A cytosolic factor is required for mitochondrial cytochrome c efflux during apoptosis

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Abstract

Treatment of HL-60 cells with staurosporine (STS) induced mitochondrial cytochrome c efflux into the cytosol, which was followed by caspase-3 activation and apoptosis. Consistent with these observations, *in vitro* experiments demonstrated that, except for cytochrome c, the cytosol of HL-60 cells contained sufficient amounts of all factors required for caspase-3 activation. In contrast, treatment of HCW-2 cells (an apoptotic-resistant HL-60 subclone) with STS failed to induce significant amounts of mitochondrial cytochrome c efflux, caspase-3 activation, and apoptosis. *In vitro* assays strongly suggested that a lack of cytochrome c in the cytosol was the primary limiting factor for caspase-3 activation in HCW-2 cells. To explore the mechanism which regulates mitochondrial cytochrome c efflux, we developed an *in vitro* assay which showed that cytosolic extracts from STS-treated, but not untreated, HL-60 cells contained an activity, which we designated 'CIF' (cytochrome c-efflux inducing factor), which rapidly induced cytochrome c efflux from HL-60 mitochondria. In contrast, there was no detectable CIF activity in STS-treated HCW-2 cells although the mitochondria from HCW-2 cells were responsive to the CIF activity from STS-treated HL-60 cells. These experiments have identified a novel activity, CIF, which is required for cytochrome c efflux and they indicate that the absence of CIF is the biochemical explanation for the impaired ability of HCW-2 cells to activate caspase-3 and undergo apoptosis.

Keywords: caspase-3; cytochrome c; dATP; CIF; apoptosis

Abbreviations: STS, staurosporine; CIF, cytochrome c-efflux inducing factor

Introduction

One of the key biochemical events in apoptosis is the activation of the caspase family of proteases (Alnemri *et al*, 1996; reviewed in Salvesen and Dixit, 1997). This activation involves a proteolytic cascade, as all caspase family members are initially synthesized as inactive, single polypeptide precursors and require proteolytic processing themselves to generate the two subunits that form the active protease (Salvesen and Dixit, 1997).

Caspase-3 has been used as the archetypal and most biologically relevant model for caspase activation in part because the activation of caspase-3 has been observed in many cells under a variety of apoptotic treatments (Darmon *et al*, 1995; Dubrez *et al*, 1996; Enari *et al*, 1996; Erhardt and Cooper, 1996; Han *et al*, 1996b; Martin *et al*, 1996; Schlegel *et al*, 1996). The mature caspase-3 consists of 17 kDa (p17) and 12 kDa (p12) subunits, which are produced from a 32 kDa (p32) precursor (Fernandes-Alnemri *et al*, 1994; Nicholson *et al*, 1995; Tewari *et al*, 1995a; Liu *et al*, 1996; Han *et al*, 1997). Some aspects of the mechanism by which the caspase-3 precursor is converted into the active enzyme are known and in particular endogenous caspase-3 in membrane-free cytosolic extracts from various cell lines can be activated by the addition of exogenous cytochrome c and dATP (Liu *et al*, 1996; Li *et al*, 1997) or exogenous cytochrome c alone (Han *et al*, 1997) under appropriate *in vitro* assay conditions. Since caspase precursors are present in the cytosol of cells whereas active cytochrome c is located in the mitochondria, these observations suggested that the efflux of cytochrome c from mitochondria into the cytosol during the apoptotic induction process was a key regulatory event in the activation of caspase-3.

An additional important contributor to apoptotic regulation is the Bcl-2 family of proteins (reviewed in Farrow and Brown, 1996; Yang and Korsmeyer, 1996). These proteins can be divided into apoptotic suppressors such as Bcl-2, Bcl-x_L, and Mcl-1, and apoptosis effectors such as Bax, Bad, Bak, and Bik/Nbk. The apoptotic suppressors are membrane bound, and found predominately in the outer membranes of mitochondria, the endoplasmic reticulum, and the nuclear envelope (Monaghan *et al*, 1992; Givol *et al*, 1994; Lithgow *et al*, 1994). The membrane association of these proteins suggested that they might regulate certain membrane activities. Indeed, purified Bcl-2 (Schendel *et al*, 1997), Bcl-x_L (Minn *et al*, 1997), or Bax (Antonsson *et al*, 1997) formed ion channels in membranes. More importantly, the presence of high levels of Bcl-2 protein in mitochondria caused a block in the process of cytochrome c efflux, and, as a consequence, an inhibition of caspase-3 activation and apoptosis (Kluck *et al*, 1997; Yang *et al*, 1997). This strongly suggested that the apoptotic suppressive activity of Bcl-2 and Bcl-x_L was due directly to their

inhibitory effect on the process of mitochondria cytochrome *c* efflux during apoptotic induction. Molecular and biochemical studies have demonstrated that apoptotic suppressors and effectors can physically interact with each other, form heterodimers, and antagonize each other's function (Sato *et al*, 1994). In particular, Bcl-2 activity is inhibitable by Bad (Yang *et al*, 1995) and Bax (Oltvai *et al*, 1993), and that of Bcl-x_L by Bad (Yang *et al*, 1995), Bak (Chittenden *et al*, 1995; Farrow *et al*, 1995; Kiefer *et al*, 1995), and Bax (Boise *et al*, 1993). Thus, mitochondrial cytochrome *c* efflux is a critical step in apoptotic induction, and this process appears to be controlled by the interplay of Bcl-2 family members.

