Caspase-8 Can Be Activated by Interchain Proteolysis without Receptor-triggered Dimerization during Drug-induced Apoptosis*

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Dennis Sohn, Klaus Schulze-Osthoff, and Reiner U. Jänicke
From the Institute of Molecular Medicine, Heinrich-Heine-University, D-40225 Düsseldorf, Germany

Proteases of the caspase family are thought to be activated by proteolytic processing of their inactivezymogens. However, although proteolytic cleavage is sufficient for executioner caspases, a different mechanism has been recently proposed for initiator caspases, such as caspase-8, which are believed to be activated by proximity-induced dimerization. According to this model, dimerization rather than proteolytic processing is considered as the critical event for caspase-8 activation. Such a mechanism would suggest that in the absence of a dimerization platform such as the death-inducing signaling complex, caspase-8 proteolytic cleavage would result in an inactive enzyme. As several studies have described caspase-8 cleavage during mitochondrial apoptosis, we now investigated whether caspase-8 becomes indeed catalytically active in this pathway. Using an in vivo affinity labeling approach, we demonstrate that caspase-8 is activated in etoposide-treated cells in vivo in the absence of the receptor-induced death-inducing signaling complex formation. Furthermore, we show that both caspase-3 and -6 are required for the efficient activation of caspase-8. Our data therefore indicate that interchain cleavage of caspase-8 in the mitochondrial pathway is sufficient to produce an active enzyme even in the absence of receptor-driven procaspase-8 dimerization.

Caspases, a family of aspartate-specific cysteine proteases, play an essential role in the signal transduction and effector processes of apoptosis (1–5). In healthy cells, caspases exist as inactive proforms (zymogens) consisting of a prodomain followed by two catalytically active subunits that are separated by a short linker domain. Upon activation, the prodomain is removed, and the linker domain is cleaved off resulting in the generation of the mature caspase, an active heterotetramer formed out of two large p20 and two small p10 subunits (6–7). Based on their order in cell death pathways, caspases can be divided into initiator and downstream effector caspases. Effectorcaspases, such as caspase-3, -6, and -7, generally contain only a small prodomain and cleave diverse cellular substrates (8). The initiator caspases such as caspase-8, -9, and -10, in contrast, contain a long prodomain that is used to recruit the enzymes to high molecular weight activation platforms, including the apoptosome in the mitochondrial pathway or the death-inducing signaling complex (DISC) in the death receptor pathway (9–12).

Caspase-8 is a key mediator of apoptotic signals triggered by death receptors such as CD95, TNF-R1, and TRAIL-R1/TRAIL-R2. In the case of the TRAIL receptors and CD95, caspase-8 is directly recruited into the DISC by the adapter protein FADD (11, 12). Signaling through TNF-R1, in contrast, appears to be more complex and to proceed via two sequential complexes (13, 14). It was suggested that following TNF stimulation a first complex is formed at the cell membrane, which contains TNF-R1, the adapter protein TRADD, and the NF-κB-activating signaling components RIP and TRAF2. Then, complex I leaves the receptor, and a second complex is formed in the cytosol, which recruits FADD and caspase-8 and induces cell death. In contrast to the executioner caspases that are found in the cytosol as inactive dimers, the initiator caspases are present as inactive monomers. Although executioner caspases are activated by direct proteolytic cleavage, initiator caspases are thought to require more complex activation events. Initially, receptor-driven oligomerization of caspase-8 was thought to activate this enzyme through facilitating the autoproteolysis of procaspase-8 molecules (15). Recent studies, however, have proposed that dimerization rather than interchain proteolysis is the critical event for activation of initiator caspases. It was suggested that dimerization is sufficient and a prerequisite for the activation of caspase-8 and -9, whereas proteolytic processing merely stabilizes the active caspase dimers (16, 17). In agreement with this hypothesis, it was shown that chemically induced dimerization of a non-cleaveable caspase-8 mutant yielded in an active procaspase-8 dimer that exhibited the same cleavage activity as the wild-type mature heterotetramer in vitro (17–19). However, although the current experimental observations would support this model, most of the evidence was gathered in artificial systems that might not reflect the correct in vivo situation (20).

