Caspase-3 cleaves Apaf-1 into an ~30 kDa fragment that associates with an inappropriately oligomerized and biologically inactive ~1.4 MDa apoptosome complex

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Abstract

Cytochrome c and dATP/ATP induce oligomerization of Apaf-1 into two distinct apoptosome complexes: an ~700 kDa complex, which recruits and activates caspases-9, -3 and -7, and an ~1.4 MDa complex, which recruits and processes caspase-9, but does not efficiently activate effector caspases. While searching for potential inhibitors of the ~1.4 MDa apoptosome complex, we observed an ~30 kDa Apaf-1 immunoreactive fragment that was associated exclusively with the inactive complex. We subsequently determined that caspase-3 cleaved Apaf-1 within its CED-4 domain (SVTD^{271}↓S) in both dATP-activated lysates and apoptotic cells to form a prominent ~30 kDa (p30) N-terminal fragment. Purified recombinant Apaf-1 p30 fragment weakly inhibited dATP-dependent activation of caspase-3 in vitro. However, more importantly, prevention of endogenous formation of the p30 fragment did not stimulate latent effector caspase processing activity in the large complex. Similarly, the possibility that XIAP, an inhibitor of apoptosis protein (IAP), was responsible for the inactivity of the ~1.4 MDa complex was excluded as immunodepletion of this caspase inhibitor failed to relieve the inhibition. However, selective proteolytic digestion of the ~1.4 MDa and ~700 kDa complexes showed that Apaf-1 was present in conformationally distinct forms in these two complexes. Therefore, the inability of the ~1.4 MDa apoptosome complex to process effector caspases most likely results from inappropriately folded or oligomerized Apaf-1. Cell Death and Differentiation (2001) 8, 425–433.

Introduction

Apoptosis is an evolutionarily conserved, biochemically and morphologically distinct form of cell death, characterized by an ordered dismantling of the cell. In most cases, a highly selective class of cysteine proteases, known as caspases, carry out this process. Caspases are often categorized based on their function within the cell, the length of their prodomains and their apparent substrate specificity (reviewed in[1–3]). Initiator caspases, such as caspases-8 and -9, contain long prodomains and are involved in transducing receptor-mediated and stress-induced death signals, respectively.[3,4] They activate effector caspases, such as caspase-3 and -7, which contain short prodomains and are responsible for cleavage of many cellular proteins during the execution phase of apoptosis (reviewed in[3,5]).

In stress-induced apoptosis, unknown cellular signals initiate the release of mitochondrial cytochrome c, which interacts with Apaf-1.[6,7] Apaf-1 is composed of three domains: an N-terminal caspase-recruitment domain (CARD), a CED-4 domain that contains Walker's A and B boxes, and a series of C-terminal WD repeats (WDR).[7] After associating with cytochrome c, Apaf-1 binds to dATP/ATP, undergoes a conformational change and self-oligomerizes via its CED-4 domains.[8–10] This process simultaneously exposes the CARD in Apaf-1, allowing it to recruit and facilitate processing of procaspase-9.[9–11] This complex of cytochrome c, Apaf-1 and caspase-9 is commonly referred to as the 'apoptosome'. Interestingly, Apaf-1 proteins that lack WDR appear to spontaneously oligomerize in the absence of cytochrome c and activate caspase-9, but do not activate caspase-3. Thus, the WDR appear to negatively regulate Apaf-1 oligomerization and may be required for recruitment and activation of effector caspases.[8,10,12] The precise binding site for cytochrome c on Apaf-1 has not been identified, but an interaction between cytochrome c and the WDR in Apaf-1 may induce a conformational change in Apaf-1 that exposes its CED-4 domain. In fact, WDR proteins are likely to form β-propeller structures, with the outer surfaces serving as stable platforms through which multiple proteins may sequentially and/or simultaneously interact to form complexes.[13]

The apoptosome is a very large caspase-activating complex, containing multiple copies of Apaf-1 and caspase-9. Reconstitution experiments with recombinant proteins indicate the apoptosome is ~1.4 MDa in size, whereas those conducted in native cell lysates suggest it is ~700 kDa.[14–16] However, we have recently described the formation of both these complexes in dATP-activated lysates. Significantly, however, only the ~700 kDa complex was biologically active as assessed by its ability to process and activate effector caspases.[17] In the present study, we wished to determine why the ~1.4 MDa Apaf-1

containing apoptosome complex was biologically inactive. We now report that in apoptotic cells and dATP-activated cell lysates, Apaf-1 is cleaved by caspase-3 to yield an ∼30 kDa N-terminal fragment. This p30 fragment selectively associates with the ∼1.4 MDa apoptosome complex, and addition of the purified recombinant protein to whole lysates inhibits dATP-activation of caspase-3. However, the p30 fragment is not responsible for the inactivity of the ∼1.4 MDa complex. Therefore, the physiological function of the p30 fragment during apoptosis is currently unknown. We also exclude a potential role for the endogenous caspase inhibitor, XIAP, in inhibiting the effector caspase processing activity of the ∼1.4 MDa complex. Instead, the conformational state of Apaf-1 within the ∼1.4 MDa and ∼700 kDa complexes appears to be significantly different. Therefore, the inactive ∼1.4 MDa complex is likely formed as a result of inappropriate Apaf-1 oligomerization.

