Determinants of Cytochrome c Pro-apoptotic Activity

THE ROLE OF LYSINE 72 TRIMETHYLATION

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Cytochrome c released from vertebrate mitochondria engages apoptosis by triggering caspase activation. We previously reported that, whereas cytochromes c from higher eukaryotes can activate caspases in Xenopus egg and mammalian cytosols, iso-1 and iso-2 cytochromes c from the yeast Saccharomyces cerevisiae cannot. Here we examine whether the inactivity of the yeast isoforms is related to a post-translational modification of lysine 72, N-e-trimethylation. This modification was found to abrogate pro-apoptotic activity of metazoan cytochrome c expressed in yeast. However, iso-1 cytochrome c lacking the trimethylation modification also was devoid of pro-apoptotic activity. Thus, both lysine 72 trimethylation and other features of the iso-1 sequence preclude pro-apoptotic activity. Competition studies suggest that the lack of pro-apoptotic activity was associated with a low affinity for Apaf-1. As cytochromes c that lack apoptotic function still support respiration, different mechanisms appear to be involved in the two activities.

Cytochrome c has been identified as an important participant in apoptosis. In living cells, cytochrome c is present in the intermembrane space of mitochondria, where it plays an indispensable role in respiration. During apoptosis, however, cytochrome c translocates to the cytosol by mechanisms that are still unclear (reviewed in Refs. 1 and 2). In the cytosol, cytochrome c binds to Apaf-1 in a dATP/ATP-dependent manner (3), precipitating the oligomerization of Apaf-1 (4–8). The ensuing recruitment and activation of caspase-9 results in activation of further caspases, such as caspase-3, which orchestrate the final packaging of the apoptotic cell (see, e.g., Refs. 9–11).

Cytochromes c are encoded by nuclear genes and translated in the cytosol. The resulting apocytochromes c are subsequently translocated through the outer mitochondrial membrane. In the intermembrane space, heme is covalently attached by thioether linkages to two cysteine residues at positions 14 and 17 of the protein, a reaction catalyzed by the enzyme, cytochrome c hemoprotein. Heme attachment is accompanied by a transition from a partially extended to a more compact conformation. Cytochrome c import depends neither on the electrochemical potential across the inner mitochondrial membrane nor on the presence of ATP (12). The sequences involved in targeting cytochrome c to mitochondria have been investigated in yeast and do not involve specific N-terminal sequences (13).

The complete sequences of 96 eukaryotic cytochromes c have been determined, constituting a rich source of information for evolutionary analysis and for evaluating structure-function relationships (14). A total of 35 out of 103–115 residues are conserved in cytochromes c from animals, plants, and fungi, with 17 of these 35 invariant residues found in a region spanning residues 68–92 (15). In contrast, the N-terminal regions are highly variable; vertebrate cytochromes c begin with acetyl glycine at position 1, whereas the others have N-terminal extensions that may or may not be acetylated (Fig. 3A).

Cytochromes c are subject to the same co-translational processing events as can occur with other proteins synthesized in the cytosol, including cleavage of N-terminal methionine residues and N-terminal acetylation. These co-translational processes are dependent on the sequences of the N-terminal regions and are to some extent conserved in different species. For example, vertebrate cytochromes c are at least partially acetylated when expressed in yeast, although native yeast cytochromes c are not acetylated (16). Furthermore, mutant forms of yeast cytochromes c are acetylated when they have the appropriate N-terminal sequences (17).

Specific lysine residues are methylated in cytochromes c from certain eukaryotes, including fungi, plants, and some protozoa, but not in animals (18, 19). Cytochromes c from many fungi, including Saccharomyces cerevisiae, contain trimethyllysine at the single position 72 (20). It is believed that apocytochrome c is trimethylated co- or post-translationally by a specific methyltransferase before uptake by mitochondria (21, 22). In fact, vertebrate cytochromes c are at least partially trimethylated when expressed in yeast (16, 19). The biological function and importance of cytochrome c methylation is unknown, and mutant forms of iso-1-cytochrome c lacking trimethyllysine 72 retain nearly full activity in vivo (19, 23).

