Importance of the redox state of cytochrome *c* during caspase activation in cytosolic extracts

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The export of cytochrome *c* from mitochondria to the cytoplasm has been detected during apoptosis. Addition of cytochrome *c* to cytosolic extracts can activate the caspases, suggesting that this export could be an important intracellular signal for initiating the apoptotic programme. We have investigated the mechanism of caspase activation by cytochrome *c*. Mitochondrial cytochrome *c* normally shuttles electrons between complexes III and IV of the electron transport chain. Interaction with these complexes is dependent on electrostatic interactions via a poly-lysine binding pocket. Cytosolic caspase activation was only observed with intact holocytochrome *c*, and increasing the ionic composition of the extracts prevented activation, suggesting that stringent allosteric interactions between cytochrome *c* and other cytoplasmic factors are necessary. Cytochrome *c* was fully reduced within 5 min of addition to the cytosolic extracts. Potassium ferricyanide could maintain cytochrome *c* in an oxidized state, but care was taken to use ferricyanide at concentrations where its polyanion effect did not cause interference. The oxidized form of cytochrome *c* was able to activate the caspases. We conclude that reduced cytochrome *c* will function in the cytoplasm; however, its reduction is not a critical step, and electron transfer from cytochrome *c* to its cytoplasmic-binding partner(s) is not necessary in the pathway leading to apoptosis.

INTRODUCTION

Caspases are a class of cysteine proteases that play a critical role during apoptosis [1,2]. They are normally present as proenzymes in the cytoplasm. Upon activation, the caspases cleave a variety of cellular substrates after aspartate residues, thereby initiating the orderly dismantling of the cell. Activation involves proteolytic conversion of a procaspase zymogen to the active form. This occurs rapidly after triggering of apoptosis, and is seen with a wide variety of apoptotic stimuli. Little is known about the earliest steps involved in procaspase activation. The best-characterized system is ligation of the Fas (CD95/APO-1) receptor, where protein–protein interactions via 'death domains' initiate the recruitment and activation of procaspases-8 and -10, which are able to cleave, and thereby activate, other members of the caspase family [3]. In the majority of other systems, however, the pathway to caspase activation is unclear.

It has recently been reported that addition of dATP and cytochrome *c* to cytosolic extracts is sufficient to initiate caspase activation [4]. Subsequently, it has been shown that cytochrome *c* is released from mitochondria into the cytoplasm of apoptotic cells, and that this may occur at a sufficiently early stage to be an important trigger of the apoptotic programme [4–6]. However, the mechanism of this proposed cytochrome *c* release is currently unclear. Mitochondria have received considerable attention in recent times with regard to their involvement in apoptosis [7]. Interest has centred around the opening of mitochondrial transition pores and the release of various proapoptotic factors. Cytochrome *c* may be released this way; however, a more specific transport of cytochrome *c* could also occur. Regardless of the mechanism, the anti-apoptotic protein Bcl-2 can block cytochrome *c* release, providing further support for the importance of this mechanism in apoptosis [5,6].

We have utilized the *in vitro* model with cytoplasmic extracts to investigate the mechanism by which cytochrome *c* activates the caspases. In its traditional role in the respiratory chain, cytochrome *c* shuttles electrons between complexes III and IV on the inner mitochondrial membrane. It has a haem group to carry electrons, and a highly conserved tertiary structure which mediates electrostatic interactions with the target complexes and the membrane [8]. Here we investigated the importance of these features, with a particular interest in determining if the redox state of cytochrome *c* is an important component in the pathway leading to caspase activation. Related haem compounds or modified cytochrome *c* were ineffective triggers, and increasing the ionic strength of the cytosol impaired the ability of cytochrome *c* to activate the caspases, indicating that critical protein–protein interactions were involved. We also showed that cytochrome *c* in the cytosol functioned while in its reduced state. However, the oxidized form could also function effectively, indicating that redox reactions of cytochrome *c* were not necessary for caspase activation.

