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Calcium-dependent viral internalization is required for adenovirus type 7 induction of IL-8 protein

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Abstract

The host response to adenovirus (Ad) infection involves induction of cytokines in lung epithelia. We have demonstrated induction of the lung neutrophil chemokine interleukin-8 (IL-8) by Ad7, a major lung pathogen, in A549 lung epithelial cells and lung tissue through activation of the Erk signaling pathway. However, the mechanism of IL-8 induction is still unclear. In this paper, we first showed that Ad7 viral gene expression is not essential for IL-8 induction as psoralen-UV inactivation of Ad7 did not affect IL-8 mRNA induction or IL-8 protein induction in A549 cells. We then inhibited internalization of Ad7 by treatment of A549 cells with EGTA in calcium-free medium during exposure to Ad7. We verified that this treatment inhibited Ad internalization by confocal microscopy, FACS analysis and Ad E1A and fiber mRNA expression. Preventing internalization by calcium depletion did not inhibit Erk activation by Ad7. However, calcium-dependent internalization was required for IL-8 protein production in Ad7 exposed cells. This is not likely due to an effect of calcium depletion on downstream Erk signaling or IL-8 protein production since calcium depletion did not block IL-8 protein production stimulated by PMA, and because addition of EGTA subsequent to Ad7 internalization also did not prevent Ad induction of IL-8. These studies indicate that Ad7 internalization is calcium-dependent and is required for IL-8 protein induction upon Ad7 infection. Ad7 induction of Erk is independent of calcium and does not require virus internalization.

Keywords: Adenovirus; Internalization; IL-8; Calcium; Erk; EGTA

Introduction

Adenovirus serotype 7 (Ad7) is a significant human pathogen that is especially problematic among children and adults in crowded living conditions. Fifty one human Ad serotypes have been identified and are categorized into six subgroups (A–F) based on their DNA sequence and resistance to neutralization by antisera to other known Ad serotypes. Ad7 belongs to the B subgroup of Ad. This subgroup in humans causes particularly severe lower respiratory tract infections. Likewise, Ad7 infections are the most common cause of febrile respiratory tract infection and pneumonia in military recruits, and have been responsible for 90% of the recruit hospitalizations for pneumonia (US Medicine, 2000). In young children, mortality rates for Ad pneumonia reach 23–29% (Brown et al., 1973; Carballal et al., 2002). Clinically, severe Adenovirus pneumonia causes respiratory failure with acute respiratory distress syndrome (Klinger et al., 1998).

Adenovirus initiates infection by attaching the Ad fiber to a primary receptor on the cell surface. The Coxsackie and adenovirus receptor (CAR) is the high-affinity receptor for all human Ad subgroups except subgroup B (Bergelson et al., 1997; Roelvink et al., 1998). In this subgroup CD46 is the primary receptor for most serotypes except for Ad7 and Ad3 (Marttila et al., 2005). Subsequent to fiber binding, the viral penton base protein binds to a secondary receptor, $\alpha v\beta 3$, $\alpha v\beta 5$ (Wickham et al., 1993), or $\alpha v\beta 1$ integrins, depending on the

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cell type (Freimuth, 1996; Huang et al., 1996; Li et al., 2001). After adsorption, the virus is internalized in a dynamin-dependent manner via receptor-mediated endocytosis using clathrin-coated pits (Duncan et al., 1978; Roelvink et al., 1998; Varga et al., 1991). The virus remains in the endocytic vesicle as the vesicle matures from an early endosome stage to a later endosome stage. The virus penetrates into the cytosol by lysis of the endosome. The intracellular trafficking of Ad subgroup B (e.g., Ad7) differs from that of subgroup C (e.g., Ad5) in that Ad5 rapidly escapes from endocytic compartments following infection whereas Ad7 accumulates in organelles (Miyazawa et al., 2001). The native trafficking pathway for Ad7 involves residence in late endosomes and lysosomes, with information encoded in the Ad7 fiber acting as a pH-dependent trigger for membrane lysis and escape to the cytosol (Miyazawa et al., 1999). The virion translocates to the nuclear membrane and disassembles at the nuclear pore. Finally, Ad DNA is released into the nucleus and early viral genes are expressed (Bridge and Pettersson, 1995; Greber et al., 1996; Seth, 1994).

Interleukin-8 (IL-8 or CXCL8) was the first characterized neutrophil chemotactic factor and is a prototypic example of the class of cytokines, called chemokines, which exhibit chemotactic activity for a limited spectrum of leukocytes (Mukaida, 2003). Neutrophil infiltration occurs rapidly and in a dose-dependent manner to the injection of human IL-8 in all animal species tested (Baggiolini et al., 1994; Larsen et al., 1989; Leonard et al., 1991). IL-8 plays a central role in the pulmonary innate immune response to bacterial, fungal and viral lung infections (Balish et al., 1999; Matsukawa et al., 2000; Tsai et al., 2000). However, uncontrolled IL-8 production can lead to intense tissue injury and inflammatory damage in the lung, and is thought to be central in the pathogenesis of acute respiratory distress syndrome (Baughman et al., 1996; Donnelly et al., 1993). The alveolar epithelium is one of the major sources of IL-8 during lung infection. The epithelium is the initial contact point for airway pathogens, and releases chemokines immediately following exposure to pathogens (reviewed in Diamond et al., 2000). The lung epithelia, therefore, plays an important role in activation of the innate immune system.

We have reported that the human alveolar type II cell line, A549, releases IL-8 upon infection with Ad7 by extracellular signal-regulated kinase (Erk) activation (Alcorn et al., 2001; Booth and Metcalf, 1999). We have also confirmed the importance of Erk activation in Ad7-mediated IL-8 induction in human and bovine lung slice organ culture models (Booth et al., 2004). However, it is still not clear which step during attachment and internalization activates the Ras/Raf/Erk pathway and induces IL-8.

In this report, we demonstrate that an Ad7 virus preparation in which viral gene expression is inactivated still induces IL-8 from A549 cells. Inhibition of Ad internalization, in calcium-free conditions with EGTA, slightly delayed but did not significantly inhibit activation of Erk but completely inhibited IL-8 protein production into the media. Additional experiments showed that calcium depletion did not cause sequestration of IL-8 protein in Ad7 infected cells, and did not block IL-8 induction or release from PMA-stimulated cells. Thus, calcium-dependent Ad internalization appears to be a critical event in IL-8 protein production in Ad7 infected cells. Overall, the results suggest that signal pathway activation from Ad is not internalization-dependent, but viral internalization is required for IL-8 protein induction upon Ad7 infection.

