The short prodomain influences caspase-3 activation in HeLa cells

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Proteolytic activation of caspases is a key step in the process of apoptosis. According to their primary structure, caspases can be divided into a group with a long prodomain and a group with a short prodomain. Whereas long prodomains play a role in autocatalytic processing, little is known about the function of the short prodomain, for example the prodomain of caspase-3. We constructed caspase-3 variants lacking the prodomain and over-expressed these in HeLa and yeast cells. We found that removal of the caspase-3 prodomain resulted in spontaneous proteolytic activation of the protein when expressed in HeLa cells. This

processing was only partially autocatalytic, as demonstrated by a catalytically inactive caspase-3 mutant. Co-expression of the anti-apoptotic protein XIAP (X-chromosome-linked inhibitor of apoptosis protein) completely blocked the observed spontaneous activation, which excluded a direct involvement of caspase-8. Our findings indicate that the short prodomain of caspase-3 serves as a silencing component in mammalian cells by retaining this executioner caspase in an inactive state.

Keywords: apoptosis, TNF α , XIAP.

INTRODUCTION

Apoptosis is a process of controlled cell death that plays an important role in tissue homeostasis of multicellular organisms. Over the last few years, it has become evident that the proteolytic activation of certain cysteine proteases, collectively called caspases [1], is a pivotal step in the apoptotic process in almost all cell types examined. Caspases-1, -4, -5 and -11 seem to function primarily in the processing of inflammatory cytokines, but the other ten known caspase species are involved in the execution of cell death in response to apoptotic stimuli. Caspases have a strict requirement for cleavage of Asp–Xaa bonds at the P₁ position. The substrate specificity of the individual caspases is determined mainly by the sequence of the three amino acids preceding the P₁ position, with P₁ being the most critical [2,3].

Caspases are synthesized as single-chain inactive zymogens with an N-terminal prodomain plus a large and a small catalytic subunit [4,5]. Activation of the zymogens is achieved through proteolytic cleavage at sites identical with the caspase-recognition motifs, generating the active heterodimeric enzyme. This suggests that caspases can process themselves or other caspase zymogens, most likely in an ordered cascade [6]. This mechanism may serve to amplify the apoptotic signal. Depending on their attributed functions within the apoptosis-related caspase cascade, these enzymes can be subdivided into a group of initiator caspases (including caspase-2, -8, -9 and -10), and a group of executioner caspases (including caspase-3, -6 and -7). The initiator group is mainly activated by an autocatalytic mechanism after a prodomain-mediated dimerization of the proenzymes, which is initiated by apoptotic stimuli. In the case of caspase-8 processing after engagement of the tumour necrosis factor- α (TNF α) receptor 1 or CD95 cell-death receptors, the procaspase is recruited to the cytosolic region of the receptor by association with the adaptor molecules Fas-associated death-domain protein (FADD)/TNF α -receptor-1-associated death-domain protein (TRADD) through homotypic interaction of their respective death-effector domains [7,8]. This aggregation is considered sufficient for procaspase-8 activation [9–11]. A different acti-

vation mechanism has been described for activation of caspase-9. Here, the ATP/cytochrome-*c*-dependent interaction of the caspase prodomain and the respective domain of the apoptotic-protease-activating factor 1 (Apaf-1) protein (CARD–CARD interaction; where CARD is caspase-recruitment domain) places the catalytic site of the caspase in close proximity to its internal cleavage site, resulting in an autocatalytic activation [12,13]. The executioner caspases act mainly downstream in the caspase pathway and they are activated, at least in part, by initiator caspases. In the execution phase, the activation of caspase-3 represents one of the key points in the transmission of the apoptotic signal because (i) caspase-3 cleavage activity results in a variety of morphological and biochemical features of apoptosis [14–16], (ii) caspase-3 seems to be able to cleave other executioner caspases, and (iii) so further amplifies the response to an apoptotic stimulus [17]. Furthermore, the central role of caspase-3 was underlined by the observation that the activated caspase-3 also cleaves the initiator caspases-9 and -2 [17], indicating its role as an amplifier in a positive-feedback loop.

