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Protein Kinase C Signaling in Adenoviral Infection

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Abstract

Activation of protein kinase C (PKC), a serine/threonine protein kinase, ubiquitously influences cellular signal transduction and has been shown to play a role in viral entry. In this study, we explored a role for PKC in human adenovirus type 37 infection of primary human corneal fibroblasts, a major target cell for infection. We sought evidence for an interaction between PKC activation and two potential downstream targets: cSrc kinase, shown previously to play a critical role in adenovirus signaling in these cells, and caveolin-1, reported earlier to be important to entry of adenovirus type 37. Infection of fibroblasts increased PKC α phosphorylation and translocation of PKC α from the cytosol to caveolin-1 containing vesicles. Virus-induced phosphorylation of both cSrc and AKT was abolished in cell lysates pretreated with calphostin C, a chemical inhibitor of PKC. Inhibition of PKC also reduced virus associated phosphorylation of caveolin-1, while inhibition of cSrc by the chemical inhibitor PP2 reduced only caveolin-1 phosphorylation, but not PKC α phosphorylation, in lipid rafts. These results suggest a role for PKC α upstream to both cSrc and caveolin-1. Phosphorylated PKC α was found in the same endosomal fractions as phosphorylated cSrc, and PKC α was present to a greater degree in caveolin-1 pull downs from virus infected than mock infected cell lysates. Calphostin C also reduced early viral gene expression, indicating that PKC α activity may be required for viral entry. PKC α plays a central role in adenovirus infection of corneal fibroblasts and regulation of downstream molecules, including the important lipid raft component caveolin-1.

Graphical abstract

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Protein kinase C (PKC) is a family of ~80 kDa phospholipid-dependent serine/threonine kinases with 10 isoforms.¹ All share a common structure with an N-terminal regulatory domain, C-terminal catalytic domain, and a hinge region joining the two. Conventional PKC isoforms, including α , β I, β II, and γ , are activated by diacylglycerol (DAG) requiring calcium, while the novel isoforms δ , ϵ , η , and θ are activated by DAG and do not require calcium. Atypical isoforms include λ and ζ that require neither DAG nor calcium for activation. Interaction with endomembranes is a key aspect of conventional PKC function. Activation of phospholipase $C\beta$ induces generation of DAG and inositol 1,4,5-triphosphate (IP_3), with subsequent release of calcium from the endoplasmic reticulum. Together, DAG and calcium induce the movement of PKC from the cytosol to endomembranes where its kinase function is released. As a major upstream signaling molecule, PKC has been shown to play a critical role in the cellular entry of viruses, including rhabdovirus, alphavirus, poxvirus, herpesvirus,² influenza virus,³ and respiratory syncytial virus.⁴ PKC isoforms play a central role in signaling events leading to alterations in the cell membrane and cytoskeleton, including the formation of caveolae,⁵ which have been implicated in the entry of filovirus,⁶ human enterovirus,⁷ echovirus,⁸ and human immunodeficiency virus.^{9,10} Human adenovirus type 37, an important cause of keratitis, was recently shown to infect corneal fibroblasts by use of caveolae.¹¹

Human adenoviruses utilize at least two distinct sets of cell surface receptors for attachment and internalization into target host cells. An initial interaction between the adenovirus capsid fiber knob with a cellular receptor including the coxsackie adenovirus receptor (CAR),¹² GD1a glycan,¹³ major histocompatibility class I,¹⁴ desmoglein,¹⁵ and CD46¹⁶ enables a secondary interaction between arginine–glycine–aspartate (RGD) amino acid sequences in the adenoviral penton capsomer and target cell integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.^{17,18} The secondary interaction induces activation of focal adhesion kinase, cSrc, and phosphoinositide 3-kinase (PI3K) that mediate endocytosis of the virus.^{19,20} The same kinases act as key signaling nodes to prolong cell survival, thus enabling viral replication,²¹ and induce expression of cytokines by infected cells.^{22–27} In particular, cSrc is the kinase identified most upstream in the pathway initiated by adenovirus infection of corneal cells.

Multiple lines of evidence point to functional interactions between PKC and cSrc,^{28–35} and we sought to explore the possibility of upstream control of cSrc activation by PKC in the earliest stages of infection by adenovirus. We demonstrate herein that adenovirus infection results in activation of PKC α , which then translocates to caveolin-1 containing vesicles, also containing cSrc. Furthermore, inhibition of PKC α activity results in reduced cSrc phosphorylation and decreases adenovirus early gene expression. Our data are consistent with an endosomal signalosome induced by adenovirus infection, in which PKC α and cSrc act as master kinases for the control of a myriad of cellular responses to viral infection.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Antibodies to total PKC α , phospho-cSrc, total AKT, phospho-AKT, phospho-PKC α , total caveolin-1, and isotype controls (antirabbit or antimouse) were obtained from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Delaware, CA). Antibodies to

total cSrc and phospho-caveolin-1 were purchased from EMD Millipore (Billerica, MA) and BD Bioscience (San Jose, CA). Antibody to actin was obtained from Thermo Scientific (Cambridge, MA). Inhibitors to PKC (calphostin C) and cSrc (PP2) were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated goat antirabbit and goat antimouse IgG were obtained from Santa Cruz Biotechnology. Lipofectamine RNAiMAX and OptiMEM cell culture medium were obtained from Invitrogen Life Technologies (Carlsbad, CA).