Various studies have proposed that caspase-8 is not only activated in the death receptor pathway, but can be also activated independently of death receptors, for instance during genotoxic stress-induced apoptosis (21–31) or by direct cleavage through other proteases including the CTL protease granzyme B and human immunodeficiency virus type 1 protease (32, 33). During stress-induced apoptosis triggered by anticancer drugs or ionizing irradiation, it was proposed that caspase-8 activation is mediated in a postmitochondrial event by the prior cleavage through caspase-6 (29). In all these studies, the proteolysis of caspase-8 has been considered sufficient evidence for the activation of caspase-8 that was proposed to

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‡ To whom correspondence should be addressed: Institute of Molecular Medicine, University of Düsseldorf, Bldg. 23.12, Universitätsstrasse 1, D-40225 Düsseldorf, Germany. Tel.: 49-211-811-5894; Fax: 49-211-811-5892; E-mail: kso@uni-duesseldorf.de.

The abbreviations used are: DISC, death-inducing signaling complex; TNF, tumor necrosis factor; fmk, fluoromethyl ketone; AMC, amionomethylcoumarin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid.
trigger a feedback amplification loop through the cleavage of Bid (23, 25, 28). Hence, in such a death receptor-independent activation pathway, caspase-8 was proposed to act as an executioner caspase. These studies, however, could not distinguish whether caspase-8 processing resulted in the gain of enzymatic activity or merely reflected a substrate cleavage event. Moreover, activation through interchain cleavage would be incompatible with the recent proximity-induced dimerization model, which should result in a processed but catalytically inactive caspase-8 monomer.

To address the question whether caspase-8 becomes indeed catalytically active in the mitochondrial death pathway, we now employed an in vivo affinity labeling approach using the biotinylated caspase-inhibitor biotin-VAD-fmk that only detects active caspases. We demonstrated that caspase-8 is indeed activated independently of death receptors and that both caspase-6 and -3 are required for the generation of the mature caspase-8 enzyme.

**MATERIALS AND METHODS**

**Cell Lines and Reagents**—All cell lines including MCF-7 breast cancer cells, the leukemic T cell lines Jurkat and H9, and human B lymphoblastoid SKW6.4 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml (all from PAA Laboratories, Linz, Austria). The CD95-resistant Jurkat subline, Jurkat-R, was generated by continuous culture in the presence of anti-c-CD95 mAb (1 μg/ml, Biocheck, Munich, Germany) for 6 months (22). FADD- and caspase-8-deficient Jurkat cells as well as Jurkat cells overexpressing Bel-2 were kindly provided by J. Blenis (Harvard Medical School, Boston, MA) and H. Walczak (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. Biotin-VAD-fmk (bixin-Val-Ala-Asp-(OMe)-fluoromethyl ketone) and biotin-DEVD-CHO (bixin-Asp-Glu-Val-Asp-aldehyde) were purchased from ICN (Eschwege, Germany) and Calbiochem. Biotinylated proteins were captured with streptavidin-conjugated agarose beads and used for immunoblotting with anti-caspase-8 antibodies. The cleavage reaction was incubated at 37 °C after addition of the recombinant caspases at the indicated amounts. 25-μg aliquots of the reaction mixture were then taken in 30-min intervals and analyzed by SDS-PAGE and subsequent Western blot analysis. For affinity labeling of caspase-8 following in vitro caspase cleavage, the non-methylated agent biotin-DEVD-CHO was used. Caspase-8 from Jurkat cell lysates was cleaved with caspase-3 as described above. After the removal of the His-tagged recombinant caspases with nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany), the supernatant was incubated for 1 h with biotin-DEVD-CHO. Biotinylated proteins were captured with streptavidin-conjugated agarose beads and used for immunoblotting with anti-caspase-8 antibodies.