Two different pathways have been described for the proteolytic activation of caspase-3: first, a direct caspase-8-mediated cleavage [18], and second, activation by caspase-9-dependent cleavage involving a cytochrome-*c*/Apaf-1-mediated oligomerization of the initiator caspase [13,19,20]. The pathway preferentially used in receptor-mediated apoptosis depends on the amount of active caspase-8 [21]. The mature caspase-3 is generated from a 32-kDa zymogen by a sequential two-step mechanism. The initial cut occurs at an IETD/S cleavage site producing the small p12 subunit and a p20 peptide. p20 is further processed at an ESMD/S site in what is most likely an autocatalytic process, whereby the prodomain is removed, resulting in the generation of the mature large p17 subunit [22]. The active enzyme is composed of two heterodimers of the generated p12 and p17 subunits [23].

In contrast with the initiator caspases, the executioner caspase zymogens contain short prodomains. Whereas the long prodomains of the initiator caspases have been ascribed an important role during the autocatalytic processing, the short prodomain

Abbreviations used: TNF α , tumour necrosis factor- α ; CARD, caspase-recruitment domain; FADD, Fas-associated death-domain protein; Apaf-1, apoptotic-protease-activating factor 1; afc, 7-amino-4-trifluoromethylcoumarin; XIAP, X-chromosome-linked inhibitor of apoptosis protein; GFP, green fluorescent protein.

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of caspase-3 was found to have no influence on the enzymic activity when expressed recombinantly in bacteria or in experiments *in vitro* using cytosolic extracts [18]. Extending these experiments, we focused our studies on the function of the short prodomain of this executioner caspase in the natural cellular environment. We generated different caspase-3 expression plasmids, including truncated constructs lacking the prodomain, and expressed these in HeLa cells. Our data suggest that the prodomain suppresses the spontaneous activation of the inactive zymogen in mammalian cells.

EXPERIMENTAL

Cell culture and transfection

HeLa Tet-Off cells were obtained from Life Technologies and cultured in minimal essential medium at 37 °C with 10 % fetal calf serum in the presence of 5 % CO₂. The cells were grown to a maximum density of 1.3×10^5 cells/cm². Subsequently they were rinsed with PBS and treated with trypsin/EDTA (0.05 % trypsin/0.02 % EDTA in PBS) for 5 min at 37 °C. Transfection experiments were performed as described previously [24]. Briefly, HeLa cells were seeded at a density of 2×10^4 cells/cm² into a 3.5-cm cell-culture dish 20 h prior to transfection. Transfection of cells was carried out by the lipofection method using SuperFect reagent (Qiagen). For preparation of the transfection mixture for one dish, 2 µg of the respective plasmids was diluted with serum-free cell-culture medium to a total volume of 100 µl and mixed with 5 µl of SuperFect reagent. The transfection mixture was incubated at room temperature for 15 min and subsequently supplemented with 600 µl of cell-culture medium (minimal essential medium) containing 10 % fetal calf serum. After washing the cells with PBS, 700 µl of the transfection mixture was added to the cells. Following 2–3 h of incubation, transfection medium was replaced by washing with PBS and adding fresh cell-culture medium.

For tetracycline-regulated expression experiments, the anti-biotic was added directly at the start of transfection using various concentrations as described below. After transfection, the cells were cultured for 16 h before further treatment. For apoptosis induction, cycloheximide was added (100 µM final concentration) 15 min prior to supplementation of the culture medium with recombinant murine TNFα (100 ng/ml final concentration). The cells were exposed to this mixture for 3.5 h. Before harvesting, the cell-culture plates were centrifuged at 900 g for 5 min, washed with ice-cold PBS and centrifuged again.

Cells were harvested by adding 75 µl of hypotonic lysis buffer (25 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefablock and pepstatin, leupeptin and aprotinin, 1 µg/ml each) and scraping with a rubber policeman. Cellular extracts were prepared by freezing the cells in liquid nitrogen and thawing rapidly at 37 °C (three cycles) followed by a centrifugation step (20000 g, 5 min).

To determine the nuclear manifestation of apoptosis, cells were stained with Hoechst 33342 (10 µg/ml in PBS). The nuclear morphology was analysed using a fluorescence microscope.

SDS/PAGE and immunoblotting

For Western blotting, cell extracts (60 µg total protein) were separated on a 4–20 % polyacrylamide gel and transferred on to a nitrocellulose membrane (Schleicher and Schuell). After blocking for 1 h at room temperature (5 % skimmed milk in Tris-buffered saline containing 0.1 % Tween 20), the membrane was incubated overnight (4 °C) with a rabbit anti-caspase-3 antibody (H277, Santa Cruz) at a 1:1000 dilution in blocking buffer

followed by a horseradish-peroxidase-coupled secondary antibody (Jackson Immuno Research) at a 1:20000 dilution for 45 min at room temperature. The blot was washed with Tris-buffered saline containing 0.1 % Tween 20 for 3 × 10 min after each incubation. The immunoreactive proteins were visualized with the ECL[®] kit (Amersham Pharmacia Biotech). To confirm equal loading, the protein concentration was determined with the bicinchoninic acid assay (BCA, Pierce).