We have described the isolation of a stable clonal variant, HCW-2, of the human promyelocytic leukemia cell line, HL-60 (Han *et al*, 1996a). In striking contrast to the parental cells, which were very apoptotic sensitive, HCW-2 cells were highly resistant to the apoptotic effects of many treatments (Han *et al*, 1995; 1996a). The resistance of HCW-2 cells to diverse apoptotic treatments suggested that it was unlikely that the cells were defective in multiple individual pathways for the transduction of different apoptotic signals, but rather were defective in a common event(s) that was induced by many apoptotic signal transduction pathways. We demonstrate here that HCW-2 cells are defective in the caspase-3 activation process due to a lack of mitochondrial cytochrome *c* efflux during apoptotic induction. In addition, we discovered the presence of a cytochrome *c*-efflux inducing factor (CIF) in HL-60 cells, which was activated by staurosporine (STS) treatment and able to induce mitochondrial cytochrome *c* efflux in an *in vitro* assay system. CIF activity was not detected in HCW-2 cells. CIF is thus identified as an important regulator of cytochrome *c* efflux and the lack of CIF in HCW-2 cells results in a severe block in the mitochondrial cytochrome *c* efflux process and thus the inability of these cells to activate caspase-3 and apoptosis.

Results

The status of caspases in HL-60 and HCW-2 cells during apoptotic induction

The status of caspase-1, caspase-2, and caspase-3 in HL-60 and HCW-2 cell lines during apoptotic induction by STS was investigated. Both cell lines expressed detectable caspase-2 and caspase-3 but not caspase-1 (Figure 1A). In untreated cells, caspase-2 and caspase-3 existed, as expected, as inactive precursors in the cytosol. Treatment of HL-60 cells with 5 μ M STS for 4 h selectively induced proteolytic activation of caspase-3 as demonstrated by the disappearance of the caspase-3 precursor (p32) and the appearance of the p17 subunit of the mature caspase-3 (Figure 1A). In addition, caspase-3 activity was easily detected in the HL-60 cytosol (Figure 1B). In contrast, with the same treatment, neither caspase-2 nor caspase-3 were activated in HCW-2 cells (Figure 1A and B). DAPI staining of HL-60 cells treated with STS for 4 h revealed that almost all of the cells were apoptotic as illustrated by granular

Figure 1 Selective activation of caspase-3 in HL-60 cells during apoptosis induction. HL-60 and HCW-2 cells were either left untreated or treated with 5 μ M STS for 4 h and S-100 cytosolic extract was prepared from the cells. (A) Western blot analyses of the status of caspase-1, caspase-2, and caspase-3 in the cells during STS treatment. The caspase-3 precursor is indicated as p32, and the mature subunit of the active caspase-3 is indicated as p17. (B) Caspase-3 activity in the S-100 extract from untreated (Ctrl) and STS-treated (STS) cells. Activity was measured using the CLONTECH ApoAlert™ CPP32 Fluorescent Assay Kit as described in Materials and Methods

bodies of highly condensed DNA in the nuclei (compare Figure 2A and B). DAPI staining of STS-treated HCW-2 cells revealed no signs of apoptosis (compare Figure 2C and D). Thus, the apoptotic-resistant phenotype of HCW-2 cells was associated with a defect(s) in the pathway leading to caspase-3 activation.

In vitro caspase-3 activation using cytosolic extracts from HL-60 and HCW-2 cells

We next attempted to determine the limiting factors for caspase-3 activation in HL-60 and HCW-2 cells using S-100 cytosolic extracts and an *in vitro* caspase-3 activation assay (Liu *et al*, 1996; Han *et al*, 1997; Li *et al*, 1997). The addition of cytochrome *c* to the S-100 cytosolic extract from HL-60 cells induced proteolytic activation of the endogen-

Figure 2 Identification of apoptotic cells by DAPI staining. HL-60 (A, B) and HCW-2 (C, D) cells were either left untreated (A, C) or were treated with 5 μ M STS for 4 h (B, D). The cells were fixed, stained with 100 ng/ml DAPI and then the cells were washed and photographed using a fluorescent microscope

ous caspase-3 as illustrated by the disappearance of the 32 kDa (p32) precursor of caspase-3 and the appearance of the 17 kDa (p17) subunit of the mature caspase-3 in Western blot analyses (Figure 3). Thus, the cytosol of HL-60 cells contained sufficient amounts of all factors, with the sole exception of cytochrome *c*, for caspase-3 activation.

Unexpectedly, caspase-3 processing also occurred with HCW-2 extracts (Figure 3). In contrast to HL-60 cells, activation of the endogenous caspase-3 in the HCW-2 S-100 cytosolic extract was not only dependent upon exogenous cytochrome *c* but also required exogenous dATP (Figure 3). These results strongly suggested that the

apoptotic defect in HCW-2 cells was not in caspase-3 itself, but in an upstream signalling event since extracts derived from HCW-2 cells were permissive for the induction of caspase-3 whereas intact HCW-2 cells were refractory.

