Apocytochrome \(c\) Blocks Caspase-9 Activation and Bax-induced Apoptosis*

Received for publication, September 12, 2002, and in revised form, October 15, 2002
Published, JBC Papers in Press, October 18, 2002, DOI 10.1074/jbc.M209369200

Angel G. Martin and Howard O. Fearnhead‡†

From the Apoptosis Section, Regulation of Cell Growth Laboratory, NCI, National Institutes of Health, Frederick, Maryland 21702

Complex networks of signaling pathways control the apoptotic response and, therefore, cell survival. However, these networks converge on a common machinery, of which the caspase cysteine proteases are key components. Diverse apoptotic stimuli release holocytochrome \(c\) from mitochondria, allowing holocytochrome \(c\) to bind apoptotic protease activating factor-1 (Apaf-1), which in turn binds caspase-9 both activating this caspase and forming an Apaf-1/caspase-9 holoenzyme. Cytochrome \(c\) lacking heme (the apo form) cannot support caspase activation, although the reason for this has not been studied. Here we show that apocytochrome \(c\) still binds Apaf-1 and that it can block holo-dependent caspase activation in a cell-free system. In addition we show that overexpression of apocytochrome \(c\) blocks Bax-induced apoptosis in cells. Thus it is possible to modulate cell survival by interfering with the Apaf-1/cytochrome \(c\) interaction. Given the key role played by Apaf-1/cytochrome \(c\) in the apoptotic process, and the role of apoptosis in degenerative disease, this interaction may serve as a novel therapeutic target.

Apoptosis is a process fundamental to normal development and tissue homeostasis, and so it is no surprise that deregulation of this process is associated with a variety of diseases from cancer to autoimmunity. As a consequence, there are efforts to manipulate the machinery that drives the apoptotic process for therapeutic gain. Central to the death machinery are a family of cysteine proteases called caspases. These proteases are expressed as inactive precursors (zymogens) that are activated by proteolytic cleavage (1). Caspases can cleave and thereby activate other caspases, but inactive caspases can also undergo autocatalytic activation when recruited into multiprotein complexes (reviewed in Ref. 2). For example, ligands such as Fas or TNFα bind to their cognate receptors causing the formation of a protein complex called the death-inducing signaling complex (DISC) that contains and activates caspase-8 (3) or -10 (4). Once active these “initiators” can, in turn, activate “effector” caspases (such as caspase-3, -6, and -7) (1) that cleave a wide range of cellular substrates (5). It is this second wave of proteolysis that brings about the morphological and biochemical changes of apoptosis.

Although in some instances caspase-8 can apparently activate caspase-3 directly, in other cases caspase-8 acts indirectly by activating Bid, which releases cytochrome \(c\) from mitochondria into the cytosol. Like caspase-8, caspase-2, which is activated by different apoptotic stimuli, can also generate as yet unknown factors that release cytochrome \(c\) (6, 7). In the cytosol cytochrome \(c\) interacts with apoptotic protease activating factor-1 (Apaf-1), which then binds and activates caspase-9 (8), forming an Apaf-1/caspase-9 holoenzyme that activates the effector caspases (9). Thus activation of the Apaf-1/caspase-9 holoenzyme may play a critical role in integrating and amplifying the signals from diverse apoptotic stimuli.

Apaf-1 is a 130-kDa protein with several recognizable domains. At the N terminus there is a caspase interaction domain (or CARD) and a nucleotide binding domain, whereas at the C terminus there are a series of WD40 repeats (10). Pro-apoptotic signals cause the release of cytochrome \(c\) from the mitochondria into the cytosol, allowing it to bind Apaf-1. Indirect data indicate that cytochrome \(c\) binds to a region within the WD40 repeats (11, 12). It has been suggested that the WD40 repeats hinder caspase-9 binding by interacting with the CARD and that this inhibition is relieved by cytochrome \(c\) binding, allowing Apaf-1 to form a large complex (the apoptosome) that recruits and activates caspase-9 (13, 14). Structural studies have shown that the apoptosome contains seven Apaf-1 molecules, arranged radially, with their CARDs forming a central hub where caspase-9 binds (12). Thus, the binding of Apaf-1 to cytochrome \(c\) is a key step in caspase-9 activation.

