Activation of caspases triggered by cytochrome c in vitro

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Abstract Previous studies have shown that Apaf-1 and caspase-9 in the presence of cytochrome *c* and dATP can form an initiating complex for an apoptotic protease cascade. We have developed a cytochrome *c*-dependent in vitro system in which caspases downstream of this initiation complex are activated. The activation of caspase-9 from zymogen form to active dimeric protease requires intrinsic enzymatic activity. In contrast, caspase-3 and caspase-7 zymogens are proteolytically processed by active caspase-9. Activation of the above caspases is blocked by a dominant negative form of caspase-9. The in vitro system displays surprising specificity in that other caspases, including 1, 2, 4, 8, 10, and 13, are not activated.

Key words: Apoptosis; Cytochrome *c*; dATP; Apaf-1; Caspase; Cell-free

1. Introduction

Apoptosis, or programmed cell death, is an evolutionarily conserved process central to the normal development and homeostasis of multicellular organisms [1]. Previous genetic studies of the nematode *Caenorhabditis elegans* identified three core components of the cell death machinery: CED-3, CED-4 and CED-9. CED-3 and CED-4 function to kill cells whereas CED-9 protects cells from death [2–6]. These central players of cell death in the nematode are conserved in vertebrates. CED-3 is equivalent to the emerging family of mammalian cysteine proteases termed caspases that cleave substrates following an Asp residue [7–16]. CED-4 is a nucleotide binding protein, potentially an ATPase, that possesses significant homology to the mammalian Apaf-1 [17,18]. CED-9 has sequence and functional similarity to anti-apoptotic members of the mammalian Bcl-2 family [19–23]. CED-4 interacts with the single polypeptide zymogen form of CED-3 and promotes its autoprocessing to the active dimeric death protease. CED-9 can either inhibit this process by binding to CED-4 or directly blocking active CED-3 by acting as a pseudosubstrate [18,24].

This molecular coupling of cell death components in the worm is emulated in mammalian cells [3,25,26]. In response to certain apoptotic stimuli, cytochrome *c* is released from mitochondria [27–29]. The released cytochrome *c* induces the formation of a caspase activating complex (CAC) in the presence of dATP [25]. Present in the CAC is a mammalian CED-4 homologue, Apaf-1, that directly binds the zymogen form of caspase-9 via a homophilic interaction involving CARD (caspase recruitment domain) motifs. The CARD domain is a collection of highly conserved residues in the amino terminus of molecules recruited to complexes signaling apoptosis [30]. Once assembled, caspase-9 is activated by an undefined mechanism. Active caspase-9 can proteolytically process the zymogen form of downstream caspases, such as caspase-3, leading to their activation [25,26]. However, whether the processing of caspase-9 is through an autocatalytic mechanism and whether other caspases (especially other CARD-containing caspases) are activated remains an open question and is the subject of the present study.

Herein we report a simple in vitro system that demonstrates cytochrome *c*-induced processing of caspase-9, downstream activation of caspase-3 and -7 and cleavage of the death substrate PARP (poly (ADP-ribose) polymerase) [31,32]. Importantly, the processing of caspase-9 from single polypeptide zymogen to active dimeric protease was an autocatalytic event dependent upon intrinsic protease activity. The processing of the aforementioned caspases could be effectively blocked by a dominant negative (catalytically dead) version of caspase-9, confirming that caspase-9 is upstream of caspase-3 and caspase-7. Surprisingly, none of the other CARD-containing caspases, including caspase-1, -2, -4, -8, -10 and -13, were processed. These data suggest that the caspase cascade triggered by cytochrome *c* and Apaf-1 possesses exquisite specificity and leads only to the activation of caspase-3, -7 and -9. The other CARD-containing caspases must be activated by a different Apaf-1-like molecule or involve a totally different mechanism.

2. Materials and methods

2.1. In vitro caspase activation assay

Rabbit reticulocyte lysates (TNT Quick T7 Master Mix, L1170) were purchased from Promega. Typically, 4 μl ^{35}S-labeled caspase or PARP was mixed with 6 μl of TNT lysate or 6 μl of in vitro translated cold dominant negative caspase-9 in a 30 μl reaction containing processing buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dATP (optional), 0.1 mM PMSF (optional) and 1 mM DTT [25]). After addition of 0.2–0.5 μg of cytochrome *c* (Sigma), the reactions were incubated at 30°C for 60–90 min. Samples were boiled in SDS sample buffer prior to resolution by SDS-polyacrylamide electrophoresis. Labeled proteins and their cleaved products were detected by autoradiography.

All expression constructs were epitope-tagged at their C-terminus and have been described previously [33–36].

