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Cleavage of Human Cytosolic Phospholipase A_2 by Caspase-1 (ICE) and Caspase-8 (FLICE)

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The activation of caspases appears to play a key role in programmed cell death. An increasing number of substrates have been identified that are cleaved by caspases. In a previous study, we have reported that human $cPLA_2$ is proteolytically inactivated during apoptosis through cleavage by a caspase-3-like activity. Here, we show that in cotransfection experiments the previously identified cleavage site at Asp_{522} can be used by a wide variety of caspases belonging to different subfamilies. The formation of additional fragments implied differences in cleavage site usage between the closely related caspases-3 and -7. A different cleavage pattern of $cPLA_2$ was observed with caspase-1. Mutational analysis identified the caspase-1 cleavage site at Asp_{459} within the sequence YQSD/N. Most interestingly, we found that even caspase-8, an upstream component of the proposed caspase cascade, cleaves $cPLA_2$ *in vitro*. The presence of multiple cleavage sites warrants proteolysis and inactivation of the proinflammatory $cPLA_2$ during apoptosis. © 1998 Academic Press

The highly conserved cell suicide program called apoptosis is essential for the elimination of "unwanted" cells (for review, see 1–3). Members of the ICE/CED-3 protease family play a key role in the induction and execution of programmed cell death. These now called caspases (4) are characterized by their unique substrate cleavage site C-terminally of an aspartic acid residue (5). The caspase family can be divided by phylogenetic analysis and substrate specificity into three subfamilies (6). The recent discovery of mitochondria being involved in the induction of apoptosis (7, 8) has led to an alternative definition of subfamilies. Upstream or initiator caspases (caspase-1, -2, -8, and -10) are activated independently of mitochondria. Second level or effector caspases (most prominently caspase-3, -6, and -7) act downstream of the mitochondrial checkpoint or are directly activated by first level caspases (9, 10). Caspase-9 appears to be the key mediator of the mitochondrial-initiated caspase cascade (11, 12).

Caspase-1 (interleukin-1β converting enzyme, ICE) cleaves the biologically inactive precursor form of the proinflammatory cytokine interleukin-1β (IL-1β; 13, 14). The generation and release of the mature, functional IL-1β is a crucial step in inflammation (15). This is most impressively demonstrated by the resistance of ICE-deficient mice against the endotoxin-induced septic shock syndrome (16). Caspase-1 appears to be activated by caspase-11, since caspase-11 knockout mice demonstrate a defect in activating caspase-1 (17). The participation of caspase-1 in programmed cell death is controversially discussed. Overexpression of caspase-1 results in apoptosis (18), while transient expression of antisense caspase-1 cDNA blocks cytotoxicity induced by cross-linking of CD95 (Fas, APO-1) (19). Caspase-1-deficient mice, however, develop normally and the ex vivo response of cells to various apoptotic stimuli is indistinguishable from cells derived from wild-type animals (6). As exception, thymocytes from caspase-1 knockout mice showed a subtle resistance to apoptosis induced by CD95 ligand (20). Caspase-8 is activated by engagement of receptors belonging to the tumor necrosis factor receptor-family and plays a non-redundant role in the death-induction by these receptors (21).

The search for caspase substrates has led to the identification of a great number of proteins that can be cleaved by different caspases. The vast majority of them are cleaved by second level caspases. Upstream caspases mainly process second level caspases themselves and thereby initiate a caspase cascade (9). Only a few unrelated cellular proteins have been identified as substrates for upstream caspases. Substrates of

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Abbreviations used: Chaps, 3-[(3-cholamidolpropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; ICE, interleukin-1β converting enzyme; IPTG, isopropylthiogalactoside; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; PARP, poly(ADP-ribose) polymerase; $cPLA_2$, cytosolic phospholipase A_2; TNF, tumor necrosis factor.

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caspase-1 include pro-IL-1β and IL-1α (15), interferon-γ-inducing factor (22), and PITSLRE kinases (23). The recently identified caspase-8 substrate BID links caspase-8 activity and mitochondrial damage (24, 25).

