Caspase-9 Can Be Activated without Proteolytic Processing*

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The recombinant form of the proapoptotic caspase-9 purified following expression in Escherichia coli is processed at Asp615, but largely inactive; however, when added to cytosolic extracts of human 293 cells it is activated 2000-fold in the presence of cytochrome c and dATP. Thus, the characteristic activities of caspase-9 are context-dependent, and its activation may not recapitulate conventional caspase activation mechanisms. To explore this hypothesis we produced recombinant forms of procaspase-9 containing mutations that disabled one or both of the interdomain processing sites of the zymogen. These mutants were able to activate downstream caspases, but only in the presence of cytosolic factors. The mutant with both processing sites abolished had 10% of the activity of wild-type, and was able to support apoptosis, with equal vigor to wild-type, when transiently expressed in 293 cells. Thus caspase-9 has an unusually active zymogen that does not require proteolytic processing, but instead is dependent on cytosolic factors for expression of its activity.

Apoptosis, the ordered dismantling of animal cells that results in their death and removal from the organism, requires specific proteolysis of a subset of cellular proteins, mediated by caspases. Many apoptotic responses are initiated by activation of the apical caspases-8 or -9, the former by recruitment to ligated cell surface receptors belonging to tumor necrosis factor receptor-1 family (1, 2), and the latter by recruitment to Apaf-1, in the presence of ATP or dATP, following delivery of cytochrome c (cyto-c)(1) from mitochondria (3). Activation of either of these two initiator caspases can lead to activation of the executioner caspase-3 (casp-3), by direct proteolysis of the casp-3 precursor (4, 5). Indeed all caspase zymogens are thought to be activated by limited proteolysis within a linker segment which results in generation of the characteristic large and small subunits of the catalytically active enzyme. Thus it has been specifically demonstrated that casp-1, -3, -7, and -8 gain marked increases in activity after proteolytic processing (6–9). This is typical of proteolytic enzymes from all families and catalytic classes. As pointed out by Neurath (10), the zymogens are stored in an inactive form to prevent adventitious proteolysis before it is needed at the required site of action. Frequently protease zymogens are activated by other proteases, in a cascade mechanism that results in either amplification or localization to specific sites. There are few exceptions to the rule that zymogen activation requires proteolysis, and these exceptional zymogens, such as tissue plasminogen activator (tPA), usually have specific allosteric modulators that allow them to become active at the required site, without proteolysis.

The initiator casp-8 has some of these exceptional properties. Casp-8 initiates apoptotic signaling induced by specific ligation of death receptors (reviewed in Refs. 11 and 12). Recruitment of procasp-8 to the cytosolic face of ligated Fas or tumor necrosis factor receptor-1 results in its autoactivation by a mechanism involving clustering of zymogen molecules (9, 13). The zymogen possesses ~1% of the activity of the activated enzyme, and it is hypothesized that this small amount of activity of the zymogen is sufficient to drive cleavage of clustered casp-8 zymogen molecules to the active form. Thus casp-8 has a zymogenicity ratio of 100 (defined as the ratio of the activity of the enzyme to the zymogen). Nevertheless, proteolysis of the casp-8 zymogen is thought to be required for its activation (9, 13).

In light of the casp-8 activation studies, it has been hypothesized that the initiator caspases, including caspases-8 and -9 (14) and Caenorhabditis elegans CED-3, share a common mechanism of activation (15). This would require clustering zymogens that possess a small amount of activity in their single chain forms, with production of the active protease dependent on proteolytic processing of the zymogen. In turn, this would mean that preventing proteolysis of the zymogen in the interdomain linker should inactivate apoptotic signal transmission. We tested this hypothesis for casp-9 by utilizing a cell-free system (16) that allows dissection of the requirements for zymogen activation.

EXPERIMENTAL PROCEDURES

Materials—Acetyl-DEVD-p-nitroanilide (Ac-DEVD-pNA) and carboxbenzoxyl-Val-fluoromethyl ketone (Z-Val-FMK) were from Bachem. Fluorogenic 7-amino-4-trifluoromethyl coumarin (FMK) fluoromethyl ketone; pNA, p-nitroanilide; PAGE, polyacrylamide gel electrophoresis; casp, caspase(s); tPA, tissue plasminogen activator; MALDI-TOF, matrix-assisted laser desorption-time-of-flight.
Approximately 36 h after transfection, the cells were fixed with 0.5% six-well tissue culture dishes using calcium phosphate precipitation. or wild-type plasmids plus 0.1 mM briefy, 2.5 ECL kit (Amersham Pharmacia Biotech) using rabbit anti-casp-3 and bilon-P (Millipore) (23). Western blot analysis was performed with the (Pierce) according to the manufacturer's protocol or blotted to Immo-

eration of activity can be followed by release of pNA from Ac-DEVD-DEVD-AFC as described previously (8, 20, 21). Alternatively, the gen-

