Ubiquitination and Degradation of the Anti-apoptotic Protein ARC by MDM2*

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Current evidence shows that cardiomyocyte apoptosis plays a central role in the pathogenesis of myocardial disease and that reactive oxygen species is critically responsible for mediating cardiomyocyte apoptosis in both ischemia-reperfusion injury and dilated cardiomyopathy. ARC (Apoptosis Repressor with Caspase recruitment domain) is an anti-apoptotic protein that is found abundantly in terminally differentiated cells such as cardiomyocytes. The ARC knock-out mouse developed larger infarcts in response to ischemia-reperfusion and transitioned more rapidly and severely to dilated cardiomyopathy following aortic constriction. In addition, ARC protein levels are decreased in human dilated cardiomyopathy and when cardiomyocytes are exposed to oxidative stress in vitro, but the mechanisms regulating ARC protein levels are not known. Here we show that degradation of ARC is dependent on the p53-induced ubiquitin E3 ligase, MDM2. Oxidative stress reduced ARC levels and up-regulated MDM2. MDM2 directly accelerated ARC protein turnover via ubiquitination and proteasomal-dependent degradation. This activity requires a functioning MDM2 ring finger domain because the MDM2C465A mutant was unable to direct ARC degradation. Furthermore, ARC degradation requires MDM2, because MDM2 knock-out fibroblasts showed defective ARC degradation that could be rescued by MDM2. Proteasomal inhibitors rescued both MDM2 and H2O2-induced degradation of ARC and inhibited cardiomyocyte apoptosis. Dilated cardiomyopathic hearts from mice that have undergone transverse aortic banding have increased MDM2 levels associated with decreased ARC levels. We conclude that MDM2 is a critical regulator of ARC levels in cardiomyocytes. Prevention of MDM2-induced degradation of ARC represents a potential therapeutic target to prevent cardiomyocyte apoptosis.

Cardiac myocyte apoptosis, implicated in ischemia-reperfusion injury and chronic heart failure (1), plays a central role in the pathogenesis of cardiac diseases. A causal role in the pathogenesis of chronic heart failure has been demonstrated by several different genetic mouse models. For example, cardiac-restricted expression of a pro-caspase-8 fusion protein (2) produced very low but significantly raised levels of cardiomyocyte apoptosis (0.023 versus 0.002% in controls) and resulted in a lethal dilated cardiomyopathy. Conversely, systemic administration of broad spectrum caspase inhibitors starting before cardiac decompensation rescued apoptosis, abrogated cardiac dilatation, and significantly ameliorated contractile dysfunction (2). Notably, even though the level of apoptosis in this mouse was 5–10 times lower than found in human hearts with end stage dilated cardiomyopathy, this was sufficient alone to produce the cardiomyopathic phenotype (1).

Increasing evidence also supports a critical role for reactive oxygen species in mediating pro-apoptotic signals in cardiomyocytes that result in chamber dilatation and pump dysfunction, for example following transverse aortic constriction (3–6). In cardiomyocytes, apoptosis-inducing factor (AIF), which is a ubiquitous flavoprotein localized in the mitochondrial intermembrane space, provides protection against apoptosis induced by oxidative stress. AIF-deficient mice subjected to aortic banding showed increased cardiomyocyte apoptosis and accelerated progression to decompensated left ventricular function compared with wild-type mice (6).

