Reduced Apaf-1 levels in cardiomyocytes engage strict regulation of apoptosis by endogenous XIAP

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Overexpression studies have identified X-linked inhibitor of apoptosis protein (XIAP) as a potent inhibitor of caspases. However, the exact function of endogenous XIAP in regulating mammalian apoptosis is less clear. Endogenous XIAP strictly regulates cytochrome *c*–dependent caspase activation in sympathetic neurons but not in many mitotic cells. We report that postmitotic cardiomyocytes, unlike fibroblasts, are remarkably resistant to cytosolic microinjection of cytochrome *c*.

The cardiomyocyte resistance to cytochrome *c* is mediated by endogenous XIAP, as XIAP-deficient cardiomyocytes die rapidly with cytosolic cytochrome *c* alone. Importantly, we found that cardiomyocytes, like neurons, have markedly reduced Apaf-1 levels and that this decrease in Apaf-1 is directly linked to the tight regulation of caspase activation by XIAP. These data identify an important function of XIAP in cardiomyocytes and point to a striking similarity in the regulation of apoptosis in postmitotic cells.

Introduction

Although studies in cell lines have identified the main components of the mammalian apoptotic pathway, the differences in the regulation of apoptosis in various primary cells remain largely unexplored. The critical executors of apoptosis are the caspase proteases (Fuentes-Prior and Salvesen, 2004). A major mechanism by which caspases become activated during apoptosis in mammalian cells involves the convergence of signaling pathways at the mitochondria to induce the release of cytochrome *c*. Once in the cytosol, cytochrome *c* binds to Apaf-1 to induce its oligomerization and recruit procaspase-9 to form the apoptosome complex. This results in the activation of caspase-9, which then activates caspase-3, which in turn cleaves various cellular proteins to induce rapid cell death (Hengartner, 2000; Wang, 2001).

The cytochrome *c*–dependent caspase activation pathway can be regulated by the inhibitor of apoptosis family of proteins (IAP; Salvesen and Duckett, 2002). These proteins, which include X-linked IAP (XIAP), cellular IAP (cIAP) 1 and 2, and neuronal apoptosis inhibitory protein, can block apoptosis by directly binding to activated caspases. Addition of purified IAPs blocks cytochrome *c*–mediated caspase activation in vitro, and overexpression of IAPs inhibits caspase activation

and apoptosis in a variety of cells (Deveraux and Reed, 1999). However, endogenous IAPs appear to be far more restricted in their ability to regulate apoptosis after cytochrome *c* in mammalian cells. In many mitotic cells, such as HeLa cells, human embryonic kidney 293 cells, and primary fibroblasts, cytosolic accumulation of cytochrome *c* alone is sufficient to induce rapid caspase activation and apoptosis. In these cells, addition of cytochrome *c* to cytosolic lysates triggers robust caspase activation in vitro (Liu et al., 1996; Kluck et al., 1997; Hu et al., 1999), and microinjection of cytochrome *c* is sufficient to induce rapid cell death in intact cells (Li et al., 1997; Brustugun et al., 1998; Juin et al., 1999; Chang et al., 2000). These results argue against a strict postcytochrome *c* regulation of apoptosis by IAPs in mitotic cells. In contrast, neonatal sympathetic neurons are remarkably resistant to cytosolic microinjection of cytochrome *c* (Deshmukh and Johnson, 1998; Neame et al., 1998). In these neurons, the resistance to cytochrome *c* is attributable to strict control of caspase activation by endogenous XIAP. Unlike wild-type neurons, XIAP-deficient sympathetic neurons die rapidly with cytosolic cytochrome *c* alone (Potts et al., 2003).

