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Caspase-Mediated Inhibition of Human Cytosolic Phospholipase A2 During Apoptosis

Sabine Adam-Klages, Ralf Schwandner, Silke Lüschen, Sandra Ussat, Dirk Kreder, and Martin Krönke

Activation of cytosolic phospholipase A2 (cPLA2) is an essential step in the initiation of the cascade of enzymatic reactions leading to the generation of proinflammatory lipid mediators. Hence, the regulation of cPLA2 is a key event in the induction of inflammatory responses. cPLA2 is activated, in part, by apoptotic stimuli such as TNF or Fas ligand. Apoptosis, however, does not provoke an inflammatory response. Here, we demonstrate that cPLA2 is cleaved by caspase-3 and/or a related caspase in HeLa cells undergoing apoptosis. Mutation of a predicted caspase-3 cleavage site abolishes cPLA2 processing both in vitro and in intact cells. The 70-kDa cleavage product of cPLA2 itself has no catalytic function, while inhibition of cleavage results in an increased enzymatic activity. Additionally, overexpression of the 70-kDa fragment appears to produce a dominant negative effect on endogenous cPLA2 activity. In HeLa cells, cPLA2 activity was dispensable for the course of apoptosis. We cannot rule out, however, that cPLA2 activity is involved in the induction of apoptosis in other cell types. Taken together, our results suggest that the enzymatic activity of cPLA2 is specifically inhibited by caspase-mediated cleavage during apoptosis. The inactivation of cPLA2 represents a previously unrecognized mechanism for avoiding an inflammatory reaction against apoptotic cells. The Journal of Immunology, 1998, 161: 5687–5694.

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induction of programmed cell death may coincide with the generation of inflammatory mediators.

In this report, we show that cPLA₂ is cleaved during apoptosis and that this cleavage is most likely conducted by caspase-3 or a protease with similar substrate specificity. The processing of cPLA₂ abolishes its catalytic activity, thereby inhibiting a proinflammatory signal potentially activated in parallel with the apoptotic signal and ensuring physiologic death without inflammation.

Materials and Methods

Reagents

Highly purified human TNF (3 × 10⁷ UI/mg) was provided by G. Adolf (Bender, Vienna, Austria). The mAb against human cPLA₂ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-Fas Ab was purchased from Coulter Immunotech (Healeah, FL), and the mAb against PARP was originally obtained from Dr. G. Poirier (McGill University, Quebec, Canada). The caspase inhibitors Z-VAD.fmk, Ac-DEVD.CHO, and Ac-YVAD.cmk were purchased from Bachem (Torrance, CA). The calcium ionophore A23187 (Sigma, St. Louis, MO) was dissolved in ethanol at 1 mM. Cycloheximide (CHX), etoposide, and daunomycin were purchased from Sigma (St. Louis, MO). C2-ceramide from Tularik (South San Francisco, CA). cDNAs encoding caspase-3, -4, and -7 were generously provided by Dr. D. Goeddel (Tularik, South San Francisco, CA). Oligonucleotides used for PCR were: for caspase-3, 5'-TGGCTATTGACTGAAGTAGAGTTCC-3'; for caspase-4, 5'-GGCAGAAGG-3'; for caspase-7, 5'-GAGTGGCTTCCATTTTCAATTGCC-3'.

Plasmids

The cDNA encoding human cPLA₂ was subcloned into the eukaryotic expression vector pRK5 (26). The truncated form of cPLA₂ (PLA₂-D523) was obtained by cloning the N-terminal Sal I fragment of the cPLA₂ cDNA into the expression vector pEF.Bos (27). The expression plasmids for caspase-1 and caspase-8 were generously provided by Dr. D. Goeddel (Tularik, South San Francisco, CA). cDNAs encoding caspase-3, -4, and -7 were obtained by PCR using a cDNA library from U937 cells and subcloned into pRK5. Oligonucleotides used for PCR were: for caspase-3, 5'-ATAAAAGGATATCCGAGAAGACCTG-3' and 5'-CCACAAACCATCCTTCTTGTAG-3'; for caspase-4, 5'-AGAGGCTGTTGCTTATGCAGG-3' and 5'-CTTGGTCTTCCATTCTTACTCC-3'; and for caspase-7, 5'-TGGAACAGTTGCAAGATGCAAGG-3' and 5'-TGGTATCTGAACTGAAGTACTGTTCC-3'.