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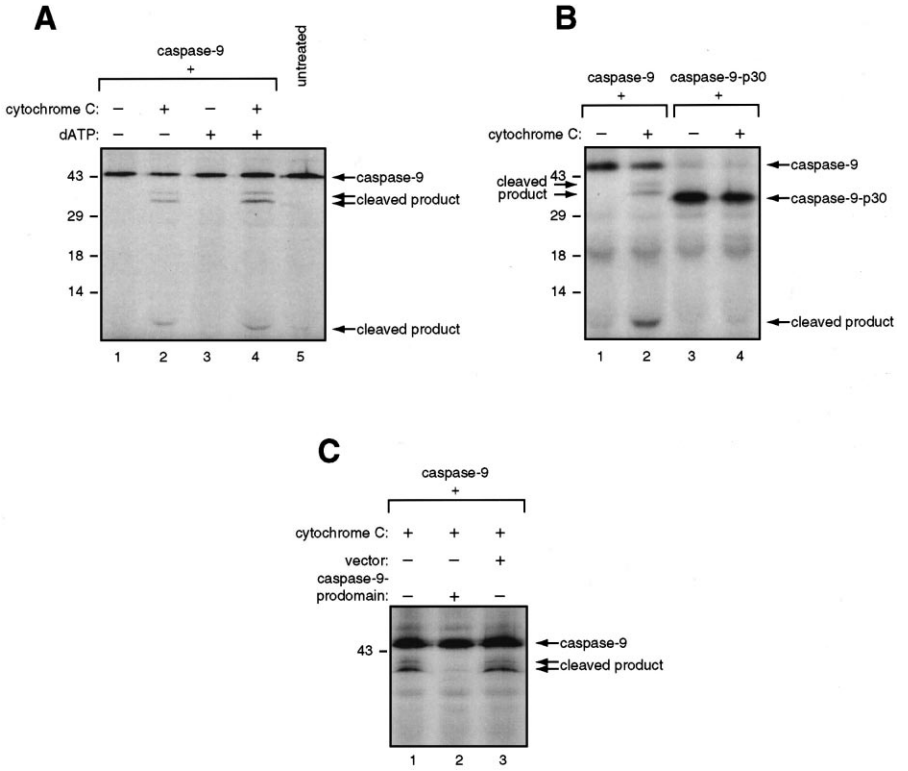

Fig. 1. Activation of caspase-9 in vitro. A: Caspase-9 is processed in a cytochrome *c*-dependent manner. The reactions containing ^{35}S-labeled caspase-9 were performed in the presence (+) or absence (−) of cytochrome *c* and dATP as indicated (see Section 2). Caspase-9 precursor and its cleaved products are indicated on the right. The size markers are indicated on the left. B: Caspase-9-p30 is not processed. The reactions containing ^{35}S-labeled caspase-9 (lanes 1, 2) and a ^{35}S-labeled truncated version, caspase-9-p30 (aa 140 C-terminus; lanes 3, 4) were performed as described in Section 2. C: Caspase-9 processing is inhibited by its own prodomain. Four μl of ^{35}S-labeled caspase-9 were mixed with 6 μl of lysate either containing a vector or a construct expressing the prodomain of caspase-9 (aa 1–170) in a 30 μl reaction as indicated. The reactions were performed as described in Section 2.

3. Results and discussion

3.1. Caspase-9 processing requires intrinsic protease activity and an intact prodomain

To determine if reticulocyte lysate retained an ability to respond to apoptotic stimuli, we asked if exogenously added cytochrome *c* was capable of triggering processing of caspase-9 zymogen. Surprisingly, caspase-9 was processed in a cytochrome *c*-dependent manner (Fig. 1A; lanes 2, 4), presumably via an endogenous Apaf-1-like activity that was present in the lysate. Additional dATP was not required for this reaction to proceed (Fig. 1A; lane 2). A truncated version of caspase-9 lacking the CARD-containing prodomain was not processed (Fig. 1B; lane 4), consistent with a requirement for a CARD-mediated interaction between caspase-9 and Apaf-1 [25,26]. Substantiating this notion, inclusion of the prodomain of caspase-9 acted to significantly attenuate the processing of native caspase-9 (Fig. 1C; lane 2). To determine whether the activation of caspase-9 was an autocatalytic process, a dominant negative form in which the catalytic cysteine essential for activity had been altered to an alanine was tested. This mutation completely abolished caspase-9 processing (Fig. 2; lane 2 vs. 5), confirming that the activation of caspase-9 triggered by cytochrome *c* required intrinsic catalytic activity.

