The Adapter Protein Apoptotic Protease-activating Factor-1 (Apaf-1) Is Proteolytically Processed during Apoptosis*

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Kirsten Lauber‡, Helga A. E. Appel‡, Stephan F. Schlosser‡, Michael Gregor‡, Klaus Schulze-Osthoff‡, and Sebastian Wesselborg‡¶

From the ‡Department of Internal Medicine I, University of Tübingen, D-72076 Tübingen, Germany and the ¶Department of Immunology and Cell Biology, University of Münster, D-48149 Münster, Germany

Apoptotic protease-activating factor-1 (Apaf-1), a key regulator of the mitochondrial apoptosis pathway, consists of three functional regions: (i) an N-terminal caspase recruitment domain (CARD) that can bind to procaspase-9, (ii) a CED-4-like region enabling self-oligomerization, and (iii) a regulatory C terminus with WD-40 repeats masking the CARD and CED-4 region. During apoptosis, cytochrome c and dATP can relieve the inhibitory action of the WD-40 repeats and thus enable the oligomerization of Apaf-1 and the subsequent recruitment and activation of procaspase-9. Here, we report that different apoptotic stimuli induced the caspase-mediated cleavage of Apaf-1 into an 84-kDa fragment. The same Apaf-1 fragment was obtained in vitro by incubation of cell lysates with either cytochrome c/dATP or caspase-3 but not with caspase-6 or caspase-8. Apaf-1 was cleaved at the N terminus, leading to the removal of its CARD H1 helix. An additional cleavage site was located within the WD-40 repeats and enabled the oligomerization of p84 into a ~440-kDa Apaf-1 multimer even in the absence of cytochrome c. Due to the partial loss of its CARD, the p84 multimer was devoid of caspase-9 or other caspase activity. Thus, our data indicate that Apaf-1 cleavage causes the release of caspases from the apoptosome in the course of apoptosis.

There exist at least two major pathways leading to the apoptotic demise of the cell: the extrinsic death receptor pathway and the intrinsic cell death pathway. In death receptor-mediated apoptosis, the adapter protein FADD plays a central role in the formation of the death-inducing signaling complex, in which FADD recruits procaspase-8 to the active trimeric death receptor. Procaspase-8 is then autocatalytically activated and in turn cleaves and activates the downstream cascade of effector caspases (1–4). Likewise, the adapter protein Apaf-11 constitutes the central element in the intrinsic pathway that can be triggered by the release of cytochrome c from the mitochondrial (5, 6). The release of cytochrome c can be initiated either through death receptor-mediated activation of the Bcl-2-related protein Bid (7, 8) or independently of this pathway by other proapoptotic Bcl-2 members such as Bcl-xL, Bad, Bak, Bax, Noxa, and Bim, which may be triggered by apoptotic stimuli such as anticancer drugs and irradiation (9–14). Conversely, antiapoptotic members of the Bcl-2 family such as Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1 block the mitochondrial release of cytochrome c and the subsequent activation of caspases and apoptosis (9–11). In the cytosol, cytochrome c together with dATP binds to the mammalian CED-4 homologue Apaf-1, which subsequently recruits and activates procaspase-9 via a mutual interaction of their caspase recruitment domains (CARDs) (6). Caspase-9 in turn activates downstream effector caspases. Thus, caspase-9 constitutes the central initiator caspase for the mitochondrial pathway as caspase-8 represents it for the death receptor pathway. In analogy, the adapter protein Apaf-1 plays a similar role for the activation of the mitochondrial pathway as FADD does it in the death receptor pathway. In addition, there is some evidence that pro- and antiapoptotic Bcl-2 proteins may interfere with mechanisms other than the release of cytochrome c (15, 16). In the nematode Caenorhabditis elegans, the proapoptotic Bcl-2 homologue Egl-1 antagonizes CED-9, an antiapoptotic Bcl-2 protein that is directly bound to and inhibits the Apaf-1 homologue CED-4 (17). Although in mammalian cells most Bcl-2 proteins do not directly bind to Apaf-1 (18), other CED-4 related proteins might exist, which in analogy to C. elegans do not require cytochrome c but are directly modulated by Bcl-2 members.

