

## ABSTRACT

**HARRIS, VIRGINIA GAIL** The Intracellular Targeting of cPLA<sub>2</sub>. (Under the direction of Scott M. Laster). Tumor necrosis factor- $\alpha$  (TNF) is an inflammatory cytokine that can induce apoptosis in virus-infected cells, susceptible tumor cells, and cells whose transcriptional or translational processes have been disrupted. The apoptotic pathway that is activated by TNF is dependent on the activity of the enzyme cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). This enzyme cleaves arachidonic acid from membrane phospholipid, which in turn causes mitochondrial dysfunction leading to cell death. The goal of this research project was to identify the specific intracellular membrane to which cPLA<sub>2</sub> binds during TNF-induced apoptosis. The membrane site for cPLA<sub>2</sub> interaction during apoptosis would be a possible target for anti-apoptotic drug development. Three methods were tested in an attempt to identify the membrane location, including; immunofluorescence, biochemical fractionation, and GC/MS. Our results with immunofluorescence suggested that cPLA<sub>2</sub> translocates to the nuclear membrane during apoptosis. The identity of the nuclear membrane was confirmed by staining with an antibody to the nuclear pore complex. In addition, cPLA<sub>2</sub> staining was noted within the nucleus, perhaps indicating an interaction with chromatin, and small areas of punctate staining were noted in the perinuclear region. Biochemical fractionation indicated that the association of cPLA<sub>2</sub> with the nuclear membrane was calcium-dependent since this association could not be stabilized in the absence of calcium. GC/MS, which was used in an attempt to find amputated phospholipids remaining in membranes as a result of cPLA<sub>2</sub> activity, instead revealed higher levels of arachidonic acid suggesting that cells may

increase the synthesis or repair of arachidonic acid containing membranes following the activation of cPLA<sub>2</sub>. Finally, our results revealed several novel, lower molecular weight forms of cPLA<sub>2</sub> that were associated with nuclei in a calcium-independent fashion. Taken together these results suggest that it will be important to understand the mechanisms controlling the association of cPLA<sub>2</sub> with nuclei.

**THE INTRACELLULAR TARGETING OF cPLA<sub>2</sub>**

by  
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Chair of Advisory Committee

## **DEDICATION**

I dedicate this work to my grandfather, who not only inspired me to pursue my education, but has also made it financially possible. He is the most amazing person I have ever met, and I have learned so much from him.

## **BIOGRAPHY**

Virginia Gail Harris was born in November of 1977 in Richmond, Virginia to Gary and Jean Harris. She lived in Midlothian, Virginia until 1990, when she moved to Asheboro, North Carolina. In 1995 Virginia graduated from Asheboro High School. In 1999 she graduated from North Carolina State University with a Bachelor of Science in Animal Science. After graduating, Virginia worked at a horse farm in Raleigh for a year. She enrolled in the masters program in the Department of Microbiology at North Carolina State University in July of 2000, where she began her training under the direction of Dr. Scott Laster.

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## INTRODUCTION

### **Tumor Necrosis Factor- $\alpha$**

Tumor necrosis factor- $\alpha$  (TNF) is a 17 kDa inflammatory cytokine responsible for many immune and inflammatory responses. TNF was first described by Carswell et al. (1) as a substance isolated from serum of mice infected with *Bacillus Calmette-Guerin* and treated with endotoxin, for its killing of certain tumor cell lines (1). Endotoxin has, in fact, been shown to be a strong inducer of TNF from monocytes. A wide variety of cells are able to produce TNF, including B cells, T cells, NK cells and adipocytes, however, macrophages and monocytes are the primary TNF producing cells (2). TNF is translated as a 233 amino acid precursor, which contains a hydrophobic sequence. This sequence is then cleaved proteolytically, resulting in the mature 157 amino acid form (17 kDa) (2). A 26 kDa bioactive, cell membrane associated form of TNF is proteolytically cleaved into 17 kDa TNF and 14 kDa cleavage products. The 17 kDa form being the active secreted form, and the 14 kDa form being membrane associated (3-7). Studies using LPS for stimulation have shown that the expression of TNF is regulated at both transcriptional and translational levels. Treatment of cells with bacterial endotoxin results in a 3-fold increase in the rate of transcription, a 100-fold increase in levels of mRNA content, and a 10,000-fold increase in levels of protein secretion (2). Post-transcriptional regulation of gene expression is affected by a highly conserved sequence in the 3' untranslated region of TNF. Brown and Beutler (8) showed that in resting cells, the 3' untranslated region inhibits translation, however translation is increased when cells are activated.

## **TNF Receptors**

TNF signaling in the cell occurs through two TNF receptors, TNFR-1, and TNFR-2, 55 and 75 kDa respectively (2). Both TNFR-1 and TNFR-2 have an extracellular pre-ligand-binding assembly domain, which is involved in the pre-complexing and trimerization of the receptors (6). TNFR-1 contains a death domain, approximately 80 amino acids, located at the carboxyl end of the receptor, which is involved in cell death signaling (2, 6). These receptors are expressed on virtually all nucleated cells (6). The majority of TNF's biological activities are initiated through TNFR-1 binding, which begins a series of signal cascades, leading to the activation of both cytosolic pathways, and the two transcription factors, NF- $\kappa$ B and c-JUN (9). These transcription factors induce the expression of genes that have many functions, including cell growth, death and development, as well as immune and inflammatory responses (9). The role of TNFR-2 in biological processes is less understood than that of TNFR-1. TNFR-2 is known to have functions in the proliferative response, and, although it lacks a death domain, is involved in the regulation of TNF-induced apoptosis (6).

The two TNF receptors are part of a large TNF receptor family. Classification is based on homology in the extracellular domain, involving the arrangement of particular cysteine residues, which are important for ligand binding (10). Other than nerve growth factor (NGF), TNF receptor family ligands are homologous to TNF and include lymphotoxin- $\alpha$ /TNF $\beta$ , CD40L, and CD30L, as well as many others (6, 10). Some TNF receptor family members contain a death domain (DD), mentioned above, while others do

not. TNFR-1, CD95/APO-1/Fas, and TRAIL receptor-1/DR4 do contain a DD, while TNFR-2, lymphotoxin  $\beta$  receptor, and RANK lack a DD (6, 10).

## **The Activities of TNF**

### **TNF, Apoptosis, and Anti-Viral Immunity**

As previously mentioned, TNF was originally described for its ability to kill certain tumor cell lines, and it was proposed as a general anti-cancer agent (1). This ability was later found to be true only for certain tumor lines, and in fact, the majority of cells lines are resistant to TNF-induced apoptosis (11, 12). Although TNF-sensitive cell lines are rare in number, the study of these cell lines has pointed investigators towards one of the major *in vivo* activities of TNF. Recent studies have shown that resistance to TNF is determined by an autocrine resistance response. The binding of TNF to its receptors triggers expression of anti-apoptotic gene products that inhibit the apoptotic pathway (13). Cell lines that are susceptible to TNF are typically defective in the resistance response and as a result die from apoptosis. Discovery of the resistance response, and cells that are defective in this response, led investigators to ask whether there are natural *in vivo* situations, such as during viral infection, where inhibition of transcriptional responses might be important for immunity. Subsequent experiments with adenovirus, herpes simplex virus and HIV suggest that the killing of virus infected cells by TNF is indeed important for controlling virus infection (14-16). A large number of different viruses have also now been shown to encode anti-apoptotic resistance genes indicating that the viruses themselves “consider” it important for their infected host cell

to remain resistant to TNF-induced apoptosis.

### **TNF and Innate Immunity**

TNF has many functions in innate immunity within the body. It is a key cytokine involved in coordinating the body's defense system against pathogens. In the innate immune response, TNF is involved in many of the first responses against invasion, and is released at the site of infection and effects vascular permeability to fluids, proteins, and immune cells (17). This allows the immune defense access to the infected tissues, after which the effect is reversed, with blood clots forming to prevent spread of the infection. TNF is also involved in the acute-phase response, inducing the synthesis of acute-phase proteins, which have an action similar to that of antibodies, but with wider pathogen specificity (17). During an infection, TNF also activates endothelial cells, leading to the induction of cell-adhesion molecules involved in recruitment of inflammatory cells to the site of infection. There are many other innate responses that TNF is responsible for, including an increase in body temperature, and the migration of dendritic cells to the lymph node for maturation, leading to a more pathogen specific immune response (17).

### **TNF and Bacterial Immunity**

TNF is also very important in the body's defense against bacterial invasion. It is able to activate macrophages, which then secrete more TNF, and once activated become more efficient at phagocytosis. TNF also induces activated macrophages to produce the antimicrobial agent nitric oxide (17). As mentioned above, TNF also activates the endothelium, which allows specific immune cells to reach the site of infection. The

maturation of dendritic cells is another important function of TNF in defense against bacterial invasion (17).

### **TNF and Disease**

While TNF is important in the defense against pathogens, overproduction is involved in a number of pathological situations. The physiological effect of TNF varies depending on the amount and timing of TNF released in the body. Acute, high dose exposure to TNF can cause an increase in vascular permeability, adrenal hemorrhage, fever and shock (17, 18). Chronic, lower doses of TNF can also have a negative effect on the body, leading to cachexia, which involves weight loss, anorexia, and dehydration (18).

TNF has also been implicated in many autoimmune diseases, such as Rheumatoid Arthritis, Inflammatory Bowel Disease (IBD) and Insulin Dependent Diabetes Mellitus (IDDM) (19-21). TNF has been found in the synovial membrane of patients with Rheumatoid Arthritis and it has been shown to cause cartilage and bone resorption in affected joints (19). TNF's involvement in IBD is demonstrated by studies showing IBD patients have high TNF levels in serum, mucosa and stool as compared to non-IBD subjects, and that treatment of IBD with anti-TNF antibody has proven very successful (20, 22-25). TNF is also involved in type I diabetes mellitus, or insulin dependent diabetes mellitus, an autoimmune disease characterized by the destruction of pancreatic  $\beta$  cells, resulting in a lack of insulin (21, 26). Initially, macrophages and dendritic cells invade the islets, followed by B cells, T cells and natural killer cells.  $\beta$  cell cytotoxic T

cells cause destruction of the islets. TNF is involved as an inflammatory cytokine, contributing to the inflammatory condition of the islets, as well as in destruction of islets through TNF-receptor mediated apoptosis (21).

TNF is also involved in the autoimmune pathology of inflammatory liver damage. Overproduction of TNF is thought to be a part of many liver pathologies, such as liver failure, liver allograft rejection, chronic hepatitis B virus infection and alcohol hepatitis (27, 28). Certain hepatitis B virus proteins have been shown to sensitize cells to TNF-mediated apoptosis, leading to extensive liver damage in many hepatitis B patients (28). An increase in the number of TNF receptors, and in the level of TNF, has been shown to correlate with the rate of progression of liver damage seen with hepatitis B and C virus, as well as alcohol hepatitis (27, 29).

### **The Apoptotic Signaling Pathway**

The apoptotic signaling cascade that is induced by TNF involves many components. Upon binding of the trimeric TNF to the extracellular portion of TNF-R1, TNF-R1-associated death domain protein (TRADD) associates with the intracellular portion, and recruits receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2), and Fas-associated death domain protein (FADD) to the site (9, 30, 31). TRAF2 is involved in events that lead to the activation of p38 and c-Jun amino terminal kinase (JNK), which phosphorylates c-Jun (9, 31). FADD recruits caspase-8 to the receptor site, which initiates a pro-apoptotic caspase cascade, cleaving the effector pro-caspases 3, 6, and 7 into active caspases. (9). The active caspase 3 is then able to cleave



its many substrates, which include, poly(ADP-ribose) polymerase (PARP), DNA-PKCs, and lamin B (32). These molecules then trigger a variety of downstream effects involved in cell death, including DNA fragmentation, nuclear condensation and mitochondrial inactivation (9).