Cytosolic phospholipase A_2 ($cPLA_2$) is an 85-kDa enzyme that plays an important role in the induction and maintenance of inflammatory processes (for review, see 26, 27). Previous studies have shown that $cPLA_2$ is cleaved by a caspase-3-like activity at the sequence motif DELD (amino acids 519–522) during apoptosis induced by various stimuli. This cleavage leads to the inactivation of its enzymatic function (28, 29). During these experiments a different cleavage pattern was observed when caspase-1 was cotransfected in HEK 293 cells along with $cPLA_2$.

In this report, we show that $cPLA_2$ can be cleaved by several other caspases generating various cleavage products. Furthermore, we identify an YQSD motif within the amino acid sequence of $cPLA_2$ as caspase-1 cleavage site. Furthermore, we demonstrate *in vitro* cleavage of $cPLA_2$ by caspase-8, which represents a characteristic initiator caspase. Thus, $cPLA_2$ cleavage and inactivation seems to be assured independently of the set of caspases involved in apoptosis in different cell types or after different stimuli.

MATERIALS AND METHODS

Reagents. Highly purified human TNF (3×10^7 units/mg) was provided by G. Adolf (Bender, Vienna, Austria). The monoclonal antibody against human $cPLA_2$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody was purchased from Pharmingen (Hamburg, Germany). Human recombinant caspase-3, -6, -7, and -8 were also obtained from Pharmingen.

Cell culture, transfections, and Western blot analysis. HEK 293 cells were originally obtained from the American Type Culture Collection and were grown in DMEM without HEPES (Biochrom, Berlin, Germany) supplemented with 10% FCS, 2 mM glutamine, and 50 μg/ml each of streptomycin and penicillin. Transfection was performed using the calcium phosphate precipitation method (30). Western blot analysis was performed as previously described (28).

Plasmids. The cDNA encoding wild-type human $cPLA_2$ was a generous gift from Dr. L.-L. Lin (Genetics Institute, Cambridge, MA). The $cPLA_2$ mutant PLA_2-D_{522}/A (previously called PLA_2-D/A) and cDNAs encoding caspases-3, -4, and -7 have been described (28). The expression plasmids for caspase-1 and -8 were generously provided by Dr. D. Goeddel (Tularik Inc., South San Francisco, CA). For prokaryotic expression, the cDNA encoding human $cPLA_2$ was amplified by PCR using Pfu-polymerase (Stratagene, Heidelberg, Germany) and the primers 5′-CTCAGATCTGTCATTTATAGATCC-3′ and 5′-CTCGTCGACGTACATGAACTATGC-3′. The PCR product was subcloned as *Bgl*II–*Sal*I fragment in frame with the N-terminal S-tag into the prokaryotic expression vector pET29a (Novagen, Madison, WI). The cDNA encoding caspase-1 was subcloned as a *Sal*I–*Hin*dIII/blunt fragment in frame with the N-terminal T7-tag into the bacterial expression vector pET21b (Novagen).

Expression in Escherichia coli. The prokaryotic expression constructs for caspase-1 and $cPLA_2$ were cotransformed into the provided E. coli strain BL21(DE3)pLysS (Novagen). Coexpression of the T7/caspase-1 and the S-tag/$cPLA_2$ fusion proteins was induced with 1 mM isopropylthiogalactoside (IPTG) for 4 h according to the instructions provided by the manufacturer. Bacterial pellets were subjected to one cycle of freezing/thawing and resuspended in 50 mM Tris (pH 8.0) and 2 mM EDTA containing the protease inhibitor mix Complete (Boehringer-Mannheim, Penzberg, Germany). After addition of Triton X-100 to a final concentration of 0.1%, cells were incubated for 10 min on ice and sonicated to shear the DNA. After centrifugation at 13,000 rpm for 20 min at 4°C, the protein concentration in the supernatants was determined using the Coomassie reagent (Pierce, Rockford, IL). Caspase-1 expression was confirmed using an antibody directed against the T7-tag purchased from Novagen. Equal amounts of protein were used for the analysis of $cPLA_2$ cleavage by immunoblotting as described previously (28).

