We have investigated the role of the mitochondrial pathway during cell death following serum and nerve growth factor (NGF)/dibutyryl cyclic AMP (Bt2cAMP) withdrawal in undifferentiated or NGF/Bt2cAMP-differentiated PC12 cells, respectively. Holocytochrome c, Smac/DIABLO, and Omi/Htra2 are released rapidly following trophic factor deprivation in PC12 cells. Bel-2 and Akt inhibited this release. The protection, however, persisted longer in differentiated PC12 cells. In differentiated, but not undifferentiated cells, Bel-2 and Akt also inhibited apoptosis downstream of holocytochrome c release. Thus, undifferentiated PC12 cells showed marked sensitivity to induction of apoptosis by micro-injected cytochrome c even in the presence of NGF, Bel-2, or Akt. In contrast, in differentiated cells these factors suppressed cell death. Consistent with these observations, in vitro processing of procaspase 9 in response to cytochrome c was observed in extracts from undifferentiated but not differentiated cells expressing Akt or Bel-2. Endogenous caspase 9 was cleaved during cell death, whereas dominant negative caspase 9 inhibited cell death. The results from determining the role of inhibitors of apoptosis (IAPs) suggest that acquisition of inhibition by IAPs is part of the differentiation program. Ubiquitin-ΔN-AVPI Smac/DIABLO induced cell death in differentiated cells only, c-IAP-2 is unregulated in differentiated cells, whereas X-linked IAP levels decreased in these cells coincident with cell death. Moreover, expressing X-linked IAP rendered undifferentiated cells resistant to microinjected cytochrome c. Overall, the inhibitory regulation, of cell death at the level of release of mitochondrial apoptotic factors and at post-mitochondrial activation of caspase 9 observed in differentiated PC12 cells, is reduced or absent in the undifferentiated counterparts.

Cell death is essential for the development of the nervous system, where neurogenic precursor cells are produced in excess and then eliminated at specified times during the migration and differentiation of distinct populations of neurons (1–3). Conversely, suppression of the cell death is vital for the maintenance of non-dividing neurons after terminal differentiation.

Compelling experimental evidence that apoptosis is critical for nervous system development has emerged from in vivo studies in mice carrying null mutations for various components of the apoptotic machinery such as Bcl-xL, caspases 3 and 9, and Apaf-1 genes (4–7). In all such cases, gross organizational abnormalities in developing CNS contribute to the observed prenatal lethality. Thus, in the absence of Bcl-xL, excessive death of differentiating neuronal cells occurs in brainstem and spinal cord. Embryonic mice with null mutations in Apaf-1, caspase 3, or caspase 9 display morphological CNS abnormalities, as a result of supernumerary cells expanded ventricular zone and forebrain protrusions occur. The general inference from detailed analyses in such mice, as well as in the double knock-out mutant mice such as Bcl-xL−/−/caspase 3−/− and Bcl-xL−/−/caspase 9−/−, is that cell death in CNS development not only serves to match the size of a neuronal population to its target field but is also vital for global morphogenesis of the nervous system. Additionally, such observations suggest that the mechanisms responsible for early cell death in neural progenitor cells may differ from those in post-mitotic neurons. In neural progenitor cells, caspase 3 or 9 function is independent of Bcl-xL regulation, whereas in post-mitotic neurons, Bcl-xL acts upstream of caspases 9 and 3 in an epistatic manner (8–10). Apoptotic pathways are also activated during inappropriate neuronal death that occurs in acute and chronic neurodegenerative diseases. For example, activated forms of caspases are observed in degenerating neurons following stroke and in Parkinson or Alzheimer diseases (11, 12).

Because caspases are the final implementers of apoptosis, tight control of caspase activation is crucial for long term survival of post-mitotic neurons. Caspases are synthesized as inactive zymogens that are catalytically activated by specific proteolytic cleavage, either by the action of upstream caspases or, in the case of the apical caspases, through autoactivation following their assembly into multimeric protein complexes (13–15). Mitochondria play a key role in orchestrating activation of one key apical caspase, caspase 9. In response to many mechanistically diverse pro-apoptotic triggers, mitochondria release multiple pro-apoptotic effectors from their inter-membrane space. One of these is holocytochrome c (hcC), which, once in the cytosol, forms a complex with and activates the Apaf-1-caspase-9 holoenzyme to generate an active "apopto-
some" (16). Upon receipt of an apoptotic signal, mitochondria synchronously release all of their cytochrome c (17), although the exact molecular mechanism of its translocation remains unresolved (18–20). Several other apoptogenic factors are also released from mitochondria during cell death, among them Smac/DIABLO and Omi/HtrA2, which both act by binding to and inhibiting IAP function, releasing the activities of caspases-9, 3, and 7 (21–25). Substantial experimental evidence indicates that pro-apoptotic Bcl-2 family members such as Bax, Bak, and Bad promote hcC release and, therefore, trigger cell death, whereas the anti-apoptotic members Bcl-2 and Bcl-xL prevent cell death by inhibiting hcC release. In addition, exogenous survival factors such as NGF and insulin-like growth factor-1 inhibit cytochrome c release, in part through an Akt-dependent phosphorylation and deactivation of Bad (24, 25).

Non-differentiated, proliferating PC12 (pheochromocytoma) cells undergo cell death upon withdrawal of serum. However, they can be rescued by acute treatment with various survival factors such as insulin-like growth factor-1 or NGF or by administration of Bt2cAMP (26, 27). In addition to signaling survival, some of these factors also induce differentiation of PC12 cells, although any mechanistic relationship between survival and differentiation is unclear. Although caspase-mediated cell death following withdrawal of serum or NGF in PC12 cells and in primary sympathetic neurons has been well studied (28–30), whether differentiation has a role in regulation of caspases has not been analyzed.

