ABSTRACT

BRYANT, ANNA MINNIS. β,β'-Iminodipropionitrile-Induced Movement of Vimentin Intermediate Filaments to the Perinuclear Region is Insufficient to Inhibit $cPLA_2$ Activity. (Under the direction of Scott M. Laster).

Cytosolic phospholipase A_2 ($cPLA_2$) is an important enzyme in the biosynthetic pathway for prostaglandins; inflammatory molecules that contribute to conditions such as fever, rash, asthma, and even tumor development. The regulation and manipulation of this pathway have important medical implications for the understanding and treatment of a number of inflammatory conditions. Our lab discovered that Adenovirus (Ad) is capable of inhibiting production of PGE_2 through inhibition of $cPLA_2$-mediated release of arachidonic acid (AA), the first substrate in the prostaglandin pathway. This effect correlated with abnormal translocation of the enzyme with the intermediate filament protein vimentin to the perinuclear region. In this study, we examined the effect of artificially-induced vimentin translocation on $cPLA_2$ activity by treating with the drug 3',3'-iminodipropiontrile (IDPN). IDPN caused the same relocalization of vimentin and $cPLA_2$ as seen in Ad-infected C3HA murine fibroblasts. However, we did not observe suppression of release of AA in PMA stimulated IDPN-treated cells as was observed with adenovirus. We conclude, therefore, that the movement of vimentin is not sufficient for the inhibition of PGE_2 release found in Ad-infected cells. Our data suggest a more complex mechanism than mislocation is responsible for Ad-mediated inhibition of $cPLA_2$ activity. Further investigation into this effect will allow us to better characterize the regulation of $cPLA_2$ during both Ad infection and homeostasis.

β,β'-Iminodipropionitrile-Induced Movement of Vimentin Intermediate Filaments to the Perinuclear Region is Insufficient to Inhibit $cPLA_2$ Activity

by
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BIOGRAPHY

Anna M. Bryant was born in 1982 to Patrick and Deborah Minnis in Newport News, Virginia. She attended Hampton Roads Academy for high school, graduating in 2000. She attended Old Dominion University and graduated in 2005 with her Bachelor's of Science. Her undergraduate studies focused on the anatomy and immunology. The following year, she worked under Dr. Robert Ratzlaff at ODU studying the newly discovered PaV1 virus, an infectious agent found in the Caribbean spiny lobster. In 2007, Anna entered the graduate program in Microbiology at North Carolina State University and began her work under Dr. Scott Laster.

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INTRODUCTION

Inflammation and Lipid Mediators

Inflammation

Inflammation is a complex biological response resulting from injury or illness. Its purpose is to protect and rebuild the compromised areas. For example, inflammation can help by increasing blood flow so that immune cells can gain access to injured tissue. Unfortunately, inflammation does not always result in a healthful conclusion. For this reason, it is implicated in a number of diseases affecting all parts of the body. Many of the typical allergic symptoms are due to inflammation, and chronic illnesses such as atherosclerosis are caused partly by sustained inflammatory reactions. Asthma, a disease which currently afflicts around 300 million people worldwide, is a result of abnormal inflammation of the airways, which restricts breathing (1). Some symptoms of Alzheimer's disease may be due to the production of damaging inflammatory mediators produced in the brain that leads to destruction of brain tissue, and anti-inflammatory drugs have been shown to delay the onset of Alzheimer's disease (2). Inflammatory bowel disease, including ulcerative colitis and Crohn's disease, is still not fully understood, but it is well-recognized that atypical immune cell responses and inflammatory molecules contribute greatly to the pathogenesis (3).

The complexity of inflammation lies in the numerous effector, or signaling, molecules and cell types involved, which include immune cells such as macrophages and neutrophils, as well as non-immune cells. Signaling molecules fall into several different categories. Protein

mediators of inflammation are cytokines and chemokines, while lipid mediators include eicosanoids such as prostaglandins, thromboxanes, and leukotrienes. Prostaglandins are found in nearly all tissue types, while thromboxanes are only present in platelets, where they function in clotting, and leukotrienes are notably involved in regulating respiratory function (4). The multifaceted functions of eicosanoids have made them targets for a number of drug therapies.

Role of prostaglandins in inflammation

The prostaglandins, which include PGE_2, $PGF_2\alpha$, and PGI_2, are secreted from cells upon stimulation by signaling molecules. They then bind to prostaglandin receptors, initiating paracrine and autocrine signaling cascades that result in regulation of other inflammatory mediators, intracellular Ca^{2+} concentrations, and cell growth factors, among others. Some of the common symptoms of inflammation, such as redness, pain, and swelling, are due to the vasodilation caused by prostaglandins. PGE_2 is the most commonly produced prostaglandin, and has been implicated in suppression of inflammatory cytokines and plays a role in T-cell differentiation (5).

Prostaglandin biosynthesis

Arachidonic acid (AA) is the first molecule produced in the biosynthetic pathway for all eicosanoids, which are processed into their final 20-carbon form through a series of enzymatic reactions. Cytosolic phospholipase A_2 ($cPLA_2$) catalyzes the hydrolysis of the sn-2 acyl bond of phospholipids to produce arachidonic acid, which is then processed by

cyclooxygenases 1 and 2 (COX-1 and COX-2) to the intermediates PGH_2 or hydroperoxy-eicosatetraenoic acid. Finally, specific synthases catalyze the formation of prostaglandins, leukotrienes or thromboxanes. COX-1 is constitutively expressed in most cell types, while COX-2 is induced in response to various stimuli. Classic NSAIDs such as aspirin target both enzymes, while rofecoxib (Vioxx®) and celecoxib (Celebrex®) are specific COX-2 inhibitors. However, both types of inhibitors have been found to cause dangerous side effects. COX-1 inhibition from NSAIDs leads to gastric complications, while COX-2-specific inhibition can lead to cardiovascular issues including cardiac arrest and ischemic stroke (6). Greater understanding of regulation of the prostaglandin biosynthetic pathway is needed to create safer and more effective drug therapy for inflammatory illness.

$cPLA_2$ activation and regulation

Control of prostaglandins occurs mainly through regulation of their biosynthesis. The enzymes responsible for each stage in the biosynthetic pathway are regulated through several mechanisms. Cytosolic phospholipase A_2 ($cPLA_2$) is a member of the phospholipase A_2 group of proteins, which also includes a secretory phospholipase and a Ca^{2+}-independent phospholipase. $cPLA_2$ is a constitutively expressed protein in most cell types, and its activation requires phosphorylation at several sites as well as an increase in intracellular Ca^{2+} concentration. Phosphorylation is believed to be essential for altering the molecular shape and allowing for interaction with its substrates (6, 7). Various MAP kinases are responsible for this phosphorylation, and the most important site appears to be Ser505; loss of phosphorylation at this site eliminates AA production (7). Upon activation, $cPLA_2$

translocates to one of several cellular membranes. The Ca^{2+} binding domain, or C2 domain, at the N-terminal region of the enzyme, regulates this movement. Upon binding to membranes, ceramide-1-phosphate and other docking proteins may be involved in activating and maintaining the position of $cPLA_2$ in the lipid membrane (8, 9). The combination of these and potentially other yet undiscovered regulatory mechanisms allows $cPLA_2$ to move to different cellular membranes.