### **Resistance to TNF-Induced Apoptosis**

While binding of TNF to its receptors initiates a pro-apoptotic signal within the cell, it also initiates an anti-apoptotic signal. This resistance pathway is largely controlled by the action of the transcription factor NF- $\kappa$ B. TNF-induced activation of NF- $\kappa$ B relies on phosphorylation of I $\kappa$ B's, inhibitory proteins which keep NF- $\kappa$ B in the cytoplasm until the activation pathway is triggered (30). The I $\kappa$  Kinase complex phosphorylates the I $\kappa$ B's, initiating their degradation, allowing NF- $\kappa$ B to move to the nucleus (9). Once activated, NF- $\kappa$ B induces TNF resistance genes as well as several genes involved in inflammatory responses, including IL-6, IL-8, and IL-2 (33). These NF- $\kappa$ B induced resistance genes include manganese superoxide dismutase, and a gene that is found to encode proteins that reduce the activity of JNK, Gadd45<sup>B</sup> (34). Studies using phosphatase inhibitors also point to a phosphatase being involved in TNF resistance (15). Much of the knowledge about the importance of NF- $\kappa$ B pathway originated from studies of mice deficient in NF- $\kappa$ B. Knockout studies have been performed analyzing the importance of the various subunits of NF- $\kappa$ B and show differing levels of immunological disfunction ranging from mild to embryonic lethality, depending on the particular subunit

deficiency. Most subunits appear to be necessary for the normal immune functions, but without RELA (p65), embryos at day 16 die of massive apoptosis in the liver (33). NF- $\kappa$ B is therefore highlighted as a critical player in resistance to TNF-induced apoptosis.

### **Cytosolic Phospholipase A<sub>2</sub>**

There are several forms of PLA<sub>2</sub>, a low molecular mass secretory form, a calcium independent form and the 85 kDa cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). cPLA<sub>2</sub> selectively cleaves arachidonic acid from the *sn*-2 position of phospholipids. Arachidonic acid can then be used to generate lipid mediators of inflammation by the cyclooxygenase and the 5-lipoxygenase pathways (15, 35, 36). The production rate of arachidonic acid, and therefore the activity of cPLA<sub>2</sub>, is considered the rate limiting step in the synthesis of the lipid mediators of inflammation (35, 37). Studies with cPLA<sub>2</sub> deficient mice have demonstrated that cPLA<sub>2</sub> is necessary for the production of eicosanoids (prostaglandins and leukotrienes) (38, 39). To understand the role cPLA<sub>2</sub>, and the eicosanoids play in the allergic response, cPLA<sub>2</sub> wild type and deficient mice were studied for their response to a type I allergic response (anaphylaxis). Upon stimulation of the allergic response, granule contents such as histamine and serotonin, and lipid mediator such as the eicosanoids are released and cause many physiological responses. The deficient mice had significantly decreased allergic responses as compared to the wild type, illustrating the importance of cPLA<sub>2</sub> in allergy (38). Prostaglandins are involved in normal reproduction, and when mice lacking in cPLA<sub>2</sub> were bred, females were unable to deliver live pups. Pups could be brought to term if females were given a

progesterone-receptor antagonist (38).

The regulation of cPLA<sub>2</sub> is complex and not fully understood. A suggested method of regulation involved both calcium and phosphorylation. Initially an increase in cytosolic calcium would induce translocation of cPLA<sub>2</sub> from its resting location in the cytosol to its target membrane. At the membrane cPLA<sub>2</sub> binds using its N-terminal calcium-dependent lipid binding domain (CaLB), allowing the enzyme access to phospholipids. Phosphorylation of specific serine residues by mitogen activating protein kinases would then increase the enzyme activity (35, 36, 40). This theory is supported by evidence showing that an increase in intracellular calcium causes translocation to membranes within the cell, and that the activity of the enzyme increases by 2- to 3-fold when phosphorylated at serine 505 (41). Although there is a major role for calcium in activation of cPLA<sub>2</sub>, in certain cell types Phorbol 12-myristate 13-acetate (PMA) alone can cause an increase in phosphorylation, catalytic activity, and arachidonic acid release without an increase in intracellular calcium (35). Phosphorylation of four serine residues, Ser-437, Ser-454, Ser-505, and Ser-727, in cPLA<sub>2</sub> has been shown. Studies have shown that mutation of Ser-505 or Ser-727 to an alanine results in lower release of arachidonic acid after activation with a calcium ionophore and PMA (42).

Translocation from the cytosol to membranes is an important step in the activity of cPLA<sub>2</sub>, therefore, various studies have been performed, using inflammatory ligands, that investigate this membrane targeting. Others have shown translocation to the nuclear envelope and the endoplasmic reticulum in response to the calcium ionophore A23187

(43). Liu et al. (40) demonstrated a movement of cPLA<sub>2</sub> from cytosol to the plasma membrane, endoplasmic reticulum and the nuclear membrane in glomerular epithelial cells in response to PMA and the calcium ionophore, ionomycin.

### **cPLA<sub>2</sub> and Apoptosis**

A number of recent investigations have indicated that the activity of cPLA<sub>2</sub> is critical to TNF-induced apoptosis. Voelkel-Johnson et al. (13) have shown in a variety of cell lines that the level of cPLA<sub>2</sub> expression correlates with the ability of sensitizing agents to induce susceptibility to TNF. In addition, Hayakawa et al. (44) showed that TNF resistant sublines, derived from TNF sensitive cell lines, had dramatic decreases in levels of cPLA<sub>2</sub>. When these resistant cell lines were transfected with cPLA<sub>2</sub>, their sensitivity to TNF was restored. Similar results were seen in an experiment by Voelkel-Johnson et al., (13) when sensitive melanoma cell lines were transfected with cPLA<sub>2</sub>, their susceptibility to TNF and CHI increased. Adenovirus induced susceptibility to TNF was also shown to be dependent on the activity of cPLA<sub>2</sub>, as experiments using anti-sense oligonucleotides to suppress cPLA<sub>2</sub> showed a dramatic drop in sensitivity (45).

There are two hypotheses to explain cPLA<sub>2</sub>'s role in apoptosis, sphingomyelinase activation and mitochondrial inactivation. Neutral sphingomyelinases are able to hydrolyze sphingolipids, generating the important lipid messenger ceramide (46). Ceramide has been shown to be necessary for TNF-induced apoptosis through work with ceramide inhibitors (47). In MCF7 and L929 cells, treatment with TNF has been shown to result in two pathways of ceramide production. One pathway can be inhibited by

glutathione, a thiol-containing cellular antioxidant. The other pathway involves *de novo* synthesis of ceramide and is inhibited by fumonisin. When both of these pathways are blocked, cell death is significantly delayed, suggesting a role for ceramide, as well as sphingomyelinases, in TNF-induced apoptosis (47).

A potential role for cPLA<sub>2</sub> in apoptosis involves membrane bound neutral sphingomyelinases, which hydrolyzed membrane sphingomyelin, leading to ceramide production. This hydrolysis leads to activation of some of the MAP kinases, which are involved in the activation of cPLA<sub>2</sub> (46, 48). Studies using TNF-sensitive and TNF-resistant cell lines have demonstrated a role for cPLA<sub>2</sub> and arachidonic acid in neutral sphingomyelinase activation (49, 50). In HL-60 cells it has been demonstrated that TNF stimulated arachidonic acid release, and subsequently activation of neutral sphingomyelinases (49). It was then shown that arachidonic acid itself could reproduce the activation effect seen with TNF-induced activation of neutral sphingomyelinases (49). The cell line C12 is lacking in cPLA<sub>2</sub>, and therefore does not produce arachidonic acid in response to TNF. This cell line was unable to produce ceramide, which is generated by the activated neutral sphingomyelinases. Restoration of cPLA<sub>2</sub> to the cells also restored arachidonic acid production and neutral sphingomyelinase activity (50).

The other hypothesis of cPLA<sub>2</sub> involvement in apoptosis is mitochondrial inactivation. In WEHI-231 cells, cPLA<sub>2</sub> activation correlates with an antigen receptor driven apoptosis. In this model, apoptosis is independent of caspase activity. It appears that cPLA<sub>2</sub> activity, in response to apoptotic stimuli, leads to disruption of the

mitochondrial function and depletion of ATP (51). Signals that rescue the cell from apoptosis cause a downregulation of cPLA<sub>2</sub> activity (51). Other experiments have implicated cPLA<sub>2</sub> and arachidonic acid in the collapse of mitochondrial membrane potential, which is believed to be an integral part of TNF-induced apoptosis (52, 53). It has been shown that arachidonic acid directly causes opening of the mitochondrial permeability transition pore, leading to release of cytochrome c and cell death (54).

The goal of our studies is to identify the intracellular membrane target of cPLA<sub>2</sub> during TNF-induced apoptosis. As discussed earlier, TNF is important in many processes throughout the body and has implications in many diseases. Gaining a better understanding of the activation of cPLA<sub>2</sub> can serve to provide possible targets for therapeutic drugs by opening up areas to prevent enzyme activation, cleavage of arachidonic acid, or synthesis of mediators of inflammation. To achieve our goal of understanding the intracellular targeting during apoptosis, we employed three techniques. The first utilized fluorescence to visualize cPLA<sub>2</sub> within the cell. We then used biochemical fractionation to analyze subcellular fractions of the cell. Lastly, we performed GC/MS analysis of fatty acid extracted from cells to investigate the content of cellular membranes.

## **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 Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and maintained at 37°C in 8% CO<sub>2</sub>. Media and reagents, unless otherwise indicated, were purchased from Sigma. Cycloheximide was purchased from Calbiochem (La Jolla, CA). Acridine Orange was obtained from Molecular Probes (Eugene, Oregon). The Nuclear Pore Complex antibody was purchased from Abcam (Cambridge, UK). <sup>3</sup>H-arachidonic acid ([5,6,8,9,11,12,14,15-<sup>3</sup>H(N)] was purchased from PerkinElmer Life Sciences, Inc. (Boston MA). Free fatty acids were purchased from Alltech (State College, PA). The Protein assay kits were purchased from Pierce (Rockford, IL).

### **Arachidonic Acid Release Assay**

For the Arachidonic Acid release assay, C3HA mouse fibroblast cells were plated at a concentration of  $1 \times 10^5$  cells/well in a 12-well flat-bottom tissue culture plates (Fisher, Pittsburgh, PA) in 600ul of assay media (Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotic/antimycotic) per well and labeled overnight with 0.1 µCi/ml <sup>3</sup>H-arachidonic acid. After incorporation, media was removed and cells were washed 2 times with Hank's Balanced Salt Solution (HBSS). 600ul of assay media was added to the wells and cells were allowed to incubate for 2 hours. After two hours cells were again washed with HBSS, then assay media alone, or

assay media with treatment where appropriate, was added for the indicated amount of time. After treatment, 300ul of media was removed from each well, spun to remove debris, and 200ul of the media was added to 5ml scintillation cocktail for counting (Beckman model LS 5801, Fullerton, CA). Results from counting were multiplied by three to give a complete sample count. To determine maximum incorporation, 10ul of 10% SDS was added to max wells and then 600ul were removed for counting. All points were conducted in triplicate.

### **Immunofluorescent Staining**

For staining with anti-cPLA<sub>2</sub> mAb, the procedure was as follows. Briefly, 2.5 X 10<sup>4</sup> cells/well were plated in 8 well chamber slides (Nalge Nunc International, Naperville, IL) and allowed to adhere overnight. Cells were treated as indicated, then media was removed and wells were washed twice with 600ul of Phosphate Buffered Saline (PBS). 10% formaldehyde/PBS was then added to wells for 15 minutes, after which the cells were again washed twice in PBS. Cells were stained with anti-cPLA<sub>2</sub> mAb (Santa Cruz, Santa Cruz, CA SC-454) at a 1:500 dilution in PBS with Saponin (0.1 g/ml) and Normal Goat Serum (20%) for 30 minutes. Cells were then washed 2 times with PBS, then incubated with rhodamine conjugated goat anti-rabbit Ab at a 1:100 dilution in PBS with Saponin and Normal Goat Serum for 30 minutes. After incubation cells were again washed 2 times with PBS, then mounted in 10% glycerol. Microscopy was conducted on a Zeiss Axioscop (Oberkochen D7082) and images were captured and processed by a Spot<sub>TM</sub> CCD camera and software (Diagnostic Instruments, Inc., Sterling Heights MI).