We have previously shown that NGF/Bt2cAMP-induced differentiation leads to these cells becoming terminally and irreversibly differentiated, and, upon withdrawal of NGF and Bt2cAMP, they die asynchronously by apoptosis (26). In this study, we have investigated the role of cytochrome c/caspase 9 activation during cell death induced by trophic factor withdrawal in undifferentiated and NGF/Bt2cAMP-differentiated PC12 cells. In addition, the regulation of this cell death pathway by the pro-survival effectors Akt and Bcl-2 was examined.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Stably Expressing Cell Lines—Undifferentiated PC12 cells were cultivated in RPMI 1640 medium containing 10% fetal calf serum and 5% horse serum. They were induced to differentiate in modified L15 medium containing 5% fetal calf serum, 10% horse serum, and 50 ng/ml NGF (75 form, Roche Applied Science) as previously described (26). After 3 days, 200 μg Bt2cAMP (Sigma) was added, and the cells were differentiated for a further 4–5 days. Cell death was induced in undifferentiated cells (50–60% confluent) by serum withdrawal for 24–48 h. In differentiated cells, apoptosis was triggered by removal of NGF/Bt2cAMP from the medium.

HcC-2 (human bcl-2) cDNA inserted in pcDNA CMV expression vector was electroporated into PC12 cells. G418-resistant hBcl-2 clones were selected, and expression of hBcl-2 protein was verified by Western blot analysis. DNA transfection into the GP + packaging cell line, and ectropic virus-containing supernatant was harvested and filtered. PC12 cells were treated for 5 h with this virus supernatant containing 8 μg/ml Polybrene. The virus supernatant was then replaced with RPMI/serum medium, and 24 h later the cells were selected in G418 (250 μg/ml) for a further 3 weeks, after which individual colonies were used for further analyses.

cDNA encoding the dominant negative caspase 9 C287S point mutant fused to the FLAG epitope (C9DN) (31) was inserted into pcDNA3.1 vector and transfected into PC12 cells by LipofectAMINE treatment, and the G418-resistant pools of clones were selected. Expression of C9DN was verified by immunoblot analysis. G418-resistant PC12 cells in the presence of doxycycline (Sigma, 0.5 μg/ml) transfected with cDNA encoding for mouse XIAP in pN21 tetracycline-repressible vector, were selected for XIAP function in undifferentiated cells. Parallel cultures of cells were transfected with empty plasmid vector and used as controls (PC12/C).

Microinjection—Undifferentiated and differentiated PC12 cells were cultivated on glass-bottomed coverslip dishes (MatTek Corp.). Cytochrome c (Sigma) solution was freshly prepared at desired concentrations and then mixed thoroughly with 0.4% (final concentration) rhodamine-dextran (Sigma), a fluoroscent marker. In differentiated cells, with appropriate NGF/Bt2cAMP was withdrawn just prior to microinjections. Doxycycline was withdrawn 16 h prior to microinjections of XIAP/PC12 cells. After microinjections, the medium was replaced and cell death monitored at timed intervals. Microinjection parameters were kept constant: an automated Eppendorf microinjection system was used in which the pressure was held at 120 hepatopascals and time 0.2 s. Approximately 200–350 cells were microinjected for each study, and culture medium was replaced immediately after microinjections. Viability of microinjected cells was monitored by direct fluorescent microscopy at various time points. At high (×63 or ×100) magnification, rhodamine fluorescence was easily detectable in the cytoplasm and neurites of differentiated cells but excluded from the nuclei. The characteristic morphology of apoptosis, rounding up of cells accompanied by homogenous distribution of the rhodamine marker and fragmentation of nuclei and/or cytoplasm into apoptotic bodies, allowed us to quantify the apoptotic versus viable cells.

Transient Transfections—Undifferentiated and differentiated PC12 cells, cultivated in 4-well multidishes, were transfected with 0.2 μg of pEGFP plus 0.8 μg of Ub-AN-AVP1-Myc or Ub-AN-MVPI Smac/DIABLO-Myc cloned in pcDNA3, using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Medium containing LipofectAMINE 2000 was removed after 6 h and replaced with appropriate normal medium. 24 h post-transfection, the cells were deprived of trophic support for 6 or 24 h and after PBS wash, were fixed with 4% paraformaldehyde. The cells were treated with 0.1% SDS/methanol/E600 and cell death was quantified by counting apoptotic nuclei of GFP-positive cells. It was verified that GFP-positive cells also express Ub-AN-AVP1-Myc or Ub-AN-MVPI Smac/DIABLO-myc by immunofluorescence using 9E10 anti-myc antibody.

Biochemical Methods—Soluble cytoplasmic and membrane fractions were prepared as described previously (32). Briefly, both undifferentiated and differentiated PC12 cells were cultivated in 15 cm culture dishes. Cells in culture dishes were rinsed twice using ice-cold phosphate-buffered saline (PBS). They were harvested by adding a small volume (100 μl) of cold sucrose-supplemented cell extract buffer (300 mm sucrose, 10 mm HEPES (pH 7.4), 5 mm MgCl2, 5 mm EGTA, 1 mm dithiothreitol, 10 mm cytochalasin B, and 1 mm phenylmethylsulfonyl fluoride) to culture dishes, scraped, and pelleted. The pellets were resuspended in the above buffer, incubated on ice for 30 min, homogenized, and centrifuged at 14,000 × g for 15 min. The supernatants comprising the cytoplasmic/soluble fraction and pellets (heavy membrane fraction) were separated and stored at −80 °C. Total cell lysates were prepared, after PBS wash, by directly adding Laemmli buffer containing protease inhibitors to the samples; the samples were boiled and centrifuged. Total protein was quantified by the method of Bradford. The antibodies used were cytochrome c (antibody at 1:1000 dilution; BD Pharmingen, clone 7H8:2C12), cytochrome oxidase IV (antibody at 1:1000 dilution, Molecular Probes), Smac/DIABLO antibody at 1:1000 dilution (22), caspase 9 (antibody at 1:1000 dilution, Stressgen), c-IAP-2 (antibody at 1:1000 dilution, Santa Cruz Biotechnology), and XIAP (antibody at 1:1000 dilution (22)). The Smac/DIABLO antibody was made against mature N-terminal region (Cancer Research, UK), and the specificity was verified by peptide blot. For immunoblot analyses, 14, 12, or 10% SDS-PAGE gels were loaded with lysates containing appropriate concentrations of total protein. After transfer to Immobilon P membrane and incubation with primary and secondary antibodies, the signals were developed using enhanced chemiluminescence kit (Amersham Biosciences).

