Phosphatidylinositol 3-Kinase Is Required for Rhinovirus-induced Airway Epithelial Cell Interleukin-8 Expression*

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Rhinovirus (RV) is a common cause of asthma exacerbations. The signaling mechanisms regulating RV-induced airway epithelial cell responses have not been well studied. We examined the role of phosphatidylinositol (PI) 3-kinase in RV-induced interleukin (IL)-8 expression. Infection of 16HBE14o—human bronchial epithelial cells with RV39 induced rapid activation of PI 3-kinase and phosphorylation of Akt, a downstream effector of PI 3-kinase. RV39 also colocalized with cit-Akt-PH, a citrogen-tagged fluorescent fusion protein encoding the pleckstrin homology domain of Akt, indicating that 3-phosphorylated PI accumulates at the site of RV infection. Inhibition of PI 3-kinase and Akt attenuated RV39-induced NF-κB transactivation and IL-8 expression. Inhibition of PI 3-kinase also blocked internalization of labeled RV39 into 16HBE14o—cells, suggesting that the requirement of PI 3-kinase for RV39-induced IL-8 expression, at least in part, relates to its role in viral endocytosis.

Rhinovirus (RV) 2 is a single-stranded RNA virus from the Picornaviridae family responsible for the majority of common colds. Viral infections trigger the majority of asthma exacerbations (1, 2), and RV accounts for 60% of virus-induced exacerbations (1). RV is also an important trigger of chronic obstructive pulmonary disease (COPD) exacerbations (3, 4). The precise mechanisms by which RV induces asthma or COPD exacerbations are unknown, but numerous studies suggest a role for IL-8, a CXC chemokine with the neutrophil-attractant Glu-Leu-Arg (ELR) motif. IL-8 and neutrophils are found in the nasal secretions and sputum of patients with RV-induced asthma exacerbations (5–9). Further, the number of neutrophils correlates with the level of IL-8 (7, 9). RV induces IL-8 expression in cultured airway epithelial cells (10–12). Increased neutrophil and IL-8 levels are a feature of asthma exacerbations (13, 14) and COPD exacerbations (15–17). Together, these data suggest that RV may stimulate asthma exacerbations by inducing bronchial epithelial cell production of IL-8, leading to a neutrophilic inflammatory response.

The human RVs include more than 100 serotypes that are divided into two groups based on their cellular receptors. Intercellular adhesion molecule (ICAM)-1 is the airway epithelial cell receptor for major subgroup RVs (e.g. RV14, RV16, and RV39), whereas the low density lipoprotein receptor functions as the receptor for minor subgroup RVs (e.g. RV1B and RV2). Thus, antibodies to ICAM-1 inhibit major subgroup RV infection of epithelial cells (18).

ICAM-1 is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily. In endothelial cells, ICAM-1 cross-linking increases phosphorylation and activation of pp60 Src (19, 20) and pp125 focal adhesion kinase (21). The p85 regulatory subunit of class IA PI 3-kinase serves as a substrate for both Src (22) and focal adhesion kinase (23, 24), suggesting that ICAM-1 ligation by RV could also activate PI 3-kinase. Other viruses with different membrane receptors also activate PI 3-kinase. Adenovirus attachment and endocytosis, via the coxsackievirus and adeno virus receptor, promotes phosphorylation of focal adhesion kinase and the p85 regulatory subunit of PI 3-kinase, as well as activation of the PI 3-kinase p110 catalytic subunit, in SW480 human colon adenocarcinoma cells (25). Endocytosis of adeno-associated virus, which binds to heparan sulfate proteoglycan, activates PI 3-kinase in HeLa cells (26). Finally, respiratory syncytial virus activates PI 3-kinase in A549 lung carcinoma cells (27). Taken together, these data strongly suggest that RV binding to ICAM-1 could induce IL-8 expression in part by activation of PI 3-kinases.

