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p53 Initiates Apoptosis by Transcriptionally Targeting the Antiapoptotic Protein ARC[▽]

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p53 plays an important role in regulating apoptosis. However, the molecular mechanism by which it initiates the apoptotic program still remains to be fully understood. Here, we report that p53 can transcriptionally target the antiapoptotic protein, apoptosis repressor with caspase recruitment domain (ARC). Our results show that reactive oxygen species and anoxia lead to the up-regulation of p53 expression. Concomitantly, ARC is down-regulated at both the protein and mRNA levels. Knockdown of p53 expression can attenuate the decreases in ARC protein and mRNA levels, indicating that ARC down-regulation is a consequence of p53 activation. Strikingly, p53-induced ARC repression occurs in a transcription-dependent manner. We further demonstrate that the p53 up-regulated modulator of apoptosis (PUMA) and Bad are up-regulated in response to the stimulation with reactive oxygen species or anoxia, and p53 is responsible for their up-regulation. ARC can interact with PUMA or Bad via its N terminus. Such an interaction displaces the association of PUMA or Bad with Bcl-2. ARC repression by p53 leads to its failure to counteract the proapoptotic activity of PUMA and Bad. Thus, our data reveal a novel p53 apoptotic pathway in which it initiates apoptosis by transcriptionally repressing ARC.

It is well established that p53 can trigger cell death via apoptosis in many cell types, but the molecular mechanisms by which p53 induces apoptosis still remain to be fully understood (19, 31, 58). p53 can transactivate a variety of apoptotic factors, such as Bax (43), p53 up-regulated modulator of apoptosis (PUMA) (45, 63), Bad (24), and p53-regulated apoptosis-inducing protein 1 ($p53^{AIP1}$) (49). p53 also can translocate to mitochondria, where it binds to Bcl-2, thereby counteracting the antiapoptotic function of Bcl-2 (39, 42, 54). In addition, p53 can stimulate reactive oxygen species production (33, 52) and Fas/CD95 to redistribute to the cell surface (2). Thus, it appears that p53 may use multiple pathways to convey its death signals.

Although a variety of proapoptotic factors have been identified to be the downstream mediators of p53, there are a few reports showing that the antiapoptotic factors can be the transcriptional targets of p53. Survivin, an antiapoptotic protein, has been reported to be negatively regulated by p53 (22). Apoptosis is controlled by a complex interplay of pro- and antiapoptotic factors. The central role of p53 in controlling apoptosis necessitates the identification of the antiapoptotic factors that are regulated by p53 and the elucidation of the molecular mechanisms by which they participate in regulating p53-dependent apoptosis.

Apoptosis is essential for normal development and maintenance of tissue homeostasis. In the cardiovascular system, for example, apoptosis participates in shaping the cardiac and vascular structures during early morphogenesis and in regulating the growth of differentiated cardiovascular tissues at the later developmental stage (13, 51). However, excessive apoptosis has been shown to be related to cardiac diseases, such as myocardial infarction, cardiomyopathy, cardiac hypertrophy, and anthracycline-induced cardiotoxicity (5, 12, 21, 25, 28).

p53-dependent apoptosis is implicated in the response of cardiomyocytes to a variety of stimuli. Blockade of the vacuolar proton ATPase induces p53-dependent apoptosis in cardiomyocytes (36). Hypoxia-induced apoptosis of cardiomyocytes is regulated by intracellular signaling pathways activated by p53 (3, 35, 61). Angiotensin II may promote cardiomyocyte apoptosis by activation of p53 (1). Insulin-like growth factor 1 protects cardiomyocytes from apoptosis by attenuating p53 transcriptional activity for Bax, angiotensinogen, and angiotensin I receptor (30). Reactive oxygen species can activate p53 (57). Hence, in order to keep the heart intact in both structure and function, it is necessary to identify the molecules that can affect p53-dependent apoptotic signaling pathways.

Intriguingly, several proapoptotic factors have been found to be expressed abundantly in the heart compared to other organs or tissues. These include Bak (11), caspase 9 (55), and Fas/CD95 (23, 60). In order to maintain the cellular equilibrium of a terminally differentiated organ like the heart, the high expression pattern of proapoptotic factors evolutionarily would require an adequate expression pattern and activity of antiapoptotic factors. Indeed, ARC is the first antiapoptotic protein so far identified to be highly expressed in cardiac and skeletal muscle tissues. ARC can block both the extrinsic and intrinsic death pathways (46). It may specifically inhibit the activation of caspase 2 and caspase 8, thereby inhibiting apoptosis induced by a variety of stimuli requiring the engagement of these caspases (29). ARC is also able to inhibit cytochrome

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c release (8, 20) and maintain mitochondrial membrane potential (48). Because of the wide involvement of p53 and the central role of ARC in regulating the apoptotic program in cardiomyocytes, it is reasonable to consider whether there is cross talk between p53 and ARC.

Our present work revealed that p53 up-regulation and ARC down-regulation simultaneously occur in the apoptotic cascades of hydrogen peroxide and anoxia. In an attempt to find out whether their occurrence and their molecular consequences are related, we identified that p53 could negatively regulate ARC expression in a transcriptional manner. The disappearance of ARC led to the initiation of the p53 apoptotic program. Our data suggest the existence of a cross talk between p53 and ARC.

MATERIALS AND METHODS

Cell treatment and viability assay. H9c2 cardiomyocytes, a fetal cardiomyocyte-derived cell line (CRL-1446; American Type Culture Collection) were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The primary neonatal rat cardiomyocytes were isolated from the hearts of Wistar rats and cultured as described elsewhere (57). Hydrogen peroxide treatment was performed as described elsewhere (32). In brief, the cultured cells were incubated at 37°C for 1 h in Hanks' balanced salt solution containing the indicated concentrations of hydrogen peroxide. The reaction was stopped by removing the Hanks' balanced salt solution containing hydrogen peroxide. Anoxia was performed as described elsewhere (40). In brief, cells were placed in an anoxic chamber with a water-saturated atmosphere composed of 5% CO_2 and 95% N_2. Cell death was determined by trypan blue exclusion, and the numbers of trypan Blue-positive and -negative cells were counted on a hemocytometer.

Constructions of rat ARC promoter and mutants, PUMA and Bad mutants, adenovirus ARC, adenovirus p53, ARC antisense oligonucleotides, and p53 RNA interference (RNAi). The ARC promoter (1,342 bp) immediately before the ARC translation starting code ATG was amplified from rat cardiomyocyte DNA using PCR. The forward primer was 5′-ACAAAGCGTCTGTCACATATG-3′. The reverse primer was 5′-TTGGGCTATATCAAGAAGGAGAGA-3′. The PCR fragment was cloned into the pcDNA 3.1 vector (Invitrogen) and then subcloned into the reporter plasmid pGL4.17 (Promega). Mutations of the two putative p53 binding sites (BS1 wild type, 5′-GGGCTTGCCT-3′, and mutant, 5′-GGGACCACCT-3′; BS2 wild type, 5′-GAGCATGGGG-3′, and mutant, 5′-GAGACCAGGG-3′) were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene). ARC mutants with the deletion of amino acids 10 to 30 (ARCΔ10–30), 31 to 50 (ARCΔ31–50), 51 to 70 (ARCΔ51–70), and 71 to 90 (ARCΔ71–90) and PUMA and Bad mutants without the BH3 domain were generated by PCR using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's instructions. All constructs were sequenced to check that only the desired mutations had been introduced. AdARC and Adp53 were constructed using the Adeno-X expression system (Clontech) according to the manufacturer's instructions.