In Vitro Procaspase 9 Processing—Procaspase 9 cDNA in pBluescript was linearized with EcoRI, transcribed with T7 RNA polymerase, translated, and radiolabeled with 35S-labeled methionine/cysteine (Amersham Biosciences) using the Tnt-Coupled transcription/translation rabbit reticulocyte lysate system (Promega). Processing of in vitro-translated procaspase 9 product by the cytoplasmic extracts prepared from PC12 cells was assayed essentially as described previously (31). Briefly, 1.5 μl of procaspase 9 product was incubated at 30 °C for 30 min with cytoplasmic extracts containing 20 μg of protein (prepared as described above for hcC analysis) in the presence or absence of cytochrome c (10 μM) and dATP (1 mM); all in 10-ml total volume. Laemmli buffer was added, and the samples were boiled and fractionated by SDS-PAGE analysis followed by autoradiography.

RESULTS

Cytochrome c Release in Undifferentiated PC12 Cells following Withdrawal of Serum—The kinetics of subcellular redistribu-
Fig. 1. Kinetics of holocytochrome c (hcC) release and cell death in undifferentiated (UD) PC12 control (PC12/C), PC12/Bcl-2, and PC12/Akt cells. A, soluble cytosolic fraction (SCF) and membrane fractions (MF) were prepared from serum containing 0, 4, 8, 18, 24, 36, and 48-h serum-deprived cells. An aliquot (whole cell lysate, wcl) was taken from serum-containing cells before fractionation and solubilized by adding 0.1% Triton X-100. 20 μg of total protein of SF and whole cell lysate (wcl) and 10 μg of MF was used for analysis of cytochrome c content. The autoradiograms of a representative experiment are shown. i, hcC in SCF and MF of PC12/C cells; ii, hcC immunoblot was stripped and reprobed with anti-cytochrome oxidase IV antibody; the protein is present only in MF; iii, hcC in SCF and wcl of PC12/Bcl-2 and PC12/Akt cells deprived of serum up to 48 h. The SCF blots were reprobed with actin as control for protein loading. The autoradiogram signals of SCF blots were densitometrically quantified (bottom left panel) using Gel Analyst software program. These results, which represent data from 3–5 experiments for each cell type: PC12/C (solid square), PC12/Bcl-2 (solid diamond), and PC12/Akt (solid circle), are expressed as % increase in hcC, mean ± S.E. using the 0 h value as control. *, p value < 0.05, PC12/Bcl-2 or Akt versus PC12/C (Student’s t test, two-tailed). B, undifferentiated PC12/C (solid square), PC12/Bcl-2 (solid diamond), and PC12/Akt (solid circle) cells were deprived of serum for 18, 24, 48, and 72 h, fixed with 4% paraformaldehyde, and nuclei were stained with 1 μg/ml Hoechst 33258. Apoptotic nuclei were quantified by analyzing five random fields, each field comprising ~150 nuclei. Each time point was assayed in quadruplicate; the results are expressed as % mean ± S.E. of at least three experiments. p values: 8, < 0.05 and **, 0.01; PC12/Bcl-2 and Akt versus PC12/C cells (Student’s t test, two-tailed).

bution of hcC from mitochondria to cytosol in PC12 cells following withdrawal of trophic support was first analyzed. The amount of hcC in the membrane fraction containing intact mitochondria (verified by electron microscopy) versus the soluble cytoplasmic fraction was determined by immunoblotting. Proliferating undifferentiated PC12 cells maintained in high serum exhibited negligible cytoplasmic hcC. However, withdrawal of serum resulted in a rapid increase in cytosolic hcC, evident by 4 h and persisting at all the time points tested (Fig. 1A, panel i). In contrast to hcC, the integral mitochondrial protein, cytochrome oxidase IV was retained within the membrane fraction (Fig. 1A, panel ii). The cytosolic accumulation of hcC precedes cell death that begins around 18 h after serum deprivation (Fig. 1B).

Because Akt and Bcl-2 can suppress the release of hcC from mitochondria and inhibit apoptosis in several cell types (25, 33), we next assayed cytochrome c translocation following serum deprivation in PC12 cells constitutively expressing either active v-Akt-gag (PC12/Akt) or hBcl-2 (PC12/Bcl-2). Each study was conducted in two independent clones in which expression of either v-Akt-gag or hBcl-2 had been previously verified by immunoblotting (data not shown). In addition, the activated status of v-Akt-gag was confirmed with an antibody specific for phosphorylated Akt at Ser-473 (data not shown). The PC12/Akt and PC12/Bcl-2 clones used each expressed comparable levels of v-Akt-gag or hBcl-2 significantly inhibited cell death in undifferentiated PC12 cells (Fig. 1B). For example, upon withdrawal of serum for 24 h, we observed 40 ± 5.5% cell death in control cells compared with <5% in either Akt- or Bcl-2-expressing clones. However, such protection was not absolute, because more extended 72-h serum deprivation elicited some 30% death in both PC12/Bcl-2 and PC12/Akt cells (compared with 80% in PC12 control cells) (Fig. 1B). Immunoblot analysis of hcC levels in serum-deprived PC12/Akt and PC12/Bcl-2 cells showed that cytoplasmic hcC levels only rise ~18 h after withdrawal of serum (Fig. 1A, panel iii). The quantification of signals revealed that the accumulation of hcC in cytoplasm of these cells was less than in control PC12 cells. Analysis of hcC in whole cell lysates showed comparable levels of hcC in PC12/control, PC12/Bcl-2, and PC12/Akt cells. Thus both Bcl-2 and Akt act to
inhibit and delay the otherwise rapid translocation of hcC observed in control PC12 cells.