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EXPERIMENTAL

Materials

Jurkat T-lymphocytes were obtained from the European Type Culture Collection. Rabbit anti-p17 antibodies of caspase-3 were kindly donated by Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Pointe-Claire-Dorval, Quebec, Canada). Rabbit anti-(caspase-7) antibodies were raised in our laboratory against the peptide CKPDRRSSFVPSLFSKK from the p20 subunit of caspase-7 [9]. Goat anti-(rabbit IgG)–horseradish peroxidase was from Pierce (Rockford, IL, U.S.A.). Enhanced chemiluminescence system was from Amersham Corp. (Buckinghamshire, U.K.). Acetyl-L-aspartyl-L-glutamyl-

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L-valyl-L-aspartic acid α-(4-methylcoumaryl-7-amide) (DEVD-AMC) was from Peptide Institute, Inc. (Osaka, Japan), ATP was from Boehringer Mannheim (Germany), potassium ferricyanide from BDH (Poole, England) and potassium ferrocyanide from Riedel-de Haën A.G. (Hannover, Germany). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Cell and extract preparation

Jurkat cells were cultured in RPMI-1640 media with 10% (v/v) heat-inactivated fetal calf serum and 2 mM glutamine, 100 units/ ml of penicillin and 100 mg/ml streptomycin. Cultures were incubated at 37 °C in humidified air with 5% CO_2, and kept in exponential growth phase. Cells were harvested at 500 g for 5 min, and S-100 extracts were prepared as described by Liu et al. [4]. The buffer included 20 mM Hepes (pH adjusted to 7.5 with KOH), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT) and was supplemented with 0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ ml aprotinin and 25 μg/ml N-acetyl-leucine-leucine-norleucine. After preparation, extracts were either used immediately, or stored at −80 °C until required. Protein concentrations varied from 2–5 μg/μl of extract, and were determined using the BioRad protein assay with BSA as standard.

Western blots

For protein detection with Western blot, samples were solubilized for 3 min at 100 °C in Laemmli's SDS/PAGE sample buffer. Extracted polypeptides were resolved at 130 V on 12% (w/v) SDS/PAGE gels and electrophoretically transferred to nitro-cellulose (0.2 μm) for 2 h at 100 V. Membranes were blocked overnight in 50 mM Tris (pH 7.5) with 500 mM NaCl, 1% (w/v) BSA and 5% (w/v) non-fat dried milk. They were then probed with anti-p17 antibodies (1:5000) in blocking solution without milk, followed by 1 h with secondary IgG (1:10000) in an identical solution, and then detected by enhanced chemiluminescence according to the manufacturer's instructions. Membranes were stripped and reprobed with anti-(caspase-7) antibodies (1:5000).

Measurement of caspase activity

The measurement of DEVD-AMC cleavage was modified from Nicholson et al. [10]. Extract samples (9 μl) were added to microtitre plates with 50 μl of assay buffer [100 mM Hepes (pH 7.25)/10% (w/v) sucrose/5 mM DTT/$10^{-6}\%$ NP-40/ 0.1% (w/v) CHAPS] and 50 μM acetyl-DEVD-AMC. Substrate cleavage to release free aminomethylcoumarin (AMC) ($\lambda_{\text{excitation}}$ 355 nm, $\lambda_{\text{emission}}$ 460 nm) was monitored with time at 37 °C. Units of fluorescence were converted to pmol of AMC using a standard curve generated with free AMC.

Cytochrome c reduction and apocytochrome c

The redox status of cytochrome c was determined by monitoring A_{550} on a Shimadzu UV/visible spectrophotometer with the bandwidth setting at 0.5 nm. Apocytochrome c was prepared as described by Fisher et al. [11].