ARC antisense oligonucleotides were synthesized to inhibit endogenous ARC expression. The sequences of phosphothioate-modified antisense oligonucleotides targeted to ARC were as follows: ARC-AS, 5′-TGGGCATGGAGGGTC ATAGCT-3′; S-ARC-AS, 5′-GTAGGCTGAGGTCGATCGGTA-3′; ARC-S, 5′-AGCTATGACCCTCCATGCCCA-3′. The specificity of the oligonucleotides was confirmed by comparison with all other sequences in GenBank using Nucleotide BLAST. There was no homology to other known rat DNA sequences.

The p53 RNAi sense sequence was 5′-CACATGACTGAGGTCGTGA-3′; the antisense sequence was 5′-TCACGACCTCAGTCATGTG-3′. The scrambled p53 RNAi sense sequence was 5′-GACGTATGCAGAGTCGTCA-3′; the scrambled antisense sequence was 5′-TGACGACTCTGCATACGTC-3′. p53 RNAi-I sense sequence was 5′-CTTACCAAGGCAACTATGG-3′; the antisense sequence was 5′-CCATAGTTGCCTTGGTAAG-3′. The scrambled p53 RNAi-I sense sequence was 5′-ATGCAATCGGCTTAGCCAA-3′; the scrambled antisense sequence was 5′-TTGGCTAAGCCGATTGCAT-3′. They were cloned into the p*Silencer* Adeno 1.0-CMV vector (Ambion) according to the manufacturer's instructions.

Adenovirus infection. All viruses were amplified in 293 cells. Cells were infected at the indicated multiplicity of infection (MOI) for 60 min. After washing with phosphate-buffered saline (PBS), culture medium was added and cells were cultured until the indicated time.

Establishment of cells stably expressing ARC mutants. H9c2 cells seeded in a 12-well plate were transfected with ARC mutants, including ARCΔ10–30, ARCΔ31–50, ARCΔ51–70, and ARCΔ71–90 by using the Effectene transfection kit (Qiagen) according to the manufacturer's instructions. Transfected cells were selected in medium containing 1 mg/ml neomycin for 28 days. Cells stably expressing ARC mutants were confirmed with anti-ARC antibody (against the C terminus of ARC; Chemicon).

Immunoblot analysis. Cells were lysed for 1 h at 4°C in a lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol [DTT], 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Equal protein loading was controlled by Ponceau Red staining of membranes. Blots were probed using primary antibodies. The anti-PUMA (1:200) and the anti-Bad (1:200) antibodies were from Santa Cruz Biotechnology. The anti-Bcl-2 antibody (1:500) was from BD Pharmingen. The anti-p53 monoclonal antibody (1:400) was from Calbiochem. The anti-ARC antibody (1:1,000) was from Chemicon. The rabbit immunoglobulin G control (1:200) was from Antigenix America Inc. After four washes with PBS-Tween 20, horseradish peroxidase-conjugated secondary antibodies were added. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

Preparation of mitochondrial fractions. Mitochondrial fractions were prepared as described elsewhere (33). Briefly, cells were washed twice with PBS, and the pellet was suspended in 0.2 ml of buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose) containing a protease inhibitor cocktail. The cells were homogenized by 12 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 × *g* for 5 min at 4°C. The supernatants were centrifuged at 10,000 × *g* for 15 min at 4°C to collect mitochondria-enriched heavy membranes.

Immunoprecipitation and in vitro protein binding assay. Immunoprecipitation was conducted as described previously (34). The samples were precleared with 10% (vol/vol) protein A- or protein G-agarose (Roche) for 1 h on a rocking platform. Specific antibodies were added and rocked for 1 h. Immunoprecipitates were captured with 10% (vol/vol) protein A- or protein G-agarose for another hour. The agarose beads were spun down and washed three times with NET/NP-40 buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.1% NP-40). The antigens were released and denatured by adding SDS sample buffer. Immunoblot analysis was performed as described above.

An in vitro protein binding assay was performed as described elsewhere (4, 44). In brief, recombinant ARC was produced as described previously (34). His-tagged PUMA or Bad in pcDNA3.1 was expressed in HEK293 cells and purified by using the ProBond purification system (Invitrogen). ARC (0.4 μg) was incubated with 0.4 μg of either PUMA or Bad in 50 μl of binding buffer (142 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.4], 0.5 mM DTT, 1 mM EGTA, 0.1% NP-40, and a protease inhibitor cocktail) at 4°C for 2 h. Immunoprecipitation was performed as described above.

ChIP analysis. Chromatin immunoprecipitation (ChIP) was performed as described elsewhere (56) with modifications. In brief, cells (0.5×10^8) were washed with PBS and incubated for 10 min with 1% formaldehyde at room temperature. The cross-linking was quenched with 0.1 M glycine for 5 min. Cells were washed twice with PBS and lysed for 1 h at 4°C in a lysis buffer. The cell lysates were sonicated into chromatin fragments with an average length of 500 to 800 bp as assessed by agarose gel electrophoresis. The samples were precleared with protein A-agarose (Roche) for 1 h at 4°C on a rocking platform, and 10 μg specific antibodies was added and rocked overnight at 4°C. Immunoprecipitates were captured with 10% (vol/vol) protein A-agarose for 4 h. Before use, protein A-agarose was blocked twice at 4°C with salmon sperm DNA (1 μg/ml) that had been sheared to a 500-bp length and bovine serum albumin (1 μg/ml) overnight. PCRs were performed with the primers that encompass p53 BS1 or BS2 of the rat ARC promoter. The oligonucleotides were as follows: BS1 (corresponding to a 183-bp fragment) forward, 5′-TCATAATAGGTAGGCAGTAGC, and reverse, 5′-CCTGTACCAAACCGTAGGAAC; BS2 (corresponding to a 182-bp fragment) forward, 5′-GAAGAATCCCGAGAGTATG, and reverse, 5′-CATA CCTCCCTTCTGAACC.

Luciferase assay. Cells were seeded in 12-well plates (6×10^4 cells/well). They were transfected with the plasmid constructs using the Effectene transfection kit (Qiagen). Each well contained 0.3 μg luciferase reporter plasmids, 5 ng SV-*Renilla* luciferase plasmids as the internal control. Cells were harvested at the indicated time after transfection for the detection of luciferase activity using the Dual Luciferase reporter assay kit (Promega) according to the manufacturer's

instructions. Twenty μl of protein extracts was analyzed in a luminometer. Firefly luciferase activities were normalized to *Renilla* luciferase activity.

Northern blot analysis and quantitative real-time PCR. Northern blot analysis was performed as described elsewhere (32). Prehybridization was conducted at 42°C for 4 h in prehybridization buffer: 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2% blocking reagent, 50 mM sodium phosphate, pH 7.4, 7% (wt/vol) SDS, and 0.1% (wt/vol) *N*-laurylsarkosine. Hybridization was performed in the same buffer and at the same temperature for 24 h with digoxigenin-labeled ARC or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For chemiluminescence detection, the membrane was blocked for 30 min in 2.5% blocking reagent. The membrane was then incubated for 30 min with antidigoxigenin antibody conjugated with alkaline phosphatase. After two washes with 100 mM maleic acid buffer containing 0.3% Tween 20, CSPD substrate solution was added to the membrane and incubated for 10 min.

For quantitative real-time PCR, RNA was prepared with TRIzol reagent (Invitrogen). Total RNA was processed to cDNA by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR using Power SYBR Green PCR master mix (Applied Biosystems) was carried out in triplicate in a 7500 Fast real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. The sequences of ARC primers were as follows: forward, 5′-CGGAAACGGCTGGTAGAAAC-3′; reverse, 5′-TGGCATGCTCACAGTTTGCT-3′. Analysis was performed using the software supplied with the instrument. Primers for GAPDH were as follows: forward primer, 5′-TGTTCCAGTATGACTCTACC-3′; reverse primer, 5′-TG GGTTTCCCGTTGATGACC-3′. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis.