**RESULTS AND DISCUSSION**

**Treatment of Jurkat Cells with Anti-CD95 or Etoposide Results in Caspase-8 Processing**—To examine whether the caspase-8 processing that was observed following anti-cancer drug-induced apoptosis also results in the generation of catalytically active caspase-8 molecules, we employed the Jurkat T-cell line that is a commonly used and established cellular model system for the elucidation of apoptotic pathways. First, we determined the time-dependent induction of apoptosis using the anti-cancer drug etoposide and as a control the agonistic CD95 antibody. As expected, both agents efficiently induced apoptosis of Jurkat wild-type cells, whereas only etoposide but not anti-CD95 was able to trigger apoptosis in Jurkat-R cells, a subclone that had lost CD95 expression, or in FADD-deficient Jurkat cells (Fig. 1A). Etoposide-induced cell death proceeded with similar kinetics in all Jurkat subclones confirming previous reports (22, 23) and indicating that etoposide-induced cell death proceeds independently of death receptors. Apoptosis induction in Jurkat wild-type cells (Fig. 1B) as well as in the CD95-resistant counterparts (see Fig. 3) was accompanied by the processing of caspase-8 and -3. Interestingly, whereas triggering of the death receptor pathway by the agonistic CD95 antibody resulted in the appearance of both a p16 and p18 caspase-8 fragment, etoposide treatment produced only the p16 subunit (Fig. 1B). Even though the CD95-resistant counterparts (see Fig. 3) was accompanied by the processing of caspase-8 and -3. Interestingly, whereas triggering of the death receptor pathway by the agonistic CD95 antibody resulted in the appearance of both a p16 and p18 caspase-8 fragment, etoposide treatment produced only the p16 subunit (Fig. 1B). Although it was shown that caspase-8 processing in Jurkat cells that are classified as so-called type II cells results first in the generation of the p16 fragment before the p18 subunit appears (36), the exact reason for the lack of the p18 caspase-8 fragment in etoposide-treated cells is presently unknown. It is, however, tempting to speculate that the appearance of the p16 fragment is closely associated with activation of the mitochondrial pathway, whereas the generation of the p18 fragment requires DISC formation.

**Immunoprecipitation of Caspase-8 and -3**—Having established the conditions for anti-CD95- and etoposide-induced apoptosis and caspase processing, we wanted to assess whether proteolytic processing of caspase-8 in etoposide-treated cells produced the catalytically active enzyme. Therefore, we first analyzed the cleavage of the preferred caspase-8 substrate, IETD-AMC, in extracts of anti-CD95-treated Jurkat cells. As expected, those extracts displayed a significant increase in...
cleavage activity of both IETD-AMC and DEVD-AMC, the preferred caspase-3 substrates (Fig. 2A). However, although the DEVD-AMC cleavage activity was substantially reduced following caspase-3 immunodepletion, indicating that caspase-3 is indeed the main DEVD cleaving caspase, depletion of caspase-8 resulted only in a slight reduction of IETD-AMC cleavage activity (Fig. 2A). In addition, the depletion of caspase-3 had a reproducibly even stronger impact on IETD-AMC cleavage activity than the depletion of caspase-8 itself. This is probably because of the fact that caspase-3 can efficiently cleave IETD-AMC as well as to our observation that the active p16/p18 caspase-8 fragment was co-precipitated with caspase-3 (Fig. 2B). On the other hand, no caspase-3 was detected in immunoprecipitates of caspase-8 (Fig. 2B). Together, these results indicate that measurement of the IETD-cleavage activity in whole apoptotic cell extracts does not allow us to exactly determine the contribution of caspase-8 as also other caspases, such as caspase-3 and -6, are able to cleave this substrate (37). Although these caspases do not cleave IETD-AMC as efficiently as caspase-8, they are activated in considerably larger amounts and have a higher specific activity and therefore cleave a substantial proportion of the IETD-AMC substrate (38). To more directly assess the catalytic competence of caspase-8 in apoptotic cells, caspase-8 and as a control also caspase-3 were immunoprecipitated from untreated and anti-CD95-treated Jurkat cells and tested directly in the substrate cleavage assay. Surprisingly, whereas immunoprecipitated caspase-3 exhibited significant DEVDase activity as well as IETD-
AMC cleavage activity when compared with the cleavage activity obtained from whole apoptotic cell extracts, immunoprecipitated caspase-8 was only slightly more active than untreated control extract (Fig. 2A). The failure of immunoprecipitated caspase-8 to convincingly cleave IETD-AMC was not because of an inefficient precipitation, as both antibodies used almost completely precipitated the full-length caspase zymogens as well as the proteolytically processed fragments of caspase-8 and -3, respectively (Fig. 2B). Also the general procedure of the immunoprecipitation technique does not seem to inhibit caspase activity as, firstly, caspase-3 precipitates were fully active (Fig. 2A), and, secondly, addition of either the caspase-8 antibody or protein G-Sepharose to cell extracts of anti-CD95-stimulated Jurkat cells changed neither IETD-AMC nor DEVD-AMC cleavage activity (data not shown). Even when we used two additional monoclonal or polyclonal antibodies that successfully precipitated caspase-8, we were not able to detect significant IETDase activity in these precipitates (data not shown).