Cytochrome \(c\) is encoded by a nuclear gene and following synthesis, the apo form (lacking heme) is imported into mitochondria where heme lyase catalyzes heme addition, making the holoprotein. In vitro studies have shown that apocytochrome \(c\) cannot support caspase activation (15), presumably a mechanism for preventing inadvertent caspase activation in cells. In mitochondria the heme group of cytochrome \(c\) allows the protein to shuttle electrons, but several studies indicate that this activity is not relevant for caspase activation. First, the redox state of cytochrome \(c\) does not affect caspase activation (16), and second; holocytochrome \(c\) in which the iron is replaced with zinc still activates caspases (17). Mutational analysis of cytochrome \(c\) indicates that there are several sites necessary for cytochrome \(c\)-induced caspase activation (18, 19). These data suggest that the role of heme is to constrain cytochrome \(c\) structure and so correctly present multiple binding sites to Apaf-1.

It is clear that from a biological standpoint that cytochrome \(c\)-dependent apoptosis can be regulated at several different stages. For example, bel-2 blocks cytochrome \(c\) release (15), heat shock proteins 90 and 70 can interfere with apoptosome

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ To whom correspondence should be addressed. Tel.: 301-846-6140; Fax: 301-846-1666; E-mail: hfearnhead@ncifcrf.gov.
† The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; Apaf-1, apoptotic protease activating factor-1; CARD, caspase recruitment domain; GST, glutathione S-transferase; CBP, calmodulin binding protein; DEVD-AFC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-fluoromethyl coumarin; CHX, cycloheximide; FasR, Fas receptor; Ni2+, nickel-nitritretriacetic acid; AFU, arbitrary fluorescence units; Cyt.c, cytochrome c; XIAP, x-linked inhibitor of apoptosis protein; PBS, phosphate-buffered saline; CMV, cytomegalovirus.

Printed in U.S.A.

This paper is available on line at http://www.jbc.org

View Article Online

Downloaded from http://www.jbc.org/ on July 25, 2018

50834

Vol. 277, No. 52, Issue of December 27, pp. 50834–50841, 2002

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Fax: 301-846-1666; E-mail: hfearnhead@ncifcrf.gov.
Apocytochrome c Blocks Apoptosis

50835

formation (20–22), and Inhibitor of Apoptosis Proteins can bind and inhibit caspases (23). This core apoptotic machinery is also modulated by a number of survival signaling pathways involving Raf, mitogen-activate protein kinase, and Akt that can act both upstream (24) and downstream (25–28) of cytochrome c release. From a pharmacological standpoint, blocking apoptosis has largely relied on inhibiting caspase activity. Thus, synthetic caspase inhibitors have been used in attempts to ameliorate cell death in experimental models of several diseases (29).

To date the reason apocytochrome c cannot drive caspase activation has not been studied. Using a cell-free system, we show here that, although apocytochrome c cannot activate caspases, it still binds Apaf-1. Moreover, the apo form blocks caspase activation by the holo form of cytochrome c. Extending our cell-free studies, we show that overexpression of cytochrome c in cells leads to an accumulation of the apo form of cytochrome c in the cytosol of transfected cells and inhibition of Bax-induced apoptosis. Thus the cytochrome c/Apaf-1 interaction may serve as a novel therapeutic target.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Human Super Fas ligand, human recombinant TNFα, and cycloheximide (CHX) were purchased from Sigma. A rat monoclonal antibody (18H2), a gift from D. Huang (Walter and Eliza Hall Institute of Medical Research), against Apaf-1 was used. Monoclonal anti-caspase-9 antibody (1, 2) was from Novus Biologicals Inc. Monoclonal antibodies against denatured (catalogue number 556433) and native (holo, catalogue number 556432) cytochrome c were purchased from BD Pharmingen. Antibody against caspase-3 (RDI-CPP32Abm) was from Research Diagnostics Inc.

