2D). Thus, ARC's CARD is required for its binding to Fas, FADD, and procaspase-8.

To assess whether ARC's interactions with Fas and FADD are direct, immunoprecipitation-immunoblots were performed using in vitro transcribed translated proteins. ARC was shown to interact directly with Fas and with FADD (data not shown). In contrast, neither Fas nor FADD interacted with an irrelevant protein (anthrax toxin receptor ectodomain). We next mapped the ARC binding domains in Fas and FADD using bacterially expressed recombinant proteins that had been purified on glutathione-sepharose columns (Figure 2E). GST, GSTFas, or GSTFasΔDD (lacking the DD) were mixed with purified recombinant ARC from which the GST moiety had been excised. Following immunoprecipitation with anti-ARC, immunoblotting was performed with anti-GST. The same approach was used with GSTFADDΔDD (lacking the DD) and GSTFADDΔDED (lacking the DED). We were unable to express full-length GSTFADD in bacteria. These experiments confirmed that ARC binds directly to Fas and FADD and demonstrated that the DD in each of these proteins is critical for ARC binding. Taken together with the experiments in Figure 2D, these data indicate that the binding of ARC to Fas and FADD is mediated by heterotypic death-fold interactions involving ARC's CARD and the DDs of Fas and FADD.

To evaluate the functional significance of ARC's binding to Fas and FADD, we determined its effect on formation of the DISC (Medema et al., 1997). DISC assembly was first assessed in HEK293 cells transfected with vector, wild-type ARC, or mutARC (Figure 3A). An agonistic Fas antibody was used both to activate Fas and to immunoprecipate the DISC, following which immunoprecipitates were resolved on SDS-PAGE and immunoblotted with antibodies against various DISC components and ARC. Immunoprecipitates from vector transfected cells that had been stimulated with the agonistic Fas antibody showed the presence of Fas, FADD, and procaspase-8 and its cleavage products. In contrast, immunoprecipitates from cells expressing wild-type ARC and treated with the agonistic Fas antibody included ARC, but the abundances of FADD and procaspase-8 were

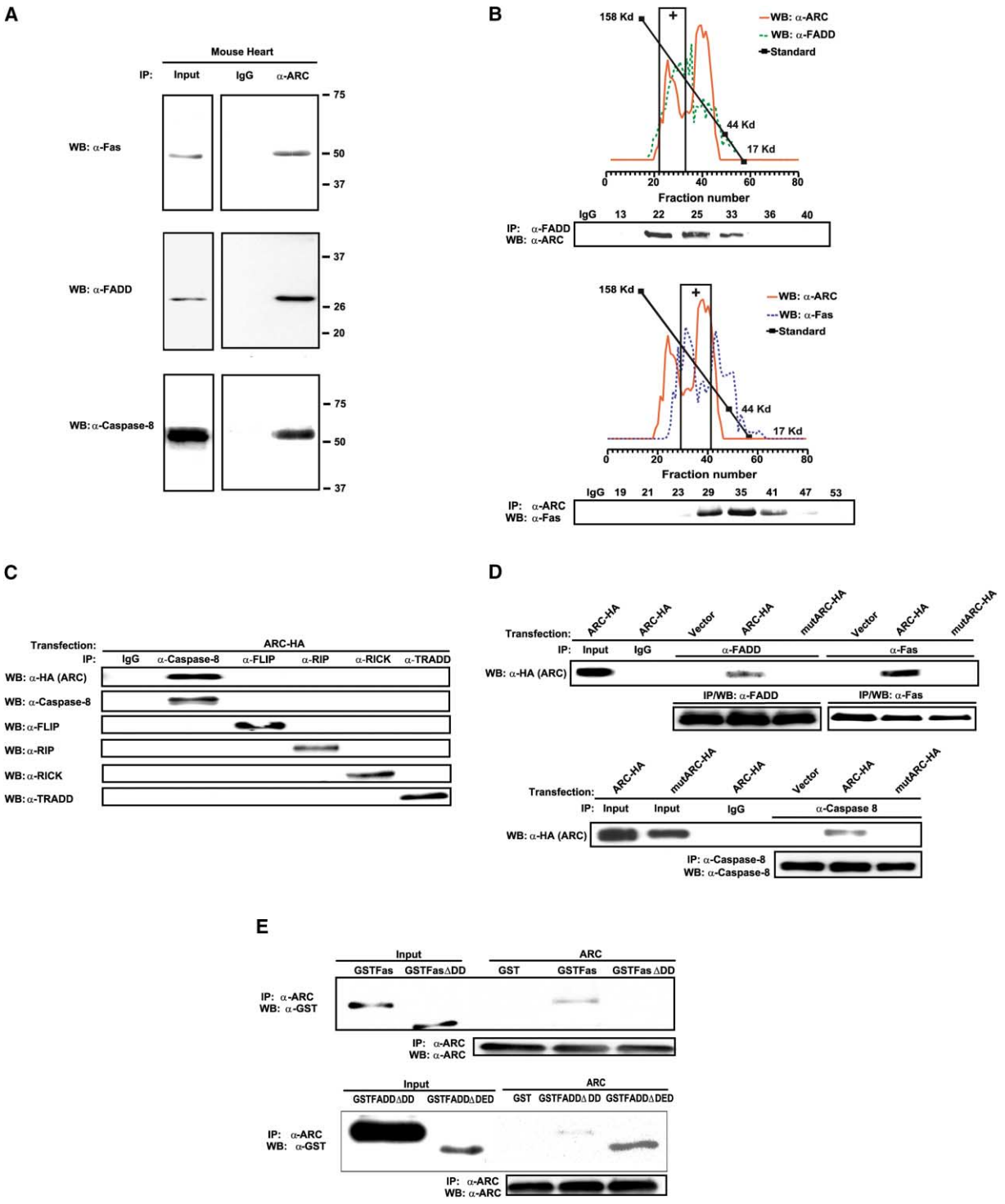

Figure 2. Heterotypic Death-Fold Interactions Mediate ARC's Binding to DISC Components

(A) Endogenous ARC interacts with endogenous Fas, FADD, and procaspase-8. Immunoprecipitation-immunoblots of mouse heart lysates as indicated. Input lanes show immunoblots of lysates prior to immunoprecipitation.