Immunocytochemical staining for hcC corroborated the above immunoblot analysis. PC12 control (PC12/C), PC12/Bcl-2, and PC12/Akt cells in the presence of serum showed punctate mitochondrial hcC staining evident as a rim around the nucleus. In contrast, serum deprivation of PC12/C for as little as 8 h resulted in many cells showing diffuse cytosolic hcC labeling that persisted throughout the period of serum deprivation. PC12/Bcl-2 and PC12/Akt cells deprived of serum for 8 h retained a punctate mitochondrial hcC distribution, which nonetheless became diffuse and cytosolic after 24-h deprivation (data not shown).

Cytochrome c Release in Differentiated PC12 Cells following Withdrawal of NGF/Bt2cAMP—Like their undifferentiated counterparts, differentiated PC12 cells deprived of NGF and Bt2cAMP also showed cytosolic hcC, which was evident by 4 h of deprivation, and persisted to 48 h (Fig. 2A, panel i), in contrast cytochrome oxidase IV was observed only within the membrane fraction (Fig. 2A, panel ii). Again, the release of hcC preceded cell death (Fig. 2B). Release of hcC from mitochondria was confirmed by immunocytochemical staining of equivalent cells, which showed that control differentiated PC12 cells retained punctate somatic and neuritic staining, whereas diffuse hcC staining was already evident in some cells by 4–6 h following deprivation of NGF/Bt2cAMP. By 24 h of factor deprivation, many cells displayed both diffuse hcC cytoplasmic pattern and nuclear morphology characteristic of apoptosis (data not shown).

In undifferentiated PC12 cells, as described above, both Bcl-2 and Akt delayed hcC translocation to cytoplasm for ~18 h. No significant increase in cytosolic hcC was observed until 48 h in either differentiated PC12/Bcl-2 or PC12/Akt cells deprived of NGF/Bt2cAMP (Fig. 2A, panel iii). 48 h after withdrawal of NGF/Bt2cAMP, about 20% of cell death was observed in these cells (Fig. 2B). Immunocytochemical analysis revealed a punctate mitochondrial distribution of hcC in cell soma and neurites in 24 h factor-deprived cells that was similar to that in undeprecated control cells (data not shown). Overall these results indicate that expression of either Bcl-2 or Akt in factor-deprived differentiated PC12 cells suppresses hcC release and this for a longer period than in factor-deprived undifferentiated cells.

Undifferentiated and Differentiated PC12 Cells Display Different Sensitivities to Microinjected Cytochrome c—Release of hcC implies a role of caspase 9 (and caspase 3) pathway during trophic factor deprivation. To clarify any role increased cytosolic hcC might have in PC12 cell death, undifferentiated (proliferating) and differentiated PC12 cells were each microinjected with holocytochrome c together with dextran-conjugated rhodamine as a fluorescent marker. In parallel experiments, the same number of cells was microinjected with dextran-rhodamine marker alone as a control for trauma from microinjections. Subsequent cell fate was then followed after various times by fluorescence microscopy. In both undifferentiated and differentiated PC12 cells, apoptosis was induced, and its extent, dependent on the dose of cytochrome c. However, undifferentiated cells were far more sensitive to induction of apoptosis by a given dose of microinjected cytochrome c. Thus, at 2 h, 40% of the injected undifferentiated cells (10 μM cytochrome c) had died, whereas there was no significant increase in the percentage of cell death in the differentiated population. However, by
21 h, some 42% of microinjected differentiated PC12 cells had died, indicating that cytochrome c was eventually pro-apoptotic, albeit with greatly delayed kinetics (Fig. 3A). This differential sensitivity to microinjected hcC was also observed in cells microinjected with the higher concentration (25 μM) of cytochrome c, although with more rapid cell death kinetics in both undifferentiated and differentiated cells (data not shown), confirming that cytochrome c-induced cell death is dose-dependent. These data are consistent with the notion that differentiated PC12 cells, unlike their undifferentiated counterparts, can to some extent be protected by intracellular signals capable of ameliorating cell survival even in the presence of exogenous cytochrome c.