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itself. The rate is greatly amplified upon addition of cyto-c/dATP, which activates casp-9 in cytosolic extracts (3, 26). While the activity of the ΔCARD casp-9 decreases slightly upon addition of cyto-c/dATP, full-length casp-9 recapitulated the activation rate of the endogenous material, even when added at 20 nM, equivalent to the endogenous casp-9 concentration. This is equivalent to a 2000-fold amplification of activity relative to the action of fully processed casp-9 on purified procasp-3 and demonstrates that binding to a cytosolic factor, probably the reported casp-9 activator Apaf-1 (26), is essential for the activity of casp-9 and not just for its activation. This is consistent with the demonstration that cleavage of casp-9 in cytosolic extracts devoid of cyto-c failed to accelerate caspase activation (8).

**RESULTS AND DISCUSSION**

**Purified Recombinant Casp-9 Is Maximally Active Only in the Presence of Cytosolic Components**—Recombinant casp-9 was obtained in the fully processed form, consisting of two chains caused by autolytic cleavage at Asp^115^, as determined by N-terminal Edman degradation and MALDI-TOF analysis of the isolated material (Fig. 1, A and B). The CARD domain-depleted form of casp-9 was obtained in the same way, with the same internal cleavage site. Both purified proteins had very low activity when measured with Ac-LEHD-AFC and contained only 1–5% active sites as judged Z-VAD-FMK titration. Thus the recombinant material is largely inactive compared with other caspases such as 3, 6, 7, and 8, which normally demonstrate 50–100% active sites following expression and purification by the same procedure (5). Moreover, activation of the natural substrate procasp-3 was extremely slow, leading us to speculate that the recombinant casp-9 forms purified from E. coli are not equivalent to those operating in vivo. To test this we immuno-depleted endogenous casp-9 from a cytosolic extract made from 293 cells, and reconstituted with the recombi-

Quantitative immunoblot analyses revealed that the concentration of casp-9 in the extract was approximately 20 nM (not shown). Addition of 200 nM (10-fold excess of the endogenous concentration) of the interdomain cleaved forms of ΔCARD or full-length casp-9 to the depleted extract resulted only in limited induction of DEVD-AFC cleaving activity (Fig. 1C). In this assay, the cleavage of DEVD-AFC measures the activity of pro-casp-9 that is activated by casp-9 in cytosolic extracts and thereby acts as an indirect measure of the activity of casp-9 itself. The rate is greatly ampliﬁed upon addition of cyto-c/dATP, which activates casp-9 in cytosolic extracts (3, 26). While the activity of the ΔCARD casp-9 decreases slightly upon addition of cyto-c/dATP, full-length casp-9 recapitulated the activation rate of the endogenous material, even when added at 20 nM, equivalent to the endogenous casp-9 concentration. This is equivalent to a 2000-fold amplification of activity relative to the action of fully processed casp-9 on purified procasp-3 and demonstrates that binding to a cytosolic factor, probably the reported casp-9 activator Apaf-1 (26), is essential for the activity of casp-9 and not just for its activation. This is consistent with the demonstration that cleavage of casp-9 in cytosolic extracts devoid of cyto-c failed to accelerate caspase activation (8).
40% of the wild-type level (Fig. 2A).

Interestingly, the linker site mutants contained a small amount of aberrantly processed material that was determined by N-terminal sequence analysis and MALDI-TOF to be due to cleavage between Ser307 and Pro308 (Fig. 1A). The amount of this material relative to the D315A/D330A single chain zymogen was determined to be 5.1 (±1.2)% based on quantitative image analysis of Coomassie-stained gels. The origin of this cleaved material is a mystery, but appears to require a competent catalytic site in casp-9, since it was absent from the C287A mutant. The presence of this aberrantly processed material raises two possibilities which pertain directly to whether proteolytic processing of casp-9 is required for activation. Is the small amount of aberrant material the activator, not the residual 95% of unprocessed material in the D315A/D330A cleavage site mutant? This is unlikely since an amount of wild-type processed casp-9 equal to the aberrantly processed material was only able to generate 9% maximal activity, as opposed to the 40% attained by the D315A/D330A cleavage site mutant (Fig. 2A). Moreover, if casp-9 requires cleavage for activation, it should compete with unprocessed material (D315A/D330A) for the activator complex. Indeed, when we simulated this by adding 2 nm wild-type processed casp-9 to 18 nm catalytic mutant (C287A), activation was abolished, presumably because the catalytic mutant prevents recruitment of processed casp-9 to the activator complex. Therefore we are left with a second possibility: the unprocessed D315A/D330A mutant is directly responsible for generating activity. To test whether the aberrant material increased during the incubation, Western blot analysis was conducted on cytosolic extracts that had been activated by cyto-c/dATP (Fig. 2C). No increase in the amount of the aberrant material, quantitated by image analysis, could be observed. This rules out adventitious proteolytic activation of the D315A/D330A mutant during the course of the assay and confirms that casp-9 can become active without proteolytic processing.