Cell Culture and Viruses

Primary corneal fibroblasts were grown from human donor corneas as previously described.³⁶ Cells from multiple donors were pooled, and the cell monolayers used at passage two. The protocol for use of corneas from deceased human donors was approved by the Massachusetts Eye and Ear Infirmary Human Studies Committee and is consistent with the principles expressed in the Declaration of Helsinki. In some experiments, cell cultures were pretreated with the PKC inhibitor calphostin C (1 μ M), the cSrc inhibitor PP2 (10 μ M), or DMSO (control), for 3 h prior to infection. Human adenovirus type 37 strain GW obtained from American Type Culture Collection (ATCC, Manassas, VA) was grown in A549 cells (CCL-185, ATCC), a human alveolar epithelial cell line, in minimum essential media (MEM) with 2% FBS, penicillin G sodium, streptomycin sulfate, and amphotericin B. Virus was purified by a cesium chloride gradient prior to use. In some instances, the virus was conjugated to Cy3 dye (GE Healthcare, Piscataway, NJ)³⁷ to enable visualization of the viral capsid for confocal microscopy, as previously described.³⁸ One milligram of Cy3 dye was reconstituted in 1 mL of 0.1 M sodium bicarbonate (pH 9.3) and conjugated to virus at a concentration approximately equal to 10^{12} Ad particles/mL; reconstituted Cy3 dye was 20% of the final solution. The mixture was incubated for 30 min in the dark with gentle mixing, followed by dialysis overnight to remove the excess dye. Virus labeled with Cy3 grew to equal titer as unlabeled virus (data not shown).

Immunoblot

Adenovirus type 37 and mock infected corneal cells were lysed with cell lysis buffer (Cell Signaling, Beverly, MA) containing protease inhibitor cocktail (Sigma, St. Louis, MO) and incubated at 4 °C for 5 min. Cell lysates were centrifuged at 13000g for 10 min at 4 °C. The protein concentration of each supernatant was measured by BCA analysis (Pierce, Rockford, IL) and equalized. Lysates were boiled in 1× sodium dodecyl sulfate (SDS) sample buffer and separated by 4–20% Tris-glycine gel (Invitrogen) and transferred onto nitrocellulose membranes (BioRad, Hercules, CA) and immunoblotted. Antibody reactivity was determined with enhanced chemiluminescent reagents (Amersham Bioscience, Piscataway, NJ) using horseradish peroxidase-coupled secondary antibodies. Densitometry was performed on images generated on a Kodak Image Station 4000R (Rochester, NY).

Immunoprecipitation

Lysates obtained from adenovirus type 37 infected or mock infected primary keratocytes (200 μ g) were precleared with protein A-agarose beads (Thermo-Fisher) for 60 min. Cleared supernatants were mixed with 3 μ g of anticaveolin-1 or isotype control (antirabbit) and 3 μ g of anti-PKC α or isotype control (antimouse) antibodies in buffer containing 50 mM Tris

HCl (pH 8.5), 50 mM NaCl, 1% NP40, protease inhibitor cocktail, and rocked at 4 °C overnight. Then 30 μ L of protein A agarose was added for an additional 2 h at 4 °C. Immunoprecipitates were washed three times with buffer containing 50 mM Tris HCl, 50 mM NaCl, 1% NP40, and protease inhibitor cocktail, and proteins were eluted by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples were run on 4–20% Tris-glycine gel and immunoblotted.

Confocal Microscopy

Corneal fibroblasts were grown on slide chambers (Nunc, Rochester, NY) and infected with Cy3 labeled adenovirus type 37 for the indicated times, and then partially fixed in 0.05% paraformaldehyde for 10 min, washed in PBS containing 2% FBS, and permeabilized in 0.1% Triton X-100 for 5 min. After 30 min blocking in 3% FBS-PBS, the slides were incubated in 5 μ g/mL of anti-PKC α , anticaveolin-1, or isotype control antibodies for 1 h at RT, washed three times in 1 $\times$ PBS containing 2% FBS. Slides were then incubated with Alexa Fluor 488 or 568 conjugated secondary antibody (Molecular Probes, Eugene, OR), washed three times in 1 $\times$ PBS containing 2% FBS, fixed in 2% paraformaldehyde, and mounted using Vectashield (Vector labs, Burlingame, CA) mounting medium containing DAPI. Images were taken in a Leica SP5 confocal microscope using a 63 $\times$ glycerol immersion objective. For inhibition experiments, cells were pretreated with calphostin C (1 μ M) for 3 h and then infected with Cy3 labeled adenovirus type 37 for 30 min, processed as above, and incubated in phalloidin conjugated with Alexa Fluor 488 for 30 min at RT, washed, and mounted. Images were taken in a Leica SP5 confocal microscope using a 60 $\times$ glycerol immersion objective. For quantitative analysis, ImageJ was used. Three frames from three separate experiments were chosen in a masked fashion to quantitate Cy3 labeled virus within cells.

