ABSTRACT

PERERA, PUBUDUNI SUDARIKA Multiple Forms of $cPLA_2$. (Under the direction of Scott M. Laster). Cytosolic Phospholipase $A_2\alpha$ is an important inflammatory molecule that preferentially hydrolyzes phospholipids containing arachidonic acid at the *sn*-2 position, which in turn is processed to generate lipid mediators of inflammation such as prostaglandins, leukotrienes and platelet activating factor. Hence, $cPLA_2\alpha$ plays a vital role in the pathophysiology of inflammatory and degenerative diseases. $cPLA_2\alpha$ is also known to mediate apoptosis via arachidonic acid, which causes mitochondrial dysfunction leading to cell death. The focus of this investigation was the identification and characterization of a possible secondary form of $cPLA_2\alpha$ selectively associated with the nucleus. Gaining knowledge about the identity of a possible secondary form may give new insight to the mechanism of activation of $cPLA_2\alpha$, which can then lead to the development of novel therapeutic agents that can act as inhibitors of $cPLA_2\alpha$ and its physiological effects. Two lines of questioning were employed within the scope of this study; (1) what portion of the full-length $cPLA_2\alpha$ is forming the possible secondary form? (2) what is the mechanism of generation of the secondary form? Two methods were utilized to address the above questions: (1) A panel of protease inhibitors targeting several major classes of proteases was employed to selectively inhibit each group independently in order to determine their effect on the generation of the nuclear species and (2) Performing structural analysis of $cPLA_2\alpha$ using a panel of antibodies with varying recognition sites along the length of the protein in order to generate a putative map of the nuclear forms. It was observed that nuclei contain three distinct forms of nuclear $cPLA_2\alpha$ ranging in molecular weight from 103kDa, 78kDa to

70kDa. The structural analysis revealed that the 78kDa and the 70kDa nuclear-associated proteins are both amino-terminal fragments of the full-length cPLA$_2\alpha$ where the 78kDa protein may potentially encompass residues 42-444 of the full-length cPLA$_2\alpha$, while the 70kDa may share the majority of its residues with the 78kDa protein. Furthermore, the protease inhibitor assays have indicated that the generation of the nuclear proteins may be due to a proteolytic cleavage event that is mediated by the concerted action of a cathepsin protease, a serine protease and caspase-3. Taken together, these results indicate that gaining knowledge about the identity of a possible secondary form of cPLA$_2\alpha$ and hence the activation of the protein would lend immensely to the development of therapeutic agents in the treatment of inflammatory diseases.

MULTIPLE FORMS OF $cPLA_2$

by

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Chair of Advisory Committee

DEDICATION

I dedicate this work to my parents who have always been the source of all my inspiration. I am eternally grateful for their love, support and guidance and most of all for all they have had to sacrifice to help me realize my dreams.

BIOGRAPHY

Pubuduni Sudarika Perera was born in February of 1978 in Sri Lanka to Solomon and Padmini Perera. She lived in Sri Lanka until 1988, when she moved to Saudi Arabia with her family. In 1994, she moved to the United States of America and in 1995 graduated from East Rochester High School, Rochester, NY. In May of 1999 she graduated from University of Rochester with a Bachelor of Science in Molecular Genetics. After graduating, Sudarika worked as a research technician in the Department of Dermatology, Strong Memorial Hospital, Rochester, NY. She enrolled in the masters program in the Department of Microbioloy at North Carolina State University in July of 2001, where she began her training under the direction of Dr. Scott Laster.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Phospholipase A_2 (PLA_2) Isoforms

PLA_2s are a group of diverse lipases that catalyze the hydrolysis of the ester bond in the *sn*-2 position of membrane glycerophospholipids thus releasing a free fatty acid and a lysophospholipid (1). PLA_2s are divided into four distinct families. The first family consists of the secretory PLA_2s, where 10 isozymes have been characterized that have a molecular weight of 14kDa (1, 2). The second family is the Ca^{2+}-sensitive cytosolic PLA_2 family, which has three isozymes identified to date and plays an essential role in the hydrolysis of arachidonic acid, the precursor for a number of eicosanoids (2). The third family, the Ca^{2+}-independent PLA_2s ($iPLA_2$) contains two enzymes and is thought to play a significant role in membrane phospholipid remodeling (3). The fourth family is the platelet-activating factor (PAF) acetylhydrolase (PAF-AH) group. This group contains four enzymes that demonstrate specificity towards PAF and phospholipids with oxidized acyl groups (4-8). The cytosolic PLA_2s are of greater significance due to their capacity to directly associate with signal transduction elements such as G-proteins and kinases (9).

Cytosolic PLA_2 Isoforms

There are three cytosolic phospholipase genes that have been cloned thus far: $cPLA_2\alpha$, $cPLA_2\beta$, and $cPLA_2\gamma$ (5). $cPLA_2\alpha$ or the Ca^{2+}-dependent cytosolic phospholipase A_2 was initially characterized from macrophage cell lines, platelets and various tissue types (11-17). The $cPLA_2\beta$ and $cPLA_2\gamma$ sequences were obtained from the expressed sequence tag

database by using the sequence of cPLA$_2\alpha$ as a search query (9,18,19). The cloned cDNAs of cPLA$_2\alpha$, cPLA$_2\beta$, and cPLA$_2\gamma$ encode proteins of 749 amino acids, 1012 amino acids and 541 amino acids respectively (10). The three cPLA$_2$ isoforms demonstrate some homology but are different enough in their primary sequences that they can be differentiated by the use of specific antibodies raised against sequences unique to each isoform. All three isoforms contain two homologous catalytic domains and a lipase consensus sequence (10). Both cPLA$_2\alpha$ and cPLA$_2\beta$ contain an amino-terminal C2 domain important in Ca^{2+}-dependent binding to substrate (10). However, this C2 domain is absent from the sequence of cPLA$_2\gamma$ (10). Biochemical studies indicate that cPLA$_2\beta$ and cPLA$_2\gamma$ have different regioselectivity than cPLA$_2\alpha$; cPLA$_2\beta$ is less selective for cleavage at the *sn*-2 position than *sn*-1 and cPLA$_2\gamma$ demonstrates both PLA$_1$ and PLA$_2$ activities (10). Furthermore, cPLA$_2\gamma$ prefers arachidonic acid at the *sn*-2 position only 2- to 3.5-fold to other fatty acids (10). In contrast, cPLA$_2\alpha$ hydrolyzes phospholipids containing arachidonic acid at the *sn*-2 position with greater than 20-fold preference over other fatty acids (10). Therefore, cytosolic phospholipase A$_2\alpha$ is considered one of the most important PLA$_2$ isoforms for the central role it plays in the generation of lipid mediators of inflammation by providing free arachidonic acid from the *sn*-2 position of membrane phospholipids.

The cPLA$_2$a Gene

The Ca^{2+}-sensitive cytosolic PLA$_2\alpha$ was initially characterized and purified from a murine macrophage cell line by Leslie et al. in 1988 (11). The cDNAs of both human and murine cPLA$_2\alpha$ were later cloned from macrophage cell line libraries (20-22). Furthermore,

the promoter region of the rat and human cPLA$_2\alpha$ genes were isolated in the early 1990s (20-22). The gene that encodes cPLA$_2\alpha$ spans over 137kb and it has been mapped to the q arm of chromosome 1 (23,24). It comprises 18 exons and 17 introns and encodes a protein of 749 amino acids with a predicted molecular mass of 85.2kDa (23,25). The 5'-flanking region of the cPLA$_2\alpha$ gene contains elements typical of a housekeeping gene such as the lack of a TATA box or CAAT box (24). However, this region is unlike a housekeeping gene in that it is not GC rich and has no SP1 sites, which would recruit transcription factors in the absence of a TATA box (24). This region also features a series of CA repeats distinct from a housekeeping gene (24). In 1995 Miyashita et al. identified a 27bp 5' flanking region containing a polypyrimidine tract that is important for the low level constitutive expression of the cPLA$_2\alpha$ gene (24). Subsequently in the year 2000, deletion analysis of a 3.4kb fragment of the human cPLA$_2$ promoter carried out by Dolan-O'Keefe and collaborators demonstrated the existence of two regions that control the basal expression levels of the cPLA$_2\alpha$ gene, the first of which is located between binding sites –3446 to –2271 and the second is located between binding sites –543 to –215 in the 5'-flanking region (23). Moreover, the CA repeat, which is centered around the position –220 when deleted was able to increase promoter activity of the 3.4kb promoter construct by as much as 40-50 percent, thus indicating that this region may act as a negative regulatory element (23). The cPLA$_2\alpha$ promoter provides low level and constitutive expression of the gene allowing for a steady supply of protein that can respond to external stimuli (24). However, the main regulation of the cPLA$_2\alpha$ gene appears to be at the post-transcriptional level. In addition, there is extensive

data that indicate that the cPLA$_2\alpha$ protein is subject to a number of post-translational regulation events (24).

The cPLA$_2\alpha$ Protein

The cDNA of cPLA$_2\alpha$ encodes a protein of 749 amino acids with a predicted molecular weight of 85.2kDa. The cPLA$_2\alpha$ protein migrates as a 100-110kDa protein on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10). The cPLA$_2\alpha$ monomer conforms to a two-domain ellipsoidal shape with dimensions of ~100Å $\times$ 55Å $\times$ 45Å (26). The NH_2-terminal domain comprising residues 16-138 shows significant homology with a number of proteins, such as protein kinase C, GTPase-activating protein (GAP), synaptotagmin and phospholipase C, all of which bind phospholipid membranes via a Ca^{2+}-dependent pathway (27). Calcium activation of cPLA$_2\alpha$ is mediated through this domain and it is responsible for the Ca^{2+}-dependent translocation of cPLA$_2\alpha$ from the cytosol to the membrane compartment, which allows for the co-localization of the catalytic domain with the membrane substrate (21,28). Thus, this region is named the Ca^{2+}- dependent lipid-binding domain (CaLB). The structure of the CaLB domain has been solved (29,30) and it has been confirmed that it is a member of the C2 family. Hence, the CaLB domain is also referred to as the C2 domain. The CaLB domain is connected to the catalytic domain by the residues, 139-143 (29,30). The region composed of residues 188-253 demonstrates significant homology with the catalytic center of phospholipase B (PLB) enzyme from *Penicillium notatum* (31). Within this region is a stretch of 5 amino acids, Gly-Leu-Ser^{228}-Gly-Ser, around a conserved serine residue, Ser-228, that aligns with the lipase consensus

sequence, Gly-X(Leu)-Ser-X(Gly)-Gly, which is present in many serine esterases and neutral lipases, including PLB (2,27). The central Ser-228 is known to be the active site nucleophile (2). Substituting Ala for Ser-228 produces a catalytically inactive cPLA$_2\alpha$ enzyme, thus confirming that Ser-228 is a catalytic center of cPLA$_2\alpha$ (2). Furthermore, replacement of Asp-549 with Ala also produces a catalytically inactive enzyme, suggesting that Asp-549 is also involved in the catalytic mechanism (2). cPLA$_2\alpha$ differs from other neutral esterases and lipases in that it doesn't require histidine for its catalytic activity (2). In addition to Ser-228 and Asp-549, a third residue, Arg-200 was also shown to be required for catalysis (26). Thus, the catalytic mechanism of cPLA$_2\alpha$ appears to be mediated through a serine-acyl intermediate and employs an unusual catalytic dyad formed by Ser-228 and Asp-549, where Ser-228 acts as the active-site nucleophile (31, 33-36). The most notable structural feature of cPLA$_2\alpha$ is the active site funnel, which extends one-third of the way into the catalytic domain and houses the catalytic dyad formed by Ser-228 and Asp-549 (26). The active site funnel is covered by a lid structure that must be displaced in order to allow the substrate access to the active site (26). The last remarkable feature of the cPLA$_2\alpha$ protein sequence is the presence of a number of consensus phosphorylation sites for serine/threonine and tyrosine protein kinases (20). Serine residues 437 and 454 can be phosphorylated, however their functional relevance remains unclear since they are not evolutionary conserved among different species (10). A third residue that is phosphorylated is Ser-727. Ser-727 in contrast to Ser-437 and Ser-454 is conserved among different species (37,38). Nevertheless, its functional significance has also eluded the scientific community. The most significant of the phosphorylated serine residues is Ser-505, since Ser-505 phosphorylation appears to be

required for maximal activation of cPLA$_2\alpha$. Furthermore, Ser-505 is conserved among evolutionary distinct species, such as the human, chicken, mouse, rat and zebrafish (37,38).