In vitro $cPLA_2$ and PARP cleavage assays. Human PLA_2-WT or the PLA_2-D_{522}/A mutant cDNAs were used for *in vitro* transcription/translation employing the SP6-coupled TNT Reticulocyte Lysate System (Promega, Madison, WI) and [^{35}S]methionine (Amersham, Freiburg, Germany). *In vitro* cleavage using commercially available recombinant caspases was performed by incubating 1.5 μl *in vitro* translated $cPLA_2$ with freshly thawed aliquots of the respective caspases in dilutions tenfold higher than suggested earlier (31). Reactions were carried out in a final volume of 15 μl AMC buffer (20 mM Pipes [pH 7.2], 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, 10 mM DTT, 1 mg/ml Pefablock SC) for 1 h at 37°C. The reaction was stopped by adding 5 μl 4× SDS sample buffer. Radioactive $cPLA_2$ proteins were analyzed on a 12.5% SDS–PAGE. The gels were fixed in 10% acetic acid, dried, and exposed on Kodak BioMAX films.

Purified recombinant caspases in equivalent dilutions as described above were incubated with 18 μg cytosolic extracts from HEK 293 cells in AMC buffer for 1 h at 37°C. Cleavage of endogenous PARP was analyzed on Western blots using the anti-PARP antibody.

Site-directed mutagenesis. The $cPLA_2$ mutant PLA_2-D_{459}/A was generated using the QuikChange site-directed mutagenesis kit (Stratagene) by replacing the aspartic acid at position 459 by alanine (GAT→GCT, oligonucleotides 5′-GGAAGTGACTATCAAAGTG-CTAATCAAGCAAGTTGG-3′ and 5′-CCAACTTGCTTGATTAGC-ACTTTGATAGTCACTTCC-3′) following the protocols provided by the supplier. The introduced point mutation was verified by DNA sequencing.

RESULTS

Cleavage of $cPLA_2$ by caspase-1. Previous studies revealed that human $cPLA_2$ is cleaved by a caspase-3-like activity in apoptotic cells. An additional cleavage pattern was detected in HEK 293 cells overexpressing caspase-1 (28). To investigate cleavage of $cPLA_2$ by caspase-1 in greater detail, we cotransfected wild-type $cPLA_2$ or the mutated form PLA_2-D_{522}A with caspase-1 and caspase-3 into HEK 293 cells and analyzed the resulting fragments on immunoblots using an antibody directed against the N-terminal portion of $cPLA_2$. Cotransfection with caspase-3 led to the generation of a 70-kDa fragment (p70) of $cPLA_2$, while coexpression of caspase-1 led to a 58 kDa cleavage product (p58) as previously described (28, and Fig. 1A). Stimulation of the cells with tumor necrosis factor (TNF) enhanced the activity of caspase-3 as demonstrated by increased amounts of p70 and the appearance of an additional $cPLA_2$ cleavage product of about 56 kDa molecular mass (p56; Fig. 1A). In contrast, $cPLA_2$ cleavage by caspase-1 was not further enhanced by TNF (Fig. 1A). Cleavage by caspase-1 was not abrogated by the mu-