Under normal conditions, $cPLA_2$ is found diffuse in the cytoplasm, although in subconfluent cells, it may exist in the nucleus (10). Confluence can also affect the translocation of $cPLA_2$ under stimulation; confluent endothelial cells treated with histamine show $cPLA_2$ localized at the plasma membrane as well as the nucleus, while subconfluent cells showed $cPLA_2$ at the nucleus only (10). Fractionation studies have demonstrated $cPLA_2$ binding to the nuclear membrane (11), the Golgi apparatus (12, 13), and the endoplasmic reticulum (14). To which membrane $cPLA_2$ translocates can vary based on cell type. Grewal, et al. found that $cPLA_2$ moves to the nuclear membrane in A23187-stimulated EA.hy.926 epithelial cells, whereas similarly stimulated A549 epithelial cells showed $cPLA_2$ concentrated near the Golgi (14). Other enzymes in the eicosanoid synthetic pathway have been found to localize at the nuclear envelope with $cPLA_2$, perhaps allowing for proximity of all intermediates to their respective catalysts to streamline the process (15). Exactly how $cPLA_2$ moves throughout the cell is likely different based on the source of stimulation, although it is known to interact with a number of different proteins**.** Nakatani, et al. showed that the intermediate filament protein vimentin binds $cPLA_2$ via its head domain (16). Cells without vimentin show an increase in

PG production when the intermediate filament protein is introduced into the cell, and disruption of the binding between the two proteins inhibits AA release (16). These results demonstrate an important role for vimentin in regulating the activity of $cPLA_2$.

Vimentin

Structure and Function

Vimentin is a 60 kDa protein that forms type III intermediate filaments in cells of mesenchymal and ectodermal origin. As with other intermediate filaments, vimentin has an alpha-helical rod domain flanked by the two non-helical domains. The amino- and carboxy-terminal regions are conserved among intermediate filaments, while the rod domain varies considerably (17). Vimentin is responsible for many different cellular functions. In adipocytes, vimentin rearranges into a cage-like structure surrounding lipid droplets and plays a role in MAP kinase signal transduction (18). Vimentin is essential for spreading and adhesion in macrophages (19) and fibroblasts (20), with the filaments constantly reassembling at the cell's edge. Vimentin is also important for resistance to shear stress in endothelial cells (21) and maintaining cellular shape and integrity in epithelial and other cell types (22). Nuclear shape and integrity are also supported by an intact vimentin network (22).

Vimentin, like other intermediate filaments, associates with the other major cytoskeletal proteins actin and tubulin. Intracellular staining shows the overlap of intermediate filament and microtubule networks, and collapse of microtubule networks by nocodazole will result in a collapse of the vimentin cytoskeleton (23). Furthermore, vimentin is known to move along

MT tracks using the molecular motors dynein and kinesin (24). Kinesin is responsible for plus-end directed movement (towards the cell's membrane), and inhibition of this protein will result in accumulation of vimentin at the nuclear interface. Specifically, vimentin binds the heavy chain and the 62-kDa light chain of kinesin. Dynein-dynactin complexes direct vimentin movement towards the nucleus; when dynactin is disrupted through overexpression of dynamitin, vimentin is found primarily clustered at the cell's periphery (24).

Vimentin association with actin is less studied than the interaction with MT, but is known to be important. For example, cells with intact vimentin-actin interactions are stiffer than those with that interaction disrupted (25). Furthermore, *vim*$^{-/-}$ mice have changes to their actin cytoskeleton (26), and *in vitro* disruption of vimentin results in loss of stress fiber formation (27). Vimentin is also involved in the subcellular localization of a number of cellular components. RhoA-binding kinase alpha, for example, colocalizes with vimentin, and will translocate in the cell even without microtubules present (28). 14-3-3 proteins, which include cell-cycle control proteins, bind directly to vimentin's head domain and may be transported throughout the cell as vimentin moves along MT tracks (29). Vimentin association with these signaling molecules broadens its function in the cell to include an important role in mediating signal transduction through molecule trafficking and scaffolding.

Numerous studies have shown the importance of vimentin in regulation of organelle translocation in cells. Vimentin is known to bind to the Golgi, mitochondria, and the endoplasmic reticulum in various cell types. Disruption of vimentin via microtubule collapse results in reorganization of the Golgi apparatus, and several proteins have been discovered to

mediate the binding of the Golgi to vimentin IF, including MICAL-1 (30) and the Golgi protein formiminotransferase cyclodeaminase (FTCD) (31). Styers, et al showed that treatment of cells with Brefeldin A, an antibiotic that inhibits transport of proteins from ER to Golgi and can induce retrograde transport towards the nucleus, resulted in bundling and nuclear localization of vimentin in vimentin-expressing SW13 cells (32). This indicates that vimentin may be bound to these membranes and a functional part of the transport process. Katsumoto, et al. found structural links between vimentin and the endoplasmic reticulum (33). Using electron microscopy, they visualized attachments between vimentin and the endoplasmic reticulum as well as the Golgi and mitochondria. One linker protein, plectin, is possibly the link between vimentin and mitochondria. Plectin, a linker molecule that binds vimentin, was proven by Winter, et al. to insert in the outer membrane of mitochondria. In cells lacking plectin, mitochondrial shape was altered (34). Furthermore, immunofluorescence showed that plectin colocalizes with mitochondria and vimentin. Minin and Moldaver inhibited mitochondrial motility through phosphorylation of vimentin, and interactions with other IF have been shown to impact mitochondrial function (20).

Movement: phosphorylation and cleavage

Vimentin is a dynamic protein, and the filaments exist in several states as they relocalize throughout the cell. Individual vimentin proteins form rudimentary squiggles, which then form short filaments, which then line up end to end to create the full-length vimentin filaments.

Vimentin is phosphorylated at a number of different sites by various kinases. Phosphorylation at specific amino acid residues seems to be associated with certain cellular states. For example, phosphorylation at Ser55 occurs during early mitosis, and allows for binding of other kinases that further phosphorylate vimentin later on (35). Ser72 is phosphorylated by Aurora-B during cytokinesis, allowing the movement of vimentin towards the cleavage furrow and proper segregation of the filaments upon separation (36).

Vimentin is further regulated through cleavage. Notably, the protease calpain, a Ca^{2+} activated enzyme, is known to cleave vimentin into both large and small fragments (37). Furthermore, caspases-3 and -8 cleave vimentin, and this cleavage disrupts nuclear membrane integrity and is correlated with apoptosis (38).

Adenovirus

Structure and infectious cycle

Adenovirus was first isolated in 1953 from adenoid tissue culture lines. The virus is mostly associated with mild respiratory infections in infants and children, but is also a cause of acute respiratory disease (ARD). ARD affects military recruits, and adenovirus vaccines have been explored as a means of reducing the incidence of ARD. Adenovirus also causes conjunctivitis, and in rare instances can cause hepatitis, myocarditis, and gastroenteritis. It can cause tumors in laboratory animals, and as such, has contributed much to the understanding of oncogenic viruses (39).

The virus is an icosahedral, non-enveloped, double-stranded DNA virus with over 50 human serotypes. It has a capsid composed of hexon capsomeres, with penton proteins sticking out from the surface. Adenovirus infection begins with attachment of the knob domain of the penton protein binding to the Coxsackie and Adenovirus receptor (CAR). A secondary interaction between an alpha-*v* integrin on the cell surface and the penton base protein of the virus capsid induces signaling pathways that cause reorganization of the actin cytoskeleton to allow for uptake of the virus through clathrin-mediated endocytosis. Acidification of the endosome allows disassembly of some of the viral proteins. The virus is then transported to the nucleus, where the viral genome enters via nuclear pores (39).