Tissue Distribution of cPLA$_2$a

cPLA$_2\alpha$ is a widely expressed gene as confirmed by RT-PCR analysis. cPLA$_2\alpha$ is expressed in a wide variety of adult human tissues. cPLA$_2\alpha$ mRNA was found to be abundantly expressed in brain, lung, heart, spleen, pancreas, placenta, and kidney (19,39). Furthermore, many different cell types are also known to express cPLA$_2\alpha$, namely, macrophages, neutrophils, platelets, endothelial cells, vascular smooth muscle cells, alveolar epithelial cells, renal mesangial cells, fibroblasts and keratinocytes (2,40). Interestingly, mature T and B lymphocytes do not express cPLA$_2\alpha$, while thymocytes and immature B cells do (41). Taken together, the above information indicates that cPLA$_2\alpha$ is a ubiquitously expressed protein that may take part in a host of cellular functions.

Enzymology of cPLA$_2$a

The enzymatic activity of cPLA$_2\alpha$ serves as the key control point in the biosynthesis of a number of inflammatory mediators. cPLA$_2\alpha$ is the only PLA$_2$ isoform that demonstrates a marked preference for arachidonate-containing phospholipids (10). cPLA$_2\alpha$ selectively cleaves arachidonic acid at the *sn*-2 position of membrane glycerophospholipids initiating the production of such eicosanoid lipid mediators as prostaglandins and leukotrienes (42,43). Experiments utilizing recombinant cPLA$_2\alpha$ and membrane vesicles demonstrated that cPLA$_2\alpha$ preferentially hydrolyzes arachidonoyl phospholipids by >20-fold over other fatty acids (21,44-46). Phospholipids containing α-linolenic acid or eicosapentaenoic acid are also

suitable substrates for cPLA$_2\alpha$, however, due to their low abundance in natural membranes, arachidonate-containing phospholipids are the main substrate for cPLA$_2\alpha$ under physiological conditions (27).

In vitro enzyme assay systems have confirmed that cPLA$_2\alpha$ does not require Ca^{2+} for catalysis (10). However, submicromolar concentrations of Ca^{2+} are necessary for the binding of the enzyme to its membrane substrate since the presence of Ca^{2+} significantly enhances the affinity of the CaLB domain for the membrane surface (2,10). There is some evidence that suggests that cPLA$_2\alpha$ is catalytically active toward monomeric phospholipids in the absence of Ca^{2+}, but that Ca^{2+} is required for the cleavage of phospholipids in vesicles or natural membranes (20,21,28). The Ca^{2+} requirement for the hydrolysis of membrane phospholipids can be overcome by introducing high concentrations of salt, which facilitate the formation of hydrophobic interactions between enzyme and substrate (33,47). Experiments utilizing active-site directed inhibitors and site-directed mutagenesis have identified the essential residues for the catalytic activity of cPLA$_2\alpha$ as Ser-228, Asp-549 and Arg-220 (31,36,48).

The proposed model for the catalytic mechanism of cPLA$_2\alpha$ is as follows: Upon increase of intracellular Ca^{2+} levels, cPLA$_2\alpha$ undergoes Ca^{2+}-dependent membrane translocation, at which point a single phospholipid molecule can bind within the narrow cleft of the active site such that the *sn*-2 ester bond is in close proximity to the active site nucleophile, Ser-228. The phosphate moiety of the head group is stabilized by Arg-200. Asp-549 activates Ser-228 by abstracting a proton during its nucleophilic attack at the *sn*-2 ester bond forming an acyl (arachidonoyl) enzyme complex. The acyl-enzyme complex is then hydrolyzed by water to produce free arachidonic acid. The cPLA$_2\alpha$ enzyme can then

dissociate from the membrane surface or bind to another substrate molecule, repeating the cycle (26).

In addition, cPLA$_2\alpha$ also displays other catalytic activities; namely, sn-1 lysophospholipase and transacylase activities (33,35,49-51). Leslie et al. first demonstrated that cPLA$_2\alpha$ exhibits *sn*-1 lysophospholipase activity toward 1-acyl-lysoPC in 1991 (49). However, there have been contrasting reports concerning the Ca^{2+} requirement for this activity. It was demonstrated by Sharp et al. that the mutation of Ser-228, the catalytic center of cPLA$_2\alpha$ also blocks the lysophospholipase activity, suggesting that both catalytic activities are present in one catalytic center (31). The transacylase activity of cPLA$_2\alpha$ was reported by Reynolds et al. who demonstrated that cPLA$_2\alpha$ catalyzed transacylation in a CoA-independent manner, forming 1,2-dipalmitoyl-phosphatidylcholine and glycerophosphorylcholine from two molecules of 1-palmitoyl-lysophosphatidylcholine (33). However, the transacylase activity is relatively weak, constituting about 5 to 8% of the lysophospholipase activity, thus its physiological significance is not certain (27).

Regulatory Mechanisms

cPLA$_2\alpha$ enzyme activity has been known to be stimulated by a wide variety of factors such as cytokines, mitogens, endotoxins, hormones, antigens, neurotransmitters and extracellular matrix constituents (27,52). In addition, a number of physical and stressful stimuli such as oxidation, UV light, hyperglycemia and shear stress have also been shown to induce cPLA$_2\alpha$ activity (10). The induction of cPLA$_2\alpha$ activity is regulated by a number of mechanisms that involve increases in Ca^{2+} concentration, activation of kinases such as MAPK and PKC and, guanine nucleotide-binding protein(s) (G protein) (10,52-54).

Furthermore, suppression of cPLA$_2\alpha$ expression appears to be mediated by glucocorticoids and seems likely due to the presence of a potential glucocorticoid response element in the promoter region of the cPLA$_2\alpha$ gene (22,24). There is some evidence that suggest that IL-4 may also have inhibitory effects on cPLA$_2\alpha$ expression (55).

cPLA$_2\alpha$ is also known to undergo some transcriptional regulation. It has been demonstrated that levels of cPLA$_2\alpha$ mRNA and protein are responsive to a wide variety of extracellular stimuli, including a number of cytokines such as tumor necrosis factor-α, interleukin-1 and interferon-γ (52,56-59). The promoter region of the human cPLA$_2\alpha$ gene lacks a TATA box, which leaves the cPLA$_2$ gene dependent on alternative sequences to assemble the transcription machinery (22,24,60). A common finding among TATA-less promoters is a GC-rich region with SP1 sites, which have been demonstrated to recruit transcription factors (24). However, the cPLA$_2\alpha$ gene promoter not only lacks SP1 sites but is also not GC rich, suggesting that other sequences may be acting as 'initiator' sequences (24). The human cPLA$_2\alpha$ gene promoter does contain several putative possible binding sites for AP-1, AP-2, NF-κB, NF-IL-6, PEA3, OCT, C/CEBP and GRE (glucocorticoid response element) (10). The cPLA$_2\alpha$ gene contains a 5' flanking region that has a 27bp polypyrimidine tract that controls the basal level expression of the gene (24). In addition a 48bp CA repeat appears to have an inhibitory effect on transcription of the cPLA$_2\alpha$ gene (23). There is some evidence that suggests that the cPLA$_2\alpha$ gene may also be subjected to post-transcriptional regulation. The cPLA$_2\alpha$ mRNA may be made unstable by a series of AUUUA sequences that lie within the 3' untranslated region (61).

Phosphorylation also plays an important role in the regulation of cPLA$_2\alpha$ activation. The cPLA$_2\alpha$ protein contains a number of consensus phosphorylation sequences for serine/threonine kinases and tyrosine kinases (10). It has been observed that stimulation of cells with various agonists that induce arachidonic acid release can also cause the phosphorylation of cPLA$_2\alpha$ (52,62). In many cases, the phosphorylation appears to be mainly on serine residues, in particular, Ser-437, Ser-454, Ser-505 and Ser-727 (37). To date, the physiological relevance of the phosphorylation of serine residues, 437, 454 and 727 is not known. However, phosphorylation on Ser-505 is known to increase the intrinsic activity of cPLA$_2\alpha$ by severalfold (63,64). Ser-505 is located within a consensus sequence for MAP-kinase directed phosphorylation (63,64). Experiments utilizing cultured cells and in vitro kinase assays have demonstrated that cPLA$_2\alpha$ is indeed a substrate for mitogen-activated protein kinases also referred to as extracellular signal-regulated kinase (ERK) (10). Furthermore, mutation of Ser-505 to an alanine abolishes the phosphorylation and the accompanied increase in arachidonic acid release (10). It appears that the maximal activation of cPLA$_2\alpha$ occurs upon sustained phosphorylation of Ser-505 by a member of the MAP kinase family (63,65-67). However, phosphorylation alone may not be sufficient for cPLA$_2\alpha$ activation, since in the absence of an increase in intracellular Ca^{2+} levels, phosphorylated cPLA$_2\alpha$ fails to release arachidonic acid (21,52). Thus, maximal cPLA$_2\alpha$ activation requires sustained phosphorylation by a MAP kinase along with an increase in intracellular Ca^{2+} levels (68).

A second level of post-translational regulation that cPLA$_2\alpha$ undergoes is the translocation from cytosol to membrane in a Ca^{2+}-dependent manner in order to colocalize with its phospholipid substrate. The previously mentioned Ca^{2+} dependent lipid binding domain located in the N terminus of the protein mediates this translocation from cytosol to membrane substrate (10). It has been observed that the CaLB domain binds two Ca^{2+} ions spaced 4 Å apart within a site known as the Ca^{2+}-binding region (CBR) (26,29). As described earlier, increases in Ca^{2+} levels markedly enhance the cPLA$_2\alpha$ activity. Ca^{2+} levels of 0.3-1μM induce binding of cPLA$_2\alpha$ to membranes (21,69).