(B) Size exclusion chromatography of mouse heart lysates. Fractions were assayed for ARC (red), FADD (green), and Fas (blue) by immunoblotting (elution profiles). Molecular mass markers: bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa). Fractions were assayed for ARC interactions using immunoprecipitation-immunoblots (blots). The rectangles in the elution profiles indicate fractions in which ARC interacts with FADD or Fas.

(C) ARC's interactions with Fas, FADD, and procaspase-8 are specific. Immunoprecipitation-immunoblots of HEK293 cells transfected with ARC showing that ARC does not interact with some other death-fold proteins.

(D) ARC's interactions with Fas, FADD, and procaspase-8 require ARC's CARD. Immunoprecipitation-immunoblots of HEK293 cells transfected with the indicated ARC constructs.

(E) ARC interacts directly with Fas and FADD through their DDs. Purified recombinant proteins were mixed and immunoprecipitation-immunoblots performed as indicated.

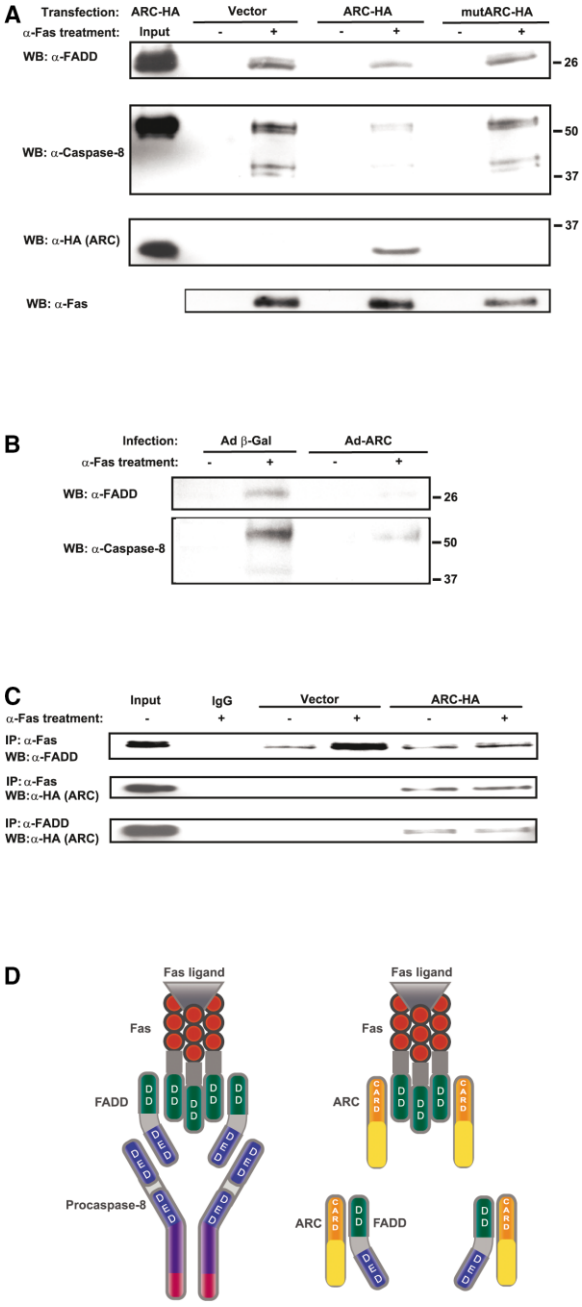

Figure 3. Heterotypic Death-Fold Interactions Disrupt DISC Formation

(A) ARC, but not mutARC, interferes with DISC assembly. HEK293 cells were transfected with the indicated constructs, following which cells were treated or not treated with an agonistic Fas antibody. The DISC was immunoprecipitated with protein A agarose beads and immunoblotted with the indicated antisera.

(B) ARC inhibits DISC assembly in primary neonatal rat cardiac myocytes. Cells were transduced with recombinant adenoviruses expressing ARC or β-galactosidase and the DISC assembly assay performed.

(C) ARC inhibits the increase in Fas-FADD binding induced by Fas activation. HEK293 cells were transfected with the indicated constructs and stimulated or not stimulated with an agonistic Fas antibody. Immunoprecipitation-immunoblots were performed as indicated.

(D) Proposed model by which ARC's heterotypic death-fold interactions interfere with DISC assembly.

markedly decreased despite the same amount of immunoprecipitated Fas. Immunoprecipates from cells expressing mutARC and treated with the agonistic Fas antibody, on the other hand, contained no ARC but exhibited restored amounts of FADD and procaspase-8. Thus, ARC inhibits DISC assembly. To further test this conclusion, we examined the effect of FKBP-ARC on this process in the presence or absence of dimerizer. As shown previously, FKBP-ARC's cytoprotection is markedly diminished by forced dimerization (Figure S2B). Consistent with this, FKBP-ARC inhibited DISC assembly in the absence, but not presence, of dimerizer (Figure S4). Third, we demonstrated ARC's inhibition of DISC assembly in primary neonatal rat cardiac myocytes (Figure 3B). These experiments demonstrate that ARC interferes with DISC assembly in a CARD-dependent manner.