Constructs—The human cytochrome c coding sequence was cloned into EcoRI/EcoRI sites of the pHA-tat vector that provides an N-terminal Histag tagged by a tat fragment tag (pHA-tat-Cyt. c). To obtain GST-tagged forms of cytochrome c, its coding sequence was cloned into BamHI/EcoRI sites on pGEX-4T1 (Amersham Biosciences). For calmodulin binding protein (CBP)-tagged forms, it was cloned into Smal/EcoRI sites of Cal-n vector (N-terminal tag) or Cal-c vector (C-terminal tag, Stratagene). Human heme lyase cDNA was cloned into BamHI/XhoI sites of pET 33b (+) (pET-heme lyase) for bacterial expression.

pEBB-XIAP was kindly provided by Dr. C. Duckett (University of Michigan Medical School). Cytochrome c cDNA was cloned into BamHI/NotI sites of pEBB to construct a vector that allows mammalian expression of a GST-tagged form of cytochrome c. pCDNA3-1-Bax, pCMV-CD20, and pCMV-Puma were obtained from Dr. K. Vousden (Cancer Research UK, Beatson Laboratories, Glasgow, UK).

Recombinant Cytochrome c Expression and Purification—BL21 bacteria was transformed with either pHA-tat-Cyt. c alone (to express the apo form) or in combination with pET-heme lyase (to express the holo form). Positive clones were selected for expression of the recombinant proteins. For cytochrome c production 80 ml of LB media was inoculated and grown overnight at 37 °C to saturation. That culture was diluted to 4 liters and grown for 1 h at 37 °C. Recombinant protein expression was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Ivivtrogen) and incubation continued for 16 h at 30 °C. Bacterial cells were collected by centrifugation (6000 × g for 15 min) and resuspended in native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1 mM antipain, and pepstatin A, 10 μg/ml). Cells were lysed by three freeze-thaw cycles in liquid nitrogen and centrifuged at 100,000 × g for 60 min to obtain an S-100 extract (∼30 mg/ml protein by Bradford assay).

Cytochrome c Depletion of 293 Cell Extracts—293 cell extract was depleted of endogenous cytochrome c essentially as described (31). Briefly, extract (30 mg/ml) underwent three, 2-h rounds of batch incubation with 0.25 volume of SP-Sepharose (Amersham Biosciences) at 4 °C. After each incubation, extract was recovered by centrifugation (100 × g, 5 min, 4 °C). Depletion of cytochrome c was confirmed by immunoblotting and assaying for caspase activation.

Caspase Activation Assay—Equine (Sigma) or recombinant cytochrome c was then added at the indicated concentrations, and extracts were incubated with 1 mM ATP or D-ATP as indicated at 37 °C for 60 min. After this time caspase activity was determined by mixing 2 μl of extract with 200 μl of assay buffer (PBS, 10% glycerol, 0.1 mM EDTA, 2 mM diithiothreitol, and 20 μg/ml Ac-DEVD-afc (BIOMOL)) and measuring the change in fluorescence (excitation, 400 nm; emission, 508 nm) at 30 °C using a Cytofluor 2000. The rate of AFC formation was used to calculate the caspase-3-like activity in the extract, expressed as FU generated/minute.

Transfections and Induction of Apoptosis—For transfection experiments, 1 × 105 U2-OS cells per 6-cm dish were seeded and incubated overnight. LipofectAMINE Plus (Invitrogen) was used to transfect cells with Bax (0.25 μg/plate), GST, or GST cytochrome c (0.5–5 μg/plate) according to the manufacturer’s instructions. After 48 h the incidence of apoptosis was assessed by flow cytometry.

To test whether apocytochrome c blocked Fas- or TNFα-induced apoptosis, U2-OS cells were first co-transfected with cytochrome c and a CD20 expression vector. CD20 expression was used to assess apoptosis in only transfected cells. 24 h after transfection cells were treated with Fas ligand (10 ng/ml) for 12 h to induce apoptosis. After this time, adherent and floating cells were pooled and apoptosis was assessed by flow cytometry. To test whether apocytochrome c blocked TNFα-induced apoptosis co-transfected U2-OS cells were treated 24 h after transfection with TNFα (10 ng/ml) + CHX (10 μg/ml) for 3 h, washed, and further incubated for 10 h with fresh medium. After this time adherent and floating cells were pooled and apoptosis was assessed by flow cytometry.

Assessment of Apoptosis by Flow Cytometry—Floating cells were recovered and pooled with adherent cells harvested by trypsinization. Cells were resuspended in PBS containing 1% Triton X-100, 50 μg/ml propidum iodide, and RNase A and stained for 30 min at room temperature. After this time the percentage of cells with hypodiploid DNA content was determined by flow cytometry.