Results

An ∼30 kDa protein selectively associates with the ∼1.4 MDa apoptosome complex

We recently described the formation of two Apaf-1 complexes in dATP-activated THP.1 lysates: a biologically active ∼700 kDa complex and an inactive ∼1.4 MDa complex.[17] In the current studies, in control lysates, Apaf-1 and procaspase-9 eluted off the Superose-6 column in fractions 18–24 ($M_r \sim 158,000$) and 18–26 ($M_r \sim 60,000$), respectively (Figure 1A). Following dATP activation of the lysate for 1 h, Apaf-1 oligomerized into ∼1.4 MDa and ∼700 kDa apoptosome complexes (Figure 1B, fractions 6–8 and fractions 10–16, respectively). Procaspase-9 was processed to its catalytically active large subunits and was associated with both the ∼1.4 MDa and ∼700 kDa apoptosome complexes or was present as the free enzyme (Figure 1B). All DEVDase activity eluted in fractions 21–28 ($M_r \sim 60,000$) (Figure 1C), along with fully processed caspase-3 (data not shown). When both the ∼1.4 MDa and ∼700 kDa complexes were assayed for their ability to process and activate exogenously added effector caspases, only the ∼700 kDa complex produced significant DEVDase activity (Figure 1C). These results were essentially identical to those previously reported.[17] However, upon dATP activation we also observed the formation of an Apaf-1 immunoreactive ∼30 kDa protein, which was selectively associated with the inactive ∼1.4 MDa complex (Figure 1B, fractions 6–7). Consequently, we hypothesized that this ∼30 kDa protein (p30) may be a caspase-mediated cleavage product of Apaf-1 and may act as an inhibitor of the ∼1.4 MDa complex.

Effector caspases cleave Apaf-1 into an ∼30 kDa fragment in dATP-activated lysates and apoptotic cells

In order to test the hypothesis that Apaf-1 was cleaved by caspases and to determine if the cleavage was cell-type specific, lysates from both THP.1 and HEK 293 cells were activated with dATP in the presence or absence of caspase inhibitors. The p30 fragment was observed 15 min after

activation and was inhibited by both DEVD.CHO and Z-VAD.FMK, indicating that cleavage of Apaf-1 was caspase-dependent (Figure 1D,E). In addition, the p30 fragment was observed in THP.1 cells treated with either TPCK (75 μM), etoposide (10 μM), or Ara-C (20 μM), and the amount of the p30 formed was consistent with the degree of apoptosis (Figure 1F).

Recombinant caspases-3 and -7 cleave purified Apaf-1 at SVTD$^{260/271}$↓S

Since DEVD.CHO potently inhibits the effector caspases-3 and -7, as well as the initiator caspase-8,[18] we next incubated purified unactivated Apaf-1 with either recombinant caspases-3, -7 or -8. Both caspases-3 and -7 processed Apaf-1 in a concentration-dependent manner into several identifiable fragments, the most prominent of which was the p30 fragment previously observed in dATP-activated cell lysates (Figure 2A,B). Of note, ∼5–10 nM concentrations of active caspases-3 and -7 were sufficient to process Apaf-1 and were below the concentration of active caspase-3 observed in dATP-activated THP.1 lysates. At the same concentrations, caspase-8 demonstrated no ability to process Apaf-1 (Figure 2C). Moreover, a role for caspase-6 was excluded because it did not process purified Apaf-1, and the caspase-6 selective inhibitor, VEID.CHO, did not inhibit Apaf-1 cleavage in THP.1 lysates (data not shown). The p30 fragment was confirmed to be an N-terminal fragment of Apaf-1 because Flag-Apaf-1 expressed in HEK 293 produced a Flag-p30 fragment following incubation with active recombinant caspase-3 (Figure 2D). Since the p30 fragment was derived from the N-terminus of Apaf-1, we estimated the probable caspase cleavage site motif to be SVTD^{260}↓S in Apaf-1/Apaf-1L or SVTD^{271}↓S in Apaf-1XL, which contains an additional eleven amino acids between the CARD and CED-4 domains (Figure 2E). To confirm this cleavage site, we utilized a truncated form of Apaf-1 (residues 1–530; ∼62 kDa) which could be more easily mutated and *in vitro* transcribed/translated. Caspase-3 cleaved wild-type [^{35}S]Apaf-530 into two fragments, one corresponding to the first 260 amino acids (p30 fragment) and the other corresponding to the remainder of the molecule, including the C-terminal His6 tag (Figure 2E, lanes 2–4). The latter fragment appeared to undergo a second cleavage event to form a slightly smaller C-terminal fragment (Figure 2E, asterisk, lanes 3–4). In contrast, the mutated [^{35}S]Apaf-530 (D260A) was not cleaved at all by caspase-3 (Figure 2E, lanes 5–8). Thus, caspase-3 cleaves Apaf-1 within its CED-4 domain (SVTD↓S), downstream of the Walker's B box (Figure 2E).