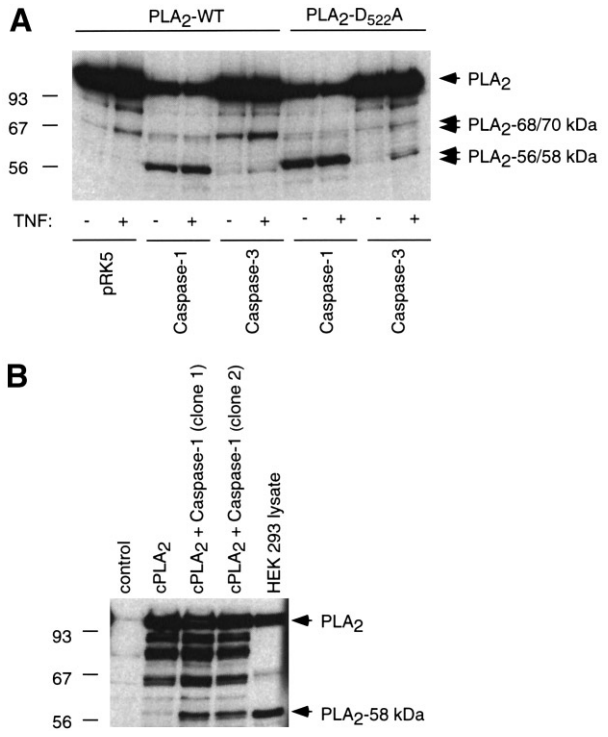

FIG. 1. Cleavage of $cPLA_2$ by caspase-1. (A) HEK 293 cells were transfected with expression plasmids for human PLA_2-WT or PLA_2-$D_{522}A$, in combination with the expression vector pRK5, caspase-1, or caspase-3. Lysates from untreated or TNF-treated cells were analyzed by immunoblotting using the anti-$cPLA_2$ antibody to visualize PLA_2 cleavage products. (B) Bacterial lysates from E. coli cells transformed with either the prokaryotic $cPLA_2$ expression construct alone or in combination with the caspase-1 expression construct were analyzed in immunoblots with the anti-$cPLA_2$ antibody. One clone was analyzed for $cPLA_2$ expression only, while two separate clones were analyzed for coexpression of $cPLA_2$ and caspase-1. A lysate from HEK 293 cells overexpressing $cPLA_2$ and caspase-1 was loaded to compare the migration of the 58 kDa cleavage product. A lysate from uninduced bacteria was used as a negative control. The positions of molecular mass standards (in kDa) are indicated at the left.

tation at aspartic acid residue 522 of $cPLA_2$, which destroys the caspase cleavage motif DELD. In contrast, mutation of the DELD motif completely blocks the generation of the 70 kDa $cPLA_2$ fragment by caspase-3 (Fig. 1A). These data indicate, that caspase-1 and caspase-3 cleave $cPLA_2$ completely independent from each other, and that the caspase-1 cleavage site has to be located upstream from the caspase-3 cleavage site.

The 58-kDa $cPLA_2$ fragment observed in cells overexpressing caspase-1 could be either the result of direct cleavage by caspase-1, or rather generated by other endogenous proteases that are activated by caspase-1. To distinguish between these two alternatives, we analyzed whether recombinant caspase-1 can process $cPLA_2$ *in vitro*. Lysates from bacteria expressing caspase-1 prepared as described earlier for prokaryotic expression of caspase-3 (28) did not produce any $cPLA_2$ cleavage products when incubated with *in vitro* translated $cPLA_2$ (data not shown). Purified recombinant active caspase-1 is very unstable due to rapid dissociation of the subunits and autolytic degradation of the small subunit (32). Therefore, we coexpressed caspase-1 and $cPLA_2$ in bacteria and analyzed for $cPLA_2$ cleavage. As shown in Fig. 1B, a 58-kDa fragment reactive with the anti-cPLA2 antibody was observed only in bacteria coexpressing caspase-1. The 58-kDa cleavage product generated in bacteria comigrated with p58 present in a lysate from HEK 293 cells overexpressing $cPLA_2$ and caspase-1 (Fig. 1B).

Identification of the caspase-1 cleavage site within $cPLA_2$. Examination of the amino acid sequence of $cPLA_2$ revealed a putative cleavage site for caspase 1 at residues 456-459 (YQSD; 33), a position consistent with the generation of the observed p58 independently of caspase-3 cleavage (Fig. 2A). This motif is almost completely conserved in $cPLA_2$ from human, mouse and rat. To test whether the YQSD motif is indeed the cleavage site recognized by caspase-1, we generated an additional mutant form of human $cPLA_2$ by replacing aspartic acid residue 459 with alanine (PLA_2-$D_{459}A$).

