

# Activation of caspases triggered by cytochrome *c* in vitro

Guoha Pan<sup>1,a</sup>, Eric W. Humke<sup>1,2,b,c</sup>, Vishva M. Dixit<sup>c,\*</sup>

<sup>a</sup>Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

<sup>b</sup>Department of Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

<sup>c</sup>Department of Molecular Oncology, Genentech, Inc., 1 DNA Way, Bldg. 10, Room 290, Mail Stop 40, South San Francisco, CA 94080, USA

Received 25 February 1998

**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.

© 1998 Federation of European Biochemical Societies.

**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 <sup>35</sup>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<sub>2</sub>, 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].

\*Corresponding author. Fax: +1 (650) 225-6443.

E-mail: dixit@gene.com

<sup>1</sup>These authors contributed equally to this report.

<sup>2</sup>Fellow of the Medical Scientist Training Program, supported by the MSTP NIH Training Grant 5T32 GM07863-16 and Pathology Departmental Funds.

This work was supported by the National Institute of Health Grant R01 AG13671.

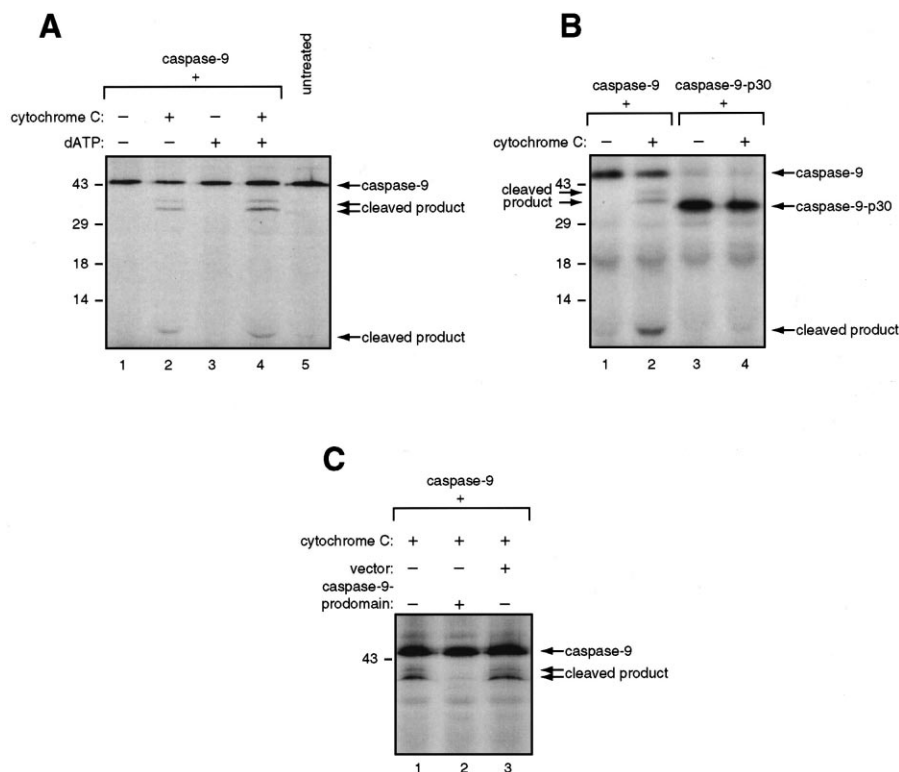


Fig. 1. Activation of caspase-9 in vitro. A: Caspase-9 is processed in a cytochrome *c*-dependent manner. The reactions containing  $^{35}$ S-labeled caspase-9 were performed in the presence (+) or absence (–) of cytochrome *c* and dATP as indicated (see Section 2). Caspase-9 precursor and its cleaved 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  $\mu$ l of  $^{35}$ S-labeled caspase-9 were mixed with 6  $\mu$ l of lysate either containing a vector or a construct expressing the prodomain of caspase-9 (aa 1–170) in a 30  $\mu$ l 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.

#### 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*.

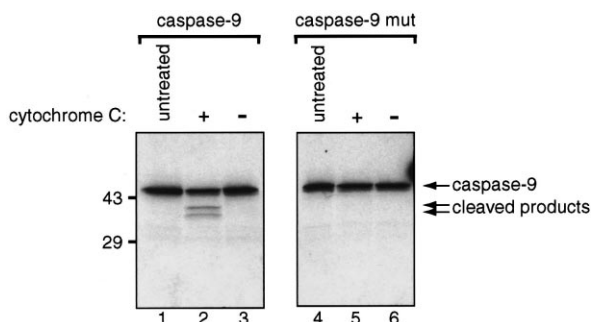


Fig. 2. Caspase-9 processing requires intrinsic catalytic activity.  $^{35}$ S-labeled caspase-9 (lanes 1–3) or its dominant negative version (caspase-9 mut, lanes 4–6) were assayed. Only the large cleaved products are shown.