Recombinant p30 weakly inhibits dATP-activation of caspase-3

In order to determine the biological effects of the p30 fragment, we cloned, expressed and purified the protein for subsequent studies. We initially verified that recombinant p30 could associate with the ∼1.4 MDa complex when added to lysate that was subsequently dATP-activated (data not shown). Next, we assessed the ability of purified Apaf-1L and p30 to activate caspase-9 in a pure recombinant system using *in vitro* transcribed/translated [^{35}S]procaspase-9, cytochrome *c* and dATP. Interestingly, while recombinant
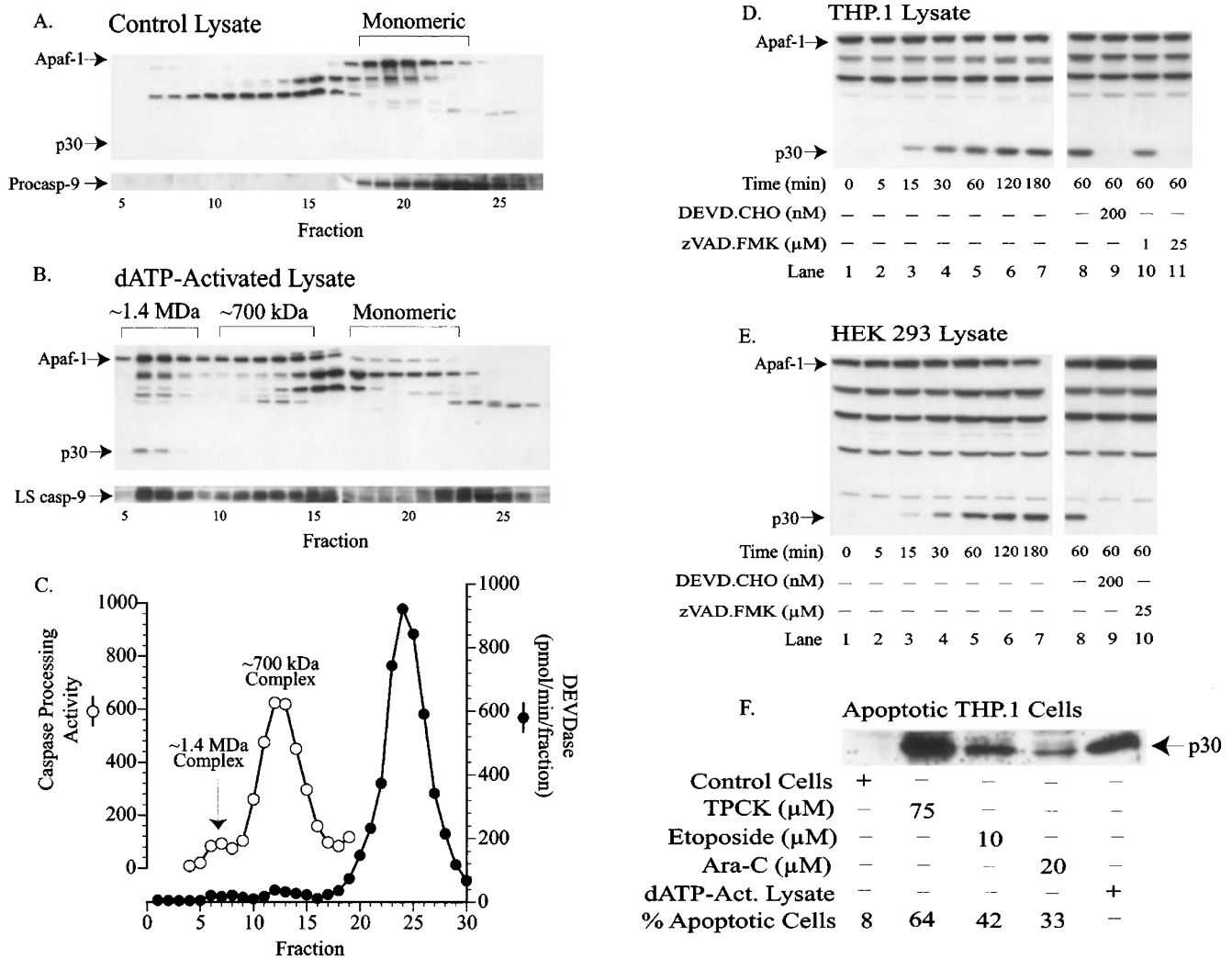
Figure 1 An ~30 kDa protein selectively associates with the inactive ~1.4 MDa apoptosome complex. (**A**) Control THP.1 lysate (10 mg/ml) and (**B**) lysate activated with dATP/MgCl₂ (2 mM) for 1 h at 37°C were loaded onto a Superose 6 HR 10/30 column. The ~1.4 MDa (fractions 6–8) and ~700 kDa (fractions 11–16) apoptosome complexes were separated as described in Materials and Methods. A portion of each column fraction was mixed with 10 × SDS loading buffer and analyzed by SDS–PAGE/immunoblotting for Apaf-1 or caspase-9. Caspase-9 was detected either as its unprocessed zymogen (Procasp-9) or as its processed large subunits together with the prodomain (LS casp-9). (**C**) Column fractions from dATP-activated lysates were assayed for DEVDase activity (●). The ~1.4 MDa and ~700 kDa apoptosome complexes were also examined for their ability to process and activate exogenously added effector caspases (○). (**D**) THP.1 or (**E**) HEK 293 dATP-activated cell lysates were preincubated on ice with either DEVD.CHO or Z-VAD.FMK for 1 h prior to dATP activation. All incubations were quenched with the addition of 2 × SDS gel loading buffer, and 25 μg of lysate was subsequently subjected to SDS–PAGE. An Apaf-1 antibody raised against amino acids 10–254 (CARD/CED-4 region) was then utilized for immunoblotting. (**F**) Lysates obtained from THP.1 cells treated with various apoptotic stimuli were immunoblotted for the presence of the p30 Apaf-1 fragment. Apoptosis was assessed by externalization of phosphatidylserine

Apaf-1L readily activated caspase-9 (Figure 3A, lanes 1–4), the recombinant p30 was incapable of activating caspase-9 (Figure 3A, lanes 5–7). Since the p30 fragment would be expected to bind procaspase-9 through a CARD-CARD interaction similar to Apaf-1L, we hypothesized that the p30 fragment might behave as a competitive inhibitor of caspase-9 activation. Indeed, addition of purified p30 to THP.1 lysates slightly inhibited dATP-mediated activation of caspase-3 by caspase-9, as demonstrated by inhibition of caspase-3 processing and decreased DEVDase activity (Figure 3B). Thus, the Apaf-1 p30 fragment appeared to compete weakly with endogenous full-length Apaf-1 for procaspase-9 and

consequently decreased the number of available active Apaf-1/caspase-9 complexes. However, as the concentrations of p30 required to inhibit caspase activation were far above those generated endogenously, the physiological significance of this result is presently unclear.

Immunodepletion of caspase-3 prevents association of the ~30 kDa fragment with the ~1.4 MDa complex