Detection of caspase 8 activity. Caspase 8 activity was detected using an assay kit (R&D Systems). The assay procedures were according to the kit instructions.

Statistical analysis. Paired data were evaluated by Student's *t* test. A one-way analysis of variance was used for multiple comparisons. A P value of <0.05 was considered significant.

RESULTS

ARC down-regulation and p53 up-regulation simultaneously occur upon stimulation with hydrogen peroxide or anoxia. Reactive oxygen species (such as hydrogen peroxide) and anoxia play an important role in the development of apoptosis-related cardiac diseases, including myocardial infarction and heart failure (17). To understand the role of ARC and p53 in regulating cell death induced by hydrogen peroxide and anoxia, we analyzed their expression levels. ARC protein levels were down-regulated whereas p53 protein levels were up-regulated upon hydrogen peroxide treatment (Fig. 1A). A similar result was observed in cells exposed to anoxia (Fig. 1B). We determined whether ARC is able to influence the cell fate upon stimulation with hydrogen peroxide or anoxia. ARC could prevent cell death induced by hydrogen peroxide (Fig. 1C) and anoxia (Fig. 1D). These data indicate that both hydrogen peroxide and anoxia are able to induce ARC down-regulation and p53 up-regulation, and the modulation of ARC levels can alter the cell fate upon treatment with hydrogen peroxide or anoxia.

p53 can negatively regulate ARC expression levels. To understand whether there is a link between p53 up-regulation and ARC down-regulation, we first detected whether inhibition of endogenous p53 can influence ARC protein levels. Inhibition of p53 was achieved by using RNA interference (RNAi) (p53RNAi). Scrambled p53RNAi (p53-S-RNAi) was used as the control. p53RNAi but not p53-S-RNAi could attenuate the decrease in ARC protein levels in response to stimulation with hydrogen peroxide (Fig. 2A) or anoxia (Fig. 2B). We next detected whether ARC mRNA levels also could be altered by modulating p53. Hydrogen peroxide (Fig. 2C) and anoxia (Fig. 2D) led to a decrease in ARC mRNA levels, revealed by quantitative real-time PCR and Northern blotting. p53RNAi but not p53-S-RNAi could block the decrease in ARC mRNA levels. Thus, it appears that p53 can negatively regulate ARC at both the protein and mRNA levels.

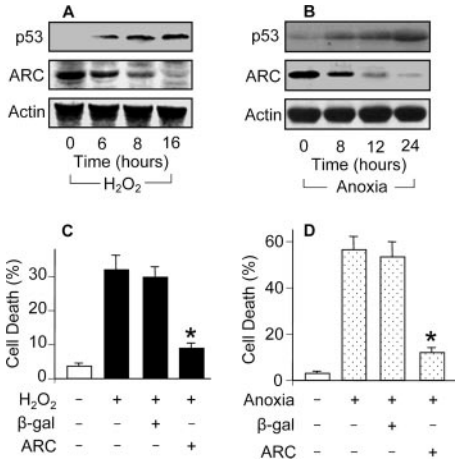

FIG. 1. Hydrogen peroxide and anoxia induce a decrease in ARC levels and an elevation in p53 levels. (A and B) H_2O_2 and anoxia induce p53 up-regulation and ARC down-regulation. H9c2 cells were treated with 0.1 mM H_2O_2 (A) or exposed to anoxic conditions (B). Cells were harvested at the indicated time for analysis of ARC and p53 by immunoblotting using the anti-ARC antibody or anti-p53 antibody, respectively. The protein loading was illustrated by α-actin, using an anti-α-actin antibody. A representative result of three independent experiments is shown. (C) ARC prevents H_2O_2-induced cell death. H9c2 cells were infected with the adenovirus harboring wild-type ARC cDNA (AdARC) or β-galactosidase cDNA (Adβ-Gal) at an MOI of 80. At 24 h after infection, cells were treated with 0.1 mM H_2O_2. Cell death was detected 24 h after H_2O_2 treatment. *, $P < 0.01$ versus H_2O_2 alone. (D) ARC prevents anoxia-induced cell death. Cells were infected with AdARC or Adβ-Gal at an MOI of 80. At 24 h after infection, cells were exposed to anoxic conditions. Cell death was detected 24 h after anoxia. *, $P < 0.01$ versus anoxia alone. Data in panels C and D are expressed as means ± standard errors of the means of three independent experiments.

ARC and p53 are functionally related. To ask whether there is a functional impact between ARC and p53 in cell death, we first detected whether p53 participates in conveying the death signals of hydrogen peroxide and anoxia. p53RNAi but not p53-S-RNAi could attenuate cell death induced by hydrogen peroxide (Fig. 3A) or anoxia (Fig. 3B), indicating that cell death occurred in a p53-dependent manner. We next detected whether ARC influences p53-induced cell death. In the enforced expression model, p53 could induce a decrease in ARC protein levels. Expression of exogenous ARC could compensate the reduction of ARC levels caused by p53. Concomitantly, ARC could prevent p53-induced cell death (Fig. 3C).

Further, we detected the susceptibility of cells to death when endogenous ARC expression is inhibited. We employed antisense oligonucleotides to inhibit endogenous ARC. Pretreatment with ARC antisense oligonucleotides (ARC-AS) but not ARC sense oligonucleotides (ARC-S) and scrambled ARC antisense oligonucleotides (S-ARC-AS) caused the cells to be more susceptible to death upon hydrogen peroxide treatment (Fig. 3D). We finally tested whether it is p53 that mediates the cell death in the absence of ARC. Knockdown of p53 led to a reduction in the percentage of cell death upon hydrogen per-

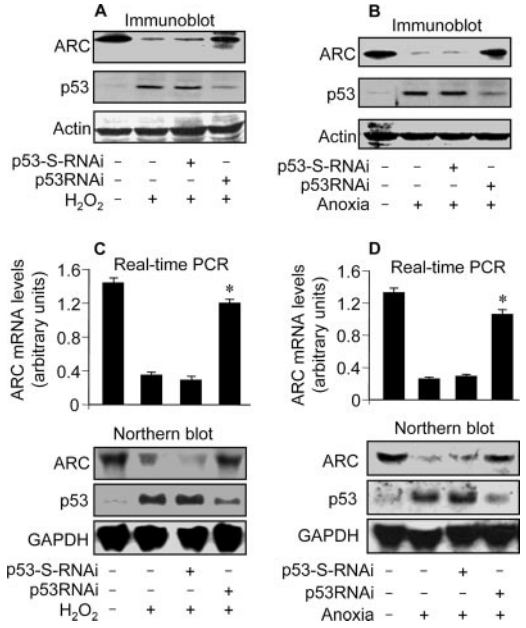

FIG. 2. p53 negatively regulates ARC expression at the protein and mRNA levels. (A) Inhibition of endogenous p53 attenuates the decrease in ARC protein levels induced by H_2O_2. H9c2 cells were infected with the adenoviral vector harboring p53RNAi (Adp53RNAi) or p53-S-RNAi (Adp53-S-RNAi) at an MOI of 50. At 24 h after infection, cells were treated with 0.1 mM H_2O_2. At 8 h after H_2O_2 treatment, cells were harvested for detecting ARC or p53 by immunoblotting with the anti-ARC antibody or the anti-p53 antibody, respectively. (B) Inhibition of endogenous p53 attenuates the decrease in ARC protein levels induced by anoxia. Cells were infected with Adp53RNAi or Adp53-S-RNAi. At 24 h after infection, cells were exposed to anoxic conditions. At 12 h after anoxia, cells were harvested for detection of ARC or p53 by immunoblotting with anti-ARC antibody or anti-p53 antibody, respectively. (C) Inhibition of p53 attenuates the decrease in ARC mRNA levels induced by H_2O_2. Cells were treated with H_2O_2 as described for panel A. Quantitative real-time RT-PCR (upper panel) and Northern blotting (lower panel) were employed to analyze ARC mRNA levels. The levels of ARC mRNA analyzed by quantitative real-time RT-PCR were normalized to that of GAPDH. Data are expressed as means ± standard errors of the means. *, $P < 0.01$ versus H_2O_2 alone. (D) Inhibition of p53 attenuates the decrease in ARC mRNA levels induced by anoxia. Cells were exposed to anoxia as described for panel B. Quantitative real-time RT-PCR (upper panel) and Northern blotting (lower panel) were employed to analyze ARC mRNA levels. The levels of ARC mRNA analyzed by quantitative real-time RT-PCR were normalized to that of GAPDH. Data are expressed as means ± standard errors of the means. *, $P < 0.01$ versus anoxia alone. Representative results from one of three independent experiments are shown.