All PI 3-kinases share a catalytic core domain that interacts directly with ATP, PI, and the PI 3-kinase inhibitor LY294002. Members of the PI 3-kinase family are divided into three classes according to their structure and in vitro substrate specificity, as reviewed in Ref. 28. Class IA PI 3-kinases α, β, and γ are tightly bound with a regulatory subunit (p85α, p85β, or p55γ) that contains Src homology 2 domains with affinity for phosphotyrosine residues in pYXXM motifs found in growth factor receptors, their substrates, and adaptor proteins. The major substrate for the class I PI 3-kinases in vivo appears to be PI(4,5)P2, and the major product appears to be PI(3,4,5)P3 (29). When activated, class IA PI 3-kinases regulate cellular functions by recruiting PI(3,4,5)P3-binding proteins to the plasma membrane. Most of these proteins, the prototype of which is the serine threonine kinase Akt, bind to 3-phosphorylated PIs through a pleckstrin homology (PH) domain.

We therefore examined the contributions of PI 3-kinase and Akt to RV-induced responses in human airway epithelial cells. We found that infection with RV39 induced rapid activation of PI 3-kinase and phosphorylation of Akt, as well as accumulation of 3-phosphorylated PI at the site of RV infection. Inhibition of PI 3-kinase and Akt attenuated RV39-induced nuclear factor (NF)-κB transactivation and IL-8 expression. Inhibition of PI 3-kinase also blocked internalization of labeled RV39, suggesting that the requirement of PI 3-kinase for RV39-induced IL-8 expression, at least in part, relates to its role in viral endocytosis.

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Experimental Procedures

Cell Culture—16HBE14o—human bronchial epithelial cells originating from bronchial epithelial tissue transfected with pSVori—containing the origin-defective SV40 genome (30) were provided by Dr. Steven White (University of Chicago). The cells were grown in minimum essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 200 mM of L-glutamine.

Differentially primary human bronchial epithelial cell cultures obtained from lung transplant donor tracheas (University Health Network, Toronto, Canada) were cultured at an air-liquid interface, as described (31). Thawed passage 1 cells were seeded into 12-mm collagen-coated Transwell-clear inserts (Costar, Cambridge, MA). The cells were grown under submerged conditions in bronchial epithelial cell growth medium (Clonetics) containing epidermal growth factor (25 ng/ml), bovine pituitary extract (65 ng/ml), all-trans-retinoic acid (5 × 10^{-8} M), bovine serum albumin (1.5 μg/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), gentamycin (50 μg/ml), and amphotericin (50 μg/ml). At confluence, the cells were shifted to a 1:1 mixture of bronchial epithelial cell growth medium and Dulbecco’s modified Eagle’s medium containing all the above growth factors and hormones and grown at airliquid interface to promote mucus production. The epithelium is pseudostatified with ciliated cells interspersed among mucus secreting cells. Periodic acid Schiff staining shows the presence of goblet cells and luminal glycoproteins, and immunostaining with antibody to tracheo-bronchial mucus has confirmed that the periodic acid Schiff-positive material is mucin. These cultures also show an acceptable transepithelial electrical resistance of 500–540 Ω/cm².

RV Infection—RV39 was obtained from American Type Culture Collection (Manassas, VA). Viral stocks were generated by infecting HeLa cells with RV in serum-free medium until 80% of the cells were cytopathic. For most experiments, HeLa cell lysates were harvested at 4 °C. To remove soluble factors of HeLa cell origin, RV was concentrated and partially purified by centrifugation with a 100,000 molecular weight cut-off Centricon filter (2,000 rpm for 2 h at 4°C; Millipore, Billerica, MA) (32). Intact virus fails to go through the filter and is concentrated. For experiments measuring PI 3-kinase activation or phosphorylation of Akt, we harvested HeLa cell supernatants rather than lysates to further decrease the possibility of contamination by HeLa cellular proteins. For fluorescent labeling (see below), partially purified and concentrated RV39 (as above) was overlayed on a sucrose pad consisting of 30% sucrose, 1 m NaCl, and 20 mM Na-Hepes (pH 7.5) and ultra centrifuged (65,000 rpm, 402,000 × g max for 2 h at 16°C). The supernatant was carefully removed, and the solid clear pellet was resuspended in 1 ml of phosphate-buffered saline, divided into aliquots, and stored at −80°C.