Furthermore, subcellular localization of cPLA$_2\alpha$ has been the focus of numerous studies. Fractionation experiments using calcium ionophore-stimulated macrophages demonstrated that cPLA$_2\alpha$ translocates from the cytosol to the perinuclear envelope and the ER in a Ca^{2+}-dependent manner (70-72). It was observed that upon activation of rat mastcytoma RBL-2H3 cells with Ca^{2+} ionophore, which leads to a permanent rise in Ca^{2+} concentration, most of the cPLA$_2\alpha$ translocated to the nuclear envelope and remained there for the entire duration the ionophore was present (71). However, upon activation with IgE/antigen stimulation, which only leads to a transient increase in Ca^{2+} levels, cPLA$_2\alpha$ translocated rapidly and transiently to the nuclear membrane, only to return to the cytosol within a period of 30 minutes (71). In both cases, the arachidonic acid release was comparative to the kinetics and the duration of binding to the nuclear membrane (71). Lin et al. demonstrated that when CHO cells are stimulated with the calcium ionophore, A23187, cPLA$_2\alpha$ translocates to the nuclear membrane and ER, but not to the plasma membrane (72). Furthermore, it was also observed that a cPLA$_2\alpha$ mutant lacking a functional CaLB domain

but possessing the catalytic domain was unable to translocate to the nuclear membrane upon cell activation (72). However, a cPLA$_2\alpha$ mutant with a mutated Ser-505, the site of MAP kinase phosphorylation, was able to translocate to the nuclear membrane, thus demonstrating that the translocation and phosphorylation of cPLA$_2\alpha$ are regulated independently of each other (27). In addition, both types of mutations blocked arachidonic acid release. Hence, cPLA$_2$-mediated arachidonic acid release is dependent on both translocation and phosphorylation (27).

The above mentioned studies suggest that the nuclear envelope and the ER may be the major sites for arachidonic acid release. A point of particular importance is that the intermediate enzymes in the prostanoid biosynthetic pathway, COX-1 and COX-2 are also localized within the ER and the perinuclear region, respectively (73). This localization would spatially allow cPLA$_2\alpha$ to supply arachidonic acid to the downstream enzymes of eicosanoid biosynthesis.

A number of studies have focused on G proteins as yet another component involved in the regulation of cPLA$_2\alpha$ activation. In 1994 Winitz et al. reported that a functional G_{i2} protein is necessary for full activation of cPLA$_2\alpha$ (53). It was shown that the overexpression of a dominant negative G_{i2} mutant blocked cPLA$_2\alpha$ activation without affecting either Ca^{2+} mobilization or MAP kinase activation, thus suggesting that a functional G protein is required for cPLA$_2\alpha$ activation in addition to phosphorylation and a rise intracellular Ca^{2+} levels (53).

Cellular Roles of cPLA$_2$ a

Activation of cPLA$_2\alpha$ by a wide variety of agonists, growth factors and cytokines leads to the hydrolysis of membrane phospholipids and the subsequent release of the precursor substrate - arachidonic acid - for the production of eicosanoids and platelet-activating factor (PAF). Eicosanoids and PAF play a critical role in the pathophysiology of inflammation, allergy and other disorders, such as global cerebral ischemia (2). Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), cause the activation of cPLA$_2\alpha$, which correlates closely with increased production of prostaglandins, leukotrienes and PAF. IL-1 is thought to be important in the pathogenesis of glomerular inflammation and injury, which is accompanied by increased production of prostaglandins (25). TNF-α like IL-1 causes rapid phosphorylation of cPLA$_2\alpha$ and thus subsequent activation leading to the production of prostaglandins, leukotrienes and PAF. Numerous factors are capable of causing the activation of cPLA$_2\alpha$, including, macrophage colony stimulating factor, bacterial lipopolysaccharide and interferons (25). Furthermore, there is also evidence that suggests a role for cPLA$_2\alpha$ as a mediator of the cytotoxic effects of TNF-α and thus a role in programmed cell death. A study done by Hayakawa et al. demonstrated that expression of a cloned cPLA$_2\alpha$ in a TNF-resistant cell line caused the sensitization of that cell line to the cytotoxic effects of TNF (76). There is evidence that suggests that the arachidonic acid released by cPLA$_2\alpha$ in response to TNF-α leads to the generation of the second messenger, ceramide. Ceramide is thought to lead to cell death by inducing the mitochondrial permeability transition (PT), which is a regulated permeability increase to solutes with molecular masses up to 1,500 Da by controlling the opening of a

high conductance channel, the permeability transition pore (PTP) (52,84). The PTP opening is thought to mediate apoptosis caused by oxidative stress and anoxia through ATP depletion and dysregulation of Ca^{2+} homeostasis (84). Furthermore, the PTP opening is thought to release cytochrome *c*, which plays an important role in capase-9 activation and apoptosis-inducing factor, which in turn causes nuclear degradation (84). Thus, the above findings suggest that cPLA$_2\alpha$ may play a critical role in the cellular responses occurring during inflammatory and degenerative processes.

Lower Molecular Weight Forms of cPLA$_2$

A number of investigators to date have identified the presence of several lower molecular weight forms of the full-length cPLA$_2\alpha$ protein. Nalefski et al. and Roshak et al. have identified the presence of several immunoreactive bands at ~70kDa, ~50kDa and between 50kDa and 60kDa within cytosolic fractions (28,74). cPLA$_2\alpha$ is also known to undergo cleavage by the apoptosis activator proteins, caspases, which are cysteine proteases related to the interleukin 1β-converting enzyme. Adam-Klages et al. (1988) reported that during apoptosis, cPLA$_2\alpha$ is most likely cleaved by caspase-3 or a protease with similar substrate specificity (77). These investigators identified a cleavage site for cysteine proteases within the sequence motif DELD at amino acids 519-522, which is strikingly similar to the caspase-3 cleavage site, DEVD within its substrate poly(ADP-ribose) polymerase (PARP) (77). In addition, this group was able to identify a putative cleavage product of ~70kDa and reported that the cleavage of cPLA$_2\alpha$ inactivated the enzyme. In contrast, Wissing et al. 1997 reported that the cleavage of cPLA$_2\alpha$ by caspase-3 generated an active form of the enzyme (79). In 1998, Luschen et al. reported that cPLA$_2\alpha$ could also be cleaved by caspase-1 and

caspase-8. They placed the caspase-1 cleavage site at Asp-459 within the sequence YQSD/N and the caspase-8 cleavage site at the caspase-3 cleavage site at Asp-522, within DELD, and they reported the presence of a 58kDa cleavage product in cells overexpressing caspase-1 and a 70kDa product generated by caspase-8 (78). Interestingly, In 1996 Sierra-Honighmann et al. reported the presence of a smaller size (70kDa) cPLA$_2\alpha$ in the nuclear extract of subconfluent cells (75). The presence of cPLA$_2\alpha$-derived fragments and particularly a distinct nuclear species of cPLA$_2\alpha$ raises a number of questions as to their identity and catalytic activity. To date, there is no conclusive evidence as to the identity of the cPLA$_2\alpha$-derived fragments and specifically that of the nuclear associated species.

The purpose of this investigation was to identify and characterize several nuclear forms of cPLA$_2\alpha$ that has been observed to be consistently present in the nuclear fraction. Thus, the questions addressed are the following: (1) what portion of the full-length cPLA$_2\alpha$ is forming the nuclear proteins? (2) what is the mechanism of generation of the nuclear proteins? As mentioned above, cPLA$_2\alpha$ plays a vital role in the pathophysiology of inflammatory and degenerative disease states. Gaining insight into the nature of a possible secondary form of cPLA$_2\alpha$ can serve to elucidate the activation of cPLA$_2\alpha$. This can lead to the usage of cPLA$_2\alpha$ as a target for novel therapies and inhibitors of cPLA$_2$ may be useful as therapeutic agents in the treatment of inflammatory diseases. To address the first question, protease inhibitor assays were employed. A panel of protease inhibitors targeting several major classes of proteases was employed to selectively inhibit each group independently in order to determine their effect on the generation of the nuclear proteins. The second question was addressed by performing structural analysis of cPLA$_2\alpha$ using a panel of antibodies with

varying recognition sites along the length of the protein in order to generate a putative map of the nuclear-associated proteins.

Materials and Methods

Cell Culture and Reagents

C3HA is a 3T3-like murine fibroblast cell line kindly provided by L. Gooding, (Emory University, Atlanta, GA). Cells were cultured in Dulbeco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and maintained at 37^{o}C in 8% CO_2. Media and reagents, unless otherwise indicated, were purchased from Sigma. A general cathepsin protease inhibitor was purchased from ICN Biomedicals, Inc. Enzyme Systems Products (Aurora, OH). Caspase-1 inhibitor (Ac-YVAD-CMK) and caspase-3 inhibitor (Z-DEVD-FMK) and a proteasome inhibitor (MG-132) were purchased from Calbiochem (La Jolla, CA). Three Rabbit polyclonal antisera raised against denatured human cPLA$_2\alpha$, residues 42-58 of human cPLA$_2\alpha$ and residues 445-460 of human cPLA$_2\alpha$ were generously donated by Dr. J. Clark, Genetics Institute (Cambridge, MA). A mouse monoclonal IgG raised against residues 1-216 of the amino terminal domain of human cPLA$_2\alpha$ was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). A rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the C-terminal residues, 734-749 of human cPLA$_2\alpha$ was purchased from Cell Signaling Technology (Beverly, MA). A protein assay kit was purchased from Pierce (Rockford, IL).

Nuclei Isolation and Immunoblotting

1 X 10^6 cells (subconfluent) were plated in 100 mm diameter tissue culture plates and allowed to adhere overnight. The following day, cells were treated as indicated. After treatment, Sigma Nuclei EZ Prep Nuclei Isolation Kit was used to obtain whole cell lysates

as well as nuclei preps according to the manufacturer's instructions. Briefly, after treatment, cells were washed with PBS, then 1ml or 2ml of lysis buffer was added to the cells. Cells were then scraped into a 15ml conical and vortexed, set on ice for 5 minutes and 300µl was collected as the whole cell lysate, which was either used immediately or stored at –70°C for later use. Nuclei were collected by centrifugation at 500 X g for 5 minutes. Supernatant represented the cytosolic fraction, which was either used immediately or stored at –70°C for later use. The pellet was resuspended in 2 ml of lysis buffer and centrifuged again to wash the nuclei. Following centrifugation, the nuclei pellet was resuspended in 200µl of storage buffer and used immediately or stored at –70°C for later use. For immunoblotting purposes, 8% Tris-glycine gels were loaded with on average ~15µg of protein and was subjected to SDS gel electrophoresis using the NOVEX system (San Diego, CA) for 1.5 hours at 30mA. After transfer to nitrocellulose membrane and blocking in a 3% powdered milk solution, the protein was probed with the indicated antiserum and the appropriate horseradish peroxidase conjugated secondary antibody. The bands were visualized using the SuperSignal chemiluminescence kit (Pierce, IL).

Antibody Mapping Analysis

Cells were plated at 1 X 10^6 24hr prior to harvesting in 100 mm diameter tissue culture plates. Cells were subjected to the EZ Nulei Isolation kit to obtain whole cell lysates and nuclei. The protein from the two cellular fractions was then subjected to SDS-PAGE and immunoblotting. The protein was detected using the panel of antibodies composed of rabbit polyclonal antisera raised against denatured human cPLA$_2\alpha$, residues 42-58 of human cPLA$_2\alpha$ and residues 445-460 of human cPLA$_2\alpha$, rabbit polyclonal antiserum raised against

a synthetic peptide corresponding to the C-terminal residues, 734-749 of human cPLA$_2\alpha$ and mouse monoclonal IgG raised against residues 1-216 of the amino terminal domain of human cPLA$_2\alpha$. For the detection of all antisera except the monoclonal antibody, a secondary horseradish peroxidase-coupled goat anti-rabbit antibody purchased from Sigma was used. For the detection of the monoclonal antibody a goat anti-mouse IgG purchased from Sigma was used. The banding pattern was visualized by chemiluminescence.