Apoptosis Repressor with Caspase recruitment domain (ARC) is a ~22-kDa protein expressed at high levels in heart and skeletal muscle (7). ARC has been reported (a) to interact with caspase-8, Fas, and FADD and to inhibit apoptosis induced by caspase-8 and receptor-induced apoptosis by Fas and TNFR1, and (b) to interact with Bax and to inhibit Bax activation and apoptosis involving the mitochondrial pathway (8, 9). ARC overexpression inhibited oxidant stress-induced cell death in H9c2 cells by preserving mitochondrial function (10) and protected against ischemia-reperfusion injury, where perfusion of TAT-ARC into isolated rat hearts reduced both creatine kinase release and infarct size (11). More recently, ARC-deficient mice were found to develop both worsened progression to dilated cardiomyopathy following aortic banding and larger infarcts following ischemia-reperfusion compared with wild-type controls (12). Indeed, tissue from human dilated cardiomyopathic fibroblast; E3, ubiquitin-protein isopeptide ligase.
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hearts showed decreased levels of ARC compared with normal control hearts. Notably, this difference was demonstrated by Western blot, but not by Northern blot, indicating that the reduction of ARC in cardiac dilatation is most likely to be regulated post-transcriptionally (12). Although ARC protein levels crucially determine cardiomyocyte apoptosis, the mechanisms regulating ARC protein levels are not known. Here, we identify that the p53-induced ubiquitin E3 ligase, mouse double minute 2 (MDM2), is responsible for inducing ARC proteasomal-dependent degradation by ubiquitination. ARC protein level is reduced in cardiomyocytes exposed to oxidative stress, while MDM2 is concurrently up-regulated. ARC degradation requires MDM2 and its ring finger domain. The proteasomal inhibitors lactacyctin and MG132 significantly inhibited ARC degradation induced by MDM2 or oxidative stress and inhibited cardiomyocyte apoptosis. Dilated cardiomyopathic hearts from mice that have undergone transverse aortic banding have increased MDM2 levels associated with decreased ARC levels.

EXPERIMENTAL PROCEDURES

Cell Culture, Expression Plasmids, Transient Transfection, and Cell Treatment—H9c2 rat embryonic cardiac cells, NIH3T3 mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and maintained at 60–80% confluence. MDM2/p53 double knock-out MEFs were obtained from Dr. G. Lozano (M. D. Anderson Cancer Center). Wild-type MEFs were MDM2+/+ /p53+/+. The human MDM2 expression plasmid was obtained from Dr. M. Oren (Weizmann Institute of Science), and site-directed mutagenesis (Stratagene) was performed to obtain the MDM2C464A mutant plasmid using the following primer pair, 5′-GGCCCTGCTTTACAGCTGCAAGAAGCTAAGAAAGG-3′ and 5′-GCTTCTTTGACGCTGTAAAGCGGCATTAAAGATGCC-3′. SIAH1 cDNA was cloned onto the pcDNA3.1 expression plasmid (Invitrogen) from a mouse cardiac cDNA preparation. All sequences were verified by DNA sequencing. The ARC expression plasmid (pcDNA3.1/ARC-HA) is as previously described (8). Transfection was performed using Effectene (Qiagen) according to the manufacturer’s protocol. To induce oxidative stress, cells were incubated with 400 μM H2O2 for the indicated time points. Cells were additionally preincubated with lactacyctin for 1 h prior to H2O2 or incubated with lactacyctin 18 h after transient transfection followed by a further 18-h incubation.

Death Assays—For measuring apoptotic index, cells were incubated with the nuclear dyes Hoechst (H22358) and propidium iodide for 15 min, and photomicrographs were taken using fluorescence microscopy. H22358 stains all nuclei, whereas propidium iodide stains only dead/apoptotic nuclei. Apoptotic index was quantified by the ratio of condensed nuclei/total nuclei in at least 10 fields where each field contained at least 50 nuclei. Results were obtained from three independent experiments and shown as mean ± S.D. To demonstrate DNA laddering, cells were lysed in lysis buffer (10 mm Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 100 mM NaCl, 0.5% SDS) and DNA was extracted with phenol-chloroform and electrophoresed in a 1% agarose gel.