All titrations were performed by infecting confluent HeLa monolayers with serially diluted RV (range, undiluted to 10^{-7}) and assessing cytopathic effect 5 days after infection. The cells were infected with RV39 for 1 h (or less, depending on experimental design) at 33 °C. Unless otherwise noted, 16HBE14o—cells infected with RV39 at a multiplicity of infection (MOI) of 1.0. Mucociliary differentiated cultures were infected at a 50% tissue culture infectivity dose (TCID₅₀/ml) of 6 × 10³ to 3 × 10⁶. TCID₅₀ values were determined by the Spearman-Karber method (33). For experiments including sham infection, the cells were incubated with an equal volume of HeLa cell lysate or supernatant. In some experiments, purified RV39 was UV-irradiated on ice for 30 min using a UVB-CL-1000 cross-linker at 1200μJ/cm² (32). The effect of UV irradiation was confirmed by the absence of a cytopathic effect on HeLa cell monolayers.

Selected cultures were pretreated for 1 h with the chemical PI 3-kinase inhibitor LY294002 in dimethyl sulfoxide (both from Sigma) or carrier alone. In other experiments, the cells were incubated with anti-ICAM-1 antibody (20 μg/ml; Serotec, Raleigh, NC) for 1 h prior to infection, or tumor necrosis factor (TNF)-α neutralizing antibody (0.1–1.0 μg/ml) (R & D, Minneapolis, MN) immediately after infection. As a control, equal concentrations of irrelevant mouse anti-human IgG (Sigma) were used.

Measurement of IL-8 Protein Levels—The cells were grown to 80% confluence, serum-starved for 24 h, and then infected with RV39 for 1 h. Inoculum was then replaced with serum-free medium. Conditioned medium was collected 48 h post-infection, centrifuged to remove cell debris, and then frozen at −80°C. For mucociliary cultures, RV was applied to the apical surface for 1 h. Forty-eight hours post-infection, apical washings and basolateral supernatants were collected from the basolateral compartment. IL-8 protein levels were measured by enzyme-linked immunosorbent assay (ELISA; R & D Systems).

Measurement of IL-8 Promoter Activity and NF-kB Transactivation—The −162/+44 fragment of the human IL-8 promoter subcloned into luciferase (−162/+44 H1100/Luc) was obtained from Dr. Allan Brasier (34). NF-kB reporter plasmid was purchased from Stratagene (La Jolla, CA). pRL family Renilla luciferase plasmid was purchased from Promega (Madison, Wisconsin). pSG5-Myc-Δp85, a dominant-negative class IA PI 3-kinase, and pSG5 p1103.3:3K-CAAX, an activated PI 3-kinase with a C-terminal farnesylation signal from H-Ras, which causes its membrane localization (35), were obtained from Dr. Julian Downward (Cancer Research UK London Research Institute). CS2+myr-Akt K/D, which expresses a constitutively membrane-localized, C-terminally truncated, catalytically inactive Akt1 (amino acids 1–647), and pCS2+PHDDAkt, which expresses an activated Akt1 (amino acids 131–480) in which Ser177 and Ser181 were replaced by alanines to Asp177 and Thr181 were altered to Asp by PCR mutagenesis, were provided by Dr. Anne Vojtek (University of Michigan) (36). Deletion mutants of inhibitor of κB (IκB)-α encoding amino acids 37–317 (IκBαΔN) and 1–242 (IκBαΔC) were provided by Dr. Dean Ballard (Vanderbilt University) (37). A dominant-negative IκB kinase (IKK)-β, in which Ser172 and Ser180 were replaced by alanines (38), was obtained from Dr. Anning Lin. 16HBE14o—cells were grown to 50% confluence, washed in OptiPrep (Invitrogen), and incubated with plasmid DNA, Lipofectamine, and Optimem (Invitrogen). After 4 h, the solution was replaced with minimum essential medium supplemented with 10% fetal bovine serum. The next morning, the cells were serum-starved for 24 h. The cells were then treated with RV39 for 1 h. Inoculum was replaced with fresh serum-free medium, and the were cells harvested for analysis 24 h post-infection. Luciferase activity was measured using a luminometer. Changes in promoter activity were normalized for transfection efficiency by dividing luciferase light units by Renilla luciferase light units. The results were then reported as fold increase over the empty vector/untransfected control. Expression of genetic mutants was verified by immunoblotting (data not shown).