Results

Adenovirus gene expression is not necessary for IL-8 gene induction

To determine if Ad viral gene expression is essential for IL-8 induction during Ad infection of A549 cells, Ad7 was inactivated by long wave irradiation in the presence of psoralen. The treatment cross-links and inactivates Ad DNA with less damage to Ad surface protein than short-wave UV irradiation used by others or γ-irradiation which we used in our prior study. (Booth and Metcalf, 1999). Inactivation was determined by performing a plaque assay in comparison to a sample of untreated virus stock. Psoralen-UV inactivation typically resulted in a near complete loss of cytotoxicity. Equivalent doses of inactivated and live virus were used for experiments as determined by comparative Ad hexon ELISA assays (see Materials and methods). Near confluent A549 cells were exposed to 50 MOI of either Ad7 or psoralen-UV inactivated Ad7 (Ps-Ad7). Viral diluent (PBS plus 10% glycerol) was used for mock infection and PMA (100 ng/ml) was used as a positive control. No Ad E1A mRNA was detected at 2 and 4 h post-infection with Ps-Ad7. A trace amount of E1A mRNA was found after 8 h. This could be due to trace amounts of active virus or may be due to DNA repair by the host cells. RT-PCR revealed IL-8 mRNA induction at 2 and 4 h post-infection despite ablation of viral gene expression (Fig. 1A). IL-8 mRNA was quantified and normalized using the corresponding GAPDH mRNA levels for each sample. This revealed that induction of IL-8 mRNA was similar at 2 and 4 h post-infection for normal and inactivated viruses. In addition, there was no obvious delay in IL-8 mRNA induction (Fig. 1B). Taken together, these observations indicate that IL-8 mRNA induction is independent of Ad gene expression.

We then investigated the response of IL-8 protein induction to Ps-Ad7. A549 cells were infected by 50 MOI of Ad7 or Ps-Ad7 as described earlier. At various incubation times from 4 to 24 h, supernatants were collected and assessed for IL-8 protein levels by ELISA (Fig. 2). Ad7 infection of A549 cells caused a four- to six-fold increase in IL-8 production above mock-infected cells, as expected. Exposure to Ps-Ad7 caused a similar, but slightly lower, induction of IL-8 protein. This is presumably due to some viral protein damage during UV treatment. The amounts of IL-8 induced by both preparations of Ad7 were approximately 25% of the amounts induced by 100 ng/ml of PMA. From these experiments, we conclude that Ad gene expression is not necessary for IL-8 protein induction by Ad7.

Inhibition of Ad internalization by EGTA

In order to determine the effect of various agents and conditions on Ad7 viral internalization into A549 cells, we developed a flow cytometry (FACS) internalization assay. Ad7 was labeled with the fluorescent dye Cy3 (Cy3-Ad7), and FACS and confocal microscopy in the presence or absence of trypsin were used to assess viral internalization. Preliminary experiments showed that labeling virus with the fluorophore did not affect Ad infectivity (data not shown).

At 4 °C, virions still attach to the host cell surface, but internalization was prevented. Our FACS assay and confocal images confirmed that at 4 °C Ad binding occurred (Figs. 3B and I) but internalization was blocked as almost all cell associated Cy3-Ad7 fluorescence was removed by trypsin treatment (Figs. 3C, J). However, at 37 °C in the absence of inhibitors, internalization occurred because the Cy3-Ad7 fluorescence became trypsin-resistant, i.e., internalized (Figs. 3E, L).

EGTA chelates free divalent calcium cation (Ca^{2+}) in the extracellular medium. In the process of examining reagents that could efficiently inhibit virus internalization, we found that calcium-free media with 100 μM EGTA and 10% dialyzed fetal calf serum reliably inhibited virus internalization. These conditions caused a slight decrease in virion binding at 4 °C. In contrast, calcium depletion inhibited internalization dramatically at 37 °C (Figs. 3E vs. G). This result was also confirmed by confocal microscopy (Figs. 3L and N). Together, these data demonstrate that extracellular calcium depletion mildly inhibits Ad binding but greatly diminishes Ad7 internalization.

To verify that Ad7 internalization was inhibited under calcium-free conditions, semi-quantitative RT-PCRs of Ad E1A, Ad fiber and GAPDH were performed. E1A and fiber mRNA amounts were normalized to the corresponding GAPDH values for each sample. Under normal conditions, significant amounts of E1A were observed at 2 h after infection (Fig. 4). Under calcium-free conditions, E1A and fiber expression were significantly delayed and did not reach the expression level seen in cells infected for 2 h under normal conditions even after an additional 6 h of infection.

There are several agents that have been considered to be inhibitors of viral internalization. Some of these agents, for example, wortmannin and LY294002 block internalization and alter intracellular signaling (both are potent inhibitors of the phophoinositide-3-OH kinase pathway), and, thus, would be inappropriate for this study which sought to determine the effects of Ad internalization and not signaling pathway activation on IL-8 induction by Ad7 (Li et al., 1998). We also tested other agents, including jasplakinolide, and cytochalasin D, which were reported to prevent Ad internalization by inhibiting functional actin polymerization. Unfortunately, these agents did not cause significant inhibition of Ad7 internalization as determined by FACS or confocal microscopy. The most reliable method in our hands to inhibit Ad7 internalization, which did not affect Erk signaling or IL-8 induction by other stimuli, specifically PMA, was calcium depletion. Thus, we chose this method to examine the importance of Ad7 internalization in IL-8 induction.

Erk is activated by adenovirus without viral internalization and external Ca^{2+}

Our previous work showed that infection with Ad7 rapidly induced Erk activation as exhibited by phosphorylation, and that Erk activation was necessary for Ad7 induction of the IL-8 promoter, IL-8 mRNA levels, and IL-8 protein (Alcorn et al., 2001). We next sought to determine whether Erk activation by Ad7 required virus internalization. Cell extracts were prepared from mock-infected, Ad7 infected, or PMA stimulated cells. Erk activation was analyzed by SDS-PAGE followed by immunoblotting using an antibody recognizing dually phosphorylated Erk (Fig. 5). Erk activation by Ad7 occurred rapidly after infection in normal conditions, and reached 60% of that seen with PMA. Prevention of internalization by calcium depletion did not affect the degree of Erk activation by Ad7, although there was a slight delay in maximal Erk activation in calcium-free conditions (Fig. 5). These results demonstrate that activation of Erk by Ad7 does not require virus internalization or external calcium.

Calcium-dependent Ad7 internalization is required for IL-8 protein induction

We then determined whether inhibiting Ad7 internalization under calcium-free conditions prevented IL-8 protein induction. A549 cells were exposed to 50 MOI of Ad7, virus-free buffer or PMA (100 ng/ml) as a positive control. Experiments were conducted under calcium-repleted conditions or in calcium-free media with 100 μM EGTA and 10% dialyzed fetal calf serum. Cell culture supernatants were analyzed for IL-8 protein by ELISA (Fig. 6A). In calcium-free conditions, IL-8 production was reduced to background levels at all times after infection. With calcium in the media, IL-8 production increased four-fold after cells were exposed to Ad7 for 24 h, consistent with our previous findings (Alcorn et al., 2001).

To examine whether EGTA inhibits IL-8 production through mechanisms other than the effect on Ad internalization, 100 μM of EGTA was added to the media 1 h after Ad7 infection of A549 cells (Fig. 6B). There was no effect of this subsequent calcium depletion on IL-8 protein induction by Ad. Furthermore, PMA induction of IL-8 production was not affected by extracellular calcium depletion prior to, and during stimulation. These results suggest that calcium depletion did not damage the cellular response to stimuli or IL-8 protein export.