Cell culture and transfection

HEK 293 cells and HeLa cells were originally obtained from the American Type Culture Collection (Manassas, VA). Both cell types were grown in DMEM without FBS (Biochrom, Berlin, and cDNA) 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 (28).

Western blot analysis

About 16 h after transfection, cells were detached using DETDA, lysed in TNE buffer (20 mM Tris [pH 8.0], 140 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitors; Promega, Boehringer, Mannheim, Germany). After precipitating cell debris from 5 ml of 14,000 rpm, protein concentrations were determined in the cytosolic supernatants using a Coomassie reagent (Pierce, Rockford, IL). From each lysate, 20 μg of total protein was separated on a 10% SDS-PAGE and transferred to a nitrocellulose filter (0.45 μm; Macherey-Nagel, Düren, Germany). Filters were blocked overnight in PBST (PBS containing 10% FCS, 2 mM glutamine, and 50 μg/ml each of streptomycin and penicillin). Transfection was performed using the calcium phosphate precipitation method (28).

In vitro cleavage assays

Human PLA₂-WT cDNA or the PLA₂-D/A mutant cloned into the expression vector pRK5 were used for in vitro transcription/translation employing the SP6-coupled TNT Reticulocyte Lysate System (Promega, Madison, WI) and [35S]methionine (Amersham).

To obtain cytosolic extracts from HEK 293 or HeLa cells containing active caspases, cells were detached using EDTA 18–24 h after transient transfection and lysed in ACHE buffer (10 mM HEPES [pH 7.4], 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM Pefabloc SC (Boehringer)) by four cycles of freezing/thawing followed by repeated passing through a 23-gauge needle. Cell debris was removed by centrifugation at 4°C for 30 min at 14,000 rpm. Protein concentrations in the cytosolic supernatants were determined using the Coomassie reagent. Total protein (7.5 μg) from the ACE extracts were incubated with 1.5 μl of in vitro-translated cPLA₂ in a final volume of 15 μl ACHE assay buffer (20 mM Tris [pH 7.5], 0.1 mM EDTA, 10 mM DTT, 1 mg/ml Pefabloc SC) for 2 h at 30°C. The reaction was stopped by adding 5 μl of 4× SDS sample buffer. Radioactive cPLA₂ proteins were analyzed on a 12.5% SDS-PAGE. The gels were fixed in 10% acetic acid, dried, and exposed on Kodak BioMAX films.

Bacterial extracts were prepared 5 h after IPTG induction by resuspending the cell pellet from 50 ml of culture in 3 ml of caspase buffer (20 mM Pipes [pH 7.2], 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM DTT, 300 μg/ml lysozyme, and 1 μg/ml leupeptin) and lysing the bacteria by four cycles of freezing/thawing followed by sonication for 30 s. Debris was removed by centrifugation at 4500 rpm for 20 min at 4°C. Protein concentration in the supernatant was determined using the Coomassie reagent. In vitro cleavage of 35S-labeled cPLA₂ by bacterially expressed caspase-3 was performed essentially as described for cleavage by HEK 293 cell lysates with the following modifications: 1 μg of bacterial proteins was used with an incubation time of 0.5 h.

Site-directed mutagenesis

The cDNA encoding human cPLA₂ was subcloned into the mutagenesis vector PALTER-1 (Promega). Using the oligonucleotide-directed in vitro mutagenesis kit (Promega) a single amino acid (aspartic acid at position 522) was replaced by alanine (GAT→GCT). cDNA encoding 5'-GATCATTGTCTTGATTGTGTA-3' to generate PLA₂-D/A following the protocols provided by the supplier. The introduced point mutation was verified by DNA sequencing.

Arachidonic acid release

To test different mutant forms of human cPLA₂ for enzymatic activity AA release assays were performed essentially as described (23). HEK 293 cells were transiently transfected in triplicates with expression constructs for various cPLA₂ proteins and labeled 6 h after transfection with 1 μCi/ml medium [5,6,8,9,11,12,14,15-3H]AA (150–230 Ci/nmol, 1 μCi/ml; Amersham). After overnight incubation, cells were washed twice times with medium and stimulated as indicated in the figure legends. Released radioactivity from the supernatants was quantified by liquid scintillation counting.