RESULTS

Post-S-100 cytosolic extracts were prepared from Jurkat T-lymphocytes harvested during the exponential phase of growth [4]. Cytochrome c (400 nM) and dATP (1 mM) were added to this extract, and caspase activity was determined at various intervals. Western-blot analysis showed sequential cleavage of
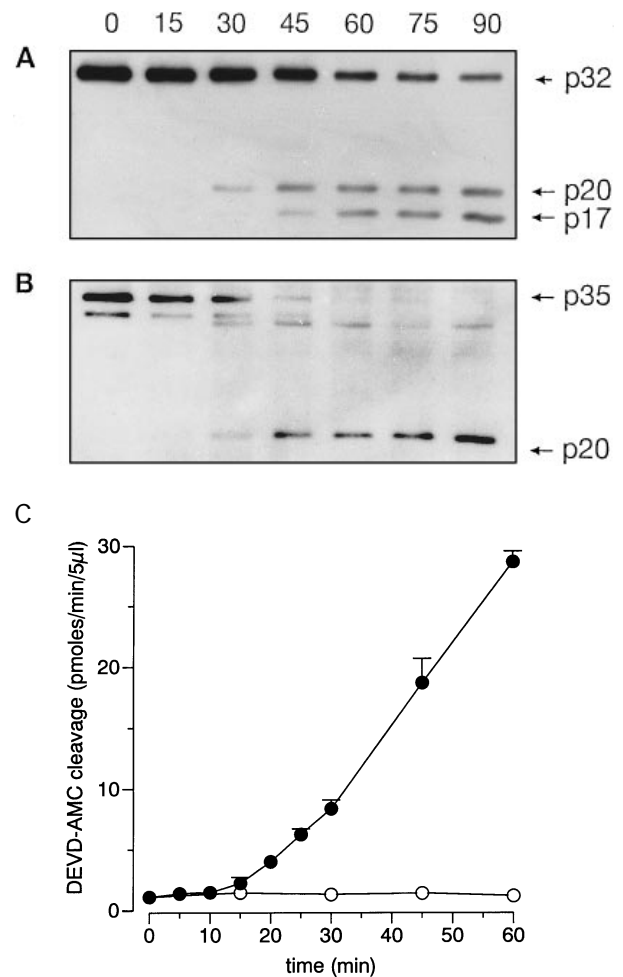

Figure 1   Time course of caspase activation by cytochrome c

Extracts were treated with cytochrome c (400 nM) and dATP (1 mM), and samples were taken at the indicated times. Western-blot analysis of (**A**) caspase-3 and (**B**) caspase-7. (**C**) Rate of DEVD-AMC cleavage at each time (means ± S.E.M. of triplicate determinations). Results are representative of 3 independent experiments. Symbols: ●, treated; ○, untreated.

the 32 kDa procaspase-3 to the p20 and p17 fragments (Figure 1A). Cleavage of 35 kDa procaspase-7 to the active p20 subunit was also detected (Figure 1B). Both processes were time-dependent and involved a lag-phase for procaspase activation. No cleavage could be detected at 15 min, but activation of the caspases was evident at 30 min.

To quantify total caspase activity in the extracts, we assayed the cleavage of the fluorophore AMC from the acetyl-Asp-Glu-Val-Asp tetrapeptide (DEVD) [10]. Caspase-3 and caspase-7 are two of the major DEVD-cleaving caspases, but other related caspases may also contribute. With this assay, a small amount of activity was detectable at 15 min, which increased rapidly after that time (Figure 1C).

Various cytochromes or haem proteins were used to determine if they could substitute for cytochrome c. We tested haemoglobin, cytochrome P-450 (2E1) and cytochrome $b5$; however, all were unsuccessful. In addition, various modifications to cytochrome c, including removal of the haem group resulting in the apoprotein, haem alone (haemin), or microperoxidase-11 (the haem group of cytochrome with amino acids 11–21) could not substitute for the fully functional cytochrome c. Biotinylated cytochrome c from
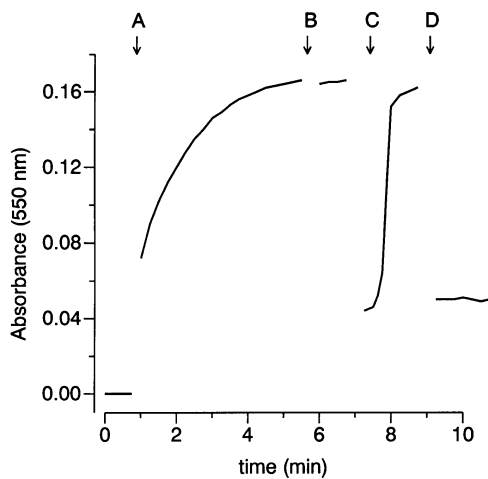
Figure 2   Reduction of cytochrome *c* in S-100 extracts