In experiments using TNFα or Fas, CD20 staining was used to identify cytochrome c-transfected cells. In this case, adherent and floating cells were pooled and incubated with anti-CD20 antibody coupled to fluorescein isothiocyanate (50 μg/ml in PBS) for 1 h at 4 °C. Cells were then fixed with 100% methanol for 16 h and stained with 50 μg/ml propidum iodide in the presence of RNase A and the percentage of CD20-positive cells with hypodiploid DNA content determined by flow cytometry.

Immunoblotting—Proteins were blotted onto polyvinylidene difluoride, and membranes were blocked in Tris-buffered saline with 0.2% Tween 20 and 4% milk powder. Immunoblotting for Apaf-1 (antibody 18H2), caspase-9 (Novus antibody 1–2), caspase-3 (RDI-CPP32Abm), or cytochrome c (BD Pharmingen, 556433) was carried out using the primary antibody at 1:1000, and the appropriate secondary coupled to horseradish peroxidase was used at 1:3000. Bands were detected by ECL (Amersham Biosciences).

Cytochrome c Localization—U2-OS cells were grown on glass cover-slips prior to transfection with GST or GST cytochrome c. 48 h post-transfection mitochondria were labeled by incubation with 100 nM of the cell permeable probe MitoTracker Red (Molecular Probes) for 1 h at 37 °C. Then cells were fixed in 1% formaldehyde and permeabilized with 0.2% Triton X-100. Fixed cells were incubated with either an antibody (diluted 1:100) that recognizes only the holo form) or anti-denatured (diluted 1:100, recognizes apo and denatured holocytochrome c) cytochrome c antibody. A secondary antibody coupled to Alexa Green (Molecular Probes) and diluted 1:500 in PBS with 2% bovine serum albumin and 0.2% Triton X-100 was used to label cytochrome c.

RESULTS

Recombinant Cytochrome c and Cell-free Caspase Activation—in the current model of Apaf-1-dependent caspase activation, caspase-9 binding to the caspase interaction domain of...
Apaf-1 is inhibited by the Apaf-1 WD40 domains. Cytochrome c binding to Apaf-1 relieves this inhibition, allowing caspase-9 to bind and autoactivate (13, 14). Here, a cell-free system was used to study the ability of cytochrome c to activate caspases. This cell-free system consists of a 293 cell extract that is depleted of endogenous cytochrome c as previously described (15). Depletion of cytochrome c is achieved by incubating cell extracts with a cation-exchange resin; because of the basic nature of cytochrome c, this protein binds the resin, whereas Apaf-1, caspase-9, and caspase-3 do not (Fig. 1A). This depleted extract has no detectable caspase-3-like activity but activates caspase-3 in a ATP/dATP- and cytochrome c-dependent fashion when incubated at 37 °C (Fig. 1B).

To further investigate this mechanism our initial goal was to better define the cytochrome c binding sites on Apaf-1. To accomplish this, His-tagged forms of cytochrome c were expressed in bacteria to produce a protein to use as an affinity reagent to capture Apaf-1. Because the holo form of cytochrome c is competent for caspase activation but the apo form is not, cytochrome c was either transfected alone (to produce the apo form) or co-transfected with heme-lyase (to produce the holo form) (32). In both cases the apo form, and the holo form were enriched of Apaf-1 (30) were incubated with 10 μl of recombinant cytochrome c bound to Ni²⁺-NTA-agarose beads (1 μg/μl) for 30 min at the temperature indicated. Proteins bound to the beads were resolved by SDS-PAGE, and Apaf-1 was detected by immunoblotting using a rat monoclonal antibody to Apaf-1. The input lane represents 10% of the amount of extract incubated with beads.