We next determined whether blocking Ad7 internalization prevented induction of IL-8 protein production or prevented IL-8 export in calcium-free conditions. This was determined by measuring intracellular IL-8 protein levels in Ad7 and PMA exposed cells by Western blot (Fig. 7). The fungal metabolite, brefeldin A (BFA), inhibits export from the distal Golgi compartment to the cell surface (Miller et al., 1992). We used BFA-treated cells as a positive control in a determination of

whether IL-8 protein sequestration could be responsible for the lack of IL-8 induction in cells exposed to Ad7 under calcium-free conditions. As shown in Fig. 6, in the presence of BFA (5 μg/ml), large amounts of IL-8 are sequestered inside cells treated with Ad7 or PMA. In the absence of BFA, A549 cells do not appreciably store IL-8, even with Ad7 and PMA stimulation. Most importantly, there is no IL-8 sequestered in cells stimulated by Ad7 in calcium-free conditions, indicating IL-8 protein is not induced by Ad7 when virus internalization is inhibited by these conditions. The fact that calcium depletion does not prevent induction of IL-8 by other stimulus strongly suggests that internalization is a critical factor in IL-8 induction by Ad7.

Discussion

Induction of cytokines by Ad is important in the inflammatory response to wild-type infection and exposure to Ad based gene therapy vectors. To modulate the consequences of infection or Ad vector exposure, it will be important to understand how the virus stimulates the cytokine response.

We reported previously that γ-irradiated inactivated Ad7 failed to induce IL-8 from A549 cells. We feel this is due to the higher energy of γ-irradiation, resulting in destruction of the surface proteins of the virus (Booth and Metcalf, 1999). Subsequent to our study, Cotton et al. demonstrated that psoralen-UV inactivated Ad eliminates virus replication and transcription while maintaining biologic activity as evidenced by endosomolytic activity of the virus capsid (Cotten et al., 1994). It is a perfect model to study the effect of virus entry into host cells in the absence of virus gene expression. Our work confirms that Ad7 viral gene expression is not necessary for induction of the IL-8 protein, and thus, an earlier step in the viral infectious pathway is responsible for induction. Therefore, viral gene expression by Ad7 is not required for stimulation of the innate immune cytokine response. We do not propose that the mechanism of WT Ad-mediated inflammation is identical to that of Ad vectors. However, our results are consistent with data from studies by Muruve and Schnell who demonstrated that administration of psoralen-UV inactivated Ad5 vectors in mice and monkeys caused cytokine induction and significant inflammation in vivo (Muruve et al., 1999; Schnell et al., 2001).

We also demonstrate that calcium-mediated processes are important in Ad internalization. The cytoplastic Ca^{2+} concentration is tightly regulated. The host cell has two potential sources of Ca^{2+}: (1) entry from the external medium and (2) release from internal stores. The Ca^{2+} ionophores A23187 and thapsigargin increase intracellular calcium availability through release from Ca^{2+} stores. These agents enhance IL-8 gene expression in monocytes, neutrophils, T cells and colonic epithelial cells (Kuhns et al., 1998; Okamoto et al., 1994; Wilson et al., 1993; Yu et al., 2001). Therefore, increasing intracellular calcium levels enhance IL-8 expression. This calcium response does not appear to be due to influx from the media. EGTA chelates, and thus, lowers extracellular Ca^{2+} availability (Bers, 1982). In similar experiments as described, EGTA does not inhibit IL-8 expression. However, pretreatment with the intracellular calcium chelator BAPTA significantly decreases IL-8 responses (Ratner et al., 2001). In a study of induction of cytokines by Ad, BAPTA completely blocks Ad5 induced TNF-α expression in macrophages (Zsengeller et al., 2000). Taken together, these studies suggest that intracellular calcium levels fundamentally affect cytokine responses, while extracellular calcium levels do not. Thus, our findings showing that extracellular calcium depletion inhibits Ad7 induction of IL-8 are not due to an effect of calcium depletion on IL-8 production per se, but instead are due to the effect of depletion on Ad7 internalization. This is further supported by the fact that extracellular calcium depletion did not affect IL-8 protein induction by PMA and that adding EGTA 1 h after infection in our system did not affect IL-8 induction by Ad7. Our finding that WT Ad7 internalization is inhibited by calcium depletion is consistent with data showing that calcium is involved in Ad uptake in epithelial cells (Greber et al., 1997). We have confirmed that calcium is required for internalization, and determined the effect of blocking this process on the innate immune cytokine response to Ad.

Our data show that induction of the Erk pathway by Ad is solely a consequence of an early noninternalization dependent step in the Ad infectious pathway, likely engagement of a receptor on host cells by Ad capsid proteins. We also demonstrate that calcium-dependent internalization is required for IL-8 protein induction. The importance of pathogen internalization in the cytokine inflammatory response depends on the type of pathogenic agent and the type of cells studied. Induction of IL-8 by *streptococci* in human epithelial cell lines is thought to be due to interaction of the bacteria with cell surface receptors (Vernier et al., 1996). In the case of viral-mediated activation of cellular defense proteins, early steps in the infectious pathway are not always important in protein induction. In airway epithelial cells infected by human rhinovirus (HRV), human beta-defensin (HBD)-2 production is not induced by viral receptor engagement or viral RNA internalization, but instead is triggered by viral replication and is mimicked by transfection of cells with synthetic dsRNA (Proud et al., 2004).

In this report of A549 cells infected by Ad7, we conclude that Ad7-host cell receptor engagement is sufficient for Erk signaling pathway activation, but insufficient for the host cytokine response, and thus, virus internalization plays an important role in IL-8 production. Although Erk pathway activation is necessary for IL-8 induction by Ad7, there must be additional cellular signals activated by subsequent steps in the Ad infectious pathway that are necessary for IL-8 induction by Ad7.

IL-8 production is tightly regulated in the absence of external stimulation. Three different mechanisms are considered important for maximal IL-8 generation: (1) derepression of the gene promoter; (2) transcriptional activation of the gene by NF-κB and JUN-N-terminal protein kinase pathways; and (3) stabilization of the mRNA by the p38 mitogen-activated protein kinase pathway (Hoffmann et al., 2002). We have previously demonstrated that mRNA stabilization is not the key factor in induction of IL-8 by Ad7 from A549 cells, although it does