(**A**) Cytochrome *c* (4 µM) was added to extracts and its A_{550} was monitored. (**B**) Addition of 1 mM potassium ferrocyanide. (**C**) Addition of 0.5 mM potassium ferricyanide. (**D**) Addition of a further 0.5 mM potassium ferricyanide. Results are representative of several independent experiments. Further experimentation showed that the faster re-reduction of cytochrome *c* after addition of low concentrations of ferricyanide (also apparent in Figure 5) occurred because ferrocyanide considerably enhances the rate of cytochrome *c* reduction by DTT (results not shown).

Sigma (St. Louis, MO, U.S.A.) was also ineffective in this respect, emphasizing the stringency for an unmodified protein.

The requirement for a redox-active cytochrome *c* was investigated. Commercial cytochrome *c* is present in the oxidized form. Upon addition to cytosolic extracts, it was quickly reduced, as assessed by an increase in A_{550} (Figure 2). Complete reduction
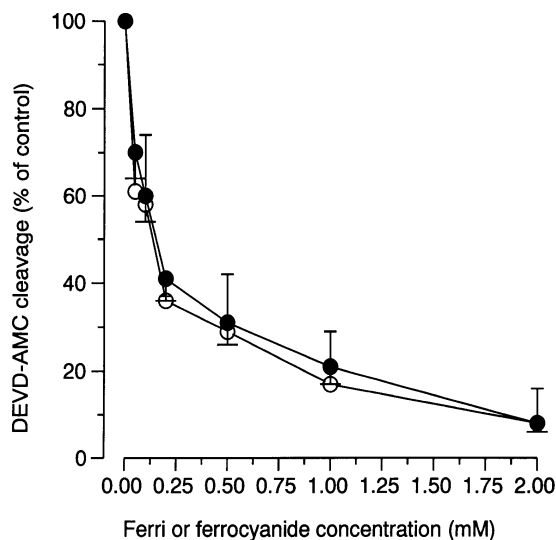

Figure 3   Effect of ferricyanide on cytochrome-*c*-mediated caspase activation

Ferricyanide (●) and ferrocyanide (○) were added to extracts with cytochrome *c* (400 nM) and dATP (1 mM). Samples were incubated for 45 min before being collected and assayed for the amount of DEVD-AMC cleavage. Results are the means ± S.E.M. of 4 independent experiments.
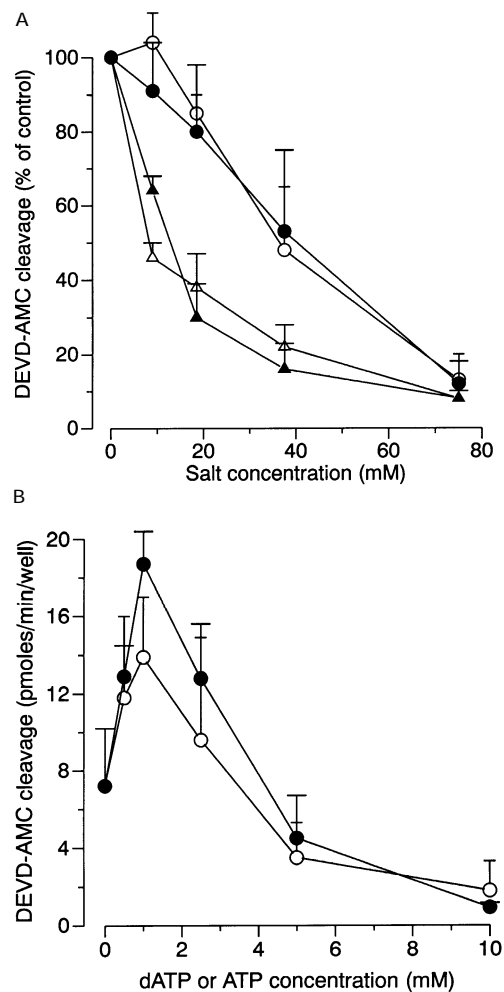