Caspases-3 and -7 are frequently viewed as redundant caspases because they often cleave the same substrates *in*
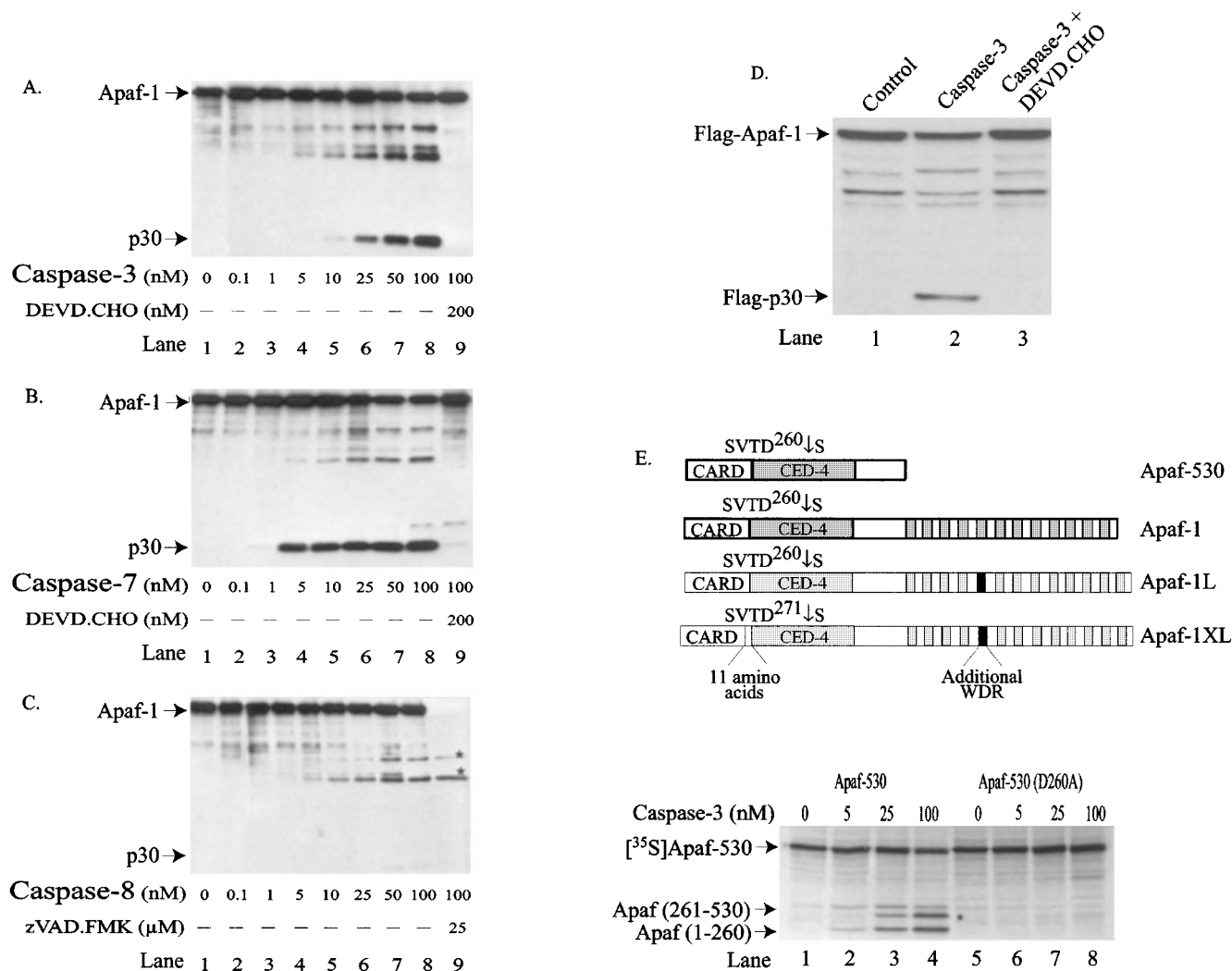
Figure 2 Apaf-1 is cleaved by caspase-3 at SVTD$^{260/271}$↓S. (**A – C**) Recombinant caspases-3, -7 and -8 (0.1 – 100 nM) were incubated with purified full-length Apaf-1 (~200 ng) for 1 h at 37°C and Western blotted for Apaf-1 fragments. Non-specific immunoreactive bands are denoted by an (*), including two minor contaminants present in the caspase-8 preparation. (**D**) Lysates from HEK 293 cells expressing NH$_2$-terminal Flag-Apaf-1 were incubated with active caspase-3 (100 nM) for 1 h at 37°C. Afterwards, Flag-Apaf-1 fragments were separated by SDS – PAGE and immunoblotted using an anti-Flag antibody. (**E**) Wild-type, truncated Apaf-530 and the site-directed mutant Apaf-530 (D260A) were incubated with active caspase-3 and analyzed by SDS – PAGE/autoradiography. The schematic represents truncated Apaf-530, various splice forms of Apaf-1, and the location of the caspase-3 cleavage site in each protein. In both Apaf-1L and Apaf-1XL, an additional WDR is shown in black, and in Apaf-1XL, the additional eleven amino acids between the CARD and CED-4 domains is shown in white

vitro. However, a number of studies suggest these caspases may play distinct roles in dATP-activated cell lysates and apoptotic cells.[15,19,20] Consequently, in order to determine the contribution of caspase-3 in producing the Apaf-1 p30 fragment and to assess the effects of blocking its formation on apoptosome function, we immunodepleted caspase-3 from lysates prior to dATP activation. Following dATP activation and separation by gel filtration, caspase-3 depleted lysate exhibited a >95% loss in DEVDase activity (Figure 3D). The remaining DEVDase activity co-eluted with processed caspase-7 in fractions 19 – 23 (Figure 3D and data not shown). Importantly, the p30 fragment was no longer associated with the ~1.4 MDa apoptosome complex (Figures 3C and 1B, compare fractions 6 – 7). In contrast, immunodepletion of caspase-7 did not significantly inhibit the