**Fig. 3. In vivo peptide affinity labeling of active caspases during apoptosis.** Jurkat wild-type cells (type II, A), H9 cells (type I, B) as well as Jurkat subclones deficient in CD95 (Jurkat-R), FADD (C), or caspase-8 (D), or Jurkat cells overexpressing Bcl-2 (D) were left untreated or stimulated with anti-CD95 (1 μg/ml, 5 h) or etoposide (100 μM, 9 h). After the incubation period, active caspases were labeled in vivo by the addition of biotin-VAD(OMe)-fmk. Cell extracts were prepared (input lane), and the biotinylated proteins were captured with streptavidin-agarose beads (streptavidin lane). The streptavidin beads and 25 μg of protein from the remaining supernatant and input were separated by SDS-PAGE and assayed for the activation of caspase-8 and -3 by Western blotting. For the detection of the streptavidin-captured intermediate p41/43 form of caspase-8 from H9 cells, a longer exposure time of the film was required, which is shown in a separate section of the gel. Asterisks denote nonspecific protein bands that are detected by the caspase-8 antibody also in caspase-8-deficient Jurkat cells (D).
Bcl-2 prevents processing of caspase-8, -3, and Bid. The indicated cell lines were treated for 15 h with etoposide (100 

Aliquots of the Jurkat cell extracts incubated for 2 h in the presence or absence of both caspase-3 and -6 were also used for affinity labeling with the non-methylated reagent, biotin-DEVD-CHO, confirming that the in vitro cleaved caspase-8 becomes proteolytically active (Fig. 3, right panel). D, Bcl-2 prevents processing of caspase-8, -3, and Bid. The indicated cell lines were treated for 15 h with etoposide (100 

mu g/mL), and the cell extracts were analyzed for the processing of caspase-8, -3, and Bid.

not shown). Our observation is consistent with findings from another group2 but in slight contrast to a very recent report (39). These authors demonstrated IETDase activity of precipitated caspase-8; however, only precipitated caspase-8 from untreated and apoptotic cell extracts were compared without relating these activities to the complete activity of the apoptotic lysates. Nevertheless, based on our results, the immunoprecipitation technique does not seem to be suitable for the determination of whether caspase-8 is active or not, at least in our hands. It appears that this technique captures either only inactive subunits or that caspase-8 is rendered inactive during the precipitation procedure, perhaps through an induced conformational change or due to the failure to precipitate other components that might be required for its activity.