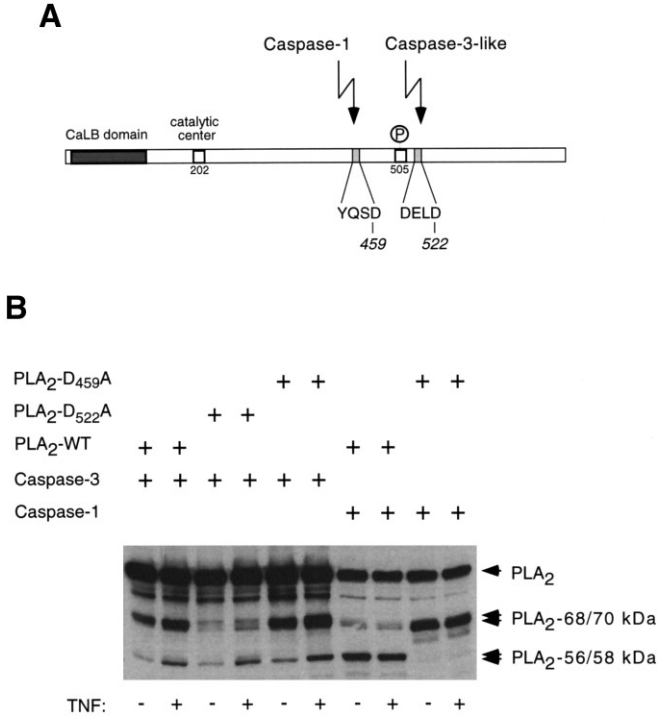

FIG. 2. Mapping of the caspase-1 cleavage site within $cPLA_2$. (A) Schematic representation of the primary structure of human $cPLA_2$. The calcium binding domain (CaLB), the catalytic center, and the phosphorylation site at serine residue 505 are marked. Cleavage sites for caspase-1 and caspase-3-like proteases are indicated. (B) HEK 293 cells were transfected with expression constructs for PLA_2-WT, PLA_2-$D_{522}A$, or PLA_2-$D_{459}A$, in combination with caspase-1 or -3. Cells were treated with TNF or left untreated. Cellular lysates were analyzed by immunoblotting using the anti-$cPLA_2$ antibody to visualize PLA_2 cleavage products.