The first genes to be transcribed are the early genes- E1 through E4. E1A and E1B are well-characterized, and are involved in control of host cell cycle, among other functions. For example, E1A binds to the retinoblastoma protein (Rb), which controls cell cycle progression to the S phase. Rb binds to E2F, a transcription factor. It prevents E2F binding to DNA and activating transcription unless phosphorylated, upon which it releases E2F. When E1A binds to the hypophosphorylated Rb, E2F loses access to its binding site on Rb and is released to activate transcription. In this way, E1A pushes the cell towards synthesis of DNA, which allows for transcription and replication of its own genome. E1A also acts a transactivator of the Adenovirus genome. It activates transcription of E1B, which acts a transactivator of viral genome transcription and binds the tumor suppressor protein p53. In this way, E1B prevents the cell from undergoing p53-induced apoptosis (39).

Once the early phase of replication is complete, the viral genome is replicated. The late phase of replication involves transcription of the 5 late genes, as well as production and assembly of adenovirus capsid proteins. Proteins begin assembling in the cytoplasm and the full capsid is completed in the nucleus, where DNA is inserted. The virions may accumulate in the nucleus, or the cell will lyse to release the progeny (39).

Adenovirus and inflammation

Adenovirus infection causes a rapid and strong innate immune reaction, with accompanying inflammation. While most adenovirus infections cause mild symptoms, and can be cleared within 10 days, infections with serotypes 4 and 7 can cause lethal levels of inflammation (39). As adenovirus is explored as a vector for vaccines and gene therapy, the mechanisms by which the virus can cause inflammation must be understood in order to reduce harmful side effects. In 1999, the clinical trial of an adenovirus vector for enzyme replacement therapy resulted in the death of a subject due to massive inflammation in the liver, the site of injection of the vector (40). Inflammatory responses to adenovirus can be initiated in the earliest stages of infection, without expression of viral genes. There are numerous pathways activated in both immune (macrophages, dendritic cells) and non-immune cells (A549, HeLa, C3HA). Toll-like receptors (TLRs) recognize molecular patterns, and when activated, lead to production of inflammatory cytokines and interferons in Ad-infected cells (41). Ad-mediated activation of TLR9 in plasmacytoid dendritic cells leads to type 1 interferon production (42), and in peritoneal macrophages stimulates release of IL-6 (43). Involvement of MAP kinase pathways, including activation of ERK1/2 and p38, leads to production of

PGE_2 in synoviocytes (44). Alveolar macrophages are stimulated by adenovirus infection to produce TNF-α, IL-6, MIP-1α, and MIP-2 within 6 hours post-infection (42).

Previous work in our lab characterized the PG response elicited by Ad5 *in vitro* in C3HA cells. $cPLA_2$ is activated rapidly, and AA release is strong within the first two hours of infection. After 18 hours of infection, however, the release of both AA and PGE_2 decreases to control levels (45). Furthermore, the release of AA and PGE_2 is suppressed when cells are exposed to inflammatory stimulants such as LPS, PMA, A23187, and IL-1. This inhibition occurred despite normally increased intracellular Ca^{2+} levels, continued phosphorylation of $cPLA_2$, and expression of COX-2. Immunofluorescent studies revealed that the movement of $cPLA_2$ in response to PMA was altered in Ad5-infected cells compared to mock-infected cells. In uninfected cells, $cPLA_2$ moves to the perinuclear region upon activation, giving the cells a bright halo around the nucleus where it is ringed by $cPLA_2$ molecules. In Ad5-infected cells, however, staining after treatment with PMA reveals no change in the location of $cPLA_2$ compared to unstimulated Ad5-infected cells (45). Further studies showed that the position of vimentin, but not that of tubulin or actin, was altered during Ad5 infection. Nakatani, et al. showed that vimentin bound to the C2 domain of $cPLA_2$ via its head domain (16). Disruption of this binding resulted in lower AA and PGE_2 release. These authors demonstrated a crucial role for vimentin in regulating $cPLA_2$ activity. Ad-induced movement of vimentin could, therefore, represent a mechanism by which $cPLA_2$ activity is inhibited. We hypothesized that vimentin caused translocation of $cPLA_2$ to an abnormal cellular compartment that resulted in its inactivity. To test this, we used the drug β,β'-

iminodipropiontrile (IDPN) to mimic the translocation of vimentin seen during Ad infection. We then examined the movement of the ER, Golgi, and $cPLA_2$ and the release of AA upon stimulation with PMA. Our results showed that vimentin movement can cause relocalization of $cPLA_2$, the ER, and the Golgi, but is insufficient to inhibit the release of AA.

MATERIALS AND METHODS

Reagents

All medium and chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The murine $cPLA_2$ mAb (sc-454) and murine monoclonal IgG vimentin Ab (sc-32322) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The Ad E1A murine mAb (MS-588-P1) was purchased from Labvision Corp., Fremont, CA. The murine monoclonal vimentin IgM Ab (ab203046), rabbit polyclonal giantin Ab (ab24586), and the rabbit polyclonal calreticulin Ab (ab4) were purchased from Abcam, Cambridge, MA. The AlexaFluor488 IgG2a specific (A-21131), AlexaFluor 488 IgM specific (A-21042), AlexaFluor 546 IgG2b specific (A-21143), AlexaFluor IgG (H+L) specific (A211030) goat anti-mouse Abs and the AlexaFluor 488 IgG (H+L) specific (A11008) goat anti-rabbit Ab were purchased from Molecular Probes, Eugene, OR. Tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit were obtained from Sigma-Aldrich. Radiolabeled compounds were purchased from DuPont NEN. Phorbol 12-myristate 13-acetate (PMA) was purchased from Biomol International, Plymouth Meeting, PA.

Cell Culture

C3HA is a murine fibroblast cell line that was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C and 8% CO_2.

Virus and infection

For these experiments, Ad5 virus stocks were prepared using standard methods with A549 cells and titrated using a plaque-forming assay. For virus infections, cell were plated overnight, washed with PBS, and incubated with 10 pfu/cell for 2 hours in media containing 2% FBS. Infection medium was replaced with medium containing 10% FBS and cells were incubated for indicated times. Infection rates were monitored with anti-E1A staining and were typically 80%.

Immunofluorescence Microscopy

Cells were plated on 8-well glass chamberslides (Nalge Nunc International), incubated overnight to achieve 80% confluence (1×10^4 cells/well), infected with Ad5 for indicated time point and/or treated with indicated concentrations of IDPN, washed two times with PBS, and fixed for 20 min in 10% formaldehyde in PBS. The cells were permeabilized with 0.5% Triton-X, blocked with 10% goat serum and incubated with primary Ab in 2% BSA for 60 min. Cells were washed with PBS and incubated with secondary Abs for 30 min. Cells were washed twice and mounted in 10% glycerol. Microscopy was conducted on a Zeiss

Axioscop 2 Plus, and images were captured and processed by a Spot charge-coupled device camera and software (Diagnostic Instruments).