Apaf-1 consists of three different domains: (i) the N-terminal protein interaction domain CARD, which can bind via a homophilic interaction to the CARD of procaspase-9; (ii) a nucleotide-binding domain that shares homology with the C. elegans homologue CED-4; and (iii) a C-terminal regulatory domain containing multiple repeats of tryptophan and aspartate residues, also known as WD-40 repeats (19). Recent studies support a model in which the WD-40 repeats mask the CARD of Apaf-1 and thereby obstruct the binding of Apaf-1 to procaspase-9. It is thought that, following its release from mitochondria, binding of cytochrome c to Apaf-1 removes the WD-40 repeat region, which together with dATP enables Apaf-1 oligomerization via a mutual interaction of the CED-4-like regions. Oligomerized Apaf-1 in turn is able to recruit procaspase-9 via a homophilic CARD/CARD interaction (20–26). The model of induced proximity has been proposed to explain how the first proteolytic signal is produced after clustering of the inactive caspase-9 precursor. According to this model, multimolecular aggregation of Apaf-1 enforces a...

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‡ These two authors share equal senior authorship.

§ To whom correspondence should be addressed: Dept. of Internal Medicine I, Eberhard-Karls-University, Otfried-Müller-Str. 10, D-72076 Tübingen, Germany. Tel.: 49-7071-29-84113; Fax: 49-7071-29-5865; E-mail: snwessel@med.uni-tuebingen.de.

¶ The abbreviations used are: Apaf, apoptotic protease-activating factor; CARD, caspase recruitment domain; AMI, 7-amino-3-methyl-4-coumarin; PAGF, polyacrylamide gel electrophoresis; zVAD-fmk, benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; GFP, green fluorescent protein.

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Caspase activities were determined by incubation of cell lysates with sodium citrate, 0.1% Triton X-100, 50 mM modified EDTA, 0.1 mM dithiothreitol. The release of aminothiolate was measured in a kinetic by spectrophotometry using an excitation wavelength of 360 nm and an emission wavelength of 475 nm. Caspase activity was determined as the slope of the resulting linear regressions and expressed in arbitrary fluorescence units per minute.

**MATERIALS AND METHODS**

**Cells and Reagents**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium, and all other cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10 mM HEPES (all from Life Technologies, Inc.). Cells were grown at 37 °C in a 5% CO2 atmosphere and maintained in log phase. Caspase-8-deficient Jurkat cells and the parental Jurkat cell line A3 were kindly provided by J. Blenis (Harvard Medical School, Boston, MA) (30). Stable transfectants of Jurkat cells overexpressing Bcl-2 were a gift from H. Walczak (Heidelberg, Germany). HeLa cells stably transfected with caspase-3 and the parental Jurkat cell line A3 were kindly provided by J. Blenis (Harvard Medical School, Boston, MA) (30). Stable transfectants of Jurkat cells overexpressing Bcl-2 were a gift from H. Walczak (Heidelberg, Germany). HeLa cells stably transfected with a fusion construct of the prodomain of caspase-9 (Pharmingen) that detected the 84-kDa fragment were provided by Y. A. Lazebnik, Cold Spring Harbor, NY (34), mouse monoclonal antibodies against caspase-3 and caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies directed against caspase-8 (BioCheck) or caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies against caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies against caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies against caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies against caspase-9 (kindly provided by Y. A. Lazebnik, Cold Spring Harbor, NY) (34), mouse monoclonal antibodies against caspase-9 (kindly provided by Y. A. 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etoposide (Etopo); purified fractions were dialyzed in 40 mM Tris-HCl, pH 7.9, 500 mM NaCl, 50% glycerol and examined for homogeneity by SDS-PAGE and Coomassie Blue staining. The activity of the purified caspases was monitored in a fluorimetric assay using the synthetic substrates Ac–Coomassie Blue staining. The activity of the purified caspases was monitored in a fluorimetric assay using the synthetic substrates Ac–DEVD-AMC for caspase-8, Ac–VEID-AMC for caspase-3, and Ac–IETD-AMC for caspase-6.

RESULTS

Different Apoptotic Stimuli Induce the Proteolytic Degradation of Apaf-1—We have recently demonstrated that anticancer drugs and staurosporine induce the activation of the mitochondrial pathway in the absence of death receptor signaling (31, 35–37). Interestingly, when Jurkat cells were stimulated with agonistic antibodies against CD95 or through the mitochondrial pathway with the anticancer drugs etoposide, mitomycin C, or staurosporine, the processing of Apaf-1 into an 84-kDa fragment could be observed (Fig. 1A). This processing involved the activation of caspases, since the addition of the broad spectrum caspase inhibitor zVAD-fmk prevented the proteolytic cleavage of Apaf-1. The very same stimuli also induced caspase activation and subsequent induction of apoptosis (Fig. 1, B and C).