Mitochondrial cytochrome *c* efflux in HCW-2 cells is blocked during apoptotic induction

Cytochrome *c*, which normally resides within the mitochondria, must be extruded into the cytosolic compartment prior to caspase-3 activation (Kluck *et al*, 1997; Yang *et al*, 1997). Correspondingly, we observed that treatment of HL-60 cells with STS induced a time-dependent accumulation of cytochrome *c* in the cytosol (Figure 4A), which preceded activation of caspase-3 (Figure 4A and B). The accumulation of cytochrome *c* in the cytosol of STS-treated HL-60 cells correlated well with the loss of mitochondrial cytochrome *c* (Figure 4C). In contrast, treatment of HCW-2 cells with STS resulted in the appearance of very little cytochrome *c* in the cytosol, which correlated with an insignificant loss of mitochondrial cytochrome *c* (Figure 4C). Thus, the mitochondrial cytochrome *c* efflux process in HCW-2 cells was severely defective and this provided an explanation for the inability of these cells to activate caspase-3.

Activation of a cytosolic cytochrome *c*-efflux inducing factor (CIF)

To study the regulation of the mitochondrial cytochrome *c* efflux process we developed an *in vitro* assay system (see Materials and Methods). Incubation of purified mitochondria from HL-60 cells with the S-100 cytosolic extract from STS-treated (5 μ M, 1 h) HL-60 cells rapidly induced complete cytochrome *c* efflux (Figure 5A). Incubation of mitochondria with buffer or the S-100 cytosolic extract from untreated HL-60 cells failed to induce cytochrome *c* efflux (Figure 5A). Thus, treatment of HL-60 cells with STS activated a specific cytosolic factor(s), which we termed 'CIF' (cytochrome *c*-efflux inducing factor).

Figure 3 *In vitro* activation of the endogenous caspase-3 in the S-100 extracts from HL-60 and HCW-2 cells. HL-60 or HCW-2 S-100 extracts (200 μ g) were incubated with an aliquot of buffer alone (Control), 0.4 μ g of cytochrome *c* (Cyt. *c*), 1.5 mM dATP, or a combination of cytochrome *c* and dATP for 60 min at 37°C (see Materials and Methods). The reaction mixtures were then subjected to Western blot analyses. p32 indicates the 32 kDa caspase-3 precursor and p17 indicates the mature 17 kDa caspase-3 subunit

Incubation of the S-100 cytosolic extract at 45°C for 60 min completely inactivated CIF activity (Figure 5B) whereas the presence of up to 2 mM thio-ATP (S-ATP), a non-hydrolyzable ATP analog, had no effect on CIF-induced mitochondrial cytochrome *c* efflux (Figure 5C). Therefore, CIF activity was highly heat-labile and apparently independent of ATP hydrolysis.

Figure 4 Kinetics of cytosolic cytochrome *c* accumulation and caspase-3 activation. (A) Equal numbers of HL-60 cells were either left untreated (0) or were treated with 5 μ M STS for 1, 2, 3 and 4 h. After each treatment, the S-100 cytosolic extract was prepared from the cells. The samples were then subjected to Western blot analyses for cytochrome *c* and caspase-3. (B) Alternatively, the presence of caspase-3 activity was measured using the caspase-3 activity assay (see Materials and Methods and Figure 1). (C) STS induces mitochondrial cytochrome *c* efflux in HL-60 but not in HCW-2 cells. Equal numbers of HL-60 and HCW-2 cells were either left untreated or were treated with 5 μ M STS for 4 h. The cells were then fractionated into cytosolic and mitochondrial fractions and subjected to Western blot analyses for cytochrome *c*

Figure 5 Characterization of CIF. (A) CIF induces mitochondrial cytochrome *c* efflux *in vitro*. Purified mitochondria from HL-60 cells were incubated either with basic buffer (basic buffer), S-100 extract from untreated HL-60 cells (Ctr S-100), or the S-100 extract from HL-60 cells that had been treated with 5 μ M STS for 1 h (STS S-100) for the indicated times. At the end of each incubation, the reaction mixture were separated into pellet (mitochondria) and supernatant. The cytochrome *c* distribution in these two fraction was then analyzed by immunoblotting. Note that the STS S-100 (supernatant, 0 min lane) already contained cytochrome *c* due to STS-induced mitochondrial cytochrome *c* efflux into the cytosol in HL-60 cells prior to the preparation of the S-100 extract. (B) CIF activity is heat-labile. Freshly prepared STS S-100 from HL-60 cells was left on ice (0°C), or incubated at 37, 41, or 45°C for 60 min. The samples were centrifuged at 12500 $\times g$ for 5 min to remove denatured proteins. The supernatant was used to incubate mitochondria for 15 min at 37°C to test the presence of CIF activity. (C) CIF activity is independent of ATP hydrolysis. The STS S-100 extract was incubated without or with 0.5 mM, or 2 mM of thio-ATP (S-ATP) for 10 min at 20°C and then CIF activity was determined

CIF does not alter the amounts of Bcl-2, Bad, Bak, and Bax proteins in mitochondria

Cytochrome *c* efflux during apoptosis induction is believed to be regulated by the Bcl-2 family of proteins (Kluck *et al*, 1997; Yang *et al*, 1997). Therefore, the status of Bcl-2, Bax, Bad, and Bak proteins in HL-60 mitochondria before and after treatment with CIF (i.e., the S-100 from STS-treated HL-60 cells) was determined. CIF-induced cytochrome *c* efflux from purified HL-60 cell mitochondria did not cause detectable changes in the levels of the mitochondrial-associated Bcl-2, Bad, Bak, and Bax proteins (Figure 6).