Nondetergent Isolation of Lipid Rafts

Cornea fibroblasts were treated with calphostin C or PP2, washed thoroughly, and infected with purified adenovirus type 37 at a multiplicity of infection (MOI) of 10 (for synchronous infection) or mock infected with virus-free dialysis buffer. After infection, lipid raft fractions were isolated using detergent free methods. Briefly, cells were washed once in ice cold 1 $\times$ PBS, suspended in 500 mM Na₂CO₃ and lysed by 20 strokes in a prechilled dounce homogenizer. Further disruption of cell membranes were achieved by passing the lysates through a 23-gauge needle five times, followed by sonication for 15 s three times. The subsequent lysates were mixed with an equal volume of 90% sucrose-MBS (25 mM MES (2-*N*-morpholinoethanesulfonic acid) plus 0.15 M NaCl (pH 6.5)), overlaid on equivalent volumes of 35% sucrose-MBS–Na₂CO₃ and 5% sucrose-MBS–Na₂CO₃, and centrifuged for 118 h at 4 °C. 500 μ L fractions were collected, and the protein concentrations were measured and equalized. Lysates were subjected to SDS-PAGE and immunoblotted as above.

Endosome Isolation

Flotation gradient fractionation of post nuclear supernatants were performed with 62%, (2.351 M) 35%, (1.177 M) and 25%, (0.806 M) sucrose gradients.³⁹ One hour post viral

infection or mock infection with dialysis buffer, cells were collected and postnuclear supernatants were prepared with the NucBuster protein extraction kit (Novagen, San Diego, CA). Postnuclear supernatants were then mixed 1:1 with 62% sucrose, layered over with 1.5 vol of 35% sucrose and 1 vol of 25% sucrose, and centrifuged for 1 h at 149000g at 4 °C. Fifty microliter fractions were collected from the top of each gradient and subjected to Western blot analysis and used to determine cSrc kinase activity (as below).

siRNA Transfection

The siRNA for PKC α was purchased from Ambion (Foster City, CA). The control scRNA (On Target Plus control pool) was purchased from Thermo Scientific. 100 picomoles of RNA was transfected into cells using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. At 48 h post transfection, cells were infected with adenovirus type 37 for 4 h and then analyzed for mRNA expression for PKC α and adenovirus type 37 E1A using real-time RT-PCR. For Western blot analysis, equal amounts of protein were separated in a 4–20% gradient gel (Invitrogen) and immunoblotted with antibodies against PKC α and actin.

Polymerase Chain Reaction

For reverse transcriptase PCR, cells were infected with virus at an MOI of 10. Following 1 h incubation, cells were washed twice with PBS and incubated an additional 4 h with fresh media. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA concentrations and quality were analyzed on a Bio-Rad Smart-Spec Plus (Bio-Rad, Hercules, CA). To remove any genomic DNA contamination, RNA was treated with Turbo DNase (Ambion). For synthesis of cDNA, 2 μ g of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using an oligo(dT) 15 primer (Promega). A reaction without reverse transcription was run with each experiment to rule out the possibility of amplification of contaminating genomic DNA in the PCR step. The cDNA product was amplified by PCR in a total volume of 25 μ L of PCR mix, including 12.5 μ L of 2 $\times$ PCR master mix (Promega), 8.5 μ L of ddH₂O, and 1 μ L of each primer (10 pmol). The PCR reaction was performed using the following cycling parameters: 95 °C for 5 min for the initial denaturation step, 30 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, followed by the final extension at 72 °C for 10 min and then kept at 4 °C until analysis. The amplification products were analyzed by gel electrophoresis on 1% agarose gel. PCR products were visualized after ethidium bromide staining using a Kodak Image Station 4000R. The primers used for PCR amplification in reverse transcriptase PCR were as follows: for GAPDH (GenBank Acc. no. NM002046.3) forward: 5' ATCATCCCTGCCTCTACTGG3'; reverse: 5' GTCAGGTCCACCACTGACAC3'; for adenovirus type 37 E1A 13S (GenBank Acc. no. DQ900900) forward: 5' GCTGGGACCTACACTTCAGG3'; reverse: 5' TGTAACACAGAGCGCAGGAG3'.