The generation of DEVD-pNA cleavage as a function of time provides a measure of instantaneous casp-3 activation. Casp-9 mutants that can be cleaved at the Asp315 autoprocessing site (wild-type casp-9 and D330A) demonstrate kinetics very similar to endogenous casp-9, but mutants that cannot be cleaved at Asp315 (D315A and D315A/D330A) cause DEVD-pNA cleavage more slowly (Fig. 2B). However, in the D315A mutant there is a significant beneficial effect of being able to form a two chain enzyme by cleavage at Asp330. Thus, the rate of caspase activation increases significantly as an increasing amount of caspase activity is generated, presumably due to a feedback of casp-3 onto the Apopase-9 site of casp-9 as proposed by Srinivasula et al. (14).

Because the activation of procasp-3 by casp-9 is a complex process, it is difficult to derive an exact quantitation of the activity of casp-9. However, the cumulative caspase activity caused by endogenous casp-9 is 2.5-fold that of the double mutant, and this occurs about four times faster for the endogenous casp-9 than for the double mutant (Fig. 2, A and B). The product of these two measures of activation indicates that the double mutant has at least 10% of the activity of endogenous casp-9 when both are allowed to activate under identical conditions in cyto-c-programmed cytosols. Thus, since the casp-9 D315A/D330A mutant can be considered a zymogen, the zymogenicity ratio, which describes the activity of an active enzyme relative to its latent precursor, is at most 10.

Cleavage Site Mutants Do Not Act as Dominant Negative Inhibitors of Endogenous Casp-9—These results combine to suggest that the cleavage site mutants would not act as dominant negative inhibitors and that they indeed should support apoptosis. Only the catalytic mutant (C287A) acts as a dominant negative inhibitor of caspase activation.
apoptosis to the level observed for the wild-type and D330A, whereas the catalytic mutant casp-9 (C287A) failed to induce more apoptosis than the empty vector control. Only the catalytic mutant acted to suppress casp-9 activation in vivo, presumably by competing with endogenous casp-9 for binding to the cytosolic activators.

**Zymogenicity and Casp-9**—Several caspases undergo proteolytic processing when expressed in *E. coli*, probably due to intrinsic cysteine properties of the zymogens which, though low, are sufficient to allow autolysis under the high concentrations obtained in expression (4, 5). However, unlike other caspases which demonstrate substantial activity when purified from *E. coli*, casp-9 appears to be largely inactive until it is delivered to the correct cellular environment. It is not clear whether the endogenous cysteic factors (presumably Apaf-1 in the presence of cyto-c/dATP) bind casp-9 and then release it in an active conformation or whether casp-9 must remain attached to retain activity. Experiments with purified cysteic factors should answer this question. Nevertheless, proteolysis of casp-9 does not seem to be required for its activation. Certainly, processing in the interdomain linker, characteristic for other caspases, enhances casp-9 activity, but only by 10-fold, thus the zymogenicity ratio of casp-9 is about 10. On the other hand, binding to cytosolic factors enhances activity 2000-fold to give a robust enzyme capable of activating available procasp-3 in a few minutes. In the natural cellular environment it is clear that casp-9 is processed during apoptosis, as demonstrated in (14) for example, and this can occur either due to an autoprocessing cleavage at Asp330 or by trans-processing by casp-3 at Asp230. However, in light of our data it would now be necessary to distinguish between processing (which may be adventitious) and activation, which does not appear to derive its driving force simply from proteolytic processing.

These observations on the relation of proteolytic processing to zymogen activation are reminiscent of tPA, which demonstrates a very small zymogenicity ratio, 2–10 (27). However, upon binding to fibrin, the ability of tPA to activate its physiologic substrate plasminogen increases several thousandfold (28). Presumably enzymes such as tPA and casp-9 have abolished the requirement for proteolysis as a mechanism of substantially increasing their activities, because allosteric regulators substitute this function, fibrin for tPA and Apaf-1 for casp-9. In the case of tPA, specific side chain interactions, absent in other members of the chymotrypsin family, allow activity of the zymogen. However, in the absence of a molecular structure of the casp-9 zymogen, few clues are available to explain the high activity of the unprocessed protein. One clue is suggested by the structure of active casp-1 and casp-3, each composed of two catalytic units thought to arise from dimerization of monomeric zymogens (reviewed in Ref. 5). If activation of zymogens of the initiator caspases-8 and -9 and CED3 operates by clustering, then the clustering phenomenon may be explained by adapter-driven homodimerization of monomers. In the case of casp-9, the putative dimerization would be envisaged to activate the protease, and from a mechanistic point of view, though not necessarily from a biologic one, proteolysis is an epiphenomenon. It will be interesting to determine whether similar processing-independent mechanisms for activation apply to the other initiator caspases.

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