HCW-2 cells lack CIF activity

The lack of cytochrome *c* efflux in HCW-2 cells during apoptosis induction (Figure 4C) suggested that either (i)

Figure 6 CIF activity does not alter the amount of mitochondrial-associated Bcl-2, Bad, Bak, and Bax proteins. Mitochondria from HL-60 cells were incubated for 0 or 15 min with the S-100 extract from HL-60 cells that had been treated with 5 μ M STS for 1 h. The samples were then separated into the supernatant (S) and mitochondrial pellet (P). The status of cytochrome *c*, Bad, Bak, Bax, and Bcl-2 were determined by Western blot analyses

HCW-2 mitochondria were highly resistant to CIF activity or (ii) that HCW-2 cells lacked CIF activity. To experimentally test these hypotheses we performed heterologous cytochrome *c* efflux assays. When HCW-2 cell mitochondria were incubated with S-100 cytosolic extract from STS-treated HL-60 cells cytochrome *c* efflux was rapidly induced (Figure 7A). In addition, when S-100 cytosolic extract from either untreated or STS-treated HCW-2 cells was incubated with HL-60 cell mitochondria no efflux of cytochrome *c* was observed (Figure 7B). Together, these results strongly suggested that HCW-2 cells lacked CIF activity and that the absence of this activity was directly related to the lack of cytochrome *c* efflux and consequently to the lack of caspase-3 activation and the apoptotic-resistant phenotype of HCW-2 cells.

dATP levels in the S-100 extracts from HL-60 and HCW-2 cells

Finally, we investigated why activation of caspase-3 in the S-100 extract from HCW-2 cells required not only cytochrome *c* but also exogenous dATP whereas caspase-3 activation in the S-100 extract from HL-60 cells required only the addition of exogenous cytochrome *c* (Figure 3). The dATP requirement was dose-dependent, and a minimum of 100 μ M exogenous dATP was required for a significant amount of the endogenous caspase-3 in the HCW-2 S-100 cytosolic extract to be activated in the presence of cytochrome *c* (Figure 8A). The dATP requirement appeared to be very specific, because no other deoxynucleotides or nucleotides could replace its function (Figure 8B).

The non-requirement for exogenous dATP in the caspase-3 activation assay using the HL-60 S-100

cytosolic extract (Figure 3) suggested either that HL-60 cells already contained a sufficient amount of dATP in the cytosol to meet the requirement for caspase-3 activation or that the reaction was dATP independent. To test these hypotheses, the effect of apyrase, which metabolizes deoxynucleoside or nucleoside di- and triphosphates into deoxynucleoside or nucleoside monophosphates (Lebel *et al*, 1980; Laliberte *et al*, 1983) on the process of cytochrome c-dependent activation of the endogenous caspase-3 in the HL-60 S-100 cytosolic extract was investigated. As a control, the effect of 5'-nucleotidase, which converts deoxynucleosides or nucleoside monophosphates into deoxynucleosides or nucleosides, was also investigated. When apyrase, but not 5'-nucleotidase, was added to the S-100 cytosolic extracts from HL-60 cells, subsequent addition of cytochrome c to the assay system no longer induced caspase-3 activation (Figure 8C). This result suggested that dATP was also required for the activation of caspase-3 in the HL-60 S-100 cytosolic extract and that the lack of the need to add exogenous dATP to these extracts was probably due to a sufficient endogenous level.

To confirm this hypothesis, we determined the concentration of dATP in the HL-60 and HCW-2 extracts. The dATP concentration in the S-100 extracts from HL-60 and HCW-2 cells was 409.5 μ M and 98.3 μ M, respectively (Table 1). Using these values, the concentration of the endogenous dATP in the caspase-3 activation assay was calculated to be 327.6 μ M, and 78.6 μ M for HL-60 and HCW-2 cells, respectively (Table 1). These results indicated that, unlike the parental HL-60 cells, HCW-2 cells contained a much lower level of dATP in their cytosol. This low level of dATP in the HCW-2 S-100 extract may be the reason why exogenous dATP was required for the activation of caspase-3 *in vitro*.

Figure 7 HCW-2 mitochondria are responsive to CIF activity. (A) Incubation of the S-100 from STS-treated HL-60 cells with purified mitochondria from HCW-2 cells rapidly induced cytochrome c efflux. (B) HCW-2 cells lack CIF activity. S-100 extract was prepared from untreated HCW-2 cells (Ctr S-100) or HCW-2 cells that had been treated with 5 μ M STS for 2 h (STS S-100), and used in an incubation with purified mitochondria from HL-60 cells for 0, 10, 20 or 30 min at 37°C. The status of cytochrome c was then determined by immunoblot analysis

Discussion

Caspase-3 was selectively activated in HL-60 cells undergoing apoptosis and the activation process was preceded and mediated by mitochondrial cytochrome c efflux into the