For real-time quantitative PCR, total RNA was extracted from treated cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was then treated with DNase I (1 unit) (New England BioLabs, Ipswich, MA) at 37 °C for 1 h. Two micrograms of the DNase treated RNA was subjected to reverse transcription with M-MLV reverse

transcriptase (Promega) and oligo dT₁₅ primers (IDT). Primers for PKC α , caveolin-1 and E1A were designed using Primer3 plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and synthesized by IDT (Table 1). cDNAs were diluted 1:10 and 1 μ L used for qRT-PCR with Fast SYBR Green master mix (Thermo Fisher) under the conditions: 40 cycles at 95 °C (30 s), 60 °C (1 min), 72 °C (30 s), and a final extension at 72 °C (10 min). A nontemplate control and endogenous control (human GAPDH) were measured for relative quantification. The relative expression levels were calculated by the 2^{-CT} method against untransfected/uninfected controls.⁴⁰

Src Kinase Assay

Src activity in endosomal fractions was assessed by the ProFluor Src-Family kinase assay (Promega). Two micrograms of pooled endosomal fractions # 7–10 (peptide substrate) and 25 μ L of 100 μ M ATP was added to the reaction buffer and incubated for 60 min at room temperature, followed by the addition of protease reagent for another 60 min at RT. The reaction was then stopped by addition of stabilizer reagent. In the presence of active kinase, the peptide substrate is phosphorylated, effectively blocking the protease activity and resulting in low fluorescence of the substrate. When there is no kinase activity, the peptide substrate remains unphosphorylated, and the protease will remove all amino acids from the peptide substrate and liberate the highly fluorescent rhodamine 110. Therefore, kinase activity is inversely proportional to fluorescence. The resulting fluorescence was read with at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Statistical Analysis

The results of at least three independent experiments were first checked for normality and then subjected to analysis by ANOVA with $\alpha = 0.05$. All analysis was performed in SAS (Cary, NC).

RESULTS

PKC α Activation and Translocation in Adenovirus Infection

As a first step toward the study of PKC in adenovirus infection of corneal cells, we subjected serum starved, primary, human corneal fibroblasts in culture to Western blot analysis for one PKC isoform of each activation group (conventional, novel, and atypical) (Figure 1A). PKC α was identified in fibroblasts at the expected 80 kDa molecular mass by use of an isoform specific antibody, while bands for PKC θ and PKC ζ were not seen. To determine whether PKC α was activated by infection with adenovirus, we infected the cells with 10 viral infectious units per cell (multiplicity of infection, MOI = 10) for 1 h, or mock infected with virus-free dialysis buffer, and immunoblotted with antibody against phosphorylated PKC α . Other cells were pretreated with the PKC inhibitor, calphostin C^{41,42} (1 μ M for 3 h), before infection. Viral infection increased phosphorylation of PKC α relative to total PKC α , and this effect was diminished by calphostin C pretreatment (Figure 1B). Densitometry analysis performed on three consecutive Western blots showed a significant increase in PKC α phosphorylation and confirmed the effect of calphostin C on PKC α phosphorylation in adenovirus infection (Figure 1C; $p < 0.05$). Previously, activation of PKC α by TPA was shown to induce movement of the protein from its association with the plasma membrane of

MCF7 cells to caveolae.⁴³ Therefore, we sought to determine if adenovirus infection would alter the cellular localization of activated PKC α . Using immunofluorescence microscopy at 15 and 30 min post infection, PKC α appeared to move from the plasma membrane to intracellular vesicles over the first 30 min post infection (Figure 1D).

PKC α Activation Occurs Upstream of cSrc and AKT in Adenovirus Infection and Is Important to Viral Entry

We have previously shown that the tyrosine kinase cSrc and the threonine kinase AKT are both activated within 1 h in adenovirus infected fibroblasts and mediate chemokine expression and cell survival, respectively.^{21,23} Because PKC α was activated so rapidly (within 15 min) after infection (Figure 1D, above), we sought to determine if there was any role for PKC α in the activation of cSrc and AKT. At 1 h after infection, calphostin C reduced phosphorylation of cSrc (Y416; Figure 2A) and AKT (T308; Figure 2B) relative to DMSO pretreated controls, consistent with a signaling pathway in infected cells in which PKC α acts upstream of cSrc and AKT. Densitometry analysis of three consecutive Western blots confirmed the findings (Figure 2C,D; $p < 0.05$). Because cSrc has previously been shown important to adenoviral entry in corneal fibroblasts,^{11,21} we performed confocal microscopy on cells infected with adenovirus that had been pretreated with either DMSO or calphostin C prior to infection for 1 h with Cy3 labeled virus (Figure 2E), and then quantified the Cy3 red signal by ImageJ (Figure 2F). Significantly less Cy3 label was evident in calphostin C pretreated cells ($p < 0.05$), consistent with a role for PKC α in adenovirus entry.