Figure 4   Effect of ionic strength on cytochrome-*c*-mediated caspase activation

(**A**) NaCl (●), KCl (○), Na$_2$SO$_4$ (▲) and Na$_2$HPO$_4$/NaH$_2$PO$_4$ at pH 7.5 (△) were added to extracts immediately before cytochrome *c* (400 nM) and dATP (1 mM) addition. Samples were incubated for 45 min before being collected and assayed for the amount of DEVD-AMC cleavage present. (**B**) A range of dATP (●) and ATP (○) concentrations were added along with 400 nM cytochrome *c*. Results are the means ± S.E.M. of 3 independent experiments.

was achieved within 5 min, i.e. approx. 10 min before significant caspase activity was detected.

Potassium ferricyanide was used to reoxidize the cytochrome *c* (Figure 2). The absorbance does not return to zero because oxidized cytochrome *c* also absorbs light at 550 nm. At concentrations exceeding 1 mM, ferricyanide was able to maintain the cytochrome *c* in its oxidized form (Figure 2). However, at lower doses, ferricyanide would initially oxidize the resident cytochrome *c*, but reduction would resume after a short interval (Figure 2). Monitoring the loss of absorbance of ferricyanide at 420 nm showed that, as expected, it was rapidly reduced to ferrocyanide (results not shown).

The ability of ferricyanide-oxidized cytochrome *c* to activate the caspases was investigated. Ferricyanide at 1 mM blocked caspase activation by over 80 % (Figure 3), and had no effect on the caspases when added at the end of the incubation period, indicating that it did not interfere with the activity of active caspases (results not shown). However, concentrations below 1 mM, which were not sufficient to maintain cytochrome *c* in its
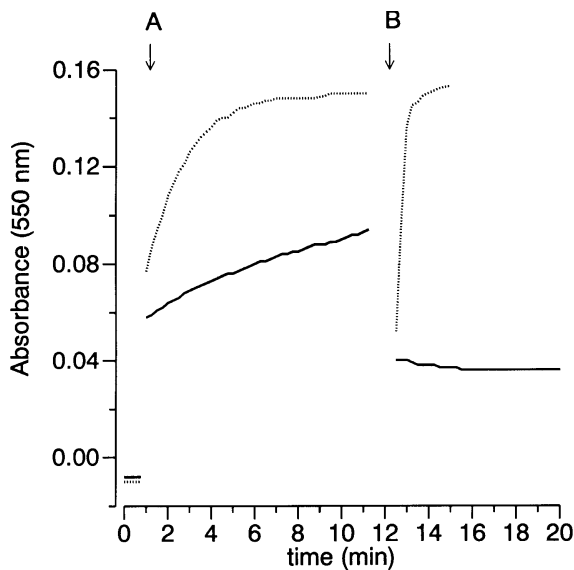
Figure 5   Cytochrome *c* reduction in the absence of DTT

DTT was left out of the S-100 extract buffer, and the rate of cytochrome *c* reduction at 550 nm (solid line) was compared with extract containing 1 mM DTT added back (dotted line). (**A**) Addition of 4 μM cytochrome *c*. (**B**) Addition of 50 μM ferricyanide. Results are representative of several independent experiments.

Figure 6   Effect of ferricyanide on cytochrome-*c*-mediated caspase activation in DTT-deficient extracts

Ferricyanide ($\bullet$) and ferrocyanide ($\bigcirc$) were added to extracts immediately before cytochrome *c* (400 nM) and dATP (1 mM). Samples were incubated for 45 min before being collected and assayed for the amount of DEVD-AMC cleavage present. Results are the means $\pm$ S.E.M. of 4 independent experiments.

oxidized form (Figure 2), were also effective at blocking caspase activation (Figure 3). Furthermore, although the addition of potassium ferrocyanide did not alter the redox state of cytochrome *c* (Figure 2), this compound was equally efficient in blocking caspase activation (Figure 3). Therefore it appears that the iron hexacyanide complexes were having an effect on cytochrome *c*, independent of their ability to alter its redox state.

