Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis

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Despite the potential of the inhibitor of apoptosis proteins (IAPs) to block cytochrome *c*–dependent caspase activation, the critical function of IAPs in regulating mammalian apoptosis remains unclear. We report that the ability of endogenous IAPs to effectively regulate caspase activation depends on the differentiation state of the cell. Despite being expressed at equivalent levels, endogenous IAPs afforded no protection against cytochrome *c*–induced apoptosis in naïve pheochromocytoma (PC12) cells, but were remarkably effective in doing so in neuronally differentiated cells. Neuronal differentiation was also accompanied with a marked reduction in Apaf-1, resulting in a significant decrease in apoptosome activity. Importantly, this decrease in Apaf-1 protein was directly linked to the increased ability of IAPs to stringently regulate apoptosis in neuronally differentiated PC12 and primary cells. These data illustrate specifically how the apoptotic pathway acquires increased regulation with cellular differentiation, and are the first to show that IAP function and apoptosome activity are coupled in cells.

Introduction

Activation of the caspase proteases is a crucial event at which cells become committed to die by apoptosis (Denault and Salvesen, 2002). In most mammalian cells, caspases become activated when apoptotic signaling pathways converge at the mitochondria to trigger the release of cytochrome *c*. Cytochrome *c*, Apaf-1, and pro-caspase-9 oligomerize to form the apoptosome complex, resulting in the activation of caspase-9. This results in the activation of caspase-3, inducing rapid apoptosis and cell death (Wang, 2001).

The inhibitor of apoptosis proteins (IAPs) are a family of proteins that can block apoptosis by directly binding to activated caspases (Deveraux and Reed, 1999). Mammalian IAPs include NAIP, cIAP-1, cIAP-2, and XIAP, and are characterized by the presence of 1–3 baculovirus IAP repeat domains (Salvesen and Duckett, 2002). Addition of purified IAPs to cell extracts blocks cytochrome *c*–mediated caspase activation (Deveraux and Reed, 1999). Likewise, overexpression of IAPs inhibits apoptosis after the point of cytochrome *c* release in cells (Duckett et al., 1998; Ekert et al., 2001). IAPs themselves can be inhibited by proteins such as Smac/Diablo and HTRA2/Omi, which translocate from the mitochondria to the cytosol after apoptotic stimuli (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001; Shi, 2002).

Despite their identification and widespread expression, however, the critical function of endogenous IAPs and their inhibitors in regulating apoptosis in mammalian cells remains unclear. Mice carrying deletions in either XIAP or Smac genes exhibit no gross morphological defects (Harlin et al., 2001; Okada et al., 2002). Importantly, in many mitotic cells, such as HeLa cells, HEK 293 cells, and fibroblasts, the endogenously expressed IAPs appear incapable of regulating apoptosis postcytochrome *c*, as cytosolic cytochrome *c* alone is sufficient to activate caspases and induce 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).

In contrast, endogenous IAPs are highly effective in regulating caspase activation in postmitotic sympathetic neurons (postnatal day 5 [P5]), as these neurons are remarkably resistant to cytochrome *c* and undergo apoptosis only when IAP inhibition is relieved (Deshmukh and Johnson, 1998; Neame et al., 1998; Deshmukh et al., 2002). Recently, XIAP was identified as the critical IAP regulating caspase activation in sympathetic neurons. Although cytosolic cytochrome *c* is unable to induce apoptosis in wild-type neurons, it does so rapidly in XIAP-deficient neurons (Potts et al., 2003).

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Abbreviations used in this paper: E16, embryonic day 16; IAP, inhibitor of apoptosis protein; P5, postnatal day 5; PC12, pheochromocytoma.
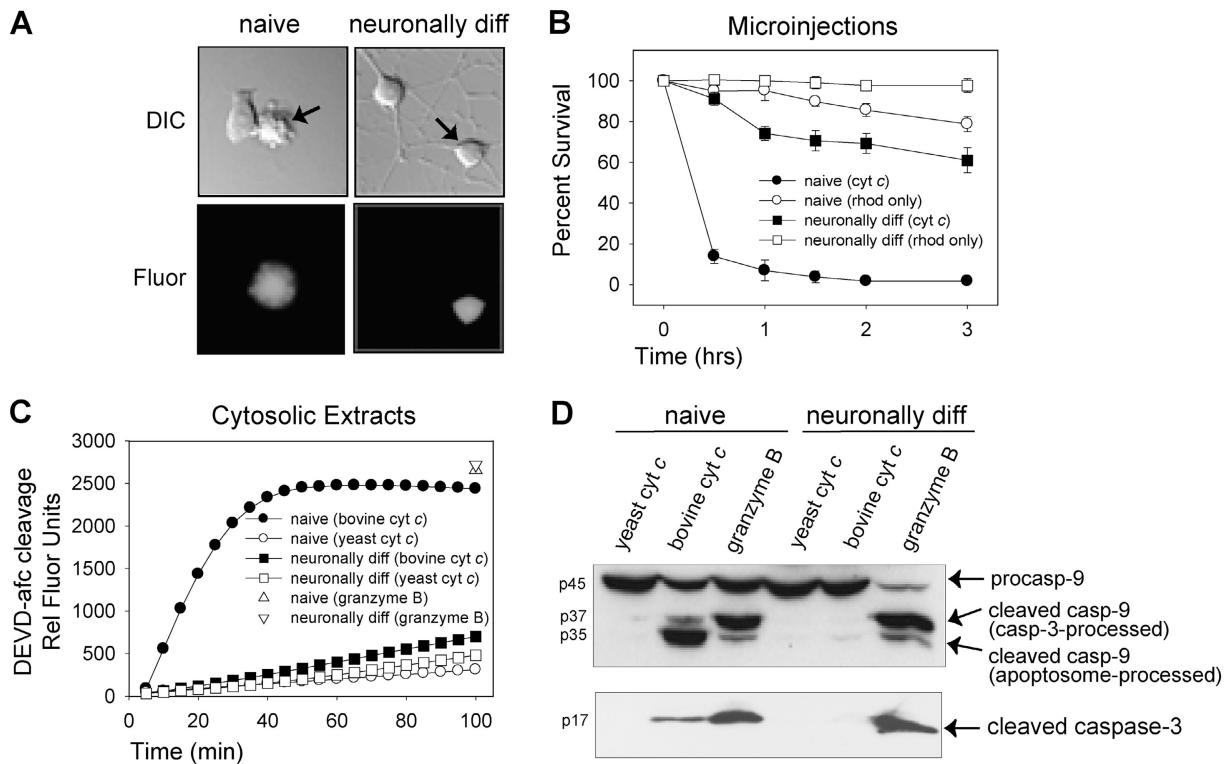
Figure 1. **Addition of exogenous cytochrome *c* induces caspase activation and apoptosis in naïve but not neuronally differentiated PC12 cells.** (A) Naïve and neuronally differentiated PC12 cells were injected with 10–15 mg/ml cytochrome *c* or rhodamine dextran alone. Injected cells (arrows) were identified by the presence of rhodamine dextran coinjected with the cytochrome *c*. (B) Viability of naïve and neuronally differentiated cells at multiple times after the injections. Data shown are mean ± SEM of three independent experiments. (C) Cytosolic lysates from naïve and neuronally differentiated PC12 cells were assessed for caspase activation after the addition of 10 μM cytochrome *c* by measuring cleavage of the fluorogenic caspase substrate DEVD-afc. 10 μM yeast cytochrome *c* or 7 U granzyme B was added to lysates as a negative or positive control, respectively, for caspase activation. (D) Western analysis showing the status of caspase-9 (top) and caspase-3 (bottom) cleavage in cytosolic lysates from naïve and neuronally differentiated cells treated as described in C. Data shown are representative of at least three independent experiments.