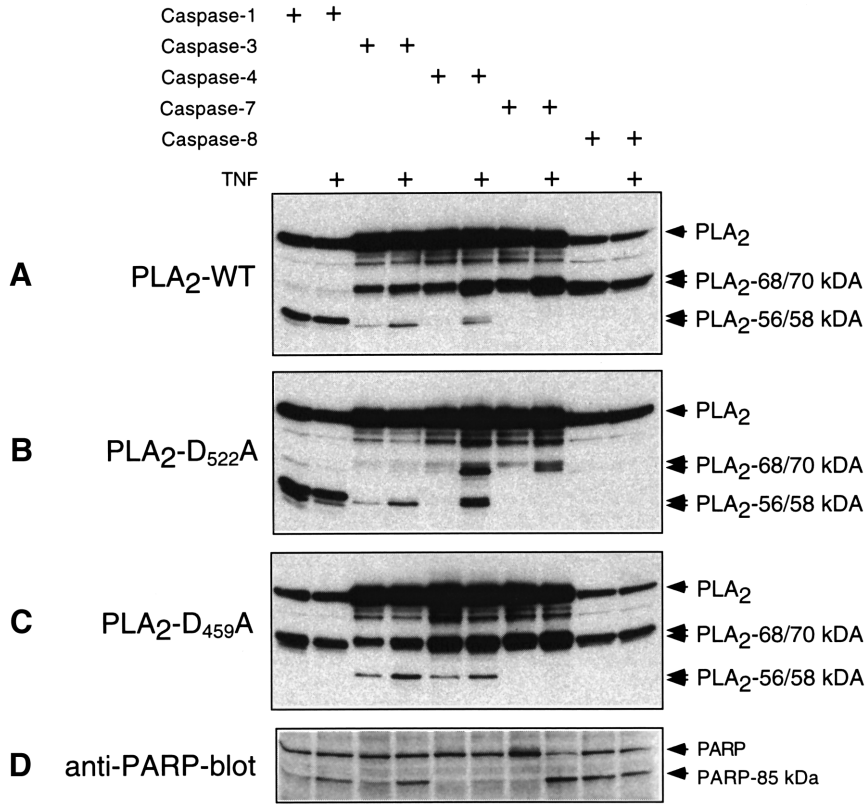

FIG. 3. Analysis of $cPLA_2$ cleavage *in vivo* by overexpression of various caspases. HEK 293 cells were transfected with expression plasmids for PLA_2-WT (A), PLA_2-$D_{522}A$ (B), or PLA_2-$D_{459}A$ (C), in combination with caspase-1, -3, -4, -7, or -8. Cells were treated with TNF where indicated. Cellular lysates were analyzed in immunoblots with an anti-$cPLA_2$ antibody to visualize cleavage $cPLA_2$ cleavage products. (D) The identical lysates as used in (A) were analyzed by immunoblotting using the anti-PARP antibody. The positions of PARP and the 85 kDa cleavage product are marked at the right.

This mutant $cPLA_2$ protein was coexpressed in HEK 293 cells with caspase-1 or caspase-3, and the resulting cleavage products were analyzed by Western blotting. While wild-type $cPLA_2$ was processed by caspase-1 leading to the previously observed p58, cotransfection of PLA_2-$D_{459}A$ with caspase-1 led to the generation of a 68 kDa fragment (p68; Fig. 2B). This product might result from cleavage by caspase-1 at a different, less suitable site or by another caspase activated by overexpression of caspase-1. The formation of p56 in TNF-treated cells overexpressing caspase-3 was not abrogated by the mutation at aspartic acid 459 (Fig. 2B), pointing to a distinct independent cleavage site for caspase-3 in close proximity to the caspase-1 cleavage site apart from aspartic acid residue 522. These data strongly indicate that the YQSD motif is a potential caspase-1 cleavage site.

Cleavage of $cPLA_2$ after overexpression of diverse caspases in vivo. To further investigate cleavage of $cPLA_2$ by various caspases belonging to different subfamilies, we cotransfected HEK 293 cells with wild-type $cPLA_2$ or both mutants with cDNAs encoding caspase-1, -3, -4, -7, and -8. The resulting cleavage patterns are shown in Fig. 3 and summarized in Table 1. In detail: Caspase-1 generated mainly p58 and, to a much lesser extent p68. A minor band at 54 kDa did not appear reproducibly in every experiment. The mutation at $D_{459}A$ abolished the appearance of p58, leading to p68 only, as described above. The mutation $D_{522}A$ or treatment with TNF had no influence on caspase-1 cleavage. Caspase-3 generated mainly p70, which disappeared when the cleavage site at aspartic acid residue 522 was mutated. A minor cleavage product (p68) and the TNF-enhanced p56 were not affected

TABLE 1

Representation of Cleavage Fragments Generated by Coexpression of Various Caspases with Human $cPLA_2$

	p70	p68	p58	p56
Caspase-1		+	++	
Caspase-3	++(T)	+		+(T)
Caspase-4	++(T)	+(T)	+(T)	+(T)
Caspase-7	++(T)	+(T)		
Caspase-8	++			

Note. [++], main cleavage product; [+], minor cleavage product or fragment that only appears after mutation of the main cleavage site; [(T)], enhanced by TNF treatment.

by any of the two mutations. Caspase-4 also generated mainly p70, while after TNF stimulation other products (p56, p58, and p68) could be detected in addition. The point mutations at aspartic acid residues 522 and 459 abolished the appearance of p70 or p58, respectively. Caspase-4 appeared to be the most efficient protease in this coexpression system. Caspase-7, although related to caspase-3, differed from the latter in generating p68 after TNF stimulation more abundantly, but not p56. Caspase-8 generated exclusively p70, which was not detected using the $D_{522}A$ mutant form of $cPLA_2$. Two $cPLA_2$ species at about 90 kDa and 100 kDa likely represent alternatively folded or unspecifically degraded proteins, since they were detected regardless of caspase overexpression or TNF-treatment.