Northern Analysis—Total RNA was prepared from primary human mucociliary-differentiated tracheal epithelial cell cultures using TRIzol reagent (Sigma), electrophoresed on 1.25% SeaKem Gold agarose gels (Reliant RNA gel system; Cambrex, Rockland, MN), and then transferred onto Immobilon-NY Plus membranes (Millipore, Bedford, MA). A probe template was prepared from RNA extracted from primary cells by reverse transcription-PCR with the IL-8 primers 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′ (sense) and 5′-TCT CAG CCC TCT
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TCA AAA ACT TCT C-3’ (antisense). 32P-Radiolabeled probe was prepared using Ready-To-Go DNA labeling beads (-dCCTP) (Amersham Biosciences). Probe hybridization was performed with ultrahyb buffer as recommended by the manufacturer (Amersham Biosciences).

**PI 3-Kinase Assay**—After RV infection, 16HBE14o− cells were immunoprecipitated with anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Inc.). The signals were amplified and visualized with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and chemiluminescence solution (Pierce).

**Fluorescent Microscopy**—For fluorescent labeling of RV39, 0.4 ml of 0.1 M NaHCO3 was added to a 0.1 ml aliquot of purified, concentrated virus to raise pH to 8.5. The virus was then incubated in the dark for 1 h with 250 μg of Alexa-fluor 555 carboxylic acid succinimidyl ester, an amine-reactive probe (Molecular Probes, Eugene, OR). The reaction was quenched with 0.5 ml of 1 M Tris buffer (pH 8.0), and labeled virus was dialyzed three times against Tris buffer (in the dark, 12 h at 4 °C). The virus was concentrated by precipitation with a final 6% (w/v) polyethylene glycol 8000 precipitation in 0.5 M NaCl for 4 h at 4 °C, and centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant fluid was completely aspirated, and the virus was resuspended in 0.1 ml of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin for stabilization. The plates were dried, and activity was assessed by autoradiography.

**Immunoblotting**—After the relevant treatment, 16HBE14o− cells were lysed, cellular proteins were resolved by 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. The membranes were probed with antibodies against Ser32/36 phospho-IκBα, total IκBα, Ser473 phospho-Akt, total Akt, p85α, p110α, and p110β (Cell Signaling, Beverly, MA), or p85α, PI 3-kinase (Upstate Biotechnology, Inc.). The signals were amplified and visualized with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and chemiluminescence solution (Pierce).

**Electrophoretic Mobility Shift Assay**—After the relevant treatment, 16HBE14o− cells were infected with Alexa fluor-labeled virus (1 × 10⁶ TCID₅₀/ml) or an equal volume of media (Sigma) for 24 h, lysing the cells by freeze-thaw, and concentrating by centrifugation with a Centricon filter, as above. 16HBE14o− cells were then infected with [35S]methionine-labeled RV39 for either 1 h at 33 °C. Forty-eight hours after infection, supernatants were collected for measurement of IL-8 protein by ELISA. Sham-infected cultures were incubated with an equal volume of HeLa cell lysate B. Cells were transfected with the −162/−44 fragment of the full-length human IL-8 promoter subconcloned into a luciferase reporter, infected with RV39, and incubated for an additional 24 h. C. RV39 infection increases IL-8 expression in mucociliary differentiated human tracheal epithelial cells. After RV39 infection, apical washings and basolateral supernatants were collected, and IL-8 was measured by ELISA. D. RV infection of mucociliary-differentiated cultures increases IL-8 mRNA expression. Total RNA was collected 24 h after RV infection and probed for IL-8. 28S and 18S rRNA are also shown. The plots shown are typical of three separate experiments. *p < 0.05 versus sham (ANOVA).

**IKK Assay**—Endogenous IKK was immunoprecipitated from cell extracts with an anti-IKKα antibody (Santa Cruz Biotechnology). Activity of the immune complex was assayed in 30 μl of kinase buffer in the presence of 10 μM ATP, 5 μCi of [γ-32P]ATP, and glutathione S-transferase-IκBα (3 μg/sample) as a substrate (30 °C for 15 min), as described (38, 41). The reactions were terminated with 4X Laemmli sample buffer. Samples were resolved by 10% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane by semidy membrane transfer. After Ponceau staining, the membrane was exposed to film, and autoradiography was performed using anti-IKKα antibody.