[^{3}H]AA release assays

2.5 x 10^4 cells were plated into 24-well flat-bottom tissue culture plates (Fisher Scientific) and labeled overnight with 0.1 μCi/ml [^{3}H]AA. The following morning, the cells were washed twice with HBSS, allowed to recover for an additional 2 hours, and washed again before treatment. At indicated time points, 275-μl aliquots of medium were removed from the wells and centrifuged to remove debris. A total of 200 μl of the supernatant was removed for scintillation counting (Beckman Coulter model LS 5801), and total [^{3}H]AA release was calculated by multiplying by a factor of 2. Each point was performed in triplicate, and maximum radiolabel incorporation was determined by lysing untreated controls with 0.01% SDS and counting the total volume.

RESULTS

Adenovirus causes abnormal relocalization of vimentin filaments

The goal of this study was to examine the role that vimentin played in the observed suppression of $cPLA_2$ activity and suppression of AA release during Ad infection. We used the drug IDPN to test our hypothesis that movement of vimentin during Ad infection was responsible for the observed change in localization of the ER, Golgi, and $cPLA_2$, and that this movement caused inactivity of $cPLA_2$. Figure 1 shows the change in distribution of vimentin typically seen after 18 hours of infection with Ad. As seen in panel A, vimentin is distributed evenly throughout the cell in uninfected cells. After 6 hours infection, the intermediate filament is still largely diffuse throughout the cytoplasm. However, by 18 hours post-infection, vimentin filaments can be found concentrated near or clustered exclusively at the perinuclear area. This pattern of movement was identical to $cPLA_2$ relocalization observed in earlier experiments (45).

β,β'-iminodiproprionitrile induces vimentin filament collapse to perinuclear region

To test our hypothesis, we first altered the typical subcellular location of vimentin by treating cells with β,β'-iminodipropionitrile (IDPN). IDPN, the structure of which is shown in Figure 2, is an intermediate filament-specific inhibitor that does not affect the structure of microfilaments or microtubules (46) but which has been shown to cause the reorganization of vimentin to the perinuclear region. IDPN is related to aminodipropionitrile, a compound that

inhibits lysyl oxidase (47). The precise mechanism by which IDPN inhibits vimentin is unknown, but its effects on vimentin have been studied (46-48). In addition, the effects of IDPN are considered selective for intermediate filaments since in fibroblasts and adipocytes, IDPN causes perinuclear collapse of the vimentin filaments while leaving microtubules and microfilaments intact (46, 48). In order to use IDPN in our experiments we first optimized the concentration needed to cause reorganization of intermediate filaments without resulting in toxicity. Over a range of concentrations, we found 0.75% IDPN to produce a collapse of the vimentin filaments without disrupting general cellular morphology (Fig. 3). At lower concentrations, there was little discernible change in the distribution of intermediate filaments. However, at higher doses, the cells rounded up and lost their normal shape, likely indicating a toxic effect.

We next determined the optimal length of time for treatment with IDPN. Over a range of times, we discovered that 2 hours of treatment with 0.75% IDPN resulted in a very similar redistribution of vimentin as that seen in Ad5-infected cells. Vimentin was primarily located in the perinuclear region, with a few filaments radiating outward (Fig. 4). Longer treatment times resulted in similar morphology to treatment with stronger doses of IDPN, while at earlier time points, there was no visible change in vimentin distribution.

IDPN-mediated vimentin collapse is accompanied by relocalization of cPLA$_2$ and major organelles

To test our hypothesis that vimentin movement caused relocalization of cPLA$_2$, we performed an immunofluorescence assay on IDPN-treated cells with staining for both vimentin and cPLA$_2$. If vimentin was indeed responsible for cPLA$_2$ perinuclear translocation, cPLA$_2$ should be visible primarily in the perinuclear region upon IDPN treatment. As shown in Figure 5, control cells show a typical organization of vimentin filaments that radiate outwards from the nucleus. IDPN-treated cells, however, have almost all of their vimentin condensed to the perinuclear region. cPLA$_2$ follows a similar pattern with diffuse distribution in control cells but nearly exclusive localization at the nucleus with IDPN treatment. These results suggest that selective vimentin redistribution results in concomitant cPLA$_2$ movement.

Since the mechanism behind IDPN inhibition of vimentin is unknown, we were interested in whether the effect on cPLA$_2$ was selective or if the positions of other major cellular components was also altered. Vimentin is known to bind to the major organelles: the Golgi, ER, and mitochondria (20, 30-34). Furthermore, previous work in our lab demonstrated that the ER and Golgi both colocalized with vimentin after 18 hours of infection with Ad5 (50). If this colocalization was due to an association with vimentin rather than a distinct viral mechanism, we would expect that IDPN would cause redistribution of these organelles. Therefore, we used immunofluorescence to test the effect of IDPN on subcellular localization of the Golgi apparatus and the ER. As seen in Figure 6, the Golgi relocalizes in IDPN-

treated cells to become more concentrated at the perinuclear region. A similar effect is seen in Ad5-infected cells at 18 h.p.i. (50). Figure 7 shows the effect of IDPN treatment on the localization of the ER. As expected, staining for the organelle shows a distinct perinuclear pattern of staining during this treatment.

IDPN-mediated vimentin collapse inhibits basal levels of cPLA$_2$ activity

Since IDPN-mediated vimentin collapse caused movement of cPLA$_2$ to similar cellular compartments as during Ad5 infection, we hypothesized that it would also cause inhibition of AA release. To test this hypothesis, C3HA cells were labeled with [^{3}H]AA, and treated with IDPN for 2 hours, with PMA added during the final 30 minutes of treatment. Overall, our experiments showed that IDPN treatment suppressed the constitutive release of AA, but not the amount of AA released in response to PMA. Figure 8 shows a representative experiment, where IDPN treatment caused a 0.71-fold reduction of release of [^{3}H]AA compared to control cells. However, the PMA-induced [^{3}H]AA release was similar in cells treated with control medium or IDPN (1.78-fold and 1.91-fold increase, respectively). Taken together, these data suggest that vimentin translocation may indeed be the cause of cPLA$_2$ translocation in Ad-infected cells, but that the movement of cPLA$_2$ to the perinuclear region is not sufficient for suppression of cPLA$_2$ responsiveness.