Comparative Analysis of Apoptotic cPLA$_2$a Fragmentation and Nuclear Proteins

Cells were plated at 1 X 10^6 24hr prior to harvesting in 100 mm diameter tissue culture plates. Cells were treated with TNF (10 ng/ml) and CHI (25mg/ml) for 6hr and the adherent and non-adherent cells were harvested separately and control nuclei were isolated using EZ Nulei Isolation kit. The protein was then subjected to SDS-PAGE and immunoblotting. The protein was detected using a rabbit polyclonal antiserum raised against denatured human cPLA$_2\alpha$. The banding pattern was visualized using chemiluminescence.

Half-life Assay

For the half-life assay, cells were plated at 1 X 10^6 24hr prior to harvesting in 100 mm diameter tissue culture plates. Cells were treated with CHI (25mg/ml) for 0hr, 6hr, 12hr and 24hr and nuclei and whole cell lysates were obtained using the EZ Nuclei Isolation Kit and subjected to SDS-PAGE and immunoblotting. The protein was detected using a rabbit polyclonal antiserum raised against denatured human cPLA$_2\alpha$. The banding pattern was visualized using chemiluminescence.

Protease Inhibitor Assays

For the protease inhibitor assays, cells were plated at 1 X 10^6 in 100 mm diameter

tissue culture plates or 0.3 X 10^6 in 60mm diameter tissue culture plates 24hr prior to harvesting. Cells were treated with the indicated protease inhibitor at the indicated concentration and length of time. Nuclei were isolated using the EZ Nuclei Isolation Kit and subjected to SDS-PAGE and immunoblotting. The protein was detected using a rabbit polyclonal antiserum raised against residues 42-58 of human cPLA$_2\alpha$. The banding pattern was visualized using chemiluminescence.

Results

Chapter I Characterization of Nuclear cPLA$_2$α

Detection of cPLA$_2$α in the Nuclear Fraction of C3HA Cells

In resting cells cPLA$_2$α is found free in the cytosol. The goal of these investigations is to identify an alternative subcellular localization of cPLA$_2$α in unstimulated cells. The method utilized to address this question is a biochemical fractionation coupled with western blot analysis to determine the presence of cPLA$_2$α in cytoplasmic and nuclear fractions. A polyclonal antiserum raised against residues, 42-58 was used for detection. Figure 1 shows the subcellular distribution of cPLA$_2$α in untreated C3HA cells. Lane A, which represents a whole cell lysate prepared using a commercially available kit designed for nuclear isolation demonstrates that the anti- cPLA$_2$α antiserum reacts with an approximately 97kDa protein, which was previously shown to comigrate with recombinant cPLA$_2$α.

To test whether the alternative subcellular localization of cPLA$_2$α is nuclear-associated, a commercially available kit manufactured by Sigma Co. was utilized to separate the cell components into cytosolic and nuclear fractions. This kit is known to retain the nuclear membrane as well as the endoplasmic reticulum. Lane B of Figure 1 shows the cytosolic fraction, whose banding pattern is similar to that of the whole cell lysate, showing the ~97kDa band. Lane C, the nuclear fraction, demonstrates a striking difference in the banding pattern of cPLA$_2$α. In the nuclear fraction, the polyclonal antiserum reacts with three highly immunoreactive proteins; a protein that is of a slightly higher molecular weight (~103kDa) than that of the full-length cPLA$_2$α of 97kDa, a ~78kDa and a ~70kDa protein.

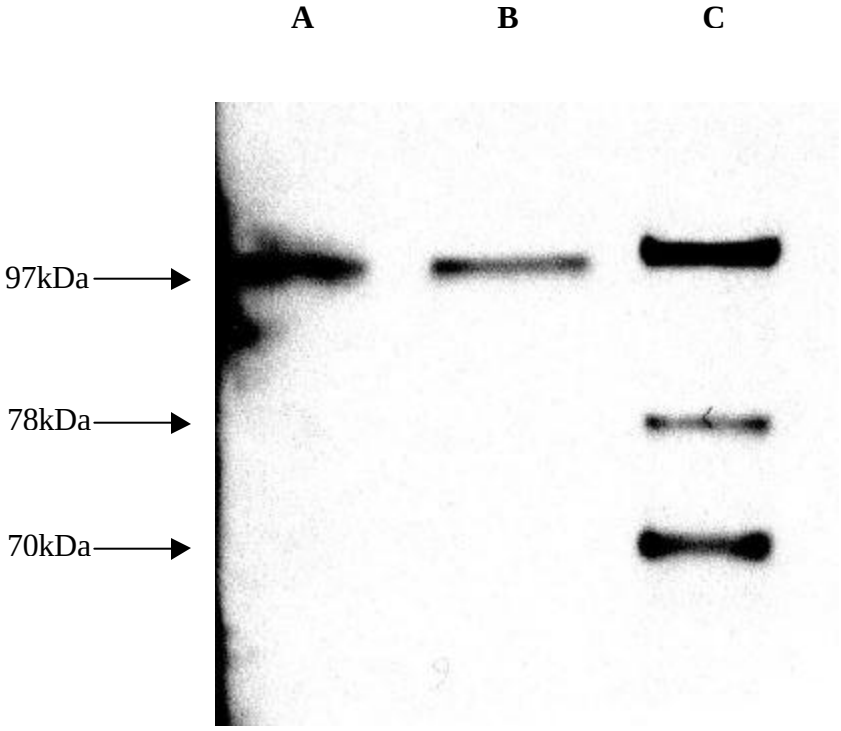

Figure 1. Subcellular distribution of $cPLA_2$ in nuclear and cytosolic fractions. 1 X 10^6 cells were plated 24hr ahead. Nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 16.5µg protein of each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lanes A represent whole cell lysate, B represent the cytosolic fraction and C represent the nuclei.

The strength of the reaction of the antiserum with these proteins suggests that they are likely different forms of cPLA$_2\alpha$. The failure to detect the lower molecular weight bands in the whole cell lysate may be due to the fact that the nuclear samples represent an approximately 5-fold enrichment over the whole cell lysate. Thus, these results demonstrate the presence of three distinct nuclear forms of cPLA$_2\alpha$. Furthermore, the 78kDa and the 70kDa nuclear forms may represent cPLA$_2\alpha$-derived fragments or alternative spliced transcripts.

Antibody Mapping of cPLA$_2$a in whole cell lysates of C3HA Cells

As mentioned above, the ~78kDa and the 70kDa proteins appear to be fragments of the full-length cPLA$_2\alpha$ molecule. Thus, a question that needs to be addressed is what portion of the full-length cPLA$_2\alpha$ is composing the nuclear forms. In order to address this question, a panel consisting a monoclonal antibody and four polyclonal antisera with varying recognition sites along the length of cPLA$_2\alpha$ was obtained :(1) A polyclonal antiserum raised against denatured cPLA$_2\alpha$ (2) A monoclonal antibody (mAb) raised against residues 1-216 (3) A polyclonal antiserum raised against residues 42-58 (4) A polyclonal antiserum raised against residues 445-460 and (5) A polyclonal antiserum raised against residues 734-749. These studies were performed to demonstrate that these 4 antisera and the mAb do indeed react with the full-length cPLA$_2\alpha$ protein in order to confirm that they are cPLA$_2\alpha$-specific and to ensure that subsequent analysis done with them would not give non-specific data. As shown in Figure 2, in whole cell lysates, all four polyclonal antisera and the mAb do react with a ~97kDa protein, the full-length cPLA$_2\alpha$ protein. Thus, these results indicate that they are

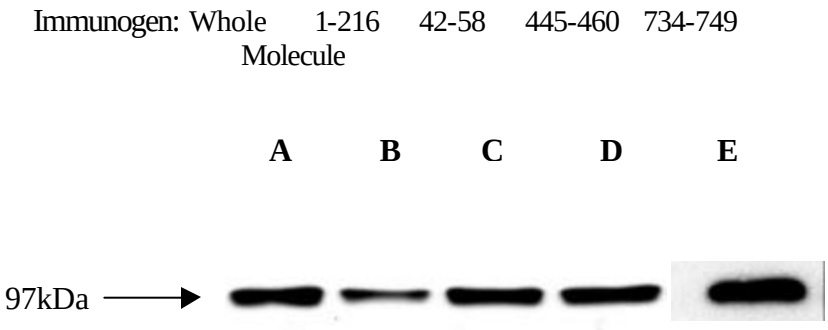

Figure 2. Antibody mapping of $cPLA_2$ in whole cell lysates. 1 X 10^6 cells were plated 24hr ahead. Each sample was resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using the above panel of four antisera and the mAb. Lanes A through E represent whole cell lysate from untreated cells.

cPLA$_2\alpha$-specific and can therefore be used for subsequent mapping analysis to address what portion of the full-length cPLA$_2\alpha$ is present within the nuclear forms.

Antibody Mapping of Nuclear-Associated cPLA$_2$a in C3HA Cells

Since the results from Figure 1 indicated that the nuclear forms may be fragments of cPLA$_2\alpha$, the following investigations were carried out in order to map the portion of the full-length cPLA$_2\alpha$ that is present in the nuclear forms using the above-mentioned four antisera and the mAb. Nuclei that were isolated using a commercially available kit were resolved by western blotting and detected using the aforementioned panel. As shown in Figure 3, a ~78kDa protein was detected by both the polyclonal antiserum raised against the whole molecule of cPLA$_2\alpha$ and the polyclonal antiserum raised against residues 42-58. However, a ~78kDa protein was not detected by the polyclonal antisera raised against residues 445-460 or residues 734-749, nor by the monoclonal antibody. Thus, these data indicate that the 78kDa protein may be an N-terminal fragment of the full-length cPLA$_2\alpha$. The antiserum raised against residues 42-58 detects a prominent protein at ~70kDa, which appears to migrate at the same molecular weight as the ~70kDa protein recognized by the polyclonal antiserum raised against denatured cPLA$_2\alpha$. This protein was not detected by the polyclonal antiserum raised against residues 445-460 or residues 734-749, nor by the monoclonal antibody. These results are indicative of the 70kDa also being an amino-terminal fragment of cPLA$_2\alpha$ sharing most of its residues with the 78kDa protein. In addition, full- length