The observation that apoptosis is strictly controlled by endogenous IAPs in sympathetic neurons, but not in many mitotic nonneuronal cells, is consistent with the general notion that apoptosis may be under tighter regulation in postmitotic cells such as neurons because these cells do not divide and need to last for the life of the organism. However, this raises two questions. First, is the stringent postcytochrome *c* regulation of apoptosis by IAPs a fundamental mechanism that becomes engaged when cells undergo neuronal differentiation? Second, how is it that the endogenous levels of IAPs are ineffective in regulating apoptosis in many mitotic cells, but become very effective in neurons despite similar levels of expression? Here, we examined these questions in the model of differentiating rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976; Greene, 1978), as well as in primary fibroblasts and neurons.

Results

Cytochrome *c* is sufficient to activate caspases and induce apoptosis in naïve but not in neuronally differentiated PC12 cells

PC12 cells can exist either as mitotic naïve cells, or can be differentiated with NGF treatment into postmitotic, neuronal-like cells (Greene and Tischler, 1976; Greene, 1978). Both naïve

and neuronally differentiated PC12 cells undergo a Bcl-2 inhibitable, caspase-dependent apoptosis in response to a variety of apoptotic stimuli (Mesner et al., 1992; Batistatou et al., 1993; Pittman et al., 1993; Mills et al., 1995; Troy et al., 1996). To determine whether cellular differentiation of mitotic cells into postmitotic neurons induces a change in the postcytochrome *c* regulation of apoptosis, we examined whether cytosolic cytochrome *c* was sufficient to induce apoptosis in naïve or neuronally differentiated PC12 cells. Naïve PC12 cells were differentiated with NGF for 12 d, during which the cells become postmitotic and extend neurites (Fig. 1 A) (Greene and Tischler, 1976; Pittman et al., 1993).

Cytosolic microinjection of cytochrome *c* induced extensive cell death within 30 min in naïve cells. In contrast, upon neuronal differentiation, these cells became remarkably resistant, with 90% of cells viable 30 min after microinjection of cytochrome *c* (Fig. 1, A and B). Even 3 h after the injections, >60% of neuronally differentiated cells remained alive (Fig. 1 B), with viability slowly decreasing at later (12–24 h) time points (unpublished data). This resistance to cytochrome *c* was acquired gradually, with 50% of maximum resistance seen only after 8 d of differentiation with NGF (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200406073/DC1).

We examined whether the differential ability of cytochrome *c* to induce apoptosis in naïve and neuronally differen-

neuronally differentiated

A Cytosolic Extracts

- bovine cyt *c*
- ▲ bovine cyt *c* + AVPI-Smac
- ■ bovine cyt *c* + MVPI-Smac

B Microinjection

naive

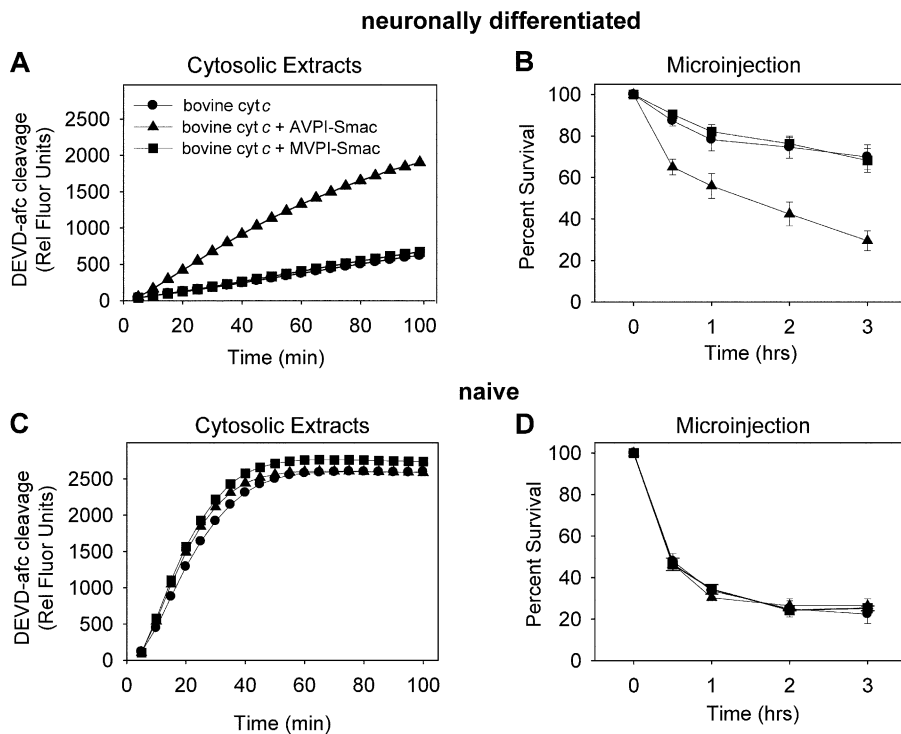**C** Cytosolic Extracts

D Microinjection

Figure 2. **Exogenous addition of Smac that can relieve IAP inhibition permits cytochrome *c* to activate caspases and induce death in neuronally differentiated PC12 cells.** Neuronally differentiated (A and B) or naïve (C and D) PC12 cells were treated with either cytochrome *c* alone, cytochrome *c* and wild-type AVPI-Smac, or cytochrome *c* and mutant MVPI-Smac. These proteins were either added to cytosolic lysates prepared from naïve or neuronally differentiated cells (Cyt *c*, 10 μM; Smac, 1 μM) and assessed for their ability to activate caspases by the DEVD-afc cleavage assay (A and C) or injected into intact naïve or neuronally differentiated cells (Cyt *c*, 10 mg/ml; Smac, 1 mg/ml) and assessed for their ability to induce cell death (B and D). Data shown are mean ± SEM (B and D) or representative (A and C) of at least three independent experiments.

tiated PC12 cells was also seen in cell-free extracts. Although addition of cytochrome *c* was sufficient to induce robust caspase activation in cytosolic lysates of naïve cells, no significant caspase activation was detected in cytosolic lysates of neuronally differentiated cells (Fig. 1 C). Control yeast cytochrome *c* protein, which is unable to bind Apaf-1 (Ellerby et al., 1997; Kluck et al., 2000), did not activate caspases in either naïve or neuronally differentiated cells. We examined whether the resistance to cytochrome *c* in neuronally differentiated cells was because of a deficit in procaspase-3. Levels of procaspase-3 were in fact found to be slightly higher in neuronally differentiated cells compared with naïve cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200406073/DC1) (Rong et al., 1999). Importantly, addition of granzyme B, which can directly activate caspase-3 (Trapani and Sutton, 2003), induced robust caspase activation in lysate from both naïve and neuronally differentiated PC12 cells (Fig. 1 C). Thus, the inability of cytochrome *c* to activate caspases in neuronally differentiated cells was not because of a defect in caspase-3.

Apoptosome-mediated cleavage of caspase-9 generates a 35-kD and a 12-kD fragment (Liu et al., 1996; Cain et al., 2002). Activated caspase-9 then cleaves and activates caspase-3, which can also feedback on unprocessed procaspase-9 to cleave it into a 37-kD and a 10-kD fragment (Srinivasula et al., 1998; Zou et al., 2003). In naïve cell lysates, the addition of bovine cytochrome *c*, but not yeast cytochrome *c*, induced significant processing of caspase-9 and caspase-3 (Fig. 1 D). In contrast, in neuronally differentiated PC12 cell lysates, consistent with the inability of cytochrome *c* to induce apoptosis, no significant processing of caspase-9 or caspase-3 was detected. Addition of granzyme B induced robust and equivalent processing of caspase-9 and caspase-3 in both naïve and neuronally differentiated PC12 cells (Fig. 1 D). Together, these results show

that although cytosolic cytochrome *c* was sufficient to activate caspases and induce death in naïve cells, upon postmitotic differentiation these cells became markedly resistant as shown in both intact cells and in cell-free extracts.