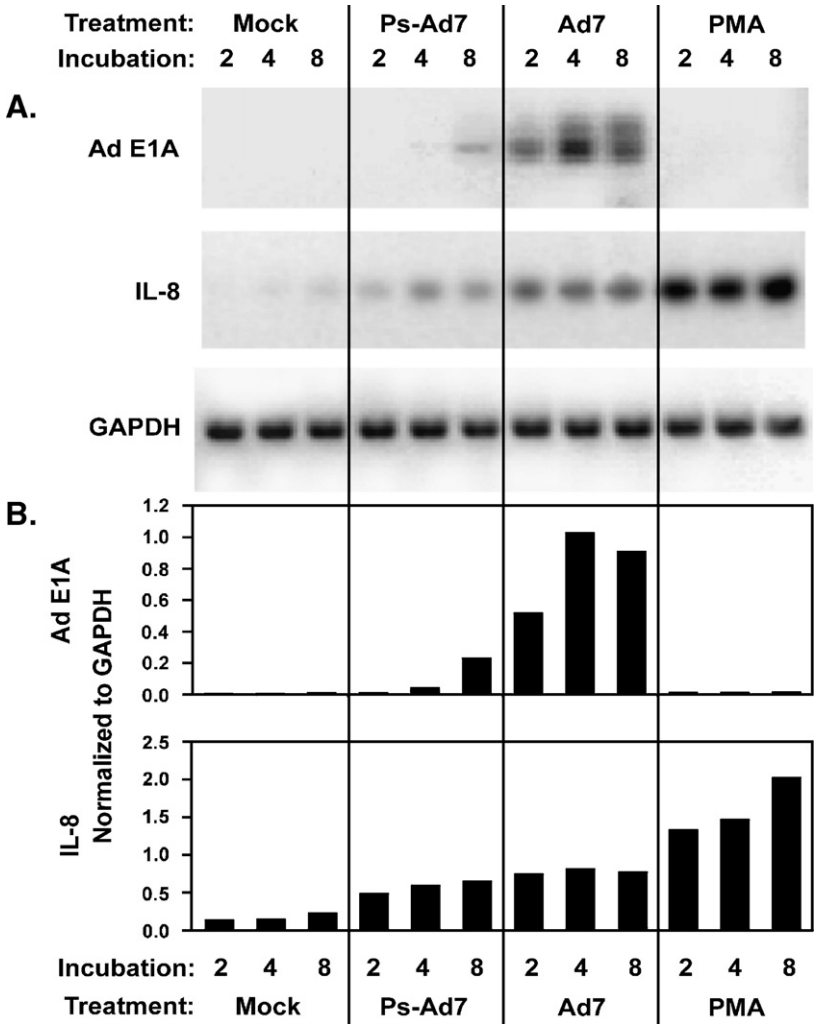


Fig. 1. Psoralen-UV inactivated Ad7 (Ps-Ad7) induces IL-8 mRNA in A549 cells. Ad stocks were inactivated using psoralen and UV irradiation to eliminate the possibility of viral DNA expression and replication. A549 cells were stimulated with either Ad7 or Ps-Ad7 at 50 MOI. Mock-treated cells received an equivalent volume of sterile virus dialysis buffer and positive control cells were stimulated with 100 ng/ml PMA. After incubation for the indicated times, the cells were harvested for whole RNA preparation. (A) Semi-quantitative RT-PCR of Ad E1A, IL-8 and GAPDH. (B) E1A and IL-8 mRNA amounts were normalized to the corresponding GAPDH values for each sample.

occur. Our work also demonstrated that promoter stimulation, likely through transcription factor activation, is more important for this process. In this light, our finding that internalization is required for IL-8 induction suggests that transcription factor activation resulting in promoter activation also requires internalization. Of note, in human upper respiratory epithelial cells infected by *streptococci*, adhesion of *streptococci* alone may be sufficient to stimulate nuclear translocation of the transcription factor NF-κB, while bacterial internalization is required for a sustained nuclear translocation of this transcriptional factor (Medina et al., 2002). A similar effect of Ad internalization on NF-κB or other transcription factors important in IL-8 promoter activation would be a likely cause of the findings presented here.

There are two cellular receptors engaged by adenovirus capsid proteins. The globular head of the Ad7 fiber protein attaches to an as yet unknown cellular receptor (Marttila et al., 2005). Subsequently, the penton base binds αvβ3, αvβ5, or αvβ1 integrins depending on the cell type. It is unknown whether stimulation of the Ad7 fiber receptor stimulates signaling pathways. However cross-linking of CD46, the fiber receptor used by other subgroup B Ad except Ad7, induces Erk activation (Zaffran et al., 2001). Also, stimulation of the other known Ad fiber receptor, CAR, used by nonsubgroup B Ad, induces the Erk pathway. It is well known that integrin clustering causes Erk activation (Juliano et al., 2004). Of particular relevance to our study, stimulation of the αvβ3 integrins activates Erk in smooth muscle cells (Clemmons and Maile, 2005). Thus, our findings showing Erk activation upon attachment of Ad to A549 cells could be due to stimulation of the fiber receptor or integrins. Our current study shows that Erk activation is not sufficient for IL-8 induction by Ad7, as when we arrest the Ad infectious pathway at the step important for activation, but prevent progression of the virus, IL-8 induction does not occur. The requirement for a divalent cation in Ad7 internalization is consistent with a role of penton base

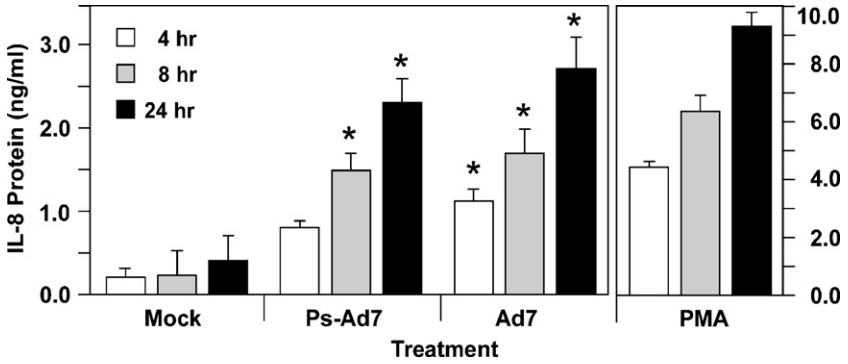


Fig. 2. Psoralen-UV inactivated Ad7 induces IL-8 protein release from A549 cells. Ad stocks were inactivated to eliminate the possibility of viral DNA expression and replication. A549 cells were stimulated with either Ad7 or Ps-Ad7 at 50 MOI. Mock-treated cells received an equivalent volume of sterile virus dialysis buffer and positive control cells were stimulated with 100 ng/ml PMA. After incubation for the indicated times, the media supernatants were collected for IL-8 ELISA. Data represent mean±SEM from three experiments. (*$P<0.05$ compared to corresponding mock-treated cells).

interaction with integrins. The interaction between penton base and integrins could be important in induction of IL-8 synthesis.

The fact that viral internalization is necessary for the innate immune cytokine response, as manifested by induction of the neutrophil chemotaxin IL-8, is important. It suggests that to modulate the acute inflammation that occurs during WT adenovirus infection, it is not necessary to block binding of the virus capsid to its cognate receptors. Instead, blockade of virus internalization should be sufficient to modulate the adverse inflammatory effects of infection. This approach would still prevent viral entry, and thus, diminish the ability of the virus to replicate and cause disseminated infection.

Materials and methods

Cells and virus

The human pulmonary epithelial cell line, A549, obtained from the American Type Culture Collection (ATCC, Manassas, VA, CCL-185) was used in binding and internalization experiments and to propagate and quantify adenovirus. The A549 cell line has the characteristics of alveolar type II cells. A549 cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 80 μg of gentamicin/ml. Ad7, also obtained from the ATCC (ATCC VR-7), was propagated and CsCl gradient ultracentrifugation purified as previously described (Alcorn et al., 2001; Booth et al., 2004). The titer of viable virus was quantified by plaque assay and the purified Ad7 preparations were stored at −80 °C.