For Nuclear Pore Complex staining cells,  $2.5 \times 10^4$  cells/well were plated in 8 well chamber slides and allowed to adhere overnight. Cells were treated as indicated, then media was removed and wells were washed twice in PBS. Cells were then fixed in 100% methanol for 2 minutes, and then 4% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were then washed twice with PBS and permeabilized in 0.2% Tween-20 in PBS for 30 minutes. After permeabilizing, cells were again washed twice in PBS. Cells were stained with 1:500 NPC Ab (Abcam, Cambridge, UK) in 3% BSA/PBS for 1 hour. After 1 hour, cells were washed twice in PBS and stained in rhodamine conjugated goat anti-rabbit Ab in 3% BSA/PBS for 30 minutes. Cells were then washed twice with PBS and mounted in 10% glycerol and staining was examined.

For analysis using Acridine Orange  $2.5 \times 10^4$  cells/well were plated in 8 well chamber slides and allowed to adhere overnight. Cells were then washed 2 X with PBS and treated as indicated. After treatment cells were again washed, then stained with  $1\mu\text{M}$  Acridine Orange in assay media for 15 minutes at room temperature. After staining cells were washed with PBS and mounted in 10% glycerol and staining was examined.

### **Nuclei isolation and Immunoblotting**

$1 \times 10^6$  cells were plated in 150 mm tissue culture plates and allowed to adhere overnight. The following day, cells were treated as indicated. After treatment Sigma's Nuclei EZ kit was used to obtain whole cell lysates and nuclei preps according to manufactures' instructions. Briefly, after treatment, cells were washed with PBS, then 2 ml of lysis buffer was added to the plate. Cells were scraped into a 15ml conical and

vortexed, set on ice for 5 minutes and then the nuclei were collected by centrifugation at 500 X g for 5 minutes. Supernatant represented the cytosolic fraction and was stored for later use. The pellet was resuspended in 2 ml of lysis buffer and centrifuged again to wash the nuclei. After centrifugation, the nuclei were resuspended in 200µl of storage buffer, vortexed and used immediately or stored at  $-70^{\circ}\text{C}$  for later use. For immunoblotting 8% Tris-glycine gels were loaded with 15 µg of protein that was subjected to SDS gel electrophoresis using the NOVEX system (San Diego CA) for 1 h at 30 mA. After transfer and blocking, protein was probed using a rabbit polyclonal rabbit antiserum raised against human cPLA<sub>2</sub> (1/1000 dilution) that was generously donated by Dr. J. Clark, Genetics Institute (Cambridge, MA). A secondary horseradish peroxidase-coupled goat anti-rabbit antibody was purchased from Sigma and bands were visualized using the SuperSignal chemiluminescence kit (Pierce, Rockford, IL).

### **Subcellular Fractionation**

For subcellular fractionation,  $2 \times 10^6$  C3HA cells were plated in a 150mm tissue culture dish (Fisher, Pittsburgh, PA) and allowed to adhere overnight. Plates were washed with PBS two times, then assay media and appropriate treatments were added for the indicated time. After treatment media was removed and plates were placed on ice. Plates were washed 2 times with ice cold PBS. One ml of ice cold homogenization buffer [50mM Hepes/0.25 M sucrose/1mM EDTA/1mM EGTA/20µM leupeptin/20µM pepstatin/0.1 mM PMSF (pH 7.4)] was added to each plate. Cells were scraped into an ice-cold tissue homogenizer and disrupted with 25 strokes of the homogenizer. Disrupted

cells were centrifuged at 1300 X g for 10 minutes to pellet unbroken cells. The resulting supernatant was centrifuged at 100,000 X g for 1 hour. The supernatant from the high speed spin, representing the cytosol, and pellet containing microsomal membranes, which were resuspended in homogenization buffer, were used for immunoblotting.

### **Fatty Acid Analysis**

2 X 10<sup>6</sup> C3HA cells were plated in 150mm tissue culture dishes and allowed to adhere overnight. Cells were treated as indicated. After treatment cells were washed 2 times with PBS. Then 1 ml of homogenization buffer (see previous section) was added, cells were scraped into a tissue homogenizer and disrupted with 25 strokes. Disrupted cells were spun for 1 hour at 100,000 X g to pellet cellular membranes. The resulting membrane pellet was resuspended in 500µl of enzyme reaction buffer (10mM calcium chloride; 100mM Tris-HCl, pH 8.9; 0.8% NP-40) and 200µl's of Naja Naja sPLA<sub>2</sub> (2.5 units/µl) was added. The reaction was allowed to continue for 4 hours at 25 °C. After 4 hours the reaction was stopped with the addition of 50µl of 100mM EDTA. To extract the fatty acids, 2 ml of Dole's reagent (Isopropanol:Heptane:Sulfuric acid; 90:10:1) was added, and the sample was vortexed. 750µl of ddH<sub>2</sub>O and 1.2 ml's Heptane were added and the samples were vortexed again. Samples were then centrifuged at 3000 rpm for 15 minutes and the top organic layer was removed. Silicic acid was added, the sample vortexed and spun at 1000 rpm for 5 minutes. The resulting supernatant was placed in a clean tube and dried under N<sub>2</sub> gas. The fatty acids obtained from the extraction were then esterified using Boron trifluoride/Methanol (14% w/v) (Alltech, State College, PA).

Briefly, 0.5ml of reagent was added to the fatty acids and boiled for 2 minutes. The sample was cooled to ambient temperature, then 3 ml of petroleum ether and 2 ml of water were added. The methyl esters were extracted by vigorous shaking, and the petroleum ether layer was removed, dried over anhydrous sodium sulfate and dried under N<sub>2</sub> gas. Samples were brought up in Hexane and run on a GC/MS-QPS5000 (Shimadzu, Kyoto, Japan). The column used was a J and W Scientific DB5MS, with a length of 30 meters, an inner diameter of 0.25 mm, and 0.25 µm film. The method parameters were an initial temperature of 35 °C, for 5 minutes, and then increasing to a run temperature of 200 °C. The program length was 35 minutes and the carrier gas was Helium. Data was analyzed using the CLASS-5000 program (Shimadzu). Peaks were identified by comparison with the National Technical Information Service (NTIS) library.

## Results

### The Release of $^3\text{H}$ -Arachidonic Acid From C3HA Cells Treated With TNF and CHI

The murine fibroblasts cell line C3HA is normally resistant to TNF-induced apoptosis. Resistance in C3HA cells can be overcome, however, by treatment with the translational inhibitor cycloheximide (CHI). Induction of apoptosis in C3HA cells by treatment with TNF and CHI is dependent on the activation of the enzyme cPLA<sub>2</sub>, which selectively cleaves arachidonic acid from membrane phospholipid. The activity of cPLA<sub>2</sub> can be monitored by measuring the release of  $^3\text{H}$ -arachidonic acid into the culture media from prelabeled cells. Figure 1 shows the time course of  $^3\text{H}$ -arachidonic acid release from C3HA cells treated with TNF (10/ng/ml) and/or CHI (25 mg/ml). Treatment with only TNF causes a small, rapid increase in radiolabel release that ceases typically after 2 hours. CHI exerts an opposite effect, causing rapid suppression of radiolabel release. The suppressive effect of CHI lasts approximately four hours after which values parallel untreated controls. Again, neither of these compounds when used individually can induce apoptosis in C3HA fibroblasts. A completely different effect is seen when C3HA cells are treated with TNF and CHI simultaneously, which does induce apoptosis. As shown in Figure 1, the release of  $^3\text{H}$ -arachidonic acid begins after 1 hour and continues in an almost linear fashion throughout the experimental period. We have shown previously (13) that the arachidonic acid released from dying C3HA cells stems from the activity of cPLA<sub>2</sub>.

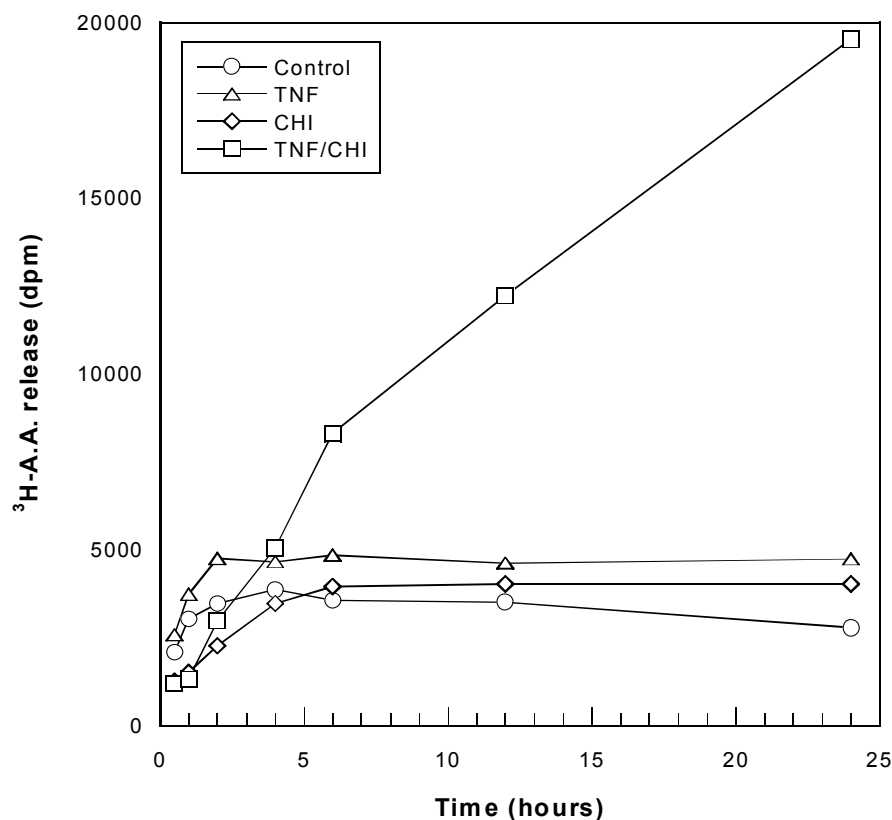


Figure 1. TNF-induced arachidonic acid release. C3HA cells were labeled at 1:1000 and plated at  $1 \times 10^5$  cells/well. Cells were cultured overnight and treated with 20 ng of TNF and/or 25  $\mu\text{g}$  of CHI for varying amounts of time. After treatment, media was counted on a scintillation counter to determine  $^3\text{H}$ -arachidonic acid release. All points were performed in triplicate.

### **Visualization of Intracellular cPLA<sub>2</sub> by Immunofluorescence**

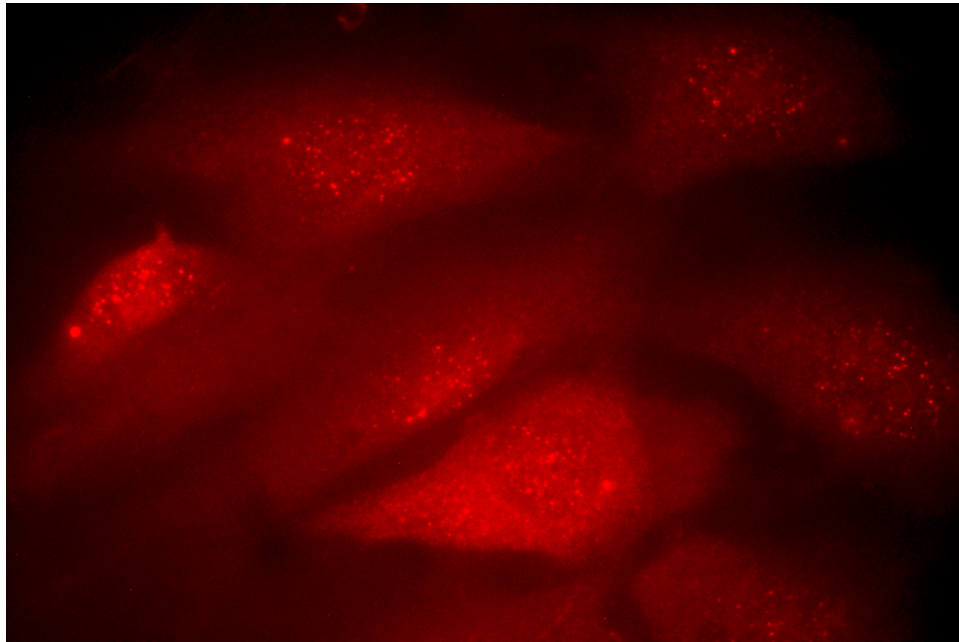
In resting cells cPLA<sub>2</sub> is found free in the cytosol. To exert its phospholipase activity cPLA<sub>2</sub> must move to and bind an intracellular membrane. The goal of these studies is to identify the membrane target for cPLA<sub>2</sub> in cells as they undergo apoptosis. One method we used to approach this problem was immunofluorescence. Cells were treated, fixed, and then stained with a murine mAb to cPLA<sub>2</sub> followed by rhodamine coupled rabbit anti-mouse secondary Ab. As shown in Figure 2A, in control cells, we found a diffuse pattern of cPLA<sub>2</sub> staining with some areas of punctate staining noted. A similar pattern of staining was noted in C3HA cells treated with TNF and CHI for 1 hour (Fig. 2B). In contrast, in cells that had been treated with TNF and CHI for 2 hours, we found a substantial decrease in staining in the peripheral cytosol and an increase in punctate staining in the nuclear/perinuclear area of the cell (Fig. 3C). Large “aggregates” of cPLA<sub>2</sub> also became visible in the cytosol and within the nuclei. Figure 2D shows that staining in the peripheral cytosol decreased even further after 3 hours of treatment (Fig. 2D) and the intranuclear “aggregates” of cPLA<sub>2</sub> became more visible. In addition, a ring structure, believed to be the nuclear membrane also became visible after three hours of treatment. Taken together these results suggest that during the apoptotic activation of cPLA<sub>2</sub> the enzyme migrates to the perinuclear region of the cell, binds the nuclear membrane, enters the nucleus and forms large aggregates within the nucleus.