Resistance to cytochrome *c*–induced apoptosis in neuronally differentiated cells is mediated by endogenous IAPs

In primary sympathetic neurons, the resistance to cytochrome *c*–induced apoptosis is mediated by endogenous IAPs (Deshmukh et al., 2002; Potts et al., 2003). To determine whether a similar IAP-mediated regulation was promoting resistance to cytochrome *c* in neuronally differentiated PC12 cells, we examined whether addition of Smac, an IAP inhibitor, could relieve the inhibition and permit cytochrome *c* to activate caspases in these cells. Addition of wild-type mature Smac (AVPI-Smac) and cytochrome *c* together resulted in increased caspase activation in cytosolic extracts from neuronally differentiated cells that are otherwise resistant to addition of cytochrome *c* or AVPI-Smac alone (Fig. 2 A; unpublished data). The concentration of Smac used in these experiments was maximal, as a 10-fold increase in Smac did not result in any further increase in caspase activation in cell extracts. Importantly, unlike wild-type AVPI-Smac, mutant MVPI-Smac, which is unable to bind to IAPs and inhibit their function (Chai et al., 2000), did not enhance cytochrome *c*–mediated caspase activation in these cell extracts (Fig. 2 A).

We also examined whether excess Smac could allow cytochrome *c* to induce death in intact neuronally differentiated cells. Coinjection of cytochrome *c* with wild-type AVPI-Smac induced significantly greater death in neuronally differentiated cells than seen with injection of cytochrome *c* alone or mutant MVPI-Smac and cytochrome *c* together (Fig. 2 B). These results show that en-

Figure 3. **Naïve and neuronally differentiated cells express similar levels of IAPs.** (A) Protein levels of XIAP, cIAP-1, cIAP-2, NAIP, and lactate dehydrogenase (LDH) were examined in naïve and neuronally differentiated PC12 cells (equal total protein loaded) by Western analysis. (B) Quantitation of protein levels from three independent experiments are shown as mean ± SEM. (C) Marked reduction in apoptosome activity with neuronal differentiation. Cytosolic lysates from naïve and neuronally differentiated cells were treated with either bovine cytochrome c alone (10 μM), yeast cytochrome c as a control (10 μM), or bovine cytochrome c with 1 μM of either wild-type AVPI-Smac or mutant MVPI-Smac. Cleavage of caspase-9 and caspase-3 in these treated extracts was examined by Western analysis.
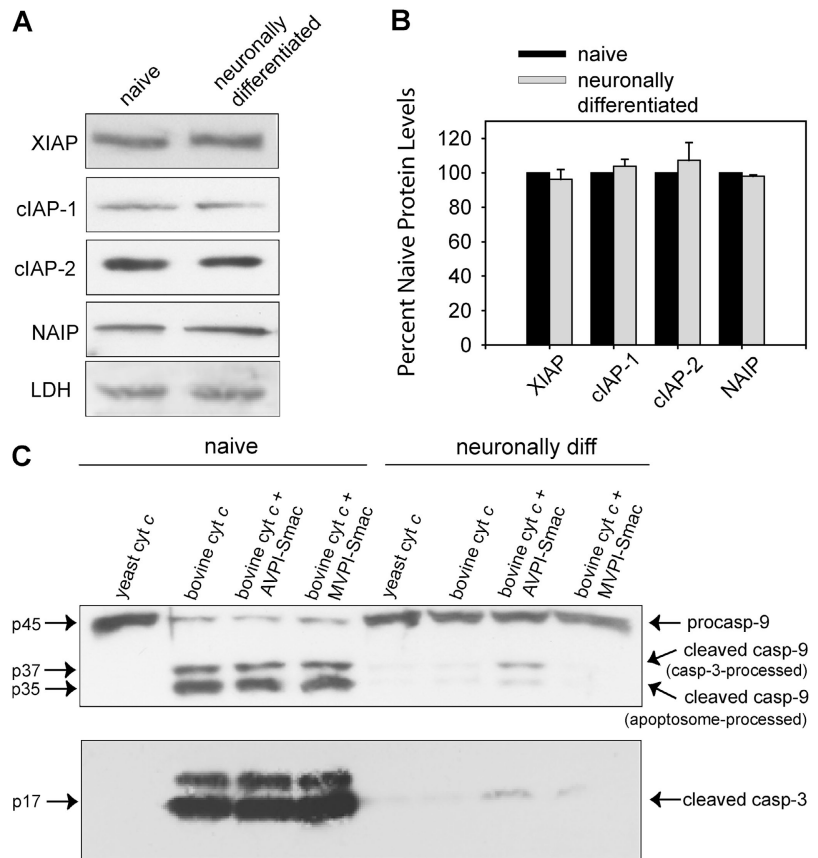
dogenous IAPs effectively inhibited cytochrome c–mediated caspase activation and death in neuronally differentiated cells. In contrast, endogenous IAPs did not appear to regulate apoptosis in the naïve, undifferentiated cells. Not only was cytochrome c alone sufficient to induce rapid cell death in naïve cells, but inhibiting IAPs with addition of Smac did not enhance the rate of caspase activation or cell death in these cells (Fig. 2, C and D).

Apoptosome becomes highly inefficient in neuronally differentiated cells

The selective ability of endogenous IAPs to block caspase activation in neuronally differentiated but not naïve cells was not due to elevated expression of IAPs in the neuronally differentiated cells. Naïve and neuronally differentiated PC12 cells expressed similar levels of multiple IAPs, including XIAP, cIAP-1, cIAP-2, and NAIP (Fig. 3, A and B).

Because inhibition of IAPs with Smac permitted cytochrome c to activate caspases in neuronally differentiated cells (Fig. 2), we examined the processing of caspases when Smac was added to the naïve and neuronally differentiated extracts. Cytochrome c–mediated activation of the apoptosome was very efficient in naïve cell extracts as the apoptosome-processed, p35-kD form of processed caspase-9 predominated over the caspase-3 feedback-cleaved, p37-kD form of caspase-9 (Fig. 3 C). Because cleavage of caspase-9 may not necessarily correlate with caspase-9 activity (Stennicke et al., 1999; Srinivasula et al., 2001), we also examined the processing of caspase-3 as an indicator of caspase-9 activity. Cytochrome c induced robust

processing of caspase-3 in naïve cells, and relieving IAPs with Smac did not enhance this processing (Fig. 3 C).

In contrast, the apoptosome appeared highly inefficient in neuronally differentiated cells as very little caspase-9 processing, neither apoptosome mediated nor feedback cleaved by caspase-3, was detected when cytochrome c was added to these extracts (Fig. 3 C). Relieving IAP inhibition with wild-type AVPI-Smac in neuronally differentiated extracts permitted cytochrome c to activate a limited amount of caspase-3, as some caspase-3 feedback-cleaved form of caspase-9 (p37) was detected under these conditions (Fig. 3 C). Importantly however, the apoptosome remained highly inefficient even under these conditions, as no significant increase in the apoptosome-mediated processing of caspase-9 (p35) was observed even when Smac was added to neuronally differentiated extracts. Furthermore, the amount of processed caspase-3 that was observed in neuronally differentiated cells with Smac addition, although adequate to induce cell death, was significantly lower than seen in naïve cells (Fig. 3 C). Thus, although Smac addition resulted in an increase in caspase-3 activation sufficient for cell death in neuronally differentiated cells, a marked decrease in the efficiency of apoptosome activity occurred when naïve cells became neuronally differentiated.