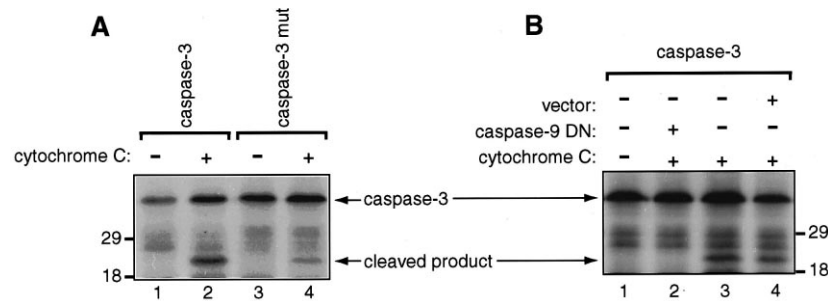


Fig. 3. Caspase-3 processing in vitro. A: Both caspase-3 and its catalytically inactive form, caspase-3 mut, are cleaved. B: Cleavage of caspase-3 is inhibited by dominant negative caspase-9 (caspase-9 DN). Four  $\mu$ l of  $^{35}$ S-labeled caspase-3 were mixed with 6  $\mu$ l of lysate containing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

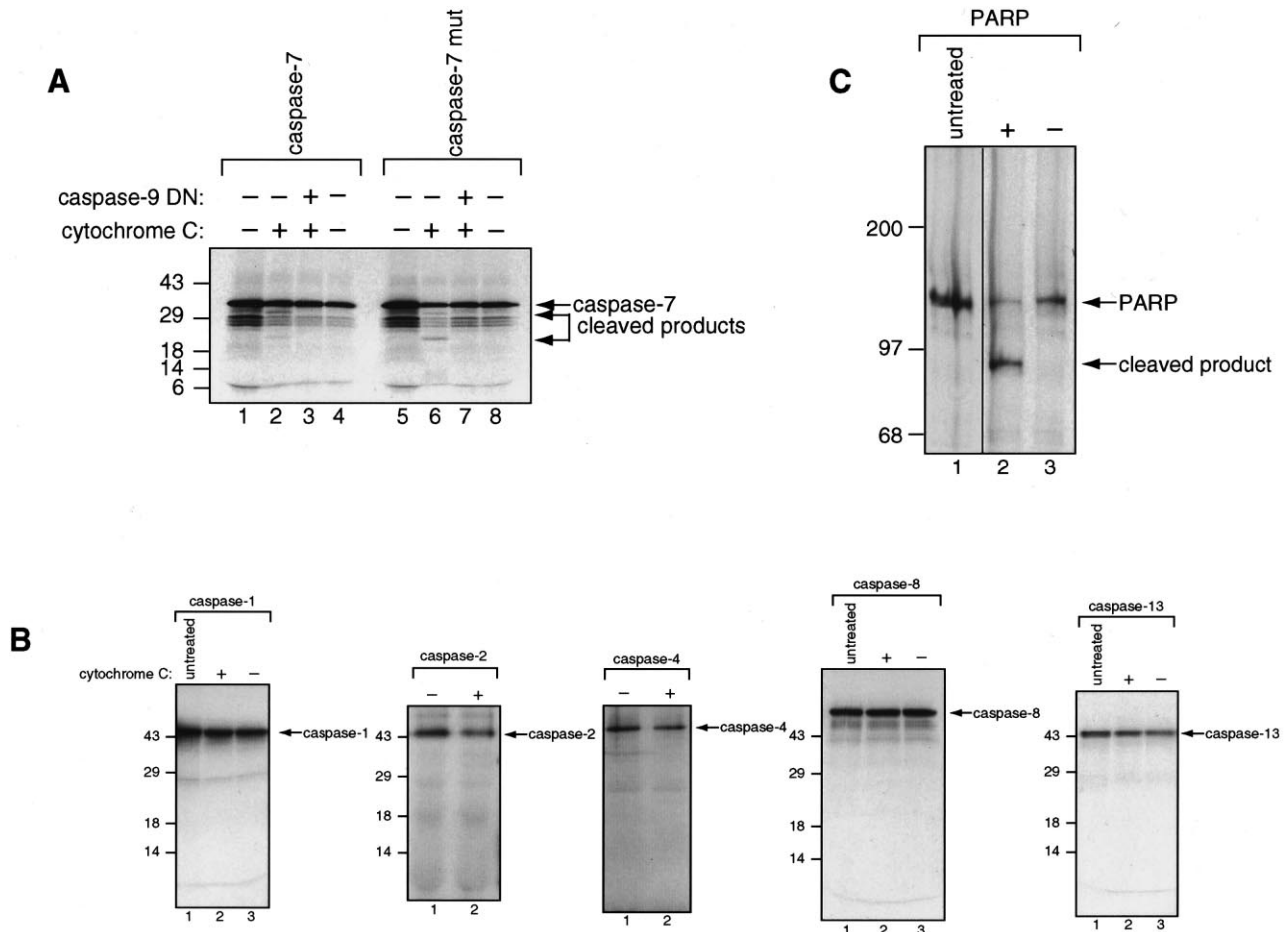


Fig. 4. Cleavage of additional caspases and PARP. A: Caspase-7 processing triggered by cytochrome *c*. Both caspase-7 and its catalytically inactive version (caspase-7 mut) were tested. Cleavage of caspase-7 is inhibited by dominant negative caspase-9 (A, lanes 3 and 7). B: Other CARD-containing caspases 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.