The selective ability of endogenous XIAP to strictly regulate apoptosis after cytochrome *c* in sympathetic neurons but not in many mitotic cells is not attributable to differences in XIAP expression levels. Instead, as we recently reported, neuronal differentiation is accompanied by a marked reduction in Apaf-1 levels, resulting in a significant decrease in apoptosome activity (Wright et al., 2004). Importantly, this decrease in

Apaf-1 is directly linked to the increased ability of endogenous XIAP to inhibit apoptosis in neurons. Restoring Apaf-1 levels is sufficient to eliminate XIAP's strict regulation of caspases and permit cytochrome *c* alone to induce rapid apoptosis in sympathetic neurons (Wright et al., 2004). Thus, the effectiveness of XIAP in regulating apoptosis in mammalian cells can be set simply by modulating Apaf-1 activity, without the need to directly affect XIAP expression or function.

An increased control of apoptosis is advantageous for neurons, as they are postmitotic and last for the life of organisms. For example, the strict regulation of caspase activation by XIAP may protect neurons from accidentally undergoing apoptosis if cytochrome *c* is released from damaged mitochondria. Arguably, such an increased regulation of apoptosis could be evolutionarily beneficial for other terminally differentiated cells that survive the life of organisms as well.

To test this hypothesis, we examined whether cytochrome *c*–mediated caspase activation is strictly regulated in primary cardiomyocytes. Cardiomyocytes, like neurons, are postmitotic cells with limited regenerative potential that last for the life of organisms (Anversa et al., 2002). Consequently, apoptotic death of cardiomyocytes is a significant factor that contributes to heart failure (Gill et al., 2002; Clerk et al., 2003; Foo et al., 2005; Garg et al., 2005). Our results show that because of low Apaf-1 levels, cytochrome *c*–dependent caspase activation is strictly regulated by endogenous XIAP in cardiomyocytes, just as in sympathetic neurons. The selective ability of endogenous XIAP to tightly regulate caspase activation in cardiomyocytes and neurons points to a common mechanism that ensures the long-term survival of postmitotic cells.

Results and discussion

Cardiomyocytes are resistant to cytosolic microinjection of cytochrome *c*

Sympathetic neurons, unlike many mitotic cells, are remarkably resistant to cytosolic cytochrome *c*. To determine whether terminally differentiated cardiomyocytes display the same resistance, we microinjected primary mouse neonatal cardiomyocytes with cytochrome *c*. As a control, we also microinjected fibroblasts isolated from the same animals. Bovine cytosolic cytochrome *c* alone was sufficient to induce rapid cell death in fibroblasts, with 90% of fibroblasts dying within 30 min of the injections (Fig. 1). In contrast, bovine cytochrome *c* alone was incapable of inducing cell death in cardiomyocytes, as >90% of cardiomyocytes remained viable even 10 h after the cytochrome *c* injections (Fig. 1). Control injections with yeast cytochrome *c*, which is incapable of activating caspases (Ellerby et al., 1997), did not induce death in either fibroblasts or cardiomyocytes. Thus, neonatal cardiomyocytes, just like sympathetic neurons, were markedly resistant to cytosolic injection of cytochrome *c*.

Western analysis indicated that the neonatal cardiomyocytes expressed Apaf-1, caspase-9, and caspase-3 (Fig. 2 A). This result argues against the possibility that cytochrome *c* was unable to induce death in cardiomyocytes because of a defi-
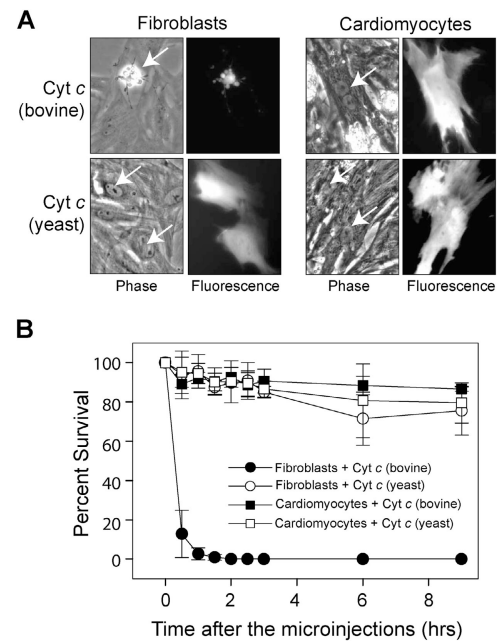