What features of cytochrome c affect its pro-apoptotic function? As expected, heat denaturation abolishes activity (24). The heme moiety is required, as holocytochrome c, which lacks...
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A heme group, possesses no pro-apoptotic activity (25). Surprisingly, however, the redox capacity of holocytochrome c, which is central to the molecule’s function in oxidative phosphorylation, is not required for apoptotic activity, as redox-inactive zinc- and copper-substituted cytochromes c exhibit strong pro-apoptotic activity (24). Microperoxidase, an enzyme-degraded product of cytochrome c, consisting of the heme group and approximately 11 amino acids, has no pro-apoptotic activity (24). Collectively, these findings indicate that certain structural features, rather than redox activity, of the holoprotein are required for its pro-apoptotic activity.

Particularly interesting is the complete lack of pro-apoptotic activity of cytochromes c from the yeast S. cerevisiae (24, 26) despite extensive sequence similarity between the yeast and mammalian proteins. S. cerevisiae expresses two isoforms of cytochrome c, iso-1 and iso-2, which constitute 95% and 5% of the total cytochrome c complement (27), and which possess similar respiratory activities (28). Here we investigate the molecular basis for the lack of yeast cytochrome c pro-apoptotic activity, with particular regard to the N-ε-trimethylation of lysine 72.

EXPERIMENTAL PROCEDURES

Materials—Cytochromes c from bovine, horse, pigeon, and tuna heart (95% purity) were obtained from Sigma. S. cerevisiae iso-1 (N52I) and iso-2 cytochromes c were fractionated as described previously (29), whereas three unfractionated preparations of iso-1/iso-2 mixtures (~95:5) from MMY290 normal yeast were prepared in two separate laboratories (Sherman, Bredesen) by standard methods (29) or purchased from Sigma. Escherichia coli-expressed horse heart cytochrome c (HHCc; courtesy of H. Roder and M. P. Kirchmnikov) and wild-type iso-1 (72/37/102T) were produced as described previously (30).

Trimethylated HHCc and non-trimethylated K72R/C102T iso-1 cytochrome c were purified as described previously (29) after expression in a yeast strain (B6748) deficient in both the iso-1 and iso-2 genes. Mass spectroscopic analysis (31) of the yeast-expressed HHCc gave a single detectable peak of mass 12,357, whereas that of native protein gave a single peak of mass 12,355. This indicated that both proteins had the initiator methionine residue removed and the N-terminal glycine acetylated, but that the yeast-expressed protein was also trimethylated (additional 42 Da). Confirmation that lysine 72 trimethylation was responsible for the increased mass of yeast-expressed HHCc came from mass spectroscopic analysis of tryptic digests. Native HHCc tryptic digest contained a fragment of mass 2082 (residues 56–72; GITWSSSGLY) of the same mass shifted to 2125. Together, these analyses indicated that the yeast-expressed HHCc was close to 100% trimethylated on lysine 72 and, as reported previously (16, 32, 33), contained no modification compared with native protein.

Yeast-expressed rat cytochrome c (RHc) was expressed and purified by Koehy et al. (32). It is estimated to be only about 20–50% trimethylated, based on peak sizes of trimethylated and non-trimethylated chymotryptic peptides LENPKKY (residues 68–74) separated by reverse-phase HPLC (32). When expressed in yeast, both trimethylated and N-acetylated of RHc are less efficient than that of HHCc (16). N-Acetylated (25% of total) and non-acetylated fractions of this partially trimethylated RHc were separated by cation-exchange HPLC and referred to as fractions I and II, respectively (32). N-Acetylation was indicated by NMR of each fraction and by Edman degradation of the chymotryptic peptide GDVEKKKIF (residues 1–10) separated by reverse-phase HPLC chromatography. Thus, yeast-expressed RHc fractions I and II are N-acetylated and non-acetylated, respectively, but both are equally trimethylated (32).

Concentration of cytochrome c stock solutions were determined using the A_{280}, of fully reduced samples (dithiothreitol added in excess) and the molar extinction coefficient, 27.7 × 10³ M⁻¹ cm⁻¹.

Mass Spectrometry—Mass spectrometric analyses were carried out on a Voyager Elite mass spectrometer (PerSeptive Biosystems). Horse heart myoglobin (16952 Da) was used as an external standard. The positions of the protease cleavage sites in the cytochrome c sequence were identified by considering the molecular masses of the polypeptide fragments detected by mass spectroscopic analysis and the specificity of trypsin.