Etoposide-generated Caspase-8 Fragments Are Active—To circumvent the problems using the immunoprecipitation procedure, we employed an alternative approach by labeling apoptotic cells in vivo with the biotinylated caspase inhibitor VAD-fmk (biotin-VAD-fmk). This inhibitor easily penetrates cell membranes and only binds active caspases that can then be captured using streptavidin-conjugated-agarose beads. Jurkat cells were first treated with either the anti-CD95 antibody or with etoposide followed by incubation for an additional hour in the presence of biotin-VAD-fmk. The detection of the active caspase-8 fragments (p21/p19/p17/p12) induced by both death stimuli served as a positive control for the induction of apoptosis as well as for the efficient labeling and isolation procedure of the active caspases (Fig. 3A, lower panels). No active caspase-3 subunits were pulled down from untreated control cells, validating the specificity of this technique. Most importantly, biotin-VAD-fmk-labeled caspase-8 fragments were present in extracts of anti-CD95-treated as well as in etoposide-treated Jurkat cells indicating that both death stimuli generate active caspase-8 (Fig. 3A, upper and middle panels). Consistent with our results shown in Fig. 1, etoposide treatment resulted only in the generation of the p16 caspase-8 fragment, whereas both the p16 and the p18 fragments could be detected in cells treated with the agonistic CD95 antibody (Fig. 3A, middle panels). Similar results were also obtained in type 1 cells such as H9 (Fig. 3B) or SKW cells (data not shown) as well as in CD95- or FADD-deficient Jurkat cells (Fig. 3C), supporting our hypothesis that only the generation of the active p18 caspase-8 fragment requires DISC formation. Also the p41/p43 caspase-8 fragments that were generated by both death stimuli were, at least partially, labeled by biotin-VAD-fmk suggesting that also these intermediate forms represent active caspase-8 (Fig. 3, upper panels). This finding is in agreement with several reports demonstrating that active initiator caspases are already generated via dimerization or oligomerization events even in the absence of complete processing of their proforms (16–19). However, considering that the input lanes contain approximately only 5% of the protein amount used for the pull downs in the respective streptavidin-captured lanes, very little active caspase-8 p41/p43 fragments could be detected. This is further emphasized by the observation that the relative amounts of the p41/p43 caspase-8 fragments before (input) and after (supernatant) the pull down did not differ significantly from each other, demonstrating that the majority of the caspase-8 intermediate fragments are not active regardless of whether they were generated following DISC or apoptosome formation (Fig. 3, upper panels).

Biotin-VAD-fmk-labeled and hence active caspase-8 fragments were also detected in etoposide-treated CD95- and FADD-deficient Jurkat cell lines (Fig. 3C) but not in Jurkat cells overexpressing Bcl-2 or caspase-8-deficient Jurkat cells (Fig. 3D). These results not only verify the specificity of our pull-down experiments but also demonstrate clearly that caspase-8 can be activated via the mitochondrial death pathway independently from death receptor signaling and DISC formation. In addition, these results also argue against an involvement of FADD in the mitochondrial pathway acting perhaps as a scaffolding protein in a secondary activation complex, as it was suggested for apoptosis induction through TNF receptor-1 (14).

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2 M. E. Peter, personal communication.
Both Caspase-3 and -6 Are Required to Generate the p16 Caspase-8 Fragment—So far our data provide strong evidence that direct interchain proteolysis of caspase-8 most likely in the absence of a death receptor-mediated dimerization platform is sufficient to produce active caspase-8. In the mitochondrial pathway, caspase-8 is believed to be proteolytically processed and thereby activated by caspase-6 (29, 40, 41). However, all of these reports could only detect the p41/p43 intermediate caspase-8 fragments but failed to convincingly demonstrate the presence of the p16/p18 caspase-8 fragments following incubation with caspase-6. In contrast, another report concluded that caspase-6, at least in vitro, does not activate caspase-8, because the caspase-6-generated p41/p43 caspase-8 fragments were not labeled by biotin-VAD-fmk (16). Based on our present results demonstrating that only the p16/p18 caspase-8 fragments were successfully labeled by biotin-VAD-fmk, whereas the p41/p43 intermediate fragments of caspase-8 were only very barely lytically process caspase-8 to yield the p41/p43 intermediate fragments, the p16 caspase-8 fragment was only very barely detectable (in the case of caspase-6) or not at all (in the case of caspase-3) (Fig. 4A). When both caspase-6 and -3, however, were added to Jurkat cell extract, an efficient generation of the large p16 subunit of caspase-8. For this purpose, we incubated cell extracts from untreated Jurkat cells with either caspase-6 or -3 alone or with a combination of both. Remarkably, although either caspase alone was able to proteolytically process caspase-8 to yield the p41/p43 intermediate fragments, the p16 caspase-8 fragment was only very barely detectable (in the case of caspase-6) or not at all (in the case of caspase-3) (Fig. 4A). When both caspase-6 and -3, however, were added to Jurkat cell extract, an efficient generation of the p16 caspase-8 subunit could be observed suggesting that both caspases are required for the activation of caspase-8 in vitro (Fig. 4A). Similar results were obtained with extracts of FADD-deficient Jurkat cells (Fig. 4B). Moreover, incubation of the cell extracts with caspase-3 and -6 not only resulted in the correct processing but also in the generation of caspase-8 fragments that could be affinity-labeled with the biotinylated caspase substrate (Fig. 4A, right panel).