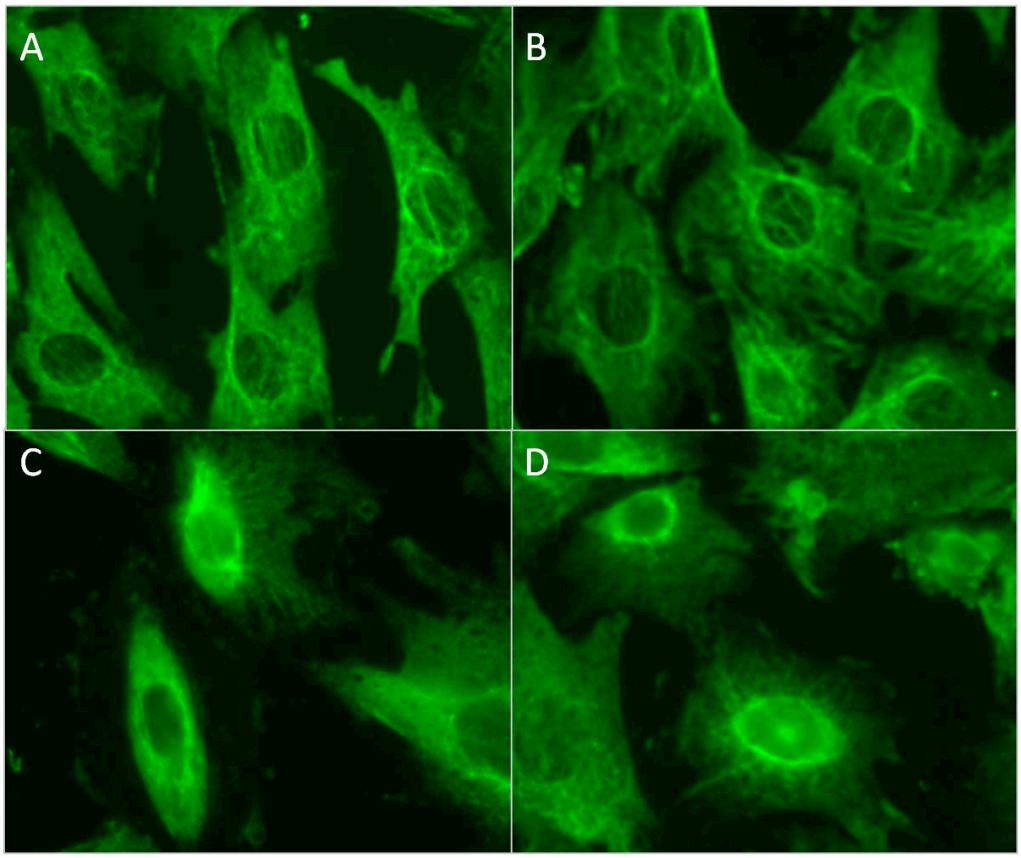

Figure 1. Ad5 infection induces changes in the subcellular localization of vimentin. Fluorescent micrographs showing the intracellular position of vimentin in mock- and Ad5-infected C3HA cells. Cells were infected for 6 (B) or 18 (C,D) hours and then stained with a vimentin-specific antibody. Mock-infected cells (A) show vimentin filaments throughout the cytoplasm. Cells infected for 6 hrs showed a similar pattern of vimentin distribution as mock-infected cells (compare A and B). In contrast, cells infected for 18 hrs displayed bright perinuclear halos of vimentin (D), or complete accumulation of filaments in the perinuclear region (C).

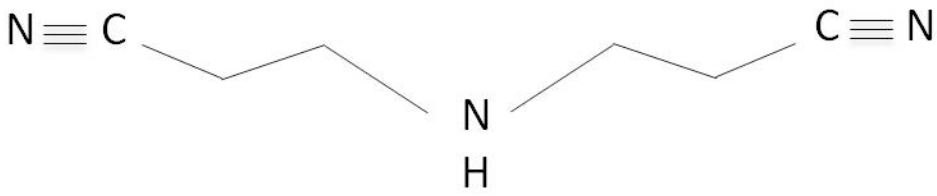

Figure 2. Structure of β,β'-iminodiproprionitrile. IDPN has a molecular weight of 123.2 g/mol, and is synthesized by a reaction of acrylonitrile and ammonia. Its structure and structure-related properties were determined using Advanced Chemistry Development, Inc. Software V11.01 (51).

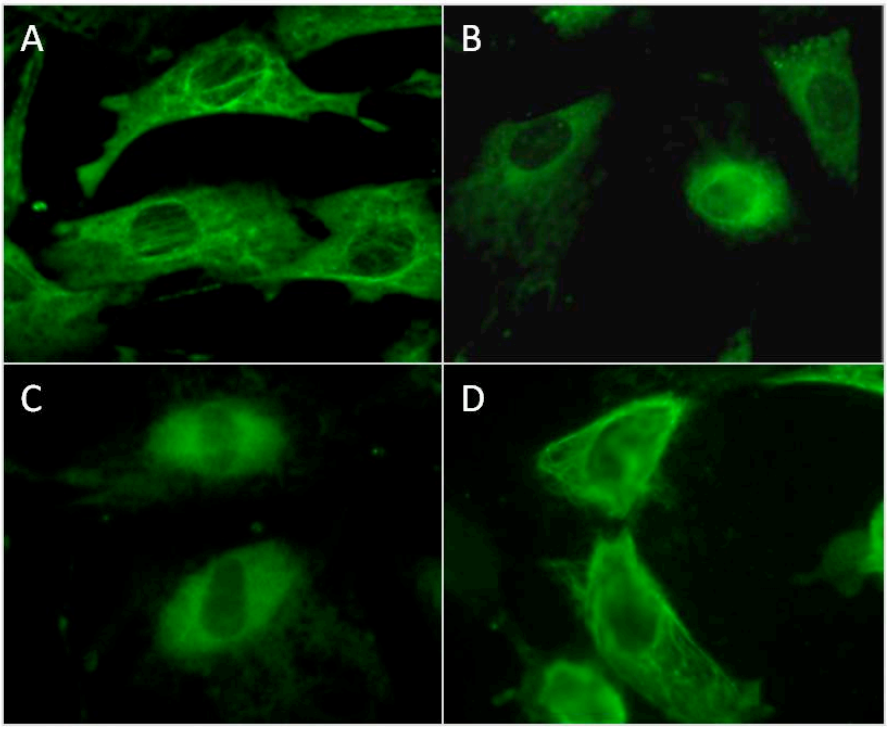

Figure 3. Effect of concentration of IDPN on vimentin distribution. C3HA cells were treated for 2 hours with media containing IDPN at concentrations (v/v) of 0% (A), 0.75% (B), 1.0% (C), and 2% (D). Higher doses of IDPN resulted in greater rounding up of cells and loss of normal morphology.

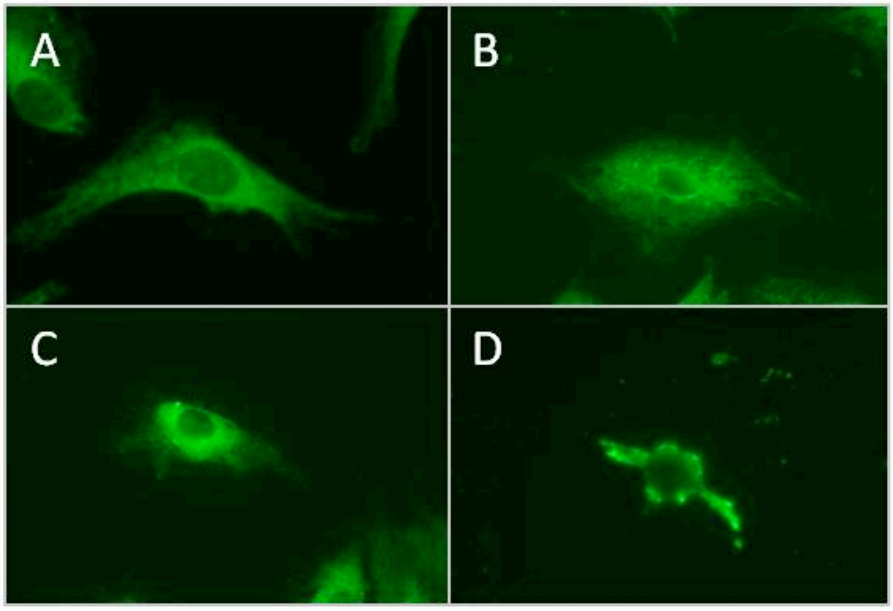

Figure 4. Effect of time on IDPN-mediated vimentin relocalization. C3HA cells were treated with growth medium (A) or growth medium supplemented with 0.75% IDPN for 1 hr. (B), 2 hrs. (C), or 4 hrs. (D). 2 hours was identified as the length of exposure that resulted in a reorganization of vimentin filaments most similar to that caused by 18-hour adenovirus infection. By 4 hours exposure, a majority of cells were misshapen and were no longer adhering to the substrate.