To determine the basis for ARC's inhibition of DISC assembly, we examined ARC's effect on the increase in Fas-FADD binding in response to Fas activation, the first recruitment step in DISC formation (Figure 3C). The DISC assembly assay was not used for this purpose because, in this assay, an agonistic Fas antibody is employed both to activate Fas and immunoprecipitate the DISC. Hence, with this approach, Fas cannot be recovered in the unactivated state, preventing assessment of the Fas-FADD interaction both before and after Fas activation. To bypass this limitation, HEK293 cells that had been transfected with vector or ARC were treated or not treated with an agonistic Fas antibody, following which, cell lysates were immunoprecipitated using a (nonagonistic) Fas antibody. These were then resolved on SDS-PAGE and immunoblotted with antibody against FADD. In vector-transfected cells, treatment with the agonistic Fas antibody stimulated a robust increase in the interaction between Fas and FADD. In contrast, in the presence of ARC, no increase in Fas-FADD binding was observed. The combined interaction (Figures 2D and 2E) and functional (Figures 3A–3C and Figure S4) data suggest a model in which ARC's disruption of DISC formation is brought about by heterotypic death-fold interactions between the CARD of ARC and the DDs of Fas and FADD that inhibit the conventional homotypic interactions between the DDs of Fas and FADD that are required for DISC assembly (Figure 3D).

Interaction between ARC's CARD and the Bax C Terminus Inhibits Bax Activation and Translocation

We have shown that ARC inhibits apoptosis induced by Bax, a direct activator of the intrinsic pathway (Figure 1B). As noted previously, the antibody array screen suggested that ARC binds Bax. Accordingly, we hypothesized that ARC's interaction with Bax mediates its inhibition of the intrinsic pathway. Therefore, we first confirmed the interaction between ARC and Bax. Immunoprecipitation of endogenous ARC demonstrated an interaction with endogenous Bax in lysates from mouse heart (Figure 4A, upper blot) as did the reciprocal immunoprecipitation (Figure 4A, lower blot). Similar results were recently reported (Gustafsson et al., 2004). Size exclusion chromatography of heart lysates showed that a significant portion of endogenous ARC coeluted and coimmunoprecipitated with endogenous Bax (Figure 4B).

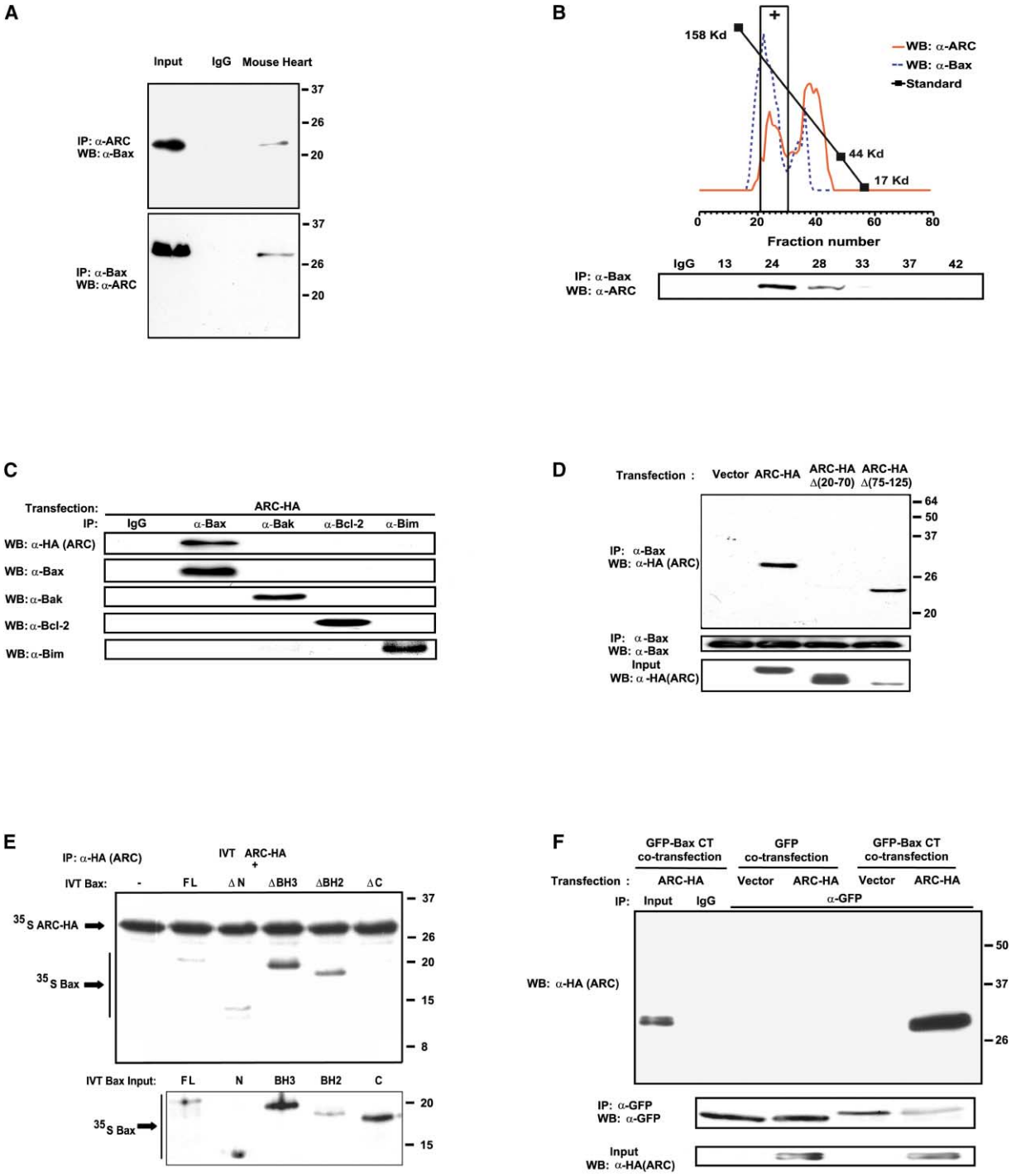

Figure 4. ARC-Bax Binding Is Mediated by ARC's CARD and the Bax C Terminus

(A) Endogenous ARC interacts with endogenous Bax. Immunoprecipitation-immunoblots of mouse heart lysates as indicated.