We next analyzed whether NGF, Bcl-2, or Akt inhibit cell death induced by microinjected holocytochrome c in either undifferentiated or differentiated PC12 cells. Interestingly, in undifferentiated PC12 cells, none of these factors exerted any measurable inhibitory effect on cell death kinetics analyzed 2 and 6 h following microinjections of 2, 10, and 25 μM concentrations of cytochrome c (Fig. 3B). In contrast, in differentiated PC12 cells, cell death induced by microinjection of 10 μM cytochrome c was significantly ameliorated by NGF/Bt2cAMP: for example, 42% ± 10% NGF/Bt2cAMP-treated cells died compared with 91% ± 4% dead cells in similarly microinjected cells deprived of NGF/Bt2cAMP (Fig. 3C). Although withdrawal of NGF/Bt2cAMP from differentiated PC12 cells alone leads to some cell death, this is significantly less than that observed following microinjection of cytochrome c (23% ± 2% control cells dead at 4 h versus 54% ± 8% cells microinjected with cytochrome c). These data indicate that increasing cytosolic cytochrome c in the absence of survival factors accelerates cell death in differentiated cells (Fig. 3C). Pre-treatment of trophic-deprived cells with the broad-specificity caspase inhibitor, benzylxycarbonyl-VAD-fluoromethyl ketone (10 μM) inhibited, although not completely, apoptosis induced by cytochrome c microinjections (Fig. 3C), in accordance with previous reports that caspases act downstream in cytochrome c-mediated cell death. In sharp contrast to undifferentiated cells, we observed that
Bel-2 and Akt suppressed cell death in differentiated PC12 cells that were deprived of NGF/Bt2cAMP and immediately microinjected with 10 μM cytochrome c (Fig. 3D). Taken together, our results show first that sensitivity to cytosolic cytochrome c decreases during PC12 cell differentiation and second that in differentiated but not undifferentiated PC12 cells, Akt and Bel-2 can suppress apoptosis downstream of cytosolic hCC.

Caspase 9 Activation Is Also Differentially Regulated between Undifferentiated and Differentiated PC12 Cells—One potential implication of our findings is that NGF/Bt2cAMP signaling regulates cell death downstream of cytochrome c release, possibly at the level of activation of caspase 9. To investigate this, we next examined the abilities of extracts derived from factor-deprived PC12 cells to induce cleavage and activation of in vitro translated procaspase 9. Procaspase 9 is cleaved into a detectable large subunit of either 35 or 37 kDa depending on the cleavage site Asp-315 or Asp-330. Processing at Asp-330 results in the autocatalytic activity of procaspase 9 with concomitant appearance of the signature 37-kDa large caspase 9 fragments (Fig. 4c). dATP does not result in the autocatalytic activity of procaspase 9 with concomitant appearance of the signature 37-kDa large caspase 9 fragments (Fig. 4c).

No procaspase 9 cleavage was induced, in the presence of exogenous cytochrome c/dATP, by cytosolic extracts from NGF/Bt2cAMP-differentiated cells maintained in NGF/Bt2cAMP: in contrast, analogous extracts derived from factor-deprived PC12 cells triggered cleavage of procaspase 9 with concomitant appearance of the signature 37- and 35-kDa large caspase 9 fragments (Fig. 4A, panel a). Only cytosolic extracts (soluble fraction) and not microsomal fractions prepared from trophic factor-deprived PC12 cells triggered cleavage of procaspase 9 with concomitant appearance of the signature 37- and 35-kDa large caspase 9 fragments (Fig. 4A, panel a).

Incubation of cytoplasmic extracts without cytochrome c and dATP does not result in the autocatalytic activity of procaspase 9 (Fig. 4A, compare panels a and b). Only cytosolic extracts (soluble fraction) and not microsomal fractions prepared from trophic factor-deprived PC12 cells triggered cleavage of procaspase 9 with concomitant appearance of the signature 37- and 35-kDa large caspase 9 fragments (Fig. 4A, panel c).

To confirm the critical role for caspase 9 in determining death of PC12 cells, first the processing of endogenous procaspase 9, during the withdrawal of trophic support, was verified both in undifferentiated and differentiated cells. The 37-kDa cleavage product of procaspase 9 was seen only in trophic-deprived lysates strongly indicating its potential role in cell death (Fig. 4D and E). Because processing of caspase 9 may not necessarily signify that it is catalytically active (34), we transfected PC12 cells with a cDNA encoding a dominant negative procaspase 9 (C9DN) that has a mutation in its active site (C287S) and has been shown to block cell death (35). PC12 cells stably expressing C9DN were prepared, and the kinetics of apoptosis in both undifferentiated and NGF/Bt2cAMP-differentiated cells was examined. Cell death induced by serum deprivation for 24 h was significantly inhibited in undifferentiated PC12/C9DN cells compared with controls (Fig. 4F). In addition, apoptosis of differentiated PC12 cells expressing C9DN and deprived of NGF/Bt2cAMP for 24 h was significantly retarded (Fig. 4G) indicating the importance of caspase 9 for this process.

Smac/DBL and Omi/HtrA2 Are Also Released from Mitochondria of PC12 Cells Deprived of Trophic Support—Recent biochemical and structural studies have revealed not only how some IAP family members inhibit proteolytic activities of initiator (caspase 9) and effector caspases (caspase 3 and 7) but also the mechanism by which this inhibition is effectively eliminated during cell death by mitochondrial proteins such as Smac/DBL and Omi/HtrA2 (34, 36). Through their BIR (baculoviral IAP repeat) domains, IAPs bind and inhibit the catalytic activity of processed forms caspases 9, 3, and 7. The mature forms of Smac/DBL and Omi/HtrA2, containing an exposed IAP binding motif (IBM), once released from mitochondria promote apoptosis by binding to IAPs and relieving caspase inhibition. To determine whether the differential sensitivity to cytochrome c that we observe between undifferentiated and differentiated cells could be related to the mechanism of IAP regulation of caspases, we first examined the release of Smac/DBL and Omi/HtrA2 from mitochondria. Withdrawal of serum or NGF/Bt2cAMP from control undifferentiated or differentiated PC12 cells, respectively, resulted in increased cytosolic levels of Smac/DBL and Omi/HtrA2, with kinetics similar to hCC release (Fig. 5A, panel i, B, panel i, and C, panels i and ii). The effect of Bel-2 and Akt on translocation of Smac/DBL and Omi/HtrA2 in factor-deprived cells was also analyzed. Deprivation of serum in undifferentiated PC12/Bel-2 and PC12/Akt cells resulted in delayed cytoplasmic accumulation of Smac/DBL (at around 18 h) (Fig. 5A, panels ii and iii), although the results of quantification showed that, like hCC, the amount released at 18 h was less than that seen in control cells. In contrast, in NGF/Bt2cAMP-deprived PC12/Bel-2 or PC12/Akt-differentiated cells, cytoplasmic accumulation of these proteins was not observed even at 24 h (Fig. 5B, panels ii and iii). These results suggest that Bel-2 and Akt co-regulate the release of hCC together with other pro-apoptotic factors from mitochondria during withdrawal of trophic support.