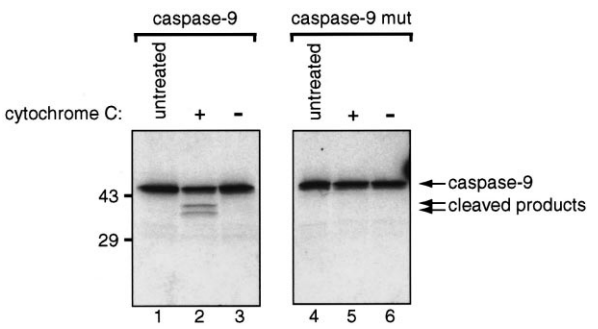

Fig. 2. Caspase-9 processing requires intrinsic catalytic activity. ^{35}S-labeled caspase-9 (lanes 1–3) or its dominant negative version (caspase-9 mut, lanes 4–6) were assayed. Only the large cleaved products are shown.

3.2. Caspase-3 processing does not require intrinsic catalytic activity and is blocked by dominant negative caspase-9

In response to cytochrome c, caspase-3 was processed (Fig. 3A; lane 2). Furthermore, a catalytically inert form of caspase-3 (catalytic cysteine altered to alanine) was similarly processed (Fig. 3A; lane 4), consistent with it being a target of an upstream caspase, presumably caspase-9. In confirmation of this, dominant negative caspase-9 (catalytic cysteine mutant) was found to completely block the processing of caspase-3 (Fig. 3B; lane 2). These observations suggest that the in vitro system faithfully reproduced an apoptotic mechanism that was responsive to cytochrome *c*.

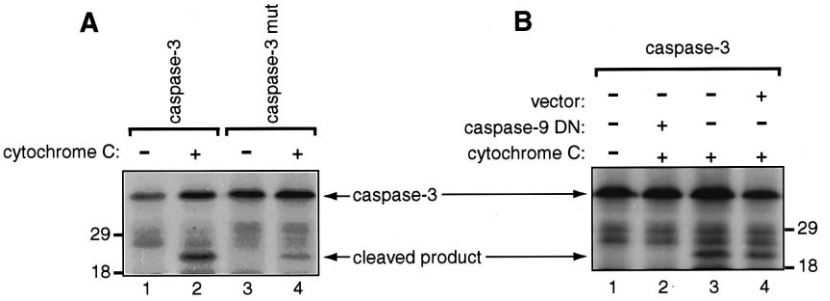

Fig. 3. Caspase-3 processing in vitro. A: Both caspase-3 and its catalytically inactive form, caspase-3 mut, are cleaved. B: Cleavage of caspase-3 is inhibited by dominant negative caspase-9 (caspase-9 DN). Four μl of ^{35}S-labeled caspase-3 were mixed with 6 μl of lysate containing cold protein from either a vector or a caspase-9 DN in vitro translation in a 30 μl reaction.

3.3. *Caspase-7 processing also does not require intrinsic catalytic activity and is blocked by dominant negative caspase-9*

We asked whether other caspases would be activated directly or indirectly when the caspase cascade was triggered by cytochrome *c*. Two non-CARD-containing caspases, caspase-7 and caspase-6 (not shown) were tested. Caspase-7 was found to be proteolytically processed (Fig. 4A; lane 2) and, like caspase-3, a catalytically inactive form was similarly cleaved (Fig. 4A; lane 6), suggesting that activation was likely mediated by an active upstream caspase, potentially caspase-9. In support of this, processing of caspase-7 was inhibited by dominant negative caspase-9 (Fig. 4A; lanes 3 and 7).

3.4. *Other caspases are not processed*

In addition to caspase-9, caspase-1, -2, -4, -8, -10 and the newly identified caspase-13 (unpublished) all contain a CARD. These caspases are believed to be recruited to their individual CACs via their CARDs [30]. It has been suggested that one or more of these caspases might substitute for cas-