association of the p30 fragment with the ~1.4 MDa apoptosome complex (data not shown). This appeared somewhat surprising given that recombinant caspase-7 was slightly more effective than caspase-3 at processing purified Apaf-1 *in vitro* (Figure 2A,B). However, caspase-7 is present at significantly lower concentrations than caspase-3 in our THP.1 cell lysates. Therefore, immunodepletion of caspase-3, but not caspase-7, effectively inhibited formation and association of the p30 fragment with the ~1.4 MDa complex. However, the ~1.4 MDa apoptosome complex was still inactive when assayed for its ability to process effector caspases and generate DEVDase activity (Figure 3D, fractions 6 – 8). In contrast, the ~700 kDa complex displayed its normal capacity to process effector caspases (Figure 3D, fractions 10 – 16). Thus, caspase-3 appeared primarily
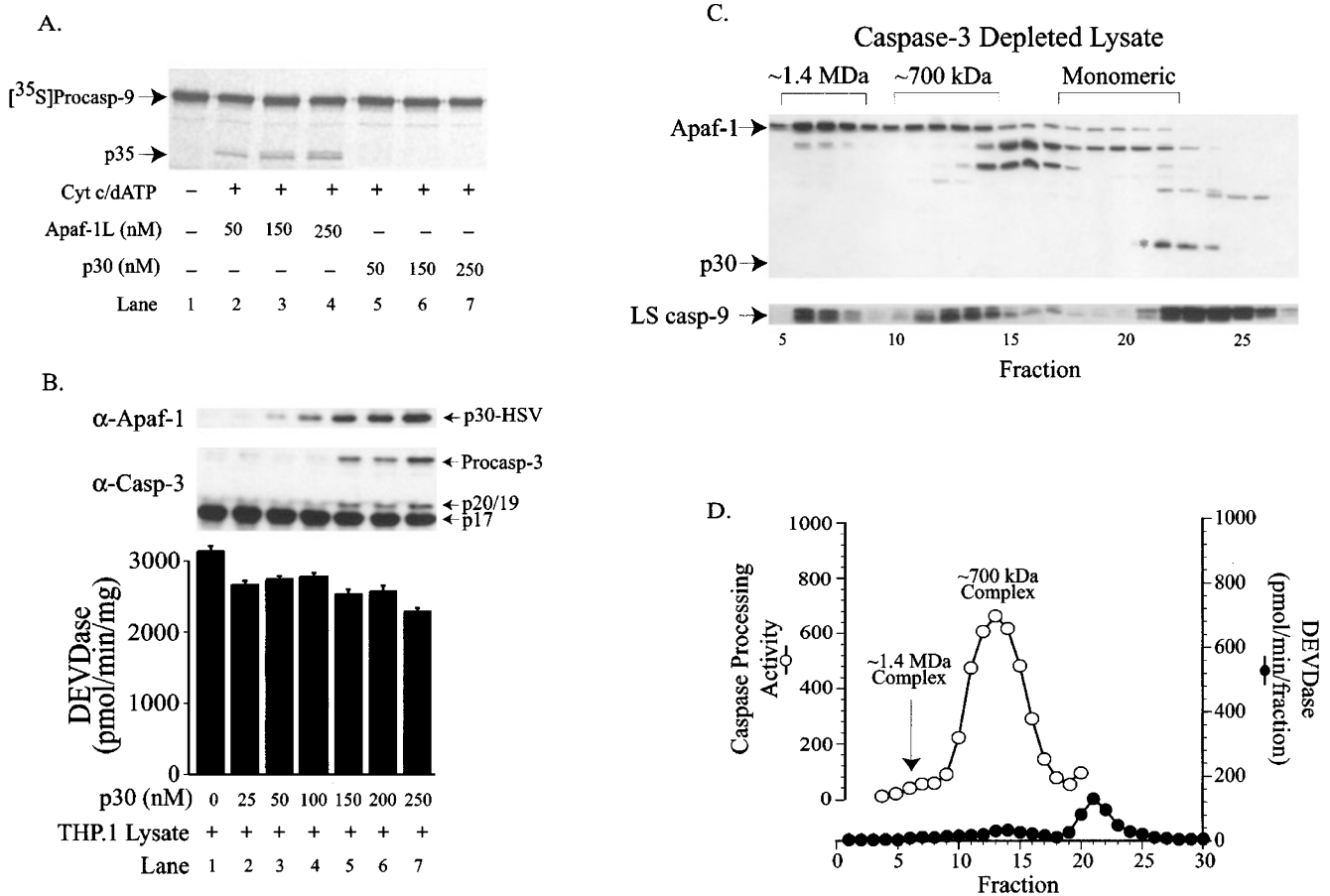
A.

B.

C.

Caspase-3 Depleted Lysate

D.

Figure 3 Endogenously produced p30 does not inhibit the $\sim$1.4 MDa apoptosome complex. (**A**) Purified Apaf-1L and p30 (0–250 nM) were incubated with in vitro transcribed/translated procaspase-9, cytochrome c and dATP for 2 h at 37°C. Trans-catalytic formation of the p35 fragment (large subunit) of caspase-9 was determined by SDS–PAGE/autoradiography. (**B**) The purified p30 fragment of Apaf-1 (0–250 nM) was mixed with naïve THP.1 lysates. The lysates were subsequently dATP-activated and both the processing and activity of caspase-3 were determined by Western blotting and fluorimetry, respectively. (**C**) THP.1 lysate was immunodepleted of caspase-3, as described in Materials and Methods, and subsequently activated with dATP/MgCl$_2$. The $\sim$1.4 MDa and $\sim$700 kDa apoptosome complexes were separated and the column fractions assayed for Apaf-1 and caspase-9 as described in the legend to Figure 1. (**D**) Column fractions were assayed for DEVDase activity ($\bullet$), and the $\sim$1.4 MDa and $\sim$700 kDa apoptosome complexes were examined for their ability to process and activate exogenously added effector caspases ($\circ$)

responsible for the cleavage of Apaf-1 and formation of the p30 fragment that associated with the large $\sim$1.4 MDa apoptosome complex. However, the immunodepletion experiments clearly demonstrated that the inability of the $\sim$1.4 MDa complex to activate effector caspases was not due to the presence of the N-terminal Apaf-1 p30 fragment or its corresponding C-terminal p105 fragment, nor was it due to caspase-3 inactivating the complex through cleavage of Apaf-1 or some other component of the apoptosome.

XIAP is not responsible for the inactivity of the $\sim$1.4 MDa apoptosome complex

In both our present and previous studies, processed caspase-9 was present in both the $\sim$1.4 MDa and $\sim$700 kDa apoptosome complexes (Figures 1B and 3C), but only the latter activated effector caspases (Figures 1C and 3D). Since IAPs, particularly XIAP, cIAP-1 and cIAP-2, are the primary endogenous inhibitors of caspase activity,[21,22] it was possible they were responsible for inhibiting the $\sim$1.4 MDa complex.