Association of PKC α with Caveolin-1 in Infection

Because of prior data showing caveolin-1 associated viral entry in corneal fibroblasts,¹¹ and in light of the apparent translocation of phosphorylated PKC α to intracellular vesicles in infection, as seen in Figure 1D, we next sought to determine if PKC α interacts with caveolin-1 after adenovirus infection. To this end, we performed caveolin-1 pull-down assays from whole cell lysates with caveolin-1 antibody and immunoblotted with antibody to phosphorylated PKC α and then stripped and reprobed the blot for caveolin-1 (Figure 3A). The results show increased phosphorylated PKC α in caveolin-1 containing precipitates at 30 min post infection, and on reprobe, also increased caveolin-1 in cell lysates after infection. A similar pull-down assay performed by precipitation with antibody against phosphorylated PKC α followed by immunoblot against caveolin-1 showed a similar result (Figure 3B). These data suggest first that both caveolin-1 and PKC α moved from the insoluble cell fraction, including the cell membrane, to the soluble, cytosolic lysate fraction upon infection, and second support a physical interaction between phosphorylated PKC α and caveolin-1 in cell lysates at 30 min after infection. Immunofluorescence microscopy was then performed to identify colocalization of these two molecules in infection (Figure 3C). In mock infection, phosphorylated PKC α and caveolin-1 both appear principally plasma membrane bound. However, at 30 min post infection, both molecules moved to the same intracellular vesicles as shown by the merged immunofluorescent image.

Effect of PKC α on Viral Gene Expression

We demonstrated previously that chemical inhibition of cSrc activity delayed adenoviral entry¹¹ and reduced early viral gene expression,²³ consistent with an effect on intracellular trafficking of adenovirus via endosomes. Because cSrc activation after infection appeared to occur downstream of PKC α , as shown in Figure 2A, we next assessed the impact of PKC α activity on viral gene expression. Cells pretreated with either calphostin C or DMSO were infected for 4 h or mock infected and subjected to reverse transcriptase PCR for E1A gene expression and GAPDH (control) (Figure 4A). As shown, E1A expression was blocked by calphostin C pretreatment, while GAPDH expression was unaffected. No E1A mRNA was detected in mock infected cells, or in cells infected but in which reverse transcriptase was omitted. Because of possible off-target effects of calphostin C, we next applied siRNA specific to PKC α . As shown in the Western blot (Figure 4B, left panel) and by quantification of three consecutive immunoblots (Figure 4B, right panel), siRNA transfection significantly reduced PKC α expression, to about 40% of what was seen in scRNA and of mock treated cells ($p < .05$). Caveolin-1 expression was not affected (data not shown). We then transfected PKC α specific siRNA or control scRNA, and assessed E1A expression by real-time reverse transcriptase PCR. At 4 h post infection, PKC α siRNA reduced E1A expression by ~80% (Figure 4C; $p < .05$).

PKC α , Caveolin-1, and cSrc Are Activated in the Same Lipid Raft Fractions after Infection

Caveolin-1 is an integral membrane protein, and a principal component of caveolae,⁴⁴ which can serve as organizing centers for cell signaling.⁴⁵ Caveolin-1 controls endocytosis and recycling of cellular integrins^{46,47} and is an upstream regulator of the AKT pathway,⁴⁸ the latter previously shown by us to prolong cell survival in the early stages of adenovirus infection.²¹ Li and coworkers previously showed the tyrosine phosphorylation of caveolin at Y14 by cSrc.⁴⁹ We sought to determine the possible interaction between activation of PKC α , cSrc, and caveolin-1 in lipid rafts of adenovirus infected cells. Detergent-free lipid raft preparations were prepared in cells pretreated with DMSO, PP2 (10 μ M), or calphostin C (1 μ M) for 3 h, prior to virus or mock infection for 1 h. Western blot analysis showed increased phosphorylated PKC α in the lipid rafts of virus infected cells, with the greatest degree of phosphorylation in fraction #4 and to a lesser degree in the adjacent fractions #3 and #5 (Figure 5A). Pretreatment with PP2 resulted in a small reduction in phosphorylated PKC α . Virus infection also increased phosphorylated caveolin-1, and when the blots were stripped and reprobed, increased total caveolin-1 and phosphorylated cSrc, in the same fractions (Figure 5B). PP2 pretreatment considerably reduced the presence of phosphorylated and total caveolin-1 and as expected, completely abrogated phosphorylated cSrc. Pretreatment with calphostin C blocked phosphorylation of cSrc (Figure 5C) and reduced phosphorylated and total caveolin-1. These results are consistent with a signalosome within infected lipid rafts including PKC α , cSrc, and caveolin-1.

PKC α Controls the Activation of cSrc in Endosomes of Viral Infected Corneal Fibroblasts

We previously demonstrated the presence of viral DNA and showed increased cSrc kinase activity in the same caveolin-1 enriched endosomes from virus infected corneal fibroblasts.