A
Fibroblasts   Cardiomyocytes
Cyt *c* (bovine)
Cyt *c* (yeast)
Phase   Fluorescence   Phase   Fluorescence

B

Figure 1.   **Cytosolic microinjection of cytochrome *c* induces death in fibroblasts but not cardiomyocytes.** Neonatal rat cardiomyocytes and dermal fibroblasts were microinjected with 25 µg/µl of bovine or yeast cytochrome *c*, and cell survival (using morphological criteria) was assessed at multiple times after the injections. (A) Phase-contrast and fluorescence photographs of the cells 3 h after the injections of cytochrome *c*. The injected cells (arrows) were identified by the presence of rhodamine dextran coinjected with the cytochrome *c*. (B) Quantitation of cell survival. Data shown are the mean ± SEM of three independent experiments.

ciency of the core components of the apoptotic machinery. In sympathetic neurons, cytochrome *c*–induced caspase activation is tightly regulated by endogenous IAPs. Addition of exogenous Smac, which inhibits IAPs, overcomes the resistance to cytochrome *c* and permits apoptosis to occur in neurons (Deshmukh et al., 2002; Potts et al., 2003). To examine whether the cardiomyocyte resistance to cytochrome *c* was also attributable to a stringent regulation by endogenous IAPs, we examined whether injection of excess Smac permitted cytochrome *c* to activate apoptosis in cardiomyocytes. Coinjection of cytochrome *c* and mature Smac, but neither alone, was remarkably effective in inducing death in cardiomyocytes (Fig. 2 B). To confirm that exogenous Smac permitted cytochrome *c* to induce apoptosis by inhibiting IAPs in cardiomyocytes, we examined whether a mutant Smac protein that cannot inhibit IAPs (Chai et al., 2000) was capable of permitting cytochrome *c* to induce death. We found that unlike wild-type Ala-Val-Pro-Ile (AVPI)–Smac, the mutant Met-Val-Pro-Ile (MVPI)–Smac was incapable of cooperating with cytochrome *c* to induce death in cardiomyocytes (Fig. 2 C). We also confirmed that the death induced by injection of cytochrome *c* and AVPI-Smac occurred as a consequence of caspase activation. Addition of the pan caspase inhibitor zVAD-FMK completely blocked the cardiomyocyte death induced by cytochrome *c* and Smac (Fig. 2 C). Together, these results show that the components of the apoptosome are present and functional in cardiomyocytes but only become permissive for apoptosis if the activity of IAPs is blocked.
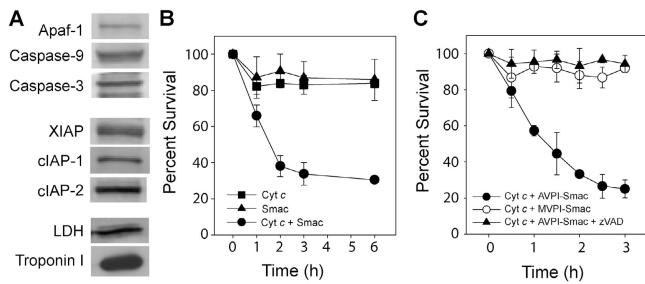
Figure 2. **Cardiomyocyte resistance to cytochrome c can be overcome with the exogenous addition of the IAP inhibitor Smac.** (A) Western blots showing that the rat cardiomyocyte cultures express the core apoptotic components (Apaf-1, caspase-9, and caspase-3) and various IAPs (XIAP, cIAP-1, and cIAP-2). LDH and Troponin I are shown as controls. (B) Rat cardiomyocytes were microinjected with 25 μg/μl of bovine cytochrome c, 1 μg/μl of wild-type mature Smac, or both, and cell survival was assessed at multiple times after the injections. (C) Rat cardiomyocytes were injected with 25 μg/μl of bovine cytochrome c and 1 μg/μl of either mature wild-type AVPI-Smac or mature mutant MVPI-Smac in the presence or absence of 50 μM of the pan caspase inhibitor zVAD-FMK. Cell survival was assessed at multiple times after the injections. Data shown are the mean $\pm$ SEM of three independent experiments.