**Measurement of RV39 Binding and Internalization**—35S-Labeled virus was generated by infecting HeLa cells with RV39 in the presence of [35S]methionine and methionine-free Dulbecco’s modified Eagle’s medium (Sigma) for 24 h, lysing the cells by freeze-thaw, and concentrating by centrifugation with a Centricon filter, as above. 16HBE14o− cells were then infected with [35S]methionine-labeled RV39 for either 1 h at
To determine virus binding, 16HBE14o- cells grown in 12-well plates were incubated with 5 x 10^4 cpm [35S]methionine-labeled RV39/well in 0.5 ml of minimum essential medium for 1 h at 4°C. The cells were washed at 4°C with PBS, and cell-associated radioactivity was measured in cell lysates by liquid scintillation counting. As a control, TNFα was incubated with neutralizing antibody. C and D, UV irradiation of RV39 attenuated but did not abolish IL-8 protein expression or promoter activity, respectively. The bars represent the means ± S.E. for three to nine experiments. *, p < 0.05 versus RV alone (ANOVA).

RESULTS

RV39 Infection Increases IL-8 Expression in Cultured Airway Epithelial Cells—16HBE14o—human bronchial epithelial cells were infected with RV39 (1 h at 33°C). Forty-eight hours after infection, cell supernatants were collected for measurement of IL-8 protein by ELISA. RV39 increased IL-8 in a concentration-dependent manner (Fig. 1A). Equal volumes of HeLa cell lysate from uninfected cells had no effect. Concentrations of RV39 as low as 0.1 MOI increased IL-8 protein secretion (data not shown).

To test whether IL-8 expression was transcriptionally regulated, cells were transfected with the —162/+44 fragment of the full-length human IL-8 promoter subcloned into a luciferase reporter, infected with RV39 (1 h at 33°C), and incubated for an additional 24 h (Fig. 1B). RV39 increased IL-8 promoter activity by about 5-fold, consistent with the notion that IL-8 is transcriptionally regulated by RV39 infection.

We tested whether RV39 infection increases IL-8 expression in differentiated human tracheal epithelial cells. Passage 1 cells, isolated from...
the tracheas of lung transplant donors, were grown at air-liquid interface for 2 weeks. After RV39 infection, washings from the apical surface and supernatants from the basolateral surface were collected, and IL-8 measured by ELISA (Fig. 1C). RV39 increased apical and basolateral IL-8 protein levels, with basolateral IL-8 increasing nearly 4-fold. Steady-state IL-8 mRNA level was also increased in cultures infected with RV39 (Fig. 1D).

To verify that the observed increases in IL-8 expression were due to RV39 infection, the cells were preincubated with anti-ICAM-1 antibody 1 h prior to RV39 infection. Equal concentrations of IgG antibody were used as a control. Preincubation with anti-ICAM-1 but not IgG attenuated IL-8 expression (Fig. 2A).

To determine whether RV39-induced IL-8 expression might result from the autocrine expression of TNFα, cells were incubated with TNFα neutralizing antibody. There was no effect of anti-TNFα on IL-8 expression (Fig. 2B). On the other hand, anti-TNFα decreased TNFα-induced IL-8 expression in a concentration-dependent manner.

Finally, IL-8 protein expression and promoter activity were assessed after infection with UV-irradiated RV39. At MOI of 1, UV irradiation attenuated but did not abolish IL-8 expression and promoter activation (Fig. 2, C and D). When the viral load was increased to MOI of 5, IL-8 protein expression was similar to that elicited by intact RV39, suggesting that viral replication was not required for the IL-8 response.

PI 3-Kinase and Akt Are Activated by RV39 Infection—After RV39 infection, 16HBE14o− cells were harvested for immunoprecipitation with anti-ICAM-1 antibody 1 h prior to RV39 infection. Equal concentrations of IgG antibody were used as a control. Preincubation with anti-ICAM-1 but not IgG attenuated IL-8 expression (Fig. 2A).

