

Rhinovirus Activates Interleukin-8 Expression via a Src/p110 β Phosphatidylinositol 3-Kinase/Akt Pathway in Human Airway Epithelial Cells[∇]

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Rhinovirus (RV) is responsible for the majority of common colds and triggers exacerbations of asthma and chronic obstructive lung disease. We have shown that RV serotype 39 (RV39) infection activates phosphatidylinositol 3 (PI 3)-kinase and the serine threonine kinase Akt minutes after infection and that the activation of PI 3-kinase and Akt is required for maximal interleukin-8 (IL-8) expression. Here, we further examine the contributions of Src and PI 3-kinase activation to RV-induced Akt activation and IL-8 expression. Confocal fluorescent microscopy of 16HBE14o– human bronchial epithelial cells showed rapid (10-min) colocalization of RV39 with Src, p85 α PI 3-kinase, p110 β PI 3-kinase, Akt and Cit-Akt-PH, a fluorescent Akt pleckstrin homology domain which binds PI(3,4,5)P₃. The chemical Src inhibitor PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine} and the PI 3-kinase inhibitor LY294002 each inhibited Akt phosphorylation and the colocalization of RV39 with Akt. Digoxigenin-tagged RV coprecipitated with a Crossside kinase likely to be Akt, and inhibition of Src blocked kinase activity. Digoxigenin-tagged RV39 colocalized with the lipid raft marker ceramide. In 16HBE14o– and primary mucociliary differentiated human bronchial epithelial cells, inhibition of Src kinase activity with the Src family chemical inhibitor PP2, dominant-negative Src (K297R), and Src small interfering RNA (siRNA) each inhibited RV39-induced IL-8 expression. siRNA against p110 β PI 3-kinase also inhibited IL-8 expression. These data demonstrate that, in the context of RV infection, Src and p110 β PI 3-kinase are upstream activators of Akt and the IL-8 promoter and that RV colocalizes with Src, PI 3-kinase, and Akt in lipid rafts.

Rhinovirus (RV) 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 (17, 22), and RV accounts for 60% of virus-induced exacerbations (17). RV is also an important trigger of chronic obstructive pulmonary disease exacerbations (28, 32).

Numerous studies suggest a role for interleukin-8 (IL-8) in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD) exacerbations. IL-8, a CXC chemokine with the neutrophil attractant Glu-Leu-Arg (ELR) motif, and neutrophils are found in the nasal secretions and sputa of patients with RV-induced asthma exacerbations (8, 9, 12, 13, 26). Further, the number of neutrophils correlates with the level of IL-8 (9, 26). RV induces IL-8 expression in cultured airway epithelial cells (34, 38, 39). Increased neutrophil and IL-8 levels are a feature of asthma (23, 24) and COPD exacerbations (1, 10, 27). Together, these data suggest that RV may stimulate asthma and COPD exacerbations by inducing bronchial epithelial cell production of IL-8, leading to a neutrophilic inflammatory response.

We have recently shown that infection of human bronchial epithelial cells with RV serotype 39 (RV39) induces rapid

phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3 (PI 3)-kinase, as well as that of Akt, a downstream effector of PI 3-kinase (21). RV39 also colocalized with Cit-Akt-PH, a fluorescent fusion protein containing the pleckstrin homology domain of Akt, indicating that PI(3,4,5)P₃ accumulates at the site of RV infection. Finally, inhibitions of PI 3-kinase activation with a chemical inhibitor and with dominant-negative p85 α each inhibited RV39-induced IL-8 expression. However, the precise mechanism by which RV activates PI 3-kinase and the specific class IA PI 3-kinase catalytic subunit involved in RV-induced Akt phosphorylation were not determined.

