Generation of Constitutively Active Recombinant Caspases-3 and -6 by Rearrangement of Their Subunits*

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Srinivasa M. Srinivasaولا‡, Manzoor Ahmad‡, Marion MacFarlane‡, Zhaowen Luo, Ziwei Huang, Teresa Fernandes-Alnemri, and Emad S. Alnemri‡

From the Center for Apoptosis Research and the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the Medical Research Council Toxicology Unit, Hodgkin Building, University of Leicester, Leicester LE1 9HN, United Kingdom

Caspases play a major role in the transduction of the apoptotic signal and execution of apoptosis in mammalian cells. Ectopic overexpression of the short prodomain caspases-3 and -6 precursors in mammalian cells does not induce apoptosis. This is due to their inability to undergo autocatalytic processing/activation and suggests that they depend on the long prodomain caspases for activation. To investigate directly the apoptotic activity of these two caspases in vivo, we engineered constitutively active recombinant caspases-3 and -6 precursors. This was achieved by making contiguous precursor caspases-3 and -6 molecules, which have their small subunits preceding their large subunits. Unlike their wild type counterparts, these recombinant molecules were capable of autocatalytic processing in an in vitro translation reaction, suggesting that they are catalytically active. They were also capable of autoprocessing and inducing apoptosis in vivo independent of the upstream caspases. Furthermore, their autocatalytic and apoptotic activities were inhibited by the pancaspase inhibitor z-VAD-fluoromethylketone, but not by CrmA or Bcl-2, thus directly demonstrating that the targets of inhibition of apoptosis by CrmA and Bcl-2 are upstream of caspases-3 and -6. Since caspases-3 and -6 are the most downstream executioners of apoptosis, the constitutively active versions of these caspases could be used at very low concentrations in gene therapy model systems to induce apoptosis in target tissues or tumors.

It has been nearly 6 years since the discovery of interleukin 1β-converting enzyme or ICE (1, 2), the first member of a growing family of cysteine proteases recently named caspases (3). So far 10 members of the human caspase family have been reported in the literature. Among these at least seven participate in the initiation and execution of apoptosis or programmed cell death, and the remaining three, in particular caspase-1 and probably caspases-4 and -5, are involved in production of the proinflammatory cytokines (4–6).

Caspases share several common features among them, in that they are synthesized as catalytically inactive zymogens that are generally activated by cleavages after specific internal Asp residues present in interdomain linkers and the ability to cleave their substrates after Asp residues. As a result certain mature active caspases, in particular those that are derived from the long prodomain caspases, can process and activate their own and other inactive caspase zymogens (7, 8). This sequential activation process is usually specific and is determined by the caspase preference toward the target P4-P1 subsite, which is present in the interdomain linker between the large and small subunits of the caspase zymogen. Interestingly, the short prodomain caspases contain target sites that are preferred by the long prodomain caspases (9). This led to the suggestion that caspases operate in a hierarchical relationship within an intracellular network of proteolytic signaling pathways or cascades (5, 6). Thus, implementation of the apoptotic program is now believed to require the participation of at least two classes of caspases, the initiator and the executioner caspases.

Three initiator or apical caspases, namely caspases-2, -8, and -10, have been implicated in apoptotic pathways triggered by the death receptors of the tumor necrosis factor receptor family (10–13). Upon ligand-induced trimerization of the death receptors, the initiator caspases are recruited through their long N-terminal prodomain by specialized adaptor molecules to form the death-inducing signaling complex (DISC) (6, 14). For example, caspase-8 and probably caspase-10 are recruited to the DISC by the adaptor molecule FADD/Mort1, whereas caspase-2 is recruited by CRADD/RAIDD and RIP (10–13). Because of the trimeric nature of the DISC, three caspase molecules are brought in close proximity to one another, which is believed to facilitate their activation by autocatalytic processing (15, 16).

Caspase-9, another long prodomain initiator caspase, is activated by binding to Apaf-1 (17). The exact mechanism by which Apaf-1 triggers activation of caspase-9 has not been defined. However, the release of cytochrome c from the mitochondria, a prerequisite for formation of the Apaf-1-caspase-9-cytochrome c complex, is believed to be triggered by many apoptotic stimuli, including those initiated by other initiator caspases (18).