Consistently observed in over five independent preparations. In addition, the holo preparation was clearly red in color (data not shown), consistent with the addition of heme to the polypeptide. A doublet was also consistently seen in all preparations whether apo or holo was purified. Moreover, a similar doublet was observed when we prepared GST-tagged cytochrome c, although in this case the doublet was ~36 kDa as expected (data not shown). The ability of recombinant cytochrome c to activate caspases while bound to their respective affinity resins was tested in a cell-free system. The holo form of cytochrome c activated caspases as well as commercially available, purified cytochrome c (Fig. 2B). The apo form did not activate caspases.
Recombinant Apocytochrome c Blocks Apaf-1—Caspase activation by the holo form indicates an interaction with Apaf-1, and this was confirmed by co-purifying Apaf-1 from the extract with tagged cytochrome c bound to nickel-agarose beads and immunoblotting for Apaf-1 (Fig. 2C). To our surprise, we observed that, although the apo form cannot support caspase activation, it still bound Apaf-1 (Fig. 2C). If Apaf-1 was incubated with comparable amounts of immobilized holo or apocytochrome c at 4 °C the holo form bound more Apaf-1 than the apo form, indicating a difference in affinity. However, this difference was minimized if the temperature was increased to 37 °C (Fig. 2C). Thus, under conditions where holocytochrome c drives caspase activation, apocytochrome c binds to Apaf-1 as well as holo, even though caspase activation fails.

To control for effects mediated by the His6 tag, a number of other tagged forms of cytochrome c (GST and calmodulin binding protein (CBP)) were produced, and their ability to bind Apaf-1 and activate caspasess was tested (Table I). Although quantitative differences in both binding and activation were observed, qualitatively different tags did not alter how holo- or apocytochrome c behaved in activation assays, i.e., holo forms promoted caspase activation while apo forms did not. In binding assays the tags did affect the amount of Apaf-1 precipitated; a C-terminal CBP tag markedly decreased the ability of apo and holocytochrome c to bind Apaf-1 below the limit of detection compared with an N-terminal CBP-cytochrome c fusion protein.

Apocytochrome c Inhibits Holo-driven Caspase Activation—Although apocytochrome c is unable to support caspase activation, its ability to bind Apaf-1 suggests that apocytochrome c may be able to modulate Apaf-1-dependent caspase activation. To test this possibility, the ability of the apo form to affect holo-dependent caspase activation was assessed. To accomplish this, apocytochrome c was added to extracts either before or after the extract was incubated with the holoprotein. Histagged apocytochrome c effectively blocked holo-induced caspase-3 activation assessed using a fluorogenic substrate (Fig. 3A) and caspase-9 processing (Fig. 3B) when added to 293 cell extracts before incubation. In contrast, the apo forms did not inhibit caspase-3 activity if added to an extract 20 min after the holo form, when caspases had already activated (Fig. 3A). Thus, the apocytochrome c acted on the activation step and not on activated caspasess, consistent with binding to, and inhibition of Apaf-1. Furthermore, titration of the amount of apocytochrome c required for inhibition showed that the concentration of the apo form necessary for inhibition of caspase activation was less than the holocytochrome c concentration required for activation (Fig. 4A). Increasing the concentration of holocytochrome c decreased the ability of the apo form to block caspase activation (Fig. 4B), suggesting that apo inhibition of Apaf-1 is reversible.

Apocytochrome c Blocks Bax-induced but Not Fas-induced Apoptosis—Having established that the apo form bound to Apaf-1 and blocked caspase activation, in a cell-free system, we tested whether the apo form could block apoptosis in cells. To test the effect of apo on apoptosis in cells, Bax, a pro-apoptotic member of the bcl-2 family was transiently expressed in an osteosarcoma cell line, U2-OS (which is readily transfectable). Bax can trigger cytochrome c release from mitochondria (33, 34) causing caspase activation. Consistent with this activity, Bax expression induced rapid apoptosis in U2-OS cells (asessed by quantifying the sub-G1 population after propidium iodide staining) that was blocked by expression of the caspase inhibitor XIAP (Fig. 5A). Co-expression of GST-cytochrome c inhibited this death in a concentration-dependent fashion (Fig. 5B). This protection from death was as good as that afforded by expression of a caspase inhibitor, XIAP (Fig. 5A). Cytochrome c tagged with green fluorescent protein also blocked Bax-induced apoptosis, although it was less efficient than GST-tagged cytochrome c (data not shown). In addition, GST-cytochrome c also blocked death induced by expression of p53-upregulated modulator of apoptosis (PUMA) (data not shown), another pro-apoptotic member of the bcl-2 family (36, 37). We also detected an inhibitory effect on Bax-induced apoptosis using a stable Bax-inducible SAOS cell line (data not shown).