Strict regulation of cytochrome c–induced apoptosis in cardiomyocytes by endogenous XIAP

Western analysis indicated that cardiomyocytes express multiple IAPs, including XIAP, c-IAP-1, and c-IAP-2 (Fig. 2 A). Despite the expression of multiple IAPs in sympathetic neurons, the removal of XIAP alone is sufficient to permit cytochrome c–dependent caspase activation in neurons (Potts et al., 2003). To determine whether endogenous XIAP is an important regulator of caspase activation in cardiomyocytes, we examined the ability of cytochrome c to induce death in cardiomyocytes isolated from XIAP-deficient mice (Harlin et al., 2001). In contrast to wild-type cardiomyocytes that are resistant to cytochrome c, the XIAP-deficient cardiomyocytes were strikingly permissive and died with cytosolic microinjection of cytochrome c alone (Fig. 3 A). 50% of XIAP-deficient cardiomyocytes were dead within 2 h of cytochrome c injections, and <15% remained alive 9 h after the injections. In contrast, 80% of wild-type cardiomyocytes remained viable even 9 h after cytochrome c injections (Fig. 3 B). The XIAP-deficient cardiomyocytes were not generally susceptible to microinjection per se, as control injections with yeast cytochrome c did not induce death in these cells.

Thus, despite the rather ubiquitous expression of XIAP in mammalian cells, these results identify cardiomyocytes as only the second mammalian cell type in which endogenous XIAP exerts a strict control over cytochrome c–dependent apoptosis. However, we cannot exclude the possibility that XIAP deficiency may simply lower the overall threshold of IAPs below a level that allows cytochrome c to activate caspases in these cells.

Markedly reduced Apaf-1 levels set the threshold for stringent regulation of apoptosis by XIAP in cardiomyocytes

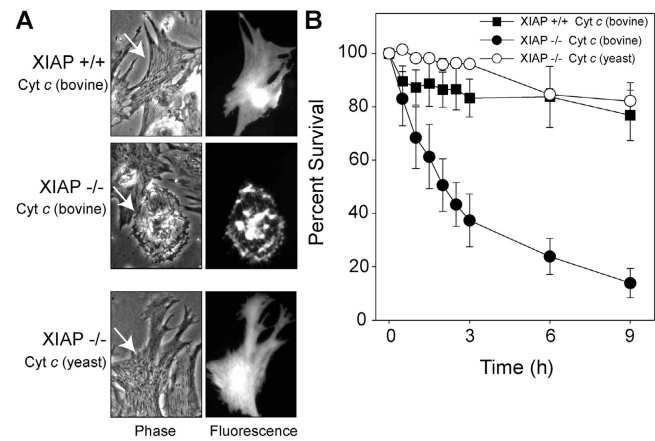We recently reported that the effectiveness of endogenous XIAP to regulate apoptosis is inversely coupled to the Apaf-1–

Figure 3. **XIAP-deficient cardiomyocytes die with injection of cytochrome c.** Cardiomyocytes isolated from XIAP-deficient ($-/-$) or wild-type ($+/+$) littermate mice were microinjected with 25 μg/μl of bovine cytochrome c. As a control, XIAP-deficient cardiomyocytes were also injected with 25 μg/μl of yeast cytochrome c. (A) Phase-contrast and fluorescence photographs of representative cells. Arrows mark the injected cells, identified by coinjection of rhodamine dextran along with cytochrome c. (B) Quantitation of cell survival at multiple times after the injections. Data shown are the mean $\pm$ SEM of three independent experiments.