However, only XIAP co-eluted with the $\sim$1.4 MDa apoptosome fractions following dATP activation of the lysate (data not shown). Therefore, THP.1 lysates were immunodepleted of XIAP (Figure 4A), activated with dATP, and fractionated as already described. XIAP was not responsible for the inactivity of the $\sim$1.4 MDa apoptosome complex as the complex remained incapable of activating effector caspases (Figure 4B, fractions 6–8.). Interestingly, removal of XIAP also had little effect on the activity of the $\sim$700 kDa complex (Figures 1C and 4B, compare fractions 10–16), suggesting that the concentration of XIAP in our THP.1 lysate preparation was either insufficient to effectively inhibit effector caspase activation or that XIAP was rendered ineffective via the action of the recently discovered IAP inhibitor, Smac/Diablo.[23,24]

Apaf-1 exhibits alternative conformations in the $\sim$700 kDa and $\sim$1.4 MDa apoptosome complexes

Since the $\sim$1.4 MDa apoptosome complex was (i) not active at any stage during its formation,[17] (ii) approximately twice the
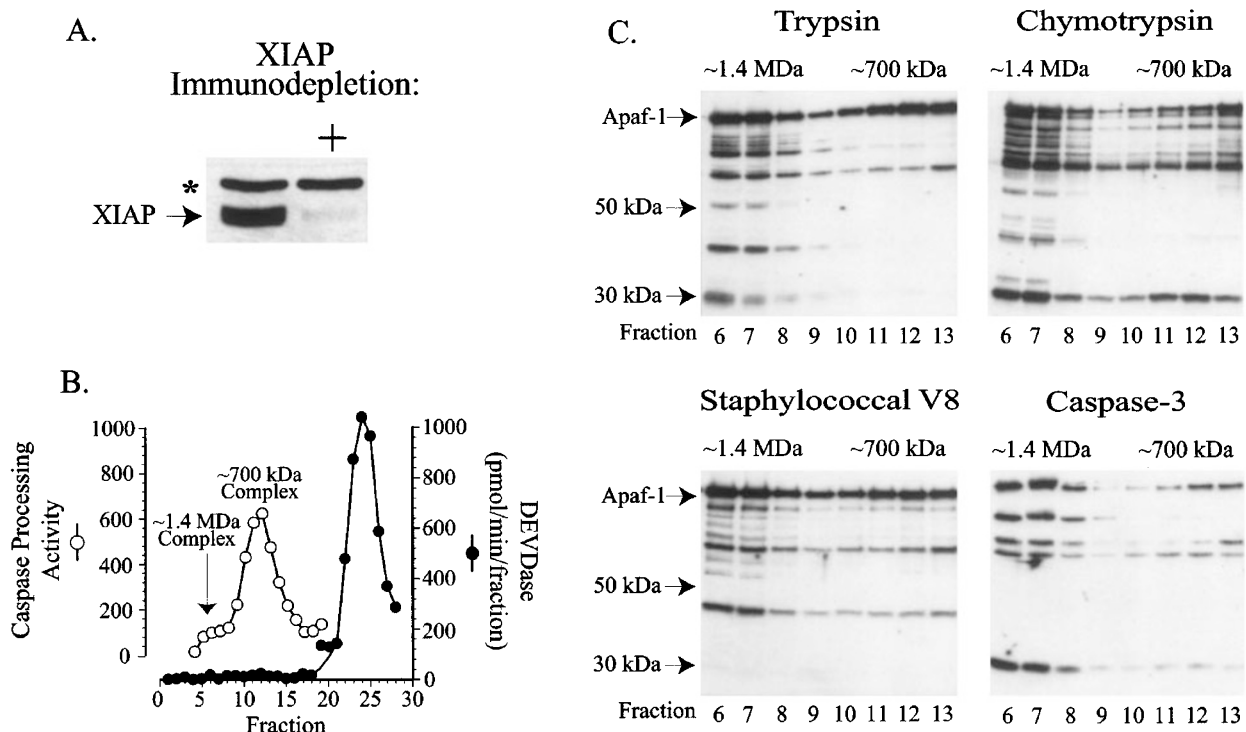
Figure 4 Inappropriately oligomerized Apaf-1, and not XIAP, is responsible for the lack of effector caspase processing activity in the ∼1.4 MDa apoptosome complex. (**A**) XIAP was immunodepleted from THP.1 lysates, as described in Materials and Methods, and immunoblotted to confirm its absence. A non-specific band (*) present in both the naïve and immunodepleted lysates confirms equal protein loading. (**B**) XIAP immunodepleted lysate was dATP-activated and separated by gel filtration as previously described. Column fractions were assayed for DEVDase activity (●) and the ∼1.4 MDa and ∼700 kDa apoptosome complexes were examined for their ability to process and activate exogenously added effector caspases (○). (**C**) Column fractions (30 μl), obtained from caspase-3 immunodepleted lysates activated with dATP (Figure 3C), were exposed to sequencing grade trypsin, chymotrypsin or staphylococcal V8 (1 ng/μl) for 15 min or to recombinant caspase-3 (100 nM) for 2 h. The reactions were quenched with 2×SDS loading buffer, and the samples were separated by SDS–PAGE and immunoblotted for Apaf-1 fragments

size of the active ∼700 kDa apoptosome complex, (iii) not directly inactivated by caspase-3 or the endogenously derived p30 fragment, and (iv) not inactivated by the presence of IAPs, we hypothesized that the ∼1.4 MDa apoptosome complex might simply be inappropriately formed. We speculated that Apaf-1 might be present in a conformational state that prevents normal recruitment/processing of effector caspases. Therefore, we isolated both ∼700 kDa and ∼1.4 MDa apoptosome complexes from dATP-activated caspase-3 immunodepleted lysates and subjected them to partial proteolysis with trypsin, chymotrypsin or staphylococcal V8 protease. Apaf-1 in the ∼1.4 MDa complex was generally more susceptible to proteolytic cleavage by all of the proteases compared to Apaf-1 in the ∼700 kDa complex (Figure 4C). In addition, the pattern of protein fragments was strikingly different. Trypsin produced at least three low molecular weight fragments (<50 kDa) from the ∼1.4 MDa complex that were not detected following proteolysis of the ∼700 kDa complex, and both trypsin and chymotrypsin produced numerous larger molecular weight fragments (∼65–125 kDa) that were primarily generated from the larger complex.