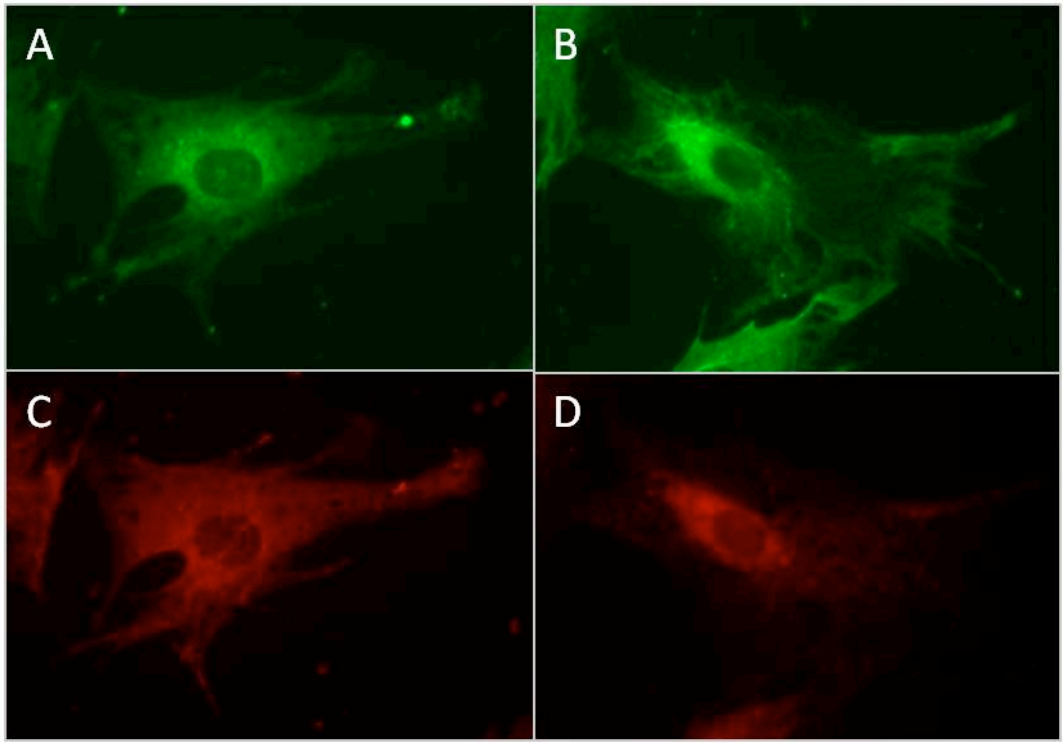

Figure 5. IDPN causes perinuclear colocalization of vimentin and cPLA$_2$. Cells treated with 0.75% IDPN or control medium for 2 hours were stained with antibodies specific for vimentin (A, B) and cPLA$_2$ (C, D). IDPN-treated cells (B, D) show accumulation and colocalization of the cPLA$_2$ and vimentin filaments around the nucleus.

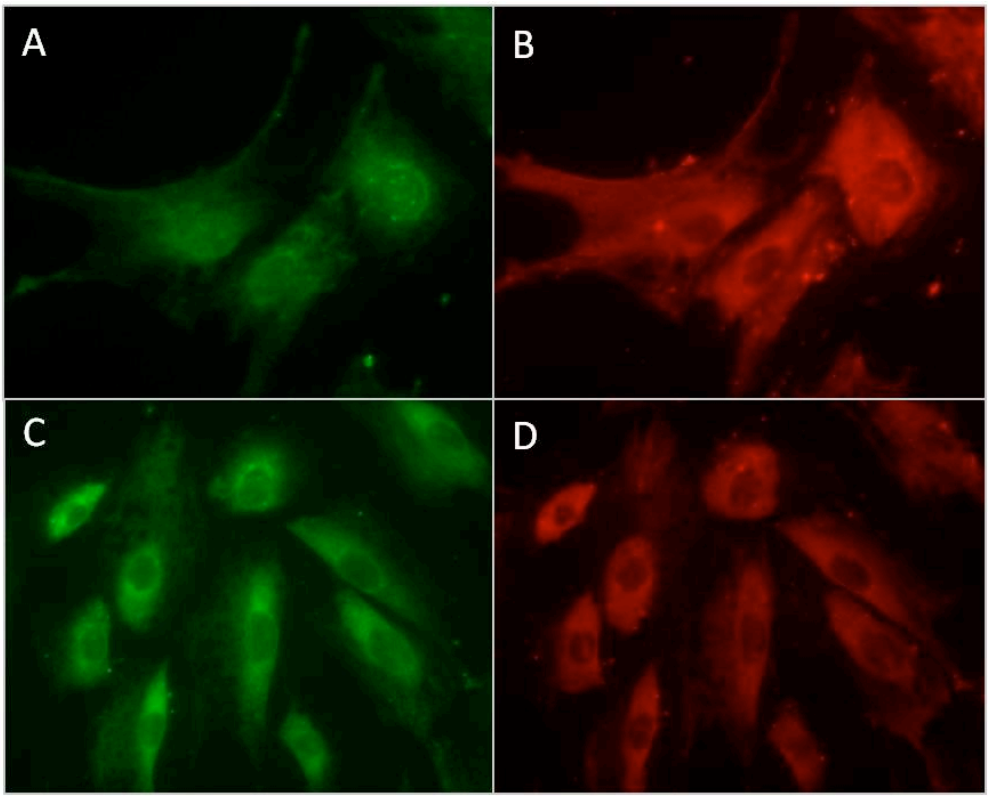

Figure 6. Vimentin and Golgi colocalize near the nucleus in IDPN-treated C3HA cells. Cells treated with 0.75% IDPN or control medium for 2 hours were stained with antibodies specific for Golgi (A, C) and vimentin (B,D). IDPN-treated cells (C,D) show accumulation and colocalization of the Golgi and vimentin filaments around the nucleus.