| Tag              | Apo Activity | Apo Binding | Holo Activity | Holo Binding |
|------------------|--------------|-------------|---------------|-------------|
| His-tat (N-term) | −            | +           | +             | +           |
| GST (N-term)     | +/−          | +           | +             | +           |
| CBP (C-term)     | −            | +           | +             | +           |
| CBP (N-term)     | −            | +           | +             | +           |

**TABLE I**

Effect of different tags and tag position on cytochrome c ability to promote caspase activation and on Apaf-1 binding
Apocytochrome c Blocks Apoptosis

**A** Titration of apocytochrome c inhibition of holocytochrome c promoted caspase activation. A, increasing amounts of recombinant soluble apocytochrome c were added with equine holocytochrome c (1 μM) to a 293 extract depleted of cytochrome c at the same time and incubated at 37 °C with 1 nm ATP. B, holocytochrome c was titrated in a parallel assay against a fixed concentration of apocytochrome c (100 nM) (open circles) or buffer (closed circles). Caspase activity is expressed as cleavage of the fluorogenic substrate Ac-DEVD AFC in arbitrary fluorescence units per minute (AFU/min).

In contrast to Bax, signaling through death receptors, e.g. via FasR or TNFR, activates caspase-8, which can activate caspase-3-independent of cytochrome c release. Fas ligand and TNFα plus cycloheximide (CHX) can induce apoptosis in U2-OS cells and therefore we tested whether death receptor-induced death was blocked by apocytochrome c. However, both TNFα/CHX and Fas induce apoptosis in the whole cell population, not just those transfected with cytochrome c, potentially masking any inhibition of death. Therefore we co-transfected cells with cytochrome c and CD20 to allow the apoptotic index of only transfected cells to be assessed. CHX is necessary for TNFα-induced apoptosis, because it inhibits protein synthesis, blocking pro-survival signals generated by TNF receptor ligation. To minimize the effect of CHX on cytochrome c expression, CHX and TNFα were added 24 h after cytochrome c transfection and only for 3 h before being washed out. Cells were then further incubated for 10 h in fresh medium after which adherent and floating cells were pooled and apoptosis was assessed by flow cytometry. Transfection of cells with apo gave limited protection from Fas and TNFα, although the amount of DNA used gave complete protection against Bax-induced apoptosis (Fig. 5B). Thus the inhibitory effects of apocytochrome c on apoptosis were most apparent when Bax activated caspasae via the "mitochondrial" pathway. These data also indicate that U2-OS cells are type I cells (38) in that they respond to death receptor ligation by inducing apoptosis via caspase-8 or -10 without mitochondrial involvement.

**Overexpressed Cytochrome c Is a Cytosolic Apoprotein**—Data from the cell-free system show that apocytochrome c binds to Apaf-1 and prevents caspase activation, consistent with the observed inhibition of Bax-induced apoptosis. However, normally apocytochrome c is imported into mitochondria where the addition of heme generates the holo form. If the mechanism suggested by the cell-free data is relevant to the effects seen in cells, the GST-cytochrome c plasmid is not converted to the holo form when expressed.

To test this, the subcellular localization of overexpressed apocytochrome c was determined by immunofluorescence using antibodies to the native and denatured protein. In control (GST-only expressing) cells the antibody to native cytochrome c produced a punctate pattern that co-localized with mitochondria identified with Mitotracker Red (Fig. 6). The antibody to
denatured cytochrome c did not give any detectable staining in these cells. In contrast, in cells expressing GST-cytochrome c, although the antibody to native cytochrome c gave mitochondrial staining identical to control cells, the antibody to denatured cytochrome c gave diffuse cytoplasmic staining (Fig. 6) plus some co-localization with mitochondria. Thus, although a proportion of tagged cytochrome appeared mitochondrial the majority was cytoplasmic. This subcellular localization of ectopically expressed cytochrome c and its persistence in the apo form are consistent with overexpressed cytochrome c blocking apoptosis by binding to Apaf-1.