We hypothesized that this was due to the polyanionic nature of the hexacyanide complexes. The ability of cytochrome *c* to perform its normal mitochondrial function depends on physical interactions with the proteins of the electron transport chain that it shuttles electrons between. The evolutionarily highly conserved conformation of cytochrome *c* includes a cluster of positively charged lysine residues which mediate these interactions [8]. The negatively charged iron hexacyanides could interfere with allosteric interactions mediated through these residues. We investigated the effect of other ions on cytochrome-*c*-mediated caspase activation. The S-100 extraction buffer already contained 20 mM Hepes, 10 mM KCl and 1.5 mM MgCl$_2$. Addition of increasing concentrations of various salts blocked caspase activation, with the phosphate and sulphate anions being particularly effective (Figure 4a).

We also investigated whether dATP and ATP would inhibit caspase activation by cytochrome *c*. Cytochrome *c* alone can trigger caspase activation, but not as efficiently as when dATP is also present. Addition of either dATP or ATP initially enhanced caspase activation by cytochrome *c*, but these polyphosphate anions acted as effective inhibitors when their concentrations rose above 2 mM (Figure 4b).

The polyanionic inhibition by ferricyanide led us to investigate the ability of other compounds to oxidize cytochrome *c*. However, those that were found to be strong enough to oxidize cytochrome *c* in the extracts would either cause cytochrome *c* to be modified or directly oxidize the caspases. Therefore we attempted to prevent the initial reduction of cytochrome *c*. DTT can effectively
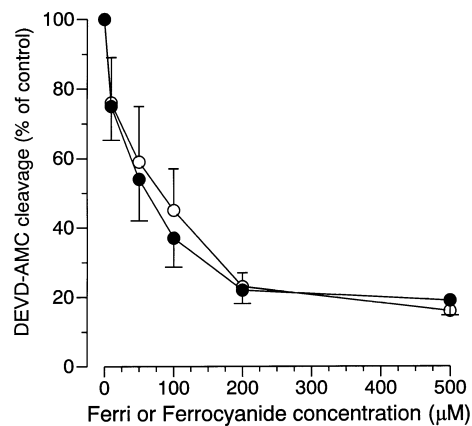
reduce cytochrome *c* (results not shown), and is present at a concentration of 1 mM in the cytoplasmic extract. Removal of DTT from the extract buffer did reduce the rate of cytochrome *c* reduction, but in its absence, complete reduction still occurred (Figure 5). Reduced glutathione (GSH) will also be present, but this is unable to reduce cytochrome *c* (results not shown). A range of inhibitors were then used in an attempt to prevent reduction in the DTT-deficient extracts. Superoxide dismutase (500 units/ml of the Cu/Zn- or Mn- or Fe-enzyme) had no effect on either reduction or caspase activation, demonstrating that superoxide plays no role in this system. Other compounds tried were ascorbate oxidase (10 units/ml), the NADPH-diapharose inhibitor dicumarol (10 μM), the flavoprotein inhibitor diphenyleneiodonium (100 μM) and finally, NADP$^+$-2',3'-dialdehyde (1 mM) and 3-aminopyridine NADP$^+$ (1 mM), inhibitors of potential NAD(P)H-utilizing cytochrome reductases that might have been present in the cytosol [12]. None of these effectively prevented cytochrome *c* reduction (results not shown).

However, one consequence of removing the DTT from the S-100 extraction buffer was that considerably less ferricyanide was required to maintain cytochrome *c* in its oxidized form (Figure 5). The concentration of ferricyanide required to maintain all of the cytochrome *c* in an oxidized state during the 45 min of the assay varied between 50 μM and 100 μM, depending on the extract preparation. At 100 μM ferrocyanide, the reduced cytochrome *c* functioned 50 % as effectively as in untreated extract (Figure 6). In the presence of 100 μM ferricyanide, the completely oxidized cytochrome *c* functioned with the same efficiency (Figure 6), indicating that cytochrome *c* reduction is not necessary for caspase activation.

DISCUSSION

Current apoptosis research has been stimulated by the newly discovered export of holocytochrome *c* from mitochondria to the cytoplasm, along with the realization that cytochrome *c* is capable of activating the cytoplasmic caspases. Various triggers result in the early detection of cytochrome *c* in the cytoplasm [5,6], but, as yet, we are unable to determine the importance of this process for subsequent apoptotic events. Further progress

requires the identification and specific inhibition of the mechanisms by which cytochrome *c* is released.