To put caspase-mediated $cPLA_2$ cleavage on perspective, immunoblots with an anti-PARP antibody were performed using the identical lysates. As shown in Fig. 3D, the only observed cleavage product of PARP was the previously described 85 kDa fragment. Cleavage of PARP occurred after transfection of caspase-8, and after overexpression of caspase-1, -3, and -7 in TNF-treated cells. Almost no cleavage was observed after overexpression of caspase-4. These results suggest that $cPLA_2$ appears to be a substrate for a broader range of caspases than PARP, and that the cleavage seen after overexpression of caspase-4 is likely not to be due to indirect activation of caspase-3/7.

In vitro cleavage of $cPLA_2$ by caspase-8. The results illustrated in Fig. 3 suggest that besides caspase-1, a further typical initiator protease, caspase-8, can use $cPLA_2$ as substrate. To investigate whether $cPLA_2$ is processed directly by caspase-8, *in vitro* cleavage assays were performed with recombinantly expressed and purified caspase-8. Three different effector caspases (caspase-3, -6, and -7) were used as controls. All caspases demonstrated comparable activity towards the artificial fluorescent substrate Ac-DEVD.AMC (data not shown). *In vitro* translated $cPLA_2$ was incubated with purified, active caspase-3, -6, -7, and -8. As shown in Fig. 4A, with the exception of caspase-6, all other enzymes, including caspase-8, were able to process $cPLA_2$. The direct cleavage of $cPLA_2$ by caspase-8 was confirmed using purified caspase-8 purchased from a different manufacturer (BioMol; data not shown). The observed cleavage products (70 and 32 kDa) correspond to both protein fragments expected after cleavage at the DELD motif. The 32 kDa fragment was not detected in Western blots with the monoclonal antibody against $cPLA_2$, since it is directed against the N-terminal portion of the protein. To test for specificity of the observed caspase-8 cleavage, we analyzed the classical caspase-3/7 substrate PARP for cleavage by purified caspase-8. In contrast to $cPLA_2$, PARP was only processed by caspase-3 and -7,

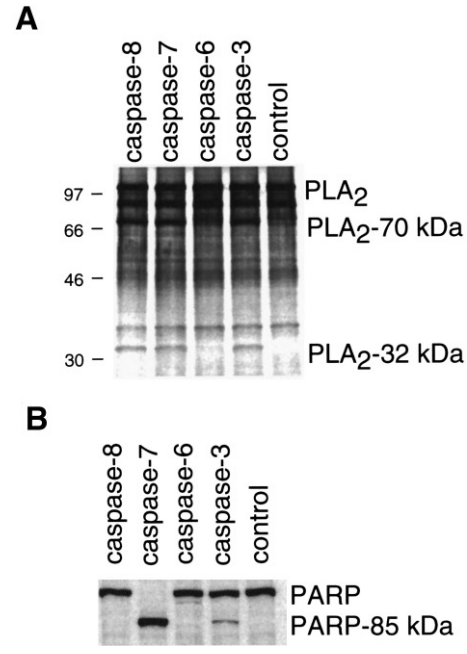

FIG. 4. Cleavage of $cPLA_2$ by recombinant caspases *in vitro*. (A) *In vitro*-translated $cPLA_2$ was incubated with buffer (control) or with dilutions of recombinant caspase-3, -6, -7, or -8. The positions of input $cPLA_2$ and the cleavage products are marked at the right. (B) Cellular lysates from HEK 293 cells containing endogenous PARP were incubated with buffer (control) or with dilutions of recombinant caspase-3, -6, -7, or -8. PARP and the resulting cleavage products were analyzed by immunoblotting using the anti-PARP antibody.

but not by caspase-8 (Fig. 4B). Thus, $cPLA_2$ represents a novel caspase-8 substrate that is not a caspase itself.

DISCUSSION

Previous reports have described caspase-3-mediated cleavage of $cPLA_2$ during apoptosis leading to the inactivation of its enzymatic activity (28, 29). Here, we have extended these findings and provide evidence that $cPLA_2$ is cleaved additionally by caspase-1 and caspase-8 *in vitro*. Thus, $cPLA_2$ is one of very few so far described proteins that can be cleaved by several caspases at multiple cleavage sites (6). Another example for multiple cleavage sites is the PITSLRE p110 protein that appears to be cleaved by caspase-1, -3, and -8 (34).