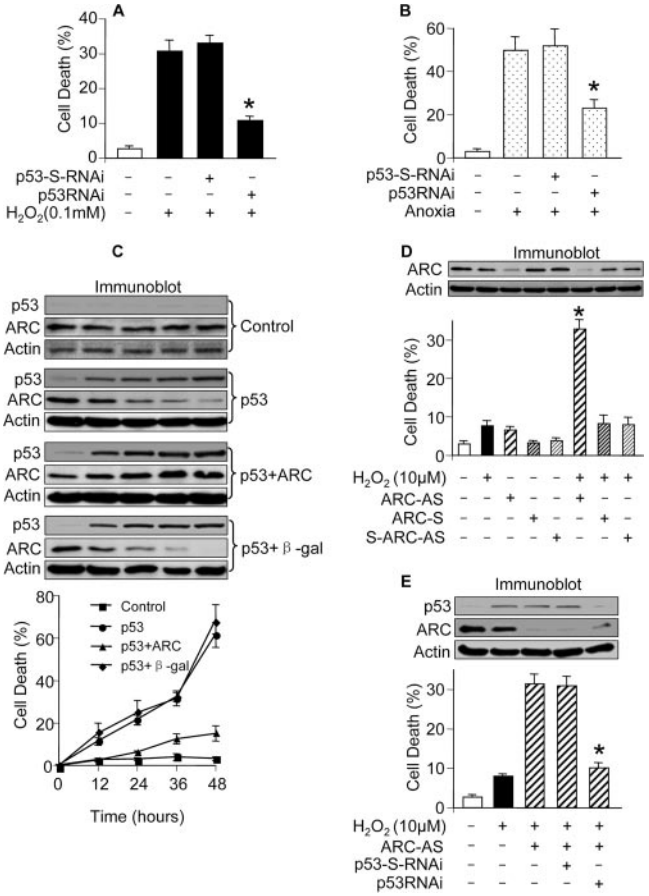

FIG. 3. ARC and p53 are functionally related. (A) Inhibition of p53 attenuates H_2O_2-induced cell death. Cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50. At 24 h after infection, cells were treated with H_2O_2. Cell death was detected 24 h after H_2O_2 treatment. *, $P < 0.05$ versus H_2O_2 alone. (B) Inhibition of p53 attenuates anoxia-induced cell death. Cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50. At 24 h after infection, cells were exposed to anoxic conditions. Cell death was detected 24 h after anoxia treatment. *, $P < 0.05$ versus anoxia alone. (C) ARC prevents p53-induced cell death. Cells were infected with Adp53 at an MOI of 50 or AdARC or Adβ-Gal at an MOI of 80. The expression levels of ARC and p53 (upper panels) and cell death (low panel) were detected at the indicated times. (D) Inhibition of endogenous ARC sensitizes H_2O_2-induced cell death. Cells were transfected with mixtures of ARC oligonucleotides (1 μM) and Lipofectin prepared according to the kit's instructions. At 24 h after transfection, they were exposed to H_2O_2. Cell death (lower panel) was detected 24 h after H_2O_2 treatment. ARC protein levels (upper panel) were detected by immunoblotting. *, $P < 0.05$ versus H_2O_2 alone. (E) Inhibition of p53 attenuates H_2O_2-induced cell death in the absence of endogenous ARC. Cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50. At 2 h after infection, they were transfected with the mixtures of ARC oligonucleotides (1 μM) and Lipofectin prepared according to the kit's instructions. At 24 h after transfection, they were exposed to H_2O_2. Cell death (lower panel) was detected 24 h after H_2O_2 treatment. The expression levels of ARC and p53 (upper panel) were detected by immunoblotting. *, $P < 0.05$ versus H_2O_2 plus ARC-AS. H9c2 cells were used to perform the experiments, and cell death was detected by trypan blue exclusion. Data are expressed as means ± standard errors of the means of three or four independent experiments.

oxide treatment (Fig. 3E). Taken together, these data indicate that ARC and p53 are functionally related.

p53 transcriptionally regulates ARC. p53 is a transcriptional factor, and the parallel alterations in p53 and ARC expression levels led us to consider whether ARC is a transcriptional target of p53. We first analyzed the consensus binding sites in the promoter region of rat ARC according to the reported consensus binding site of p53, PuPuPuC(A/T)(T/A)GPyPyPy $(N)_{0-14}$PuPuPuC(A/T)(T/A)GPyPyPy, where N stands for any nucleotide (9). The ARC promoter region contains one optimal p53 binding site (p53 BS1, −811 to −787, GGGCTTGC CTAGCAAACGCAAGGCC) and one suboptimal binding site (p53 BS2, −397 to −378, GAGCATGGGGTGCCTTG GCA) (Fig. 4A).

We next tested whether p53 can influence ARC promoter activity. The wild-type ARC promoter activity was dramatically