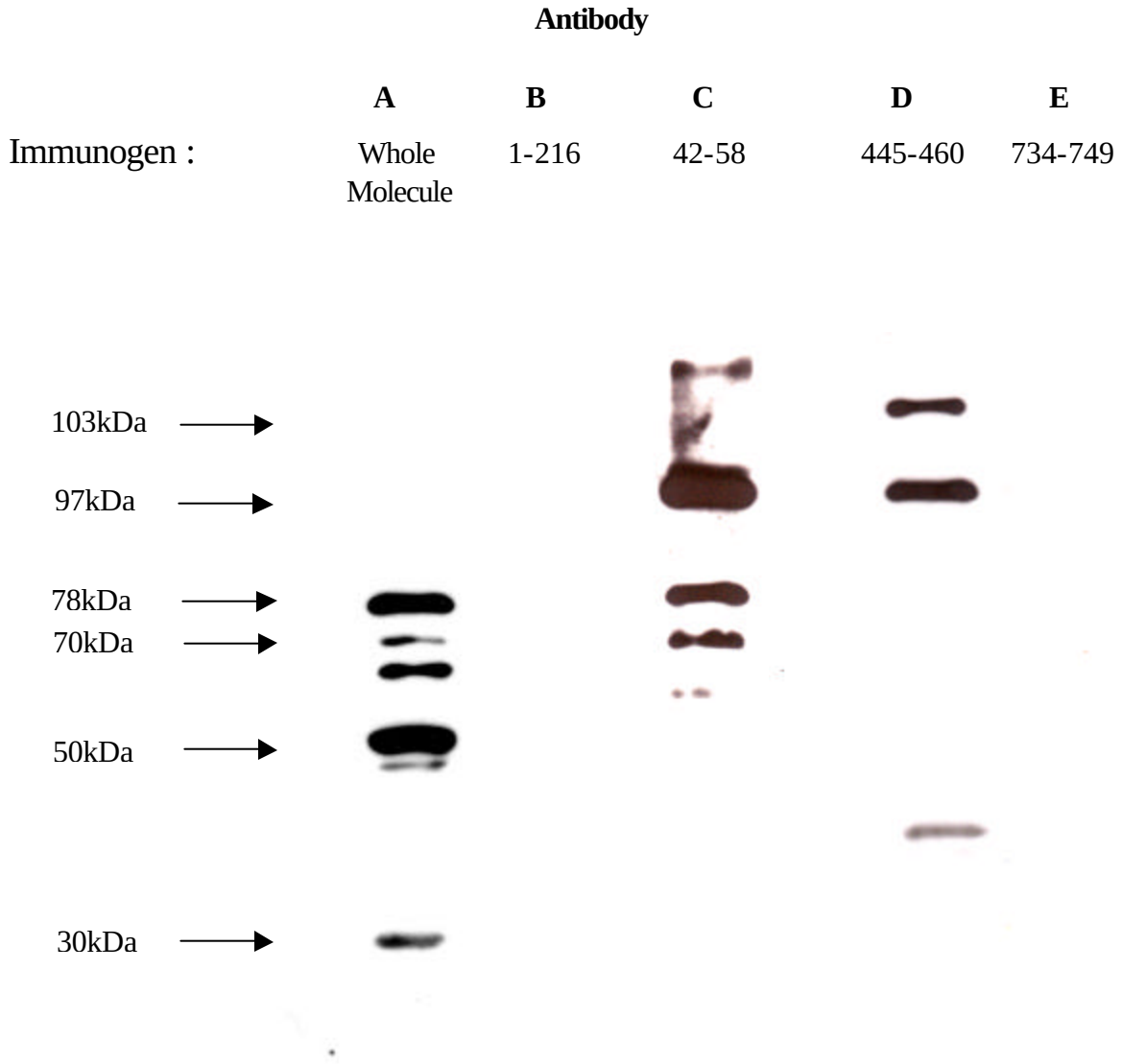

Figure 3. Ab mapping of $cPLA_2$ in nuclear fractions. 1 X 10^6 cells were plated 24hr ahead. Nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 25µg protein of each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using the above panel of Abs. Lanes A through E represent the nuclei from untreated cells.

$cPLA_2\alpha$ is only detected by the two polyclonal antisera raised against residues 42-58 and 445-460 and this may be due to the masking of the epitope recognized by the mAb and the two polyclonal antisera raised against the whole molecule and residues 734-749 by a modification such as phosphorylation or glycosylation . The polyclonal antiserum raised against denatured $cPLA_2\alpha$ also recognizes a number of smaller fragments that range in molecular weight from ~30kDa to ~60kDa, which may represent breakdown products of $cPLA_2\alpha$. Furthermore, both polyclonal antisera raised against residues 42-58 and 445-460 recognize nuclear bands that migrate at a higher molecular weight than the full-length $cPLA_2\alpha$, which migrates at ~97kDa.

Primary Sequence Information of $cPLA_2\alpha$ and Putative Map of the Nuclear Proteins

Figure 4 illustrates the integral elements of the primary sequence of $cPLA_2\alpha$ protein. The NH_2-terminal houses the CaLB/C2 domain comprising about 130 amino acids (27). Residues $GLS^{228}GS$ aligns with the lipase consensus sequence and is centered around Ser-228, the catalytic center of $cPLA_2\alpha$ (2,31). The three residues that appear to be important for the catalytic mechanism of $cPLA_2\alpha$ are Ser-228, Asp-549 and Arg-200 (2,26). $cPLA_2\alpha$ is phosphorylated mainly on four serine residues, namely, Ser-437, Ser-454, Ser-505 and Ser-727 (10,37,38). In addition, Figure 4 shows the recognition sites of the 4 antisera and 1 mAb that were utilized for the mapping experiments. The polyclonal antiserum raised against denatured $cPLA_2\alpha$ is depicted as “Polyclonal”, the monoclonal antibody raised against residues 1-216 is depicted as “monoclonal”, the polyclonal antiserum raised against residues 42-58 is depicted as “ Ab 42-58”, the polyclonal antiserum raised against residues

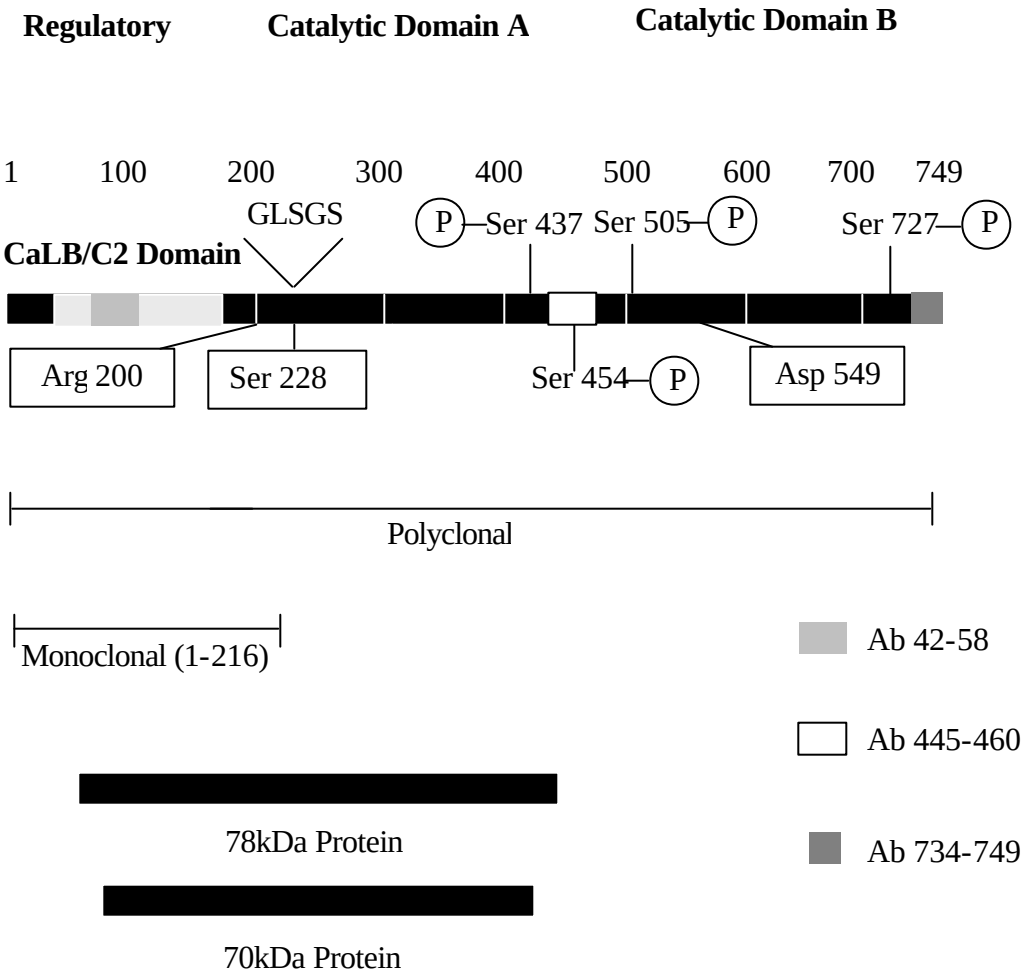

Figure 4. Schematic of Ab mapping data and 1^{o} sequence information of $cPLA_2$.

445- 460 is depicted as " □ Ab 445-460" , and the polyclonal antiserum raised against residues 734-749 is depicted as " ■ Ab 734-749." Finally, as shown in Figure 4, the putative map of the ~78kDa protein as deduced by the results of Figure 3 is a fragment that potentially extends from residue 42 to residue 444 and the putative map of the second nuclear protein (70kDa) is also depicted as an amino terminal fragment that is shorter in length than the 78kDa protein but one that shares most of its residues with the 78kDa protein.

Comparison of cPLA$_2$a Fragmentation in Apoptotic Cells with the Nuclear Proteins

cPLA$_2\alpha$ is known to be cleaved by caspase-3 during TNF-induced apoptosis producing a fragment of ~70kDa. The TNF-resistant murine fibroblast cell line, C3HA, can be sensitized to the cytotoxic effects of TNF by treatment with the translational inhibitor cycloheximide (CHI). The goal of these studies is to determine whether the caspase-dependent cleavage product of cPLA$_2\alpha$ migrates at the same molecular weight as the nuclear proteins.

As shown above, Figure 3 established that the polyclonal antiserum raised against denatured cPLA$_2\alpha$ also detects 78kDa and 70kDa nuclear species, which appear to comigrate with those recognized by the polyclonal antiserum raised against residues 42-58. Furthermore, the fragmentation of cPLA$_2\alpha$ during TNF-induced apoptosis has been established using the polyclonal antiserum raised against denatured cPLA$_2\alpha$ (85). Therefore, it was used for detection purposes in these studies as an appropriate substitute for the antiserum raised against residues 42-58. C3HA cells were treated with TNF (10 ng/ml) and CHI (25mg/ml) for 6hr and the adherent and non-adherent cells were harvested separately and nuclei were isolated using a commercially available kit.

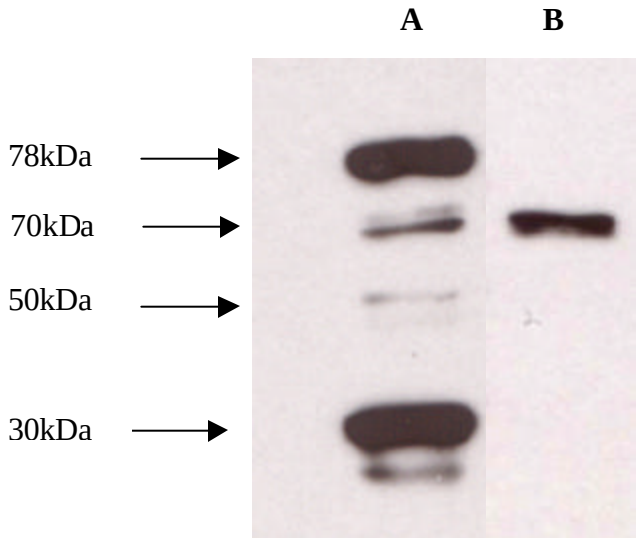

Figure 5. Subcellular distribution of $cPLA_2$ following treatment with TNF and CHI in apoptotic cells. 1 X 10^6 cells were plated 24hr ahead. Nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 31.8µg protein of each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against denatured $cPLA_2$. Lane A represents the untreated nuclei. Lane B represents whole cell lysate from adherent cells treated for 6hr.