Figure 8 The dATP requirement for caspase-3 activation. (A) In the caspase-3 activation assay (Figure 3), the HCW-2 cell S-100 extract was supplemented with 0.4 μ g of cytochrome c alone or various concentrations of dATP. The 32 kDa precursor of caspase-3 is indicated as p32. p20 indicates an intermediate product derived from p32, which is then converted into the mature p17 subunit during the caspase-3 activation process (Han *et al*, 1997). (B) In the caspase-3 activation assay, the HCW-2 cell S-100 extract was supplemented with 0.4 μ g cytochrome c and either incubated with an aliquot of buffer alone (Control) or with 1.5 mM of the indicated compound. (C) Inhibition of the cytochrome c-dependent activation of the endogenous caspase-3 in the S-100 extract from HL-60 cells by apyrase. In the caspase-3 activation assay the S-100 extract from HL-60 cells was incubated with buffer (–) or 0.4 μ g cytochrome c (+) for 60 min at 37°C. Alternatively, the S-100 extract was first incubated with 50 mU 5'-nucleotidase, or 50 mU apyrase for 5 min at 20°C. Then, 0.4 μ g cytochrome c was added to each sample and the incubation continued at 37°C for 60 min. The reaction mixtures were then subjected to a Western blot analysis for caspase-3

Table 1 Endogenous dATP Concentrations in the S-100 Extracts from HL-60 and HCW-2 cells

Cells	dATP concentration in the final S-100 Extract	Endogenous dATP concentration in the caspase-3 activation assay
HL-60	409.5 $\pm$ 46.3 μ M	327.6 $\pm$ 37.0 μ M
HCW-2	98.3 $\pm$ 22.0 μ M	78.6 $\pm$ 17.6 μ M

The data represent the average of five independent experiments

cytosol. Therefore, regulation of mitochondrial cytochrome *c* efflux was the limiting step for caspase-3 activation and thus apoptotic induction in HL-60 cells. Furthermore, we developed a reliable assay for studying how mitochondrial cytochrome *c* efflux is regulated. Using this assay, we have identified a heat-labile, STS-activatable cytosolic factor (termed CIF) in HL-60 cells, which rapidly induced mitochondrial cytochrome *c* efflux. Apoptotic-resistant HCW-2 cells failed to activate caspase-3 because there was a severe block in the mitochondrial cytochrome *c* efflux process, which was associated with a lack of CIF activity. Therefore, the apoptotic phenotypes of HL-60 and HCW-2 cells appeared to be a function of cytochrome *c* efflux regulation by CIF and the ultimate ability or inability, respectively, to activate caspase-3.

The lack of CIF activity correlates with the apoptotic-resistant phenotype of HCW-2 cells

HL-60 cells are extremely sensitive to the apoptotic effects of many different chemical and physical treatments (Martin *et al*, 1990; Boe *et al*, 1991; Ishida *et al*, 1992; Gong *et al*, 1993; Bertrand *et al*, 1994; Kiguchi *et al*, 1994; Han *et al*, 1995; 1996a). The HCW-2 cell line was isolated originally as a stable clonal variant of HL-60 by virtue of the cell's ability to survive a long-term treatment with 8-chloro-adenosine 3', 5'-cyclic monophosphate (Han *et al*, 1996a). Subsequently, HCW-2 cells were also found to be highly resistant to the apoptotic effect of other chemical and physical treatments including cycloheximide or STS exposure, and X-irradiation (Han *et al*, 1995; 1996a). Such a resistance profile indicated that HCW-2 cells were unlikely to be defective in the signal transduction pathways that mediate the apoptotic effect of each individual treatment. Rather, a defect(s) in the centralized apoptotic signal transduction pathway was most likely to be responsible for this phenotype. In particular, caspase-3 activation occurs in many cell lines under a variety of apoptotic conditions (Darmon *et al*, 1995; Tewari *et al*, 1995a; Dubrez *et al*, 1996; Enari *et al*, 1996; Erhardt and Cooper, 1996; Han *et al*, 1996b; Martin *et al*, 1996; Schlegel *et al*, 1996). Importantly, it had also been shown that the inhibition of caspase-3 activity in cells could block apoptosis (Darmon *et al*, 1995; Tewari *et al*, 1995b; Dubrez *et al*, 1996; Enari *et al*, 1996; Martin *et al*, 1996; Schlegel *et al*, 1996). Our results strongly suggest that the apoptotic-resistant phenotype of HCW-2 cells is due to their inability to activate caspase-3 during apoptosis. This is not due to a defect in caspase-3 *per se* (Figure 3) but due to the lack of CIF and the related inability to flux mitochondrial cytochrome *c* into the cytosol (Figure 7B).

The *in vitro* activation of endogenous caspase-3 in the cytosolic extract from HL-60 cells did not require exogenous dATP (Figure 3). This was due to the presence of high levels of endogenous dATP in the extract (Figure 8C and Table 1). In contrast, activation of caspase-3 in the S-100 extract from HCW-2 cells was dependent on both exogenous cytochrome *c* and dATP (Figure 3). Thus, at first glance, it appeared that the small dATP pool size in the S-100 extract from HCW-2 cells (Table 1) correlated well with the cells' *in vivo* inability to activate caspase-3. However, although small in comparison with HL-60 cells,

the actual dATP pool size in HCW-2 cells may not be a limiting factor for caspase-3 activation *in vivo*. The concentration of endogenous dATP in the caspase-3 activation assay using the HCW-2 S-100 extract was calculated to be approximately 78.6 μ M (Table 1). In a typical assay, when the S-100 extract from HCW-2 cells was used, at least 100 μ M of additional exogenous dATP was required for the activation of a significant amount of caspase-3 (Figure 8A). This suggested that the presence of approximately 180 μ M dATP is required for caspase-3 activation. However, when the S-100 extract was prepared from HCW-2 cells, the ratio of the final lysate volume to the volume of the original wet cell pellet was at least 2:1 (see Materials and Methods). This corresponds to a twofold dilution of the cytosolic components in the S-100 extract. If this dilution factor is taken into consideration, then the actual dATP concentration in the cytosol of intact HCW-2 cells should be at least 160 μ M. Accordingly, at this dATP concentration significant caspase-3 activation can probably occur when a sufficient amount of cytochrome *c* is available (Figure 8A). Therefore, the dATP pool size in HCW-2 cells may not be a limiting factor for caspase-3 activation *in vivo*.