**Acknowledgements:** We thank other members of the Dixit lab for useful discussion, I. Jones for assistance in preparing figures, and B. Schumann for secretarial help. G.P. holds a Special Fellowship from the Leukemia Society of America. E.W.H. is a fellow in the Medical Scientist Training Program.

### References

- [1] Ellis, R.E., Yuan, J. and Horvitz, H.R. (1991) *Annu. Rev. Cell Biol.* 7, 663–698.
- [2] Hengartner, M.O. and Horvitz, H.R. (1994) *Curr. Opin. Genet. Dev.* 4, 581–586.
- [3] Chinnaiyan, A.M., O'Rourke, K., Lane, B.L. and Dixit, V.M. (1997) *Science* 275, 1122–1126.
- [4] Hengartner, M.O. (1996) *Curr. Opin. Gen. Dev.* 6, 34–38.
- [5] Wu, D., Wallen, H.D. and Nunez, G. (1997) *Science* 275, 1126–1129.
- [6] Ellis, H.M. and Horvitz, H.R. (1986) *Cell* 44, 817–829.
- [7] Thornberry, N.A. et al. (1992) *Nature* 356, 768–774.
- [8] Cerretti, D.P. et al. (1992) *Science* 256, 97–100.
- [9] Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) *Cell* 75, 641–652.
- [10] Xue, D. and Horvitz, R. (1995) *Nature* 377, 248–251.
- [11] Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) *Cell* 87, 171.
- [12] Villa, P., Kaufmann, S. and Earnshaw, W. (1997) *TIBS* 22, 388–393.
- [13] Nicholson, D. and Thornberry, N. (1997) *TIBS* 22, 299–306.
- [14] Miller, D. (1997) *Sem. Immunol.* 9, 35–49.
- [15] Salvesen, G. and Dixit, V. (1997) *Cell* 91, 443–446.
- [16] Thornberry, N.A. et al. (1997) *J. Biol. Chem.* 272, 17907–17911.
- [17] Zou, H., Henzel, W.J., Lui, X., Lutschg, A. and Wang, X. (1997) *Cell* 90, 405–413.
- [18] Chinnaiyan, A.M., Chaudhary, D., O'Rourke, K., Koonin, E.V. and Dixit, V.M. (1997) *Nature* 388, 728–729.
- [19] Hockenbery, D., Nunez, G., Millman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) *Nature* 348, 334–336.
- [20] Hengartner, M.O. and Horvitz, H.R. (1994) *Cell* 76, 665–676.
- [21] Jacobson, M.D. and Evan, G.I. (1994) *Curr. Biol.* 4, 337–340.
- [22] Vaux, D.L., Weissman, I.L. and Kim, S.K. (1992) *Science* 258, 1955–1957.
- [23] Chen-Levy, Z., Nourse, J. and Cleary, M.L. (1989) *Mol. Cell. Biol.* 9, 701–710.
- [24] Seshagiri, S. and Miller, L.K. (1997) *Curr. Biol.* 7, 455–460.
- [25] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) *Cell* 91, 479–489.
- [26] Pan, G., O'Rourke, K. and Dixit, V. (1998) *J. Biol. Chem.*, in press.
- [27] Liu, P., Kim, C.N., Yang, J., Jemerson, R. and Wang, X. (1996) *Cell* 86, 147–157.
- [28] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) *Science* 275, 1132–1134.
- [29] Yang, J. et al. (1997) *Science* 275, 1129–1132.
- [30] Hofmann, K., Bucher, P. and Tshopp, J. (1997) *TIBS* 22, 155–156.
- [31] Tewari, M. et al. (1995) *Cell* 81, 801–809.
- [32] Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) *Nature* 371, 346–347.
- [33] Duan, H., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W. and Dixit, V.M. (1996) *J. Biol. Chem.* 271, 1621–1625.
- [34] Duan, H., Orth, K., Chinnaiyan, A., Poirier, G., Froelich, C.J., He, W.-W. and Dixit, V.M. (1996) *J. Biol. Chem.* 271, 16720–16724.
- [35] Duan, H.J. and Dixit, V.M. (1997) *Nature* 385, 86–89.
- [36] Muzio, M., Salvesen, G.S. and Dixit, V.M. (1997) *J. Biol. Chem.* 272, 2952–2956.