The downstream or executioner caspases, namely caspases-3, -6, and -7, lack the long prodomain that is required for recruitment to caspase activation complexes such as the DISC or Apaf-1. Thus these caspases remain dormant until the
initiator caspases activate them by direct proteolysis (7, 8, 17). In this way cells ensure complete control over their activity. This is because, once these caspases are activated, they rapidly dismantle important structural and regulatory components of the cell, leading to the characteristic apoptotic phenotype observed in cells undergoing apoptosis (4–6).

In order to study the role of the downstream caspases in apoptosis independent of the initiator caspases, it is necessary to generate constitutively active downstream caspases. Here, we describe the engineering of two recombinant constitutively active caspases-3 and -6 precursors by switching the order of their two subunits, such that the engineered molecule mimicked a structure presented by the processed wild type active molecule. These caspases were designated reversed-caspases-3 and -6 (rev-caspases-3 and -6). Unlike their wild type counterparts, the rev-caspases were capable of autocatalytic processing in an in vitro translation reaction and rapid induction of apoptosis in vivo. The potential applications of these rev-caspases are discussed.

MATERIALS AND METHODS

Generation of cDNAs Expressing Rev-caspases-3 and -6 Precursors—cDNAs encoding rev-caspases-3 and -6 precursors were generated by PCR. The large and small subunits of caspase-3 were amplified with the following primers using the caspase-3 cDNA as a template: LS-forward, ATGGAGAACACTGAAAAACTCGAG; LS-reverse, GTACGTAATCAGCTCATCTCTCCTCTTTC; SS-forward, GGATCCATGATTGAAAGCAGACGAGCTGTGG; SS-reverse, ATCACTCTATCGTGAATAAATAAGAGTTG. The PCR products were cloned separately into the Smal site of pBluescript KS- vector. The small subunit was then excised from KS- vector with BamHI and inserted into the BamHI site of the second KS- vector, which contains the large subunit. This places the small subunit in-frame 5’ to the large subunit. Rev-caspase-6 was amplified and cloned in the KS- vector in a similar way. The following PCR primers were used with caspase-6-His6 cDNA as a template: LS-forward, ATGAGCTGCGCTCCGGG; LS-reverse, TTAATCTACTACATCCAAAGG; SS-forward, GGATCCATTGATGATAAGTTGCTCTGACATGAGTT; SS-reverse, ATCAATTTCAACGTGGTGGTGGTGGTGC. To express the rev-caspases in bacteria (BL21-DE3), their cDNAs were excised with BamHI/XhoI and subcloned into the bacterial expression vector pET28a in-frame with the His6-tag of this vector.

General Computational Methods Used in Modeling of Rev-caspase-3—General computational methods used in modeling of rev-caspase-3 were performed as described previously (19).

Expression of Rev-caspases in Mammalian Cells and Assay of Apoptosis—To express the rev-caspases in mammalian cells and assay their apoptotic activity, they were amplified with the T7-tag primer and the test cDNA under the cytomegalovirus promoter. (11). This vector allows the expression of lacZ under the Rous sarcoma virus promoter and the test cDNA under the cytomegalovirus promoter. It also allows in vitro transcription of the test cDNA from the T7-promoter. To assay for apoptosis, MCF-7 or 293 cells were transfected with the pRSC-LacZ constructs in the presence or absence of different apoptosis inhibitors. The cells were stained with 7-5-propidium iodide (PI) after transfection and examined for morphological signs of apoptosis. The percentage of round blue apoptotic cells (mean ± S.D.) were represented as a function of total blue cells under each condition (n ≥ 3).

In Vitro Translation of Caspases—Wild type and Rev-caspases were in vitro translated in the presence of [35S]methionine in rabbit reticulocyte lysate with a T7-RNA polymerase-coupled TNT kit (Promega), using the pRSC-LacZ or pET28a constructs as templates according to the manufacturer’s recommendations.

RESULTS AND DISCUSSION

Production of Constitutively Active Caspases-3 and -6 Precursors—To control their death-inducing activity, caspases are synthesized as harmless inactive single chain precursor molecules. It is necessary to cleave the caspase precursor at conserved aspartate processing sites located 12–28 amino acids C-terminal to the active site Cys to produce the active zymogen, which is a heterodimer made of the two cleavage products. In this heterodimer, the SS is derived from the C terminus of the caspase precursor, and the LS is derived from the N-terminal region. Based on the three-dimensional structure of caspase-3 (CPP32) (20, 21), the C terminus of the LS and the N terminus of the SS must be made free in order for the two subunits to interdigitate and fold properly into the final active conformation. In this conformation, the N terminus of the SS and the C terminus of the LS are separated far from each other, whereas the N terminus of the LS and the C terminus of the SS are brought closer to each other (Fig. 1A). That could explain why a contiguous caspase precursor molecule cannot freely fold into an active zymogen without cleavage between the two subunits. Based on these observations we reasoned that a contiguous molecule in which the C terminus of the LS and the N terminus of the SS are free, whereas the N terminus of the LS and the C terminus of the SS are physically linked, would enable spontaneous folding into a fully active conformation that simulates the three-dimensional structure of the processed wild type molecule (Fig. 1B).