Activation of the Mitochondrial Pathway Induces the Caspase-9-Dependent Processing of Apaf-1—Apaf-1, the central element in the mitochondrial apoptotic pathway, forms a large hetero-oligomeric complex with cytochrome c in a dATP-dependent fashion. Only the oligomeric complex is capable of activating procaspase-9, which in turn activates the executioner caspase-3 (20–26). We therefore wished to investigate the sequence of events involved in the proteolytic degradation of Apaf-1. To this end, we stimulated Jurkat cells with staurosporine for different amounts of time and determined the proteolytic cleavage of caspase-9, caspase-3, and Apaf-1 by immunoblotting. Stimulation with staurosporine induced the proteolytic activation of caspase-9 after 90 min and of caspase-3 after 120 min. Proteolytic degradation of Apaf-1 was observed at a later time point after 180 min (Fig. 2A). Inhibition of staurosporine-induced caspase activity with zVAD-fmk also abolished the degradation of Apaf-1.

To analyze whether Apaf-1 can be cleaved following activation of the apoptosome in vitro, we added cytochrome c and dATP to mitochondria-depleted S10 extracts. This procedure induced the activation of caspase-9 as early as after 20 min. The subsequent activation of caspase-3 and degradation of Apaf-1 occurred after 40 min. Again, the addition of zVAD-fmk inhibited the processing of procaspase-9 and Apaf-1 (Fig. 2B).

To further address the question of whether caspase-9 is a prerequisite for the cleavage of Apaf-1 in the mitochondrial pathway, we used HeLa cells stably expressing a dominant
negative caspase-9 mutant that consisted of the first 163 C-terminal amino acids but lacked the catalytic domain (31). Overexpression of this mutant abrogated the staurosporine-mediated processing of Apaf-1, whereas degradation was observed in vector control cells (Fig. 2C). Thus, proteolytic degradation of Apaf-1 occurred during activation of the mitochondrial pathway and was dependent on caspase-9.

**Caspase-3 but Not Caspase-8 Is Required for Apaf-1 Processing**—Recent in vitro studies have demonstrated that caspase-9 activates procaspase-3. Active caspase-3 then processes procaspase-6, which in turn can activate procaspase-8 (31, 38, 39). In order to investigate which caspase in the mitochondrial cascade was responsible for Apaf-1 cleavage, we incubated cytosolic extracts of unstimulated Jurkat cells with purified caspase-3, caspase-6, or caspase-8. As shown in Fig. 3, caspase-3 and caspase-8 but not caspase-6 were able to degrade Apaf-1 into the 84-kDa fragment in vitro. We also tested a commercial preparation of caspase-9 that did not degrade Apaf-1 (data not shown).

In the previous experiment, it could not be ruled out that the cleavage of Apaf-1 by caspase-8 was mediated indirectly via caspase-8-induced activation of endogenous caspase-3. In order to examine this possibility, we made use of Jurkat cells deficient in the expression of caspase-8 (30). Stimulation of the caspase-8-containing parental Jurkat line A3 with different stimuli such as anti-CD95, staurosporine, etoposide, and mitomycin C induced the activation of caspase-8 and caspase-3 and the cleavage of Apaf-1 (Fig. 4A), which was paralleled by the induction of apoptosis (Fig. 4B). However, in caspase-8-deficient Jurkat, we observed neither induction of apoptosis nor the cleavage of caspase-3 and Apaf-1 upon CD95 triggering (Fig. 4, A and B). In contrast to CD95, staurosporine as well as the anticancer drugs etoposide and mitomycin C induced the activation of caspase-3 and subsequent apoptosis in the absence of caspase-8 (Fig. 4, A and B). Interestingly, Apaf-1 was also processed into the p84 fragment in the absence of caspase-8, indicating that caspase-8 was not required for the degradation of Apaf-1 and most likely mediated the generation of the p84 fragment indirectly via the activation of caspase-3.

To further substantiate this point, we incubated lysates of caspase-3-deficient and caspase-3-transfected MCF7 cells with...
exogenous caspase-3, -6, and -8. As shown in Fig. 4C, only caspase-3 was able to induce the processing of Apaf-1 in lysates of caspase-3-deficient MCF7 cells, whereas in caspase-3-transfected MCF7 cells both caspase-3 and caspase-8 could cleave Apaf-1. The addition of the broad range caspase inhibitor zVAD-fmk abrogated the proteolytic cleavage of Apaf-1. Caspase-6 that in the mitochondrial pathway is downstream of caspase-3 had no effect on Apaf-1 cleavage.