(B) Size exclusion chromatography of mouse heart lysates. Fractions were assayed for ARC (red) and Bax (blue) by immunoblotting (elution profile). Molecular mass markers as in Figure 2B. Fractions were assayed for ARC interactions using immunoprecipitation-immunoblots (blot). The rectangle in the elution profile indicates fractions in which ARC interacts with Bax.

(C) ARC interacts with Bax, but not some other Bcl-2 proteins. Immunoprecipitation-immunoblots of HEK293 cells transfected with ARC.

(D) ARC residues 20–70 are required for the ARC-Bax interaction. HEK293 cells were transfected with the indicated constructs following which immunoprecipitation-immunoblots were performed as noted (upper blot). Lower blot shows input.

(E) The C-terminal 33 residues of Bax are required for ARC binding. Radiolabeled in vitro transcribed translated HA-tagged ARC and full-length (FL) or deletion mutants of Bax (see text) were mixed, immunoprecipitated with anti-HA, resolved on SDS-PAGE, and subjected to autoradiography (upper blot). Lower blot shows the input of Bax constructs.

(F) The C-terminal 33 residues of Bax are sufficient for ARC binding. HEK293 cells were transfected and immunoprecipitation-immunoblots were performed as indicated. GFP-Bax CT denotes a fusion between GFP and Bax residues 160–192.

To test the specificity of ARC's interaction with Bax, we assessed whether ARC also binds other Bcl-2 proteins. While ARC interacted with Bax, it did not bind Bak (another multidomain proapoptotic Bcl-2 protein closely related to Bax), Bcl-2 (an antiapoptotic Bcl-2 protein), or Bim (a Bcl-2 homology domain (BH) 3-only protein) (Figure 4C). Moreover, while ARC inhibited cell death induced by hydrogen peroxide, an activator of the intrinsic pathway, in wild-type and Bak$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) (Lindsten et al., 2000), it was unable to do so in Bax$^{-/-}$ MEFs (Knudson et al., 1995) (Figure S5). These data indicate that Bax is a specific target of ARC in its inhibition of the intrinsic pathway.

To map the domain in ARC required for binding Bax, immunoprecipitation-immunoblot experiments were performed using lysates from HEK293 cells transfected with ARC or ARC mutants (Figure 4D). Loss of ARC residues 20–70, which encompass the majority of the CARD, ablated the ARC-Bax interaction. As expected, this mutant failed to inhibit Bax-induced apoptosis (data not shown). To determine if the interaction between ARC and Bax is direct and to map the ARC binding domain in Bax, ^{35}S-radiolabeled in vitro transcribed translated HA-tagged ARC and Bax or Bax mutants were mixed, immunoprecipitated with anti-HA, resolved on SDS-PAGE, transferred to a PVDF membrane, and autoradiographed (Figure 4E). The immunoprecipitation-immunoblots are shown in the upper blot and the Bax inputs in the lower blot. ARC interacted directly with full-length Bax as well as with mutants lacking the N terminus (residues 2–62), BH3 (residues 63–72), and BH2 (residues 151–165). In contrast, deletion of the C terminus (residues 160–192) ablated ARC binding. To test whether Bax residues 160–192 are sufficient to mediate ARC binding, these residues were fused to GFP (GFP-Bax CT). HEK293 cells were cotransfected with ARC and GFP-Bax CT or GFP alone and coimmunoprecipitations subsequently performed. While GFP did not interact with ARC, GFP-Bax CT did (Figure 4F). These data show that ARC binds Bax directly, and this interaction is mediated by ARC's CARD and the C terminus of Bax.

Mutagenesis and structural studies have suggested that Bax is inhibited by folding of its C terminus into the hydrophobic cleft created by BH 1, 2, and 3 (Nechushtan et al., 1999; Suzuki et al., 2000; Zha et al., 1996). Bax activation involves a conformational change, which exposes an N-terminal epitope (residues 13–19) that can be detected with a Bax monoclonal antibody (clone 6A7) (Nechushtan et al., 1999). Activated Bax translocates from the cytosol to the mitochondria to trigger cell death. Since ARC inhibits Bax-induced loss of mitochondrial membrane potential and cell death (Figure 1B) and also binds to the C terminus of Bax (Figures 4E and 4F), we hypothesized that ARC inhibits Bax. To evaluate the functional significance of ARC's binding to Bax, we tested whether ARC inhibits Bax activation and translocation. Bax activation was assessed by immunoprecipitating cell lysates with Bax monoclonal antibody 6A7 followed by immunoblotting with an antibody that recognizes total Bax (Figure 5A). Transfection of HEK293 cells with Bax resulted in Bax activation, which was blocked by cotransfection of wild-type ARC, but not mutARC. Bax translocation was assessed by subcellular fractionation and immunoblotting (Figure 5B). Cotransfection of ARC decreased the amount of Bax in the heavy membrane fraction and increased the amount in the cytosolic fraction. These data show that ARC inhibits Bax activation and translocation.