Measurement of caspase-3-like activity and luciferase activity

The fluorimetric DEVD-afc (7-amino-4-trifluoromethylcoumarin) cleavage assay was carried out in microtitre plates according to the method described by Thornberry [25] with following modifications: 5 µl of cytosolic extracts was diluted 1:20 with substrate buffer [55 µM fluorogenic substrate DEVD-afc (Biomol) in 50 mM Hepes, pH 7.4/1 % sucrose/0.1 % CHAPS/10 mM dithiothreitol]. Blanks contained 5 µl of extraction buffer and 95 µl of substrate buffer. Generation of free afc at 37 °C was determined by fluorescence measurement at $t = 0/t = 30$ min using the multilabel plate reader Victor II (Wallac) set at an excitation wavelength of 390 nm and an emission wavelength of 510 nm. Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

The amount of recombinant expressed caspase-3 in HeLa Tet-Off cells was estimated measuring the activity of the co-expressed luciferase as described previously [24].

Construction of expression vectors

For the isolation of the human caspase-3 cDNA and cDNA of the anti-apoptotic protein XIAP (X-chromosome-linked inhibitor of apoptosis protein), total RNA was extracted from HepG2 cells (caspase-3) or Jurkat cells (XIAP) with the RNeasy kit from Qiagen. The cDNA was synthesized in an oligo(dT)-primed reaction with *MuLV* reverse transcriptase (Perkin Elmer) and amplified specifically by PCR using *Pfu* polymerase (Stratagene). The following primers were used: caspase-3 forward primer, 5'-CCCAGGCCGTGAGGAGTTAGC-3'; caspase-3 reverse primer, 5'-CAGCATCACTGTAACCTTGCTAATC-3'. Isolation of XIAP cDNA was performed using primers specified by Deveraux et al. [26]. The caspase-3 amplification product was cleaved subsequently at *Ban*II/*Pst*I sites and cloned into the respective sites of a pUC19 vector. This construct served as a template for the following PCRs. Expression plasmids containing (i) or lacking (ii) the caspase-3 prodomain were generated by PCR using the forward primer (i) 5'-GACGATATCATGGA-GAACTGAAACTCA-3' and forward primer (ii) 5'-GTC-GATATCATGCTCTGGAATATCCCTGGAC-3' (*EcoRV* sites are indicated by italics, translation start codons are underlined) and the reverse-sequencing primer (Pharmacia). Subsequently, the obtained fragments were cloned into the *EcoRV* site of the pBI-luc tetracycline-dependent expression plasmid (Clontech). The XIAP PCR product was first inserted into the *EcoRI*/*XhoI* sites of the BlueScript KS II vector and then subcloned for expression as a green-fluorescent-protein (GFP)-fusion protein into the pEGFP-C1 vector (Clontech) using the *Bgl*II/*Kpn*I restriction sites.

For mutation of the caspase-3 catalytic site (substitution of Cys with Ser), the respective expression construct was subjected to site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The exact nucleotide sequences of the cDNA fragments were determined by automated DNA sequencing.

RESULTS

In contrast with large prodomain initiator caspases, which are prone to autoactivation, caspase-3 is only a poor inducer of cell death when overexpressed as native protein in mammalian cells [27–29]. These observations suggest that the premature zymogen is not activated by autocatalytic cleavage. To study a possible contribution of the short caspase-3 prodomain to the suppression of proteolytic activation, we expressed caspase-3 variants with or without the prodomain in HeLa cells. The peptide sequence of the construct missing the prodomain started with a Met (resulting from the ATG start codon), directly followed by a Ser, the first amino acid of the native large subunit. By using the Tet-Off tetracycline-regulated expression system and vectors that simultaneously co-express the luciferase reporter protein, we varied

Figure 1 Expression of caspase-3 without prodomain results in spontaneous proteolytic activation