Amino Acid Numbering in Cytochromes c—To simplify nomenclature, the numbering of amino acid residues is according to the vertebrate, rather than the yeast, system (yeast iso-1 contains 5 additional N-terminal residues), and “yeast” in this work refers only to S. cerevisiae.

Preparation of Xenopus and Mammalian Extracts—Xenopus egg cytochrome c was isolated as described previously (34, 35). Cytosol from mouse T cell A1.1 hybridoma (36) and mouse embryonic fibroblast (MEF) cell lines was obtained as follows. Cells were cultured to log phase in RPMI 1640 containing 10% fetal bovine serum, harvested, and washed twice in phosphate-buffered saline prior to washing in lysis buffer (250 mM sucrose, 20 mM HEPES/KOH pH 7.5, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol, 5 μg/ml cytochalasin B, and 50 μg cycloheximide) and suspension in a volume of lysis buffer (supplemented with Complete protease inhibitor mixture, Roche Molecular Biochemicals) twice that of the cell pellet. Cells were placed on ice for 30 min prior to homogenization (100–200 strokes) with a Potter-Elvehjem Teflon-coated pestle until cell lysis was ~50% as determined by trypan blue uptake. Non-lysed cells and nuclei were removed by centrifugation twice at 300 g for 1 min. Cytosol was separated from heavy membranes (mitochondria) by centrifugation at 15,000 × g for 15 min. Cytosol contained ~5% of cellular cytochrome c as determined by Western blot comparison of the resulting cytosolic and mitochondrial fractions (data not shown; Pharmingen anti-cytochrome c antibody clone 7H8.2C12). Xenopus egg and A1.1 cytosolic extracts contained 40 and 20 mg/ml protein, respectively (Biorad method).

Cytosol Incubations with Cytochrome c—The pro-apoptotic activity of cytochromes c was assessed by their ability to activate caspases present in cytosolic extracts. Titration of cytochrome c provided a comparison of potency between variants. Generally, 1 μl of cytochrome c stock solution (or vehicle) was added to 10 μl of cytosol and incubated at 22 °C for up to 4 h. The ensuing caspase activation was assessed as follows. Extract aliquots (2 μl) were taken at several time points and incubated at 22 °C with the colorimetric peptide substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA, 40 μM; Biomol, Plymouth Meeting, PA) in 200 μl of a buffer similar to that used to make the extracts (250 mM sucrose, 20 mM HEPES/KOH, pH 7.5, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol). Production of the cleaved pNA (p-nitroanilide) was monitored at 22 °C by an increase in absorption at 405 nm (SPECRAMAX 250 spectrophotometer) and calibrated against a standard curve of pNA (Sigma). Different vehicles used for cytochrome c stock solutions prepared in different laboratories had no effect on caspase activation by HHCc; (data not shown). All cytosol incubations were supplemented with an ATP regenerating system consisting of 10 mM phosphate, 2 mM ATP, and 150 μg/ml creatine phosphokinase. A1.1 and MEF cytosolic extracts were also supplemented with 1 mM ATP.

Mitochondrial Incubation—Mitochondria were prepared from yeast iso-1 and iso-2, which constitute 95% and 5% of yeast cytochrome c, respectively. Yeast mitochondria were obtained as described previously (37, 38) from three different S. cerevisiae strains: B6748 lacks both cytochrome c genes, cyc1 (iso-1) and cyc7 (iso-2); B7553 has cyc1 integrated back at the cyc1 locus; and MMY290 is a normal control. Mitochondria and other fractions were either used immediately or stored at ~80 °C.

Mitochondrial incubations contained a mixture of Xenopus cytosol, 10% v/v light membrane, 4 × 10⁶ rat liver nuclei/μl, and the ATP-regenerating system. Mitochondria were added at 0.66, 1.0, or 3.0 mg of protein/ml. Mitochondrial protein content was compared among batches by absorption at 280 nm of mitochondrial aliquots in 0.6% SDS and calibrated using the Biuret assay. Apoptotic nuclear morphology of chromatin condensation, margination, and beading was assessed using Hoechst 33258 (bisbenzimide) staining (34).