Western Blotting, Immunoprecipitation, and Cycloheximide Chase—Cells were scraped into a lysis buffer (50 mm Tris, pH 8.0, 150 mm NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40) and protein concentration determined using the Bradford method. Equal protein amounts were resolved by SDS-PAGE and blotted with antibodies as follows: ARC (Cayman), p53 (1C12; Cell Signaling), phospho-p53(Ser-20) polyclonal (Cell Signaling), MDM2 (Calbiochem), SIAH1 (Santa Cruz), β-actin (Abcam), and ubiquitin (Abcam). Goat anti-rabbit and goat anti-mouse secondary antibodies (Jackson Laboratories) were used. For immunoprecipitation of ARC complexes, cells were incubated with the proteasomal inhibitor MG132 (10 μM) 18 h after transfection and incubated for a further 18 h before being scraped into lysis buffer. Protein concentration was determined, and 500 μg of total protein lysates were incubated overnight at 4 °C with 30 μl of protein A beads (Sigma) and 8 μg of ARC antibody (Cayman). Protein A beads were prewashed twice with lysis buffer. Following incubation, beads were washed three times with phosphate-buffered saline, and proteins were eluted with SDS loading buffer and resolved by SDS-PAGE. Cycloheximide chase was performed by incubating cells with 50 ng/ml cycloheximide for the indicated time points, following which lysates were immediately collected into lysis buffer.

Transverse Aortic Banding—Transverse aortic banding was performed on 3–4-month-old FVB/N mice as previously described (13) according to protocols approved by the Institute for Animal Studies of the Albert Einstein College of Medicine. Left ventricular (LV) dilatation was confirmed by echocardiography and LV weight/body weight measurements 12 weeks following transverse aortic banding (2).

RESULTS

H2O2 Induces Apoptotic Cell Death in H9c2 with Concurrent Decrease of ARC and Up-regulation of MDM2—Previous studies have shown that oxidative stress induced by H2O2 decreased endogenous ARC levels in rat embryonic cardiac H9c2 cells in a concentration-dependent manner (10). We found similarly that H2O2 induced significant apoptotic (50–60%) cell death in H9c2 cells (Figs. 1 and 8) and this was associated with a time-dependent decrease in ARC protein levels (Fig. 1C). Condensed nuclear morphology of apoptotic cells from H2O2 treatment is shown in Fig. 1A, and DNA laddering in similarly treated cells is shown in Fig. 1B. In myocytes, H2O2-induced apoptosis is mediated by the pro-apoptotic transcription factor p53 (14), and in this pathway, activated p53 can be stabilized via a variety of post-transcriptional means, including phosphorylation (15). We found that ARC decrease coincided with p53 phosphorylation, as represented by the appearance of a slower running band when lysates were immunoblotted for total p53 (Fig. 1C). Blotting with a phosphor-specific antibody showed that at least serine 20 phosphorylation is increased (Fig. 1C), but not phosphorylation of serine 15 (data not shown).

Because the decrease of ARC in cardiac disease appears to be post-transcriptionally controlled (12), we examined whether a target gene of the transcriptional factor p53 may be responsible for increased ARC protein degradation. We found that ARC
decrease and p53 phosphorylation coincided with an up-regulation of the MDM2 (Fig. 1C).

**MDM2 Degrades ARC Protein Directly**—MDM2 is a RING finger domain protein with ubiquitin E3 ligase activity. In the ubiquitin proteolysis system, the ubiquitin target protein complex is formed in three sequential steps (16) that comprise the ubiquitin activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin ligase, E3. E1 is a common enzyme involved in ubiquitination of all proteins, but the specificity for the protein that is ubiquitinated is usually dependent on the E3 ligase in the reaction. Further polyubiquitination of the targeted protein often involves other multiprotein complexes, but degradation of the resultant polyubiquitinated protein takes place at the proteasomal complex.

Because we found that the E3 ligase MDM2 is up-regulated concurrently with ARC decrease, we determined whether ARC protein can be directly reduced by MDM2. Overexpression of MDM2 itself led to a decrease in ARC levels (Fig. 2). In contrast, overexpression of another E3 ligase that is also a gene target of p53, SIAH1 (17, 18), does not down-regulate ARC. We further determined the effect of MDM2 on ARC protein turnover by performing cycloheximide chase. As shown in Fig. 3, turnover of endogenous ARC protein was accelerated in MDM2-transfected H9c2 compared with vector control-transfected cells, indicating that MDM2 is able to increase ARC degradation.