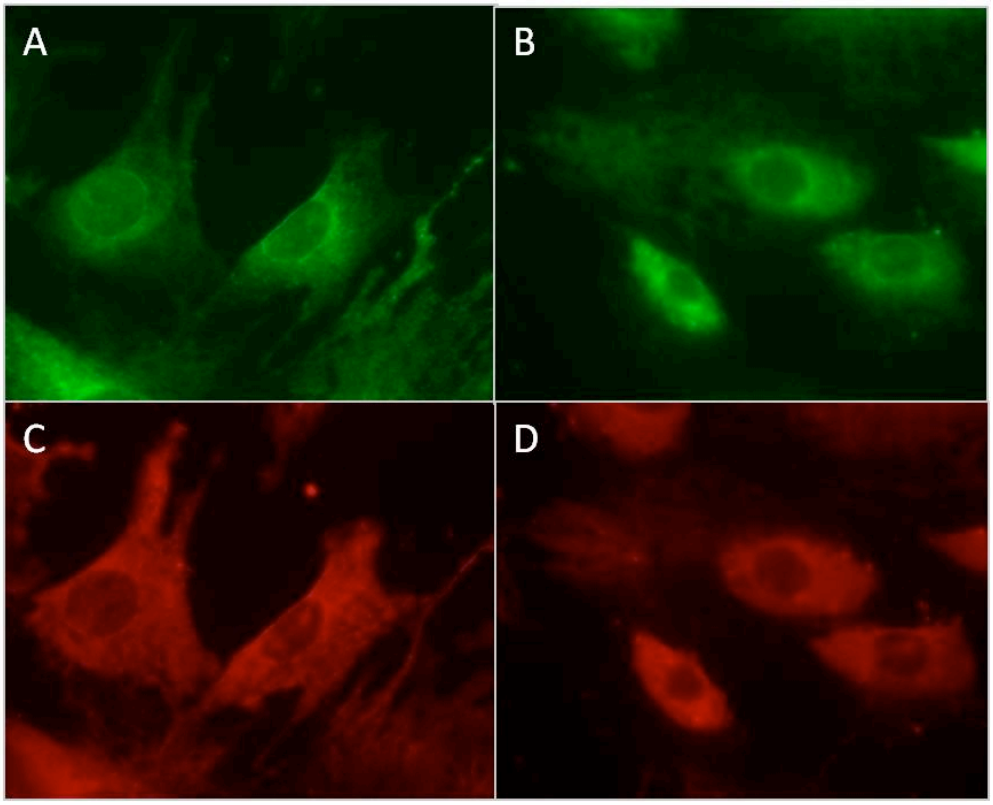

Figure 7. Vimentin and ER colocalize near the nucleus in IDPN-treated C3HA cells. Cells treated with 0.75% IDPN or control medium for 2 hours were stained with antibodies specific for endoplasmic reticulum (A, B) and vimentin (C, D). IDPN-treated cells (B, D) show accumulation and colocalization of the ER and vimentin filaments around the nucleus.

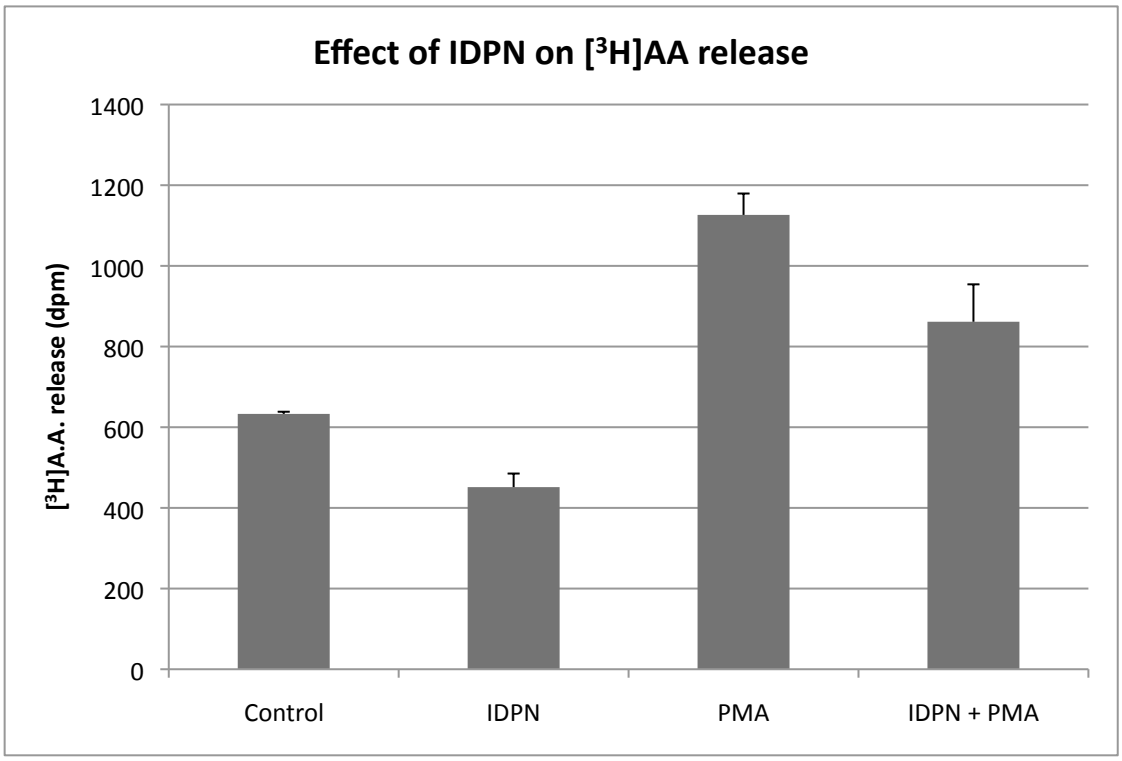

Figure 8. IDPN suppresses basal levels of [^{3}H]AA release. C3HA cells were treated for 2 hours with control medium or medium containing 0.75% IDPN. For cells treated with PMA, the medium was supplemented with 10 ng/mL PMA for the final 30 minutes of the assay. Supernatants were analyzed for [^{3}H]AA release as described in Materials and Methods. All points were performed in triplicate with SEM, and data shown is representative of three experiments.

DISCUSSION

In these experiments we examined the potential role of the intermediate filament protein vimentin in the Ad-dependent suppression of $cPLA_2$ activity. Our lab has previously shown that Ad elicits a strong PG response from infected cells in the early stages of infection, but after 6-12 hours of infection, this response is suppressed, and cannot be induced by external stimuli. This is accompanied by an abnormal translocation of $cPLA_2$ to the perinuclear region of the cell (45). Further experiments showed that not only was $cPLA_2$ translocating, but vimentin, the Golgi, and the ER were also translocating (50). In this study, we hypothesized that the relocalization and suppression of $cPLA_2$ seen in Ad-infected cells was due to vimentin-dependent movement. We treated cells with the intermediate filament-disrupting drug IDPN because it mimics the reorganization caused by Ad and used immunofluorescence to observe changes in subcellular distribution of $cPLA_2$ and various organelles. The results of our investigation partially supported our hypothesis. We did find that IDPN caused a similar pattern of vimentin, $cPLA_2$, Golgi and ER translocation supporting the notion that vimentin movement is responsible for the movement of $cPLA_2$ and the other organelles. On the other hand, the IDPN-induced movement of $cPLA_2$ did not result in the complete suppression of PMA-dependent $cPLA_2$ activity. It is likely therefore that additional factors are responsible for the Ad-dependent suppression of $cPLA_2$ activity.

There are many factors responsible for the regulation of $cPLA_2$ activity that may be the target of Ad. For example, $cPLA_2$ requires Ca^{2+} for proper activity. The binding of Ca^{2+} with acid residues in the C2 domain of $cPLA_2$ allows the enzyme to interact with lipids (52). Culver

and Laster tested for the presence of Ca^{2+} during Ad-induced $cPLA_2$ suppression and found that intracellular levels of Ca^{2+} were normal (45). However, if Ca^{2+} was unable to bind to the acid residues on $cPLA_2$, this would prevent enzyme activation. Vimentin has been shown to bind $cPLA_2$ in the same C2 domain (16) as Ca^{2+}. Therefore, while normal levels of calcium were measured in the cell, the Ad-induced interaction between vimentin and $cPLA_2$ might be sufficiently strong to interfere with the contact between Ca^{2+} and $cPLA_2$, thus inhibiting the release of AA. IDPN treatment may have produced sufficient interaction between $cPLA_2$ and vimentin to cause $cPLA_2$ translocation, but not sufficiently strong to prevent Ca^{2+} from binding the enzyme. Resolving this hypothesis would require analysis of the $cPLA_2$:vimentin interaction induced by both Ad and IDPN.