Western Blotting—To examine cytochrome c release from yeast mitochondria incubated in Xenopus egg cytosol, extract aliquots were removed at various times and centrifuged (12,000 × g, 5 min) to pellet mitochondrial membranes. Cytosolic supernatants were removed and filtered through 0.22- and 0.1-μm filters (Ultrafree-MC; Millipore) to remove any mitochondrial membrane contaminants. Equivalent mitochondrial and cytosolic aliquots were separated on 12% SDS-polyacrylamide gels. Western blots were probed with rabbit antisera to yeast cytochrome c, followed by horseradish peroxidase-labeled anti-rabbit antibody (Amersham Pharmacia Biotech), and developed with enhanced chemiluminescence (Amersham Pharmacia Biotech).
RESULTS

Limited Phylogenetic Conservation of Cytochrome c Pro-apoptotic Activity—We reported previously that vertebrate, but not yeast, cytochromes c, are able to activate caspases in Xenopus egg cytosol. HHCc (0.03–0.3 μM) was added to Xenopus cytosol and incubated for 2 h at 22 °C. At the indicated times, aliquots were tested for the ability to cleave the caspase-3 substrate DEVD-pNA. B, species comparison of cytochromes c pro-apoptotic activity in Xenopus cytosol. Cytochromes c purified from the indicated species were added at a range of concentrations to Xenopus egg cytosol and incubated as in A. DEVDase activity at 1 h is shown. C, species comparison of cytochromes c pro-apoptotic activity in cytosolic extract from mammalian A1.1 cells. Incubations were performed as in A and B except that data were taken at 2 h. Yeast preparations 1, 2, and 3 were iso-1/iso-2 mixtures obtained from normal yeast by three different sources (see “Experimental Procedures”).

Non-trimethylated Yeast Cytochrome c Lacks Pro-apoptotic Activity—To test whether yeast cytochrome c lacks pro-apoptotic activity due to the trimethylation of lysine 72, we tested the activity of non-trimethylated iso-1 cytochromes c. One iso-1 variant, J72K/C102T, is the wild type expressed in E. coli and...
Fig. 2. Isolated yeast mitochondria incubated in Xenopus extracts release cytochrome c, but do not induce apoptotic changes. Mitochondria isolated from Xenopus eggs or from yeast expressing either iso-1 or iso-2 cytochromes c (ΔCc; R6748), only iso-1 (C7553), or both iso-1 and iso-2 (MMY290), were incubated in reconstituted Xenopus extracts containing rat liver nuclei. Mitochondrial fractions were added at 0.7, 1.0, or 3.0 mg/ml and incubated at 22°C for 4 h. A, Western blot of yeast cytochrome c in cytosolic and mitochondrial fractions at 4 h. Cytosolic and mitochondrial samples each derived from 5 μl of incubated extract mix. To estimate cytochrome c concentrations, the last three lanes of the same gels were loaded with 5 μl of 1, 3, 3, and 10 μM yeast cytochrome c stocks (= 9:1 iso-1:iso-2; Sigma). Anti-cytochrome c antibody does not recognize Xenopus cytochrome c (lanes 1 and 2). B, yeast mitochondria fail to induce caspase activation or apoptotic nuclear changes in Xenopus egg cytosol. As previously determined, aliquots were assessed for DEV-Dase activity (left) and for nuclear disintegration (right). Data are representative of two experiments.

thus remains non-trimethylated due to the absence of the methylase in E. coli. A second iso-1 variant, K72R/C102T, was expressed in cytochrome c-deficient yeast (strain B-6748; see “Experimental Procedures”), and is non-trimethylated due to the substitution of arginine for lysine. Both proteins contain a C102T mutation to increase stability of the purified protein (40).

Neither of the non-trimethylated iso-1 proteins exhibited pro-apoptotic activity in Xenopus and mammalian extracts (Fig. 5). A third non-trimethylated iso-1 variant expressed in yeast, K72A/C102T, also lacked pro-apoptotic activity.2 It is unlikely that some unknown aspect of expression in E. coli is responsible for the inactivity of J72K/C102T, as E. coli-expressed HHCCc retained full pro-apoptotic activity (Fig. 6C). It is also unlikely that the C102T substitution abrogated pro-apoptotic activity, as all of the active cytochromes c in Fig. 1 possess Thr-102 (Fig. 3A). Together, these results indicate that elements of the yeast cytochrome c sequence, apart from trimethylation of lysine 72, preclude pro-apoptotic activity in vertebrate cytosolic extracts.