Cytochrome *c* efflux and CIF

The original finding (Liu *et al*, 1996) that cytochrome *c* was required for caspase-3 activation indicated that mitochondrial cytochrome *c* efflux should play a key role in apoptotic regulation, since biologically active cytochrome *c* protein is localized in the intermembrane space of mitochondria. Indeed, an early event during apoptotic induction in HL-60 cells is the efflux of mitochondrial cytochrome *c* into the cytosol, which is followed by caspase-3 activation (Yang *et al*, 1997). More interestingly, this process appeared to be regulated by the Bcl-2 family of proteins. Specifically, mitochondria in HL-60 cells that contained an excess amount of Bcl-2 protein due to ectopic over-expression of the gene were blocked in cytochrome *c* efflux (Yang *et al*, 1997). These studies suggested that the ability of apoptotic suppressors such as Bcl-2 and Bcl-x_L to block apoptosis was directly due to their ability to block mitochondrial cytochrome *c* efflux, and thus caspase-3 activation. In this study, we show that CIF, a cytosolic factor, also plays a significant regulatory role for mitochondrial cytochrome *c* efflux during apoptosis. CIF's salient features are: (i) its activity was not found in cells under normal conditions (Figure 4C and 5); (ii) it was rapidly activated by STS (Figure 4C, 5 and 7); (iii) it was very heat labile (Figure 5B); (iv) it did not require ATP hydrolysis (Figure 5C); (v) it did not alter the amount of mitochondrial membrane associated Bcl-2 family proteins (Figure 6); and (vi) it was not detected in HCW-2 cell cytosol (Figure 7B).

Therefore, we envision that treatment of HL-60 cells with STS activates CIF, which then interacts with specific mitochondrial proteins and induces cytochrome *c* efflux. The consequences are caspase-3 activation and apoptosis (Figure 9). This hypothesis suggests that Bcl-2 and related proteins may be downstream targets of CIF. Thus, CIF may inactivate Bcl-2, either by directly binding to it or by modifying the protein in some unknown fashion and this

should facilitate the efflux of cytochrome *c* from the mitochondria. If such a Bcl-2:CIF interaction does take place it apparently does so without affecting the size, stability or location of mitochondrial Bcl-2 (Figure 6). When Bcl-2 is in excess of CIF, mitochondria may be resistant to CIF. This is consistent with the observation that Bcl-2-overexpressing HL-60 cells are blocked in the process of cytochrome *c* efflux (Yang *et al*, 1997). Currently, numerous cell death effector molecules including Bax (Oltvai *et al*, 1993), Bad (Yang *et al*, 1995), Bcl-x_S (Boise *et al*, 1993), Bik/Nbk (Boyd *et al*, 1995; Han *et al*, 1996), Bid (Wang *et al*, 1996), and Hrk (Inohara *et al*, 1997) are known to interact with Bcl-2 and antagonize its activity. Therefore, some of these proteins may fulfill the function of CIF. Since HL-60 cells do not express the *bcl-x* gene (Han *et al*, 1996a), Bcl-x_S is an unlikely candidate. In addition, the mitochondria of HL-60 cells contain Bax and Bad proteins under normal (non-induced) conditions and are therefore unlikely to be candidates for CIF (Figure 6). The most interesting candidates for CIF could be Bik/Nbk, Bid, and/or Hrk. In particular, it has been demonstrated that overexpression of Bik/Nbk (Boyd *et al*, 1995; Han *et al*, 1996), Bid (Wang *et al*, 1996), or Hrk (Inohara *et al*, 1997) in cells by itself causes cell death without subjecting the cells to an apoptotic treatment. Since Bik/Nbk, Bid, and Hrk interact with and antagonize the biological function of Bcl-2, it could be speculated that one or more of these proteins may inhibit mitochondrial Bcl-2 activity and directly induce cytochrome *c* efflux, thus causing caspase-3 activation and apoptosis. We are currently using the *in vitro* cytochrome *c* efflux assay to assess whether Bik/Nbk, Bid or Hrk is consistent with being CIF.

Another plausible hypothesis is that CIF and Bcl-2 compete with each other for binding to the same target

protein in the membrane of mitochondria. The results of CIF or Bcl-2 binding, however, would have different biological results. Thus, binding of Bcl-2 to the target protein would block, whereas binding of CIF would facilitate, cytochrome *c* efflux. In this regard, mitochondrial Bax and Bad would be strong candidates for the target protein. This model is also attractive since it would explain why the simple overexpression of Bax (Oltvai *et al*, 1993) or Bad (Yang *et al*, 1995) in cells does not result in apoptosis, but is still dependent upon apoptotic induction.