Cleavage of Apaf-1 Can Be Induced Outside of the Apoptosome—The previous experiments demonstrated that death receptor and caspase-8 signaling were not required for the proteolytic degradation of Apaf-1. Next, we investigated whether Apaf-1 was cleaved as part of the apoptosome complex during activation of the mitochondrial pathway or whether Apaf-1 could be cleaved also outside of the apoptosome in the death receptor pathway. To this purpose, we stimulated Jurkat cells overexpressing the anti-apoptotic protein Bcl-2, which inhibits the mitochondrial pathway by blocking the release of cytochrome c. As demonstrated previously (40), activation of the mitochondrial pathway by anticancer drugs and staurosporine was inhibited by Bcl-2 (Fig. 5B). Overexpression of Bcl-2 also prevented staurosporine- and drug-induced degradation of caspase-3 and Apaf-1 (Fig. 5A). However, CD95-mediated activation of caspase-3 and apoptosis was only attenuated and not completely blocked by Bcl-2. Interestingly, the residual caspase activity was obviously sufficient to degrade Apaf-1 to its p84 fragment. Since Bcl-2 blocks the formation of the apoptosome and caspase activation in the mitochondrial pathway, CD95-mediated cleavage of Apaf-1 in Jurkat Bcl-2 cells occurred presumably outside of the apoptosome (Fig. 5A).

Localization of the Putative Caspase Cleavage Sites in Apaf-1—In order to map the potential caspase cleavage sites in Apaf-1, we used antibodies recognizing different structural Apaf-1 regions. As shown by immunoblot analysis (Fig. 6A), only a rabbit antiserum directed against the N-terminal amino acids 37–52 of the Apaf-1 CARD detected the p84 fragment. In contrast, antibodies generated by immunization with a peptide spanning amino acids 12–28 recognized the native p130 form but not the p84 fragment of Apaf-1 (Fig. 6A). The only potential caspase cleavage site located within the first N-terminal 36 amino acids of Apaf-1 is the motif LEKD19. Cleavage at this site removes the first 19 amino acids and would explain why antibodies recognizing an epitope at amino acids 12–28 did not detect the p84 fragment (see also Fig. 6C). To validate the specificity of the antibodies, we constructed mutants of Apaf-1 containing amino acids 1–540 or amino acids 20–540 (Fig. 6C). When the different mutants were tested in immunoblot analyses, the anti-serum recognizing amino acids 12–28 detected only Apaf-1(1–540) but not Apaf-1(20–540), whereas the antibody against amino acids 37–52 reacted with both mutants (Fig. 6B). This further indicated that a caspase cleavage site was located at the N terminus within the first 20 amino acids. However, since removal of the first 19 amino acids would reduce the molecular mass by only ~2 kDa, another caspase cleavage site must be assumed at the C terminus within the WD-40 repeat region (see Fig. 6C). Due to the abundance of aspartates and potential cleavage sites within the WD-40 repeats, the exact location of this cleavage site is hard to predict.

The Apaf-1 p84 Fragment Can Oligomerize in the Absence of Cytochrome c but Lacks Caspase Activity—The current model suggests that during apoptosis induction Apaf-1 oligomerizes and recruits procaspase-9 into a large heteromeric complex of ~700 kDa. It is thought that the WD-40 repeats mask the CARD- and CED-4-like region in the inactive Apaf-1 monomer. Binding of cytochrome c to Apaf-1 relieves the WD-40 repeat region and thus enables Apaf-1 oligomerization via its CED-4 domain and the subsequent recruitment and activation of procaspase-9 by CARD/CARD interaction (20–26). Since the p84 Apaf-1 fragment was obviously missing part of the WD-40 repeat region, it was conceivable that p84 might be able to self-oligomerize in the absence of cytochrome c. Moreover, the lack of the first 19 amino acids within its CARD region of p84 might disable its binding to procaspase-9. Consequently, we had to investigate (i) whether Apaf-1 was just another caspase substrate cleaved outside or within the apoptosome, (ii) whether the Apaf-1 fragment was part of the apoptosome and had no function or an inhibitory function, and (iii) whether p84 could oligomerize and recruit procaspase-9 independently of cytochrome c.