**Figure 2.** Immunofluorescent staining of cPLA<sub>2</sub>.  $2.5 \times 10^4$  cells/well were plated in 8 well chamber slides and cultured overnight. Cells were treated with 20 ng TNF and 25  $\mu$ g CHI for 1 hour in panel B, 2 hours in panel C, 3 hours in panel D or untreated in panel A. After treatment cells were stained with anti-cPLA<sub>2</sub> mAb and a rhodamine conjugated secondary Ab.

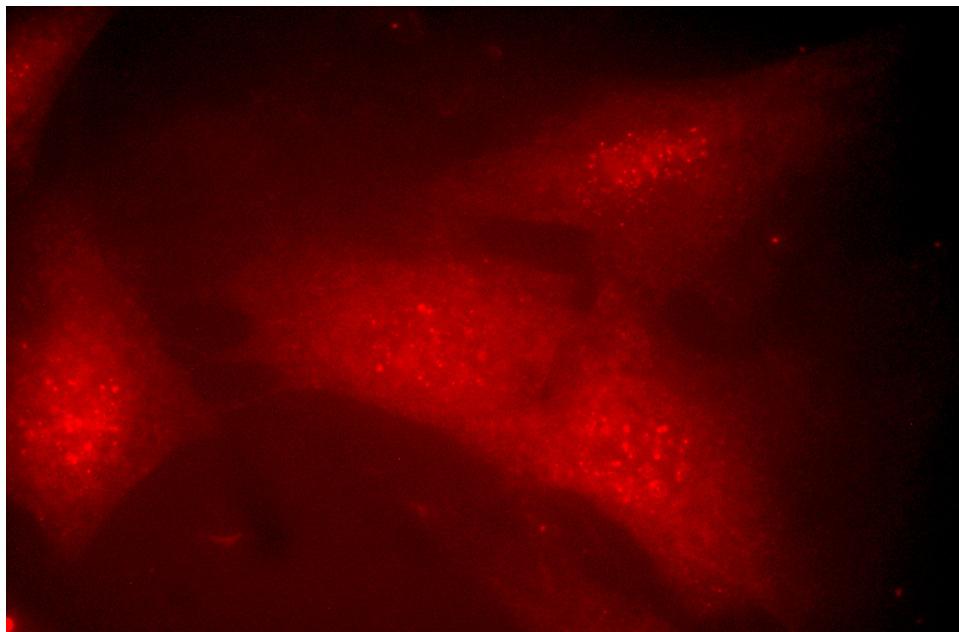


Figure 2

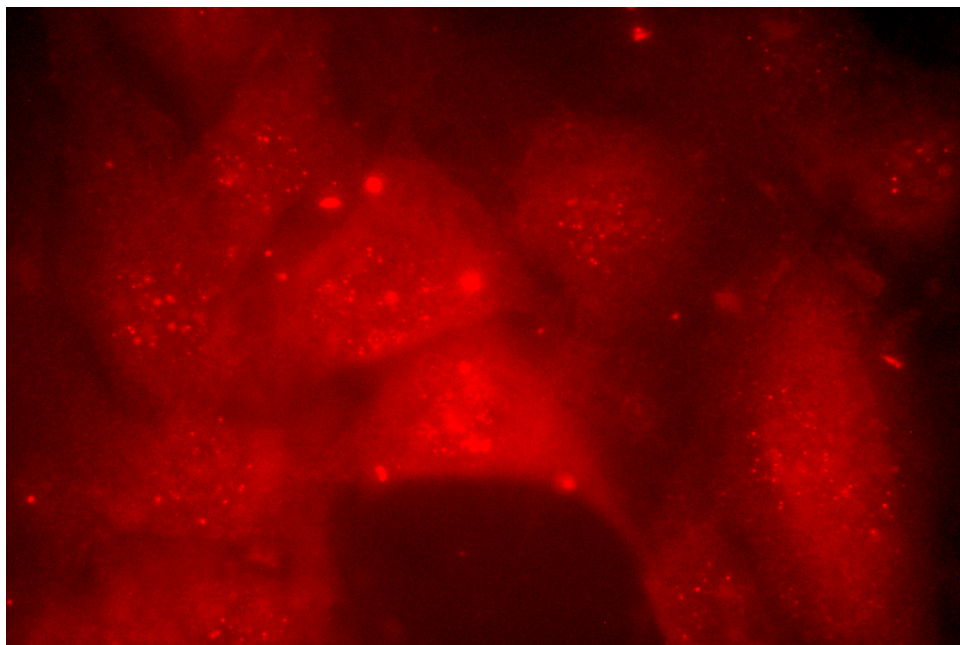
Panel A



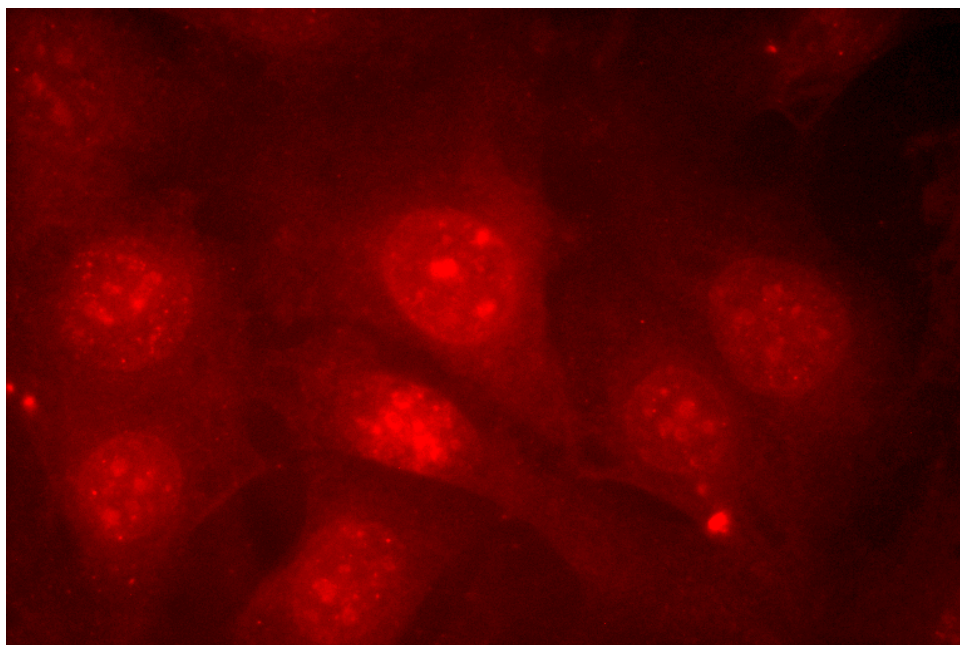
Panel B



Panel C



Panel D



## **Visualization of Nuclear Pore Complexes**

In Figure 2D, a ring-like structure became visible that we believed represented the nuclear membrane. To confirm this hypothesis, C3HA fibroblasts were stained with an antibody against a protein known to reside in the nuclear membrane, the nuclear pore complex protein (55). This protein is an important component of the nuclear pore complex, which controls the export of mRNA from the nucleus. As shown in Figure 3A, in control cells, staining with this Ab did reveal ring-like structures within C3HA fibroblasts. In addition, we also noted large areas of intranuclear staining. Similar ring-like structures were observed in cells treated with TNF and CHI for 3 hours (Fig. 3B) although clearly they became oblong and misshapen. The similar pattern of intracellular staining between this Ab and the anti-cPLA<sub>2</sub> staining pattern noted in Figure 2D strengthens the hypothesis that cPLA<sub>2</sub> is indeed translocating to the nuclear membrane.

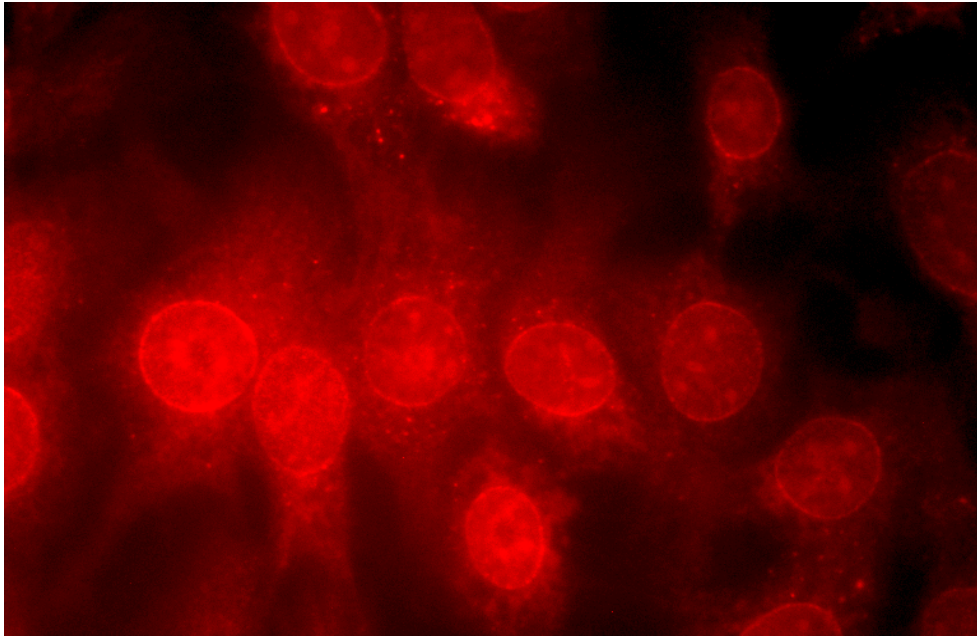
## **Visualization of DNA and RNA**

Staining with anti-cPLA<sub>2</sub> and the NPC antibodies revealed aggregates and large areas of intranuclear staining. To investigate the possibility that these represent cPLA<sub>2</sub> binding to DNA or RNA within the cell we stained C3HA with Acridine Orange. Acridine Orange intercalates into DNA and RNA, displaying green fluorescence when bound to DNA and red fluorescence when bound to RNA. Figure 4 demonstrates the staining pattern of Acridine Orange in C3HA cells. Untreated cells that were stained showed a diffuse pattern (panel A). When cells were treated with TNF and CHI for 3 hours, a more clearly defined nucleus was visible (panel B), and there was increased