The apparent sensitivity of the ∼1.4 MDa complex to proteolytic cleavage led to us to question if this complex might likewise demonstrate greater sensitivity to caspase-3 mediated cleavage. Indeed, Apaf-1 within the ∼1.4 MDa

complex (Figure 4C, fractions 6–8) was clearly more susceptible to cleavage by caspase-3 than Apaf-1 within the ∼700 kDa complex (Figure 4C, fractions 11–13). These data were compatible with the hypothesis that the ∼700 kDa apoptosome complex initially activated caspase-3, which in turn cleaved Apaf-1 within the ∼1.4 MDa apoptosome complex to form the ∼30 kDa fragment. Caspase-3 was capable of cleaving purified monomeric Apaf-1 into the ∼30 kDa fragment (Figure 2A) and recombinant p30 associated with the ∼1.4 MDa complex when added to dATP-activated lysates (data not shown). Thus, it appears that both monomeric and inappropriately oligomerized Apaf-1 are sensitive to cleavage by caspase-3 and that in either case the resulting Apaf-1 p30 fragment associates with the ∼1.4 MDa apoptosome complex. However, since the vast majority of Apaf-1 undergoes oligomerization in dATP-activated lysates prior to activation of effector caspases,[17] it appears more likely that caspase-3 targets the inappropriately oligomerized Apaf-1 in the ∼1.4 MDa complex rather than unactivated Apaf-1.

Discussion

Cytochrome *c*/dATP-dependent oligomerization of Apaf-1 is required for formation of a functional apoptosome that

processes caspase-9, followed by caspases-3 and -7.[9,10,14–16] However, this process is still somewhat ill defined. In reconstitution experiments using purified Apaf-1, caspase-9 and cytochrome *c*, Apaf-1 apparently oligomerizes into an ~1.4 MDa complex.[14,16] Nevertheless, we find that in cell lysates, this complex is relatively inactive compared with the ~700 kDa apoptosome complex.[17] However, the ~1.4 MDa apoptosome complex can be observed in apoptotic cells and appears to be dependent upon the apoptotic stimulus and the degree of apoptosis.[17] In the present studies we have attempted to explain why the ~1.4 MDa complex is inactive. In doing so, we discovered that in dATP-activated lysates and apoptotic cells, caspase-3 can cleave Apaf-1 at SVTD$^{260/271}$↓S to generate an Apaf-1 fragment that contains an intact CARD domain and a truncated CED-4 region (Figures 1 and 2). Purified p30 fragment was capable of weakly inhibiting dATP-mediated activation of caspase-3 by caspase-9 in lysates, but only did so at concentrations that are likely to exceed those produced endogenously. Consistent with these findings, immunodepletion of caspase-3 from lysates prevented formation and association of the N-terminal Apaf-1 p30 fragment and its corresponding C-terminal p105 fragment with the ~1.4 MDa apoptosome by preventing cleavage of Apaf-1. However, the ~1.4 MDa complex was formed to the same extent in both caspase-3 depleted and normal dATP-activated lysates, indicating that the p30 fragment did not 'convert' an active ~700 kDa apoptosome complex into an inactive ~1.4 MDa apoptosome complex. In addition, the ~1.4 MDa apoptosome complexes remained incapable of activating effector caspases, even in the absence of any Apaf-1 p30 fragment (Figure 3). Thus, although the biological consequences of Apaf-1 cleavage remain unknown, neither cleavage of Apaf-1 nor the resulting p30 fragment were responsible for producing or inhibiting the ~1.4 MDa complex.

Interestingly, processed caspase-9 was associated with the inactive ~1.4 MDa apoptosome complex, implying that the large complex might be capable of promoting trans-activation of procaspase-9 or alternatively, might simply recruit processed caspase-9 released from the active ~700 kDa apoptosome complex. If the former were in fact true, then the presence of a caspase-9 inhibitor, for example a member of the inhibitor of apoptosis (IAP) family of proteins such as XIAP,[21,22] might explain the inability of the ~1.4 MDa complex to activate effector caspases. However, XIAP was not responsible for the inactivity of the ~1.4 MDa complex as its removal did not restore the activity of the large complex (Figure 4). Rather, partial proteolysis of both the ~700 kDa and ~1.4 MDa complexes revealed markedly different proteolytic digestion profiles and suggested that the ~1.4 MDa apoptosome complex was inappropriately formed (Figure 4). Indeed, all of the proteases tested, including caspase-3, appeared to cleave Apaf-1 in the ~1.4 MDa complex far more efficiently than in the ~700 kDa complex. It is interesting to note that the WD repeats present in Gβ-subunits form β-propeller structures, which are very resistant to trypsin, unless they are misfolded or disturbed by particular amino acid substitutions.[25] Apaf-1 contains a large C-terminal WD40 repeat region, which has been suggested to form β-propellers and which has recently

been implicated in the recruitment of effector caspases-3 and -7 to the apoptosome.[7,12] In our studies, both trypsin and chymotrypsin produced numerous large N-terminal Apaf-1 fragments in the inactive ~1.4 MDa apoptosome complex, but not in the ~700 kDa complex (Figure 4). Thus, inappropriately oligomerized Apaf-1 in the ~1.4 MDa apoptosome complex may contain misfolded WD repeats, which are sensitive to trypsin degradation and are incapable of effectively recruiting and processing effector caspases-3 and -7. However, the specific factors that dictate inappropriate formation of the ~1.4 MDa apoptosome complex in apoptotic cells are currently unclear, but the focus of future research.

In conclusion, we have demonstrated that active effector caspase-3 cleaves Apaf-1 within the ~1.4 MDa apoptosome complex into a prominent ~30 kDa N-terminal fragment, which remains associated with the complex. However, the inability of this complex to efficiently activate effector caspases is not due to degradation and inactivation of the complex by active caspase-3 or to the presence of an Apaf-1 fragment. Moreover, IAPs do not appear responsible for its lack of activity. Instead, the inability of the ~1.4 MDa apoptosome complex to process effector caspases is likely because the complex contains inappropriately oligomerized Apaf-1.