Efficiency of apoptosome sets the threshold for IAPs to inhibit apoptosis in cells

If the differential ability of endogenous IAPs to regulate cytochrome c–mediated caspase activation in naïve and neuronally
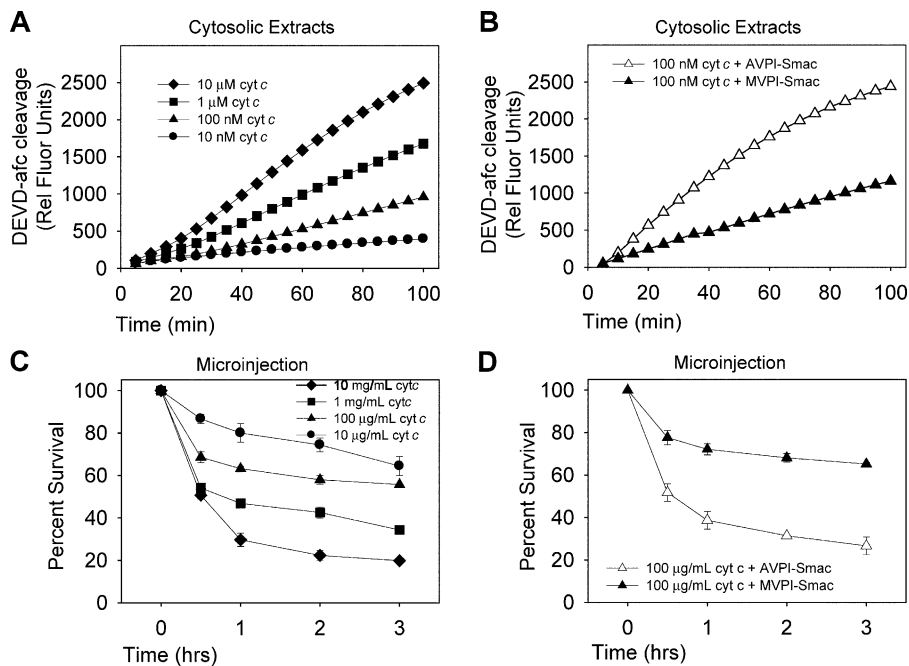
Figure 4. **Reducing the apoptosome efficiency with addition of suboptimum cytochrome c permits endogenous IAPs to effectively block caspase activation and apoptosis in naïve cells.** (A) Cytosolic lysates from naïve cells were incubated with serial dilutions of cytochrome c to identify a concentration of cytochrome c that was suboptimum for caspase activation in these extracts. (B) Naïve cytosolic extracts were incubated with a suboptimum dose of cytochrome c (100 nM) and 1 μM of either wild-type AVPI-Smac or mutant MVPI-Smac. Data shown are representative of multiple experiments. (C) Naïve PC12 cells were injected with serial dilutions of cytochrome c to identify a dose of cytochrome c that was suboptimum for inducing cell death. (D) Naïve cells were injected with a suboptimum dose of cytochrome c (100 μg/ml) and 1 mg/ml of either wild-type AVPI-Smac or mutant MVPI-Smac. Data shown are mean ± SEM of three independent experiments.

differentiated cells is linked to the differential efficiencies of the apoptosome in these two cell types, two simple predictions result from this model. First, reducing the efficiency of the apoptosome in naïve cells should allow endogenous IAPs to now tightly regulate cytochrome c–mediated caspase activation. Second, increasing the efficiency of the apoptosome in neuronally differentiated cells should eliminate the stringent IAP-mediated regulation of caspase activation seen normally in these cells.

To reduce apoptosome function in naïve cells, we simply decreased the amount of exogenous cytochrome c, thereby limiting the amount of apoptosome formation that could occur in these cells. Serial dilutions of cytochrome c were added to cytosolic lysate from naïve PC12 cells, causing a dose-dependent decrease in caspase activation (Fig. 4 A). To determine whether the reduced caspase activation was because of an increased ability of endogenous IAPs to inhibit caspase activation in these cells, we examined whether relieving IAP inhibition with Smac addition enhanced caspase activation seen with suboptimum cytochrome c. Indeed, addition of wild-type AVPI-Smac to lysates with suboptimum cytochrome c (100 nM) effectively increased the amount of caspase activation as compared with lysates with suboptimum cytochrome c alone (Fig. 4 B).

Additionally, serial dilutions of cytochrome c were injected into intact naïve cells to identify a concentration of cytochrome c that was ineffective in inducing cell death (Fig. 4 C). At the suboptimal dose of 100 μg/ml cytochrome c, ~65% of naïve cells were still viable at 3 h. Importantly, coinjection of 100 μg/ml cytochrome c with wild-type AVPI-Smac significantly enhanced the amount of cell death, with <25% of cells remaining viable at 3 h (Fig. 4 D). Together, these results indicate that reducing the activity of the apoptosome alone in naïve cells was sufficient to allow endogenous IAPs to now effectively regulate caspase activation and apoptosis.

Neuronally differentiated cells have decreased apoptosome activity because of markedly reduced Apaf-1 levels

Next, we examined whether the reduced apoptosome activity in neuronally differentiated cells was due to limiting levels of Apaf-1 or procaspase-9. We found that levels of Apaf-1, but not caspase-9, were reduced by a striking 50% when naïve cells were neuronally differentiated for 12 d (Fig. 5 A).

To determine whether the reduction in Apaf-1 levels was specifically responsible for the decreased apoptosome activity in neuronally differentiated cells, we examined the ability of wild-type, Apaf-1$^{-/-}$, and caspase-9$^{-/-}$ cell lysates to complement the deficit in neuronally differentiated cell lysates. Cytosolic lysates from either wild-type, Apaf-1$^{-/-}$, or caspase-9$^{-/-}$ fibroblast cells were incubated with neuronally differentiated cell lysates and activated in the presence of cytochrome c and dATP. Addition of wild-type or caspase-9$^{-/-}$ cell lysate to neuronally differentiated lysate allowed robust caspase activation with cytochrome c (Fig. 5 B), indicating that one or more components from these lysates were sufficient to complement the deficiency and activate caspases in neuronally differentiated cell lysate, without the need to overcome any IAP inhibition. Lysates from naïve cells also complemented the defect in neuronally differentiated cells (Fig. S2 B). In contrast, addition of Apaf-1$^{-/-}$ cell lysates failed to complement the apoptosome deficiency of neuronally differentiated cells, as no activation of caspases with cytochrome c were seen under these conditions (Fig. 5 B). The inability of Apaf-1$^{-/-}$ lysates to complement in this experiment was not because of a nonspecific defect in the preparation of these lysates, as addition of Apaf-1$^{-/-}$ lysates to caspase-9$^{-/-}$ lysates fully complemented each other and activated caspases with cytochrome c (Fig. S2 C). These complementation studies indicate that the inability of cytochrome c alone to activate caspases in neuronally differentiated cells was because of a specific reduction in Apaf-1 function in these cells.