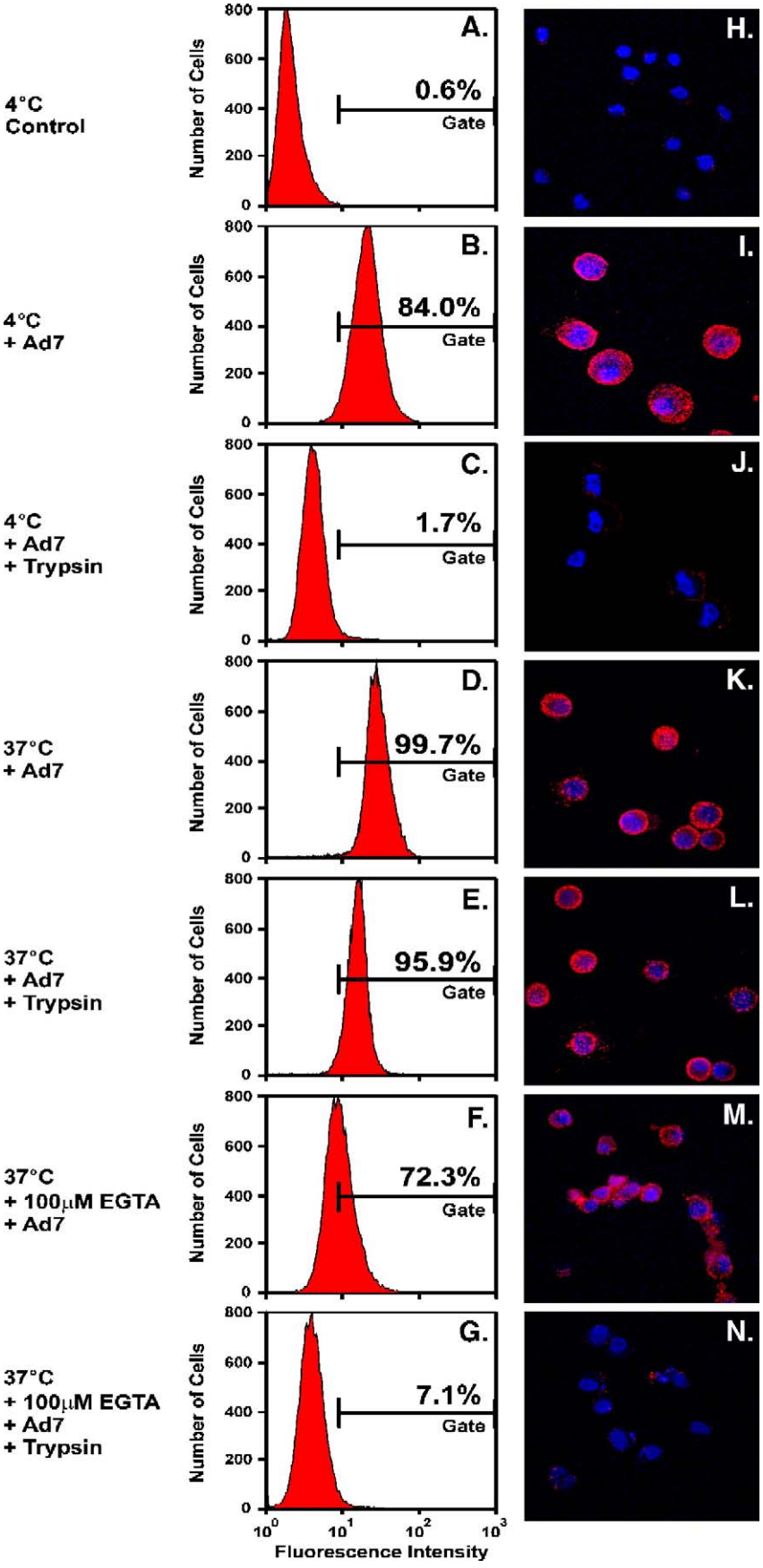
Psoralen-UV inactivation of Ad7 DNA

Ad stocks were inactivated to determine the role of viral DNA expression and replication in the induction of IL-8 (Cotten et al., 1994). In a 35-mm tissue culture dish, 1 ml of purified Ad stock was added to 1 ml of 0.5 mg/ml 8-methoxypsoralen (Sigma, St. Louis, MO) diluted in 100% glycerol (8-MOP initially dissolved in DMSO to 33 mg/ml). The mixture was then exposed to a 365-nm ultraviolet light source (Spectroline model ENF-260C; Spectronics, Westbury, NY) at a distance of 2 cm from the filter to the viral mixture for 4 h at 4 °C then mixed gently. This exposure is approximately 1.8×107 μW/cm^2 s. Following the treatment, the inactivated Ad7 was dialyzed against PBS+10% glycerol at 4 °C for 3 buffer changes. Inactivation was determined by performing a plaque assay in comparison to samples of untreated and mock-treated (treated with glycerol only) Ad7. Psoralen-UV inactivation typically resulted in a greater than eight-log loss of cytotoxicity (e.g., 1×10^{10} to $<1\times10^{2}$ pfu/ml). An Ad hexon sandwich ELISA was developed using an anti-adenovirus hexon monoclonal capture antibody and a biotinylated-anti-adenovirus hexon monoclonal secondary antibody (Chemicon, Temecula, CA). This assay was used to determine equivalent amounts of psoralen-UV irradiated and live, infectious virus. The amounts of virus used in our experiments (50 MOI) are based on a plaque assay of the live virus stock, and an equivalent amount of psoralen-UV inactivated virus as determined by the Ad hexon ELISA. Psoralen-UV-treated Ad was stored at −80 °C.

Semi-quantitative RT-PCR

To test the effect of psoralen-inactivated Ad7, A549 cells grown to 90% confluence were stimulated with either Ad7 or psoralen-inactivated Ad7 (Ps-Ad7) at a multiplicity of infection (MOI) of 50 plaque forming units (pfu)/cell. Mock-treated cells received an equivalent volume of virus dialysis buffer (sterile PBS+10% glycerol) and positive control cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma). After incubation for the indicated times at 37 °C, 5%CO_2, the media supernatants were collected for IL-8 ELISA, and the cells were harvested for preparation of whole cellular RNA. Solutions for RNA preparation were treated with diethylpyrocarbonate (1:1000).