Determination of cPLA₂ activity using phosphatidylcholine vesicles

The phosphatidylcholine vesicle assay was performed as described (23). Briefly, HEK 293 cells overexpressing cPLA₂ or its mutant forms were detached, washed once with PBS, and lysed by sonication in buffer containing 10 mM Tris (pH 7.4), and 150 mM NaCl. Protein content was measured, and equal amounts of protein (1 μg/μl) were incubated for 10 min at 37°C (pH 8.8), 4 mM CaCl₂, 2 μM 1-stearoyl-2-[14C]arachidonylphosphatidylcholine (Amersham) for 30 min at 37°C. The [14C]AA released was extracted by the method of Dole and Meinertz (29). The associated radioactivity was quantified by scintillation counting.

Cell cycle analysis

HeLa cells were transiently transfected with expression constructs for various cPLA₂ proteins and incubated for 24 h to allow for expression of cPLA₂ proteins. Cells were detached using EDTA, washed twice with cold PBS/5 mM EDTA, and resuspended in 1 ml PBS/5 mM EDTA. Cells were fixed by adding 1 ml of ethanol and incubated for 30 min at room temperature. Cells were harvested and resuspended in 0.5 ml of PBS/5 mM EDTA. RNA was removed by digestion with 20 μl of RNase A (1 mg/ml) for 30 min at room temperature. After 1 h of incubation with 0.5 ml of staining solution (500 μl of propidium iodide in PBS/5 mM EDTA), cell cycle analysis was performed by flow cytometry using a FACScalibur Analyzer (Becton Dickinson, Heidelberg, Germany).
Results

Cleavage of cytosolic phospholipase A2 during apoptosis

Analysis of the amino acid sequence of human cPLA2 revealed the presence of a putative cleavage site identified for cysteine proteases. This sequence motif (DELD at amino acids 519–522; Fig. 1A) is very similar to the characteristic cleavage site for caspase-3 within its substrate PARP (DEVD; Ref. 13). We used HeLa cells stimulated with diverse cell death-inducing agents to investigate whether cleavage of cPLA2 could be detected during apoptosis. Western blot analysis with a mAb against the N-terminal 200 amino acids of human cPLA2 revealed the appearance of a cleavage product of about 70 kDa molecular mass (Fig. 1B). Given the fact that the cPLA2 protein migrates on SDS-PAGE higher than predicted from its calculated molecular mass (30), the size of this protein fragment is in agreement with a cleavage at the putative caspase-3 motif, DELD. The amount of the 70-kDa cleavage product and, in parallel, the disappearance of the intact cPLA2 correlated well with the number of apoptotic cells determined by cell cycle analysis using flow cytometry (Fig. 1C). Cleavage of the typical caspase substrate PARP was also detected in comparable proportions (Fig. 1B).

Inhibitor studies revealed that cleavage of cPLA2 after inducing apoptosis with TNF and CHX was blocked by pretreating the cells with caspase-3-specific peptide inhibitor Ac-DEVD.CHO or Ac-YVAD.cmk. After induction of cell death with TNF/CHX for 12 h, cells were lysed, and cPLA2 cleavage was analyzed in immunoblots. The positions of molecular mass markers (in kDa) are indicated on the left.

Caspase-mediated cleavage of cPLA2 occurs in intact cells and in vitro

To further investigate the involvement of distinct caspases in the observed apoptotic cleavage of cPLA2, we used HEK 293 cells overexpressing different types of caspases. Since cPLA2
expression in HEK 293 cells is extremely low (data not shown), we cotransfected an expression construct for human cPLA2. Proteolytic processing of cPLA2 yielding the 70-kDa cleavage product could be detected in HEK 293 cells overexpressing cPLA2 after stimulation with TNF (Fig. 2A). HEK 293 cells are resistant to TNF-induced apoptosis, but appear to activate endogenous caspases after TNF treatment (data not shown). To identify specific caspases that can use cPLA2 as substrate, we used HEK 293 cells overexpressing caspase-1, -3, and -8, belonging to different subfamilies (6), in combination with cPLA2. As shown in Fig. 2B, overexpression of caspase-3 and -8 resulted in the generation of the 70-kDa cleavage product detected in apoptotic HeLa cells or after TNF treatment. Overexpression of caspase-1, however, led to a different cleavage product of ~58 kDa molecular mass (Fig. 2B).