As shown in Figure 5, the polyclonal antiserum raised against denatured cPLA$_2\alpha$ detects a 78kDa protein, a 70kDa protein, a 50kDa protein and a 30kDa protein in the untreated nuclear fraction. In the adherent cell fraction representing the apoptotic cells, this antiserum reacts with a ~70kDa protein distinct in molecular weight from the nuclear 78kDa protein but appearing to migrate at the same molecular weight as the 70kDa nuclear protein in the control nuclear fraction. Thus, these results indicate that the fragment of cPLA$_2\alpha$ generated during apoptosis is of a different molecular weight than the nuclear 78kDa protein but it may share its identity with the nuclear protein of 70kDa.

Determination of the half-life of cPLA$_2$a in CHI treated Cells

The goal of these studies was to determine the half-life of cPLA$_2\alpha$ to gain some insight into the mechanism of generation of the 78kDa protein. Learning about the half-life of cPLA$_2\alpha$ would allow us to establish the breakdown pattern of cPLA$_2\alpha$, which in turn would give information about the generation of the lower molecular weight nuclear species derived from the full-length protein. For the purpose of these studies, C3HA cells were treated with the translational inhibitor, CHI, cycloheximide (25mg/ml) and nuclei were isolated using a commercially available kit manufactured by Sigma Co. The polyclonal antiserum raised against denatured cPLA$_2\alpha$ was used for detection since it detects the 78kDa and the 70kDa nuclear species of interest. As shown in Figure 6, over a period of 24hr, with time points at 0hr, 6hr, 12hr and 24hr, in a whole cell lysate the polyclonal antiserum raised against denatured cPLA$_2\alpha$ detects a decrease in intensity of the 97kDa, full-length protein and an increase in intensity of the 78kDa protein, suggesting a diminishing of the quantity of

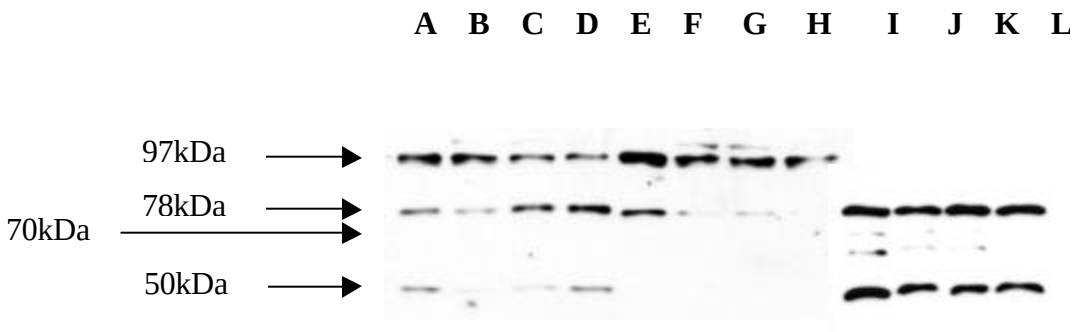

Figure 6. Half-life of $cPLA_2$ in CHI treated cells. 1 X 10^6 cells were plated 24hr ahead. Nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using an anti-$cPLA_2$ antiserum raised against denatured $cPLA_2$. Lanes A through D represent the whole cell lysate, Lanes E through H represent the cytosolic fraction and Lanes I through L represent the nuclei. Lanes A, E and I are from control cells. Lanes B, F, and J are from cells treated for 6hr. Lanes C, G, and K are from cells treated for 12hr. Lanes D, H, and L are from cells treated for 24hr.

the full-length cPLA$_2\alpha$ and an accompanied accumulation of the 78kDa protein. In the cytosolic fraction, the antiserum detects a gradual decrease in intensity of both the 97kDa and the 78kDa proteins while in the nuclear fraction the antiserum detects no apparent change in the intensity of the 78kDa protein. Furthermore, a strongly immunoreactive protein migrating at around 50kDa in the nuclear fraction also exhibits no apparent change in intensity as indicated by the polyclonal antiserum reaction. In addition three proteins in the nuclear fraction migrating at 50kDa, 60kDa and 70kDa also appear to decrease in intensity suggesting a gradual decrease in their cellular content.

Chapter II Proteolysis Investigation

Visualization of the Effects of a General Cysteine Protease Inhibitor on the Generation of the 78kDa and the 70kDa Nuclear Proteins

The goal of these studies was to determine whether a proteolytic cleavage event mediated by a cysteine protease leads to the generation of the 78kDa and the 70kDa nuclear proteins. Hence, C3HA cells were treated with the general cysteine protease inhibitor, E64 (10μM) for a period of 24hr. Nuclei were isolated using a commercial kit manufactured by Sigma Co. An anti- cPLA$_2\alpha$ antiserum raised against residues 42-58 was used for detection. As shown in Figure 7, Lane A represents the control nuclear fraction and Lane B represents the treated nuclear fraction and the antiserum reacts with the 78kDa protein from the treated and untreated fraction with the same intensity. Similarly, this antiserum also detects the 97kDa, full-length cPLA$_2\alpha$ and the70kDa protein at the same intensity in both the control and treated samples. Thus, the antiserum reactions indicate no apparent differences in the control versus treated samples, suggesting that a cysteine protease may not be involved in the generation of either of the two nuclear proteins.

Visualization of the Effects of a General Cathepsin Protease Inhibitor on the Generation of the 78kDa and the 70kDa Nuclear Proteins

Foghsgaard et al. (2002) showed that cPLA$_2\alpha$ appears to undergo cleavage by cathepsin B. Thus, the goal of these studies was to determine whether a proteolytic cleavage event mediated by a cathepsin protease leads to the generation of the two nuclear proteins.

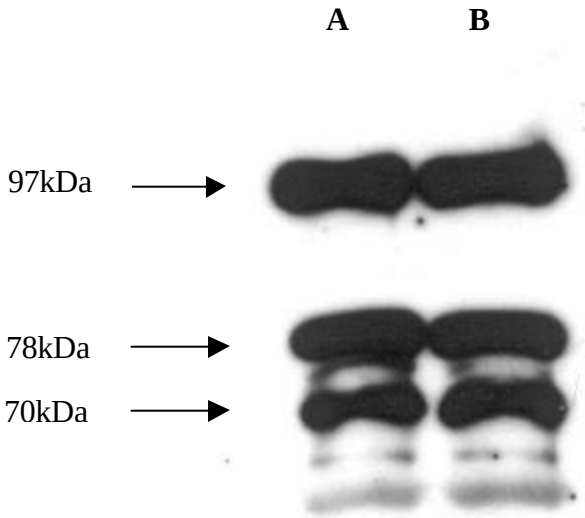

Figure 7. Subcellular distribution of $cPLA_2$ in nuclear fractions following treatment with a general cysteine protease inhibitor, E64. 1 X 10^6 cells were either treated with E64 for 24hours at a final concentration of 10μM or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 23.7μg of protein each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lane A represents nuclei from untreated control cells. Lane B represents nuclei from treated cells.

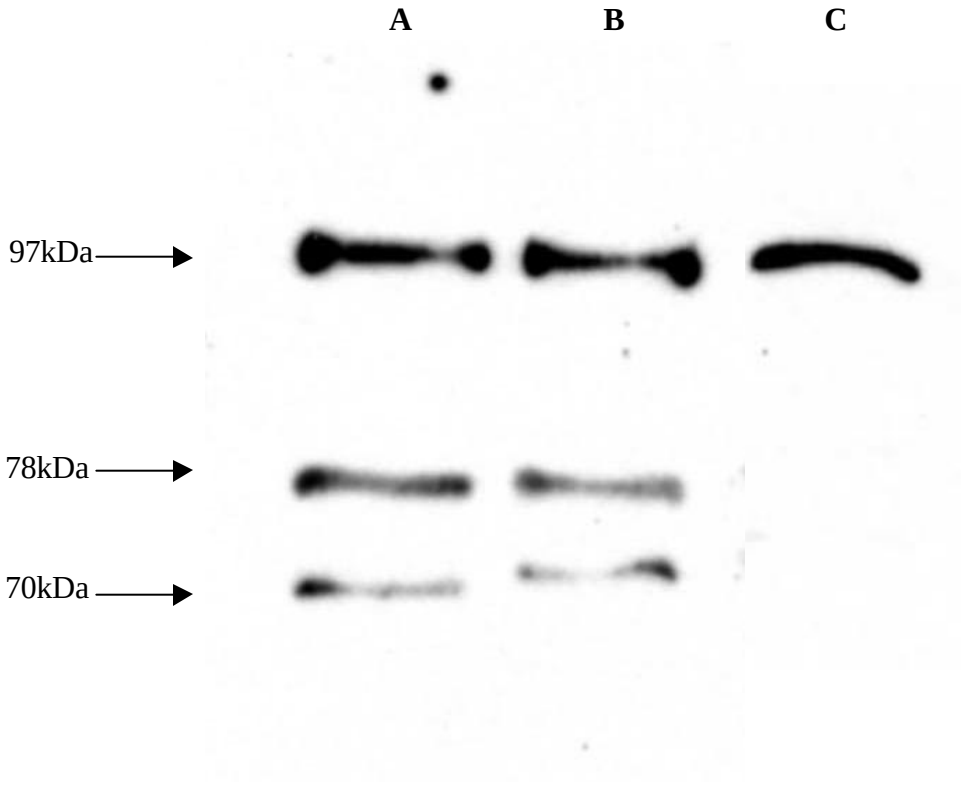

Figure 8. Subcellular distribution of $cPLA_2$ in nuclear fractions following treatment with the general cathepsin protease inhibitor, Z-FMK. 1 X 10^6 cells were either treated with Z-FMK for 24hours at a final concentration of 80µM or 160µM or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 37.5µg of protein each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lane A represents nuclei from untreated control cells. Lane B represents nuclei from cells treated with 80µM Z-FMK. Lane C represents nuclei from cells treated with 160µM Z-FMK.

For this purpose, C3HA cells were treated with the general cathepin protease inhibitor Z-FMK (80μM or 160μM) for a period of 24hr. Nuclei were isolated using a commercial kit manufactured by Sigma Co. An anti- cPLA$_2$α antiserum raised against residues 42-58 was used for detection. As shown in Figure 8, the anti- cPLA$_2$α antiserum reacts with the 97kDa protein in both the control and treated (80μM and 160μM) lanes, and it also detects the 78kDa fragment in the control and the 80μM Z-FMK treated lane. However, the antiserun fails to detect the 78kDa protein in the 160μM Z-FMK treated lane. In addition, the antiserum detects the 70kDa protein within the control and the 80μM Z-FMK treated lanes but not in the 160μM Z-FMK treated lane. These results indicate that a cathepsin protease may be directly or indirectly involved in the generation of both the 78kDa and the 70kDa nuclear proteins; that is a cathepsin protease may be directly involved in the cleavage of the full-length cPLA$_2$α or be involved in the activation/inactivation of another protein responsible for the cleavage of the full-length protein.