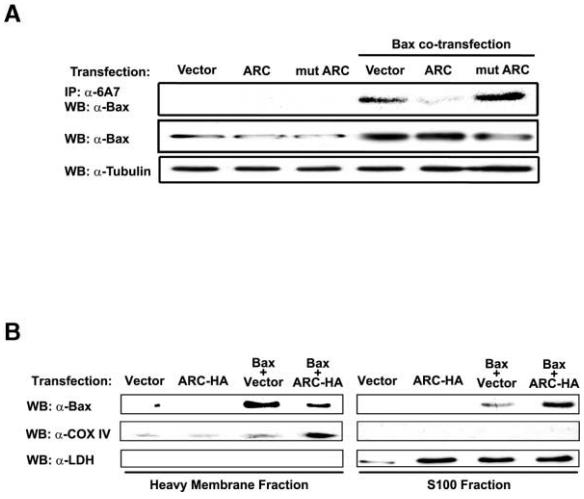

Figure 5. Interaction between ARC's CARD and the Bax C Terminus Inhibits Bax Activation and Translocation

(A) ARC, but not mutARC, inhibits Bax activation. HEK293 cells were transfected with the indicated constructs following which lysates were immunoprecipitated with an antibody that recognizes active Bax (clone 6A7) and immunoblotted with an antibody that recognizes total Bax (upper blot). Immunoblot for Bax expression (middle blot). (B) ARC blocks Bax translocation. HEK293 cells were transfected with the indicated constructs and immunoblots performed on heavy membrane and S100 fractions. COXIV and LDH are mitochondrial and cytosolic markers, respectively.

Knockdown of Endogenous ARC Facilitates DISC Assembly and Triggers Spontaneous Bax Activation and Cell Death

The studies presented above demonstrate that the interactions between ARC and Fas, FADD, and Bax occur between endogenous proteins. The experiments showing the functional effects of ARC on inhibition of DISC assembly (Figures 3A–3C) and Bax activation and translocation (Figures 5A and 5B), however, involved ARC overexpression. To define the role of physiological levels of ARC, we knocked down endogenous ARC levels in H9c2 rat myocytes using specific antisense phosphothiorate oligodeoxynucleotides. Sequences were chosen so that they did not overlap with those encoding the CARD, a motif found in other proteins. In addition, a low stringency BLAST search yielded no significant similarity between the oligodeoxynucleotide sequence and the rat database except with ARC itself. Oligodeoxynucleotide transfection efficiency was ~85% as judged by the transfer of fluorescently labeled versions of the antisense and sense oligodeoxynucleotides (data not shown). Transfection with the antisense oligodeoxynucleotide reduced ARC levels ~90% (Figure 6A, upper blot). In contrast, ARC levels did not change significantly following transfection of sense (Figure 6A, upper blot) or reverse antisense (data not shown) oligodeoxynucleotides. Furthermore, the antisense oligodeoxynucleotide did not affect the

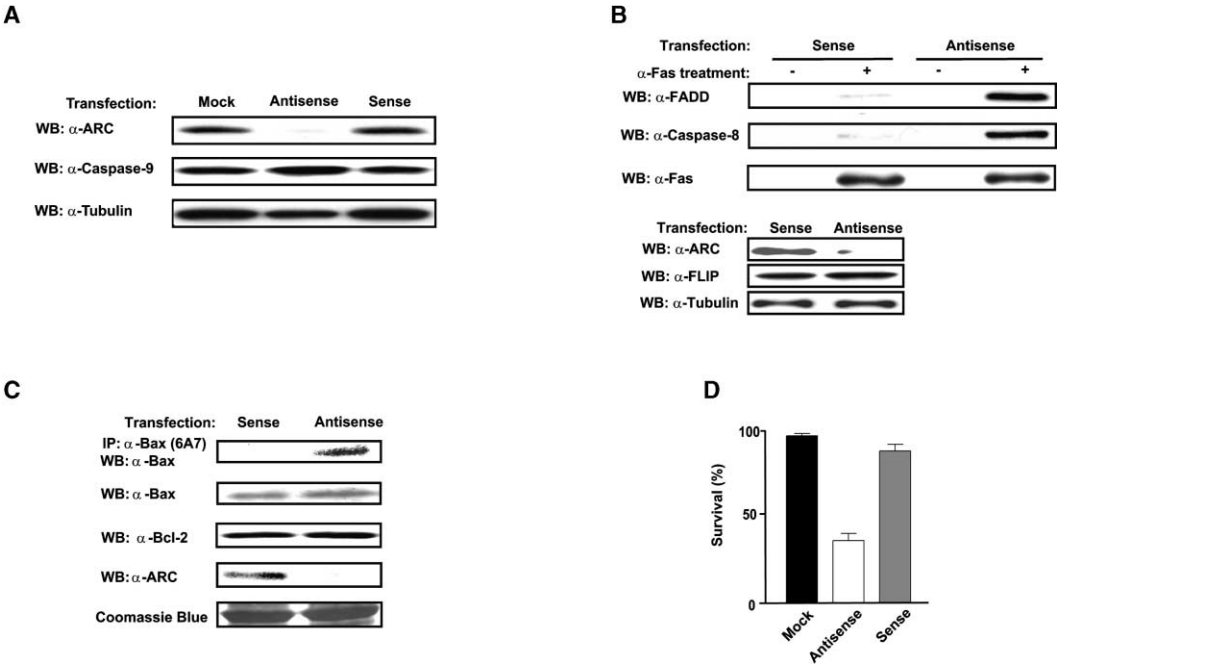

Figure 6. Knockdown of Endogenous ARC Facilitates DISC Assembly and Triggers Spontaneous Bax Activation and Cell Death

(A) Knockdown of endogenous ARC levels in H9c2 myocytes with antisense phosphothiorate oligodeoxynucleotides. Cells were transfected with antisense or sense ARC oligodeoxynucleotides and immunoblots performed for ARC and procaspase-9, another CARD-containing protein.

(B) Knockdown of endogenous ARC facilitates DISC formation. H9c2 cells were transfected with sense or antisense ARC oligodeoxynucleotides and the DISC formation assay was performed (upper blots). ARC antisense does not affect levels of FLIP, another death-fold protein that inhibits the extrinsic pathway (lower blots).

(C) Knockdown of endogenous ARC causes spontaneous Bax activation. H9c2 cells were transfected with sense or antisense ARC oligodeoxynucleotides and the Bax activation assay was performed (upper blot). Levels of total Bax were unchanged (second blot). In addition, antisense did not affect the abundance of Bcl-2, an antiapoptotic Bcl-2 protein (third blot).