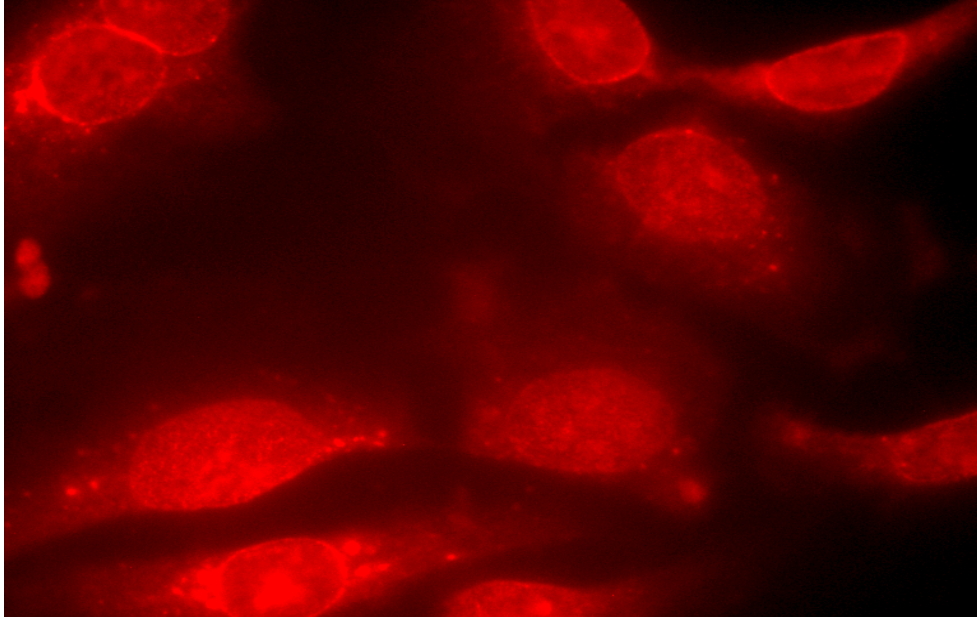
**Figure 3.** Staining of the nuclear pore complex.  $2.5 \times 10^4$  cells/well were plated in 8 well chamber slides and cultured overnight. Cells were treated with 20 ng TNF and 25  $\mu$ g CHI for 3 hours in panel B or untreated in panel A. After treatment cells were fixed in paraformaldehyde and methanol, permeabilized with Triton-X and stained with the NPC mAb. Cells were then labeled with rhodamine conjugated secondary Ab.

Figure 3

Panel A



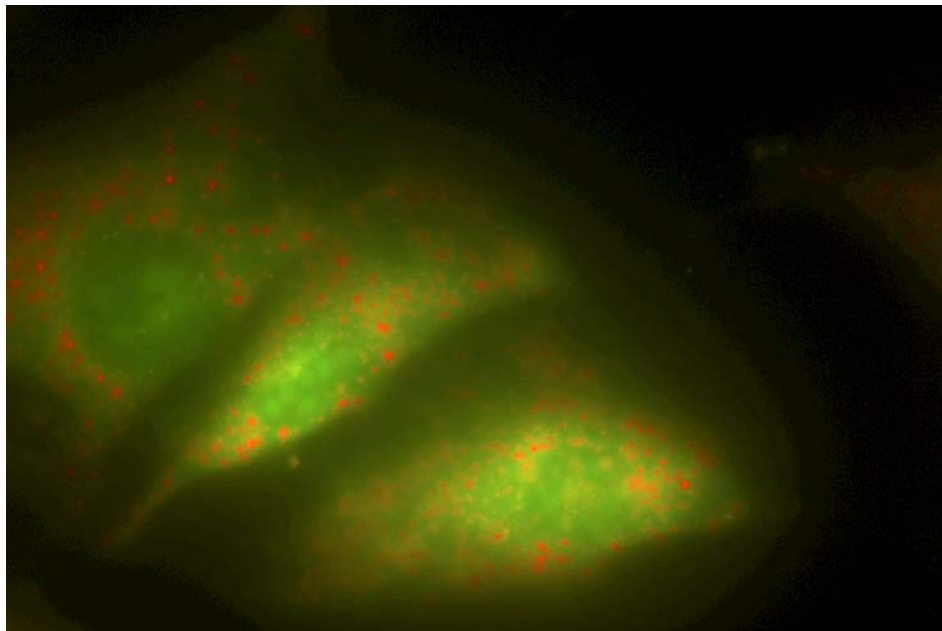
Panel B



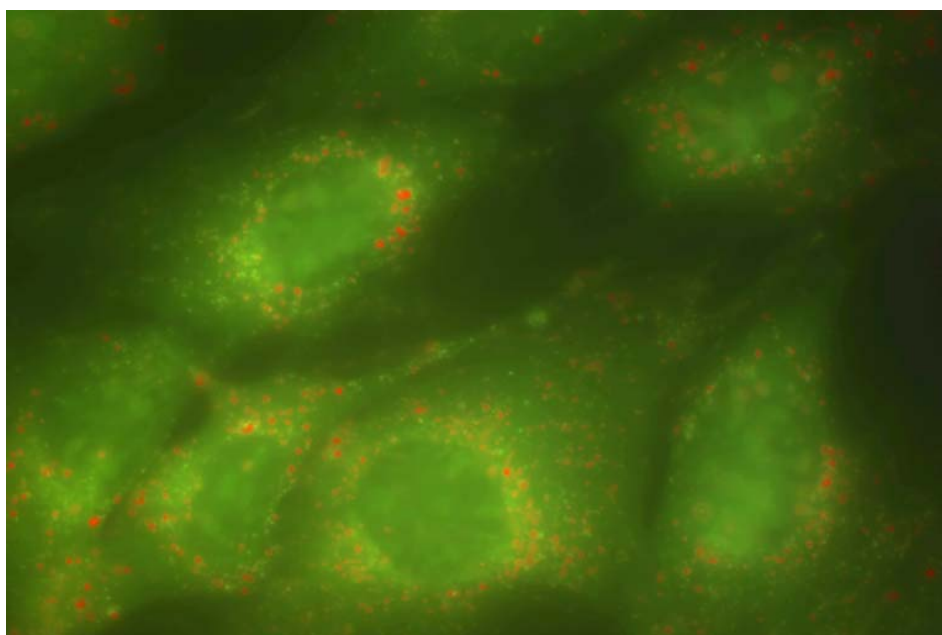
**Figure 4.** Staining with Acridine Orange.  $2.5 \times 10^4$  cells/well were plated in 8 well chamber slides and cultured overnight. Cells were treated with 20 ng TNF and 25  $\mu$ g CHI for 3 hours in panel B or untreated in panel A. After treatment cells were stained with 1  $\mu$ M Acridine Orange for 15 minutes.

Figure 4

Panel A



Panel B





perinuclear staining. Large aggregates of DNA staining were also observed within the nucleus, although they were not as bright as those seen with TNF and CHI treatment.

### **Detection of cPLA<sub>2</sub> in Nuclear and Cytosolic Extracts**

As previously stated, cPLA<sub>2</sub> must move from its resting location in the cytosol to intracellular membranes to exert its activity. We first employed fluorescent staining to analyze the intracellular location of the enzyme. Data obtained from those studies strongly suggest that cPLA<sub>2</sub> is moving to the nuclear membrane upon activation by TNF and CHI. We next used a biochemical fractionation coupled with western blot analysis as a method to test this hypothesis. A polyclonal Ab to cPLA<sub>2</sub>, raised against full-length recombinant cPLA<sub>2</sub> produced in *E. coli*, was used for detection.

As shown in Figure 5, lane A, in a whole cell lysate prepared using a commercially available kit designed for nuclear isolation, the polyclonal Ab reacts strongly with an approximately 97 kDa protein which we have previously shown comigrates with recombinant cPLA<sub>2</sub>. The polyclonal Ab also detects an approximately 70 kDa band whose identity is unknown. Various breakdown products of cPLA<sub>2</sub> have been reported (56) and the strength of the reaction of the Ab with the 70 kDa protein suggests it is likely a fragment of cPLA<sub>2</sub>. As would be expected, the same proteins are recognized in whole cell lysates prepared from C3HA cells treated with TNF and CHI for either 1.5 hours (lane B) or 3 hours (lane C). cPLA<sub>2</sub> does have a caspase cleavage site which causes the molecule to be fragmented during apoptosis, however, this cleavage



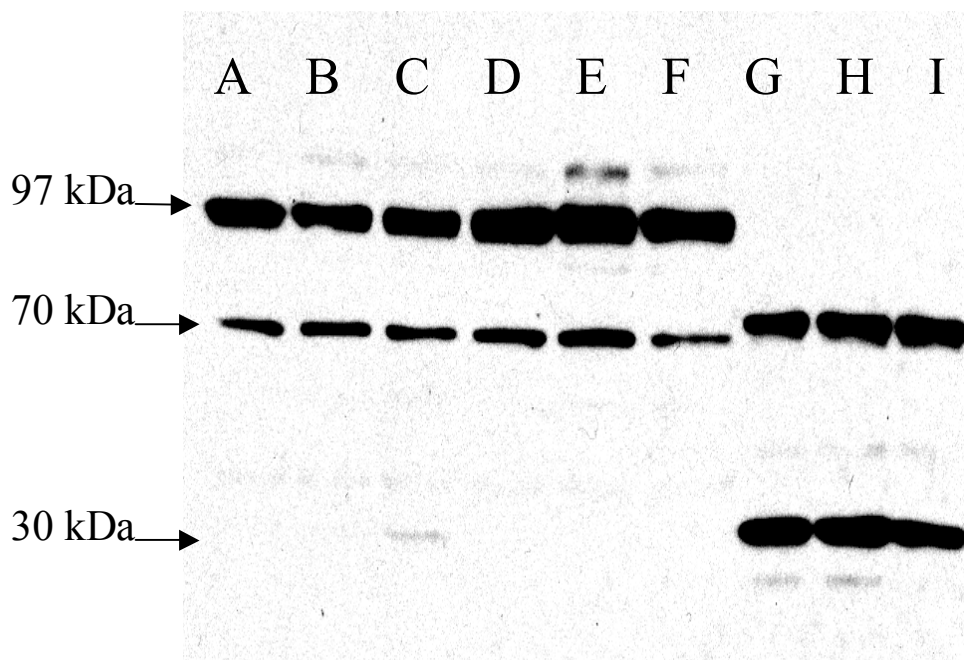


Figure 5. Subcellular distribution of cPLA<sub>2</sub> in nuclear and cytosolic fractions following treatment with TNF and CHI.  $2 \times 10^6$  cells were either treated with CHI (25  $\mu$ g) and TNF (20ng) for 1.5 hours, 3 hours or left as untreated controls. After treatment nuclei were isolated according to the protocol in the materials and methods. Nuclei pellets were resuspended and 15  $\mu$ g of protein of each sample was subjected to SDS PAGE, transferred to a nitrocellulose membrane and probed using anti-cPLA<sub>2</sub> Ab. Lanes A, B, and C represent whole cell lysates, D, E, and F represent the cytosolic fraction and G, H, and I the nuclei. A, D and G are from untreated control. B, E, and H are from cells treated for 1.5 hours, C, F, and I are from cells treated for 3 hours.

event does not occur until later in the apoptotic response (57).

To test whether cPLA<sub>2</sub> is indeed binding to the nuclear membrane during apoptosis, cells were fractionated into nuclei and cytosol using a commercially available kit obtained from Sigma Co. Lanes D-F of Figure 5 show cytosolic fractions from untreated cells and from cells treated with TNF and CHI for 1.5 or 3 hours, respectively. We did not observe any striking differences from the banding pattern seen with whole cell lysates.

Several important items were, however, noted in protein samples prepared from nuclei. First of all, as expected, nuclei from control cells (lane H) did not contain any full-length cPLA<sub>2</sub>. However, cPLA<sub>2</sub> was also not detected in nuclei prepared from cells treated with TNF and CHI (lanes I and J). These data would seem to contradict our earlier findings using immunofluorescent microscopy, which showed clear nuclear and perinuclear translocation of cPLA<sub>2</sub>. We address this contradiction below, in experiments designed to test the role of calcium in the membrane binding of cPLA<sub>2</sub>.