DISCUSSION

Cytochrome c is a nuclear gene encoding a protein that in its apo form exhibits a random coil structure and high protease sensitivity indicative of a loosely folded conformation. The apo form is taken up by mitochondria where heme lyase catalyzes the formation of the holoprotein. Holocytochrome c binds the porphyrin ring of heme via two thioether linkages at cysteines 14 and 17 and two axial ligands, histidine 18 and methionine 80. As a result the polypeptide wraps the heme group, taking a compact globular conformation. Mutational analysis of cytochrome c/Apaf-1 interactions suggests that Apaf-1 contacts holocytochrome c at several sites (18). Thus, it appears that heme constrains cytochrome c structure so that a number of binding sites are presented to Apaf-1 in the correct orientation. In the current model of caspase activation, cytochrome c binding to the WD40 repeats of Apaf-1 relieves WD40-mediated autoinhibition of Apaf-1 oligomerization (11, 13, 14). Presumably, cytochrome c binding to the WD40 repeats favors an open Apaf-1 conformation allowing Apaf-1 oligomerization and the subsequent recruitment and activation of caspase-9.

Here we have used a cell-free system to study both caspase activation and Apaf-1 binding by recombinant cytochrome c purified from bacteria. Our data show, for the first time, that apocytochrome c binds Apaf-1 but that this interaction is insufficient for caspase activation. Moreover, we show that the apo form can inhibit caspase activation driven by holocytochrome c. One concern is that a bacterial contaminant is responsible for the observed inhibition. However, Coomassie Blue staining showed that cytochrome c is the major protein present...
Apocytochrome c Blocks Apoptosis

(Fig. 2A). In addition, bacterial lysate did not inhibit holo-dependent caspase activation (data not shown) suggesting that inhibition cannot be ascribed to a bacterial contaminant.

Apocytochrome c inhibition of caspase activation is competed out by increasing the holo concentration. Thus our data indicate that the effects of apo are reversible but cannot differentiate between apo and holo binding to the same or different sites on Apaf-1. It is possible that apo, while binding at the same site or sites as holo, lacks the conformation necessary to correctly present all the binding sites to Apaf-1. As a result apocytochrome c binds only a subset of these sites on Apaf-1 at any one time, but this is insufficient to alter Apaf-1 conformation. Alternatively, the apo form may bind to a different site from holo and as a consequence reduce the affinity of Apaf-1 for the holo form. Either model accounts for both the ability of apo to drive caspase activation and its inhibition of holo-dependent caspase activation.

Our data also show that overexpression of cytochrome c is able to block apoptosis in cells. Although we cannot exclude other possibilities, our immunolocalization studies are consistent with death being blocked by the apo form of cytochrome c binding Apaf-1. There are many possible explanations for why overexpressed cytochrome c remains in the cytosol and predominantly in the apo form. It is possible that either the translocation of the outer membrane complex or the heme lyase, both required for mitochondrial import of cytochrome c (39) become saturated. Alternatively, the N-terminal tag may inhibit mitochondrial uptake or bind a cytosolic protein so trapping cytochrome c in the cytosol. Nonetheless, it appears that enough of the overexpressed cytochrome c remains in the apo form to block cell death.

Immunolocalization using an antibody against the apo form did not detect any endogenous apocytochrome c in the cytosol of the cells examined, presumably because the speed of uptake prevents accumulation in the cytosol. However, the limits of detection and the amount of apocytochrome c protein required to block apoptosis in cells are both unknown. Thus, although Bax-induced apoptosis was blocked by overexpressing the apo form, the data neither demonstrate nor exclude a physiological role for endogenous apocytochrome c in regulating caspase activation. However, physiologically relevant modulation of Apaf-1 activity has been ascribed to several other binding partners (40–43), and although none show any sequence similarity to cytochrome c, apocytochrome c may be mimicking their mechanism of action.

In the current model of caspase activation different apoptotic stimuli activate different initiator caspases that act on a common set of effector caspases (1). Until recently, caspase-8 and caspase-9 were the archetypal initiators: -8 being the apical caspase of an extrinsic or death receptor pathway and -9 being the apical caspase of an intrinsic or mitochondrial pathway triggered by cellular stress. However, some data were inconsistent with this model; in some cells induction of apoptosis by caspase-8 involves an amplification step requiring the mitochondrial pathway (44). More recently, caspase-2 has been implicated as the apical caspase for the stress stimuli usually associated with the mitochondrial pathway (6, 7). Thus although caspase-9 has not yet been excluded as the apical or initiator caspase for all apoptotic stimuli, its major role appears to be as part of an amplification step for “true” initiator caspases like -2 and -8 (7). Two different Fas-induced pathways have been described: type 1 and type 2. In type 1 cells death receptor ligation activates more caspase-8 than in type 2 cells (38), although the reason for this is not clear. High levels of active caspase-8 in type 1 cells directly activate caspase-3 and, therefore, induce apoptosis. However, in type 2 cells the weak caspase-8 activity requires a mitochondrial amplification loop to activate caspase-3 and kill cells. Our data show that, in U2-OS cells, death receptor-mediated death was less sensitive to cytochrome c inhibition than Bax-induced death. Thus, in U2-OS cells it appears that caspase-8 kills even without amplification via cytochrome c release, indicating for the first time that U2-OS are type 1 cells. Despite the specificity of apo for Bax-induced death seen in U2-OS cells, if the mitochondrial pathway serves primarily as an amplification step for different initiators, cytochrome c overexpression will block a much wider spectrum of apoptotic stimuli than tested here.