Figure 5. **A marked reduction in Apaf-1 levels causes the reduced apoptosome activity in neuronally differentiated cells.** (A) Western blots comparing the levels of Apaf-1, caspase-9, and lactate dehydrogenase (LDH) in naïve and neuronally differentiated cell lysates. Quantitation of protein levels (mean ± SEM of three independent experiments) for Apaf-1 and caspase-9 are shown. (B) The ability of exogenously added cytochrome *c* (10 μM) to activate caspases was examined in cytosolic lysates of neuronally differentiated PC12 cells alone (150 μg) or neuronally differentiated cell lysates (75 μg) mixed with cytosolic lysates from either wild-type, caspase-9$^{-/-}$, or Apaf-1$^{-/-}$ fibroblasts (75 μg). (C) Cytosolic lysate (150 μg) from neuronally differentiated cells was incubated with cytochrome *c* alone (10 μM) or cytochrome *c* with Apaf-1 protein (10 nM) and assessed for caspase activation. Western analysis of these lysates probed for caspase-9 are shown below. (D) Neuronally differentiated cells were injected with plasmid DNA encoding EGFP (50 ng/μl) and plasmid DNA for vector alone, Apaf-1, or caspase-9 (200 ng/μl). After 24 h for DNA expression, GFP-positive cells were injected with cytochrome *c* (10 mg/ml) or rhodamine dextran and assessed for viability at the indicated times after the injections. Data shown are mean ± SEM of three independent experiments.
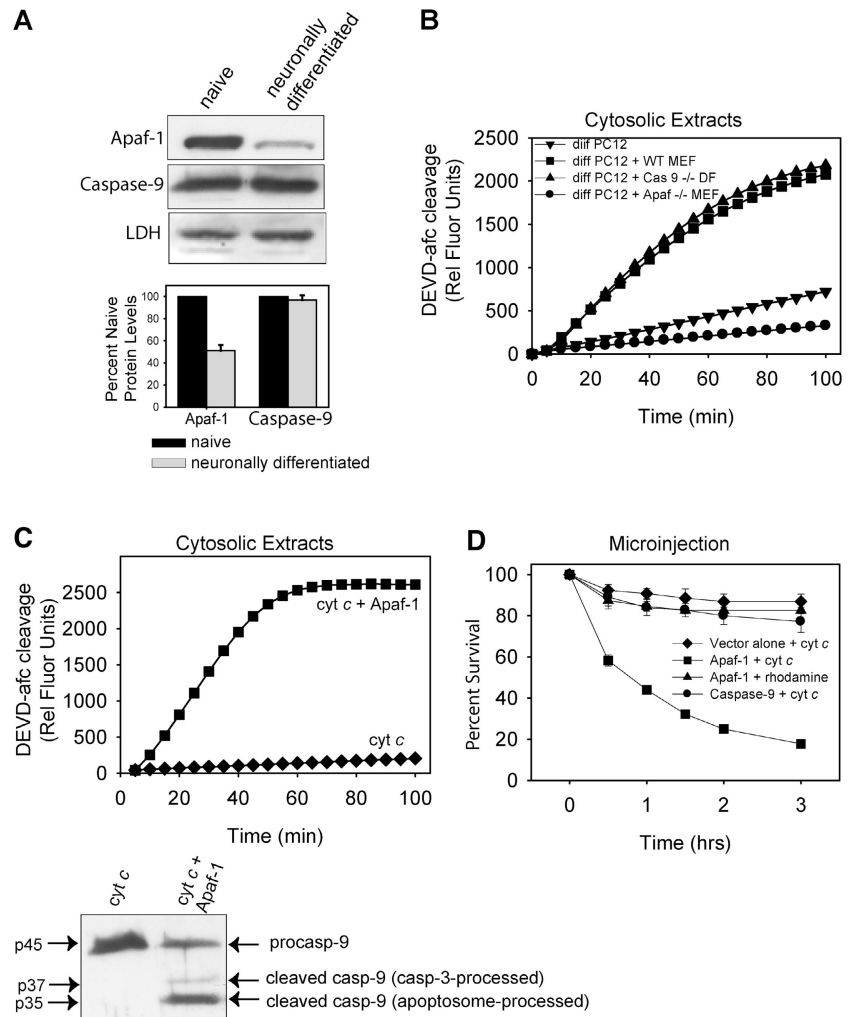
Restoration of Apaf-1 levels in neuronally differentiated cells eliminates regulation by endogenous IAPs and sensitizes cells to cytochrome *c*

If the reduced apoptosome activity was crucial in enabling endogenous IAPs to effectively regulate apoptosis postcytochrome *c* in neuronally differentiated cells, then increasing Apaf-1 levels should be sufficient to overcome the deficit in the apoptosome and permit cytochrome *c* alone to activate caspases in neuronally differentiated cells. Although cytochrome *c* alone did not induce caspase activation, addition of Apaf-1 protein was remarkably effective in permitting cytochrome *c* to induce robust caspase activation in the neuronally differentiated cell lysates, without the need to overcome IAP inhibition (Fig. 5 C). The amount of Apaf-1 protein added (10 nM) was in the physiological range for restoring Apaf-1 levels in the neuronally differentiated lysates, as it was equivalent to the amount required for 50% of maximal activation in Apaf-1 knock-out fibroblast lysates (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200406073/DC1). Also, the processing of caspase-9 seen in neuronally differentiated extracts to which Apaf-1 was added was indicative of its restored apoptosome efficiency with the p35 form (apoptosome processed) predominating just as was seen in naïve cell extracts.

We also examined whether expression of Apaf-1 permitted cytochrome *c* to induce apoptosis in intact neuronally differentiated cells. Neuronally differentiated cells were injected with plasmids expressing GFP and either Apaf-1 or vector alone. After allowing 24 h for expression, the GFP-positive cells were injected with cytochrome *c* to examine the ability of cytosolic cytochrome *c* to induce apoptosis in these cells. As anticipated, the neuronally differentiated cells expressing GFP and vector alone were resistant to cytosolic injection of cytochrome *c*. In contrast, increasing the levels of Apaf-1 permitted cytochrome *c* to induce apoptosis, as <20% of the Apaf-1–expressing cells remained viable 3 h after cytochrome *c* injections (Fig. 5 D). Injection of rhodamine dextran dye alone did not induce cell death in Apaf-1–overexpressing cells. To determine whether this effect was specific to Apaf-1, we examined whether overexpression of procaspase-9 also made the neuronally differentiated cells more vulnerable to cytosolic cytochrome *c*. No appreciable increase in cell death was observed when cytochrome *c* was injected in procaspase-9–overexpressing cells (Fig. 5 D). Thus, restoring Apaf-1 levels eliminated the stringent postcytochrome *c* regulation of apoptosis and allowed cytochrome *c* to induce apoptosis rapidly and without the need to overcome any endogenous IAP inhibition in neuronally differentiated cells.
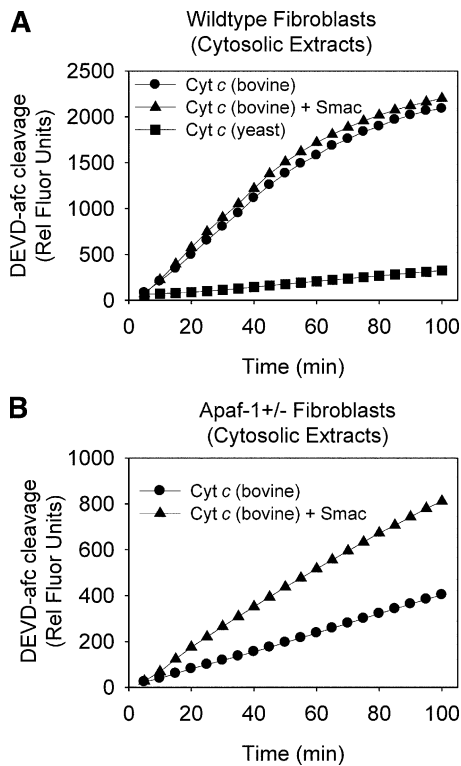
A Wildtype Fibroblasts
(Cytosolic Extracts)

B Apaf-1+/- Fibroblasts
(Cytosolic Extracts)

Figure 6. **Reduction of Apaf-1 in primary fibroblasts allows strict IAP-mediated regulation of cytochrome *c*–mediated caspase activation.** Cytosolic lysates were prepared from primary dermal fibroblasts from either wild-type (A) or Apaf-1 heterozygous (B) mice. The ability of bovine cytochrome *c* (10 μM) alone or bovine cytochrome *c* and Smac (1 μM) together to activate caspases was examined in these lysates. Yeast cytochrome *c* was added to these lysates as a negative control. Data shown are representative of at least three independent experiments.

Apaf-1 levels set the threshold for stringent postcytochrome *c* regulation of apoptosis by IAPs in primary mammalian cells

To test whether our model of coupling between Apaf-1 levels and IAP function can extend beyond the PC12 cell paradigm, we examined this in two different primary cell types. Our model predicts that reducing Apaf-1 levels in a primary cell would engage a strict regulation of cytochrome *c*–mediated apoptosis by endogenous IAPs where such a regulation is otherwise not detected. We examined this in cytosolic extracts of primary dermal fibroblasts isolated from wild-type and Apaf-1 heterozygous mice. Exogenous cytochrome *c* induced robust caspase activation in wild-type fibroblast lysates and addition of Smac did not enhance caspase activation, indicating that endogenous IAPs were ineffective in regulating caspase activation in wild-type fibroblasts (Fig. 6 A). In contrast, caspase activation with cytochrome *c* was significantly reduced in Apaf-1 heterozygous fibroblast lysates. Importantly, this reduction in cytochrome *c*–mediated caspase activation was not simply because of reduced Apaf-1 levels alone, but rather as a consequence of the resulting increased regulation by IAPs, as addition of Smac allowed greater activation of caspases in Apaf-1 heterozygous extracts (Fig. 6 B).