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FIGURE 4. 3-Phosphorylated PI accumulates at the site of RV infection. In RV39-infected 16HBE14o− cells, Alexa fluor-labeled virus colocalized with Cit-Akt-PH, as evidenced by bright yellow focal accumulations beneath the basement membrane. A, visible image of RV39-infected cells. B, confocal fluorescent image demonstrating RV39 infection (red), Cit-Akt-PH (green), and areas of colocalization (yellow-green). C, 16HBE14o− cells infected with sham HeLa cell lysates. D, 16HBE14o− cells stably transfected with enhanced green fluorescent protein and infected with labeled RV39. E, in cells pretreated with 1 μM LY294002, there was a dramatic reduction in labeled RV39 fluorescent label as well colocalization with Cit-Akt-PH. 
PI 3-kinase and Akt are required for maximal RV39-induced IL-8 expression.

**A** A, in 16HBE14o− cells, pretreatment with LY294002 (10 μM for 1 h) attenuated RV39-induced Akt phosphorylation. Untreated cells were incubated in a similar volume of carrier (Me2SO), B, pretreatment with LY294002 (1–25 μM) attenuated RV39-induced IL-8 protein expression in a concentration-dependent manner. C, LY294002 (10 μM) also blocked IL-8 protein expression in mucociliary differentiated primary cultures. D and E, 16HBE14o− cells were cotransfected with −162/+44 IL-8/Luc and a cDNA encoding either a dominant-negative form of p85, the regulatory subunit of class IA PI 3-kinase, or Akt-K/D. The bars represent the means ± S.E. for three to six experiments. *, p < 0.05; **, p < 0.005 compared with Me2SO or empty vector (ANOVA).

**FiguRe 5: PI 3-kinase Is Required for NF-κB Transactivation.**—We examined the requirement of PI-3 kinase activation for RV39-induced responses by pretreating cells with LY294002, a chemical inhibitor of PI-3 kinases. First we showed that Akt phosphorylation was inhibited by 10 μM LY294002, demonstrating that, in the context of RV infection, Akt functions downstream of PI-3 kinase (Fig. 5A). In 16HBE14o− cells, LY294002 (1–25 μM for 1 h) attenuated RV39-induced IL-8 protein expression in a concentration-dependent manner (Fig. 5B). LY294002 also blocked IL-8 protein expression in mucociliary differentiated primary cultures (Fig. 5C). LY294002 inhibits all classes of PI-3 kinase, as well as other kinases including the PI-3 kinase catalytic c domain. To examine the specific role of class I PI-3 kinase, 16HBE14o− cells were cotransfected with −162/+44 IL-8/Luc and a cDNA encoding a dominant-negative form of p85, the regulatory subunit of class IA PI-3 kinase. Expression of Δp85 decreased RV39-induced transactivation from the IL-8 promoter (Fig. 5D). We also examined the requirement of Akt for RV39-induced responses. Cells were cotransfected with −162/+44 IL-8/Luc and a kinase dead form of Akt. Expression of Akt-K/D decreased RV39-induced transactivation from the IL-8 promoter (Fig. 5E), consistent with the notion that maximal RV39-induced IL-8 expression requires activation of a class IA PI-3 kinase/Akt pathway.

**NF-κB Is Required for RV39-induced IL-8 Expression.**—16HBE14o− human bronchial epithelial cells were infected with RV39 and nuclear extracts harvested for electrophoretic mobility assay. Within 10 min, RV39 increased the binding of nuclear proteins to an oligonucleotide encoding the NF-κB consensus binding sequence (Fig. 6A), showing that RV39 induces NF-κB translocation and DNA binding. We transfected 16HBE14o− cells with a cDNA encoding NF-κB-responsive promoter elements subcloned into luciferase (NF-κB-TATA-Luc). Infection with RV39 increased NF-κB transactivation (Fig. 6B), although the level of transactivation was substantially less than that induced by 3 ng/ml TNFα. We also transfected 16HBE14o− cells with −162/+44 IL-8/Luc in which the NF-κB site was mutated. Mutation of the NF-κB site abolished responsiveness to RV39 (Fig. 6C), demonstrating that RV39-induced IL-8 expression requires NF-κB transactivation. We monitored Ser32/36 phosphorylation of IκBα following RV infection. RV39 infection increased IκBα phosphorylation while decreasing total IκBα protein abundance, consistent with degradation by the proteosome (Fig. 6D). Expression of a nonphosphorylatable N-terminal mutant of IκBα (IκBαΔN) missing the critical serine phosphorylation sites, but not a C-terminal mutant (IκBαΔC), inhibited RV39-induced IL-8 promoter activity (Fig. 6E), demonstrating that serine phosphorylation of IκBα is required for the response. Finally, expression of a dominant-negative IKKβ inhibited RV39-induced IL-8 promoter activity (Fig. 6F). Together these results demonstrate that RV39 may regulate NF-κB-dependent gene expression and that NF-κB signaling is critical for RV39-induced IL-8 expression.