DISCUSSION

To continue our investigation of the pro-apoptotic behavior of cytochrome c (24, 26), we have examined the lack of activity of the yeast isoforms, with particular regard to the role of lysine 72 N-ε-trimethylation. Cytochromes c from several higher eukaryotes can substitute for Xenopus cytochrome c to activate caspases in Xenopus egg and mammalian cytosol (Fig. 1). The phylogenetic conservation of cytochrome c pro-apoptotic activity is consistent with the high conservation of amino acid sequences and structures of eukaryotic cytochromes c (Fig. 2A; Ref. 15) and likely reflects a conserved role for these proteins in apoptosis as well as in respiration. A similar functional and structural conservation for proteins in apoptosis is observed with the Bel-2, caspase, and inhibitor of apoptosis proteins (IAP) protein families (reviewed in Ref. 41).

It was intriguing, therefore, to find that S. cerevisiae iso-1 and iso-2-cytochromes c possessed no pro-apoptotic activity (Fig. 1). Sequence comparison revealed several differences between the yeast isoforms and the pro-apoptotic cytochromes c (Fig. 3A). However, the trimethylation of lysine 72 was of special interest because lysine 72 is positioned in the most highly conserved region and is one of the lysine residues important in binding to redox partners, including cytochrome c oxidase and the cytochrome bc1 complex (42–46).

Lysine 72 is in fact important for the pro-apoptotic activity of cytochrome c, as HHCCc expressed in yeast no longer possessed pro-apoptotic activity (Fig. 4). This HHCCc is modified by trimethylation of lysine 72. As trimethylation of lysine 72 in HHCCc blocks pro-apoptotic activity, it was possible that a similar trimethylation accounted for the absence of activity of yeast cytochromes c. This was not the case, however, as non-methylated yeast variants also lacked the ability to activate caspases (Fig. 5). Collectively, the evidence suggests that non-trimethylated lysine 72 is necessary for pro-apoptotic activity, but that other elements in the yeast sequence also contribute to the lack of caspase-activating function.

Non-trimethylated yeast cytochrome c may be inactive due to either the lack of some feature(s) present in active forms, or the presence of some feature(s) negating activity. There are, in fact, several observed structural differences between yeast and tuna proteins as determined by x-ray diffraction (47). Large conformational differences between the peptide backbones of the two proteins occur in two regions. The first of these is an extended N terminus in iso-1 (Fig. 3A), which projects parallel to the rear of the molecule (47). Second, the region around residue 56 does not extend into solvent to the degree seen in the tuna protein, explainable by altered intramolecular hydrogen bonding of amino acid substitutions T40S and I57V. Additional subtle differences in the side-chain positions of conserved residues are also observed between iso-1 and tuna cytochromes c, i.e. in iso-1, the guanidinium group of the highly conserved Arg-38 is less buried than in the tuna protein due to different

2 R. Kluck, C. Szabo, E. Y. T. Chien, E. Oldfield, and D. Newmeyer, unpublished data.
intramolecular hydrogen bonds formed by the amino acid substitution L35I. Moreover, the alkyl portion of the invariant Arg-91 is positioned differently in yeast due to altered hydrogen bonding of the M65S amino acid substitution. Finally, it is possible that K13R may diminish pro-apoptotic activity (of yeast and insect proteins; Figs. 1 and 3), as surface lysine residues have been implicated in binding to both respiratory (see Introduction) and apoptotic (described here) partners.

The acetylated N terminus present in all vertebrate cytochromes c, but lacking in native yeast cytochromes c, has no effect on pro-apoptotic activity, as N-acetylated and non-N-acetylated fractions of yeast-expressed RHCc had equal pro-apoptotic activities (data not shown). Further studies using mutated vertebrate and yeast cytochromes c will aid in delineating the requirements for pro-apoptotic function in cytochrome c.