The hypothesis that both caspase-3 and -6 are required for the activation of caspase-8 was verified by employing MCF-7 cells that harbor a functional deletion in the CASP-3 gene and hence are deficient for caspase-3 protein expression (42). Similar to the results described above (Fig. 4, A and B), the p16 caspase-8 fragment was generated in MCF-7 cell extracts in the presence of both recombiant caspases (caspase-6 and -3), whereas caspase-6 alone only generated the p41/p43 fragments (Fig. 4C). On the other hand, caspase-6 alone was sufficient to produce the p16 caspase-8 fragment in extracts of MCF-7 cells stably expressing caspase-3 further supporting our hypothesis that both caspase-3 and -6 are required.

Finally, we analyzed the hierarchical caspase activation profile in etoposide-induced apoptosis and found that the processing of caspase-8 as well as the cleavage of Bid were induced in Jurkat and FADD-deficient Jurkat cells but were completely abrogated in Jurkat cells overexpressing Bcl-2 (Fig. 4D). Consistent with this we also observed that streptavidin-captured biotin-VAD-fmk did not recover any active caspase-8 or -3 fragments from these cells (Fig. 3D). Together these data not only only demonstrate that the etoposide-induced activation of caspase-8 depends entirely on caspases that are activated in a postmitochondrial event such as caspase-3 and -6 but thereby also argue against an involvement of caspase-2 that was recently implicated as an initiator caspase acting upstream of mitochondria in drug-induced apoptosis (43).

In Jurkat cells deficient of caspase-8 Bid cleavage occurred to an almost similar extent as in wild-type cells (Fig. 4D). This observation is mostly likely because of the fact that in comparison to caspase-8, Jurkat cells abundantly express caspase-3, which was shown to efficiently cleave Bid (25, 40). Interestingly, the susceptibility of Bid to cleavage by caspase-8 and -3 appears to be cell-type specific and differentially regulated by posttranslational modifications (44, 45). Thus, even though postmitochondrial activation of caspase-8 did not strongly affect Bid cleavage in our system, it might constitute a mitochondrial amplification loop by augmenting Bid cleavage and cytochrome c release in particular in response to a weak apoptotic signal. Indeed, such a positive postmitochondrial feedback loop has been recently proposed for several cell types in studies investigating the proteolytic processing of caspase-8 during death receptor-independent apoptosis (28, 40, 46–48).

Together our data demonstrated that interchain processing of caspase-8 in the absence of DISC formation, a process that occurs during the mitochondrial death pathway in vivo, is indeed sufficient to produce active caspase-8 enzyme. Thus, it is valid to conclude that the appearance of caspase-8 fragments observed in numerous death receptor-independent apoptosis systems correlates with its activity. During the preparation of our paper, Murphy et al. (39) showed that direct proteolysis of caspase-8 by the CTL protease granzyme B produces an active enzyme in vitro that displays proteolytic activity toward synthetic as well as natural caspase-8 substrates. Our results are not only supportive but further extend this finding as we show that active caspase-8 is generated during etoposide-induced apoptosis in vivo. Furthermore, we show that both executioner caspases, caspase-3 and -6 are required to process and thereby activate caspase-8 in vitro, supporting the idea that a similar process occurs also in vivo during the course of the mitochondrial death pathway. As the p41/p43 caspase-8 fragments were much more efficiently generated in the presence of caspase-6, it seems plausible that caspase-6 preferentially cleaves the linker domain between the large and small subunit of caspase-8, whereas caspase-3 removes preferentially the prodomain. Although our data do not reveal whether the observed caspase-8 fragments following etoposide treatment in vivo are active as a dimer or monomer, the observation that substrate binding promotes the formation of caspase-8 dimers supports the former view (17). Finally, our results argue against an involvement of the DISC adapter protein FADD further supporting our view of a death receptor-independent activation of caspase-8 via the caspase-3- and -6-mediated interchain proteolysis.

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