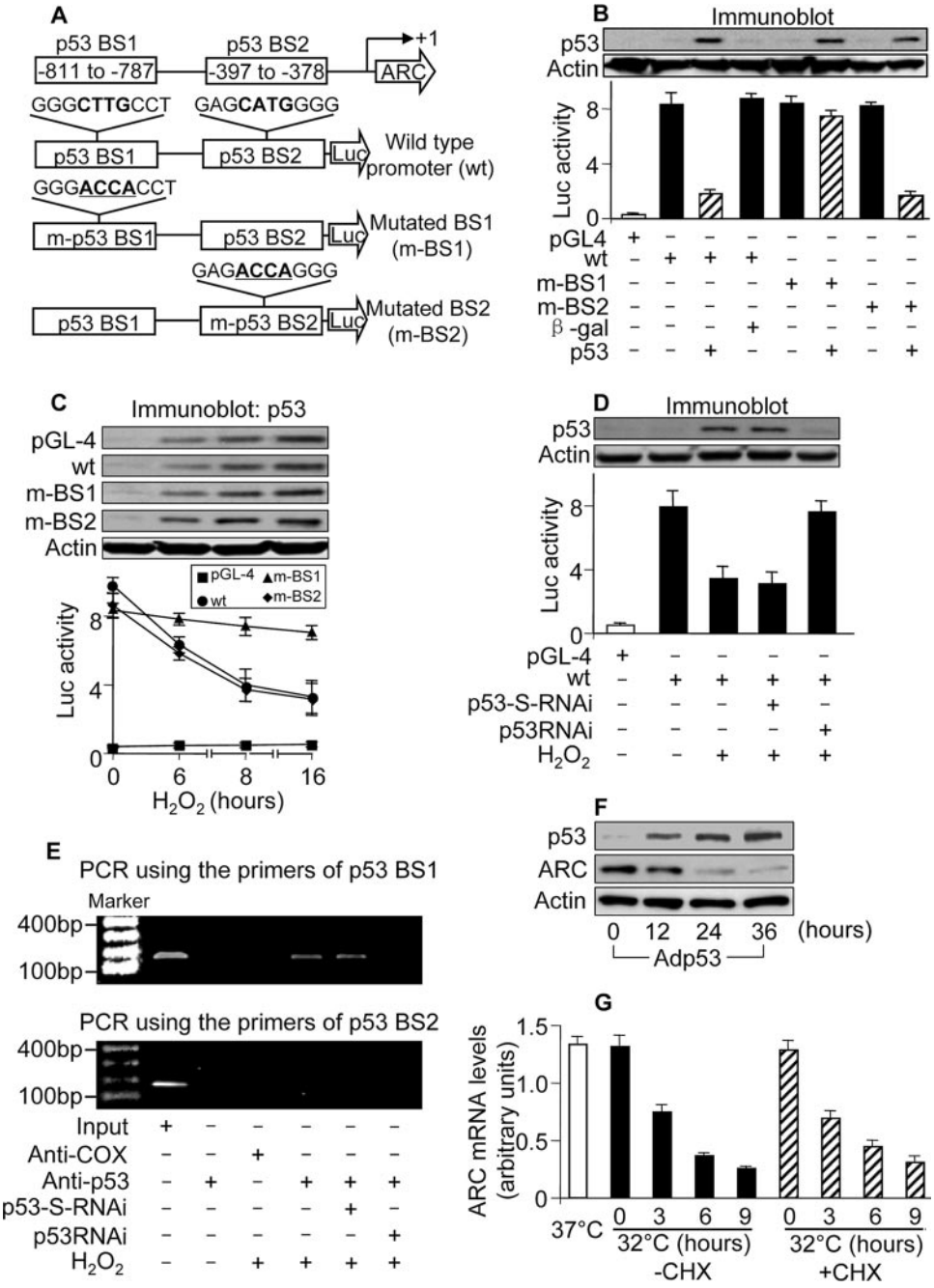

FIG. 4. p53 negatively regulates ARC promoter activity. (A) Potential p53 binding sites in the 5′-flanking sequence of the rat ARC gene. The 5′-flanking sequence of ARC contains two potential p53 DNA binding sites (indicated as BS1 and BS2). The ARC promoter region was synthesized and linked to the luciferase (Luc) reporter gene. The mutations were introduced to BS1 and BS2 (the converted nucleotides are underlined), respectively. (B) p53 represses ARC promoter activity. H9c2 cells were infected with Adp53 or Adβ-Gal at an MOI of 50. At 24 h after infection, cells were transfected with the constructs of the empty vector (pGL-4), ARC wild-type promoter (wt), or the mutated ARC promoters (m-BS1 and m-BS2). The transfection was performed using the Effectene transfection kit according to the manufacturer's instructions. The luciferase activity was determined as described in Materials and Methods. Firefly luciferase activities were normalized to *Renilla* luciferase activities. Data are expressed as the relative luciferase (Luc) activity. The expression levels of p53 were detected by immunoblotting. (C) ARC promoter activity in cells treated with H_2O_2. H9c2 cells were transfected with the constructs of pGL-4, ARC wild-type promoter (wt), m-BS1, or m-BS2. At 24 h after transfection, cells were exposed to 0.1 mM H_2O_2. The luciferase activity was determined at the indicated time. The expression levels of p53 were detected by immunoblotting. (D) p53 is responsible for the repression of ARC promoter activity upon H_2O_2 treatment. H9c2 cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50. At 24 h after infection, cells were transfected with the constructs of pGL-4 or ARC wt promoter. At 24 h after transfection, cells were exposed to 0.1 mM H_2O_2. The luciferase activity was determined 8 h after H_2O_2 treatment. The expression levels of p53 were detected by immunoblotting. (E) ChIP analysis of in vivo p53 binding to the ARC promoter. Cells were exposed to 0.1 mM H_2O_2. At 8 h after H_2O_2 treatment, cells were harvested for the ChIP analysis. Chromatin-bound DNA was immunoprecipitated with the anti-p53 antibody. Anti-cytochrome oxidase subunit V (COX) antibody was used as a negative control. Immunoprecipitated DNA was analyzed by PCR using a primer combination that encompassed p53 BS1 (upper panel) or BS2 (lower panel). (F) p53 could down-regulate ARC in primary

suppressed by p53 but not by the negative control, β-galactosidase. The introduction of mutations in p53 BS1 but not p53 BS2 resulted in the disability of p53 to repress the promoter activity, indicating that BS1 was the binding site of p53 (Fig. 4B). We also tested whether hydrogen peroxide treatment influences ARC promoter activity. A decrease in the promoter activities of wild-type ARC and the BS2 mutant, but not the BS1 mutant, was observed in cells treated with hydrogen peroxide (Fig. 4C). Subsequently, we asked whether p53 is responsible for the alterations of ARC promoter activity upon hydrogen peroxide treatment. p53RNAi but not its scrambled form could block the decrease in ARC promoter activity upon hydrogen peroxide treatment (Fig. 4D). These data indicate that p53 can influence ARC promoter activity.

A ChIP assay was employed to detect whether p53 can associate with the ARC promoter in vivo. The results showed that p53 bound to the ARC promoter region encompassing BS1 upon hydrogen peroxide treatment (Fig. 4E, upper panel). However, an association was not detectable in the ARC promoter region encompassing BS2 upon hydrogen peroxide treatment (Fig. 4E, lower panel).

To further characterize whether ARC repression by p53 occurs in a direct manner, we employed primary rat cardiomyocytes, which lose their ability to proliferate, withdraw from the cell cycle, and express a high level of ARC but an extremely low level of p53 (29, 38, 50, 57). p53 induced a decrease in ARC protein levels in the primary rat cardiomyocytes (Fig. 4F). In addition, the temperature-sensitive p53(Val135) in the presence of cycloheximide still induced a significant decrease in ARC mRNA levels (Fig. 4G), indicating that ARC is a primary target of p53. From these findings together, it appears that p53 can negatively control the transcriptional activity of ARC.

ARC binds to PUMA or Bad, displacing their associations with Bcl-2. In the following experiments we explored the molecular mechanisms by which ARC antagonizes p53-induced cell death. p53 can induce cell death through the transcription-dependent pathway. PUMA and Bad are the transcriptional targets of p53 (24, 37, 45, 63). We first detected the expression levels of PUMA and Bad upon treatment with hydrogen peroxide and anoxia. Both hydrogen peroxide (Fig. 5A) and anoxia (Fig. 5B) led to the up-regulation of PUMA and Bad blocked by p53RNAi but not p53-S-RNAi.

We next determined whether there is an association between ARC and PUMA or Bad. The associations of ARC with PUMA or Bad in cells without treatment were barely detectable (Fig. 5C, first lane, middle panel), and this can be explained by the low levels of PUMA and Bad in cells without treatment. The associations of endogenous ARC with PUMA or Bad were weak in cells treated with hydrogen peroxide or anoxia (data not shown), and this could be due to the down-regulation of endogenous ARC, as shown in Fig. 1. In contrast, the associations of ARC with PUMA or Bad could be detected in cells expressing exogenous ARC and exposed to hydrogen peroxide or anoxia (Fig. 5C). To test whether ARC can directly bind to PUMA or Bad, we analyzed whether ARC is able to bind to PUMA or Bad in vitro. The in vitro binding assay revealed that ARC could associate with PUMA (Fig. 5D, upper panel) or Bad (Fig. 5D, lower panel). To test whether stress can influence the binding of ARC to PUMA or Bad, we employed a model in which ARC and PUMA or Bad were simultaneously expressed. To exclude the influence of endogenous ARC, HEK293 cells were used to coexpress ARC and PUMA or Bad, because endogenous ARC expression in this cell line is undetectable (34, 46). The results showed that the binding of ARC to PUMA or Bad could occur in the presence or absence of stress (Fig. 5E). Taken together, these results suggest that ARC can bind to PUMA or Bad.