Differentiated but Not Undifferentiated PC12 Cells Are Sensitive to Active Smac/DBL—Proteolytic processing of Smac/DBL or Omi/HtrA2 results in the removal of mitochondrial signal peptide sequence and exposure of a novel N terminal or IBM containing the tetrapeptide sequence AVPI, which has been shown to bind to IAPs (37, 38). To elucidate whether cytochrome c sensitivity of caspase 9 pathway is dependent on AVPI proteins, undifferentiated and differentiated PC12 cells were transiently co-transfected with plasmids encoding cDNAs for GFP and ubiquitin (Ub) fused to ΔN-AVPI Smac/DBL or the inactive Ub-ΔN-MVPI Smac/DBL. Intracellular processing of ubiquitin has been shown to yield mature Smac/DBL protein with exposed tetrapeptide sequence (39). Cell death was monitored and quantified by counting Hoescht stained apoptotic nuclei of GFP-positive cells, at 8 and 24 h following withdrawal of trophic support. In undifferentiated PC12/C cells, following withdrawal of serum, increased cell death was observed at 8 and 24 h; however, there was no difference in cultures transfected with Ub-ΔN-AVPI or MVPI-smac/DBL. There was some cell death at 8 h in control cells, which may be due to other regions than N-terminal of Smac/DBL sensitizing serum-deprived cells to apoptosis (40) (Fig. 6A). Transfection of Ub-ΔN-AVPI-smac/DBL in...
factor-deprived undifferentiated PC12/Bcl-2 or PC12/Akt cells did not result in significantly more cell death compared with cells transfected with Ub-ΔN-MVPI Smac/DIABLO (Fig. 6A). In contrast, NGF/Bt2cAMP-deprived control, Bcl-2-expressing, or Akt-expressing differentiated PC12 cells were susceptible to cell death by Ub-ΔN-AVP1 Smac/DIABLO but not Ub-ΔN-MVPI Smac/DIABLO (Fig. 6B). Increased cell death in PC12/C-differentiated cells was seen even in the presence of NGF/Bt2cAMP. These results suggest that, although IBM of Smac/DIABLO is not required for cell death by trophic factor deprivation in undifferentiated cells, it plays an important role in regulating caspase activation in differentiated cells. Further, the anti-apoptotic function of Bcl-2 and Akt is antagonized by Ub-ΔN-AVP1 Smac/DIABLO in differentiated cells indicating that inhibition of Smac/DIABLO release by Bcl-2 or Akt is crucial to suppressing cell death.

Role of IAPs in Regulating Cell Death in Undifferentiated and Differentiated PC12 Cells—The above results strongly indicate that the release of mitochondrial proteins Smac/DIABLO and Omi/HtrA2 that act to relieve IAP inhibition of
Regulation of Cytochrome c/Caspase 9 Pathway

Fig. 5. Immunoblot analysis of Smac/DIABLO and omi/HtrA2 levels in undifferentiated and NGF/Bt<sub>cAMP</sub>-differentiated PC12 cells following trophic factor deprivation. A, for immunoblot analysis of Smac/DIABLO in undifferentiated (UD) cells, 20 μg of total protein of SCF was used, and the blots were probed with anti-smac antibody. Shown are representative results of Smac in: i, PC12/C cells containing serum (0) deprived of serum for times indicated (h); ii, undifferentiated PC12/Bcl-2; and iii, PC12/Akt cells deprived of serum. Results of actin are shown as control for loading. The autoradiograms were quantified and % increase in cytosolic Smac was calculated using the intensity value at 0 h as control. The results are expressed as mean ± S.E. (n = 3 ± 4).* p < 0.05 PC12/Bcl-2 and Akt versus PC12/C cells. B, Smac levels in differentiated PC12 cells (0) deprived of NGF/Bt<sub>cAMP</sub> at times indicated (h). Shown are representative results of Smac in SCF in: i, PC12/C cells; ii, PC12/Bcl-2 cells; and iii, PC12/Akt cells. Quantification of autoradiograms are represented as % increase (mean ± S.E., n = 3 ± 4) in Smac in cytosolic fraction. C, 30 μg of total protein of SCF was used for HtrA2/Omi analysis in trophic-deprived undifferentiated (i) and differentiated (ii) PC12/C for the times indicated.