To assess the effect of EGTA on Ad7 internalization A549 cells were grown to 90% confluence; the cells were washed in either complete HBSS or calcium-free HBSS, and the medium was replaced with either DMEM+10% FBS or calcium-free DMEM+10% dialysed FBS containing100 μM EGTA. Following incubation for 2 h at 37 °C, 5% CO_2; the media were replaced with fresh media and the cells were infected with 50 pfu/cell of Ad7. An equivalent volume of sterile virus dialysis


4°C
Control
A.
0.6%
Gate
H.
4°C
+ Ad7
B.
84.0%
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I.
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+ Ad7
+ Trypsin
C.
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J.
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D.
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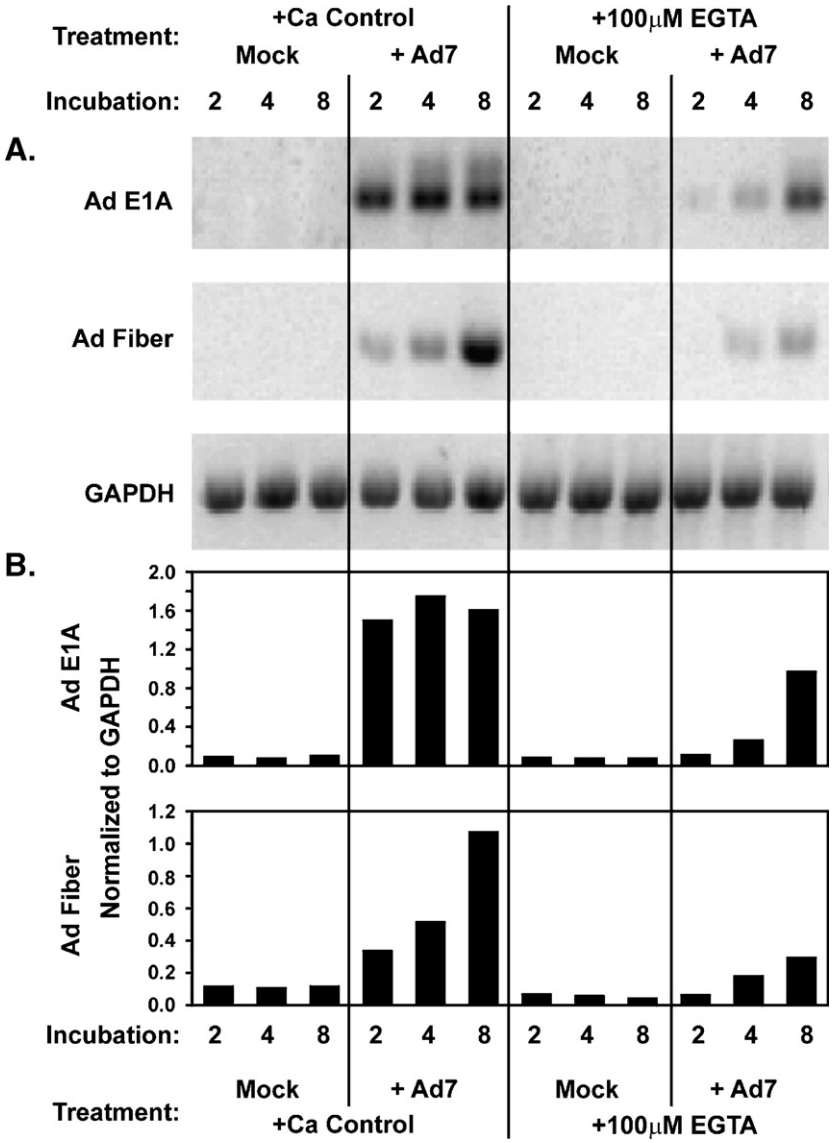

Fig. 4. Ad7 E1A and fiber mRNA expression are markedly inhibited by calcium depletion with EGTA during exposure of A549 cells to Ad7. A549 cells were exposed to Ad7 at an MOI of 50 under normal, or calcium-free conditions. After incubation for the indicated times, the cells were harvested for whole RNA preparation. (A) Semi-quantitative RT-PCR of Ad E1A, fiber and GAPDH. (B) E1A and fiber mRNA amounts were normalized to the corresponding GAPDH values for each sample.

buffer was added to mock-treated cells. After incubation for the indicated times at 37 °C, 5%CO_2, the cells were harvested for preparation of whole cellular RNA.

For semi-quantitative RT-PCR, equal masses (5 μg) of total RNA were used for cDNA synthesis using oligo(dT) primers and a commercially available kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Subsequently, equivalent amounts of the cDNA preparations were subjected to PCR amplification for 30 cycles (95 °C, 30 s; 55 °C, 1:00 min; 72 °C, 1:00 min) with the primer pairs for IL-8, Ad7 E1A, Ad7 fiber or GAPDH. The primers sequences are as follows: IL-8 sense ATGACTTCCAAGCTGGCCG, IL-8 anti-sense TTATGAATTCTCAGCCCTCTTC; Ad7 E1A sense GTTTTCCTCCGAGTGATGATGAAG, Ad7 E1A anti-sense TTATCCACAGCAGGGCGTTTC; Ad7 fiber sense GGGTCT-TACAA-TAGATGACACCG, Ad7 fiber antisense AGTG-GAGTTTTTAGGGATGAAAGG; GAPDH sense TGAAG-GTCGGAGTCAACGGATTTGGT, GAPDH anti-sense CATGTGGGCCATGAGGTCCACCAC. Following PCR, 10 μl aliquots of the reactions were run on 1% agarose gels and visualized by ethidium bromide staining. DNA bands were quantified using ImageQuant 5.0 software (G.E. Healthcare, Piscataway, NJ). E1A and IL-8 values were normalized to the corresponding GAPDH values for each sample.

Fig. 3. Extracellular calcium depletion with EGTA blocks Ad internalization. A549 cells were exposed to Cy3-Ad7 at an MOI ≅ 1000 with or without EGTA and the viruses were allowed to bind to the cells at 4 °C for 1 h. Following the incubations of selected samples with Cy3-Ad7 ± trypsin, the samples were washed in triplicate in the appropriate buffer to remove unbound virus. For FACS analysis (A–G), the percentage of cells exceeding the analytical gate was used to determine Ad binding and internalization. For confocal microscopy (H–N), the cells were resuspended, stained with DAPI and mounted on microscope slides (see Materials and methods). Panels A–G are from one experiment which are representative of 4 separate experiments.