The interaction between $cPLA_2$ and lipid membranes which allows it to cleave AA can also be influenced by other lipids in the vicinity. It is possible that Ad targets these interactions For example, ceramide-1-phosphate is a sphingolipid that acts allosterically on $cPLA_2$ to increase the time that it is bound to a membrane (5, 9). If Ad is able to disrupt the interaction between C1P and $cPLA_2$, this would decrease the ability of $cPLA_2$ to remain affixed at a membrane, decreasing its activity. There are three specific Arg and Lys residues in the C2 domain of $cPLA_2$ necessary for C1P binding, which could be disrupted by the association of the C2 domain with vimentin (9). Conversely, Ad may exert an effect on this region independent of vimentin that disrupts the C1P-$cPLA_2$ interaction.

As mentioned above, phosphorylation of serine residues is also critical for the regulation of $cPLA_2$ activity. Serine 505 is a target for the MAP family of kinases and its phosphorylation

can be essential for $cPLA_2$ activation. Culver and Laster showed that Ser505 was phosphorylated during Ad infection suggesting that blocking this reaction is not the mechanism of Ad-mediated suppression of $cPLA_2$ activity (45). On the other hand, there are several additional serines on $cPLA_2$, which are also thought to be important for enzyme activity, whose phosphorylation has not been evaluated during Ad infection. The effects of Ad infection on the phosphorylation of Ser515, which is targeted by the calmodulin-activated kinase II (6), or on the phosphorylation of Ser727, which is targeted by the MNK-1-like kinase (53) could be evaluated by phosphospecific antibodies.

Ad infection and IDPN both induced vimentin reorganization but IDPN did not cause suppression of $cPLA_2$ activity as was seen with Ad. However, we utilized a relatively superficial technique, fluorescence microscopy, to evaluate the effects of Ad and IDPN on vimentin position. It is possible that Ad infection and IDPN are actually exerting distinct effects, by distinct mechanisms, on the positions and activities of vimentin and $cPLA_2$.

The effects of Ad on the cytoskeleton have been studied extensively. Ad is known to affect the actin fibers for entry (39), using dynein to move towards the nucleus in the following minutes (54). Belin and Boulanger discovered that four different serotypes of Ad (including Ad5) induced cleavage of vimentin in the early stages of infection (55). They later demonstrated that Ad5 and Ad2 caused perinuclear clustering of vimentin in early infection, and this was absent in heat-inactivated virions (56). One mechanism underlying this effect is likely the activation of cellular kinases by the Ad E1A gene product (57). These authors showed that E1A causes transactivation of the *cdc2* gene. This gene encodes cyclin-

dependent kinase 1 (cdk1), which is responsible for phosphorylating many substrates in order to push the cell through the G1→ S transition. The researchers found that E1A effectively activates the *cdc2* promoter. Interestingly, vimentin can be phosphorylated at 3 residues by cdk1 (Ser55, Ser71, and Ser72) (58). Yamaguchi, et al. demonstrated that cdk1-phosphorylated vimentin recruits plk1, which further phosphorylates the intermediate filament. They then showed that plk1-phosphorylated vimentin is inhibited from full filament assembly (58). It is possible, therefore, that E1A-mediated activation of cdk1 leads to a chain of signaling events that prevents normal vimentin filament structure, resulting in the perinuclear clustering of vimentin and $cPLA_2$ that we have noted.

It is also possible that the perinuclear clustering of vimentin is mediated by the E1A-dependent activation of cAMP-dependent protein kinase A (PKA). In early infection, the activation of PKA has been correlated with successful viral motility towards the nucleus (59). PKA affects vimentin filament integrity; researchers have shown that vimentin reorganization occurs within 30 minutes of phosphorylation by PKA (60). They microinjected the kinase into REF-52 and CCL-146 cells and observed a shift from widely distributed vimentin filaments to clustering of the filaments around the nucleus (60). More recently, microinjection of PKA into BHK-21 cells was used to show that the kinase impairs vimentin filament formation from its subunits (61). Thus, E1A-activated PKA may cause disassembly of vimentin and redistribution to the perinuclear region.

It is also possible that vimentin reorganization in Ad infected cells occurs through activation of protein phosphatase 2A (PP2A). Turowski, et al. used okadaic acid to inhibit PP2A

activity in Hs68 fibroblasts and noted hyperphosphorylation of vimentin and filament dispersal and clustering around the nucleus (62). Ad, via several E4 transcripts, can also regulate PP2A. E4orf4 protein forms a complex with PP2A that negatively regulates its phosphatase activity (63). E4orf6 has also been found to suppress PP2A activity to disrupt host cell responses (64). Thus, E4 protein interactions with PP2A may lead to vimentin hyperphosphorylation and relocalization.

Collectively, studies with Ad reveal many possible mechanisms that may cause vimentin modification and intermediate filament reorganization in infected cells. In contrast, relatively little is known about the mechanism by which IDPN causes reorganization of intermediate filaments. Most studies of IDPN have been conducted in neurons, where IDPN impairs retrograde transport of neurofilaments (NF), leading to axonal swelling (65). In IDPN-treated neurons, researchers have found kinesin clustered with NF (66) and NF separation from the microtubules to which they are typically bound (67, 68), indicating that IDPN may possibly inhibit motor protein function. Hyperphosphorylated NF accumulated in the body of neurons from IDPN-treated rats, demonstrating a potential role for NF phosphorylation in IDPN-mediated filament collapse (69). Further examination is necessary to understand how IDPN causes vimentin collapse. The different mechanism by which it occurs in Ad infection and IDPN treatment may explain the difference in $cPLA_2$ activity under the two conditions.

The limitations of IDPN as a model for Ad-induced vimentin movement prevent a comprehensive understanding of how this movement may contribute to $cPLA_2$ inhibition.

For example, IDPN treatment must is limited to 2 hours, whereas vimentin movement in Ad infection can last up to 12 hours. We are unable to determine whether the length of time that vimentin is mobilized at the perinuclear region is important in $cPLA_2$ suppression. Exploring other models of vimentin movement may resolve the issue more firmly, and lead to a greater understanding of how the effect occurs in Ad-infected cells.

In this study, we used the compound IDPN to test whether the movement of vimentin to the perinuclear region is sufficient to explain the suppression of $cPLA_2$ activity observed during late Ad infection. Our data showed that IDPN did indeed cause the relocalization of vimentin to the perinuclear region, along with $cPLA_2$, the ER, and the Golgi, all effects seen during Ad infection. However, movement of $cPLA_2$ to the perinuclear region was insufficient to suppress AA and PGE_2 release. This research has important implications for understanding the regulation of $cPLA_2$. Additionally, the role that vimentin has in trafficking membrane-bound organelles and $cPLA_2$ will be important in treatment for inflammatory diseases.

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