In order to address these questions, we used mitochondria depleted S100 extracts and incubated them in the absence or presence of cytochrome c plus dATP or purified caspase-3. Subsequently, cellular proteins and protein complexes were separated based on their molecular weight on a Superdex-200 gel filtration column. The processing of Apaf-1 and procaspase-9 in the respective fractions was detected by immunoblotting. When untreated control lysates were separated by gel filtration, Apaf-1 eluted as a protein of ~250–300 kDa (Fig. 7A; fractions 25–27) that was not associated with procaspase-9 (fractions 29–34), similar to previous results (24). The addition of cytochrome c and dATP to S100 extracts induced the oligomerization of Apaf-1 p130 into the apoptosome of ~700 kDa...
Fig. 5. Apaf-1 can be processed outside of the apoptosome. A, overexpression of Bcl-2 inhibits the processing of caspase-3 and Apaf-1 via the mitochondrial pathway but not via the death receptor pathway. 2 × 10⁶ Jurkat cells stably transfected with the vector alone or with Bcl-2 were pretreated and stimulated as described in the legend to Fig. 4A. Cellular proteins were separated by SDS-PAGE, and the proteolytic processing of Apaf-1 and caspase-3 was detected by immunoblotting. The closed arrowheads indicate the uncleaved form and open arrowheads indicate the cleaved form of the indicated proteins. A nonspecific band is indicated with an asterisk. B, induction of apoptosis. 3 × 10⁶ Jurkat cells overexpressing Bcl-2 or the vector control were treated as described in Fig. 4B. After 20 h, induction of apoptosis was assessed by propidium iodide staining of hypodiploid apoptotic nuclei and flow cytometry. The mean values of triplicate cultures are given.

The in vitro induced cleavage of Apaf-1 in S100 extracts by exogenous caspase-3 should answer the question of whether p84 could self-oligomerize in the absence of cytochrome c and dATP and whether multimerized p84 was able to recruit procaspase-9. As shown in Fig. 7C, the addition of caspase-3 induced the formation of the ~440-kDa multimer of p84 in the absence of cytochrome c and dATP (fractions 23–25). Similarly, to the treatment with cytochrome c (see Fig. 7B, fractions 23–25), the caspase-3-induced p84 multimer was almost devoid of caspase-9; nor did it contain any p130 (Fig. 7C, fractions 23–25).

Interestingly, treatment of S100 extracts with caspase-3 induced a shift in multimerization of p130 from fractions 25–27 (as also observed in the control; see Fig. 7A) to a multimer with a higher molecular weight (Fig. 7C; fractions 20–23). This p130 multimer of Apaf-1 had a lower molecular weight than the ~700-kDa p130 multimer induced by the addition of cytochrome c and dATP. The distribution of caspase-9 upon the addition of caspase-3 was similar to the treatment with cytochrome c/dATP. However, the caspase-9-cleaved p35 form of caspase-9 was not detected in fractions 21 and 22 (compare fractions 21 and 22 in Fig. 7C with Fig. 7B). Interestingly, as upon treatment with cytochrome c/dATP, we detected the caspase-3-cleaved p37 form of caspase-9 in the high molecular weight fractions 19 and 20, although there was no coelution with the p130 or p84 oligomer of Apaf-1 (Fig. 7C). Thus, the treatment of S100 extracts with caspase-3 apparently induced the multimerization of caspase-9, which was either due to altered protein folding or its association with another high molecular complex in which Apaf-1 was not physically present.

In order to examine whether the p84 multimer also contained caspase activity, we monitored the DEVDase activity of each fraction. As shown in Fig. 7D, the majority of caspase activity upon treatment with cytochrome c and dATP eluted in fractions 31–34, corresponding to free caspases, presumably caspase-3 (Fig. 7D). In addition, the multimer of caspase-9 (p35 and p37 in fractions 19–21; Fig. 7B) and p130 constituting the apoptosome contained DEVDase activity (Fig. 7D). This activity could not be attributed to caspase-3, since caspase-3 was missing in these fractions (Fig. 7B). Treatment of extracts with exogenous caspase-3-induced caspase activity in the high molecular weight fractions 19 and 21 (Fig. 7D), which coincided with the p37 form of caspase-9 in these fractions (Fig. 7C).

However, we could not detect any caspase activity in the 440-kDa multimer of p84 (see fractions 23–25 in Fig. 7, B–D). Thus, neither the cytochrome c nor the caspase-3-induced multimer of p84 contained caspase activity. As a control, purified caspase-3 alone was separated by gel filtration and showed little if any DEVDase activity in the high molecular weight range. Lysates of unstimulated control cells were almost completely devoid of any DEVDase activity.

Taken together, we demonstrate that the p84 fragment of Apaf-1 is able to self-oligomerize even in the absence of cytochrome c and dATP. Interestingly, the p84 multimer contained neither full-length Apaf-1 nor caspase-3 and -9. The ability of the Apaf-1 fragment to self-associate into a ~440-kDa complex without cytochrome c was presumably due to the partial removal of the WD-40 repeat region. In addition, the partial loss of the CARD region could explain the inability of the Apaf-1 fragment to recruit procaspase-9 in response to caspase-3 or cytochrome c/dATP treatment.