We also examined whether the ability of endogenous IAPs to strictly regulate apoptosis in primary sympathetic neurons (at P5) (Deshmukh et al., 2002; Potts et al., 2003) was a result of decreased Apaf-1 levels with neuronal differentiation. First, we examined sympathetic neurons isolated from embryonic day 16 (E16) mice, a point at which a majority of these neurons have begun postmitotic differentiation and are responsive to NGF (Wyatt and Davies, 1995). After 2 d in culture, these cells were microinjected with cytochrome *c* and were found to undergo rapid apoptosis with cytochrome *c* alone (Fig. 7 A). In contrast, P3 sympathetic neurons maintained in culture for 2 d (P5 equivalent) were remarkably resistant to microinjection of cytochrome *c* because of strict regulation by IAPs, as described previously (Deshmukh and Johnson, 1998; Deshmukh et al., 2002). E16 sympathetic neurons maintained in culture for 8 d (P5 equivalent) also became markedly resistant to cytochrome *c* (unpublished data).

To determine whether the differential regulation of apoptosis by IAPs was linked to changes in Apaf-1 levels, we examined the levels of Apaf-1 protein in E16 and P3 sympathetic neurons after 2 d in culture. Levels of Apaf-1 were dramatically reduced in P3 as compared with E16 neurons (Fig. 7 B). In contrast, no differences in the levels of caspase-9, caspase-3, or IAPs, as well as Smac and HtrA2, were observed in E16 versus P3 sympathetic neurons (Fig. 7 B; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200406073/DC1). We examined whether increasing Apaf-1 levels specifically had the ability to overcome the strict IAP-mediated regulation of apoptosis seen in P5 sympathetic neurons. Primary P5 sympathetic neurons were injected with plasmids expressing GFP and either Apaf-1 or vector alone, and were subsequently reinjected with cytochrome *c* to examine the ability of cytosolic cytochrome *c* to induce apoptosis in these neurons. Increasing Apaf-1 levels was remarkably effective in permitting cytochrome *c* to induce apoptosis, without the need to overcome endogenous IAPs in primary sympathetic neurons. Less than 20% of Apaf-1–injected neurons were viable 6 h after cytochrome *c* injections (Fig. 7, C and D). Importantly, overexpression of caspase-9 was ineffective in changing the resistance of these neurons to cytochrome *c*. These results show that primary sympathetic neurons develop IAP-mediated resistance with neuronal maturation by specifically down-regulating Apaf-1 levels.

Together, the results in primary fibroblasts and neurons are consistent with the data in the PC12 cells and are indicative of a novel common mechanism by which the effectiveness of endogenous IAPs in regulating apoptosis is coupled to Apaf-1 levels in mammalian cells.

Discussion

Increased regulation of apoptosis by IAPs upon postmitotic neuronal differentiation

Despite their ubiquitous expression, endogenous IAPs appear to stringently regulate apoptosis in neurons, but not in many mitotic cells (Li et al., 1997; Brustugun et al., 1998; Juin et al., 1999; Wiese et al., 1999; Chang et al., 2000; Holcik et al.,
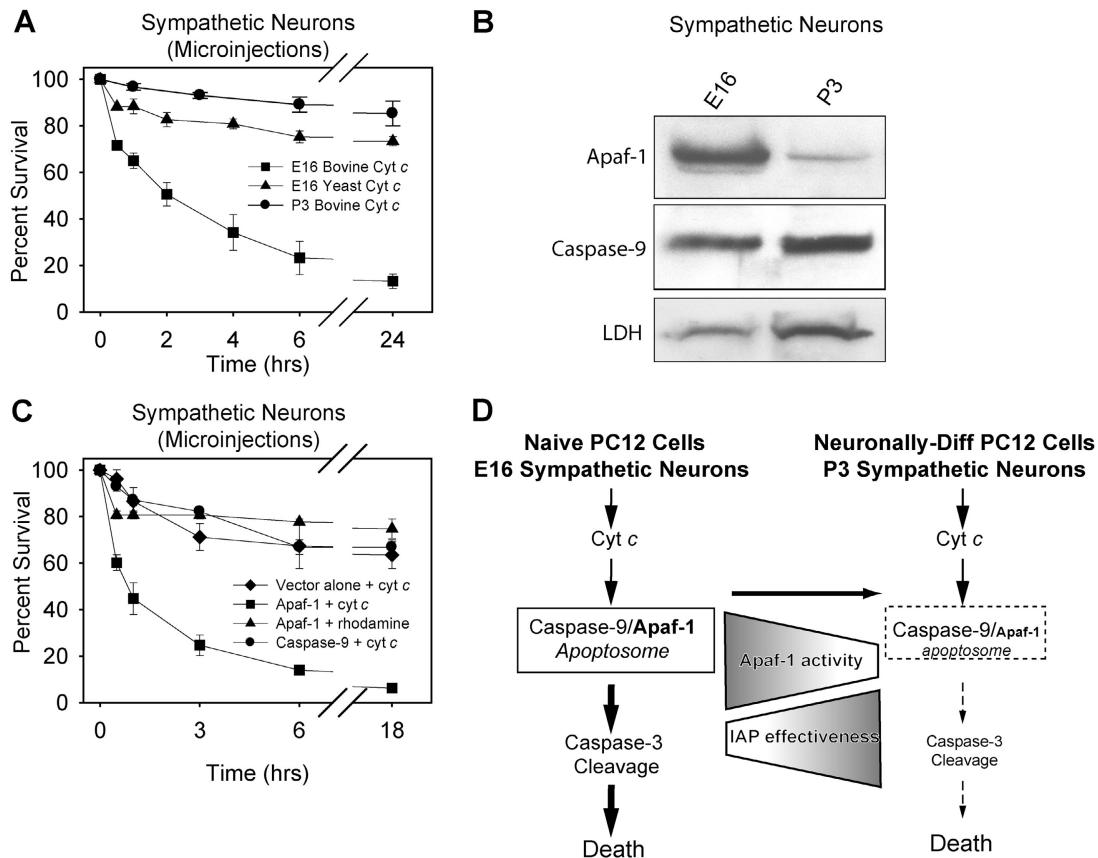
Figure 7. **Modulation of Apaf-1 levels determines the strict IAP regulation of caspases in developing primary sympathetic neurons.** (A) Sympathetic neurons from E16 and P3 mice were maintained in culture for 2 d and compared for the ability of cytochrome *c* to induce apoptosis by cytosolic microinjection of cytochrome *c*. (B) Levels of Apaf-1 and procaspase-9 were examined in whole-cell lysates of E16 and P3 sympathetic neurons after 2 d in culture. Levels of lactate dehydrogenase (LDH) were examined as a loading control. (C) Primary sympathetic neurons (from P0 mice after 5 d in culture) were injected with plasmid DNAs encoding EGFP (50 ng/μl) and vector alone, Apaf-1, or caspase-9 (200 ng/μl). After 24 h for DNA expression, GFP-positive cells were injected with cytochrome *c* or rhodamine dextran and were assessed for viability at multiple times after the injections. Data shown are mean ± SEM of three independent experiments. (D) Model showing how a marked reduction in Apaf-1 activity with neuronal differentiation is coupled to the increased effectiveness of endogenous IAPs in regulating cytochrome *c*–mediated apoptosis.