Potential upstream activators of PI 3-kinase include the tyrosine kinase p60 Src and focal adhesion kinase, each of which regulates the remodeling of the actin cytoskeleton in response to cell adhesion and integrin clustering. Upon stimulation, Src translocates from the perinuclear region of the cell to peripheral sites of integrin clustering. Src binds to its substrates via its Src homology domains, which in turn interact with phosphotyrosine-containing or proline-rich sequences. Among its substrates are Src itself (autophosphorylation at tyrosine-416), focal adhesion kinase, and the p85 regulatory subunit of PI 3-kinase (15). p85 PI 3-kinase, in turn, may form heterodimers with one of three class IA PI 3-kinase catalytic subunits (p110 α , p110 β , and p110 δ). p110 α and p110 β are ubiquitously expressed, whereas p110 δ expression is largely restricted to cells of the immune system (4).

The human RVs include more than 100 serotypes, which are

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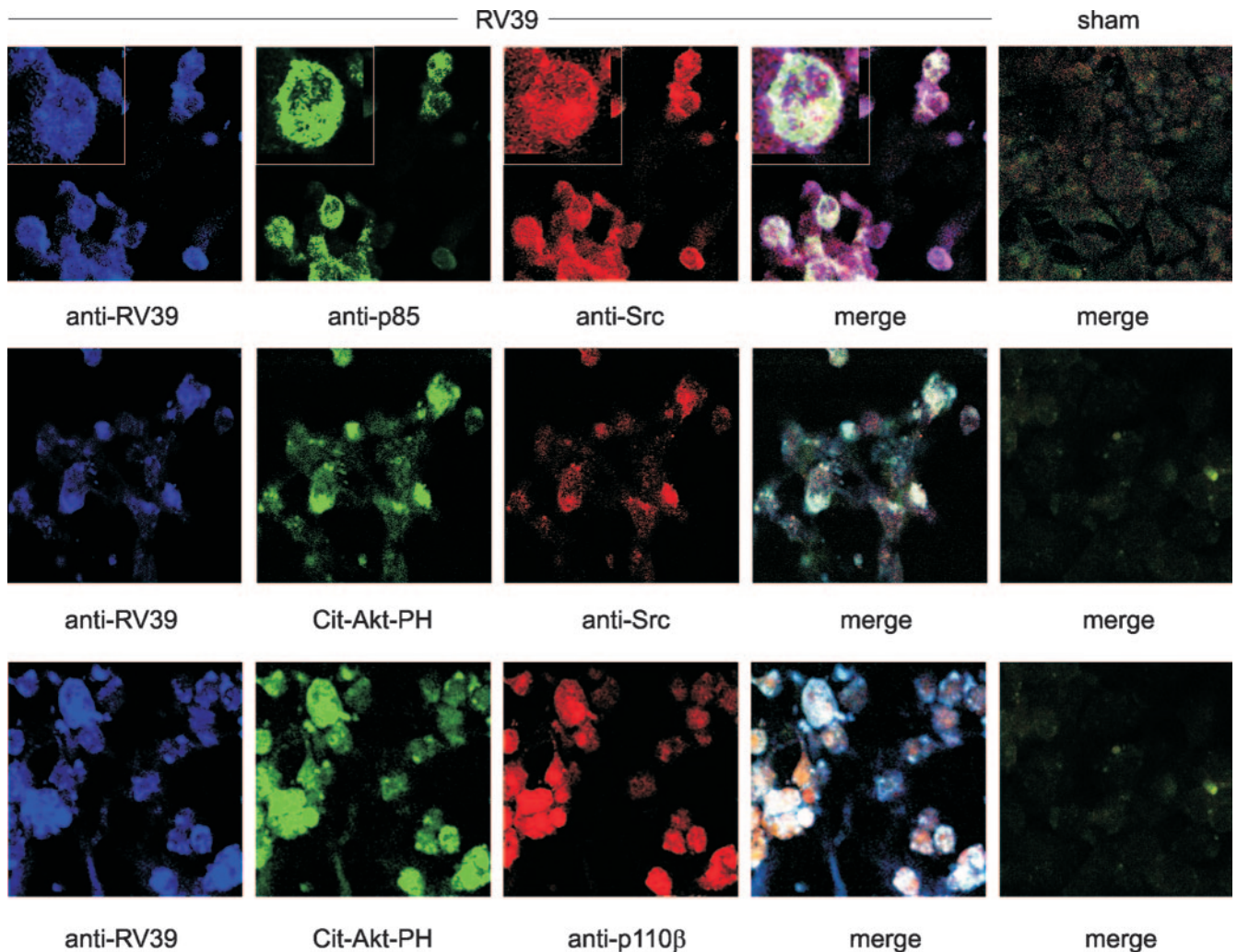