dependent apoptosome activity in cells (Wright et al., 2004). To determine whether the strict control of cytochrome c–mediated death by XIAP in cardiomyocytes is regulated by a similar mechanism, we examined the levels of Apaf-1. As a comparison, we also examined Apaf-1 levels in dermal fibroblasts. Unlike cardiomyocytes, fibroblasts show no strict regulation by endogenous XIAP and die with injection of cytochrome c alone (Fig. 1). We found that the level of Apaf-1 protein in cardiomyocytes was strikingly reduced to <25% of the Apaf-1 levels found in fibroblasts (Fig. 4). This decrease in Apaf-1 was not an artifact of maintaining cardiomyocytes in culture for 6–8 d, as similar low levels of Apaf-1 were seen in cardiomyocytes immediately after their isolation (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200504082/DC1).

We examined whether this marked difference in Apaf-1 seen between cardiomyocytes and fibroblasts was reflective of a general decrease in the apoptosome components in cardiomyocytes. However, we found no differences in caspase-9 levels between cardiomyocytes and fibroblasts. Because E2F1 has been identified as a transcriptional regulator of Apaf-1 (Moroni et al., 2001), the reduced activity of E2F1 in terminally differentiated cardiomyocytes (Flink et al., 1998) is likely to contribute to the marked reduction in Apaf-1 levels in these cells.

To determine whether the low Apaf-1 expression in cardiomyocytes was indeed responsible for the strict regulation of caspase activation by XIAP, we examined whether elevating the levels of Apaf-1 in these cells rendered XIAP ineffective. Cardiomyocytes were transfected with plasmids expressing GFP and either Apaf-1 or vector alone. After 24 h to allow for expression, the transfected cardiomyocytes (GFP positive) were injected with cytochrome c to examine the ability of cytosolic cytochrome c to induce apoptosis. Increasing Apaf-1 levels was highly effective in permitting cytochrome c alone
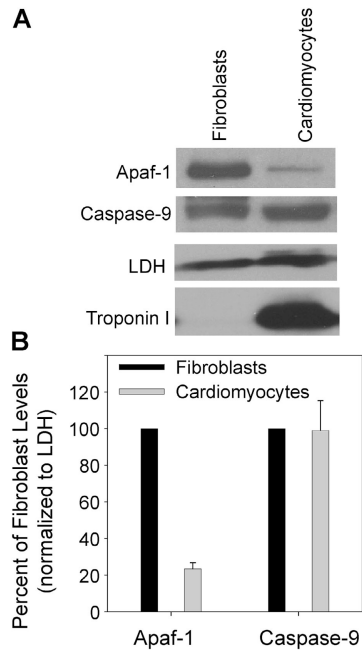
Figure 4. Apaf-1 but not caspase-9 levels are markedly reduced in cardiomyocytes in comparison to fibroblasts. (A) Western blots showing levels of Apaf-1 and caspase-9 proteins (and LDH and Troponin I as controls) in cultures of rat dermal fibroblasts and cardiomyocytes. (B) Quantitation of the data in which Apaf-1 and caspase-9 protein levels detected in cardiomyocyte cultures are expressed as a percentage of the levels (normalized to LDH) seen in fibroblast cultures. Data shown are the mean ± SEM of three independent experiments.

Figure 5. Restoring Apaf-1 levels eliminates the strict control of XIAP and permits cytochrome *c* to induce apoptosis in cardiomyocytes. Rat cardiomyocytes were transfected with plasmids expressing GFP alone (Vector/GFP), Apaf-1 and GFP (Apaf-1/GFP), or procaspase-9 and GFP (caspase-9/GFP). After 24 h, the transfected cells (identifiable by GFP expression) were microinjected with 25 μg/μl of bovine or yeast cytochrome *c* and rhodamine dextran (Cyt *c*/Rhod). (A) Fluorescence photographs (for the GFP- or Rhodamine-selective channels) of representative cells taken 2 h after the cytochrome *c* injections. Arrows point to the GFP-positive cells that were injected with cytochrome *c* and rhodamine. (B) Quantitation of cell survival (2 h after the injections) for the various conditions. Data shown are the mean ± SEM of three independent experiments.

to induce cell death in cardiomyocytes (Fig. 5). Apaf-1 overexpression alone did not affect survival, as >90% of Apaf-1–expressing cardiomyocytes remained alive when injected with the control, yeast cytochrome *c*. Also, this effect was selective for Apaf-1, as overexpression of procaspase-9, the other apoptosome component, did not overcome XIAP inhibition (Fig. 5). Thus, the ability of endogenous XIAP to strictly regulate apoptosis in cardiomyocytes was coupled to the reduced Apaf-1 levels.