Visualization of the Effects of Caspase Inhibitors on the Generation of the 78kDa and 70kDa Nuclear Proteins

It has been demonstrated by a number of investigators that cPLA$_2$α undergoes cleavage by the apoptosis activator proteins, caspases. Adam-Klages et al. (77) showed that cPLA$_2$α is cleaved by caspase-3 and later Luschen et al. (78) reported that cPLA$_2$α can also undergo cleavage by caspase-1 and caspase-8. Hence, the goal of these investigations was to determine whether a member of the caspase family mediates the generation of the two nuclear proteins. Thus, C3HA cells were treated with two caspase inhibitors Ac-YVAD-

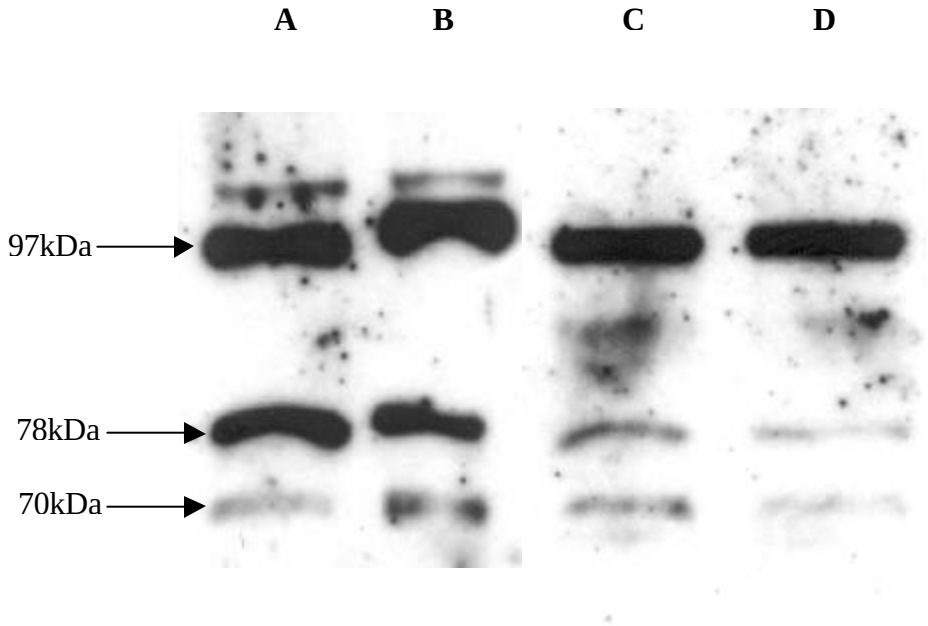

Figure 9. Subcellular distribution of $cPLA_2$ in nuclear fractions following treatment with two caspase inhibitors; caspase-1 inhibitor: Ac-YVAD-CMK and caspase-3 inhibitor: Z-DEVD-FMK. 0.3 X 10^6 cells were either treated with caspase inhibitors, Ac-YVAD-CMK and Z-DEVD-FMK for 16hours at a final concentration of 200µM or 100µM respectively or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and each sample was resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lane A and C represent nuclei from untreated control cells. Lane B represents nuclei from cells treated with Ac-YVAD-CMK. Lane D represents nuclei from cells treated with Z-DEVD-FMK.

CMK (200μM), a caspase-1 inhibitor and Z-DEVD-FMK (100μM), a caspase-3 inhibitor. Nuclei were isolated using a commercial kit manufactured by Sigma Co. An anti- cPLA$_2\alpha$ antiserum raised against residues 42-58 was used for detection. As shown in Figure 9, in the untreated controls, Lanes A and C, the antiserum reacts with 97kDa, 78kDa and 70kDa proteins. The banding pattern in the nuclear fraction treated with Ac-YVAD-CMK, the caspase-1 inhibitor is not strikingly different from that of the control lanes. However, in the nuclear fraction treated with Z-DEVD-FMK, the caspase-3 inhibitor, the antiserum reaction with the 78kDa and 70kDa proteins is weaker than that in the untreated control fraction, thus suggesting the abundance of these species is reduced. One notable observation is that in the nuclear fraction treated with Ac-YVAD-CMK there is a band that migrates at a slightly higher molecular weight than the 97kDa, the putative full-length cPLA$_2\alpha$. Thus, these results may suggest a role for caspase-3 in the generation of the 78kDa and the 70kDa proteins.

Visualization of the Effects of a Serine Protease Inhibitor on the Generation of the 78kDa and the 70kDa Nuclear Proteins

Tosyl-argenine methyl ester (TAME) has been shown to have a protective effect on cells from the cytotoxicity caused by TNF (80,81). TNF induced cytotoxicity is known to be mediated by cPLA$_2\alpha$ (76), which may indicate that cPLA$_2\alpha$ can undergo proteolytic cleavage by a serine protease. Thus, the goal of these investigations was to determine whether a serine protease has an effect on the generation of either of the two nuclear proteins. Hence, C3HA cells were treated with TAME (10mM and 20mM) for 6hr. Nuclei were isolated using a commercial kit manufactured by Sigma Co. An anti- cPLA$_2\alpha$ antiserum raised against

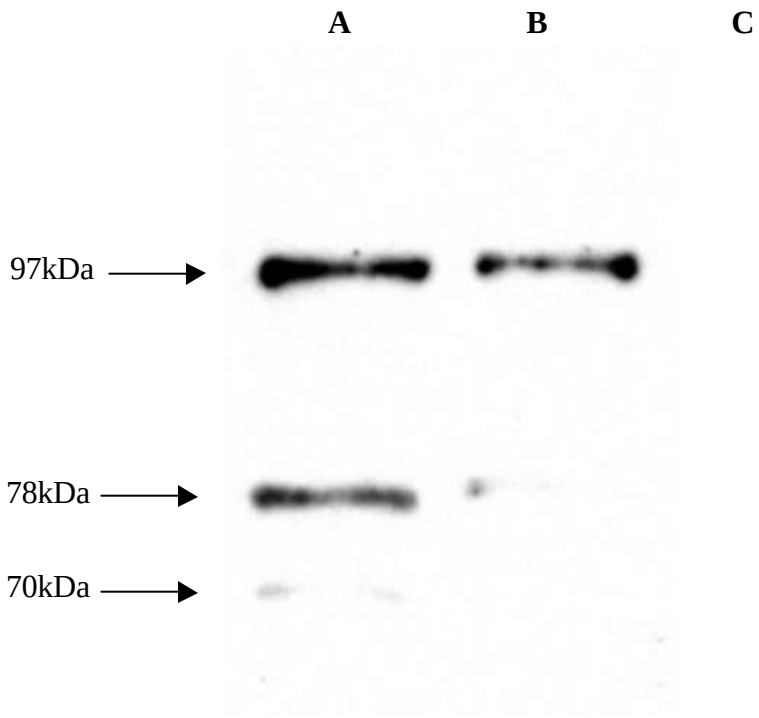

Figure 10. $cPLA_2$ in nuclear fractions following treatment with the serine protease inhibitor, TAME. 1 X 10^6 cells were either treated with TAME for 6hours at a final concentration of 10mM or 20mM or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 26.2µg of protein each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lane A represents nuclei from untreated control cells. Lane B represents nuclei from cells treated with 10mM TAME. Lane C represents nuclei from cells treated with 20mM TAME.

residues 42-58 was used for detection. As shown in Figure 10, in the control lane the antiserum reacts with 97kDa, 78kDa and 70kDa proteins. In the 10mM TAME treated lane, the antiserum reacts with the 97kDa protein, however the antiserum reaction with the 78kDa protein is distinctly weak showing a band of low intensity, while the 70kDa band is not detected at all. Finally, in the 20mM TAME treated lane the antiserum fails to react with the 97kDa, 78kDa and the 70kDa protein. These results indicate that a serine protease may not only be involved in the generation of the 78kDa and the 70kDa proteins but may also be involved in either the generation of the full-length cPLA$_2\alpha$ or nuclear-localization of the protein.

Visualization of the Effects of a Proteasome Inhibitor on the Generation of the 78kDa and the 70kDa Nuclear Proteins

Cytoplasmic protease systems such as the proteasome have been reported as being of central importance to the regulation of intracellular activities such as programmed cell death, protein kinase activities and cell-cycle progression (83). The proteasome is composed of a catalytic 20S core that is involved in the degradation of cellular proteins and antigen processing. Many cellular proteins are known to associate with the proteasome (82). Thus, the goal of these investigations is to determine whether proteolysis of cPLA$_2\alpha$ by the proteasome is involved in the generation of the 78kDa and the 70kDa proteins. C3HA cells were treated with the proteasome inhibitor, MG132 (20μM) for 4hr. Nuclei were isolated using a commercial kit manufactured by Sigma Co. An anti- cPLA$_2\alpha$ antiserum raised against residues 42-58 was used for detection. As shown in Figure 11, the pattern of protein species detected by the antiserum reaction in the control lane is strikingly similar to that of

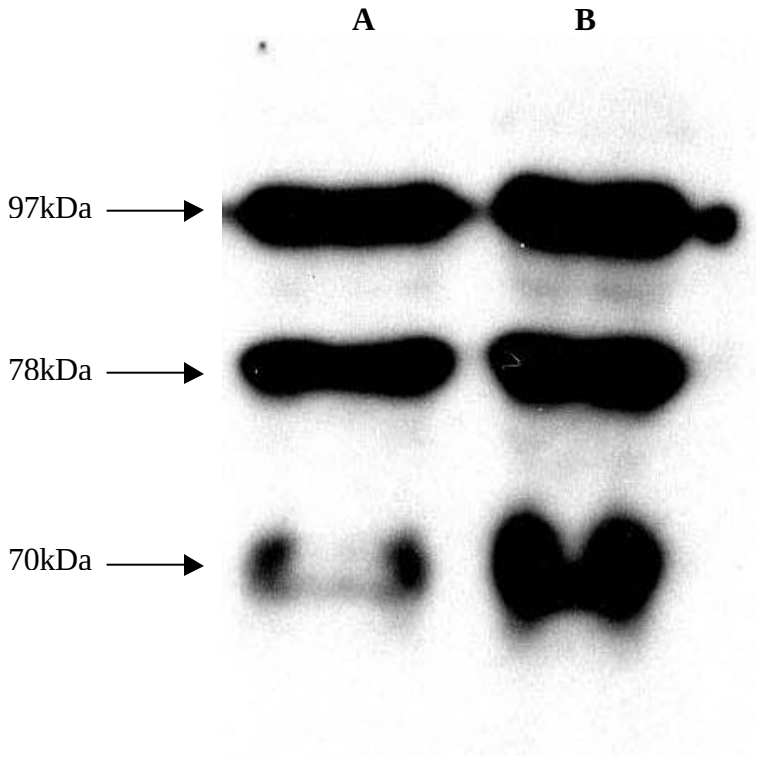

Figure 11. $cPLA_2$ in nuclear fractions following treatment with proteosome inhibitor, MG132. 1 X 10^6 cells were either treated with MG132 for 4 hours at a final concentration of 20μM or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 16μg protein of each sample were resolved by SDS PAGE, transferred to a nitrocellulose membrane and immunodetected using anti-$cPLA_2$ antiserum raised against residues 42-58. Lane A represents nuclei from untreated control cells. Lane B represents nuclei from MG132 treated cells.

the treated lane suggesting the lack of a proteasomal effect on the generation of the two nuclear proteins.