Further, we analyzed the associations of Bcl-2 with PUMA or Bad in the presence or absence of ARC. The associations of Bcl-2 with PUMA or Bad could be observed in cells exposed to hydrogen peroxide or anoxia. However, their associations disappeared in the presence of ARC. ARC could not influence the expression levels of PUMA, Bad, and Bcl-2 (Fig. 5F). These data indicate that ARC can prevent the associations of PUMA or Bad with Bcl-2.

Characterization of the binding sites between ARC and PUMA or Bad. To further confirm the association of ARC with PUMA or Bad, we analyzed the binding sites between ARC and PUMA or Bad. The binding of ARC to caspase 8 is through its CARD (29). We tested whether the CARD is responsible for ARC associations with PUMA or Bad. The binding abilities of ARC mutants to endogenous PUMA or Bad were analyzed. As shown in Fig. 6A, ARCΔ10–30 and ARCΔ71–90 but not ARCΔ31–50 or ARCΔ51–70 were able to bind to PUMA and Bad upon hydrogen peroxide treatment, indicating that amino acids 31 to 70 are responsible for the binding ability of ARC to PUMA or Bad.

To test whether such an association plays a functional role, we analyzed the ability of these mutants to inhibit cell death. ARCΔ10–30 and ARCΔ71–90 but not ARCΔ31–50 and ARCΔ51–70 retained the ability to antagonize cell death induced by hydrogen peroxide (Fig. 6A).

We finally analyzed the binding sites of PUMA and Bad to ARC. The BH3 domains of PUMA and Bad have been shown to be essential for their interactions with other proteins, such as Bcl-2 (27, 63). We tested whether the BH3 domain is necessary for PUMA or Bad to interact with ARC. Deletion of the BH3 domain in PUMA (Fig. 6B) or Bad (Fig. 6C) led to their

cardiomyocytes. The primary rat cardiomyocytes were infected with Adp53 at an MOI of 50. Cells were harvested at the indicated times after infection in order to determine ARC and p53 in immunoblot assays using their antibodies. (G) ARC is a primary target of p53. The primary cardiomyocytes were infected with the adenovirus harboring temperature-sensitive p53(Val135) and cultured either at 37°C (restrictive temperature) or at 32°C (permissive temperature) in the absence or presence of 10 μg/ml cycloheximide (CHX). CHX was added to the medium 1 h prior to temperature shift. (Cycloheximide was observed to inhibit protein synthesis by 93.02 ± 2.25% as measured by [^{35}S]methionine incorporation). ARC mRNA levels were analyzed by quantitative real-time RT-PCR. Data are expressed as means ± standard errors of three or four independent experiments.

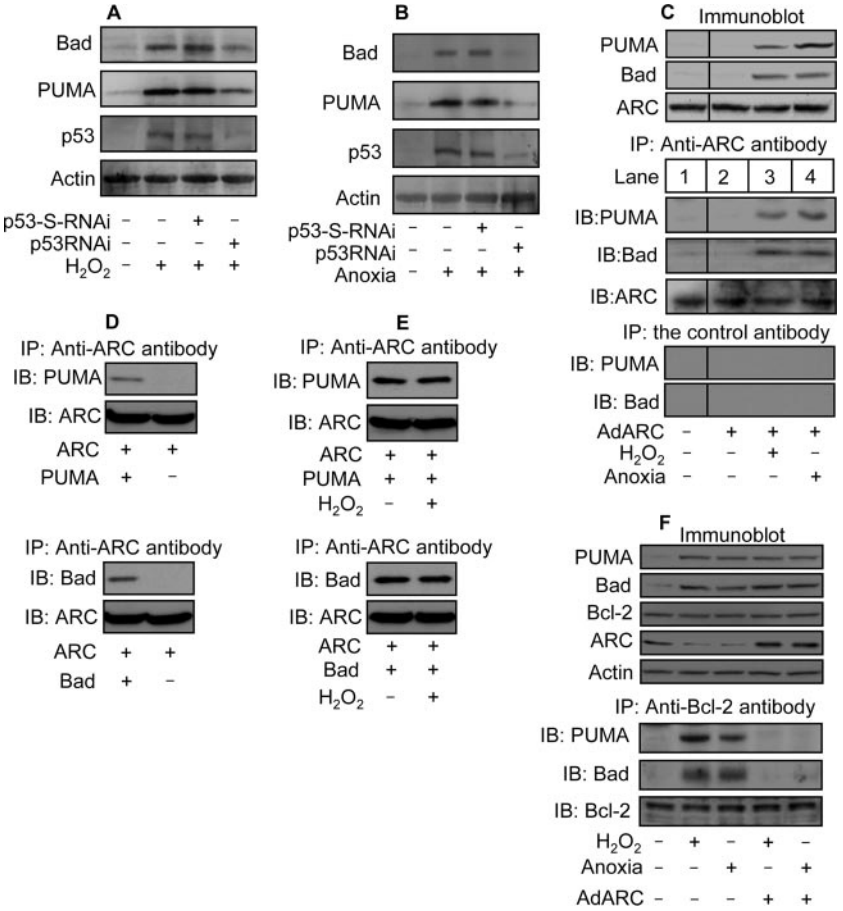

FIG. 5. ARC binds to PUMA and Bad, displacing their associations with Bcl-2. (A) PUMA and Bad are up-regulated in response to H_2O_2 treatment. Cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50. At 24 h after infection, they were treated with 0.1 mM H_2O_2. At 8 h after H_2O_2 treatment, cells were harvested for analysis of Bad, PUMA, or p53 by immunoblotting with their respective antibodies. (B) PUMA and Bad are up-regulated upon exposure to anoxic conditions. Cells were infected with Adp53RNAi or Adp53-S-RNAi at an MOI of 50 and then exposed to anoxic conditions. At 12 h after anoxia, cells were harvested for the analysis of Bad, PUMA, and p53. (C) ARC can bind to PUMA and Bad. Cells were infected with AdARC at an MOI of 80 and then treated with 0.1 mM H_2O_2 or exposed to anoxic conditions. Mitochondria were prepared 8 h after H_2O_2 treatment or 12 h after anoxia. In order to take the same amount of ARC to perform IP, 80, 15, 20, and 25 μg of mitochondrial lysates corresponding to lanes 1 to 4, respectively, were used. The levels of PUMA, Bad, and ARC in the lysates were analyzed by immunoblotting (IB; upper panel). Immunoprecipitation with rabbit anti-ARC antibody (8 μg) was followed by immunoblotting with anti-PUMA antibody or anti-Bad antibody. The membrane was stripped and reprobed with anti-ARC antibody (middle panel). To test the binding specificity, immunoprecipitation with the control rabbit immunoglobulin G was performed, followed by IB with anti-PUMA antibody or anti-Bad antibody (lower panel). (D) In vitro binding assay. Recombinant ARC was incubated with either PUMA (upper panel) or Bad (low panel) in the binding buffer as described in Materials and Methods. Immunoprecipitation with anti-ARC antibody was followed by immunoblot (IB) with anti-PUMA antibody or anti-Bad antibody. (E) Detection of ARC binding to PUMA or Bad in the presence or absence of stress. HEK293 cells were cotransfected with the constructs of ARC and PUMA (upper panel) or Bad (low panel) and then treated with 0.1 mM H_2O_2. Mitochondria were prepared 8 h after H_2O_2 treatment. Immunoprecipitation with anti-ARC antibody was followed by IB with anti-PUMA antibody or anti-Bad antibody. (F) ARC prevents the association of Bcl-2 with PUMA or Bad. Cells were infected with AdARC at an MOI of 80 and then treated with 0.1 mM H_2O_2 or exposed to anoxic conditions. Mitochondria were prepared 8 h after H_2O_2 treatment or 12 h after anoxia. The protein levels of PUMA, Bad, Bcl-2, and ARC were analyzed by immunoblotting. Aliquots of 100 μg from mitochondrial lysates were used to perform immunoprecipitation with rabbit anti-Bcl-2 antibody, followed by immunoblotting using anti-PUMA antibody, anti-Bad antibody, or anti-Bcl-2 antibody. A representative result of three independent experiments is shown.

inabilities to interact with ARC, suggesting that the BH3 domain is necessary for their associations with ARC.