¹¹ To determine signaling activity in the same endosomal compartment after infection, we

performed flotation gradient fractionation of post nuclear supernatants,³⁹ followed by Western blot analysis using phospho-specific antibodies to PKC α , cSrc, and caveolin-1 (Figure 6A). At 1 h post infection, virus infected fractions #7–10 expressed greater phosphorylated PKC α than mock infected fractions. The increase in PKC α phosphorylation was blocked by pretreatment with calphostin C. Blots were then stripped and reprobed for phosphorylated cSrc and caveolin-1. Phosphorylation of cSrc in fractions # 7–10 was increased by infection and abrogated by calphostin C. Caveolin-1 phosphorylation was also increased by infection and reduced by calphostin C. To confirm a role for PKC α in cSrc kinase activation in the same endosomal fractions, we performed a cSrc kinase assay on pooled fractions # 7–10 (Figure 6B). In this assay, fluorescence and kinase activity are inversely proportional. Cells treated with DMSO and either mock infected or virus infected, and cells treated with calphostin C in the absence of infection, served as controls. Src kinase activity in pooled fractions # 7–10 was increased by virus infection as compared to mock infection, and this increase was blocked by calphostin C treatment ($p < .05$, both comparisons). Taken together, these data suggest a pathway in which adenovirus is internalized and trafficked in corneal fibroblasts by endosomes expressing PKC α , cSrc, and caveolin-1. A schematic model of adenovirus signaling is shown (Figure 6C), comprising our findings from this and previous publications.^{11,23–26,50}

DISCUSSION

PKC plays important roles in regulation of cell growth, differentiation, apoptosis, secretion, neurotransmission, and signal transduction.^{51,52} Classical PKC signaling begins upon activation of G protein-coupled receptors and receptor tyrosine kinases,⁵² where, for example, binding of epidermal growth factor, platelet-derived growth factor, or hormones initiates a cascade leading to DAG formation and PKC activation.⁵³ Not surprisingly, viral infections can also active PKC. For example, respiratory syncytial virus infection of human bronchial epithelial cells was previously shown to induce phosphorylation of PKC α at T638.⁴

We set out to determine if PKC could be active upstream in adenovirus infection. Of the 10 or so PKC isoforms, we tested corneal fibroblasts for the expression of the most commonly studied PKC α , θ and ζ , and identified only PKC α . We then demonstrated rapid (within 15 min of infection) phosphorylation of PKC α at T638 and subsequent endosomal translocation in adenovirus infected cells, consistent with initiation of PKC α activity at the very earliest stages of viral binding and internalization, and within a time period consistent with activation prior to that of cSrc.²³ An upstream role for PKC was further suggested by the inhibitory effect of the PKC inhibitor calphostin C^{41,42} on phosphorylation of cSrc and AKT, the latter previously shown to be activated by adenovirus infection.²¹

PKC regulates the internalization and trafficking of many plasma membrane proteins, for example, the growth factor receptor c-Met,⁵⁴ the dopamine transporter,⁵⁵ the epithelial Na⁺ channel,⁵⁶ and plasma membrane integrins.⁵⁷ Phosphorylation and lipid raft-mediated internalization of another transporter, NET, is also PKC dependent.^{58,59} Before activation, PKC exists predominantly in an inactive folded structure in the cytosol. The generation of calcium and DAG initiate PKC activation, resulting in translocation of PKC from the cytosol

to the plasma membrane where PKC C1 and C2 domains bind with DAG/phosphatidylserine and calcium/anionic phospholipids, respectively.⁶⁰

In respiratory syncytial virus infection, it was shown that PKC α translocates to the plasma membrane upon infection.⁴ However, in our study, adenovirus infection induced PKC α translocation to intracellular vesicles. Caveolins are the major integral membrane protein of caveolae and include three types with a similar molecular weight of 22 kDa.^{44,61,62} It was previously reported that PKC α resides in the same plasma membrane fractions by sucrose gradient as caveolin-1.⁶³ TPA exposure induces the transport of activated PKC α from the plasma membrane to endosomes via a caveolae-mediated process.⁴³ We identified PKC α , caveolin-1, and cSrc in same lipid raft fractions after infection, where adenovirus infection induced phosphorylation of all three. Caveolin-1 was phosphorylated at Y14, in accordance with a prior report showing Src activation of caveolin-1,⁴⁹ and calphostin C reduced caveolin-1 phosphorylation, consistent with a signaling cascade from PKC to caveolin-1 through cSrc.