The cleavage site for caspase-1 has been mapped to aspartic acid residue 459 upstream of the caspase-3 cleavage site at residue 522. This is in good agreement with the consensus sequence identified as recognition motif for caspase-1 (33). We detected cleavage by caspase-1 at this site *in vitro* and in intact cells. The resulting cleavage product p58 was specific for caspase-1 and was not observed with other caspases examined under the same conditions. Whether $cPLA_2$ is cleaved in intact cells under conditions, where

caspase-1 is activated, remains to be determined. The participation of caspase-1 in the induction and/or execution of apoptosis has been controversially discussed (6). The 58-kDa cleavage product was not detected in HeLa cells undergoing apoptosis induced by several stimuli (28). This observation argues against a participation of caspase-1 in the induction of apoptosis in HeLa cells. The generation of a clearly separable, caspase-1-specific $cPLA_2$ cleavage product may allow the exploitation of $cPLA_2$ as an easily accessible indicator for the involvement of caspase-1 during apoptosis. Since caspase-3-mediated cleavage of $cPLA_2$ inactivates its catalytic function (28, 29), and since the cleavage site for caspase-1 is located upstream of the one used by caspase-3, the cleavage products generated by caspase-1 are most likely enzymatically inactive as well.

Using five caspases from different subfamilies in an coexpression assay system revealed discrete cleavage patterns of $cPLA_2$ for these caspases. Overexpression of a single caspase together with $cPLA_2$ likely leads to direct cleavage, but might additionally stimulate the apoptotic program of the cell and, thus, the activation of other caspases that eventually cleave $cPLA_2$. Thus, the results of *in vivo* cleavage assays as described here probably reflect a mixture of fragments generated by direct and indirect processing. Nevertheless, we have identified distinct differences in the cleavage patterns observed after overexpression of various caspases. One important observation was the difference in cleavage patterns seen after overexpression of caspase-3 versus caspase-7 (see Table 1). Although both caspases display an almost identical preference for the DEVD-motif (33), caspase-3 cleavage produced p56 after TNF-treatment, while caspase-7 did not. This observation could reflect a different cleavage site recognition, variances in the activation of both caspases by TNF, or differences in the activation of endogenous caspases. Another intriguing observation was that overexpression of caspase-4, belonging to the same caspase subfamily as caspase-1, did not generate p58, but led to a cleavage pattern similar to that typical for effector caspases. In contrast to the pronounced $cPLA_2$ cleavage after coexpression with caspase-4, we had not observed *in vitro* cleavage of $cPLA_2$ with cytosolic extracts from caspase-4 overexpressing cells in previous experiments (28). This contradiction could be due to a lack in accessibility of the *in vitro* translated $cPLA_2$ to caspase-4 or to a loss of active caspase-4 during the extraction procedure. Since almost no PARP cleavage could be detected in lysates of caspase-4-transfected cells, the observed $cPLA_2$ cleavage appears to be due to a direct caspase-4 cleavage rather than cleavage by endogenous caspases activated by overexpressed caspase-4.

Finally, we have provided evidence that $cPLA_2$ directly serves as substrate for caspase-8. This conclusion is supported by previous results showing that $cPLA_2$ is cleaved in HEK 293 cells treated with TNF alone (28), while we were not able to detect caspase-3 activation or cleavage of the classical caspase-3/7 substrate PARP under these conditions (S.U. and S.A.-K., unpublished results). Moreover, TNF-induced $cPLA_2$ cleavage in HEK 293 cells could be inhibited by overexpressing a dominant-negative form of caspase-8, but not by overexpressing an antisense caspase-3 construct (S. L., unpublished results). Thus, $cPLA_2$ might represent one of few substrates for caspase-8 besides effector caspases, which are activated by caspase-8 cleavage.

Taken together, our results provide evidence, that human $cPLA_2$ serves not only as substrate for the classical effector caspase-3 and -7, but can also be cleaved by caspase-1 and -8. The presence of multiple cleavage sites and the availability as substrate for various caspases might warrant cleavage and inactivation of the proinflammatory enzyme $cPLA_2$ in any context leading to apoptosis.

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