To test this hypothesis we chose the downstream caspases-3 and -6 because of their inability to undergo autocatalytic processing or induce apoptosis on their own when overexpressed in mammalian cells. We engineered contiguous caspases-3 and -6...
molecules in which the SS was fused in-frame N-terminal to the LS, and a cleavage site (DEVDG in the case of caspase-3; VEIDA in the case of caspase-6) was introduced between the two subunits (Fig. 1A). Unlike their wild type counterparts, these engineered “reversed” molecules (rev-caspases-3 and -6) now have their SS preceding their LS. Presentation of the subunits in this order should result in spontaneous folding into a structure similar to the native active caspase-3 structure (Fig. 1A). This hypothesis was examined by molecular modeling of the rev-caspase-3 based on the three-dimensional structure of caspase-3 (20, 21). Both high temperature (900 K) and room temperature (300 K) dynamic simulation of 100 ps suggested that the linker loop between the SS and the LS does not change the original structure of the LS and SS, and the new molecule may keep the same structure as the native protein (Fig. 1B).

As expected, and unlike the wild type caspases-3 and -6, rev-caspases-3 and -6 were able to undergo autocatalytic processing in an in vitro translation reaction (Fig. 2A). This processing was completely inhibited by mutation of the active site Cys to Ala of rev-caspases-3 and -6 (third and sixth lanes) and by selected caspase inhibitors (see below).

Effects of Caspase Inhibitors on the in Vitro Autocatalytic Activity of Rev-caspases-3 and -6—To test the effect of selected caspase inhibitors on the autocatalytic activity of rev-caspases-3 and -6, we translated the rev-caspases in the presence of different amounts of the inhibitors. As shown in Fig. 2B, in the presence of increasing amounts of DEVD-CHO, a decrease in the amount of cleavage products and a corresponding increase in the amount of the rev-caspase-3 precursor was observed. This corresponded to nearly 50–90% inhibition of the autocatalytic activity of rev-caspase-3 at 40–400 nM concentration. However, the same concentrations of this inhibitor had little effect on the autocatalytic activity of rev-caspase-6 (Fig. 2B). This is consistent with earlier observations that caspase-6 is poorly inhibited by DEVD-CHO (22).

On the other hand, z-VAD-fmk had nearly an equal inhibitory effect on rev-caspases-3 and -6 autocatalytic activity at the concentration used in this experiment (Fig. 2B). Nevertheless, nearly 10-fold more of z-VAD-fmk than DEVD-CHO was required to obtain complete inhibition of caspase-3 activity. Similarly, baculovirus p35 had nearly an equal inhibitory effect on rev-caspases-3 and -6 autocatalytic activity (data not shown).

Bacterially Expressed Rev-caspases-3 and -6 Can Cleave PARP and Lamin A, Respectively—At limited caspase concentration, PARP is specifically cleaved by caspases-3 and -7 but not other caspases. Similarly, lamin A is specifically cleaved by caspase-6 but not other caspases. To compare the activity of the wild type and rev-caspases-3 and -6 toward PARP and lamin A, we expressed these caspases in bacteria and then incubated them with the two substrates. As shown in Fig. 3, the activity of the rev-caspases toward these two substrates was indistinguishable from their wild type counterparts. Both caspase-3 variants (reversed and wild type), but not caspase-6 variants,
Constitutively Active Caspases-3 and -6

**Fig. 5.** Autocatalytic and enzymatic activity of the uncleavable rev-caspase-3.A, uncleavable rev-caspase-3 in the pET28a vector was* in vitro* translated in the presence of increasing concentrations of DEVD-CHO (0–0.4 μM). The translation product contains a cleavable 35-residue-long His6-T7-tag at its N terminus (*hatched box, see bar diagram*). The active site mutant rev-caspase-3 in the pET28a vector (sixth lane) was used as a control. The p32 cleavage product without the His6-T7-tag is indicated to the right. B, bacterially expressed uncleavable rev-caspase-3 can cleave DEVD-AMC. Rev-caspase-3 (Rev), uncleavable rev-caspase-3 (Rev-mod), and active site mutant rev-caspase-3 (Rev-C/A) were expressed in BL-21 bacteria and their activity (100 ng of total protein) was then assayed by spectrofluorometry with the fluorogenic DEVD-AMC substrate (50 μM). The activity as a function of time is represented by arbitrary fluorometric units. Lysates from BL-21 bacteria were used as a control.