**PI 3-Kinase Is Required for NF-κB Activation.**—We studied the requirement of PI-3 kinase for RV39-induced NF-κB transactivation. 16HBE14o− cells were also transfected with the NF-κB reporter plasmid and either pretreated with LY294002 or cotransfected with a dominant-negative form of p85, the regulatory subunit of class IA PI-3 kinase. LY294002 (10 mM) and Δp85 each decreased NF-κB transactivation (Fig. 7, A and B), suggesting that class IA PI-3 kinase is required for the response. In addition, pretreatment with LY294002 inhibited RV39-induced IκBα phosphorylation by IKK (Fig. 7C). On the
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other hand, expression of constitutively active PI 3-kinase (p110 CAAX) and Akt (PHDDAkt) were each sufficient for NF-κB transactivation (Fig. 7, D and E).

Requirement of PI 3-Kinase for RV39 Internalization—We monitored the effect of LY294002 on colocalization of Alexa fluor-labeled RV39 and Cit-PH-Akt. In cells pretreated with 10 μM LY294002, there was a dramatic reduction in labeled RV39 fluorescent label as well colocalization with Cit-Akt-PH (Fig. 4E), suggesting that PI 3-kinase is required for viral internalization.

We quantified the effect of LY294002 on binding and internalization of virus by infecting 16HBE14o− cells with [35S]methionine-labeled RV39 and measuring cell-associated radioactivity after incubation at 4 and 33 °C, respectively. There was no effect of LY294002 on RV39 binding (Fig. 8A). Preincubation with LY294002 significantly reduced RV39 internalization, as assessed by the amount of labeled virus resistant to trypsin digestion (Fig. 8B). Together these data suggest that PI 3-kinase activity is required for viral internalization.

DISCUSSION

Viral infections trigger the majority of asthma exacerbations (1, 2), and RV accounts for 60% of virus-induced exacerbations (1). RV is also an important trigger of COPD exacerbations (3, 4). Clinical studies suggest that RV may stimulate exacerbations of asthma and COPD by inducing bronchial epithelial cell production of IL-8, leading to a neutrophilic inflammatory response (5–17). Little is known about the biochemical signaling pathways regulating RV-induced IL-8 expression, however. The IL-8 response to RV16 infection in BEAS-2B airway epithelial cells is inhibited by antibodies to ICAM-1, implying that integrin-mediated pathways play a role (18). NF-κB activation has been shown to be required for a number of RV responses including airway epithelial cell expression of IL-8, IL-6, ICAM-1, and vascular cell adhesion molecule-1 (11, 32, 43, 44, 46). Pretreatment with a chemical inhibitor of p38 mitogen-activated protein kinase partially inhibits IL-8 expression in RV-infected BEAS-2B epithelial cells (47). The serine-threonine kinase inhibitor 2-aminopurine blocks double-stranded RNA-induced secretion of IL-8 (48). Previous work in this lab has shown that RV16 3C protease is sufficient to increase IL-8 response via NF-κB activation (49).