We have investigated caspase activation by cytochrome *c* arriving in the cytoplasm. This is made possible by the reported ability of exogenous cytochrome *c* to trigger caspase activity in cytoplasmic extracts [4]. First, we have shown that caspase activation is specific for cytochrome *c*; even minor modifications render it ineffective. This suggests stringent allosteric interactions with other cytoplasmic factors. The sensitivity of the system to changes in ionic strength suggests the polylysine pocket that cytochrome *c* uses to interact with its binding partners in the mitochondria may also be used in the pathway leading to caspase activation in the cytoplasm.

Our major focus was to determine whether the redox status of cytochrome *c* plays an important role in the pathway leading to caspase activation. The ultimate function of cytochrome *c* in the mitochondria is to transfer electrons between its binding partners. It was clear that exogenous cytochrome *c* was rapidly reduced upon addition to cytosolic extracts. It is highly likely that cytochrome *c* in the cytoplasm will also exist in the reduced form. It is probable that this protein leaves the mitochondria in this state, having been reduced by either complex III or mitochondrial superoxide. Any oxidized cytochrome *c* arriving in the cytoplasm would also be targeted by ascorbate or a variety of cytochrome reductases. None of the inhibitors we used was able to block cytochrome *c* reduction in our cytosolic extracts, so we were unable to use this tool to determine the importance of the redox state in caspase activation.

We used the electron acceptor potassium ferricyanide to keep the cytochrome *c* oxidized. Caspase activation did occur when all of the cytochrome *c* present in the system was oxidized. We therefore conclude that the reduction of cytochrome *c* is not a critical regulatory step in the pathway leading to apoptosis.

The experiments with ferricyanide were complicated initially by the ability of the iron hexacyanide complexes to inhibit cytochrome-*c*-mediated caspase activation, independent of their effects on the redox state of the protein. Further studies showed that the system was sensitive to ionic strength, particularly the addition of polyvalent anions. The most disruptive of these were the iron hexacyanides and the polyphosphate anions ATP/dATP. It has been shown that anions directly bind cytochrome *c* around the polylysine binding pocket [13,14]. This binding of ATP may be physiologically important for feedback inhibition of the respiratory chain [15].

The sensitivity of cytochrome *c* to increases in ionic concentration leads us to questions about whether this pathway could work under physiological conditions, or if it could be a site for regulation of the system. Polyvalent anions were the most effective; however, a large portion of intracellular anions are macromolecules and hence are unlikely to interfere. Potassium is the predominant intracellular cation, and its intracellular concentration will exceed 75 mM, the concentration at which we observed complete inhibition with KCl. However, at this con-

centration, binding of chloride to cytochrome *c* could be the major disruptive influence and chloride is only present at 5–15 mM intracellularly. The concentration of dATP is normally around 50 μM and is therefore unlikely to be present at inhibitory concentrations. In contrast, ATP is present at concentrations of 2–10 mM and, at this level, could be too high to allow the system to function. Inhibitory concentrations of ATP may differ between the intracellular milieu and our cytosolic extracts. The S-100 cytosolic extracts (2–5 mg protein/ml) are more dilute than the cytoplasm with respect to protein and other ATP-binding proteins, which may alter the sensitivity of the system to changes in ionic strength. Furthermore, the addition of exogenous cytochrome *c* may not be a good model for the cytosolic export of endogenous mitochondrial cytochrome *c*, which may be accomplished in association with other regulatory factors. More work is required before significant conclusions about ionic regulation of the system *in vivo* can be made.

In summary, cytochrome *c* will be exported to the cytoplasm where it will specifically associate with (as yet) unidentified factors. This cytochrome *c* will be in its reduced state. However, subsequent events, i.e. activation of the caspase cascade and apoptotic dismantling of the cell, are not reliant on the reduction of cytochrome *c*, or any electron transfer from the cytochrome *c* to its cytoplasmic-binding partners.

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