efficiently cleaved PARP. In contrast, both caspase-6 variants, but not caspase-3 variants, efficiently cleaved lamin A. These results demonstrate that the mature caspases generated from the reversed and the wild type constructs have similar substrate specificity for PARP and lamin A.

**Induction of Apoptosis in Mammalian Cells by Rev-caspases-3 and -6**—To determine the apoptotic activity of rev-caspases-3 and -6 in* in vitro*, we expressed them in human MCF-7 cells. Unlike the wild type caspases-3 and -6 or the C/A mutant rev-caspases-3 and -6, the rev-caspases potently induced apoptosis in nearly 90% of the transfected cells (Fig. 4). Overexpression of Bcl-2 or CrmA, which protect against different forms of apoptosis, did not significantly reduce their apoptotic activity. Nevertheless, overexpression of the baculovirus p35, which inhibits the activity of most caspases, partially protected against their apoptotic activity. Also, incubation of the transfected cells in the presence of 20 μM z-VAD-fmk dramatically reduced their apoptotic activity to nearly 30%. These data confirm previous observations that the activity of caspases-3 and -6 are downstream of the CrmA and Bcl-2 block in the apoptotic cascade and can only be inhibited by relatively high concentration of the pancaspase inhibitor z-VAD-fmk.

The activity of Rev-caspase-3 does not require separation of the two subunits—To demonstrate that the rev-caspase molecules are inherently active and do not require separation of the two subunits, we removed the DEVD site and mutated the Asp-9 and Asp-28 that are present between the two subunits of rev-caspase-3 (see Fig. 1B). However, to follow the activity of this molecule, we introduced a cleavable 35-residue-long His6-T7-tag N terminus to the IETD site (Fig. 5A). Upon* in vitro* translation of this molecule there was no evidence of cleavage between the two subunits (Fig. 5A). Nevertheless, the translated molecule was active as evident from its ability to cleave the His6-T7-tag at its N terminus to form the p32 species (Fig. 5A). Also, expression of this molecule in bacteria generated an active molecule that can cleave the DEVD-AMC, substrate (Fig. 5B). However, when the active site Cys was mutated to Ala or in the presence of 400 nM DEVD-CHO, processing of the His6-T7-tag was inhibited and only the full-length p36 species were seen (Fig. 5A, fifth and sixth lanes). These data demonstrate that when the two subunits of a caspase are rearranged in the reverse order, it is not necessary to first separate them from each other to generate an active caspase. Also, in the rev-caspases the two subunits are not derived from two precursor molecules but from a spontaneous folding of one molecule. Thus by mimicking the three-dimensional structure of the mature caspase-3, it was possible to design contiguous and active caspases-3 and -6 molecules.

In conclusion, some of the important aspects of these constitutively active caspases are their utility in* in vitro* and* in vivo* drug screening, identifying endogenous substrates and inhibitors and targeted gene therapy model systems. For example, the rev-caspase cDNAs could be introduced into transgenic mice under the control of inducible promoters to induce apoptosis selectively in target tissues. This transgenic model system could be utilized to determine the usefulness of a given caspase inhibitory drug to block the process of apoptosis induced by the rev-caspase. It could also be used to study the pathology of selective apoptosis induction in certain tissues. To identify endogenous substrates or inhibitors, the rev-caspases could be used in the yeast two-hybrid system. Because the active rev-caspase is encoded by one contiguous molecule and does not require cleavage, it could be fused to the DNA binding domain of yeast two-hybrid vectors to screen cDNA libraries for potential inhibitors of caspases. An active site mutant of the rev-caspase could also be used to screen for interacting substrates in this system. In targeted gene therapy models, rev-caspases could potentially be used to destroy unwanted cells such as tumor and autoimmune cells. Because of the importance of caspases in apoptosis and the disease processes associated with it, it is expected that these engineered rev-caspases may find additional unique and valuable applications in the future.

**REFERENCES**


Constitutively Active Caspases-3 and -6