Nuclear fractionation also revealed a completely unexpected result. As shown in Figure 5, lanes H-J, the polyclonal Ab detected two bands with molecular weights of 72 and 30 kDa, respectively. At this point it is unclear whether the 72 kDa band observed in lanes H-J is the same band seen in whole cell and cytosolic fractions. The molecular weight of this band appears slightly different in the different fractions; however, the overall chemical composition of these fractions differs considerably, which may affect its mobility through the SDS PAGE gel. The 30 kDa band observed in nuclear fractions is

clearly not seen in whole cell lysates and cytosolic fractions. Nuclear samples represent an approximately 5-fold enrichment over whole cell lysates for nuclear proteins which may explain the unique detection of this band in nuclear extracts. Again, the strength of recognition suggests that this band does indeed represent a fragment of cPLA<sub>2</sub>, although its identity is unknown.

### **Detection of cPLA<sub>2</sub> in Membrane and Cytosolic Fractions**

In Figure 5 we failed to show transfer of cPLA<sub>2</sub> from cytosol to nuclear fractions in cells triggered to undergo TNF-induced apoptosis. Several experiments were performed in an attempt to validate this finding. One possibility we considered was that cPLA<sub>2</sub>, although visually appearing to translocate to the nuclear membrane, was instead binding to endoplasmic reticulum membranes. The ER and nuclear membranes are intimately associated and microscopic observations may be misleading.

To address this hypothesis, rather than isolate nuclear and cytosolic fractions, we instead used a technique designed to isolate all cell membranes. This technique relies on mechanical disruption of cells followed by ultracentrifugation for purification of membranes, rather than detergent-based lysis followed by low speed centrifugation for isolation of nuclei. A representative figure summarizing the results of these experiments is shown in Figure 6. Again, the Ab detects similar amounts of full-length cPLA<sub>2</sub> in whole cell lysates (lanes A-C) and the 70 kDa putative fragment of cPLA<sub>2</sub> (which appeared as a doublet in whole cell lysates prepared by mechanical disruption). Minor

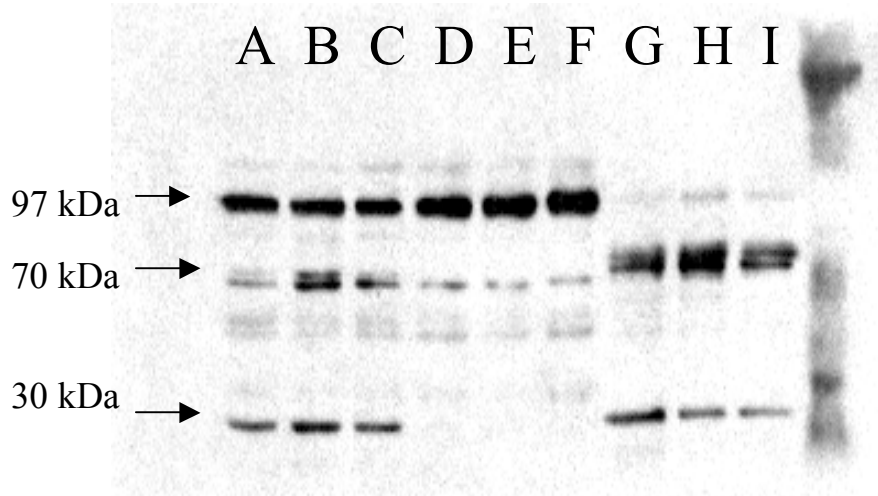


Figure 6. Subcellular distribution of cPLA<sub>2</sub> in membrane and cytosolic fractions following treatment with CHI and TNF.  $2 \times 10^6$  cells were either treated with TNF (20ng) and CHI (25  $\mu$ g) for 1.5 hours, 3 hours or left as untreated controls. Cells were disrupted with a tissue homogenizer and centrifuged for 1 hour at 100,000 X g to pellet intracellular membranes. Pellets were resuspended and 12.5  $\mu$ g of protein of each sample was subjected to SDS PAGE, transferred to a nitrocellulose membrane and probed using anti-cPLA<sub>2</sub> Ab. Lanes A, B, and C represent whole cell lysates, D, E, and F represent the supernatant fraction and G, H, and I the membrane pellet. A, D and G are from untreated control. B, E, and H are from cells treated for 1.5 hours. C, F, and I are from cells treated for 3 hours.

differences in the amount of this doublet were seen following treatment with TNF however these variations were not consistent between experiments. We also found that whole cell lysates prepared by mechanical disruption also contained the 30 kDa band, a finding clearly different from our results with the detergent based nuclear isolation kit. Cytosolic fractions (the supernatant after high speed centrifugation) contained primarily full-length cPLA<sub>2</sub> (lanes D-F) and again no difference was seen between control and treated samples. Membrane samples (the pellet following high speed centrifugation) contained the 70 and 30 kDa bands and only a small amount of full-length cPLA<sub>2</sub> (lanes G-I). Again, as with the nuclear isolation protocol, we did not observe the transfer of cPLA<sub>2</sub> to cell membranes following the induction of apoptosis with TNF. Finding the same result with both fractionation procedures makes it unlikely that our failure to observe stable membrane association results from our missing the appropriate membrane during cell fractionation. Rather, we hypothesized that the binding of cPLA<sub>2</sub> to membranes during apoptosis is calcium-dependent and that this association is lost when cells are lysed in the presence of calcium chelators.

### **Experiments to Test the Calcium Dependence of cPLA<sub>2</sub> Binding**

Calcium is an important second messenger in cells that controls many cellular functions. Many membrane-binding proteins have calcium binding domains (including cPLA<sub>2</sub>) that actually mediate the insertion and binding of those proteins to membranes. Mammalian cells have large intracellular stores of calcium and therefore, to prevent the artifactual, mass binding of cytosolic proteins to membranes cells are typically lysed in

buffers containing both EDTA and EGTA. In our cell fractionation experiments (where both chelators were used) we did not observe any translocation of cPLA<sub>2</sub> to intracellular membranes, suggesting that calcium is required for this association. We wondered, however, whether it would be possible to lyse C3HA cells in the absence of chelators, or at reduced chelator concentration, and not observe the artifactual translocation of cPLA<sub>2</sub>. If so, then it might be possible to lyse activated cells in the absence of chelators and observe the calcium-dependent, apoptosis-triggered, membrane association that we had observed by fluorescent microscopy.

A representative figure summarizing the results of these experiments is shown in Figure 7. C3HA cells were lysed at normal chelator concentration (1 mM EDTA and EGTA), at one half-chelator concentration (500  $\mu$ M EDTA and EGTA), or in the absence of both chelators. The proteins were then run on PAGE and blotted using the anti-cPLA<sub>2</sub> Ab described above. As shown in lanes A-C, whole cell lysate prepared with either normal (lane A), none (lane B) or one half (lane C) chelator concentrations did not affect the observed banding pattern. Similarly, changes in chelator concentration did not affect levels of cPLA<sub>2</sub> observed in cytosolic fractions. In contrast, changes in chelator concentration did affect levels of cPLA<sub>2</sub> observed in membrane fractions. As would be predicted, low levels of membrane-associated cPLA<sub>2</sub> are seen in the sample prepared at the normal chelator concentration (lane G). Unfortunately, removing the chelators completely (lane H) or reducing their concentration by one-half (lane I) caused a large amount of cPLA<sub>2</sub> to become membrane-associated, suggesting it would be difficult to

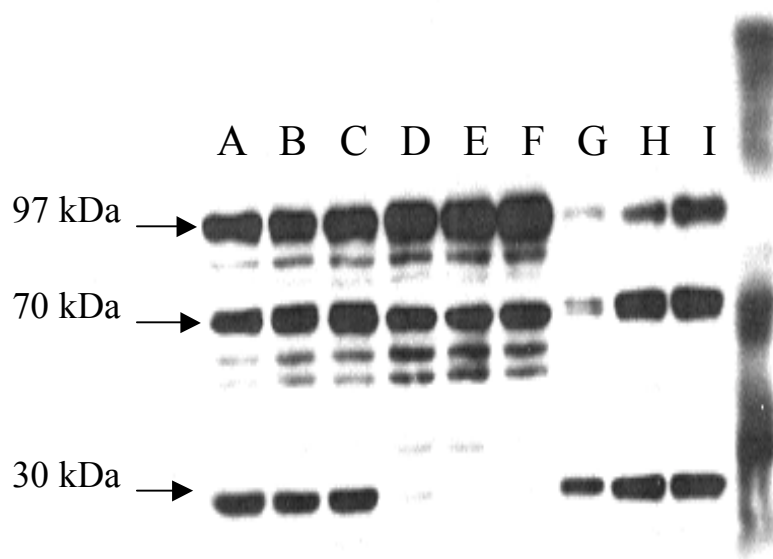


Figure 7. Subcellular distribution of cPLA<sub>2</sub> following lysis in the absence of calcium chelators. Subcellular fractionation was performed as described in the materials and methods except that for one sample the homogenization buffer was prepared without the calcium chelators EDTA and EGTA, for another sample the buffer was prepared with one half of the original concentration of chelators. After centrifugation pellets were resuspended and 15 µg of protein of each sample was subjected to SDS PAGE, transferred to a nitrocellulose membrane and probed using anti-cPLA<sub>2</sub> Ab. Lanes A, B, and C represent whole cell lysates, D, E, and F represent the supernatant fraction and G, H, and I the membrane pellet. Lanes A, D and G are from control cells fractionated using traditional levels of chelators in the buffer. Lanes B, E, and H are from cells fractionated without EDTA or EGTA in the buffer and lanes C, F, and I represent cells fractionated with 500 µM EDTA and EGTA in the homogenization buffer.

use biochemical fractionation to observe the calcium-dependent, apoptosis-triggered, membrane association of cPLA<sub>2</sub>.

Alternatively, it is possible that another unknown problem was responsible for our inability to observe the stable association of cPLA<sub>2</sub> with cell membranes. We decided, therefore, to test the effects of 12-phorbol, 13-myristic acetate (PMA) on the translocation of cPLA<sub>2</sub>. Previous studies (35) have shown that, although calcium is important, PMA can still trigger the translocation and activation of cPLA<sub>2</sub> under conditions with reduced calcium. As shown in Figure 8, in the presence of calcium chelators, we found that treatment with PMA for 10 minutes did allow us to measure an increase in full length, membrane associated cPLA<sub>2</sub>. The increased levels of membrane-associated cPLA<sub>2</sub> were also observed after 30 minutes but decreased to background levels after 1 hour. We conclude, therefore, that our isolation and purification procedures are sound and that calcium is indeed a key requirement for cPLA<sub>2</sub> membrane binding during TNF induced apoptosis.