2000; Deshmukh et al., 2002; Perrelet et al., 2002; Potts et al., 2003). In this work, we investigated whether IAP function was modulated in differentiating cells. We found that although cytosolic cytochrome *c* was sufficient to activate caspases and induce death in naïve PC12 cells, upon postmitotic differentiation these cells became markedly resistant both in intact cells and in cell-free extracts (Fig. 1). Likewise, cytochrome *c* was sufficient to induce apoptosis in sympathetic neurons at early (E16) but not in later (P3) stages of neuronal differentiation (Fig. 7). The increased regulation of cytochrome *c*–mediated apoptosis seen with postmitotic neuronal differentiation is unlikely to be a consequence of exit from cell cycle alone. Naïve PC12 cells stopped dividing and began extending neurites within 2–4 d of NGF treatment, yet became resistant to cytochrome *c* only after 10–12 d of differentiation (Fig. S1).

The marked resistance to cytosolic cytochrome *c* in neuronally differentiated cells could be overcome with excess mature Smac, but not mutant Smac that does not inhibit IAPs (Fig. 2). These results indicate that the increased apoptotic regulation seen in neuronally differentiated cells is mediated by endogenous IAPs. While this manuscript was being prepared for

submission, Vyas et al. (2004) reported a similar observation, where neuronally differentiated PC12 cells were shown to exhibit an increased IAP-mediated resistance to cytochrome *c*. These data in neuronally differentiated cells are strikingly similar to what has been observed in primary P5 sympathetic neurons, which are remarkably resistant to cytosolic cytochrome *c* because of stringent regulation by endogenous IAPs (Deshmukh et al., 2002). Physiologically appropriate stimuli such as trophic factor withdrawal are able to activate neuronal apoptosis because they induce not only the release of cytochrome *c*, but also the degradation of XIAP, the specific IAP that regulates death in these neurons (Potts et al., 2003).

Coupling of IAP function and apoptosome activity in cells

We were surprised to find that despite the striking difference in IAP function in naïve and neuronally differentiated PC12 cells, the levels of IAPs examined in these cells were similar (Fig. 3). Also, no differences in the levels of Smac and HtrA2 were observed in these cells (Fig. S2 A). How is it, then, that endogenous IAPs are capable of regulating apoptosis in neuronally

differentiated but not naïve cells? IAPs have been reported to be regulated at the posttranslational level by mechanisms that include ubiquitination and association with other proteins (Salvesen and Duckett, 2002; LeBlanc, 2003). However, our data indicate that the differential ability of IAPs to regulate apoptosis is directly linked to the activity of the apoptosome in cells. First, cytochrome *c*–induced apoptosome activity was very efficient in naïve cells but highly inefficient in neuronally differentiated cells, even under conditions where IAP inhibition is relieved (Fig. 3 C). This decrease in apoptosome activity was because of a specific reduction in Apaf-1 function, as Apaf-1 protein levels were markedly reduced in neuronally differentiated cells. The time course of reduction in Apaf-1 levels correlated well with the increasing resistance to cytochrome *c* in cells undergoing differentiation (Fig. S1, C and D). Furthermore, the apoptosome defect in neuronally differentiated cells could be fully complemented in vitro by wild-type and caspase-9–deficient lysates, but not by Apaf-1–deficient lysates (Fig. 5). Second, decreasing the apoptosome activity by using suboptimum cytochrome *c* in naïve cells enabled the endogenous IAPs to become highly effective in blocking caspase activation and cell death in these mitotic cells (Fig. 4). Third, increasing the apoptosome activity in neuronally differentiated cells, by restoring Apaf-1 levels, rendered the endogenous IAPs completely ineffective in regulating caspase activation (Fig. 5).

Thus, endogenous IAPs in naïve or neuronally differentiated cells could be modulated to either be fully effective or ineffective in regulating apoptosis simply by altering the apoptosome activity in cells. These data argue against any intrinsic differences in the IAPs in naïve and neuronally differentiated cells. Importantly, our data in Apaf-1 heterozygous fibroblasts and in neurons (Fig. 7) extend these observations to primary cells, suggesting a shared mechanism of regulating IAP function in mammalian cells.

Based on these data, we suggest a model in which the effectiveness of IAPs in controlling apoptosis is tightly coupled to the apoptosome activity in cells (Fig. 7 D). We propose that although an efficient apoptosome renders endogenous IAPs ineffective in blocking caspase activation in mitotic cells, postmitotic differentiation is accompanied by a marked reduction in Apaf-1. The specific reduction in Apaf-1 protein leads to a decrease in apoptosome activity, which allows endogenous IAPs to be highly effective in regulating apoptosis in postmitotically differentiated cells such as neurons. For example, the reduction in Apaf-1 protein would result in slow apoptosome processing of the initiator procaspase-9 and provide IAPs an opportunity to bind to activated caspase-9, and therefore prevent it from activating caspase-3 and blocking apoptosis in neuronally differentiated cells. This is supported by the data showing that although relieving IAP inhibition with Smac permitted apoptosis to occur in neuronally differentiated cells, this proceeded at a slower rate as compared with mitotic naïve cells. This is likely because of the overall slow processing of procaspases-9 as a result of reduced apoptosome efficiency in these cells (Fig. 2).

The exact mechanism by which Apaf-1 levels are reduced with neuronal differentiation is unknown. Like Apaf-1 protein, Apaf-1 mRNA is also markedly reduced with neuronal differentiation (unpublished data; Yakovlev et al., 2001). Recent reports have identified E2F1 and p53 as transcriptional regulators of Apaf-1 (Moroni et al., 2001). As the transcriptional activity of both E2F1 and p53 is decreased in neuronally differentiated cells (Galderisi et al., 2003), these are likely to contribute to the marked reduction in Apaf-1 levels seen in these cells. Additionally, although Apaf-1 protein levels are reduced by 50% upon postmitotic neuronal differentiation, other factors such as posttranslational modifications of Apaf-1 could also contribute to the dramatic reduction in Apaf-1 activity in neuronally differentiated cells.

Physiologically important functions of IAPs

The marked difference in the postcytochrome *c* regulation of apoptosis observed between naïve and neuronally differentiated PC12 cells suggests that a fundamental change in the regulation of apoptosis may occur when any cell undergoes postmitotic differentiation. A strict regulation of apoptosis by IAPs is an important safety brake that could protect against unwanted caspase activation if cytochrome *c* is accidentally released from damaged mitochondria in cells. Arguably, such a mechanism would be physiologically important in postmitotically differentiated cells such as neurons because these cells have limited regenerative potential and need to last for the lifetimes of organisms. In contrast, rapid and efficient activation of caspases in mitotic cells could be advantageous because of the potential of these cells to have dysregulated proliferation.

In these neurons, physiologically appropriate apoptosis during development occurring as a consequence of trophic factor withdrawal is dependent on both the release of cytochrome *c* and the degradation of XIAP (Potts et al., 2003). Because neither event alone is capable of activating caspases and inducing apoptosis in neurons, the lack of any developmental phenotype observed in the XIAP-deficient mice (Harlin et al., 2001) is not surprising. However, the XIAP-deficient neurons, like the NAIP-deficient neurons (Holcik et al., 2000), are likely to be more vulnerable to injury that causes direct mitochondrial damage and rapid release of cytochrome *c*.

Lastly, results in this paper allow us to make several predictions. First, cells in which IAPs are found to strictly regulate apoptosis may have reduced apoptosome activity. Indeed, we tested this prediction in primary sympathetic neurons and found a marked reduction in Apaf-1 levels accompanying neuronal differentiation. Furthermore, elevating Apaf-1 levels specifically in P5 neurons resulted in the elimination of the strict XIAP control of apoptosis that is otherwise seen in these neurons (Fig. 7). In many cancer cells where endogenous IAPs effectively block apoptosis (Deveraux and Reed, 1999; Liston et al., 2003), we predict that a reduction in apoptosome activity may have the same effective outcome as observed with elevated levels of IAPs.