**MDM2 Promotes Ubiquitination of ARC in Vivo, and a Functional MDM2 Ring Finger Domain Is Required for ARC Degradation**—Because MDM2 is an ubiquitin E3 ligase and it is able to directly induce ARC degradation, we tested the possibility that MDM2 facilitates ARC ubiquitination. ARC-deficient NIH3T3 mouse fibroblasts were transfected with ARC, with or without MDM2, and polyubiquitinated bands were detected in immunoprecipitated ARC complexes in the presence of MDM2 transfection but not in the presence of vector control transfection (Fig. 4). The MDM2 ring finger domain is responsible for its ubiquitin E3 ligase property, and a C464A mutation is known to disrupt the MDM2 ring finger domain function (19). Expression of mutant MDM2C464A did not induce ARC degradation, indicating that an intact ring finger domain is necessary for MDM2-induced ARC degradation (Fig. 5). This is despite MDM2 accumulation as a result of the ring finger mutation as MDM2 serves to autoubiquitinate and promote its own degradation (20).

**MDM2 Is Required for ARC Degradation**—To examine whether MDM2 is both necessary and sufficient for ARC deg-
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Increased MDM2 Expression Is Associated with Decreased ARC Protein Levels in Dilated Cardiomyopathic Mouse Hearts—Mechanical load is known to activate signaling cascades, including oxidative stress, which in turn predisposes to cardiomyocyte apoptosis (22), and the latter is seen during the transition from hypertrophy to dilated cardiomyopathy (23). In wild-type mice 12 weeks after transverse aortic banding we found a ~1.8-fold decrease in ARC levels on Western blot compared with sham-operated mice, and this decrease in ARC protein levels was associated with a marked increase in MDM2 expression (Fig. 8).

DISCUSSION

We demonstrate that the ubiquitin E3 ligase MDM2 is a critical regulator of ARC protein level. Oxidative stress induced by H$_2$O$_2$ resulted in cardiomyocyte apoptosis and degradation of ARC. Concurrently, p53 is activated and MDM2 levels are increased. Interestingly, in this in vitro model of H$_2$O$_2$-induced H9c2 apoptosis increased phosphorylation of p53-Ser-20 but not p53-Ser-15 is observed. It has been previously shown that Chk2 can phosphorylate Ser-20 (24) and that phosphorylation at this site enhances p53 stability and activity. In fact, phosphorylation of Ser-20 inhibits MDM2-mediated degradation of p53 (25–27), whereas this does not apply to phosphorylation of other N-terminal serine residues such as Ser-15 (28). Nonetheless, we have found that up-regulated MDM2 is able to directly induce ARC decrease mediated at least in part through increased ARC protein turnover. Whether MDM2 also induces decrease in transcription or translation of ARC is not yet known, but the former remains possible because MDM2 is also able to induce degradation of p53 (29, 30) and as a transcription factor p53 may be involved in positive feedback to up-regulate ARC protein transcription. Whether MDM2 also induces decrease in transcription or translation of ARC is not yet known, but the former remains possible because MDM2 is also able to induce degradation of p53 (29, 30) and as a transcription factor p53 may be involved in positive feedback to up-regulate ARC protein transcription. MDM2 induces ubiquitination of ARC, and MDM2-induced degradation of ARC requires a functional MDM2 ring finger domain, indicating that the E3 ligase activity is required for ARC degradation. MDM2 is both necessary and sufficient for ARC protein degradation, because ARC protein levels do not change in MDM2/p53 double knock-out MEFs and re-introduction of MDM2 in MDM2/p53 double knock-out MEFs restores ARC protein degradation.