We recently showed that adenovirus type 37 enters corneal fibroblasts via caveolin-1 associated endocytosis.¹¹ Fission of membrane-associated caveolae and their release into the cytosol are important for Gp60-activated albumin endocytosis.⁶⁴ Expression of Y14F caveolin-1 abolished the endocytosis of albumin and cholera toxin subunit B.⁶⁵ In pulmonary microvessel endothelial cells, pretreatment of cells with chemical inhibitors of Src kinases abolished the phosphorylation of caveolin-1 at residue 14.⁶⁵ We also previously reported that inhibiting cSrc activity reduces adenovirus cellular entry.²¹ In the current study, calphostin C treatment was shown to reduce viral entry and E1A gene expression by the virus, the latter likely a direct effect of reduced viral entry. Our data and these prior publications implicate PKC α activation of cSrc, and Src phosphorylation of caveolin-1, in the entry of adenovirus in cells with caveolae.¹¹

Epidemic keratoconjunctivitis is an ocular adenoviral syndrome with significant and prolonged corneal involvement,⁶⁶ in which infection of corneal fibroblasts,³⁶ and activation of cSrc,²³ have been implicated in the onset of chronic keratitis. However, the mechanism of cSrc activation upon adenovirus infection has until now been a mystery. In the present study, we demonstrate that adenovirus infection results in activation of PKC α , and that inhibition of PKC reduces cSrc and AKT activity, and inhibits viral entry. We previously showed adenovirus enters corneal fibroblasts in a caveolin-1 dependent fashion. In the present study, virus activated PKC α translocated to intracellular vesicles, also containing caveolin-1. Caveolin-1 was phosphorylated only in lipid raft fractions where both activated cSrc and PKC α were present. The phosphorylation of cSrc and PKC α occurred in same endosomal fractions, and PKC α and caveolin-1 colocalized upon infection in a PKC α phosphorylation dependent fashion. PKC α inhibition also abolished adenovirus early gene expression, consistent with a role for PKC α in viral entry. The sum of our observations suggests that PKC α activation occurs early upon adenovirus infection, where it regulates the activity of downstream effector kinases previously demonstrated to play a role in adenovirus entry, cell survival, and inflammation.

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ABBREVIATIONS

CAR	coxsackie adenovirus receptor
DAG	diacylglycerol
IP₃	1,4,5-triphosphate
MOI	multiplicity of infection
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
RGD	arginine-glycine-aspartate

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Figure 1.

Adenovirus infection of corneal fibroblasts induces activation and translocation of PKC α . (A) SDS-PAGE followed by Western blot analysis for PKC isoforms in serum starved, primary, human corneal fibroblasts shows the presence of the PKC α but not the θ or ζ isoforms. Twenty micrograms of protein was loaded in each well. Anti-GAPDH antibody was also used as a control. The right-most lane is the molecular weight marker. (B) Representative Western blot analysis for phosphorylated and total PKC α upon adenovirus infection. Mock infection of serum-starved fibroblasts with dialysis buffer (M), purified, endotoxin-free adenovirus type 37 (V) at a multiplicity of infection of 10, for 15 min, or adenovirus type 37 following pretreatment with calphostin C (Cal.C + V; 1 μ M for 3 h prior to infection) shows increased phosphorylated PKC α (p-PKC α) relative to total PKC α (t-PKC α), and reduction of p-PKC α when cells were pretreated with Cal.C. Virus-infected cells (V) were pretreated with DMSO for 3 h as a control. (C) Densitometric analysis of three consecutive Western blots as in (B), normalized to mock infected levels shows reduction in p-PKC α when fibroblasts are treated with Cal.C prior to infection (* p < 0.05).

(D) Immunofluorescence microscopy after infection shows movement of p-PKC α (green) from the plasma membrane to the cytosol within 15 min of infection, and further concentration of p-PKC α in intracytoplasmic vesicles (inset) at 30 min post infection. Isotype control (ctl) treated and others were also stained with DAPI to show cell nuclei.

Figure 2.

Viral activation of PKC α occurs upstream of cSrc and AKT and impacts viral entry in human corneal fibroblasts. (A) and (B) show representative Western blots; (C) and (D) show densitometric analysis of three blots as in (A) and (B) for phosphorylated cSrc (p-cSrc; aa: Y416) and AKT (p-AKT; aa: T308) in comparison to total cSrc (t-cSrc) and total AKT (t-AKT) after mock infection (M) with virus-free dialysis buffer, infection with purified adenovirus type 37 (V) for 1 h, or in cells pretreated with calphostin C (1 μ M) for 3 h prior to 1 h infection (Cal.C+V). All cells were serum starved and virus-infected cells (V) were pretreated with DMSO for 3 h as a control. The lower blots for t-cSrc and t-AKT were generated by stripping and reprobing the blots above them. Calphostin C pretreatment reduced phosphorylation of both cSrc and AKT otherwise increased by viral infection (* p < 0.05), consistent with an upstream role for PKC α in their activation. (E) Confocal microscopy and (F) quantitative analysis using ImageJ, of viral entry into cells at 30 min

post infection, shows reduced entry in cells pretreated with calphostin C (* $p < 0.05$). Cy3-labeled virus appears red. Cellular actin is stained with phalloidin (green).

