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 the apoptotic signal and execution of apoptosis in mammalian cells are the constitutively active recombinant 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.
Constitutively Active Caspases-3 and -6

**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, ATGGAGAAGCAGTTAAACCTGAG; LS-reverse, GATCATCACACCCTGACTGTC; SS-forward, AACACTACCATGATAAAATAGG; SS-reverse, ATCAACTGATGATAAAATAGG. The PCR products were cloned separately into the KS- vector pBluescript KS- in-frame with the His 6-T7-tag of this vector.

**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 LS-reverse primers using the pET28a constructs as templates and the test cDNA under the cytomegalovirus promoter.

**In Vitro Translation of Caspases**—Wild type and Rev-caspases were translated in vitro in the presence of [35S]methionine in rabbit reticulocyte lysate with a T7-RNA polymerase-coupled TNT kit (Promega), and the translated proteins were separated by SDS-PAGE and visualized by autoradiography.

**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 from the N-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 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 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 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 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 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 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 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 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 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 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 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 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 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 caspase precursor molecule cannot freely fold into an active zymogen without cleavage between the two subunits.
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. 1, A and C). 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 in both rev- and wild-type 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 active site Cys to Ala mutants in pRSC-lacZ constructs, were in vitro translated in the presence of [35S]methionine. The translation products were then analyzed by SDS-PAGE and autoradiography. The LS and the SS are indicated. B, rev-caspase-3 (upper panel) or rev-caspase-6 (lower panel) were in vitro translated in the presence of increasing concentrations of DEVD-CHO (0–0.4 μM) or z-VAD-fmk (0–5 μM). The translation products were then analyzed as in A.

At limited caspase concentrations, PARP is specifically cleaved by caspases-3 and -7 but not other caspases. Similarly, lamin A is specifically cleaved by caspases-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

The activity of Rev-caspases-3 and -6 is 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 His$_6$-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 His$_6$-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 His$_6$-T7-tag was inhibited and only the full-length p36 species was 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.

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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 vitro, we expressed them in human MCF-7 cells. Unlike the wild type caspase-3 and -6 or the C/A mutant rev-caspase-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.

**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 His$_6$-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 His$_6$-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 BL21 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.
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