HeLa cells were transfected with various caspase-3 constructs, or with a control vector. For induction of apoptosis, cells were treated for 3 h with 100 ng of TNF α /ml in the presence of 100 μ M cycloheximide (CHX). After transfection (20 h), cytosolic extracts were prepared by freeze-thawing. **(A)** For the measurement of caspase-3-like activity, 5 μ l of cytosolic extract was incubated for 30 min at 37 $^{\circ}$ C with 50 μ M DEVD-afc in 95 μ l of assay buffer. Increase in fluorescence intensity by afc release was measured at 510 nm. Measurements were performed in triplicate, values are an average of three experiments, and error bars indicate S.E.M. **(B)** Proteolytic processing of caspase-3 constructs was analysed by immunoblotting with an antibody directed against the p17 subunit of caspase-3. Expression of the full-length construct resulted in one band (p32) representing the full-length caspase (lane 1, reading from the left). Three bands were visible when the construct lacking the prodomain was expressed (lane 2); the band at 17 kDa (p17) represents the mature large subunit, whereas the 29-kDa band (p29) shows the unprocessed recombinant protein. The band at 32 kDa represents the endogenous caspase-3 zymogen. After TNF α treatment, full-length caspase-3 was processed to the mature enzyme as indicated by the p17 subunit. An additional fragment, marginally smaller than the full-length protein, represents a partial cleavage at the Asp-9 site in the prodomain, whereas a fragment consisting of the small and the large subunit without the prodomain did not appear (lane 3). Molecular masses are depicted to the right of **(B)**.

Figure 2 Effect of the prodomain is independent of expression level

HeLa cells were transfected with caspase-3 expression constructs (with or without prodomain) in a bidirectional pBI-L Tet vector which allows regulated expression by tetracycline supplementation. Tetracycline (Tc) at the concentrations indicated (0–25 ng/ml in **A**, 0–10 ng/ml in **B**) was added immediately after transfection. **(A)** Caspase-3 activity was measured in cytosolic extracts from transfected cells by DEVD-afc cleavage 20 h after transfection. Additionally, luciferase activity was measured as described in the Experimental section. Data represent means $\pm$ S.D. ($n = 2$). **(B)** Caspase-3 Western blot was used to demonstrate the expression level of the two caspase constructs and the associated spontaneous proteolytic processing. Equal amounts of cytosolic protein (30 μ g) were loaded into each lane. Note that for the construct without prodomain (right half), the band at 32 kDa represents the endogenous caspase-3 zymogen.

the amount of the expressed protein and were able to monitor and normalize the expression levels between different transfections.

As shown in Figure 1(A), a DEVD-afc cleavage activity, which was markedly higher compared with cells transfected with the control vector, was measured in caspase-3-transfected cells 20 h post-transfection. This demonstrates that the specific caspase-3 constructs were activated spontaneously. Furthermore, the construct lacking the prodomain showed a higher caspase activity (≈ 7 -fold) compared with its native counterpart. Although the activation level of full-length caspase-3 is much lower compared with the protein without prodomain, the spontaneous activation is not completely blocked. Interestingly, whereas TNF α treat-

Figure 3 XIAP co-expression completely and catalytic-site mutation partially inhibit spontaneous caspase-3 activation

A caspase-3 construct without prodomain [Casp-3 (—pro)] and a catalytically inactive, mutated variant [Cys-163 changed to Ser, without prodomain, Casp-3 (—pro)mutCys163] were transiently transfected in HeLa cells and co-expressed with XIAP as an enhanced-GFP-fusion protein. Caspase-3 activity was measured by DEVD-afc cleavage in medium alone or 3 h after apoptosis induction with TNF α (100 ng/ml) and cycloheximide (CHX, 100 μ M). Note that the caspase-3 Western blotting (lower panel) reveals partial processing of the mutated caspase-3 without apoptotic stimulation (lane 2, reading from the left). Data of the DEVD-afc cleavage assay are shown as means $\pm$ S.E.M. from three independent experiments.

ment causes apoptosis in about 70% of the vector-transfected control cells, the DEVD-afc cleaving activity was similar to (with prodomain) or much lower than (without prodomain) that in untreated transfected cells. Despite the high DEVD-afc cleaving activity, only about 15% of the caspase-transfected cells (determined by enhanced-GFP co-transfection) showed typical signs of apoptosis-like membrane blebbing or chromatin condensation (results not shown). When the transfected cells were exposed to TNF α /cycloheximide treatment (100 ng of TNF α /ml, 100 μ M cycloheximide) for 3.5 h, the activity of both caspase-3 variants was markedly increased (Figure 1A). Although the activity of constructs lacking the prodomain was about 2.5 times stronger after TNF α treatment, the TNF α -induced relative activity shift in cells transfected with the full-length caspase was much higher ($\approx$ 14-fold). Western-blot analysis of the two constructs with an antibody raised against the p17 subunit of caspase-3 confirmed that the prodomain-free enzyme was activated spontaneously in the transfected cells (Figure 1B).