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Figure 3. Virus infection induces physical association of phosphorylated PKCα (p-PKCα) with caveolin-1 (Cav-1) in whole corneal fibroblast lysates by immunoprecipitation assays (A) and (B), and confocal microscopy (C). Immunoprecipitation (IP) with anticaveolin-1 (A), or anti-p-PKCα (B) followed by immunoblotting (IB) shows increased p-PKCα in caveolin-1 immunoprecipitates and increased caveolin-1 in p-PKCα immunoprecipitates, respectively, at 30 min after viral infection (V), as compared to mock infection (M) with virus-free dialysis buffer. Antirabbit and antimouse IgG isotype controls did not immunoprecipitate either protein. Immunoconfocal microscopy (C) at 30 min post infection demonstrates mostly membrane bound p-PKCα (Alexa Fluor 488: green) and caveolin-1 (Cav-1, Alexa Fluor 568: red) in mock infected cells (M). Virus infected cells (V) show intracellular colocalization of p-PKCα and caveolin-1 in intracytoplasmic vesicles. Cell nuclei are stained with DAPI (blue). Original magnification: 63×.

Figure 4.

Inhibition of PKC α activity reduces early viral gene expression of adenovirus type 37 in human corneal fibroblasts. (A) Reverse transcriptase PCR at 4 h after mock (M) or viral (V) infection, with either DMSO or calphostin C (Cal.C+V) pretreatment at 1 μ M for 3 h prior to infection. Total RNA was isolated, reverse transcribed, and subjected to PCR with primers specific for E1A and the control gene GAPDH and run on a 1% agarose gel. GAPDH expression is shown, along with reverse transcriptase negative (RT-) controls. E1A expression in virus infected cells is blocked by calphostin C. (B) Representative Western blot (left) and densitometric analysis (right) of three consecutive Western blots show PKC α -

specific siRNA knockdown of PKC α protein expression in comparison to untreated (Naïve) cells, and those treated with scRNA (* $p < 0.05$). (C) Quantitative real-time PCR analysis of E1A expression at 4 h post infection shows almost complete reduction in E1A mRNA expression in cells pretreated with PKC α -specific siRNA (* $p < 0.05$).

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Figure 5.

Lipid raft preparations demonstrating increased the presence of p-PKC α , phosphorylated and total caveolin-1 (p-Cav-1 and t-Cav-1, respectively) and phosphorylated cSrc (p-cSrc) in the same raft fractions 1 h after viral infection (V) as compared to mock infection (M), and the effect of chemical inhibition of cSrc by PP2 (A) and (B), and of PKC α by calphostin C (Cal.C) in (C). (A) Western blot for p-PKC α shows increased signal upon viral infection, with slight reduction upon PP2 treatment (10 μ M, or DMSO control). (B) Western blot for p-Cav-1, with or without pretreatment by PP2 (or DMSO control), stripped and reprobed for p-cSrc and then stripped and reprobed for t-Cav-1, shows increased p-Cav-1 and p-cSrc in the

same lipid raft fractions with a reduction in phosphorylation for both in cells pretreated with PP2. t-Cav-1 in the lipid raft fractions was also slightly reduced by PP2 pretreatment. (C) Western blots on cells pretreated with either DMSO control or calphostin C ($1 \mu\text{M}$) for 3 h prior to mock or virus infection, and stripped and reprobed as in (B), show a dramatic reduction in p-Cav-1 but not t-Cav-1, and complete abrogation of p-cSrc expression by calphostin C in the same lipid raft fractions.

Figure 6.

Activation of cSrc and caveolin-1 in endosomes occurs downstream of PKC α . (A) Western blot analysis of endosomal preparations of serum starved corneal fibroblasts mock infected (M), DMSO (V), or calphostin C pretreated and virus infected (Cal.C+V) for two hr. Postnuclear extracts were subjected to endosome purification using sucrose gradient centrifugation. After SDS-PAGE, membranes were blotted with antibody to p-PKC α , then stripped and reprobed with antibody against phosphorylated cSrc (p-cSrc), and then stripped and reprobed with antibody to phosphorylated caveolin-1 (p-Cav-1). Virus infection increased expression of all three proteins, and all were markedly reduced by pretreatment with calphostin C. (B) cSrc kinase assay performed at 2 h post infection on pooled endosomal preparation fractions 7 through 10 shows increased cSrc activity in virus infection (V) than in mock infection (M) or in virus infected cells pretreated with calphostin C (Cal.C + V, $*p < 0.05$). Uninfected cells treated with only calphostin C are shown as a control. Note that in this assay, increased kinase activity manifests by reduced fluorescence. (C) Schematic of PKC α signaling initiated by adenovirus infection of corneal fibroblasts.

Table 1

PCR Primer Pairs

	forward	reverse
caveolin-1	GGGCATTACTTCGCCATTC	ACGGTGTGGACGTAGATGGA
E1A	TTCTCCCAGCGATTCAGAG	GGGCACCTCAGGATTGTCC
GAPDH	TGGGCTACACTGAGCACCAG	ACCACCCTGTTGCTGTAGCC
PKC α	TTCTTCCGGAGGATCGAGTG	TTAAGACGGGCTGTCCTCGT