Our observation that cytosolic cytochrome *c* was insufficient to induce apoptosis in cardiomyocytes is consistent with studies showing substantial translocation of cytochrome *c* to the cytosol without much detectable apoptosis in human cardiomyopathic hearts (Narula et al., 1999; Scheubel et al., 2002). Likewise, a recent study found staurosporine treatment capable of inducing cytochrome *c* release but not apoptosis in cardiomyocytes (Sanchis et al., 2003). However, this study found no detectable Apaf-1 expression in cardiomyocytes and concluded that the cardiomyocyte resistance to cytochrome *c* occurred because these cells lacked Apaf-1 (Sanchis et al., 2003). In contrast, we report here that cardiomyocytes express low levels of Apaf-1, and a consequence of this low Apaf-1 activity is the engagement of a strict control of caspase activation by endogenous XIAP. Using single cell microinjections of cardiomyocytes from wild-type and XIAP-deficient mice, we show that cytosolic cytochrome *c* is fully capable of inducing apoptosis in cardiomyocytes, provided XIAP's inhibition of caspases is relieved.
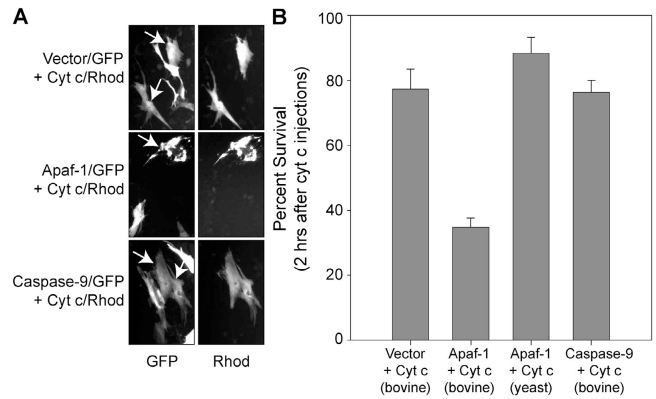
The overall similarities in the mechanisms governing the strict postcytochrome *c* regulation of apoptosis in cardiomyocytes and sympathetic neurons are striking (Potts et al., 2003; Wright et al., 2004). In both of these postmitotic cell types, but not in mitotic fibroblasts, endogenous XIAP is highly effective in controlling cytochrome *c*–dependent caspase activation. In both, this occurs as a consequence of markedly reduced Apaf-1 expression. Although cardiomyocytes and sympathetic neurons have very different physiological and morphological properties, both cell types have limited regenerative potential and require long-term survival. Apaf-1 expression is reduced even further in adult, as compared with neonatal, heart (Fig. S1 B) and brain tissues (Yakovlev et al., 2001). Low Apaf-1 expression is also seen in other tissues, such as liver and skeletal muscle, that contain a substantial number of postmitotic cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200504082/DC1; Burgess et al., 1999). Together, our data indicate that terminal differentiation triggers a marked reduction in Apaf-1 levels and the consequent engagement of a highly effective XIAP-mediated safety brake to ensure the long-term survival of postmitotic cells.

Materials and methods

Reagents
All reagents were purchased from Sigma-Aldrich or Fisher Scientific, unless otherwise stated. The Apaf-1 cDNA was a gift from G. Nuñez (University of Michigan, Ann Arbor, MI), and the caspase-9 cDNA was a gift from C. Du (Stowers Institute, Kansas City, MO). Recombinant AVPI- and MVPI-Smac proteins were generated as previously described (Potts et al., 2003).