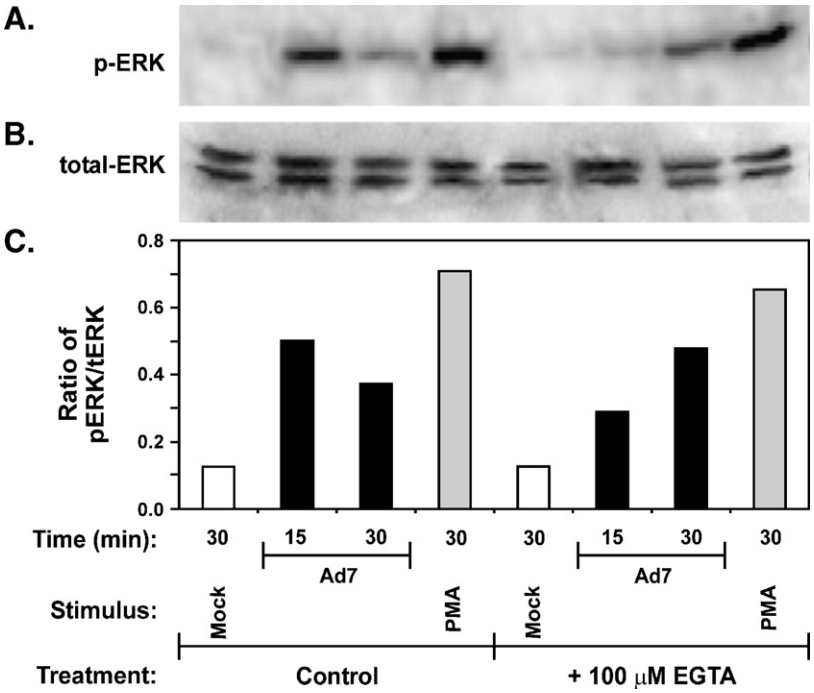

Fig. 5. Erk activation by Ad7 does not require extracellular calcium. Serum-starved A549 cells were exposed to Ad7 at an MOI of 50 for 15 and 30 min prior to preparation of cellular lysates. An equivalent volume of sterile virus dialysis buffer was added to mock-treated cells, and positive control cells were stimulated with 100 ng/ml PMA. Cells were treated normally or in calcium-depleted conditions to inhibit Ad internalization. Western blot was performed to determine phosphorylated active Erk (A) and total Erk (B). For calcium depletion, the cells were washed in calcium-free HBSS and stayed in serum-free, calcium-free DMEM containing100 μM EGTA for 2 h before infection. Erk activation is displayed as the ratio of phosphorylated active Erk to total Erk (C).

FACS analysis and confocal microscopy of Cy3 labeled Ad7

To assess Ad7 internalization by FACS and confocal fluorescence microscopy, *bis*-reactive, *N*-hydroxysuccinimido ester Cy3 fluorophore (G.E. Healthcare) was covalently conjugated to the Ad7 capsid. Ad7, 1 mg/ml (approximately 1×10^{10} PFU/ml), was reacted with Cy3 dye in 0.1 M sodium carbonate buffer (pH 9.3) for 30 min at room temperature with occasional mixing according to the manufacture's protocol for labeling proteins. Labeled virus was separated from the unconjugated dye by dialysis against multiple changes of PBS+ 10% glycerol at 4 °C. The degree of Cy3 labeling was calculated to be 2353 molecules of Cy3/Ad7 virion as derived from the absorbance values at 552 and 260 nm (Sweeney and Hennessey, 2002). Ad binding and infectivity was not diminished by the fluorophore, as assessed by plaque assay. The Cy3-Ad7 preparations were stored at −80 °C.

To prepare the cells for FACS and confocal microscopy, A549 cells in exponential growth phase were washed 3 times with calcium-free Hank's Balanced Salts Solution (HBSS), harvested from flasks using a nonenzymatic cell dissociation solution (Sigma), and resuspended in complete HBSS with 1% fetal calf serum or in calcium-free HBSS with 1% dialyzed fetal calf serum containing 100 μM EGTA. Aliquots of 100 μl containing 5×10^5cells were exposed to Cy3-Ad7 at a multiplicity of infection of approximately 1000 pfu/cell, and the virus was allowed to bind to the cells at 4 °C for 1 h. Negative control cells were exposed to an equal volume of sterile dialysis buffer. Selected samples were incubated at 37 °C for 30 min to allow for Cy3-Ad7 internalization. Surface associated Cy3-Ad7 was removed by incubating the cells in 0.25% trypsin, 2.21 mM EDTA in calcium and magnesium-free HBSS (Mediatech, Inc., Herndon, VA) for 15 min at 4 °C with gentle rocking. Following the incubations with Cy3-Ad7 and/or trypsin, the samples were washed in triplicate to remove unbound virus. The cells were fixed by incubation on ice in 300 μl freshly prepared 1% paraformaldehyde-PBS and then washed in triplicate. For FACS analysis, the cells were resuspended in 500 μl PBS supplemented with 0.1% fetal calf serum and 0.02% sodium azide. For confocal microscopy, the cells were resuspended in 200 μl of the same buffer and mounted on microscope slides using acrylic-based mounting medium containing DAPI (Invitrogen). A FACScan cytometer (BD Biosciences, San Jose, CA) equipped with an argon ion laser emitting at 488 nm and a fluorescence detector bandpass filter of 585/42 using summit Software with Cicero upgrade (Cytomation, Ft. Collins, CO) was used to assess Ad binding and internalization. A minimum of twenty thousand events were counted for each sample. Analytical gates were set so that ≤1% of negative control cells exceeded the gate. The percentage of cells exceeding the gate was used to determine Ad binding and internalization. Confocal laser-scanning microscopy was conducted using a Zeiss LSM-510META laser scanning confocal microscope equipped with a META spectral detector, a laser excitation via an Enterprise laser emitting at 364 nm, and a helium–neon laser emitting at 533 nm.

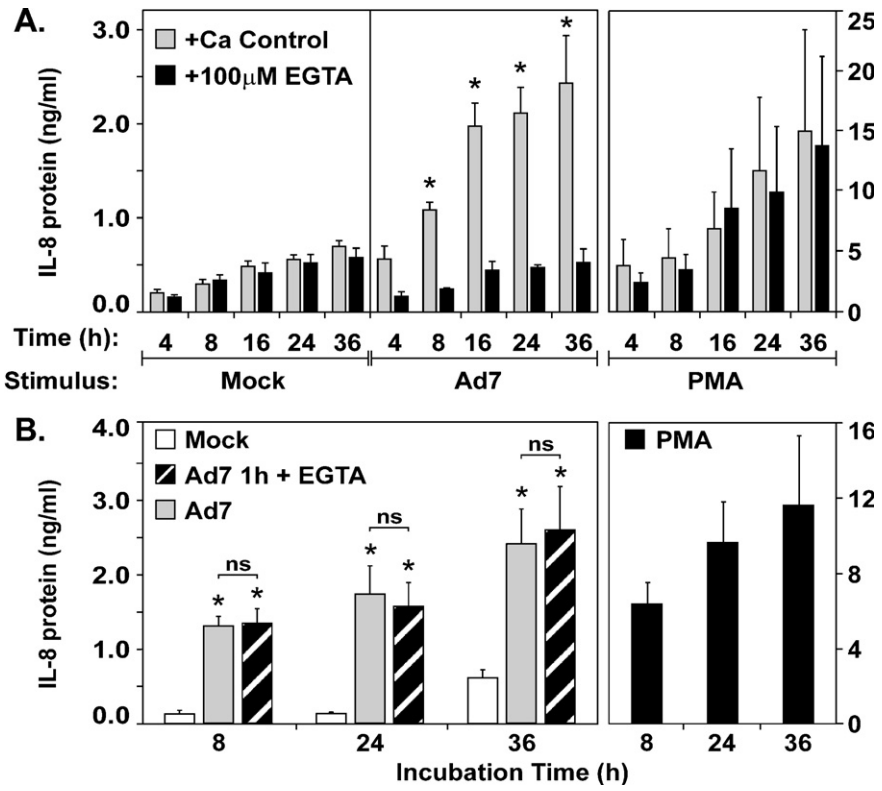

Fig. 6. IL-8 protein production from Ad7 stimulated A549 cells is dependent on calcium-mediated Ad internalization. (A) Prior calcium depletion with EGTA reduces IL-8 induction by Ad7, but not by PMA. To assess whether calcium-dependent Ad7 internalization is required for IL-8 protein induction, A549 cells were infected with Ad7, mock-treated or stimulated with PMA as described under normal, or calcium-free conditions. (B) Post-internalization calcium depletion by EGTA does not reduce IL-8 induction by Ad7. To examine whether EGTA inhibits IL-8 production through nonspecific mechanisms, 100 μM of EGTA was added to the media 1 h after Ad7 infection of A549 cells. The cells were then incubated at 37 °C for the times indicated, and ELISA was used to assess IL-8 protein levels in the media supernatants. Data represent mean ± SEM from three experiments (*$P<0.05$ compared to mock infection conditions).