caspase 9 cascade, is an important determinant of cytochrome c sensitivity in undifferentiated and differentiated cells. To ascertain the role of differentiation, we first examined for changes in levels of c-IAP-2 and XIAP as well as Smac/DIABLO and Omi/HtrA2 in undifferentiated versus differentiated cells (Fig. 7A). Quantification of the results using actin as control in each case showed that the levels of XIAP, Smac/DIABLO, and Omi/HtrA2 remain the same, however there is 41 ± 3% (n = 3) increase in c-IAP-2 levels in differentiated cells. Next we analyzed the levels of XIAP and c-IAP-2 during deprivation of trophic support. In undifferentiated PC12/C, Bcl-2, or Akt cells, XIAP protein levels were unaltered following withdrawal of serum (Fig. 7B). In contrast, in PC12/C-differentiated cells, withdrawal of NGF/Bt<sub>cAMP</sub> resulted in decreased level of XIAP at 24 h (25–35%) (Fig. 7C). This change in XIAP protein level was not seen in differentiated PC12/Akt or PC12/Bcl-2 cells following deprivation of NGF/Bt<sub>cAMP</sub> (Fig. 7C). In contrast to XIAP, there were no changes in c-IAP-2 levels (data not shown). Taken together with the above AVPI data, these results suggest that XIAP and c-IAP-2 play a role in regulating caspase 9 cascade in differentiated but not undifferentiated cells. To further examine this possibility, we constructed an inducible XIAP-tetracycline-repressible PC12 cell line to examine whether sensitivity to microinjected cytochrome c decreases in undifferentiated cells. Undifferentiated PC12 cells in the presence or absence of doxycycline were microinjected with 2, 10, and 25 μM cytochrome c, and cell death was quantified after 6 h (Fig. 7D). In sharp contrast to results obtained with PC12/C, PC12/Bcl-2, and PC12/Akt cells (cf. Fig. 3, A and B), these cells were resistant to microinjected cytochrome c. However, in the presence of doxycycline these cells were not as sensitive to microinjected cytochrome c as PC12/C cells (cf. Fig. 3, A and B). The highest concentration of doxycycline that these cells could tolerate was 0.5 μg/ml, which may not completely repress tetracycline. The results of immunoblot analysis of XIAP in the absence and presence indicate that XIAP levels are higher in doxycycline-treated cells (Fig. 7D). Nevertheless, inhibition of cell death was greater in cells in which doxycycline was withdrawn. Quantification of cell death upon deprivation of trophic support for 24 h in both undifferentiated and differentiated cells showed cell death that is less efficient in the absence than in presence of doxycycline (Fig. 7E). In PC12/C12/ XIAP cells, cell death in the presence of doxycycline was less compared with PC12/C cells (cf. Figs. 1B and 2B). Overall the results indicate that both XIAP and c-IAP-2 regulate caspase 9 and possibly caspases 7 and 3 activation in differentiated cells.

DISCUSSION

The characteristics of PC12 cells make them a useful and tractable in vitro model with which to examine the parameters regulating cell survival in both proliferating neuroectoderm- derived neoplastic cells and in differentiated neuronal cells. In this study, we have shown that hcC, Smac/DIABLO, and Omi/HtrA2 are released from the mitochondrial compartment of factor-deprived PC12 cells undergoing apoptosis. Moreover, ectopic expression of a dominant interfering mutant of caspase 9 inhibits such cell death. Thus, the mitochondrial hcC-caspase 9-cell death pathway is both activated and important in determining cell death of factor-deprived PC12 cells. We also investigated the regulation exerted by Bcl-2 and activated-Akt on hcC and Smac/DIABLO release from mitochondria and the induction of cell death by cytosolic hcC and caspase 9 activation. We find that in both undifferentiated and differentiated (post-mitotic) PC12 cells, Bcl-2 and Akt exert a suppressive effect on release of hcC and Smac/DIABLO, the effect persisting longer in differentiated cells. Moreover, in contrast to un-
differentiated cells, in differentiated PC12 cells, Bcl-2 and activated Akt also inhibit the subsequent activation of caspase 9. Further analysis concerning regulation of the caspase 9 pathway by Smac/DIABLO and XIAP indicate that in differentiated but not undifferentiated cells, the IAP binding motif of Smac/DIABLO plays an important role in activating caspase 9 pathway, most likely, by alleviating IAP inhibition. These results strongly suggest that the PC12 differentiation program alters regulation of events downstream of mitochondrial release of apoptogenic factors.

Release of hcC, Smac/DIABLO, and Omi/HtrA2 from Mitochondria in Factor-deprived PC12 Cells—In cell death induced by trophic factor deprivation, we observed high cytosolic hcC levels in both undifferentiated and NGF/Bt2cAMP-differentiated PC12 cells. In addition to hcC, the processed forms of both Smac/DIABLO and Omi/HtrA2 were also released from mitochondria during factor-deprivation in PC12 cells. The kinetic analysis revealed an early cytosolic accumulation of these proteins, already evident by 4 h of factor deprivation. However, the peak DEVD cleaving activity occurred at around 16 h (data not shown) just preceding cell death. Because we observed a significant delay before caspases were activated, even though these proteins are released rapidly from mitochondria, this indicates mitochondrial release of these factors is not the rate-limiting event for apoptosis. This result is in accordance with previous findings that, although release of hcC from mitochondria is a critical early event in caspase activation, it does not coincide with the commitment point to cell death (32, 41). Moreover, in response to apoptotic signals such as factor withdrawal, cells can withstand high cytosolic hcC levels and survive as long as caspase activation is inhibited and mitochondrial function maintained (30, 42). Other factors, for example, attainment of optimal cytoplasmic levels of these released proteins, may also play a role in determining the activation of the caspase 9 cascade. In this respect, the kinetic data show that in undifferentiated PC12/Bcl-2 and PC12/Akt cells, the release of hcC is less than in PC12/C cells.