In the present manuscript, we have shown that RV39 infection induces activation of the PI 3-kinase/Akt signaling pathway. Infection of 16HBE14o− human bronchial epithelial cells with RV39 induced rapid activation of PI 3-kinase and phosphorylation of Akt, a downstream effector of PI 3-kinase. Although PI 3-kinase has been previously shown to be activated by other respiratory viruses, in particular adenovirus (25), adeno-associated virus (26), and respiratory syncytial virus (27), we now extend this information by demonstrating colocalization of RV with cit-Akt-PH, evidence that 3-phosphorylated PI accumulates at the site of viral infection. Further, we show in both 16HBE14o− and primary mucociliary differentiated airway epithelial cells that inhibition of PI 3-kinase, either by LY294002 or expression of a dominant-negative form of the regulatory subunit of class 1A PI 3-kinase, Δp85, inhibits RV39-induced expression of IL-8.
Further, we have shown that the requirement of PI 3-kinase for RV39-induced NF-κB transactivation and IL-8 expression, at least in part, relates to its role in viral internalization. Pretreatment with LY294002 inhibited internalization of both Alexa fluor- and [35S]methionine-labeled RV39. PI 3-kinase has been previously shown to be required for internalization of adenovirus (25). Attenuation of viral internalization would be expected to reduce the amplitude of all downstream signals including, as we have found, NF-κB activation and IL-8 expression. However, PI 3-kinase, rather than simply playing a permissive role, may also actively initiate downstream signaling pathways leading to IL-8 expression. Consistent with this notion, activation of PI 3-kinase and Akt, a downstream effector of PI 3-kinase, were each sufficient for NF-κB transactivation. The blot shown was typical of two separate experiments. The bars represent the means ± S.E. for six experiments. *, p < 0.05; **, p = 0.001 compared with untreated or empty vector (ANOVA).

Further, we have shown that the requirement of PI 3-kinase for RV39-induced NF-κB transactivation and IL-8 expression, at least in part, relates to its role in viral internalization. Pretreatment with LY294002 inhibited internalization of both Alexa fluor- and [35S]methionine-labeled RV39. PI 3-kinase has been previously shown to be required for internalization of adenovirus (25). Attenuation of viral internalization would be expected to reduce the amplitude of all downstream signals including, as we have found, NF-κB activation and IL-8 expression. However, PI 3-kinase, rather than simply playing a permissive role, may also actively initiate downstream signaling pathways leading to IL-8 expression. Consistent with this notion, activation of PI 3-kinase and Akt, a downstream effector of PI 3-kinase, were each sufficient for NF-κB activation.

As noted above, NF-κB has been shown to be required for RV-induced expression of IL-8 and other pro-inflammatory molecules (11, 32, 43, 44, 46). We have extended these results by showing that N-terminal phosphorylation of IkBa and IKKβ are each required for RV39-induced IL-8 production. We have also found that expression of a dominant-
negative NF-κB-inducing kinase blocks NF-κB transactivation, suggesting that NF-κB-inducing kinase is the upstream activator of IKK in this context.3

We found in mucociliary differentiated human tracheal epithelial cells that infection with 10^6 TCID_{50} RV39 induces a reproducible 3–4-fold increase in basolateral IL-8 expression. Our data conflict with a report by Lopez-Souza and colleagues (50) showing that fully differentiated cells are resistant to RV infection and that RV fails to induce IL-8 secretion. Given the ability of RVs to readily infect human airway epithelial cells in normal individuals, the resistance of differentiated cells to RV is surprising. The explanation for this discrepancy may relate to the high transpneumal resistance of the Lopez-Souza cultures (1186 Ω/cm²). If RV enters airway epithelial cells more efficiently across the basolateral than apical membrane, as does adenovirus, this high transpneumal resistance would limit infection. However, similar to other studies (51), our cultures show an acceptable transepithelial resistance (50–540 Ω/cm²) and demonstrate normal expression of tight junction proteins (data not shown).

We did not address the mechanism of RV-induced PI 3-kinase activation. As noted above, antibody cross-linking of ICAM-1 results in the tyrosine phosphorylation of Src and focal adhesion kinase (19–21), each of which phosphorylate p85 PI 3-kinase (22–24). Also, we did not determine all of the PI 3-kinase isoforms involved in the RV-induced response. Expression of the class IA PI 3-kinase dominant-negative p85 regulatory subunit inhibited IL-8 promoter activity, demonstrating the involvement of this isoform in the regulation of transcription from the IL-8 promoter. Further, because PI(3,4,5)P3 is the major product of the regulatory subunit of PI 3-kinase (22–24), PI 3-kinase therefore constitutes a promising target for therapeutic intervention in the treatment of asthma, chronic obstructive pulmonary disease, and other chronic airways diseases that are exacerbated by RV infection.

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