### **Fatty Acid Analysis Using GC/MS**

As described above we were unable to use biochemical fractionation to monitor the intracellular movement of cPLA<sub>2</sub> during apoptosis. As an alternative, we decided to examine cell membranes for the footprint of cPLA<sub>2</sub> activity. cPLA<sub>2</sub> cleaves arachidonic acid from phospholipid and we hypothesized that membranes that were targeted by cPLA<sub>2</sub> should have reduced arachidonic acid content. Furthermore, since arachidonic acid is selectively integrated at the *sn*-2 position of phospholipids (which is where cPLA<sub>2</sub>



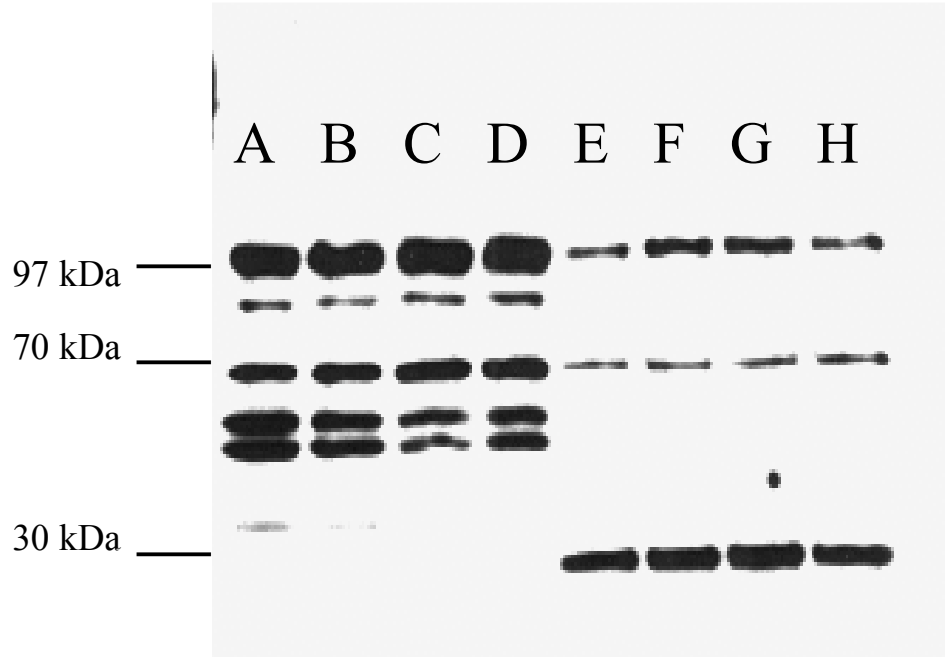


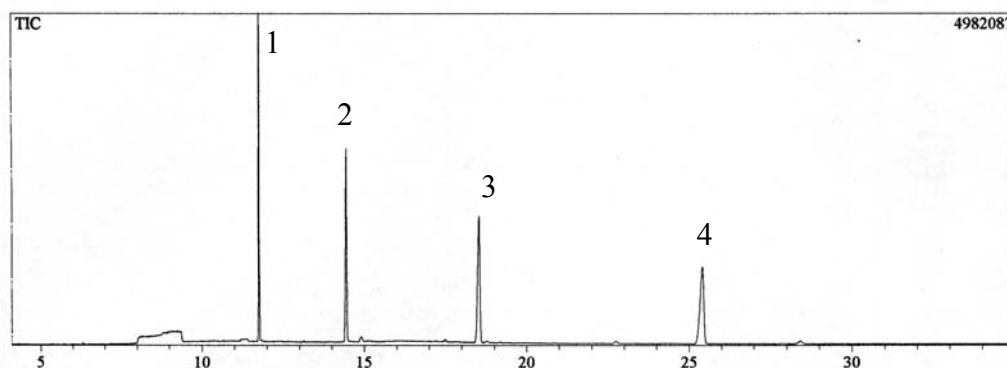
Figure 8. Subcellular distribution of cPLA<sub>2</sub> in membrane and cytosolic fractions following treatment with PMA.  $2 \times 10^6$  cells were either treated with PMA (10ng/ml) for 10 minutes, 30 minutes, 60 minutes or left as untreated controls. Cells were disrupted with a tissue homogenizer and centrifuged for 1 hour at 100,000 X g to pellet intracellular membranes. Pellets were resuspended and 20  $\mu$ g of protein of each sample was subjected to SDS PAGE, transferred to a nitrocellulose membrane and probed using anti-cPLA<sub>2</sub> Ab. Lanes A through D represent cytosolic fractions, lanes E through H represent membrane fractions. Lanes A and E are untreated controls, lanes B and F are from cells treated with PMA for 10 minutes, lanes C and G are from cells treated with PMA for 30 minutes and lanes D and H are from cells treated with PMA for 60 minutes.

cleaves) we focused our experiments on determining the amount of arachidonic acid at the *sn*-2 position. Gas Chromatography coupled with mass spectroscopy (GC/MS) was the method we used to analyze fatty acid content. Cells were treated, and either subcellular membranes or nuclei were isolated as before. Subsequently, all *sn*-2 position fatty acids were released with a non-specific form of PLA<sub>2</sub> (naja naja snake venom sPLA<sub>2</sub>). Cleaved fatty acids were then separated from undigested phospholipid using silicic acid, and a methylation reaction was performed to facilitate their movement through the GC column. Mass spectroscopy was then used to identify individual fatty acids.

Four fatty acids that are commonly found within cellular membranes were chosen for standards to calibrate the GC/MS. Figure 9, panel A is a chromatogram of these standards run as a mixture to show the resolving ability of the GC, the retention time, and the mass spectra of these compounds. Peak 1 is tetradecanoic acid, methyl ester (methyl myristate), peak 2 is hexadecanoic acid, methyl ester (methyl palmitate), peak 3 is *cis*, *cis*-9, 12-octadecadienoic acid, methyl ester (methyl linoleate), and peak 4 is all *cis*-5,8,11,14-eicosatetrenoic acid, methyl ester (methyl arachidonate).

Panel B of Figure 9 is a chromatogram of untreated C3HA cells to show the normal, *sn*-2 fatty acid content of C3HA cell membranes (obtained by mechanical disruption and high speed centrifugation). Methyl myristate, methyl palmitate, methyl

Panel A



Panel B

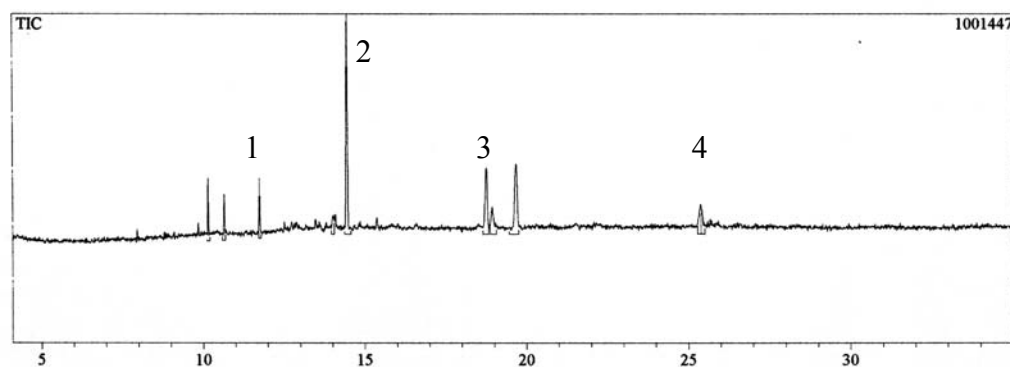


Figure 9. Analysis of fatty acid standards and controls by GC/MS. In panel A, four fatty acid standards were run on GC/MS. Peak 1 is tetradecanoic acid, methyl ester (methyl myristate), peak 2 is hexadecanoic acid, methyl ester (methyl palmitate), peak 3 is *cis*, *cis*-9, 12-octadecadienoic acid, methyl ester (methyl linoleate), and peak 4 is all *cis*-5,8,11,14-eicosatetrenoic acid, methyl ester (methyl arachidonate). Panel B is a chromatogram of untreated C3HA cellular membranes. The numbered peaks correspond to the same numbers from panel A.

linoleate, and methyl arachidonate are all clearly discernable peaks with calculable area. Several other peaks were observed, however, these were determined not to be fatty acids. To express these data quantitatively, each fatty acid was described as a percent of the four major peaks observed. A summary of these data are shown in Table 1. Typically, we found fatty acid content to be: myristate (10%), palmitate (45%), linoleate (27%), and arachidonate (17%).

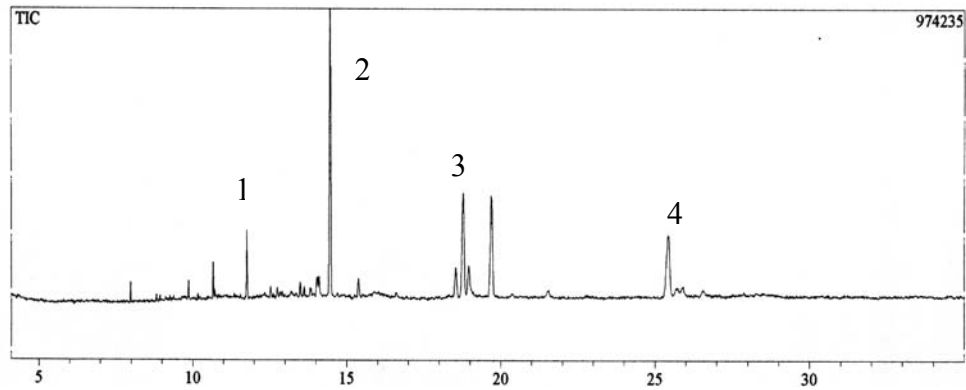
Next we examined fatty acid content in membranes from cells triggered to undergo apoptosis by TNF and CHI. As shown in Figure 10, all four major fatty acid peaks are present, and have calculable areas. Typically, we found that the contents of methyl myristate and methyl palmitate were similar to that of the untreated cells, while the content of methyl linoleate did decrease somewhat, changing 27.30% to 24.42% (mean values). Interestingly and contradicting our prediction, the amount of arachidonic acid increased when compared to untreated cells, changing from 17.44% in untreated to 21.47% in the TNF and CHI treated cells. This result was surprising, as it would be expected that treatment with TNF and CHI would cause activation of cPLA<sub>2</sub>, translocation to the membrane and cleavage of arachidonic acid. We were concerned that the sensitivity of the method may not be able to detect the change in arachidonic acid content in response to TNF and CHI, so we next used the treatment of lithium chloride and the calcium ionophore A23187. This treatment combination has been shown to cause much greater levels of arachidonic acid release than do TNF and CHI (C.C., personal communication). Treatment of C3HA cells with lithium chloride in combination with

Table 1. Summary of Data from Fatty Acid Analysis

	% Methyl Myristate	% Methyl Palmitate	% Methyl Linoleate	% Methyl Arachidonate
Untreated cells	10.43 ± 3.92	44.87 ± 3.34	27.30 ± 1.32	17.44 ± 1.77
TNF and CHI	9.93 ± 5.27	44.19 ± 4.62	24.42 ± 3.17	21.47 ± 6.72
LiCl and A23187	8.23 ± 2.14	38.62 ± 3.27	27.40 ± 6.65	25.76 ± 5.53

Table 1. Fatty acid analysis of C3HA cellular membranes. Cells were treated with either TNF and CHI, LiCl and A23187, or left untreated. Fatty acids were extracted, and then methylated. Samples were run on GC/MS and peaks were identified by comparison with the NTIS library. Data represented as mean values ± SEM.

Panel A



Panel B

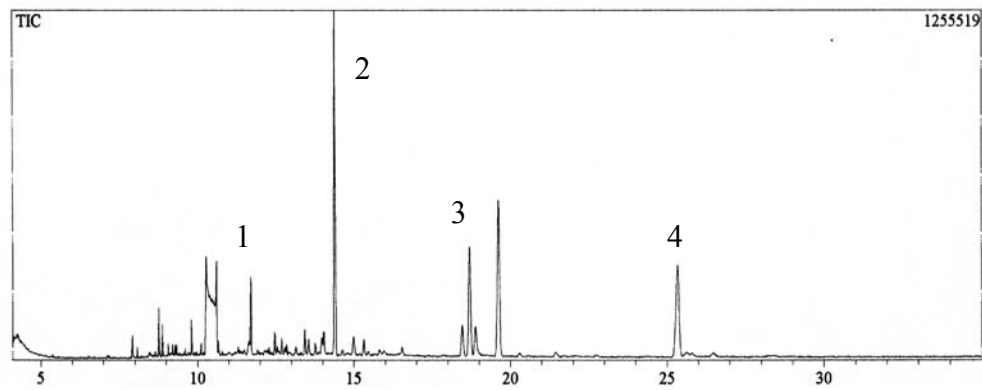


Figure 10. Fatty acid analysis of C3HA cellular membranes. Cells were treated with TNF and CHI for 6 hours, fatty acids were extracted, and then methylated (panel A). Samples were run on GC/MS and peaks were identified by comparison with the NTIS library. In panel B, cells were treated with LiCl and A23187. Samples were prepared as in panel A. For both panels, peak 1 corresponds to methyl myristate, peak 2 with methyl palmitate, peak 3 with methyl linoleate, and peak 4 with methyl arachidonate.

A23187 typically causes 100%  $^3\text{H}$ -arachidonic acid release from prelabeled cells. Again however, as shown in Figure 10, panel B the amount of arachidonic acid in cell membranes was increased over control cells.