In summary, we have characterized a biochemical pathway which is essential for apoptotic induction triggered by STS in HL-60 cells (Figure 9). In addition, we have identified the defect in the apoptotic resistant HCW-2 cell line as a failure to flux cytochrome *c*. Lastly, and most importantly, we have demonstrated that HL-60 cells possess a cytoplasmic activity, CIF, that appears to be required for cytochrome *c* efflux. The further characterization of CIF should greatly facilitate our understanding of the apoptotic mechanism.

Materials and Methods

Materials

Fetal bovine serum (FBS), EDTA, phenylmethylsulfonyl fluoride (PMSF), aprotinin, antipain, leupeptin, purified cytochrome *c* from rat heart, and dATP were purchased from Sigma Chemical, Co (St Louis, MO). Staurosporine (STS) was purchased from Kamiya Biomedical Co. (Seattle, WA). The CompleteTM protease inhibitor cocktail tablets were purchased from Boehringer Mannheim Co (Indianapolis, IN). One tablet was dissolved in 50 mL solution to give a 1 × concentration of the protease inhibitors. The caspase-3 activity assay reagents (ApoAlertTM CPP32 Fluorescent Assay Kits) were produced by CLONTECH Laboratories, Inc. (Palo Alto, CA). Human caspase-1 antibodies were purchased from Oncogene Sciences, Inc. (Uniondale, NY). Human caspase-2 and caspase-3 antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY). Monoclonal antibodies against cytochrome *c* have been described (Jemmerson *et al*, 1991). Protein quantitation reagents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). ECL Western blot analysis reagents were purchased from Amersham Life Science, Inc. (Arlington Heights, IL).

Cells

HL-60 and HCW-2 cells were cultured in RPMI 1640 medium supplemented with 20% and 10% FBS, respectively. The medium also contained 100 U/ml penicillin and 50 U/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Mitochondrial cytochrome *c* efflux and caspase-3 activation during apoptosis

Cells (1 × 10⁸) were left untreated or treated with 5 μM STS for 0, 1, 2, 3 or 4 h. At the end of each treatment, cells were pelleted by centrifugation and washed in 50 mL of phosphate-buffered saline (PBS) once. The cells were then incubated in 1 ml of the lysis buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 50 μg/ml each of leupeptin, aprotinin and antipain) on ice for 10 min.

Figure 9 A model for apoptotic induction in HL-60 cells. The model shows that CIF activity lies upstream of cytochrome *c* efflux within the cell death pathway. It is anticipated that Bcl-2 antagonizes CIF activity at a site on the outer membrane of mitochondria. The efflux of cytochrome *c* into the cytosol then activates a proteolytic cascade leading to the activation of caspase-3 (Han *et al*, 1997; Li *et al*, 1997) as long as the dATP concentration is sufficient and other appropriate factors are available

The cells were broken by five passages through a syringe fitted with a 25-gauge needle. The lysate was divided into two equal parts. To isolate mitochondria, the lysate was mixed with 13.5 ml of lysis buffer containing 0.25 M sucrose. The mixture was centrifuged at $500 \times g$ for 10 min at 4°C to pellet nuclei. The supernatant was then recentrifuged at $10\,000 \times g$ for 10 min at 4°C , and the pellet was used as the mitochondrial preparation. The other half of the initial lysate was immediately centrifuged at $12\,500 \times g$ for 5 min at 4°C to pellet nuclei and mitochondria. The pellet was discarded and supernatant was then centrifuged at $100\,000 \times g$ for 30 min at 4°C . The clarified supernatant (S-100) was used as the cytosolic extract. Fifty μl aliquots of the S-100 extracts were stored at -80°C for the caspase-3 activity assay. The mitochondrial or cytosolic fractions were lysed in a final volume of 600 μl (10% glycerol, 5% β -mercaptoethanol, and 2% SDS), and heated at 100°C for 5 min. Samples were then electrophoresed in 12% polyacrylamide gels, and blotted onto nitrocellulose filters. Cytochrome c and caspases were detected using ECL Western blot reagents.

In vitro caspase-3 activation assay

Concentrated S-100 cytosolic extract was prepared as described above, except that the ratio of the cell pellet volume to lysis buffer volume was 1:1. The extract was adjusted to 10 μg protein/ μl plus 5% glycerol and stored in small aliquots at -80°C . To activate caspase-3, 20 μl of the S-100 extract was mixed with 2 μl of cytochrome c (0.2 $\mu\text{g}/\mu\text{l}$), 1 μl of an EDTA stock solution (187 mM), and 2 μl of dATP stock solutions at various concentrations or 2 μl of H_2O to replace the dATP. The mixture was then incubated at 37°C for 1 h. The reaction was terminated by the addition of 6 μl of $5 \times$ SDS sample loading buffer and heated at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and Western blot analyses (Han *et al.*, 1997). Alternatively, 20 μl of the reaction mixture was used for a caspase-3 activity measurement using the ApoAlert™ CPP32 fluorescent assay (CLONTECH Laboratories, Inc.). The caspase-3 activity was quantitated by fluorescence at 505 nm.