Discussion

The purpose of these investigations was to identify and characterize a possible secondary form of cPLA$_2\alpha$ selectively present in the nuclear fraction. The characterization of a secondary form of cPLA$_2\alpha$ would lend immensely to the elucidation of the activation path of the protein. Gaining a better understanding of the mechanism of activation of cPLA$_2\alpha$ can then lead to the development of compounds that could act as inhibitors of cPLA$_2\alpha$ activation and therefore as novel therapeutic agents in the treatment of inflammatory diseases. The scope of our studies encompassed two major questions: (1) what portion of the full-length cPLA$_2\alpha$ is forming the possible secondary form? (2) what is the mechanism of generation of the secondary form? We utilized a biochemical fractionation coupled with western blot analysis as the basis for addressing both questions; furthermore, the antisera utilized within the study were selective for cPLA$_2\alpha$ to remove the possibility of generating false data. A means of establishing selectivity would be to analyze the pre-immune serum and the post-immune serum to ensure that the cPLA$_2\alpha$-specific antibodies are only present in the post-immune serum. In particular, to answer the first question a panel of antisera with varying recognition sites along the length of the cPLA$_2\alpha$ protein was utilized to create a putative map of the secondary form. The second question was addressed by employing a group of protease inhibitors that targeted several major classes of proteases to selectively inhibit each class independently to assess their effect on the generation of the secondary form. Thus, using biochemical fractionation and immunoblotting of cellular fractions we have demonstrated that cPLA$_2\alpha$, as expected is a predominantly cytosolic species in unstimulated cells.

Remarkably, in the nuclear fraction of unstimulated cells, we have demonstrated the presence of several highly immunoreactive nuclear species of cPLA$_2\alpha$ with electrophoretic mobilities of ~103kDa ~78kDa, 70kDa that are consistently present in the nuclear fractions. Three other proteins seen inconsistently within nuclear fractions are a 30kDa, a 50kDa and a 60kDa protein. Their inconsistent presence may indicate that they are breakdown products of cPLA$_2\alpha$. To date, several other investigators have reported the presence of a number of lower molecular weight forms of the full-length cPLA$_2\alpha$. Nalefski et al. (28) and Roshak et al. (74) have identified the presence of several immunoreactive bands at ~70kDa, ~50kDa and between 50kDa and 60kDa within cytosolic fractions. Our results are consistent in particular with reports by Sierra-Honigmann who demonstrated the presence of a lower molecular weight nuclear form of about 70kDa within subconfluent cell extracts (75). The strong intensity of the antiserum reaction with the nuclear proteins we have observed warrants the conclusion that they are indeed three distinct forms of nuclear cPLA$_2\alpha$. A key question raised by these results is the portion of the full-length cPLA$_2\alpha$ that is present within the nuclear forms.

To generate a putative map of the nuclear proteins we subjected the nuclear fraction of a whole cell lysate to immunoblotting with a panel of antisera with varying recognition sites along the length of the full-length cPLA$_2\alpha$ protein. Consequently, it was observed that the 78kDa and the 70kDa nuclear proteins were detected by the antiserum raised against denatured full-length cPLA$_2\alpha$ and the antiserum raised against residues 42-58, but not by the monoclonal antibody raised against residues 1-216, nor by the antisera raised against residues

445-460 or residues 734-749. This suggests that the two nuclear forms are amino terminal fragments of the full-length cPLA$_2\alpha$. The 78kDa nuclear-associated protein may be composed of residues spanning from at the very extremes 42 to 444, while the 70kDa may share majority of its residues with the 78kDa protein. The failure of the monoclonal antibody raised against residues 1-216 to detect the two nuclear proteins may be reasoned by the following rationalizations: (1) The epitope of the antibody raised against residues 1-216 may not be contained within either of the two nuclear proteins and (2) The epitope of the antibody raised against residues 1-216 may be masked by the three-dimensional conformation of the two nuclear proteins. Hence, in conclusion, these results are suggestive of both the 78kDa and 70kDa nuclear-associated proteins being amino-terminal fragments of cPLA$_2\alpha$, where both of which may extend no further than residue 444. While, the mapping doesn't give much insight to the residue composition of the 103kDa nuclear protein, it is logical to speculate that it is a modified version of the full-length cPLA$_2\alpha$ that may contain modifications such as phosphorylation, glycosylation or acylation and future studies need to be carried out to shed light onto the identity of this nuclear species.

cPLA$_2\alpha$ is known to be cleaved by caspase-3 during TNF-induced apoptosis. Thus, to further clarify the identity of the nuclear-associated proteins we investigated the possibility that the caspase-derived apoptotic cPLA$_2\alpha$ fragment is of the same molecular weight as either of the two nuclear-associated proteins (78kDa and 70kDa). Immunoblotting of TNF-treated and untreated nuclear fractions using an antiserum raised against denatured cPLA$_2\alpha$ that detects both the 78kDa and the 70kDa nuclear proteins of interest revealed that the

fragment of cPLA$_2\alpha$ generated during apoptosis is of a different molecular weight than the nuclear 78kDa protein and the difference in migration in SDS-PAGE between the two species may be due to two possible explanations: (1) The two species are in fact two different proteins or (2) The migration difference is due to an SDS-resistant conformational difference between the two species. Hence, further investigations are required to shed light onto the specific identity of this nuclear-associated protein. Such further investigations could consist of obtaining the primary sequence information of the purified nuclear form. However, the 70kDa band detected in the apoptotic fraction appears to migrate at the same molecular weight as the 70kDa nuclear protein. Thus, these results indicate that the nuclear 70kDa band may share its identity with the fragment of cPLA$_2\alpha$ generated during apoptosis.

Another facet we inquired into was the half-life of cPLA$_2\alpha$ in order to establish the breakdown pattern of the protein, which would then give us information about the generation of the lower molecular weight nuclear species derived from the full-length cPLA$_2\alpha$ protein. We used cellular fractions from cells treated with cycloheximide (CHI), a translational inhibitor, for immunoblotting purposes using an antiserum raised against denatured cPLA$_2\alpha$ that reacts with both the 78kDa and the 70kDa nuclear proteins of interest. The cells were treated over a period of 24 hours with time points at 0hr, 6hr, 12hr and 24hr. We observed a decrease in the cellular content of the full-length, 97kDa cPLA$_2\alpha$, which was accompanied by an increase in the quantity of the 78kDa protein. This is suggestive of a pathway where the 78kDa protein is derived from the 97kDa cPLA$_2\alpha$ instead of it being independently expressed by a process such as an alternative splicing event.

Through the course of our investigation one fundamental question we were inquiring about is the precise mechanism of generation of the nuclear-associated proteins. For this purpose a panel of protease inhibitors that targeted several major classes of proteases was employed to selectively inhibit the action of one class without impinging on the effects of another. The protease inhibitor assays have provided us with some very compelling results. The general cysteine protease inhibitor, the caspase-1 inhibitor (Ac-YVAD-CMK) and the proteasome inhibitor gave us negative, but nonetheless informative results. They each demonstrated that at the level of sensitivity we were testing, each inhibitor appeared to not be directly or indirectly involved in the generation of the 78kDa and the 70kDa proteins. However, the general cathepsin protease inhibitor, the caspase-3 inhibitor (Z-DEVD_FMK) and the serine protease inhibitor all demonstrated some very interesting activity.

Treatment with the general cathepsin protease inhibitor revealed that the generation of the 78kDa and the 70kDa nuclear-associated proteins is selectively inhibited in cells treated with the inhibitor at a final concentration of 160μM. However, the presence of the full-length, 97kDa species was not affected. Thus, these results indicate that the generation of the two nuclear proteins is dependent upon a cathepsin protease, where a cathepsin protease may be directly involved in the cleavage of the full-length cPLA$_2\alpha$ or be involved in the activation/inactivation of another protein responsible for the cleavage of the full-length protein. In addition, treatment with the caspase-3 inhibitor, Z-DEVD-FMK demonstrated a similar effect on the generation of the 78kDa and the 70kDa proteins. In cells treated with 100μM Z-DEVD-FMK, the cellular content of the 78kDa and 70kDa proteins was noticeably

reduced than in the untreated cells. Conversely, the 97kDa protein appeared unaffected by the treatment. Hence, it appears that caspase-3 may also be involved directly or indirectly in the pathway leading to the generation of the 78kDa and the 70kDa nuclear proteins. Finally, treatment with the serine protease inhibitor provided by far the most compelling results. In cells treated with 10mM TAME it was observed that the generation of the 78kDa was severely affected as evidenced by the presence of a band of extremely low intensity while the generation of the 70kDa protein appeared to be strongly inhibited. Remarkably, in cells treated with 20mM TAME, the 97kDa, the 78kDa and the 70kDa proteins were all distinctly absent, which is strongly suggestive of a role for a serine protease in the generation of not only the two nuclear-associated proteins but also in either the generation of the full-length $cPLA_2\alpha$ protein or the nuclear localization of the protein. To determine whether a serine protease is involved in the generation of the full-length- $cPLA_2\alpha$, western blotting of treated whole cell lysate can be performed to observe the presence of the protein, if it is indeed lacking in a treated whole cell lysate, this would indicate a probable role for a serine protease in the generation of the full- $cPLA_2\alpha$ protein.

Our results open up many avenues of study for future investigations. Although compelling, the results uncovered by these investigations have lead to many more unanswered questions. Future investigations can focus on isolating purified 78kDa and the 70kDa nuclear-associated proteins and subjecting them to sequence analysis to obtain the primary sequence information. This would directly answer the question of the residues that make up the nuclear-associated forms. Furthermore, in vitro cleavage of a recombinant

97kDa protein with proteases of interest can be performed to directly observe their individual roles in the generation of the two nuclear proteins. An inquiry that was beyond the scope of our investigation is the physiological function of the nuclear-associated proteins. A possible strategy to investigate the function of the 78kDa/70kDa proteins is expressing ectopic 78kDa/70kDa proteins independently in a cell line that does not express full-length cPLA$_2\alpha$ such as MCF-7S1 and such a cell line can then be radiolabeled and subjected to arachidonic acid-release assays to determine if either of the two nuclear-associated forms is indeed the active form with the phospholipase activity. Inquiring about the catalytic activity of nuclear cPLA$_2\alpha$ is of great importance; if the nuclear form is indeed the active form of the protein, then learning of the specific ligands that activate this enzyme would open new means of inhibiting its functions. Furthermore, if the generation of the nuclear form is in fact a step in the natural processing of the full-length cPLA$_2\alpha$, then inhibiting the breakdown of cPLA$_2\alpha$ would prevent its synthesis via feedback inhibition. Thus, there are many areas of study open to investigation and although the scope of our investigations left many unanswered questions, the data suggest that the 78kDa and 70kDa nuclear proteins may be fragments derived from full-length cPLA$_2\alpha$ and that they are both N-terminal fragments. The 78kDa protein may be potentially composed of residues 42 to 444, while the 70kDa protein may share most of its residues with the 78kDa protein. Furthermore, we provided some preliminary evidence that indicates the generation of the 78kDa and the 70kDa proteins may be due to a proteolytic cleavage event that is mediated by the concerted action of a cathepsin protease, a serine protease and caspase-3. cPLA$_2\alpha$ plays a vital role in the pathophysiology of innumerable

inflammatory disorders, hence gaining an in depth understanding of the mechanism of activation of $cPLA_2\alpha$ is critical in the quest for developing therapeutic agents for the treatment of inflammatory and degenerative diseases.

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