Primary cardiomyocyte and fibroblast cultures
Primary cardiomyocyte cultures were isolated from postnatal day 0–1 rats or mice by using the Worthington neonatal cardiomyocyte isolation system (Worthington Biochemical Corp.) following the manufacturer's instructions. A preplating step (for 2 h) was included to reduce the number of fibroblasts

in the cultures. Cells were then plated on laminin-coated plates in MEM with Earle's salt supplemented with 2 mM glutamine, 10% horse serum, 5% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Experiments were performed on cells 6–8 d after plating.

Primary dermal fibroblasts were isolated from postnatal day 0–1 rats as follows. The dorsal skin was removed and treated with 1 mg/ml collagenase and 2.5 mg/ml trypsin for 1 h each at 37°C. Tissue was then triturated, and the dissociated cells were plated in DME supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged once before experiments.

Microinjections and quantitation of cell survival
Cells were microinjected on 35-mm plates using Femtotip II needles (Eppendorf, Inc.) or needles pulled on a Flaming-Brown horizontal micropipette puller (Sutter Instrument Co.) and a Narashigi micromanipulator mounted on an inverted fluorescence microscope (Leica). Between 50 and 150 cells were injected for each condition in these experiments. The microinjection solution contained 100 mM KCl, 10 mM KP_i, pH 7.4, and 8 mg/ml rhodamine dextran, with 25 μg/μl bovine or yeast cytochrome c and 1 μg/μl AVPI- or MVPI-Smac in some experiments as indicated. After microinjections, viable cells were identified as those rhodamine-positive cells that continued to beat (for cardiomyocytes) and had an intact morphology. The dead cells appeared rounded and often with disintegrated membranes.

Transfections
Neonatal cardiomyocytes were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, cells were switched to antibiotic-free media and transfected with 2.5 μg DNA (0.6 μg EGFP and 1.9 μg pCDNA3 plasmids expressing Apaf-1, procaspase-9, or vector alone).

Western blots
Western blots were performed as described previously (Potts et al., 2003). Primary antibodies used were as follows: anti-XIAP (R&D Systems), anti–cIAP-1 (R&D Systems), anti–cIAP-2 (R&D Systems), anti–caspase-9 (MBL International Corporation), anti–procaspase-3 (Cell Signaling Technology), anti-Apaf-1 (Alexis Biochemicals Corp.), anti–lactate dehydrogenase (LDH; Rockland Immunochemicals), and anti–Troponin I (Chemicon). Anti–mouse/rabbit HRP-conjugated secondary antibodies were purchased from Pierce Chemical Co. Proteins were detected using ECL-Plus detection system. Protein levels were quantified by scanning blots on a Typhoon scanner (GE Healthcare) and analysis with ImageQuant software (GE Healthcare).

XIAP −/− mice
The XIAP-deficient mice were obtained from C. Thompson (University of Pennsylvania, Philadelphia, PA; Harlin et al., 2001). Our procedure for genotyping these mice was described previously (Potts et al., 2003).

Image acquisition and processing
All images were acquired by a digital, black-and-white, charge-coupled device camera (ORCA-ER; Hamamatsu) mounted on an inverted fluorescence microscope (DMIRE 2; Leica). Images were taken with a 40× oil immersion objective. The fluorescence images were taken with a tetramethylrhodamine-isothiocyanate DiL HQ filter set (rhodamine; Chroma Technology Corp.). The image acquisition software was Metamorph 5.0 (Universal Imaging Corp.). Images were scaled down and cropped in Photoshop (Adobe) to prepare the final figures.

Online supplemental material
Fig. S1 A shows that levels of Apaf-1 are not affected by time in culture, and Fig. S1 B shows that Apaf-1 levels are further decreased in adult versus neonatal cardiomyocytes. Fig. S2 shows the expression profile of Apaf-1 in various tissues from adult rat. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200504082/DC1.

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