Coexpression studies as described above might provide rather misleading results, since overexpression of a single caspase might stimulate just the apoptotic program of the cell, thereby activating other caspases that finally cleave cPLA2. To further analyze processing of cPLA2 by a specific caspase, we performed in vitro cleavage assays. In vitro translated cPLA2 was incubated with lysates from HEK 293 cells containing caspases belonging to different groups within the protein family (6), to define whether cPLA2 can serve as substrate for caspases in vitro. Cleavage of the 35S-labeled cPLA2 was observed after incubation with cytosolic extracts from cells overexpressing caspase-3, caspase-7 (Mch3, ICE-LAP3, CMH-1), and caspase-8, but not caspase-4 (ICErel-II, TX, ICH-2; Fig. 3A). Extracts from control cells did not lead to cPLA2 processing (Fig. 3A). The generated 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 mAb against cPLA2, indicating that it corresponds to the C-terminal portion of cPLA2. In vitro cleavage of cPLA2 by cell extracts containing caspase-3 was also seen using HeLa cells (data not shown). In vitro cleavage of cPLA2 after incubation with extracts from cells overexpressing caspase-3 was inhibited by the caspase-3-specific inhibitor Ac-DEVD.CHO, but not by Ac-YVAD.cmk (data not shown).

Finally, we investigated whether cPLA2 serves as a substrate for recombinantly expressed caspase-3. We could detect cleavage of cPLA2 after incubation with extracts from E. coli cells containing active caspase-3 (Fig. 4A). The size of the major cleavage products was identical to that seen with extracts from HEK 293 or HeLa cells. Additional protein fragments seen in lower quantities might be due to processing of alternatively started translation products or to nonspecific cleavage at related sites. No cleavage could be detected with E. coli lysates containing the expression vector (Fig. 4A). The activity of caspase-3 in the E. coli lysates was confirmed by an enzymatic assay using the caspase-3-specific fluorescence peptide substrate, Ac-DEVD.AMC. Detectable nonspecific cleavage was also seen using the caspase-1-specific substrate, Ac-YVAD.AMC (Fig. 4B). In vitro cleavage of cPLA2 could be inhibited by the caspase-3-specific inhibitor, Ac-DEVD.CHO, but not by caspase-1-specific inhibitor, Ac-YVAD.cmk (data not shown).

Thus, our data strongly indicate that cPLA2 is cleaved in apoptotic cells and that this cleavage is most likely conducted by caspase-3 and/or a caspase with similar substrate specificity.

**Mutational analysis of cPLA2**

To prove that the major caspase cleavage site used in apoptosis is indeed the proposed DELD sequence, we constructed a mutant form of human cPLA2 replacing aspartic acid (residue 522) with...
slightly different manner from that predicted by their calculated molecular mass (29). The truncated form, PLA₂Δ523, showed a migration pattern indistinguishable from the 70-kDa cleavage product of wild-type cPLA₂, generated in cells treated with TNF (Fig. 5). In contrast to wild-type cPLA₂ (PLA₂-WT), the mutant form, PLA₂-Δ523, was not cleaved in cells treated with TNF (Fig. 5). Identical results were obtained using HeLa cells overexpressing the truncated cPLA₂ protein, PLAs-Δ523, or PLA₂(D/A). Where indicated, cells expressing PLA₂-WT, PLA₂-D/A, or PLA₂Δ523 after stimulation with TNF, the calcium ionophore A23187, or a combination of both. In this system, increased AA release after stimulation was observed in cells overexpressing PLA₂-WT or PLA₂-D/A, but not in vector-transfected cells (Fig. 6A), while treatment with a specific inhibitor of cPLA₂ (AACOCF₃) completely inhibited the stimulated AA release (data not shown). This result indicates that the observed release of AA is due to the activity of overexpressed cPLA₂. Overexpression of the cleavage resistant PLA₂-D/A led to an increased basal activity. After stimulating the cells with TNF and, more prominently, with a combination of TNF and A23187, the rise in AA release was greater compared with wild-type cPLA₂ (Fig. 6A). This indicates that resistance to the caspase-mediated cPLA₂ cleavage leads to an increased enzymatic activity. In contrast, cells overexpressing the truncated cPLA₂ protein showed no increased AA release after stimulation. In addition, overexpression of PLA₂Δ523 exhibited a dominant negative effect on the basal level of AA release (Fig. 6A). Equal expression of cPLA₂ proteins was demonstrated by immunoblot analysis (Fig. 6A).