DISCUSSION

The adapter protein Apaf-1 forms a central element in the mitochondrial death machinery and is responsible for the recruitment and activation of initiator caspase-9. Caspase-9 in turn activates executioner caspase-3 and -7, thus instigating the caspase cascade that after cleavage of several vital proteins finally leads to cell death (41). In the present study, we observed that Apaf-1 itself is a target of caspases that are proteolytically processed from its 130-kDa full-length form into an 84-kDa fragment. In cells, Apaf-1 cleavage was observed after stimulation of the CD95 death receptor pathway as well as of the mitochondrial pathway by anticancer drugs and staurosporine.

Activation of the mitochondrial pathway by staurosporine induced the sequential proteolytic cleavage of caspase-9,
caspase-3, and subsequently Apaf-1. Apaf-1 was also cleaved in vitro, when the mitochondrial pathway was triggered directly by the addition of cytochrome c and dATP. The proteolytic processing of Apaf-1 in the mitochondrial pathway was dependent on caspase-9, since in HeLa cells stably expressing a dominant negative caspase-9 mutant Apaf-1 was not degraded. In order to identify the caspase responsible for the processing of Apaf-1, we incubated cellular extracts with purified caspases. Caspase-3 and -8 but not caspase-6 or -9 induced the proteolytic degradation of Apaf-1 from cellular extracts into its p84 fragment. In order to examine whether caspase-8-induced Apaf-1 cleavage was a direct effect or mediated by caspase-8-induced activation of downstream caspases, we performed experiments with lysates from cells deficient in single caspases. Stimulation of the cytochrome c/Apaf-1 pathway by anticancer drugs or staurosporine induced the processing of Apaf-1 also in caspase-8-deficient Jurkat cells. Moreover, recombinant caspase-8 processed Apaf-1 in cellular lysates of caspase-3-transfected MCF7 cells, whereas in caspase-3-deficient MCF7 cells the only caspase capable of cleaving Apaf-1 was caspase-3. These data indicated that caspase-8-induced processing of Apaf-1 was mediated by caspase-3 and that caspase-8 itself was not responsible for the cleavage of Apaf-1.

It was recently demonstrated that caspase-3 and -7 are part of the apoptosome (24, 26). Thus, although caspase-9 might not be directly involved in Apaf-1 cleavage, it was possible that the processing of Apaf-1 was restricted to the apoptosome and mediated by executioner caspases associated within the holoenzyme complex. However, when the mitochondrial pathway was blocked by Bcl-2 overexpression, CD95 death receptor stimulation induced a weak but detectable processing of Apaf-1. The attenuated cleavage of Apaf-1 corresponded to the decreased processing of caspase-3 during CD95 stimulation in Jurkat cells overexpressing Bcl-2 or Bcl-xL (31). Therefore, in cells overexpressing Bcl-2, stimulation of CD95 can activate caspase-3 independently of the mitochondrial pathway, indicating that Apaf-1 can be processed outside of the apoptosome.

Using antibodies directed against different regions of Apaf-1...
and Apaf-1 mutants, we localized a putative caspase cleavage site to the motif LEKD at the N terminus of the CARD. Since cleavage at this site would reduce the molecular weight by only about 2 kDa, an additional caspase cleavage must be located within the WD-40 repeats in order to generate the p84 fragment. Due to the presence of several potential cleavage sites, the exact localization of the second C-terminal site would require direct sequence analysis. The location of the two caspase cleavage sites within the CARD and WD-40 repeats may have functional consequences for the activity of Apaf-1, since both regions play important regulatory roles. Previous studies demonstrated that an Apaf-1 mutant that lacked the WD-40 repeats was able to self-oligomerize and to activate procaspase-9 without the requirement of cytochrome c and dATP (20, 21). Consequently, the partial removal of the WD-40 repeats should enable Apaf-1 p84 to assemble in the absence of cytochrome c and dATP. One way to test this possibility was to process Apaf-1 from S100 extracts in vitro with purified caspase-3.