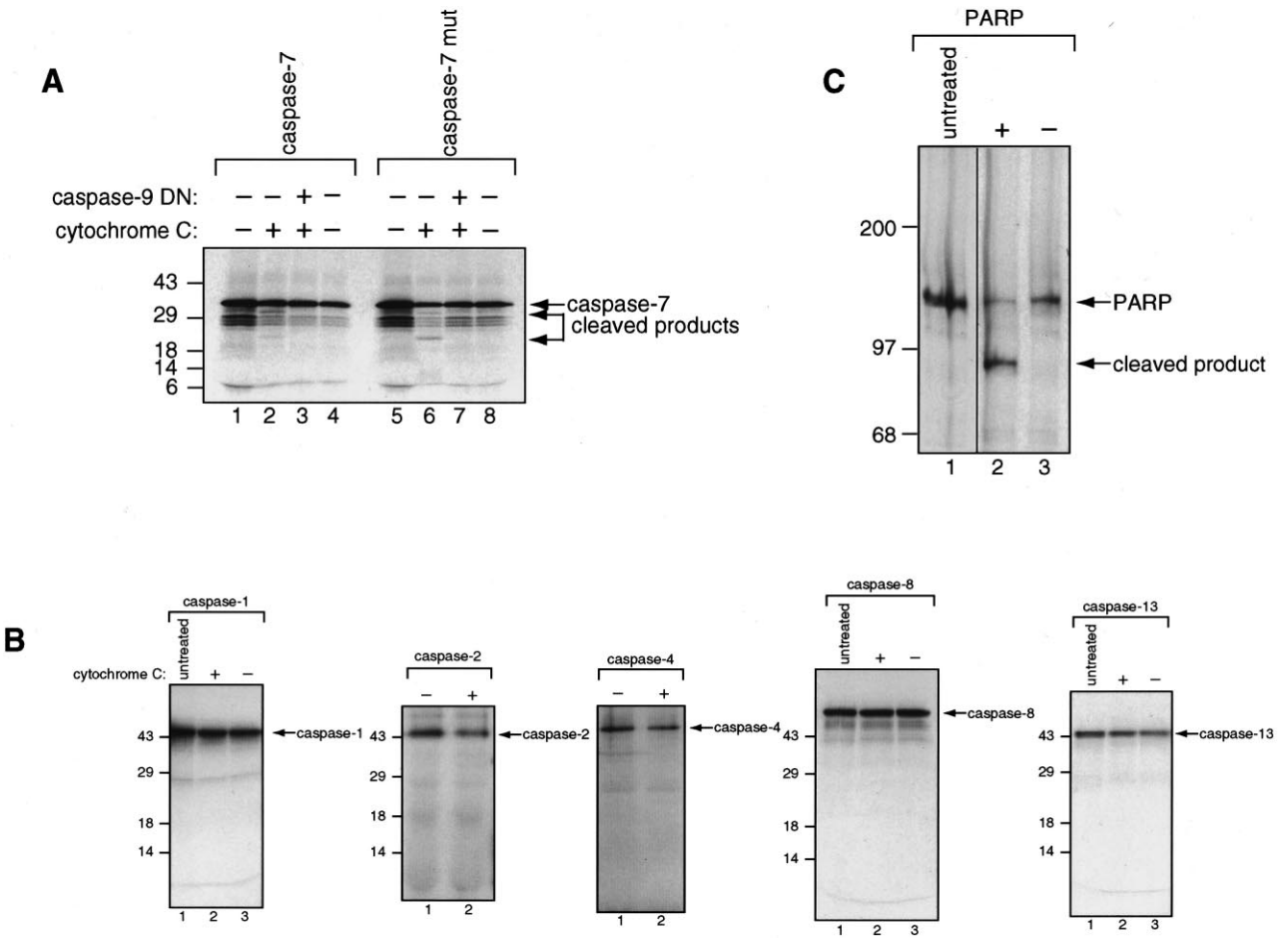

Fig. 4. Cleavage of additional caspases and PARP. A: Caspase-7 processing triggered by cytochrome *c*. Both caspase-7 and its catalytically inactive version (caspase-7 mut) were tested. Cleavage of caspase-7 is inhibited by dominant negative caspase-9 (A, lanes 3 and 7). B: Other CARD-containing caspases are not processed. Caspase-1, -2, -4, -8 and -13 were tested and none of them were cleaved. C: PARP cleavage triggered by cytochrome *c*. Full length PARP and its cleaved signature fragment are indicated.

pase-9 in tissues where they are predominantly expressed [25]. Thus, we tested whether any of these caspases was proteolytically cleaved in response to cytochrome *c*. None of these CARD-containing caspases was processed (Fig. 4B and not shown), suggesting that the CAC composed of Apaf-1 and cytochrome *c* was specific for caspase-9. Consistent with this, Apaf-1 does not interact with caspase-1, -2 and -8 [25] (data not shown). It is therefore possible that activation of other CARD-containing caspases may require their own specific Apaf-1-like molecule or is achieved by a different mechanism. Poly (ADP-ribose) polymerase, or PARP, is one of the hallmark death substrates that is cleaved when a cellular apoptotic program is engaged. Cytochrome *c*-triggered caspase activation led to the cleavage of PARP (Fig. 4C), confirming that the in vitro system using reticulocyte lysate can recapitulate the entire apoptotic cascade from initiation of caspase activation to cleavage of death substrates.

3.5. Concluding remarks

In response to cytochrome *c*, caspase-9 is proteolytically processed through an autocatalytic mechanism, leading to activation of downstream caspase-3 and caspase-7 and subsequent cleavage of the death substrate PARP. None of the other caspases were processed. The caspase cascade triggered by cytochrome *c* involves caspase-9 at the apex and caspase-3 and -7 as downstream effectors.

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