The association of PUMA with ARC leads to caspase 8 activation. ARC under physiological conditions is associated with caspase 8 (34). PUMA could bind to ARC, as shown in Fig. 5C. We asked whether PUMA can influence the association of ARC with caspase 8. The association of ARC with caspase 8 was detectable in the control cells without treatment or expressing β-galactosidase. However, their association levels were decreased in cells expressing PUMA. Enforced expression of ARC restored their association levels (Fig. 7A). Concomitantly, caspase 8 activation (Fig. 7B) and cell death (Fig. 7C) were observed in the presence of PUMA. ARC attenuated caspase 8 activation and cell death induced by PUMA. These results suggest that PUMA can influence the interaction of ARC with caspase 8.

ARC repression by p53 is involved in the death program with daunomycin. Chemotherapy agents can induce cardiomyo-

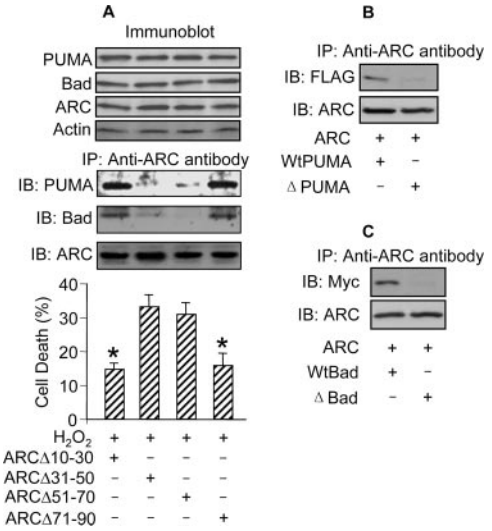

FIG. 6. Analysis of the binding sites between ARC and PUMA or Bad. (A) Analysis of the binding sites of ARC to PUMA and Bad. H9c2 cells stably expressing ARC mutants were treated with 0.1 mM H_2O_2. The protein levels of PUMA, Bad, and ARC were analyzed by immunoblotting (upper panel). Mitochondria were prepared 8 h after H_2O_2 treatment. Immunoprecipitation with anti-ARC antibody (against the C terminus of ARC) was followed by immunoblot using anti-PUMA antibody or anti-Bad antibody (middle panel). Cell death was detected 24 h after H_2O_2 treatment (lower panel). *, $P < 0.05$ versus ARCΔ31–50 or ARCΔ51–70. Data are expressed as means ± standard errors of the means of three independent experiments. (B) Analysis of the binding site of PUMA to ARC. HEK293 cells were cotransfected with the constructs of ARC and the wild-type (Wt) PUMA or PUMA mutant without BH3 domain (ΔPUMA). Both Wt-PUMA and ΔPUMA were tagged with FLAG. Immunoprecipitation with anti-ARC antibody was followed by immunoblotting using anti-FLAG antibody. (C) Analysis of the binding site of Bad to ARC. HEK293 cells were cotransfected with the constructs of ARC and the WtBad or Bad mutant without a BH3 domain (ΔBad). Both WtBad and ΔBad were tagged with Myc. Immunoprecipitation with an anti-ARC antibody was followed by immunoblot using anti-Myc antibody. A representative result of three independent experiments is shown.

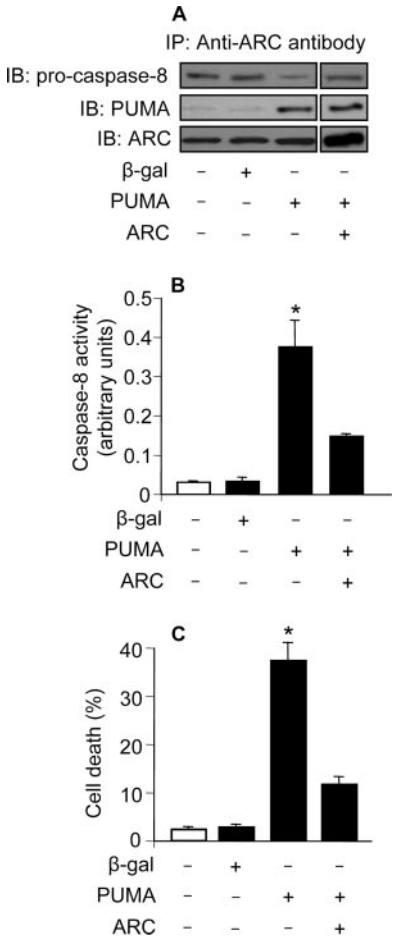

FIG. 7. PUMA binds to ARC, resulting in activation of caspase 8. (A) The association levels of ARC with caspase 8 are reduced in the presence PUMA. H9c2 cells were infected with AdPUMA, Adβ-Gal, or AdARC at an MOI of 80. At 24 h after infection, cells were harvested for the immunoprecipitation with anti-ARC antibody followed by immunoblotting (IB) with anti-caspase 8 antibody or anti-PUMA antibody. (B) Analysis of caspase 8 activity. Cells were treated as described for Fig. 7A. *, $P < 0.05$ versus control. (C) Analysis of cell death. Cells were treated as described for Fig. 7A. Cell death was detected 48 h after infection. *, $P < 0.05$ versus the control. Data are expressed as means ± standard errors of the means of three independent experiments.

cyte apoptosis, leading to heart failure (53, 62). To understand whether ARC repression by p53 is involved in apoptosis induced by chemotherapy agents, we first tested whether ARC expression levels can be altered in response to daunomycin treatment. As shown in Fig. 8A, the expression levels of p53 were up-regulated, whereas the expression levels of ARC were decreased upon daunomycin treatment. We next tested whether p53 is responsible for ARC down-regulation. Two p53 RNAi constructs were employed to inhibit p53. p53-RNAi and p53-RNAi-I but not their scrambled forms could inhibit p53 expression and attenuate the reduction of ARC expression levels (Fig. 8B, upper panels). Concomitantly, they could block daunomycin-induced cell death (Fig. 8B, lower panel). Enforced expression of ARC could prevent cell death induced by daunomycin (Fig. 8C). We finally tested whether ARC could bind to PUMA upon daunomycin treatment. The association of ARC with PUMA was observed in cells treated with daunomycin (Fig. 8D). Taken together, these results suggest that ARC repression by p53 may contribute to daunomycin-induced cell death.

DISCUSSION

Our present work shows that ARC expression levels are decreased in response to stimulation with oxidative stress, anoxia, or daunomycin. Such a decrease is caused by p53 in a transcription-dependent manner. PUMA and Bad can be transcriptionally activated by p53. ARC is able to interact with PUMA and Bad, resulting in their inabilities to bind to Bcl-2. ARC repression disrupts the equilibrium between pro- and antiapoptotic factors and, consequently, it cannot block cell death (Fig. 9). Hence, it appears that the transcriptional repression of ARC constitutes a component in the death machinery of p53.