(D) Knockdown of ARC causes spontaneous cell death. H9c2 cells were transfected with antisense or sense ARC oligodeoxynucleotides and cell viability assessed by trypan blue exclusion.

abundance of procaspase-9, another CARD-containing protein (Figure 6A, middle blot).

To test the effect of ARC knockdown on DISC assembly, H9c2 myocytes were transfected with ARC antisense and sense oligodeoxynucleotides, stimulated with an agonistic Fas antibody, and then subjected to DISC analysis as previously described. Although H9c2 cells were able to form a DISC, they did so inefficiently as illustrated in the cells transfected with the sense oligodeoxynucleotide (Figure 6B, upper three blots). In contrast, DISC assembly was robust in the antisense transfectants. Of note, similar recoveries of Fas were obtained in the two groups. In addition, immunoblots of input lysates confirmed the knockdown of ARC, but not FLIP, a DED-containing protein that also regulates the DISC (Figure 6B, lower three blots). Thus, reduction of endogenous ARC levels facilitates DISC formation in response to Fas activation.

We also evaluated the effect of ARC knockdown on Bax activation. Strikingly, reduction of endogenous ARC levels caused the spontaneous activation of Bax in the absence of an apoptotic stimulus (Figure 6C, upper blot). Bax activation occurred without changes in total Bax levels (Figure 6C, second blot). Again, ARC antisense reduced the abundance of ARC, but not Bcl-2 (Figure 6C, lower three blots). We evaluated cell viability using trypan blue exclusion following ARC knockdown. This showed that ~66% of the antisense transfectants, but only ~9% of the sense transfectants, failed to exclude trypan blue (Figure 6D). Similar results were obtained using a reverse antisense oligodeoxynucleotide control (data not shown). Thus, knockdown of ARC causes spontaneous Bax activation and cell death. These data suggest that basal levels of endogenous ARC constitutively inhibit Bax activation and are essential for survival of H9c2 myocytes under these conditions.

Maintenance of Basal ARC Levels Is Sufficient to Inhibit Bax Activation and Cell Death

Endogenous ARC levels are known to decrease in response to a wide variety of apoptotic stimuli (Ekhterae et al., 1999; Neuss et al., 2001). This is illustrated in H9c2 myocytes subjected to oxidative stress from hydrogen peroxide (Figure 7A, left) and isolated, perfused rat hearts undergoing ischemia-reperfusion, which also involves a significant component of oxidative stress (Figure 7A, right). Given our experiments showing that primary knockdown of ARC levels alone is sufficient to induce Bax activation (Figure 6C) and cell death (Figure 6D), we hypothesized that decreases in ARC levels during apoptosis may be an initiating event—rather than a consequence—of the resulting cell death. To test this hypothesis, we assessed whether maintaining ARC at approximately baseline endogenous levels would be sufficient to suppress Bax activation and cell death following an apoptotic stimulus. To accomplish this, we

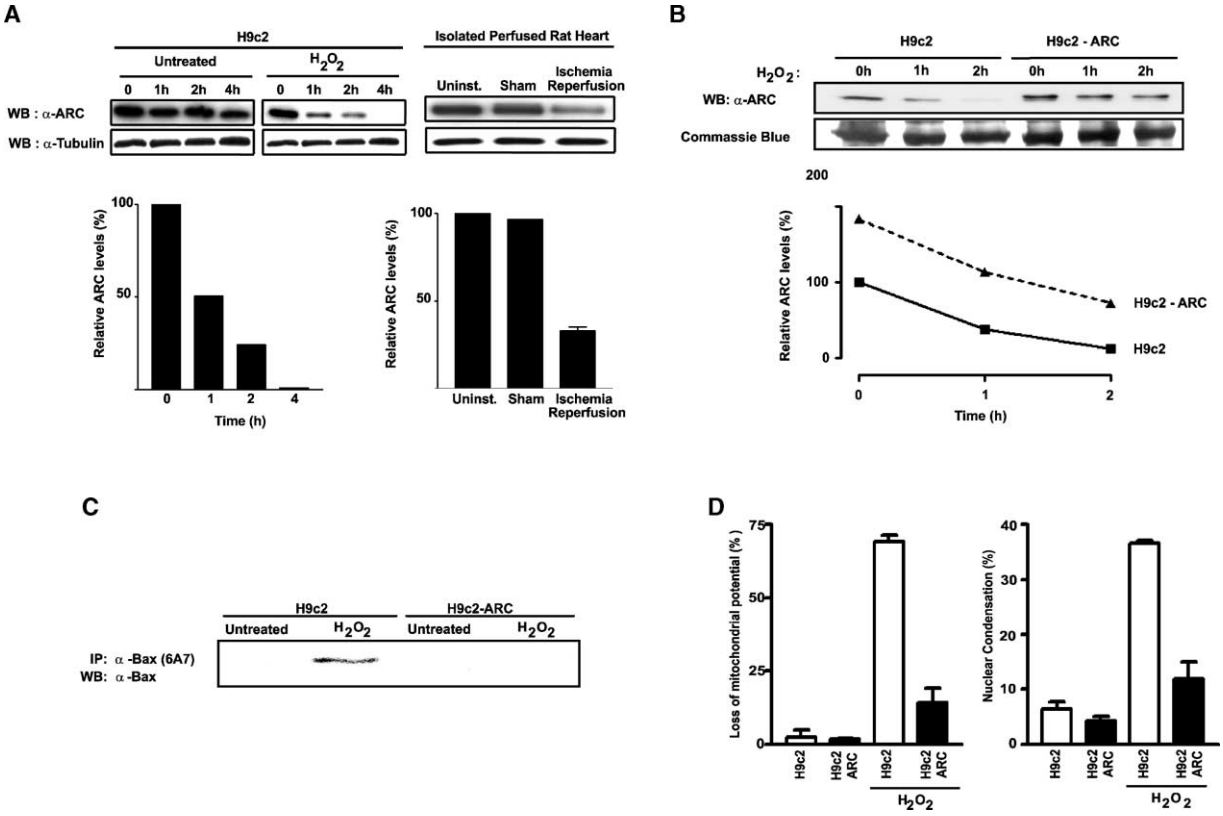

Figure 7. Maintenance of Basal ARC Levels Is Sufficient to Inhibit Bax Activation and Cell Death

(A) Endogenous ARC levels decrease during apoptosis induced by oxidative stress and ischemia-reperfusion. H9c2 cells were either treated or not treated with hydrogen peroxide for the times indicated (left). Isolated rat hearts were either uninstrumented (uninst), perfused with buffer (sham), or subjected to ischemia-reperfusion (right). ARC levels were assessed by immunoblotting.