Second, cells that are known to have reduced Apaf-1 levels may be subject to strict regulation by endogenous IAPs. Indeed, postmitotically differentiated cells such as mature cortical neurons (Yakovlev et al., 2001; Ota et al., 2002), cardiomyocytes (Sanchis et al., 2003), and skeletal muscle cells (Burgess et al., 1999) have significantly reduced or no detect-

able Apaf-1 levels, and are resistant to cytochrome *c*–induced apoptosis. Our results predict that the resistance to cytochrome *c* seen in these cells could be because of a strict regulation of caspase activation by endogenous IAPs that becomes effective because of the marked reduction in Apaf-1 levels and the subsequent reduction in apoptosome activity.

Materials and methods

Reagents
All reagents were purchased from Sigma-Aldrich or Fisher scientific, unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical Corporation. The Apaf-1 cDNA was a gift from Dr. Gabriel Nuñez (University of Michigan, Ann Arbor, MI) and the caspase-9 cDNA was a gift from Dr. Chunying Du (Stowers Institute, Kansas City, MO). Apaf-1 knockout and wild-type mouse embryonic fibroblasts were provided by Dr. Douglas Green (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Apaf-1 protein was provided by Dr. Xiaodong Wang (University of Texas Southwestern, Dallas, TX) and tested for its ability to complement the Apaf-1 knockout lysates (Fig. S3).

Cell culture
PC6-3, a subline of PC12 cells, was provided by Dr. Randy Pittman (University of Pennsylvania, Philadelphia, PA; Pittman et al., 1993). PC12 cells were maintained in their mitotic naïve state in RPMI 1640 supplemented with 10% heat-inactivated horse serum, 5% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin. Glucose was added to a final concentration of 4,500 mg/l. PC12 cells were neuronally differentiated by plating at a density of 10^4 cells/cm^2 on collagen-coated dishes in complete media supplemented with 50 ng/ml NGF. Cell culture media was replenished every 2 d, and on d 8 media was removed and replaced with serum-free RPMI 1640 (4,500 mg/l glucose) supplemented with 50 ng/ml NGF. Experiments were performed on neuronally differentiated cells at d 12 of differentiation.

Primary dermal fibroblasts were isolated from P0 mice. The dorsal skin was removed and treated with 1 mg/ml collagenase, and then with 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 and 100 μg/ml penicillin and 100 μg/ml streptomycin.

Primary sympathetic neurons were isolated from E16 and P1 or P3 mice and maintained in culture as described previously (Deshmukh and Johnson, 1998). In brief, dissected ganglia were treated with 1 mg/ml collagenase, and then with 2.5 mg/ml trypsin for 30 min each at 37°C. Ganglia were triturated, and dissociated cells were plated on collagen-coated plates in MEM with Earle's salts supplemented with 50 ng/ml NGF, 10% FCS, 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, and 3.3 μg/ml aphidicolin. Experiments were performed on cells 2–5 d after plating.

Microinjections
Cells were microinjected on 35-mm plates using Femtotip II needles (Eppendorf Inc.) or needles pulled on a Flaming-Brown horizontal micropipette puller (Sutter Instruments) and a Narashigi micromanipulator mounted on an inverted fluorescence microscope (Leica). Between 75 and 150 cells were injected for each condition in these experiments. The microinjection solution contained 100 mM KCl and 10 mM KP$_i$, pH 7.4. For injections involving plasmid DNAs, neuronally differentiated PC12 cells were injected with the DNA after 11 d of differentiation and sympathetic neurons were injected 4 d after plating. The microinjection solution included 50 ng/μl of enhanced EGFP (CLONTECH Laboratories, Inc.) plasmid and 200 ng/μl of either caspase-9– or Apaf-1–expressing plasmid DNAs. After 24 h to allow for expression, the GFP-positive cells were reinjected with cytochrome *c*. For these injections, the microinjection buffer contained rhodamine dextran with cytochrome *c* prepared fresh and diluted to various concentrations in water. Where indicated, the wild-type AVPI-Smac and the mutant MVPI-Smac proteins (Potts et al., 2003) were added to the microinjection buffer at a final concentration of 1 μg/μl. After microinjections, viable cells were identified as rhodamine-positive cells that had intact, phase-bright cell bodies (Deshmukh and Johnson, 1998).

Cytosolic extracts for caspase activation assays
Cells were harvested by scraping and the cell pellet was washed once in ice-cold PBS and resuspended in a volume of hypotonic buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 0.5 mM EDTA, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM DTT, pepstatin, leupeptin, and aprotinin) equal to three times the pellet volume. After 20 min of incubation at 4°C on a tumbler, cells were lysed by passage through a 27-gauge needle. Cells were then centrifuged for 30 min at 16,000 *g* to remove intact cells, nuclei, and heavy membranes. The lysate was centrifuged at 100,000 *g* for 1 h to remove light membranes, and the resulting supernatant was used for caspase activation assays.

In vitro caspase activation assays were performed using equal protein from naïve and differentiated PC12 cell cytosolic lysate. PC12 cell (150 μg) or dermal fibroblast (100 μg) cytosolic extracts were incubated with various concentrations of cytochrome *c* and 1 mM dATP in Apaf-1 oligomerization buffer (25 mM Hepes, pH 7.5, 50 mM NaCl, 10 mM KCl, 1.5 mM MgCl$_2$, 10% glycerol, 1 mM DTT) in a final volume of 100 μl at 37°C for 30 min. After incubation with the fluorogenic caspase substrate 50 μM DEVD-afc (Biomol Research Laboratories), the caspase activity was measured at 37°C on a Fluoroskan Ascent fluorescent plate reader (ThermoLabsystems). Where indicated 1 mM MVPI-Smac, 1 mM AVPI-Smac, 10 nM Apaf-1 protein, or 7 U granzyme B (Sigma-Aldrich) was added to the lysates.

Western blots
Western blots were performed as described previously (Potts et al., 2003). Primary antibodies used were as follows: anti-XIAP (AF822; R&D Systems); anti-cIAP1 (AF818; R&D Systems); anti-cIAP2 (AF817; R&D Systems); anti-Smac (AF7891; R&D Systems); anti-HtrA2 (AF1458; R&D Systems); anti-caspase–9 (M0543; MBL International Corporation); anti-cleaved caspase-3 (9661; Cell Signaling); anti-procaspase-3 (9665; Cell Signaling); anti-Apaf-1 (13F11; Alexis Biochemicals), and anti-NAIP (AF829; R&D Systems). Anti-Apaf-1 antibody, clone Apaf-1L91-71 (a gift of Dr. Yuri Lazebnik, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was used for the Western analysis of primary sympathetic neurons. Anti–mouse/rabbit HRP conjugated secondary antibodies were purchased from Pierce Chemical Co. Proteins were detected using the ECL-Plus detection system (Amersham Biosciences). Protein levels were quantified by scanning blots on a Typhoon scanner (Amersham Biosciences) and analysis with ImageQuant software (Amersham Biosciences).

Online supplemental material
Fig. S1 shows the time course of the development of resistance to cytosolic cytochrome *c* during neuronal differentiation of PC12 cells. Also shown is its correlation with the time course of reduction in Apaf-1 levels during neuronal differentiation. Fig. S2 includes Western blots showing levels of Smac, HtrA2, and procaspase-3 in naïve and neuronally differentiated PC12 cells. Also included are data from two cell-free complementation experiments. First, that cytosolic lysates of naïve and neuronally differentiated PC12 cells complement each other and permit cytochrome *c* to activate caspases. Second, that cytosolic lysates of Apaf-1$^{-/-}$ and caspase-9$^{-/-}$ fibroblasts complement each other and permit cytochrome *c* to activate caspases. Fig. S3 tests the functionality of the Apaf-1 protein and shows that the Apaf-1 protein can complement the Apaf-1$^{-/-}$ mouse embryonic fibroblast lysates in a cell-free caspase activation assay. Fig. S4 includes representative Western blots showing levels of various IAPs, Smac, HtrA2, and procaspase-3 in E16 and P3 primary sympathetic neurons. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200406073/DC1.

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