To examine the effect of different expression levels on the spontaneous activation of the caspase-3 variants, we varied the amount of expressed protein in HeLa Tet-Off cells with different concentrations of tetracycline in the cell-culture medium. As shown in Figure 2, we observed a suppressive effect of the prodomain independent of the expression level of the recombinant caspases. At all expression levels examined (normalized to luciferase activity), the construct lacking the prodomain displayed much higher activity (6–8-fold). The largest difference was observed at a low expression level (2.5 ng of tetracycline/ml).

The prodomain-dependent differences in the spontaneous activation at various expression levels were also confirmed by Western blot (Figure 2B). These data indicate a pivotal role of the prodomain in preventing caspase-3 activation in unstimulated cells.

To further investigate the mechanisms mediating the activation of the truncated caspase-3, we studied the effect of simultaneous overexpression of the caspase-3 (without prodomain) and XIAP in HeLa cells. XIAP is known to be a direct and specific inhibitor of active caspase-3. Furthermore, it also suppresses the caspase-9-dependent activation of the caspase-3 proenzyme [30]. As shown in Figure 3, the spontaneous activity of the truncated caspase-3, which was observed when the caspase was transfected alone, was strongly suppressed by co-expression of XIAP (Figure 3, lane 3 versus lane 1, reading from the left), indicating that this inhibitor can interrupt spontaneous maturation of the enzyme at the cleavage site between the large and small subunits. Interestingly, mutation of the catalytic site of the truncated caspase-3 by replacing Cys-163 with a Ser only partially inhibited the spontaneous cleavage of the subunits (Figure 3, lane 2). Although no significant caspase activity resulted from transient transfection of this mutated caspase construct, the Western-blot analysis demonstrated clearly a conversion of the zymogen into the p17 peptide, which was blocked by XIAP co-expression (Figure 3, lane 4). In addition, these data demonstrate that an intact catalytic site in the recombinant caspase is required for the observed increase in DEVD-afc cleaving activity in transfected cells. The extent of caspase-3 processing was increased after

induction of receptor-mediated apoptosis by post-transfectional treatment with TNF α /cycloheximide (Figure 3, lanes 5–8). The differences between the prodomain-free native form and the inactive Cys-163 mutated form were still evident, i.e. the activation rate of the native form was higher (Figure 3, lane 5 versus lane 6). Furthermore, in TNF-treated cells, caspase-3 was processed partially in spite of XIAP overexpression (Figure 3, lanes 7 and 8). However, the amount of mature p17 and the measured caspase-3 activity in cells co-transfected with XIAP were notably lower compared with cells only overexpressing the caspase (Figure 3, lane 7 versus lane 5 and lane 8 versus lane 6). In addition, when XIAP was overexpressed, no significant differences in the proteolytic processing of the native and the mutated caspase-3 forms were observed in the Western blot (Figure 3, lanes 7 and 8). Complete suppression of the spontaneous activation of caspase-3 was achieved by adding the peptide inhibitor DEVD-fmk (fluoromethylketone; results not shown). In summary, these data suggest that caspase-3 constructs lacking the prodomain are activated in cells without apoptotic stimulation by at least two XIAP-inhibitable caspase-dependent mechanisms: (i) by autocatalytic processing and (ii) by a second process that still occurs when caspase-3 is inactivated by mutation. This second process may be mediated by a low caspase activity that is most probably related to a caspase with a small zymogenicity ratio, for example caspase-9 [31]. The XIAP-mediated inhibition of the truncated caspase-3 activation makes a direct participation of caspase-8 unlikely, as caspase-8-mediated cleavage between the large and the small subunits of caspase-3 is not inhibited by XIAP [26]. However, in the case of TNF treatment, caspase-8 activity could be responsible for the observed caspase-3 processing in cells overexpressing XIAP.