Inappropriate apoptosis has been implicated in the etiology of neurodegenerative (45) and cardiovascular diseases (46), prompting the testing of anti-apoptotic molecules as potential therapies for these diseases. Thus caspase-3 inhibitors, blocking downstream of mitochondria, have been tested in a number of disease models (47–49). Upstream of caspase activation, minocycline blocks cytochrome c release and delays disease progression in a mouse model of amyotrophic lateral sclerosis (50). Although not involving a potential drug, the inhibition of neuronal death in a model of Parkinson’s disease by a dominant negative Apaf-1 (51) is perhaps most relevant to the data presented here. The effect of the dominant negative protein indicates that, although mitochondria may release several pro-apoptotic proteins besides cytochrome c, compromising Apaf-1-dependent caspase activation may confer a therapeutic benefit. Although it is as yet unclear if the ability of apocytochrome c to inhibit caspase activation interactions can be exploited for therapeutic gain, the data presented here show that intervention at this stage of the process is at least possible.

Given the lack of structure of apocytochrome c, apo-mediated inhibition of caspase activation may be mediated by a simple peptide. We are currently testing this hypothesis as the identification of an inhibitory peptide may lead to a small non-peptidic molecule that also has inhibitory activity. Apocytochrome c or inhibitors based on apocytochrome c may prove useful experimental tools for dissecting apoptotic pathways.

Acknowledgments—We acknowledge the kind help of Dr. D. Powell (Computer and Statistical Services, NCI, National Institutes of Health, Frederick, MD) in data analysis. Our sincere thanks go to Drs. P. Kaldis, J. Acharya, and A. Wu; Division of Cell Growth Laboratory and Regulation of Protein Function Laboratory, NCI, National Institutes of Health, Frederick, MD for valuable suggestions and critical review of the manuscript. We also thank N. Martin for excellent technical support.

REFERENCES

1. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
2. Herr, I., and Debatin, K. M. (2001) Blood 98, 2603–2614
3. Rischel, P. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
4. Wang, J., Chun, H. J., Wong, W., Spencer, D. M., and Lenardo, M. J. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 12884–12888
5. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
6. Robertson, J. D., Enoksson, M., Ssumela, M., Zhivotovsky, B., and Orrenius, S. (2002) J. Biol. Chem. 277, 29603–29609
7. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002) Science 297, 1352–1354
8. Li, P., Nijhawan, D., Budhiajoo, I., Srivinavula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
9. Rodriguez, J., and Lazebnik, Y. (1999) Genes Dev. 13, 3179–3184
10. Zou, H., Henzel, W. J., Liu, X., Lutschig, A., and Wang, X. (1997) Cell 90, 465–473
11. Benedict, M. A., Hu, Y., Inohara, N., and Nunez, G. (2000) J. Biol. Chem. 275, 8461–8468
12. Archan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., and Akey, C. W. (2002) Mol. Cell 9, 423–432
13. Hu, Y., Ding, L., Spencer, D. M., and Nunez, G. (1998) J. Biol. Chem. 273, 33489–33494
14. Adrain, C., Slee, E. A., Harte, M. T., and Martin, S. J. (1999) J. Biol. Chem. 274, 20855–20860
15. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Bhrad, A. M., Cai, J., Peng, T., Jones, D. P., and Wang, X. (1997) Science 275, 1129–1132
16. Hampton, M. B., Zhivotovsky, B., Slater, A. F., Burgess, D. H., and Orrenius, S. (1998) Biochem. J. 329, 95–99