The relationship between MDM2 and cardiomyocyte apoptosis was also recently investigated by Toth et al. (31). These

| Transfect: | Vector control | MDM2 |
|---|---|---|
| Chx 50 ng/mL(h): | 0 | 12 | 24 | 48 | 0 | 12 | 24 | 48 |

**FIGURE 3.** MDM2 up-regulates protein turnover of ARC. H9c2 cells were transfected with either vector control (pcDNA) or a vector expressing MDM2. Twelve hours following transfection, cyclohexamide chase was performed by incubating cells with cyclohexamide and harvested after the indicated times. Western blot analysis was performed using anti-ARC polyclonal antibody. Western blot was quantified and densitometry was normalized to β-actin for each time point and expressed as a percentage of the densitometry at time 0 h. Results represent average ± S.D. (n = 3).

radation, we examined ARC protein turnover in cells lacking MDM2. The MDM2 knock-out mouse is embryonic lethal, and MDM2$^{-/-}$ MEFs do not exist (21). We therefore expressed ARC in MDM2/p53 double knock-out or wild-type (MDM2$^{+/+}$/p53$^{+/+}$) MEFs and incubated cells with the translation inhibitor cycloheximide. ARC levels were decreased with cycloheximide in wild-type MEFs but maintained in double knock-out MEFs. Re-introduction of MDM2 to double knock-out MEFs by transient transfection restored ARC protein turnover (Fig. 6).

Proteasomal Inhibitors Rescue Both MDM2-induced and H$_2$O$_2$-induced ARC Degradation and Inhibit H$_2$O$_2$-induced Cardiomyocyte Apoptosis—Because most polyubiquitinated proteins are targeted for degradation at the proteasomal complex, we examined the effect of the proteasomal inhibitor lactacystin on ARC levels. Lactacystin rescued MDM2-induced (Fig. 5, lane 4) and H$_2$O$_2$-induced ARC degradation (Fig. 7A). Furthermore, lactacystin inhibited H$_2$O$_2$-induced cardiomyocyte apoptosis as measured by apoptotic index (Fig. 7B). Similar results were found for another proteasomal inhibitor, MG132 (not shown).
workers showed that cardiomyocytes overexpressing MDM2 acquired resistance to hypoxia/reoxygenation (H/R)-induced cell death, whereas with a specific MDM2 peptide inhibitor (PNC-28) that disrupts the p53-MDM2 interface H/R resulted in higher p53 levels and promoted apoptosis. Although this report from Toth et al. appears to contradict our current findings, we have in fact shown here that this system is perhaps more complex. Like Toth et al., we found that oxidative stress induces cardiomyocyte death accompanied by p53 activation. Because MDM2 is a critical regulator of p53 and is responsible for ubiquitination and degradation of p53 (29, 30), Toth et al. found that MDM2 overexpression results in p53 down-regulation and cardioprotection. We report here that in addition to

|          | WT MEFs | MDM2−/p53−/ MEFs |
|----------|---------|------------------|
| Transfect: |         |                  |
| MDM2 (4.5 μg) | -      | -                |
| pcDNA3.1    | 5       | 4.5              |
| Chx (50 ng/mL) | -      | +                |

**FIGURE 4. MDM2 up-regulates ubiquitination of ARC.** ARC-deficient NIH3T3 mouse fibroblasts were transfected with plasmids as indicated and incubated with MG132 for a further 18 h following transfection. Equal quantities of lysates were immunoprecipitated with anti-ARC antibody and immunoblotted using anti-ubiquitin antibody. Input lysates were immunoblotted for ARC and MDM2 to confirm transfection.

**FIGURE 5. Degradation of ARC requires a functioning MDM2 ring finger domain, and MDM2-induced ARC degradation is inhibited by lactacystin.** H9c2 cells were transfected, as in Fig. 2, with either vector control (pcDNA) or vectors transducing the expression of MDM2 or MDM2C464A (MDM2 ring finger mutant). Additionally, lane 4 represents cells that were transfected with MDM2 plasmid and incubated with lactacystin for a further 18 h following transfection.