In addition, a phosphatidylcholine vesicle assay was performed to directly assess the enzymatic activity of the cPLA₂ mutants. As shown in Fig. 6B, PLA₂-D/A exhibited a catalytic activity slightly higher than PLA₂-WT, while PLA₂Δ523 demonstrated no enzymatic activity at all. The activity of PLA₂-D/A could be completely blocked by the cPLA₂ inhibitor AACOCF₃ at a concentration of 10 μM. These data confirm that cleavage of cPLA₂ leading to the 70-kDa fragment inactivates its function.

To further corroborate that inhibition of cPLA₂ cleavage indeed leads to enhanced enzymatic activity as proposed by the higher activity of PLA₂-D/A, we inhibited caspase activity using z-VAD.fmk, an inhibitor that blocks a broad range of caspases. In Western blots, we proved that z-VAD.fmk blocked cPLA₂ cleavage. Fig. 7A shows that the appearance of the 70-kDa product was inhibited in a dose-dependent manner by z-VAD.fmk. In parallel, we performed an AA release assay that revealed a marked increase in the stimulated AA release in cells treated with an amount of z-VAD.fmk that completely blocked cPLA₂ processing (Fig. 7B).

cPLA₂ cleavage does not play a role in the apoptotic process cPLA₂ has been implicated in the induction of apoptosis by TNF (31–34). Moreover, Wissing et al. (35) reported that cPLA₂ is
activated by caspase-dependent cleavage and speculated that the presumably activated cPLA2 cleavage product might be involved in the initiation of apoptosis. To examine whether cleavage of cPLA2 by caspases influences the induction or execution of the apoptotic program, we overexpressed wild-type cPLA2 or the truncated cleavage product in HeLa cells. After stimulation with TNF/CHX, the amount of apoptotic cells was determined by cell cycle analysis using flow cytometry. Overexpression of neither PLA2-WT nor PLA2Δ523 led to induction of apoptosis by itself. In addition, the amount of apoptotic cells after treatment with TNF/CHX was not changed significantly (Fig. 8A). Thus, in HeLa cells, cleavage of cPLA2 does not appear to alter the course of apoptosis.

Moreover, blocking of cPLA2 activity by incubating HeLa cells with the cPLA2 inhibitors MAFP or AACOCF3 did not lead to a decreased cell death induced by TNF/CHX, anti-Fas Ab, or daunomycin (Fig. 8B). This confirms that cPLA2 function is unlikely to be a prerequisite for apoptosis in HeLa cells.

**Discussion**

In this report, we demonstrate that human cPLA2 is a substrate for caspase(s) during apoptosis in HeLa cells. Several lines of
substrate for caspase-3 in vitro; and 3) cleavage of cPLA₂ can be observed during apoptosis. The cleavage site has been confirmed using the cPLA₂ mutant D/A, which contains a single amino acid exchange (D522->A) and is completely cleavage resistant, ascertaining the identity of the resulting cPLA₂ fragments.

The generation of cPLA₂ mutants (PLA₂-D/A and PLA₂Δ523) allowed us to examine the consequence of the observed cPLA₂ processing on its enzymatic function. We used HEK 293 cells overexpressing the cPLA₂ mutants to test for their catalytic function, because these cells express very little endogenous cPLA₂, and show almost no cPLA₂ activity or increased AA release in response to stimulation (Fig. 6). We could show that overexpression of PLA₂-D/A leads to slightly enhanced enzymatic activity after stimulation, while the 70-kDa cleavage product demonstrated no enzymatic activity at all. The increased basal activity of PLA₂-D/A might result from an increase of apoptotic cells after transfection leading to cleavage of PLA₂-WT, but not PLA₂-D/A. However, we cannot rule out the possibility that the PLA₂-D/A mutant is intrinsically more active than PLA₂-WT. Taken together, our data clearly indicate that cPLA₂ belongs to the group of caspase substrates that become inactivated by cleavage.