How does lysine 72 trimethylation block pro-apoptotic activity? Trimethylation does not alter the positive charge of lysine at physiological pH 7.4. Trimethyllysine 72 of iso-1 has a fully extended conformation (Fig. 3B), projecting directly toward the front of the molecule in a relatively rigid position (14). Although the structure of a trimethylated mammalian cytochrome c has not been determined, the presence of three methyl groups may contribute steric hindrance sufficient to interfere with binding to Apaf-1. As respiratory function is not significantly altered by trimethylation (48–50), it appears that distinct mechanisms are involved in the mitochondrial and pro-apoptotic activities of cytochrome c. This is consistent with our previous finding that the cytochrome c redox activity, essential for respiration, is not required for apoptosis (24). Might the evolutionary selection for both respiratory and apoptotic activities explain the extremely high sequence homology of eukaryotic cytochromes c, especially if the requirements are different?

Despite findings that trimethylation does affect the molecule in subtle ways (22, 30), the biological reason for retaining the methylase through evolution in yeast has remained elusive (18, 19). Our finding that trimethylation blocks pro-apoptotic activity initially promised a resolution to this issue. However, as discussed below, in yeast there is no known apoptotic pathway downstream of cytochrome c that might need to be inhibited by trimethylation. Thus, there may remain some unknown function for methylation in yeast and plants.

The possibility that yeast cytochrome c still participates in some apoptosis-like process in yeast is unlikely, as yeast lack Apaf-1, caspases, and several other components of the apoptotic machinery (51). Some upstream apoptotic components may be present, however, as mammalian Bax or Bak can cause cell death (or decreased growth) in S. cerevisiae and Schizosaccharomyces pombe (52–56), in some cases associated with mitochondrial cytochrome c release (57). Furthermore, Bak, Bax (57, 58), and Xenopus egg cytosol (Fig. 2) can release cytochrome c from isolated yeast mitochondria. Further study will be required to determine whether this phenomenon is physiologically relevant. If so, it could imply that both yeast and metazoan cells possess a common cell death-related mitochondrial permeabilization mechanism. Within whole yeast cells, the resulting mitochondrial lesions may precipitate cell death by non-apoptotic pathways. In contrast, metazoans engage caspases to ensure the orderly packaging and removal of a potentially harmful dying cell, an issue less important to unicellular organisms.
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Competition experiments suggest the former, in that high concentrations (100 μM) of each of the non-apoptotic variants (J72-iso-1, J72K-iso-1, K72A-iso-1, trimethyl-HHCc) did not inhibit the pro-apoptotic activity of low concentrations (0.3 μM) of HHCc (data not shown). Together with reports (59) of a high affinity (K ~ 10^{11} M^{-1}) of Apaf-1 for Zn-substituted HHCc, a variant with strong pro-apoptotic activity (24), the inability of the inactive cytochromes c to compete out HHCc suggests that their affinity for Apaf-1 is low.

In conclusion, these studies show that both the yeast cytochrome c amino acid sequence and the trimming of lysine 72 preclude pro-apoptotic activity, likely by reducing affinity for Apaf-1. Cytochromes c that lack pro-apoptotic activity still retain respiratory function, indicating that distinct mechanisms are involved in the pro-life and pro-death functions of cytochrome c.

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FIG. 4. Trimmingly at lysine 72 blocks pro-apoptotic activity of horse heart cytochrome c. Either native or trimethylated (yeast-expressed) HHCc was added at the indicated concentrations to Xenopus egg (A) or mammalian cytosolic extracts (B) and incubated at 22 °C for 4 h. DEVDase activity at 2 h is shown.

FIG. 5. Non-trimethylated yeast cytochrome c lacks pro-apoptotic activity. Native HHCc, yeast-expressed K72R iso-1, E. coli-expressed HHcc or E. coli-expressed wild-type iso-1 (J72K) were added at the indicated concentrations to Xenopus egg (A) or mammalian cytosolic extracts (B) and incubated at 22 °C for 4 h. DEVDase activity at 2 h is shown.

As cytochrome c activation of caspases is mediated by complexing with Apaf-1 (3), the failure of yeast cytochromes c to activate caspases may be due either to a failure to bind to Apaf-1, or to a failure to alter Apaf-1 function once bound.
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