(B–D) Endogenous ARC levels are sufficient to suppress Bax activation and cell death in response to an apoptotic stimulus. H9c2 cells or H9c2-ARC stable transfectants were treated with hydrogen peroxide and ARC levels assessed at the times indicated (B). Bax activation (C) and loss of mitochondrial membrane potential and nuclear condensation (D) were measured after 2 hr of hydrogen peroxide treatment, at which time ARC levels were reduced to ~20% in wild-type H9c2 cells and ~80% in H9c2-ARC cells.

compared the effects of hydrogen peroxide treatment on wild-type H9c2 cells and H9c2-ARC stable transfectants that exhibit 1.8-fold overexpression of ARC. In response to hydrogen peroxide, ARC levels fell with similar kinetics in both cell lines such that, following 2 hr of treatment, they were ~80% of basal wild-type levels in the H9c2-ARC cells and ~20% of basal wild-type levels in the H9c2 cells (Figure 7B). At this time point, Bax activation was easily detectable in the H9c2 cells but suppressed in the H9c2-ARC cells (Figure 7C). Similarly, loss of mitochondrial membrane potential and cell death in the H9c2-ARC cells was markedly decreased as compared with H9c2 cells (Figure 7D). These studies demonstrate that ~baseline levels of ARC are sufficient to inhibit hydrogen peroxide-induced Bax activation and much of the resulting cell death. Taken together with the knockdown studies, these data indicate that physiological levels of ARC are both necessary and sufficient for survival in these muscle cells. These experiments also suggest that decreases in ARC levels are necessary for Bax activation and cell death in this model.

Discussion

In this study we elucidate the mechanism by which a single death-fold protein, ARC, inhibits both the extrinsic and intrinsic death pathways. Direct interactions between ARC and Fas, FADD, and Bax are key to this inhibition. These interactions are specific and demonstrable between endogenous proteins. Moreover, in contrast to the traditional homotypic death-fold interactions that mediate apoptotic signaling, ARC inhibits apoptosis by engaging in nonhomotypic death-fold interactions. ARC's binding to Fas and FADD (involving CARD-DD interactions) inhibits DISC assembly, while ARC's binding to Bax (involving a CARD-non-death-fold interaction) blocks Bax conformational activation and translocation to the mitochondria. The physiological importance of these interactions is underscored by the fact that reductions in endogenous ARC levels augment DISC formation in response to Fas activation and trigger spontaneous Bax activation and cell death in the absence of an apoptotic stimulus. Conversely, physiological levels of ARC suppress these events.

Nonhomotypic Death-Fold Interactions

Traditionally, death-fold motifs engage in homotypic interactions. This is exemplified by assembly of the Fas DISC in which Fas and FADD interact through their DDs and FADD and procaspase-8 interact through their DEDs (Boldin et al., 1996; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). Similarly, the CARDs in

Apaf-1 and procaspase-9 mediate their interaction in the apoptosome (Li et al., 1997; Qin et al., 1999). ARC's CARD also engages in homotypic death-fold interactions as shown in this study by ARC homodimerization. In contrast to these homotypic interactions, ARC also engages in nonhomotypic death-fold interactions. ARC's binding with Fas and FADD involves heterotypic death-fold interactions between ARC's CARD and the DDs of Fas and FADD. While such interactions have not been previously reported, they are consistent with recent structural analyses showing similarities between the binding surfaces of a DD and a DED from two functionally unrelated proteins, *Drosophila* Tube (containing a DD) and hamster PEA-15 (containing a DED) (Hill et al., 2004). The interaction between ARC and Bax is an atypical death-fold interaction as it is dependent on ARC's CARD and the α-helical C terminus of Bax, a non-death-fold protein. Bax was also recently shown to bind the apoptosis activator ASC (Ohtsuka et al., 2004), an interaction requiring ASC's pryin domain, another death-fold motif. Other interactions between death-fold motifs and non-death-fold proteins include the binding of PEA-15 to ERK MAPK (mediated by the DED in PEA-15) (Hill et al., 2002) and Fas to calmodulin (mediated by the DD in Fas) (Ahn et al., 2004). The ability of death-fold domains to engage in interactions other than the conventional homotypic ones may further expand the role of these motifs in apoptotic signaling. Future structural studies will be of great interest in understanding these non-traditional death-fold interactions.

A Repressor of Both Central Death Pathways

Prior work had established that ARC inhibits the extrinsic pathway. ARC's inhibition of apoptosis due to hypoxia and oxidative stress suggested that ARC may also antagonize the intrinsic pathway. The demonstration in this study that ARC blocks Bax-induced cell death provides direct evidence that ARC is indeed an inhibitor of the intrinsic pathway. Moreover, the recognition that ARC interacts with and inhibits specific components of the extrinsic and intrinsic pathways establishes ARC as a direct inhibitor of both pathways. This dual inhibition makes ARC unusual among endogenous inhibitors of apoptosis as most inhibitors neutralize only one or the other pathway. Another exception is the neuronal-specific bifunctional apoptosis repressor (BAR) (Roth et al., 2003; Stegh et al., 2002; Zhang et al., 2000). BAR interacts with procaspase-8 and Bcl-xL and Bcl-2, but the mechanism by which it antagonizes each of the death pathways has not yet been delineated.