This finding is in agreement with previously published studies on structure and function of human cPLA₂ identifying residues 200, 228, 331, and 549, located N-terminally as well as C-terminally of the cleavage site at residue 522 (Fig. 1A), to be essential for the catalytic function of cPLA₂ (20, 36). Therefore, it is highly unlikely that either one of the cPLA₂ fragments resulting from the caspase-mediated cleavage at residue 522 (the N-terminal 70-kDa or the C-terminal 32-kDa fragment) can be catalytically active. Earlier data demonstrating that the cytosol from apoptotic cells had less cPLA₂ activity than cytosol from control cells (37) also provided evidence for an inactivation mechanism of cPLA₂ in apoptosis. There is, however, a contradiction to a previous report of a caspase-dependent activation mechanism of cPLA₂ (35). These authors have generated all functional data on cPLA₂ activation by caspase cleavage in two cell lines that are highly sensitive to the inflammatory response. The cleavage of cPLA₂ followed by the inflammatory response during physiologic cell death.

FIGURE 8. cPLA₂ activation does not appear to be involved in the apoptotic pathway in HeLa cells. A, HeLa cells were transfected with expression constructs for PLA₂-WT or PLA₂Δ523, treated after 6 h with 50 ng/ml TNF or, where indicated, with a combination of 50 ng/ml TNF and 1 µg/ml CHX, and stained for cell cycle analysis after an additional 16 h. The percentage of hypodiploid apoptotic cells is indicated. B, HeLa cells were treated for 16 h with the indicated stimuli, either alone or in combination with 50 µM of MAFP or 10 µM AACOCF₃. The percentage of hypodiploid apoptotic cells is indicated. ND, not determined.

evidence indicate that the protease processing cPLA₂ is likely to be caspase-3 and/or an enzyme with similar substrate specificity: 1) the cleavage site as identified by the generation of the cleavage-resistant mutant PLA₂-D/A is almost identical with the known caspase-3 recognition sequence in PARP; 2) cPLA₂ can serve as substrate for caspase-3 in vitro; and 3) cleavage of cPLA₂ can be inhibited by the caspase-3 inhibitor Ac-DEVDCCHO, but only slightly by the caspase-1 inhibitor Ac-YVADCMK. In a previous study, Wissig et al. have also identified the DELD motif as the possible cleavage site for caspase-3 and studied cPLA₂ cleavage after cell death induced by TNF (35). Using HeLa cells stimulated with diverse cell death-inducing agents, we could show that cleavage of cPLA₂ is a generally observed phenomenon during apoptosis. The cleavage site has been confirmed using the cPLA₂ mutant PLA₂-D/A, which contains a single amino acid exchange (D522->A) and is completely cleavage resistant, ascertaining the identity of the resulting cPLA₂ fragments.

The generation of cPLA₂ mutants (PLA₂-D/A and PLA₂Δ523) allowed us to examine the consequence of the observed cPLA₂ processing on its enzymatic function. We used HEK 293 cells overexpressing the cPLA₂ mutants to test for their catalytic function, because these cells express very little endogenous cPLA₂, and show almost no cPLA₂ activity or increased AA release in response to stimulation (Fig. 6). We could show that overexpression of PLA₂-D/A leads to slightly enhanced enzymatic activity after stimulation, while the 70-kDa cleavage product demonstrated no enzymatic activity at all. The increased basal activity of PLA₂-D/A might result from an increase of apoptotic cells after transfection leading to cleavage of PLA₂-WT, but not PLA₂-D/A. However, we cannot rule out the possibility that the PLA₂-D/A mutant is intrinsically more active than PLA₂-WT. Taken together, our data clearly indicate that cPLA₂ belongs to the group of caspase substrates that become inactivated by cleavage.

This finding is in agreement with previously published studies on structure and function of human cPLA₂ identifying residues 200, 228, 331, and 549, located N-terminally as well as C-terminally of the cleavage site at residue 522 (Fig. 1A), to be essential for the catalytic function of cPLA₂ (20, 36). Therefore, it is highly unlikely that either one of the cPLA₂ fragments resulting from the caspase-mediated cleavage at residue 522 (the N-terminal 70-kDa or the C-terminal 32-kDa fragment) can be catalytically active. Earlier data demonstrating that the cytosol from apoptotic cells had less cPLA₂ activity than cytosol from control cells (37) also provided evidence for an inactivation mechanism of cPLA₂ in apoptosis. There is, however, a contradiction to a previous report of a caspase-dependent activation mechanism of cPLA₂ (35). These authors have generated all functional data on cPLA₂ activation by caspase cleavage in two cell lines that are highly sensitive to the inflammatory response (TNF). Thus, their AA release data are derived from apoptotic cells. In our hands, cells undergoing apoptosis appear to release AA nonspecifically even before cell death becomes apparent (data not shown). HEK 293 cells used in this study are resistant to apoptosis induced by TNF.