**FIG. 7.** The p84 Apaf-1 fragment can oligomerize in the absence of cytochrome c and dATP but contains no caspase activity. 20 mg of S100 extracts from unstimulated Jurkat cells were either left untreated (A), incubated with 10 μM purified cytochrome c and 1 mM dATP (B), or treated with 40 μg of purified caspase-3 (C) in a final volume of 1 ml for 30 min. Subsequently, 10 mg of protein was fractionated by size exclusion chromatography on a Superdex-200 gel filtration column as described under “Materials and Methods.” Fractions of 500 μl were collected, and aliquots of 100 μl were subjected to SDS-PAGE followed by immunoblot analysis against Apaf-1, caspase-9, and caspase-3. The filled arrowheads show the uncleaved form and open arrowheads show the cleaved form of indicated proteins. The asterisks indicate nonspecific bands in Apaf-1 Western blots. The fraction number applied to immunoblot analysis is shown at the top. Elution positions of molecular weight standards are marked with arrows at the top. The positions of the apoptosome and the free caspases are indicated with brackets at the bottom. D, an aliquot of 100 μl of the chromatographic fractions was assayed fluorimetrically for DEVDase activity as described in the legend to Fig. 1B. Caspase activity is given in arbitrary fluorescent units/min. DEVDase activity in untreated S100 lysates is shown with a dashed line and open circles, lysates treated with cytochrome c and dATP are indicated with a continuous line and closed circles, lysates treated with purified caspase-3 are shown with a continuous line and closed triangles, and purified caspase-3 without S100 extracts is shown with a dashed line and open triangles.
Indeed, caspase-3-mediated cleavage enabled the oligomerization of p84 in the absence of cytochrome c and dATP. The resulting ~440-kDa complex of p84 was not associated with the full-length form p130. However, this does not exclude the possibility that at an early time point of apoptosome formation an intermediate p84-p130 complex might occur that is further processed to the ~440-kDa p84 multimer. Thus, the 440-kDa multimer might result from the proteolytic cleavage of Apaf-1 p130 and the subsequent loss of caspase-9 from the ~700-kDa apoptosome.

In contrast to mutant Apaf-1 lacking the WD-40 repeats, which is able to recruit procaspase-9 (21, 26), the ~440-kDa multimer of p84 was not associated with caspase-9; nor did it contain any caspase activity. The inability of binding procaspase-9 was presumably caused by the removal of the Apaf-1 N terminus within the CARD region. The CARD of Apaf-1 consists of six tightly packed amphipathic α-helices (42–44). The cleavage sequence LEKD is located at the end of the α-helix H1 that is in fact composed of two smaller helices and therefore should be easily accessible for caspase cleavage. The CARD/CARD interaction site with procaspase-9 has been mapped to helices H2 and H3, which are opposite to H1 (42–44). Thus, it is conceivable that deletion of the H1 helix destroys the overall structure of Apaf-1 CARD and disables interaction with procaspase-9. This would in turn release caspase-9 from the apoptosome and leave a p84 multimer devoid of any caspase activity. It may be speculated that caspase-mediated degradation of Apaf-1 is a mechanism for the release of caspase-9 from the apoptosome. In a similar context, we observed the majority of the p35 and p37 form of caspase-9 in the monomeric fractions. However, a recent study suggested that caspase-9 is only highly active when bound in a complex with Apaf-1 serving as an allosteric activator (28). Thus, whether the DEVDase activity found in the low molecular weight fractions is attributable to caspase-9 or to executioner caspase-3, -6, and -7 is currently unclear.

Besides the functional apoptosome, two other high molecular Apaf-1 complexes have been recently described, including an inactive 1.4-MDa complex as well as a so-called microaposome of ~200–300 kDa (24, 45). The microaposome has been found to contain caspase-3 and caspase-7 but no Apaf-1 p130 and may be generated from the functional apoptosome by proteolytic degradation. It is plausible that the antibodies used in the above mentioned study failed to detect the p84 proteolytic fragment. Indeed, we noticed in the literature that several Apaf-1 antibodies have been generated against N-terminal peptides and may therefore not recognize the Apaf-1 proteolytic molecules. The cleavage site of p30 has been mapped within the CED-4-like region of Apaf-1. Since the p30 cleavage product was preferentially found within the biologically inactive 1.4-MDa complex as well as a so-called microaposome of ~200–300 kDa (24, 45), the microaposome has been found to contain caspase-3 and caspase-7 but no Apaf-1 p130 and may be generated from the functional apoptosome by proteolytic degradation. It is plausible that the antibodies used in the above mentioned study failed to detect the p84 proteolytic fragment. Indeed, we noticed in the literature that several Apaf-1 antibodies have been generated against N-terminal peptides and may therefore not recognize the Apaf-1 proteolytic fragment. Nevertheless, we consider it unlikely that the 440-kDa multimer is identical to the microaposome, due to differences in the molecular weight of both complexes and the fact that the 440-kDa multimer contained neither caspase-3 nor DEVDase activity.