The results of our GC/MS analysis were surprising. Rather than finding a decrease in arachidonic acid content of cell membranes following cPLA<sub>2</sub> activation we found that arachidonic acid content was enhanced. We hypothesized that the cell was responding to the loss of arachidonic acid from established membranes by increasing the synthesis of new membranes with high arachidonic acid content. We therefore decided to focus more specifically on the nuclear membrane, the site of cPLA<sub>2</sub> translocation as determined by immunofluorescence. Figure 11 shows a typical fatty acid profile from untreated C3HA nuclei. As can be seen, the nuclei did not contain any arachidonic acid. The lack of arachidonic acid in these nuclear fractions makes it impossible to examine treated cells for even lower levels of this fatty acid and therefore we did not continue this analysis. The lack of arachidonic acid in nuclei also raises questions concerning the activity of cPLA<sub>2</sub> when it is bound to nuclear membranes. It is also possible that cPLA<sub>2</sub> could be bound to the endoplasmic reticulum, which is closely associated with the nuclear membrane.

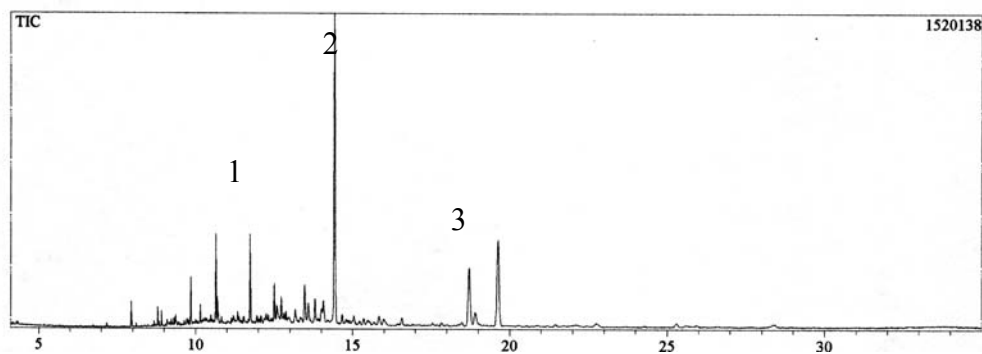


Figure 11. Fatty acid analysis of nuclei. Nuclei of untreated C3HA were isolated, fatty acids were extracted, methylated, and run on GC/MS. Peaks were identified by comparison with the NTIS library. Peak 1 corresponds to methyl myristate, peak 2 with methyl palmitate, and peak 3 with methyl linoleate.



## Discussion

The goal of these studies was to identify the intracellular membrane target for cPLA<sub>2</sub> during TNF-induced apoptosis. By identifying the site, and understanding how cPLA<sub>2</sub> interacts with that membrane, it may be possible to design compounds to inhibit cPLA<sub>2</sub> activation. These compounds would be important for preventing damage to liver, pathologies of IBD and rheumatoid arthritis, and others, during autoimmune disease. We used a number of different techniques to identify cPLA<sub>2</sub> in the membrane: immunofluorescence, subcellular fractionation and GC/MS. Each technique has shortcomings, and our goal was to use complementary techniques to avoid pitfalls.

Immunofluorescence microscopy yielded the best results. With this technique we were able to observe the translocation of cPLA<sub>2</sub> to the perinuclear region of the cell, the nuclear membrane, and intranuclearly. These results are somewhat consistent with what has been reported by others for cPLA<sub>2</sub>. Schievella et al. (43) found translocation of cPLA<sub>2</sub> to the nuclear envelope in response to the calcium ionophore A23187 and Liu et al. (40) reported movement to the nuclear membrane in response to PMA and the calcium ionophore ionomycin. While our results seem to agree with these reports, they conflict with others. Schievella (43) also reported a translocation to the endoplasmic reticulum, and Liu (40) showed movement to the endoplasmic reticulum and the plasma membrane. One possible explanation for the discrepancies is the difference in both cell lines and ligands. Other studies have used inflammatory stimuli such as calcium ionophores and PMA. These ligands may induce a different pathway of cPLA<sub>2</sub> activation than apoptotic

stimuli, possibly with different membrane targets. The response may also vary based on the cell line used, as one study used Chinese hamster ovary cells, and another used rat glomerular epithelial cells (40, 43).

In the course of our immunofluorescent studies, we observed a distinct ring like structure of staining around the nucleus. To further investigate this we used an antibody to the nuclear pore complex that is commonly used to study nuclear membrane characteristics and nuclear structure (55). Staining with this antibody showed a similar ring like structure around the nucleus. This pattern of cPLA<sub>2</sub> staining, coinciding with the staining of the nuclear pore complex, suggests that cPLA<sub>2</sub> was localizing at the nuclear membrane. We also observed increased staining at what appeared to be the intranuclear area. To investigate the possibility of cPLA<sub>2</sub> binding to chromatin within the nucleus we used Acridine Orange. Acridine Orange intercalates into DNA and RNA, displaying green fluorescence when bound to DNA and red fluorescence when bound to RNA. The Acridine Orange exhibited similar aggregates of heavy staining within the nucleus, as did the cPLA<sub>2</sub>. This does suggest that cPLA<sub>2</sub> binding to chromatin within the nucleus is a possibility, however, more investigation is necessary to determine if this is occurring. If the intranuclear aggregates are not a result of cPLA<sub>2</sub> binding to chromatin, another possible explanation for the intranuclear staining is membrane invaginations. Around the nucleus, membrane areas fold in and can form pocket like structures (58), and we may be seeing cPLA<sub>2</sub> that is still on the nuclear membrane but appears to be inside the nucleus.

Since our results from the cPLA<sub>2</sub> staining and the nuclear pore staining strongly indicate nuclear membrane binding, we investigated the possibility of nuclear binding using GC/MS analysis of fatty acid content in the nuclear membrane. The results of the GC/MS experiments with nuclei appear to be very contradictory to our immunofluorescent results, as we found an absence of arachidonic acid in the nuclei sample from GC/MS. This finding is surprising, since cPLA<sub>2</sub> must move to a membrane to exert its activity. This raises the question as to why cPLA<sub>2</sub> would translocate to a membrane without arachidonic acid. There are several possible explanations that we can consider. First, there is the potential that cPLA<sub>2</sub> is playing another role in the pathway, and may exert other activity at the membrane, possibly colocalizing with other factors involved in apoptosis. Another possible explanation is that our immunofluorescent images show binding that appears to be nuclear membrane associated, but may in fact be at the endoplasmic reticulum. The endoplasmic reticulum and the nuclear membrane are intimately associated, and the possibility does exist that what we believed to be nuclear binding is really binding to the ER. Our nuclei isolation protocol would not include the ER, and therefore isolation of nuclei would yield a different result than the fluorescence. This possibility of cPLA<sub>2</sub> binding to the ER needs further study, including organelle specific stains to identify the exact pattern of ER staining within the cell. Carbocyanine dyes and Brefeldin A both stain the endoplasmic reticulum in cells, and are two possible stains that could be utilized to more closely investigate the possibility of cPLA<sub>2</sub> binding to the ER.

Our approach to studying the translocation of cPLA<sub>2</sub> was based on complementary techniques, and included biochemical fractionation of cells. We did not find any stable membrane binding of cPLA<sub>2</sub> using this technique. For both subcellular membrane isolation and nuclei isolation, there was no translocation seen in response to TNF and CHI. We believe this to be a result of the calcium-dependent nature of TNF-induced binding of cPLA<sub>2</sub> to membranes. This would suggest that the calcium-binding domain of cPLA<sub>2</sub> (CalB) is critical, and reduces the likelihood of intracellular receptors in the binding. The premise of the biochemical fractionation method is sound, and it would be very useful in investigating the specific membrane location of cPLA<sub>2</sub>. Possibly a buffer with precise amounts of chelators vs. calcium could be devised to allow a calcium dependent association to membranes without artifactual binding. Another potential solution to the method problem involves a different technique of lysis. Schievella et al. (43) used nitrogen cavitation, possibly that would preserve membrane binding more accurately than mechanical or chemical lysis. The results of our investigation demonstrating a lack of calcium-independent binding raises the possibility of using calcium inhibiting drugs as treatments for autoimmune diseases. Although specific work on the subject has not been performed, one study has shown that treatment of nonobese diabetic mice, the model for insulin dependent diabetes mellitus, with compounds that reduce the serum levels of calcium and blocking chemicals in the body involved in increasing intracellular calcium levels, is able to prevent clinical diabetes when treatment began before the onset of insulinitis (59). Perhaps this does imply a

possible role for calcium in autoimmune disease.

As mentioned earlier, we used GC/MS analysis in our investigations. Our goal was to find a footprint of cPLA<sub>2</sub> activity, as evidenced by membrane arachidonic acid content. The results from these studies were surprising. We have extensive data showing release of arachidonic acid after treatment with TNF and CHI from cells prelabeled with <sup>3</sup>H-arachidonic acid, however, our GC/MS data of whole cell membranes showed an increase in the arachidonic acid content following cPLA<sub>2</sub> activation. After obtaining these surprising results, we tried an even stronger activator of cPLA<sub>2</sub>. Cells treated with lithium chloride and A23187, which causes a 100% release of arachidonic acid from prelabeled cells, had an even higher arachidonic acid content in their membranes. It is possible that the cells are responding to the activity of cPLA<sub>2</sub> and synthesizing new membranes, perhaps transacylase enzymes have been activated and are repairing the arachidonic acid cleavage sites with new arachidonic acid. No clear answer is present right now, but these are interesting results and should be followed up on with further work. Potentially studies with inhibitors of membrane rebuilding and synthesis of arachidonic acid could be very interesting.

Our experiments also revealed another interesting observation. Nuclear fractionation revealed several lower molecular weight forms (70 and 30 kDa) of proteins that cross-reacted with cPLA<sub>2</sub> antibody. The strength of the reaction suggests that they are indeed derived from cPLA<sub>2</sub>, and associated with the nucleus. It is possible that they represent alternatively spliced products. The gene for cPLA<sub>2</sub> has a number of exons,

however, little is known about their alternative splicing. The lower molecular weight forms could also be the result of degradation. Degradation products of cPLA<sub>2</sub> have been reported, however they were either not the same size as those seen with our fractionation or seen at very late times in apoptosis. Voelkel-Johnson et al. (57) reported cPLA<sub>2</sub> cleavage by intracellular proteases in response to TNF and CHI, however this cleavage was seen at 6 hours, whereas the fragments we observed were present in untreated cells and did not vary following treatment. Adam-Klages et al. (60) also reported a 70 kDa fragment of cPLA<sub>2</sub>, but again, their cells were treated with apoptotic stimuli for 16 hours and allowed to die. Another question is why these fragments are in the nucleus. Are they an active form of cPLA<sub>2</sub> with a biological purpose, or perhaps simply a by-product of degradation? It is interesting to note that their nuclear binding would appear to be calcium independent, raising the possibility of a receptor for these fragments. If these fragments were found to have biologically important activity, a nuclear receptor would become a possible target for drug design.

Our data suggests that it is important to understand the association of cPLA<sub>2</sub> with the nuclear membrane, and potentially the endoplasmic reticulum. The immunofluorescent observations point to the nuclear region as being a target of cPLA<sub>2</sub> in TNF-induced apoptosis. The results of our investigations left several unanswered questions. Further fluorescent studies should focus on ER markers and try to colocalize cPLA<sub>2</sub> with either the ER or nucleus using fluorescence and confocal microscopy. Confocal microscopy offers the benefit of directly examining cPLA<sub>2</sub> and the marker in

three dimensions, potentially reducing the confusion over membrane locations. More work can also be done with the biochemical studies. Perhaps a buffer can be found that would balance the need for calcium in the system without creating artifactual binding. If this difficulty could be overcome, separation of the subcellular fractions by gradient would be very informative. Markers of ER, golgi, nuclei and other organelles can be used to probe immunoblots to determine the exact membrane location of cPLA<sub>2</sub>. The GC/MS results also warrant further investigation. There are many questions about membrane rebuilding to answer, and the lack of arachidonic acid in nuclei is an important result to understand. Although our investigations did provide us with many questions to answer, we did establish that cPLA<sub>2</sub> translocates to the nucleus in a calcium-dependent fashion during TNF-induced apoptosis. These observations provide us with information, and open up new avenues of investigation. cPLA<sub>2</sub> is a key player in TNF-induced apoptosis, and understanding its regulation and activity is important in the effort to control many TNF related diseases.

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