Overexpression of the 70-kDa cleavage product of cPLA₂ not only showed no detectable enzymatic activity, but exhibited a dominant negative effect on the slight activation of endogenous cPLA₂ (Fig. 6A). A possible explanation might be that the truncated cPLA₂ could compete for binding of cofactors necessary for the activation process. Such cofactors are calcium ions stimulated by the ionophore A23187 or protein kinases phosphorylating cPLA₂ activated by TNF. Since both the calcium-binding domain (CaLB domain) and the phosphorylation site (Fig. 1) are still contained in the functionally inert PLA₂-D/A mutant, this finding is in agreement with previously published studies identifying residues 200, 228, 331, and 549 of human cPLA₂ as being essential for the catalytic function of the cPLA₂ (20, 36). Therefore, it is highly unlikely that either one of the cPLA₂ fragments resulting from the caspase-mediated cleavage at residue 522 (the N-terminal 70-kDa or the C-terminal 32-kDa fragment) can be catalytically active. Earlier data demonstrating that the cytosol from apoptotic cells had less cPLA₂ activity than cytosol from control cells (37) also provided evidence for an inactivation mechanism of cPLA₂ in apoptosis. There is, however, a contradiction to a previous report of a caspase-dependent activation mechanism of cPLA₂ (35). These authors have generated all functional data on cPLA₂ activation by caspase cleavage in two cell lines that are highly sensitive to the inflammatory response. The cleavage of cPLA₂ followed by the inflammatory response leading to high levels of truncated cPLA₂ might down-regulate the activity of the remaining intact cPLA₂. Ligands such as TNF or FasL can induce apoptosis but also activate cPLA₂ (24, 25). If both of these actions took place in the same cell, an inflammatory signal would coincide with the apoptotic program that does not lead to an inflammatory response. The cleavage of cPLA₂ followed by the competitive inhibition of the remaining cPLA₂ molecules by the cleavage product, as proposed by this study, might be an additional mechanism to block an inflammatory response during physiologic cell death.

Several studies have implied that activation of cPLA₂ is a necessary step in the signaling pathway leading to TNF-induced apoptosis. Hayakawa et al. (31) showed that a TNF-resistant clone of
L929 cells showed reduced cPLA2 expression and became TNF sensitive upon cPLA2 overexpression. Other groups have demonstrated that apoptosis of various tumor cell lines was dependent on the activity of cPLA2 (32, 33). Palombella and Vilecek (34) reported that PLA2 activity is essential for cytotoxicity, but is also essential for growth stimulation of TNF. Our data, however, clearly show that cPLA2 is cleaved and thereby inactivated in HeLa cells during apoptosis induced by different stimuli, including TNF. In addition, neither overexpression nor pharmacologic inhibition of cPLA2 demonstrated any influence on TNF/CHX-induced cell death in HeLa cells. Thus, a causative role for cPLA2 during TNF-mediated apoptosis in HeLa cells appears to be rather unlikely. In summary, the involvement of cPLA2 in TNF-induced apoptosis observed in previous studies (31–34) may thus reflect secondary or cell type-specific events.

Recently published studies of cPLA2 knockout mice did not reveal any phenotypic signs that would suggest irregularities in cell death involving processes during development, thus questioning the general involvement of cPLA2 in apoptosis (38, 39). However, cPLA2 knockout mice showed decreased brain infarct sizes after ischemic injury (39), which could reflect decreased neuronal apoptosis after ischemia, again pointing to a potential role of cPLA2 in apoptosis in select cell types.

In summary, our data indicate that cPLA2 is cleaved during the apoptotic process in HeLa cells by caspase-3 and/or a related caspase, leading to a functionally inactive, dominant negative inhibitor of its own enzymatic function. This cleavage does not appear to be involved in apoptosis but might ensure that a potentially proinflammatory enzyme is inactivated in physiologic cell death, which does not lead to an inflammatory response.

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