Very recently, Bratton et al. demonstrated that caspase-3 can cleave Apaf-1 into an ~30-kDa fragment (46). Interestingly, the p30 cleavage product was preferentially found within the biologically inactive ~1.4 MDa apoptosome, suggesting that this cleavage may selectively occur in misfolded Apaf-1 molecules. The cleavage site of p30 has been mapped within the CED-4-like region of Apaf-1. Since the p84 fragment described here should contain an intact CED-4-like domain, Apaf-1 obviously might be processed at more than two different cleavage sites. Interestingly, when Bratton et al. used purified Apaf-1 for cleavage with caspase-3 or -7 in vitro, besides the p30 product two additional higher molecular weight fragments of Apaf-1 became apparent. Unfortunately, the authors did not follow this observation in detail, and it remains unclear whether these cleavage products are related to our p84 fragment. Thus, future experiments will have to address the question of whether Apaf-1 is differentially cleaved within the active ~700-kDa and the inactive ~1.4-MDa apoptosome complex.

Another intriguing functional consequence of Apaf-1 cleav-
age concerns the role of effector caspases within the apoptosome. It has been suggested that the WD-40 repeats form propeller-like structures that may serve as interfaces for effector caspases (47). Indeed, in some studies, caspase-3 and -7 have been found within the apoptosome (24, 26). Furthermore, it was demonstrated that an Apaf-1 mutant lacking WD-40 repeats associated with and activated procaspase-9 but failed to recruit procaspase-3 and to induce apoptosis (26). These results indicate that WD-40 repeats may be involved in the recruitment of procaspase-3 to the apoptosome. Conversely, the partial removal of the WD-40 repeats during activation of the cytochrome c/Apaf-1 pathway would facilitate the release of executioner caspases such as caspase-3 from the apoptosome.

Alternatively, the degradation of Apaf-1 might serve as a negative feedback mechanism that allows the apoptotic process to be halted. Inhibitor of apoptosis proteins including XIAP can inhibit individual caspases and other apoptotic events through distinct protein domains (48). Especially in cells with low Apaf-1 and increased XIAP levels, Apaf-1 cleavage could thus even completely inhibit the propagation of a death signal. However, we cannot exclude the possibility that the cleavage of Apaf-1 constitutes a negative feedback mechanism by generating a dominant-negative Apaf-1 inhibitor, we presume that Apaf-1 cleavage has rather an apoptosis-promoting effect. During apoptosis, caspase-3 and -7 have been localized in different cellular compartments, such as for instance the cell nucleus (49, 50). In contrast, Apaf-1 is mainly localized in the cytosol (18), although it remains unclear whether the apoptosome is tethered to particular intracellular structures. Therefore, it is conceivable that a cleavage at the WD-40 repeat region and the subsequent release of effector caspases would facilitate the subcellular redistribution of caspases and their access to substrates in different cellular compartments.

In summary, we demonstrate that Apaf-1 is processed during apoptosis from its full-length p130 form into a p84 fragment that misses the CARD N-terminal helix H1 and part of the C-terminal WD-40 repeats. Cleavage at both sites is presumably responsible for the lack of caspase-9 and effector caspases in the resulting 440-kDa Apaf-1 multimer. We are aware that our data may not allow a clear interpretation of the consequences of this cleavage in vivo. On the basis of our findings, one could, however, envisage the following model (Fig. 8). A proapoptotic stimulus induces the release of cytochrome c, resulting in the nucleotide-dependent formation of an Apaf-1 oligomer. The oligomerized but not the free Apaf-1 binds to procaspase-9 and increases its activity through allosteric interaction (28). This activity is sufficient to carry out intramolecular processing resulting in fully active caspase-9. Caspase-9 then activates effector caspase-3 and -7, which are associated with Apaf-1. Caspase-3 in turn can cleave Apaf-1, which by the proapoptotic deletion of CARD and WD-40 repeats results in the release and redistribution of caspase-9 and caspase-3 from the apoptosome. Our results favor the intriguing idea that this mechanism may play a functional role for the intracellular propagation of a death signal.

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The Adapter Protein Apoptotic Protease-activating Factor-1 (Apaf-1) Is Proteolytically Processed during Apoptosis
Kirsten Lauber, Helga A. E. Appel, Stephan F. Schlosser, Michael Gregor, Klaus Schulze-Osthoff and Sebastian Wesselborg

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