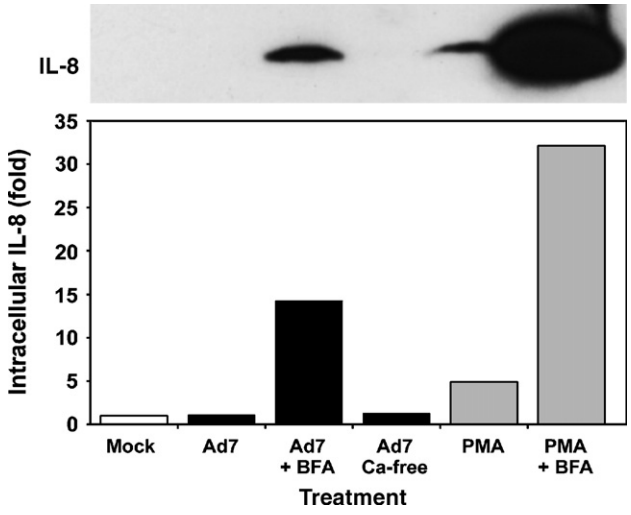

Fig. 7. IL-8 is not sequestered in A549 cells exposed to Ad7 under calcium-free conditions. A549 cells were exposed to Ad7 at an MOI of 50 for 24 h prior to preparation of cellular lysates. An equivalent volume of sterile virus dialysis buffer was added to mock-treated cells and positive control cells were stimulated with 100 ng/ml PMA. Western blot on cell extracts was performed to determine intracellular IL-8 protein. For calcium depletion, the cells were washed in calcium-free HBSS and stayed in serum-free, calcium-free DMEM containing 100 μM EGTA for 2 h before infection. Brefeldin A (5 μg/ml) was added to the samples indicated prior to Ad7 treatment or PMA exposure to block protein export.

Signaling pathway kinase assay, SDS-PAGE and immunoblotting

To determine the effect of calcium depletion on Erk signaling, A549 cells at 90% confluence on 6-well plates were incubated in serum-free DMEM overnight at 37 °C, 5% CO_2. The cells were washed in either complete HBSS or calcium-free HBSS, and the medium was replaced with either serum-free DMEM or serum-free, calcium-free DMEM containing100 μM EGTA. Following incubation for 2 h at 37 °C, 5% CO_2, the media were replaced with fresh media. The cells were stimulated with an MOI of 50 pfu/cell Ad7, and then returned to the incubator for the specified times. An equivalent volume of sterile virus dialysis buffer was added to mock-treated cells and positive control cells were stimulated with 100 ng/ml PMA.

To assess intracellular IL-8 levels, A549 cells at 90% confluence on 6-well plates were washed in either complete HBSS or calcium-free HBSS and the medium was replaced with either DMEM plus 10% FCS or calcium-free DMEM plus 10% dialyzed-FCS containing100 μM EGTA. Following 2-h incubation, the media were replaced and the cells were stimulated with MOI = 50 pfu/cell Ad7. For certain cells, protein export was blocked by adding Brefeldin A (dissolved in DMSO) to a final concentration of 5 μg/ml. Mock-treated cells and positive

control cells were treated as described above. The cells were incubated for 24 h at 37 °C, 5% CO_2.

The cells were harvested and lysed in 250 μl cold lysis buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 10 mM each EDTA; NaF; Na-pyrophosphate; 1% NP-40; 0.5% Na deoxycholate; 0. 1% SDS; 3 mM sodium vanadate; 10 μg/ml aprotinin and 10 μg/ml leupeptin) (Sigma). Cell homogenates were clarified by centrifugation at 4 °C, and 20 to 30 μg of the resultant post-nuclear lysates were mixed with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2.3% SDS) and heated to 95 °C for 5 min. The samples were separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes and then the membranes were blocked overnight in 5% powdered milk in 0.05 M Tris-buffered saline, pH 7.2. To detect activated phosphorylated Erk, the filters were immunoblotted with a rabbit polyclonal antibody specific for the phosphorylated forms of Erk (Cell Signaling Technology, Beverly, MA), and the membranes were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology). The same filter was stripped with 2% SDS in 50 mM Tris, pH 6.0, to remove the primary antibody and subsequently reprobed with polyclonal anti-Erk antibody (Cell Signaling Technology) that recognized both phosphorylated and nonphosphorylated Erk. For detection of intracellular IL-8, the membranes were immunoblotted with an anti-IL-8 neutralizing mouse monoclonal antibody (R&D Systems, Minneapolis, MN) and then were developed with horseradish peroxidase-labeled goat anti-mouse IgG (BD Biosciences). For all westerns, detection was achieved using chemiluminescent reagents (Pierce Biotechnology, Rockford, IL) and the developed membranes were exposed to X-ray film. Digital scans of the film were quantified with ImageQuant 5.0 software.

IL-8 ELISA

Media supernatants from experiments in which A549 cells were exposed to psoralen-inactivated Ad7 and from experiments involving calcium depletion of A549 cells followed by exposure to wild-type Ad7 were assessed by IL-8 ELISA. The conditions for treatment of A549 cells with psoralen-treated Ad7 are described above. For Ad7 infection of calcium-depleted A549 cells, the cells were grown overnight on 12-well plates in DMEM plus 10% FBS at 37 °C, 5% CO_2 to approximately 8.5×10^5 cells/well. The cells were washed in either complete HBSS or Ca^{2+}-free HBSS. The medium was replaced with 1.0 ml of DMEM plus 10% FBS or Ca^{2+}-free DMEM plus 10% dialysed FBS containing 100 μM EGTA and the cells were incubated for 2 h at 37 °C, 5% CO_2. Following calcium depletion, the media were replaced with identical fresh media, the cells were infected with MOI = 50 pfu/cell Ad7, and were incubated for the indicated times at 37 °C, 5% CO_2. Mock-treated cells received an equivalent volume of sterile virus dialysis buffer and positive control cells were stimulated with 100 ng/ml PMA. After incubation, the media supernatants were collected and stored at −80 °C. IL-8 ELISA was performed as previously described (Booth et al., 2004) using anti-IL-8 neutralizing monoclonal primary antibodies, biotinylated anti-IL-8 polyclonal secondary antibodies and recombinant human IL-8 protein standards (R&D Systems), and the plates were developed using avidin-horseradish peroxidase conjugate and TMB reagents (BD Biosciences).

Data analysis

Where applicable, the results are expressed as means ± standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA with Student–Newman–Keuls post hoc analysis. Significance was considered as $P<0.05$.

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