It has been shown that ARC protein levels are decreased in patients with heart failure (6) or in dilated cardiomyopathic hearts (14). At the cellular level, ARC protein levels also are

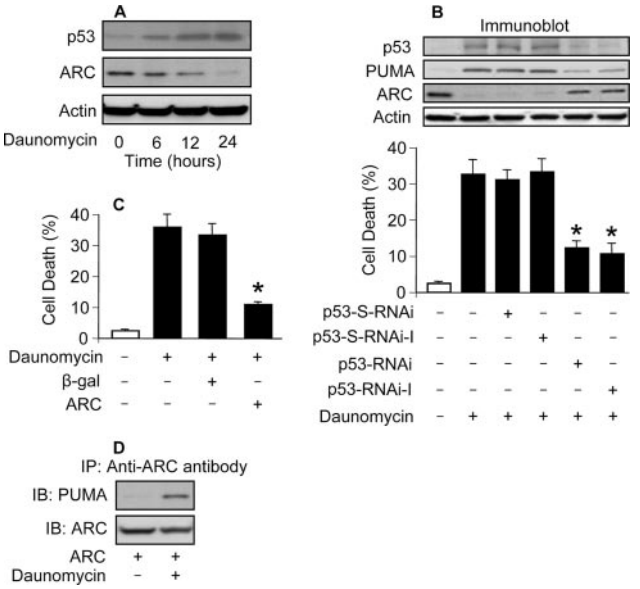

FIG. 8. p53 is responsible for ARC down-regulation in response to daunomycin treatment. (A) ARC expression levels were reduced upon daunomycin treatment. The primary rat cardiomyocytes were treated with 1 μM daunomycin. Cells were harvested at the indicated times for analysis of ARC and p53 by immunoblotting using the anti-ARC antibody or anti-p53 antibody, respectively. A representative result of three independent experiments is shown. (B) p53 is responsible for ARC down-regulation. The primary rat cardiomyocytes were infected with the adenoviral vector harboring p53RNAi, p53RNAi-I, or their scrambled forms (p53-S-RNAi and p53-S-RNAi-I) at an MOI of 50. At 24 h after infection, cells were treated with 1 μM daunomycin. At 24 h after daunomycin treatment, cells were harvested for detecting ARC, PUMA, or p53 by immunoblotting with their antibodies (upper panel) and cell death (low panel). *, $P < 0.05$ versus daunomycin alone. (C) ARC prevents daunomycin-induced cell death. The primary rat cardiomyocytes were infected with AdARC or Adβ-gal at an MOI of 80. At 24 h after infection, cells were treated with 1 μM daunomycin. Cell death was detected 24 h after treatment. *, $P < 0.05$ versus daunomycin alone. (D) ARC can bind to PUMA in response to daunomycin treatment. Primary rat cardiomyocytes were infected with AdARC at an MOI of 80 and then treated with 1 μM daunomycin. Mitochondria were prepared 12 h after treatment. Immunoprecipitation with anti-ARC antibody was followed by immunoblotting (IB) with anti-PUMA antibody. Data in panels B and C are expressed as means ± standard errors of the means of three independent experiments.

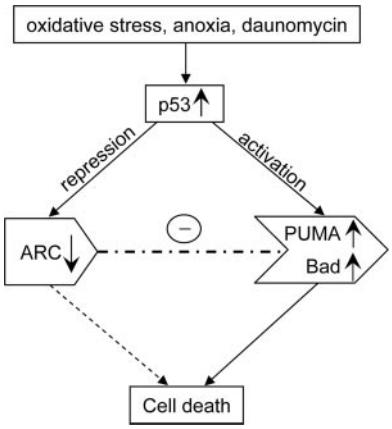

FIG. 9. Schematic model of ARC in the death program of p53. p53 transcriptionally represses ARC expression but activates the expression of PUMA and Bad. ARC can bind to PUMA and Bad. However, the down-regulation of ARC results in its failure to block PUMA- and Bad-mediated death signals.

decreased in response to stimulation with hydrogen peroxide (20, 46, 48). Most recently, it has been shown that the decrease of ARC protein levels could be due to its increased degradation (14, 47). Our present work reveals that ARC repression by p53 could contribute to its decrease. Such a discrepancy could be related to the concentrations of hydrogen peroxide. It appears that hydrogen peroxide at a high concentration predominantly activates the ARC protein degradation system (14, 47), whereas hydrogen peroxide at a low concentration predominantly triggers the p53-dependent transcriptional system, as revealed in our present study. It would be interesting to determine under which stages of cell death the programs for transcriptional repression and posttranslational degradation of ARC occur, as well as under which pathological conditions. It is of note that ARC degradation is dependent on the p53-induced ubiquitin E3 ligase MDM2 (14). Our present work shows that p53 itself can repress ARC expression. These results indicate not only that ARC, a central cardiac-protecting molecule, is a target of p53 but also that p53 can down-regulate ARC through different pathways.

Our finding that p53 transcriptionally represses ARC is supported by the distinct expression patterns of ARC and p53. Under physiological conditions, tissues or organs with a high level of p53 express a low level of ARC. By contrast, those with a high level of ARC have a low level of p53 (16, 29). ARC repression by p53 is probably necessary for maintaining tissue homeostasis. The abnormalities of p53 likely lead to the pathophysiological disorders related to ARC. For example, p53 up-regulation and ARC reduction are related to myocardial infarction and heart failure (6, 15). In contrast, some tumor cells with p53 defects, such as the breast cancer cell line MDA-MB-231 and HeLa cells, have a high expression level of ARC, which has been implicated in carcinogenesis (41, 59).

In response to p53 transcriptional activity, it has been reported that 38 genes are up-regulated and 24 genes are down-regulated (26). p53 can up-regulate proapoptotic factors such as Bax (43), PUMA (45, 63), Bad (24), etc. It can down-regulate antiapoptotic factors such as glutathione *S*-transferase α (10) and Survivin (22). In addition, the gene encoding p202, an interferon-inducible negative regulator of p53, is a target of p53-mediated transcriptional repression (7). p53-induced gene repression can occur in an indirect manner (18). The data of our present study suggest that ARC repression by p53 occurs in a direct manner. This is consistent with a previous observation that showed that p53 can induce Survivin suppression by directly binding to its promoter (22). Nevertheless, the exact molecular mechanism by which p53 transcriptionally up- or down-regulates genes needs to be fully elucidated in a future study.

PUMA and Bad are downstream apoptotic mediators of p53. PUMA is rapidly induced by p53. It is exclusively located to mitochondria and can bind to Bcl-2 and Bcl-X_L, thereby inducing cytochrome *c* release and consequent activation of caspase 9 and -3 (45, 63). Bad is able to translocate to mitochondria, where it binds to Bcl-2, resulting in cytochrome *c*

release. Recently, it has been reported that p53 can activate Bad transcription (24). Our present work reveals that both hydrogen peroxide and anoxia are able to up-regulate PUMA and Bad expression, and their up-regulation is dependent on p53. Thus, in order to initiate the apoptotic program, on the one hand p53 transcriptionally suppresses the antiapoptotic protein ARC and on the other hand it activates proapoptotic factors, such as PUMA and Bad.

Strikingly, ARC is able to bind to PUMA or Bad, leading to their failure to associate with Bcl-2. ARC also can associate with Bax (20, 46), a p53 transcription-dependent proapoptotic protein (43). Hence, ARC can block apoptosis by interacting with PUMA, Bad, or Bax, thereby quenching their mediated death signals.

The heart is an organ composed of terminally differentiated cardiomyocytes. Since cardiomyocyte loss cannot be compensated by efficient cell proliferation, induction of cell death in cardiomyocytes may lead to pathophysiological disorders. The involvement of p53 in cardiac cell death induced by a variety of stimuli makes this molecule a particularly interesting target. Interventional approaches could include the disruption of p53 itself or the modulation of its apoptotic pathway. The results revealed in our present work warrant future studies to explore the plausible beneficial effect of ARC on cardiac diseases that are related to p53-dependent cell death.

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