ARC's Inhibition of DISC Assembly

Assembly of the DISC is necessary for activation of the extrinsic pathway. The simplest model for ARC's inhibition of DISC assembly is competition for the DDs of Fas and FADD by ARC's CARD (Figure 3D). Since death-fold motifs are monovalent, this competition would be predicted to interfere with homotypic interactions between the DDs of Fas and FADD, an obligate step in DISC formation (Boldin et al., 1996; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). This model is consistent with our observation that the binding of ARC to Fas and FADD markedly inhibits Fas-FADD binding induced by death receptor activation. In contrast, an ARC mutant defective for binding Fas and FADD does not affect DISC assembly, while knockdown of endogenous ARC potentiates formation of the DISC. Although ARC's interference with the Fas-FADD interaction is sufficient to explain ARC's inhibition of the extrinsic pathway, additional mechanisms may be involved. For example, ARC's interaction with procaspase-8, which is mediated by ARC's CARD (Figure 2D) and the DED(s) of procaspase-8 (Koseki et al., 1998), may also contribute to the inhibition of procaspase-8 recruitment into the DISC. The roughly proportionate decreases in the recruitment of FADD and procaspase-8 that we observe, however, suggest that ARC's major effect on DISC assembly is the inhibition of FADD recruitment.

ARC's Inhibition of Bax Activation

Bax is thought to be held in an inactive state by the interaction of its C-terminal ninth α helix (α9) with the hydrophobic cleft created by BH 1, 2, and 3 (Suzuki et al., 2000). This is mediated both by hydrophobic interactions and hydrogen bonding (Suzuki et al., 2000). Our studies demonstrate that the binding of ARC and Bax is dependent on the CARD of ARC and the C terminus of Bax. The binding of ARC to Bax maintains Bax in its inactive conformation and inhibits Bax translocation. The fact that knockdown of ARC results in spontaneous Bax activation and cell death in H9c2 myocytes indicates that basal levels of ARC constitutively maintain Bax in an inactive state in this system. We hypothesize that the binding of ARC to Bax functions to enforce the interaction between α9 and the cleft, but the mechanism by which this is accomplished will require structural studies.

Recently, several other proteins have been found to bind and inhibit Bax. Humanin, a 24 amino acid peptide, binds Bax through a yet to be defined domain (Guo et al., 2003), while Ku70 interacts with the Bax N terminus (Sawada et al., 2003). Overexpression of each of these proteins inhibits Bax translocation and cell death. Conversely, knockdown of each potentiates cell death in response to an apoptotic stimulus. In contrast to ARC, however, spontaneous Bax activation and cell death do not occur with silencing of either Humanin or Ku70 (Guo et al., 2003; Sawada et al., 2003).

ARC as an Apoptotic Switch

Although ARC is expressed diffusely, it is enriched in cardiac and skeletal myocytes and neurons (Geertman et al., 1996; Koseki et al., 1998), long-lived cells that persist even in the face of multiple stresses. We speculate that ARC may be important in keeping these cells alive. This hypothesis is supported by our observation that knockdown of endogenous ARC levels in H9c2 myocytes causes spontaneous cell death, an effect suppressed by basal levels of ARC. Thus, endogenous ARC is essential for the survival of these cells under the conditions tested. Gene targeting studies will be required to assess the role of ARC in cell survival in vivo.

Despite their longevity, however, myocytes and neurons do die in some situations (Bialik et al., 1997; Yuan and Yankner, 2000). Our studies show that ARC levels decrease in response to apoptotic stresses, and that maintenance of even $\sim$basal levels of ARC is sufficient

to abrogate most cell death. These observations suggest the hypothesis that decreases in ARC levels may function as a critical switch in the decision to die. An understanding of the molecular nature of this switch may provide insights into cellular differentiation, aging, heart disease, and stroke.

Experimental Procedures

Constructs, Cell Culture, and Transfections

Plasmids, viruses, cell culture, and transfections are described in the Supplemental Data and can be found at http://www.molecule.org/cgi/content/full/15/6/901/DC1. Constructs encoding the FKBP-ARC fusion proteins were made using the Argent regulated homodimerization kit including AP20187, the dimerizing drug (Ariad Pharmaceuticals). Subcellular fractionation was performed as described (Bialik et al., 1999).

Antibodies and Recombinant Proteins

Antibodies and recombinant proteins are described in the Supplemental Data. Antibody arrays were obtained from Hypomatrix or made by spotting 1 μg of each antibody onto a PVDF membrane and immobilizing by drying.

Immunoprecipitations

Immunoprecipitations were performed in IP buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% [v/v] Nonidet P-40 [NP-40], 0.5% [w/v] sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin) except in the case of Bax where CHAPS buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% CHAPS containing protease inhibitor cocktail [Sigma]) was used to avoid conformational activation of Bax by NP-40 (Hsu and Youle, 1997; Sawada et al., 2003).

Size Exclusion Chromatography

Mouse heart lysate was fractionated on a Sephacryl S400 (Amersham) column at 4°C. 80 0.5 ml fractions were collected and assayed for ARC, Fas, FADD, and Bax by immunoblot. Interactions between ARC and its binding partners were assessed in fractions using coimmunoprecipitations.

DISC Assembly Assay

DISC assembly was assessed as described (Li et al., 2002; Medema et al., 1997).

Cell Death Assay

Cell death was assessed by nuclear morphology, loss of mitochondrial membrane potential, or loss of trypan blue exclusion as described in Supplemental Data.

Isolated, Perfused Hearts

Isolated, perfused heart preparations were performed as described (Tarzami et al., 2003).

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