DISCUSSION

Apoptosis-associated caspases have been classified in the literature either according to their function in the caspase cascade (i.e. initiators or executioners), or due to their primary structure (i.e. groups with long or short prodomains). Interestingly, in either case, the different criteria result in an almost congruent classification. This suggests that the specific function of a caspase is also determined by the structure of its prodomain. For the caspases with long prodomains, i.e. the initiator caspases, it has been shown that the N-terminal region is required for the recruitment to death-receptor complexes as well as for dimerization and autoprocessing [7,32,33]. Specific structures within the large prodomain, which are crucial for the proteolytic activation of the initiator caspases, have been characterized in detail. In case of caspase-8 activation, the death-effector domain integrated in the long prodomain mediates steric aggregation via interaction with the FADD adapter molecule, inducing autoprocessing of the procaspase-8 molecules [8,9,34]. A similar principle of long-prodomain-mediated autoactivation has been described for caspase-9. Here, procaspase-9 autoactivation is induced by interaction of the CARDs in the prodomain and the respective sequence of Apaf-1, followed by oligomerization of the Apaf-1 adapter molecule. By this mechanism, the procaspase is brought into the proximity of its catalytic sites, resulting in autoactivation of caspase-9 [20,35,36]. Although certain functions have already been attributed to the long prodomains, a function of the short prodomains (e.g. caspase-3 pro-peptide) has not been described until now.

The potential for autocatalytic activation of caspase-3 has been demonstrated by recombinant expression in *Escherichia coli* [18] and in the yeast *Saccharomyces cerevisiae* (T. Meergans and A. Wendel, unpublished work). However, in mammalian cells we

found, in agreement with previous reports [37,38], that transient overexpression resulted in little native caspase-3 being processed and becoming enzymically active, indicating an important contribution to the activation process of the conditions in the specific cell. We demonstrate here that the short prodomain of caspase-3 has an inhibitory function on the activation of this executioner caspase in HeLa cells. This general feature of the native full-length caspase is changed in absence of the prodomain, resulting in a proteolytic activation of the recombinant caspase. Because this inhibitory effect of the prodomain was not found in a bacterial expression system [18] or in the yeast cells (T. Meergans and A. Wendel, unpublished work), our results may indicate that factors exist that are specific for a living mammalian cell and mediate the silencing effect of the prodomain.

Spontaneous activation of caspase-3 fusion proteins has been reported previously. In this case, N-terminal fusion of the long prodomain of caspase-2 to the full-length caspase-3 resulted in caspase-3 processing most probably due to a caspase-2-prodomain-mediated oligomerization [38]. Furthermore, rearrangement of the subunits (the small subunit preceding the large subunit) generated a constitutively active enzyme that was capable of autoactivation [37]. In these preceding reports, the silencing function of the caspase-3 prodomain may have been obscured by the artificial structures resulting from these unusual fusion proteins. Our data show that deletion of the prodomain alone is sufficient for caspase-3 to become activated in the natural cellular environment. It is important to note that this finding was also valid for recombinant expression levels (regulated by the Tet-Off system) comparable with those of the endogenous zymogen.

As shown in Figure 1, the differences in the activity levels resulting from prodomain deletion were pronounced in transfected cells lacking apoptotic stimulation, but became smaller after TNF α treatment of the cells. This indicates that TNF α stimulation may initiate reactions that overcome the prodomain-associated activation barrier, whereas mechanisms responsible for processing the prodomain-free caspase may be constitutively available. Recently, Stennicke et al. described a partial activity of the caspase-9 zymogen without prior proteolytic processing [31]. Although caspase-9 activity is mainly cytochrome *c* regulated, the small remaining activity could be sufficient to activate caspase-3 lacking the prodomain. Therefore, the function of executioner caspase prodomains may consist of protection against small amounts of latent initiator caspase activity, which in turn is necessary to enable autocatalysis in response to stimulation.

The anti-apoptotic protein XIAP has been shown to specifically inhibit caspase-3 and -7 as well as caspase-9 activity. In addition, XIAP blocks the removal of the caspase-3 prodomain during caspase-8-initiated processing, but is unable to prevent caspase-8-mediated cleavage of caspase-3 between the small and the large subunit [26,30,39]. Hence, the lack of caspase-3 proteolysis into the large and small subunits in unstimulated cells co-expressing XIAP and caspase-3 (without prodomain) suggests that caspase-8 is not involved in the spontaneous activation of caspase-3. Autoactivation of recombinant caspase-3 could be involved, but is unlikely to be exclusively responsible for the spontaneous activation in HeLa cells. Although we cannot exclude that the observed partial proteolysis of the mutated construct was mediated by traces of activated endogenous caspase-3, these data suggest that the spontaneous activation of caspase-3 is not only an autocatalytical process.

To our knowledge, this is the first report showing a functional role of a short caspase prodomain. Further studies will address the identification and characterization of factors mediating the silencing effect of the caspase-3 prodomain.

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