In vitro mitochondrial cytochrome c efflux assay

Concentrated S-100 cytosolic extract was prepared from cells as described above. The extract was adjusted to 10 mg/ml protein plus 50 mM KCl, 5 mM EDTA, and $2.5 \times$ of the Complete™ protease inhibitor cocktail, and stored in small aliquots at -80°C . To purify mitochondria, cells were washed in PBS three times. The cell pellet was resuspended in two volumes of a 5 mM MgCl_2 solution, and incubated on ice for 2 min. The cells were lysed by being passed through a 25-gauge needle three times. The lysate was immediately mixed with 25 ml of a 0.88 M sucrose/5 mM MgCl_2 solution. The mixture was centrifuged at $1400 \times g$ for 10 min at 4°C to pellet the nuclei. The supernatant was re-centrifuged at $26\,000 \times g$ for 10 min at 4°C to pellet the mitochondria. The supernatant was discarded and the mitochondrial pellet was resuspended in a 0.25 M sucrose/5 mM MgCl_2 solution and incubated on ice for 5 min. During this incubation period, an aliquot of the suspension was diluted into 2 ml of the 0.25 M sucrose solution and the OD_{520} absorption of this diluted mitochondria suspension was determined. Following this, the stock mitochondria suspension was adjusted to an OD_{520} equivalence of approximately 18. Aliquots of 80 μl of the mitochondrial suspension were pipetted into microcentrifuge tubes and the mitochondria were pelleted at $12\,500 \times g$ for 1 min at 4°C . The supernatant was discarded. The mitochondria were resus-

pended in 100 μl of washing buffer (10 mM HEPES, pH 7.5, 50 mM KCl, 5 mM EDTA), incubated on ice for 2 min, and centrifuged at $12\,500 \times g$ for 1 min to pellet mitochondria. The supernatant was removed and the mitochondria were immediately used for experiments. Mitochondria were incubated in 20 μl of basic buffer (10 mM HEPES, pH 7.5, 1 mM DTT, 1 mM PMSF, 50 $\mu\text{g}/\mu\text{l}$ each of antipain, aprotinin, and leupeptin, 5 mM MgCl_2 , 5 mM EDTA, $2.5 \times$ of the Complete™ protease inhibitor Cocktail, 50 mM KCl) or the S-100 extract at 37°C for various lengths of time. To terminate the incubation, the tubes were centrifuged at $12\,500 \times g$ for 1 min. The supernatant was mixed with 5 μl of $5 \times$ SDS-loading buffer, the pellet was dissolved in 25 μl of $1 \times$ SDS-loading buffer, and the samples were heated at 100°C for 5 min. A 10- μl aliquot of each sample was subjected to electrophoresis in a 12% polyacrylamide gel and subsequently analyzed by immunoblot analysis for the presence of cytochrome c.

DAPI staining of cells

Cells were washed in PBS once and fixed in 2% paraformaldehyde-0.1% Triton X-100 PBS solution for 30 min at 4°C . The cells were then washed in PBS three times. Cells were stored at 4°C in PBS or spun onto slides in a cytospin centrifuge. Cells were stained in PBS containing 100 ng/ml DAPI for 10 min. The slides were then washed in PBS three times and nuclei staining with DAPI were observed under a fluorescent microscope. Apoptotic cells were identified as those with densely stained granular nuclear bodies.

Quantitative analysis of the dATP content in the S-100 extracts

The method originally described by Solter and Handschumacher (1969) for quantitative determination of deoxynucleoside triphosphates in cell extracts was adapted and modified for this study. Briefly, 4 μl of *Hind*III digested lambda DNA (0.5 $\mu\text{g}/\mu\text{l}$) was mixed with 5 μl of random hexamer oligonucleotides (100 ng/ μl) and 6 μl of H_2O in 1.5 ml microfuge tubes and incubated at 100°C for 5 min. The tubes were then immediately placed on ice for 5 min and subsequently shifted to room temperature for another 5 min. To determine the dATP-dependent incorporation of [α - ^{32}P]dCTP into DNA, 5 μl of $5 \times$ reaction buffer (250 mM Tris-HCl, pH 8.0, 50 mM MgCl_2 , 1 mM dCTP, 1 mM dGTP, 1 mM dTTP) and 1 μl of [α - ^{32}P]dCTP (3000 Ci/mmol, 3.3 μM) were added to the tubes. In addition, 2 μl of H_2O and/or dATP was added to the mixture to give a final concentration of 0, 5, 10, 25, or 50 μM dATP. To determine the amount of dATP in the cellular S-100 extracts, the dATP was replaced by 2 μl of the S-100 extract with various dilutions. Klenow DNA polymerase solution (2 μl at 2.5 U/ μl) was then added to each tube and the mixtures were incubated at 37°C for 5 min. The reactions were stopped by the addition of 0.5 ml of ice-cold 20% trichloroacetic acid (TCA) solution to the tubes. Sonicated salmon sperm DNA (100 μl of 5 mg/ml) was added to each tube as carrier DNA. The contents were mixed, incubated on ice for 60 min and then centrifuged at $12\,500 \times g$ for 10 min at 4°C . The supernatant was discarded and the pellet was extracted with 1 ml of the TCA solution on ice for 60 min. Finally, the tubes were centrifuged at $12\,500 \times g$ for 10 min at 4°C . The TCA solution was removed, the tip of the tube which contained the DNA pellet was cut off, and the radioactivity in the pellet was determined by scintillation counting. A standard curve of a linear relationship between the amount of dATP and [α - ^{32}P]dCTP incorporation (c.p.m.) into DNA was obtained and this and the amount of radioactive incorporation into DNA using the S-100 extract were used to determine the concentration of dATP in the extracts.

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