Materials and Methods

Preparation and activation of cell lysates/ apoptotic cells

Human monocytic THP.1 cells and human embryonic kidney (HEK) 293 cells were grown in RPMI 1640 and DMEM, respectively, supplemented with 10% heat inactivated FBS in 5% CO_2 at 37°C. Cell lysates were prepared as previously described.[15] *In vitro* activation of caspases was initiated by incubating lysates (10 mg/ml) with dATP/MgCl$_2$ (2 mM) for various times at 37°C. Exogenous cytochrome *c* was not required as it was released from mitochondria during lysate preparation. In some experiments, THP.1 cells were treated with either *N*-tosyl-*l*-phenylalanyl chloromethyl ketone (TPCK; 75 μM), etoposide (10 μM), or 1-β-D-arabinofuranosylcytosine hydrochloride (Ara-C; 20 μM) for varying times, and apoptosis was assessed by phosphatidylserine exposure as previously described.[26]

Caspase cleavage of endogenous and recombinant Apaf-1

THP.1 and HEK293 lysates (25 μg protein) were activated with dATP in the presence or absence of the caspase inhibitors, benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl-ketone (Z-VAD.FMK) or Ac-Asp-Glu-Val-Asp aldehyde (DEVD.CHO). Z-VAD.FMK inhibits all known caspases, whereas DEVD.CHO is more selective for caspases-3, -7 and -8.[18] Purified recombinant unactivated Apaf-1 (~200 ng) was incubated with active caspases-3, -6, -7 or -8 for 1 h at 37°C. All protein samples were mixed with 2× SDS loading buffer, separated by SDS–PAGE, and immunoblotted for Apaf-1 cleavage products using an antibody generously provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX, USA). This antibody was raised against the N-terminus of Apaf-1 (amino acids 10–254).[7]

Apaf-1 mutagenesis/deletions

The caspase cleavage site in Apaf-1 was determined using a truncated form of Apaf-1 (pET28a-Apaf-530; generously provided by Dr. Emad S. Alnemri).[10] Asp-260 was mutated to an Ala using Stratagene's Quik-Change kit with the following primers (mutation underlined) (5′→CAAGAGTGTTACAG$\underline{C}$TTCAGTAATGG→3′; 5′→CCATTACTGAA$\underline{G}$CTGTAACACTCTTG→3′) and confirmed by DNA sequencing. The wild-type Apaf-530 and Apaf-530 (D^{260}→A) plasmids were transcribed/translated using the T7 TNT system (Promega), and the ^{35}S-labeled proteins were incubated with recombinant caspase-3 for 2 h and analyzed by SDS – PAGE/autoradiography. This mutation corresponds to D^{271}→A in Apaf-1XL/Apaf-1WD13,[12,16] which contains an additional 11 amino acids between the CARD and CED-4 domains. While conducting these studies, Apaf-1XL was shown to be the primary form of Apaf-1 present in cells.[27] Consequently, we PCR amplified the sequence corresponding to the N-terminal cleavage product of Apaf-1XL (residues 1 – 271), using an Apaf-1XL construct (kindly provided by Dr. Gabriel Nuñez) as a template and the following primers which contained the necessary restriction sites (5′→CGGGATCCATGGATGCAAAAGCTCGAAATTG→3′ and 5′→GGGGT-ACCAGCTGTAACACTCTTGTCTCTGGTTG→3′). The PCR product was cloned into pTriEX-1 (Novagen) at the *Bam*HI/*Kpn*I sites and incorporated C-terminal HSV and His-6 tags. In addition, the D^{271}→A mutation was also included in the fragment to ensure that the HSV and His-6 tags would not be removed by caspase-3 during dATP activation experiments.

Expression and purification of human recombinant Apaf-1 (and its p30 cleavage fragment) and caspases

Sf-9 insect cells were infected with high titer baculoviral stocks encoding full-length Apaf-1.[16] Cells were harvested 48 h later and His-tagged Apaf-1 was purified on a Ni^{2+} column essentially as previously described.[16] Similarly, the p30 Apaf-1 fragment was bacterially expressed, isolated on Ni^{2+} beads, and further purified by DEAE-chromatography. Active caspases-3, -6, -7 and -8 were expressed, purified and active site titrated as previously described.[28,29]

Analysis of apoptosome complexes/immunodepletion of caspase-3 and XIAP

Control and dATP-activated lysates were fractionated by size-exclusion chromatography, using an FPLC protein purification system with a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Herts, UK). Apoptosome complexes (~700 kDa and ~1.4 MDa) were eluted using column buffer supplemented with 50 mM NaCl, assayed for their ability to process exogenously added caspases-9, -3 and -7, and immunoblotted for Apaf-1 and caspase-9 (D.R. Green, La Jolla Institute for Allergy and Immunology, San Diego, CA, USA) as previously described.[17] In some experiments, lysates were immuno-depleted of caspase-3 or XIAP prior to dATP-activation. Briefly, protein G sepharose beads (500 μl of 50% slurry; Amersham Pharmacia Biotech, Herts, UK) were blocked with 3% BSA, preincubated for 2 h with anti-caspase-3 or XIAP antibodies (1 : 50; Transduction Laboratories/Pharmingen, San Diego, CA, USA) and thoroughly washed to remove unbound antibody. Lysates (15 mg/ml) were then incubated with antibody coated beads for 2 h. Partially immuno-pleted supernatants were removed by centrifugation and subjected to a second round of immunodepletion in order to completely deplete caspase-3 or XIAP.

Partial proteolysis of ~1.4 MDa and ~700 kDa apoptosome complexes

Following dATP activation of caspase-3 immunodepleted lysates, apoptosome complexes were isolated by gel filtration as described above. A portion of each column fraction (30 μl) was incubated with either 100 nM caspase-3 for 2 h or 1 ng/ml of sequencing grade trypsin, chymotrypsin or staphylococcal V8 protease (Boehringer Mannheim, Lewes, UK) for 15 min at 37°C. Reactions were terminated with an equal volume of 2 × SDS loading buffer and analyzed for Apaf-1 cleavage products.

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