**FIGURE 6. ARC is stable in MDM2-deficient mouse embryonic fibroblasts, and this is reversed by the re-introduction of MDM2.** Wild-type and MDM2−/p53−/ double knock-out mouse embryonic fibroblasts were transfected with plasmids expressing ARC and incubated with or without cyclohexamide for a further 12 h after transfection. Additionally, lane 5 represents cells that were doubly transfected with ARC expression plasmid and MDM2 expression plasmid prior to incubation with cyclohexamide.

**FIGURE 7. Proteasomal inhibitor lactacystin blocks H2O2-induced degradation of ARC and rescues H2O2-induced apoptosis.** A, H9c2 cells were incubated with 100 μM H2O2 for 12 h with or without lactacystin. Cell lysates were analyzed by immunoblotting using anti-ARC and anti-MDM2 antibodies. Asterisk (*) represents a nonspecific band that demonstrates equal protein loading. B, H9c2 cells were incubated with 100 μM H2O2 for 12 h with or without lactacystin, and apoptotic index was determined as the ratio of condensed nuclei to total nuclei in at least 10 fields where each field contained at least 50 nuclei. Results represent the mean ± S.D. from three independent experiments. †, H2O2 treated versus H2O2 + lactacystin treated; p < 0.05.
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down-regulating the pro-apoptotic protein p53, MDM2 also down-regulates the anti-apoptotic protein ARC. Because p53 signals are proximal to the protective mechanisms of ARC (10), overexpression of MDM2 would be expected to confer protection via degradation of p53 even if simultaneously ARC is degraded by MDM2. Interestingly, the MDM2 peptide inhibitor (PNC-28) used by Toth et al., which disrupts the p53/MDM2 interface so that p53 accumulates and is rendered untargeted by MDM2, does not inhibit MDM2-induced ARC degradation (not shown). The latter suggests that the ARC/MDM2 interface is distinct from the p53/MDM2 interface and that ARC degradation despite PNC-28 could contribute in part to the increased cell death that is observed with PNC-28 treatment. Toth et al. also used a recently generated mouse model with 70% knock down of MDM2 and found that these mice responded more severely to ischemia-reperfusion than wild type. It is unknown ifARC levels are sustained at this level of MDM2 knock down, but it would appear that at this level, p53 pro-death signals exceed the protection provided by ARC.

In conclusion, MDM2-mediated degradation of ARC is a potential therapeutic target to prevent apoptosis in cardiac disease. Cardiomyocyte apoptosis occurs in human cardiomyopathy, and in the mouse myocyte apoptosis can directly cause cardiac dilatation. An anti-apoptotic protein such as ARC that is only abundantly expressed in the heart has been proposed as a suitable target for cardiac-specific therapy (1, 32). Although we have shown that inhibition of the proapoptotic complex in vitro is able to maintain ARC levels and inhibit cardiomyocyte apoptosis, it remains to be seen whether this result will translate to physiological benefits such as smaller infarcts following ischemia-reperfusion or retarded progression to dilated cardiomyopathy in vivo. Interestingly, 12-week systemic administration of proapoptotic inhibitors to spontaneously hypertensive rats successfully suppressed myocardial expression of matrix metalloproteinases and collagens and significantly reduced cardiac fibrosis (33). In that study no change in heart weight/body weight ratio was detected, but cardiac chamber dimensions were not measured. Indeed, a concurrent benefit from inhibition of cardiomyocyte apoptosis in this or another cardiomyopathic model would be worth investigating. Longer term treatment to inhibit ARC degradation may require more ARC-specific inhibitors of degradation. Furthermore, our observations with the MDM2 inhibitor (PNC-28) that specifically targets the p53/MDM2 interface, but apparently not the ARC/MDM2 interface, suggest that further investigation to map the domains on ARC and MDM2 that are required for MDM2-mediated ARCDegradation may help to identify additional small peptide MDM2 inhibitors. Indeed, there may be an opportunity to use such a peptide inhibitor to switch the regulatory control of apoptosis so that MDM2 predominantly degrades p53 (pro-apoptotic), but not ARC (anti-apoptotic), in a situation such as cardiac disease where down-regulation of myocyte apoptosis is the objective.

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