The mature Smac/DIABLO and Omi/HtrA2 proteins have an exposed tetrapeptide IAP-binding motif (IBM) at the N terminus, which by binding to XIAP, c-IAP-1, or c-IAP-2 abrogates IAP inhibition of caspase 9 and the effectors caspases 3 and 7, resulting in positive amplification of caspase loop (34). Co-release of these proteins with hcC in factor-deprived PC12 cells suggests that the inhibitors such as IAPs are removed from caspases allowing them to function in cellular demise. The removal may involve degradation of IAPs as shown in the case of XIAP by Omi/HtrA2 (43). In this regard, we find that XIAP levels decrease in differentiated PC12/C cells deprived of trophic support. Omi/HtrA2 may have other targets. For example, the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride was shown to inhibit cleavage of c-IAP-1 by Omi/HtrA2 (44). 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride has also been reported to inhibit caspase 2 processing and cell death in trophic-deprived PC12 cells (45). It is probable that the released Omi/HtrA2 that we observe activates caspase 2, because caspase 2 is cleaved after serum or NGF/Bt2cAMP withdrawal in undifferentiated and differentiated cells, respectively (data not shown). In differentiated and undifferentiated PC12 cells, the release of mitochondrial hcC, as well as of Smac/DIABLO was suppressed by Bcl-2 or constitutively active Akt. Suppression of Smac/DIABLO release by Bcl-2 and Akt presumably dampens down a feed-forward mitochondrial amplification loop ensuring that caspases are not activated. Reinforcing this proposition are the results showing that differentiated PC12/Bcl-2 and PC12/Akt cells are sensitive to Ub-AN-AVPI-Smac/DIABLO. We observed that the inhibitory effect of Bcl-2 or Akt on mitochondrial release extended for a longer period in differentiated cells compared with undifferentiated PC12 cells. This observation suggests

**FIG. 6.** Transient co-transfection of PC12 cells with GFP plus Ub-AN-AVPI or MVPI Smac/DIABLO. A, undifferentiated PC12/C, PC12/Bcl-2, and PC12/Akt cells were co-transfected with pEGFP and either Ub-AN-AVPI (solid square) or Ub-AN-MVPI (open square) Smac/DIABLO. Cell death was quantified in the presence of serum (0 h) and after 8 and 24 h of serum deprivation. The results are mean ± S.E. (n = 3). B, results of cell death following co-transfection with Ub-AN-AVPI (●) or Ub-AN-MVPI (□) Smac/DIABLO in differentiated PC12/C, PC12/Bcl-2, and PC12/Akt containing NGF/Bt2cAMP (0 h) and after withdrawal of NGF/Bt2cAMP for 8 and 24 h. The results are mean ± S.E. (n = 3). *, p < 0.05; **, < 0.01; in Ub-AN-AVPI versus Ub-AN-MVPI transfected cells (Student’s t test, two-tailed).
that differentiation has possibly modulated processes, which are involved in translocating these proteins from mitochondria to cytoplasm, and that control at this level is more stringent in differentiated cells.

Regulation of Cell Death Post-hcC Release from Mitochondria—Directly introducing cytochrome c into PC12 cells by microinjection triggers apoptosis. Such apoptosis must involve activation of caspase 9 for the following reasons: First, the ability of cytoplasmic extracts to induce in vitro processing of procaspase 9 mirrored cell death that was observed with microinjected cytochrome c. Second, endogenous procaspase 9 was cleaved in trophic factor-deprived cells. Third, cell death was suppressed by expression of a dominant negative caspase 9 mutant. Caspase 9 is activated when holocytochrome c, translocated from mitochondria, induces oligomerization of Apaf-1, a process dependent on ATP (16, 46, 47). Procaspase 9, which is either already associated with Apaf-1 as a holoenzyme (16) or recruited following hcC release, is then cleaved and activated within this “apoptosome complex.”

Undifferentiated PC12 cells exhibited markedly greater sensitivity to induction of apoptosis by microinjected cytochrome c than differentiated cells. Additionally, NGF, Bcl-2, or constitutively active Akt were ineffective in protecting undifferentiated PC12 cells against microinjected cytochrome c. The greater sensitivity cannot simply be explained by differences in surface area (differentiated cells have approximately 1.5× greater surface area) as microinjections of even low concentrations of cytochrome c induced cell death that was not inhibited by anti-apoptotic factors. In sharp contrast, resistance to microinjected cytochrome c was found in differentiated PC12 cells; there are a delay period before cell death occurred indicating that the differentiation process enables the cells to withstand, for longer periods, the lethal effects of high cytosolic cytochrome c. In addition, Bcl-2 and Akt strongly suppressed microinjected cytochrome c-induced cell death in differentiated PC12 cells. Bel-2 was more effective in this regard than Akt; however, their effects may simply be a reflection of levels of expression. The inhibitory effect that we observed in cytosolic extracts from PC12/Bcl2 and PC12/Akt cells correlates with a lack of procaspase 9 processing in response to cytochrome c addition. The specific post-mitochondrial action of AVPI-Smac/ DIABLO in differentiated cells as well as up-regulation of
c-IAP-2 suggests that the caspase 9 cascade is inhibited by IAPs in differentiated cells but not in undifferentiated cells. In this regard, the induction of chicken IAP protein, ITP, by NGF in sympathetic neurons has been shown to suppress cell death in the absence of NGF (48). The AVPI motif binds to the BIR3 domain of XIAP relieving inhibition of caspase 9, which suggests that XIAP inhibition of caspase 9 is critical for cell death regulation in differentiated PC12 cells. This may explain the greater sensitivity to cytochrome c seen in undifferentiated cells. The observation that undifferentiated XIAP-tetacycline-repressible PC12 cells were not very sensitive to microinjected cytochrome c strongly indicates that XIAP-mediated inhibition of caspase 9 is acquired during a differentiation program of PC12 cells. The observation that undifferentiated XIAP-tetacycline-repressible PC12 cells was has been reported in NGF-differentiated and -dependent sympathetic neurons (49). Moreover, it was shown recently that exogenous Smac/DIABLO could relieve this resistance to cytochrome c (50) and that deprivation of NGF results in down-regulation of XIAP (51). We have previously shown that cell death induced by deprivation of NGF/Bt2cAMP in differentiated PC12 cells is not dependent on protein synthesis (26, 52), thus it is also likely that there are differences in the regulatory mechanisms of cell death in trophic factor-deprived differentiated PC12 cells and sympathetic neurons.

In conclusion, our PC12 data raise the possibility that regulation of Cytochrome c/Caspase 9 Pathway