FIG. 1. RV39 increases the association of Src with PI 3-kinase containing membranes. 16HBE14o- cells were infected with sham HeLa cell lysates or RV39 at an MOI of 100 for 10 min. (Upper panel) Confocal fluorescent microscopy was initially performed with guinea pig anti-RV39 (blue channel in all panels), mouse anti-Src (clone GD-11 [red]), and rabbit p85 α (green). The colocalization of RV, Src, and p85 α in the merged image appears white. (Middle panel) Colocalization of RV39, Src (using rabbit anti-Src specific for the C terminus [red]), and stably expressed Cit-Akt-PH, a marker of PI(3,4,5)P₃-containing membranes (green), appears white in the merged image. (Lower panel) RV and p110 β , a catalytic subunit of PI 3-kinase (red), colocalize with Cit-Akt-PH (green), appearing white in the merged image.

divided into two groups based on their cellular receptors. Intercellular adhesion molecule-1 (ICAM-1) is the airway epithelial cell receptor for major-subgroup RVs (e.g., RV14, -16, and -39). In endothelial cells, antibody-mediated clustering causes ICAM-1 to colocalize with Src in detergent-insoluble membrane domains, i.e., lipid rafts (35). ICAM-1 cross-linking increases phosphorylation and activation of Src in endothelial cells (7, 37). It has recently been shown that RVs infect human epithelial cells via ceramide-enriched membrane platforms (11). We therefore hypothesized that the Src-mediated activation of PI 3-kinase and its downstream effector Akt is a critical event in the transduction of RV signaling.

MATERIALS AND METHODS

Cell culture. 16HBE14o- human bronchial epithelial cells originating from bronchial epithelial tissue transfected with pSVori-, containing the origin-defective simian virus genome (5), were provided by Steven White (University of

Chicago). Cells were grown in minimum essential medium supplemented with 10% fetal bovine serum and 2 mM of L-glutamine.

Human primary airway epithelial cells obtained from the tracheal trimmings of donor lungs at the time of double lung transplantation were cultured in collagen-coated plates with bronchial epithelial cell culture media (Cambrex, East Rutherford, NJ) as previously described (21, 30). First-passage cells were either grown under submerged conditions or, for selected experiments, differentiated to a mucociliary phenotype by seeding on collagen-coated transwells. After growth to confluence, cells were shifted to an air-liquid interface and maintained in a 1:1 mixture of bronchial epithelial cell culture medium and Dulbecco's modified Eagle medium for 3 weeks. The resulting epithelium was pseudostratified with ciliated cells interspersed among mucus-secreting cells.

Rhinovirus. RV39 was obtained from American Type Culture Collection (Manassas, VA). Viral stocks were generated by infecting HeLa cells with RV until 80% of the cells were cytopathic. HeLa cells were serum deprived overnight, lysates were harvested, and cellular debris was pelleted by centrifugation (10,000 \times g for 30 min at 4°C). RV was concentrated and partially purified by centrifugation with a 100,000-molecular-weight-cutoff Centricon filter (2,000 rpm at 4°C for 8 h; Millipore, Billerica, MA) (25). Similarly treated HeLa cell lysates from mock-infected cells served as controls (sham medium controls).

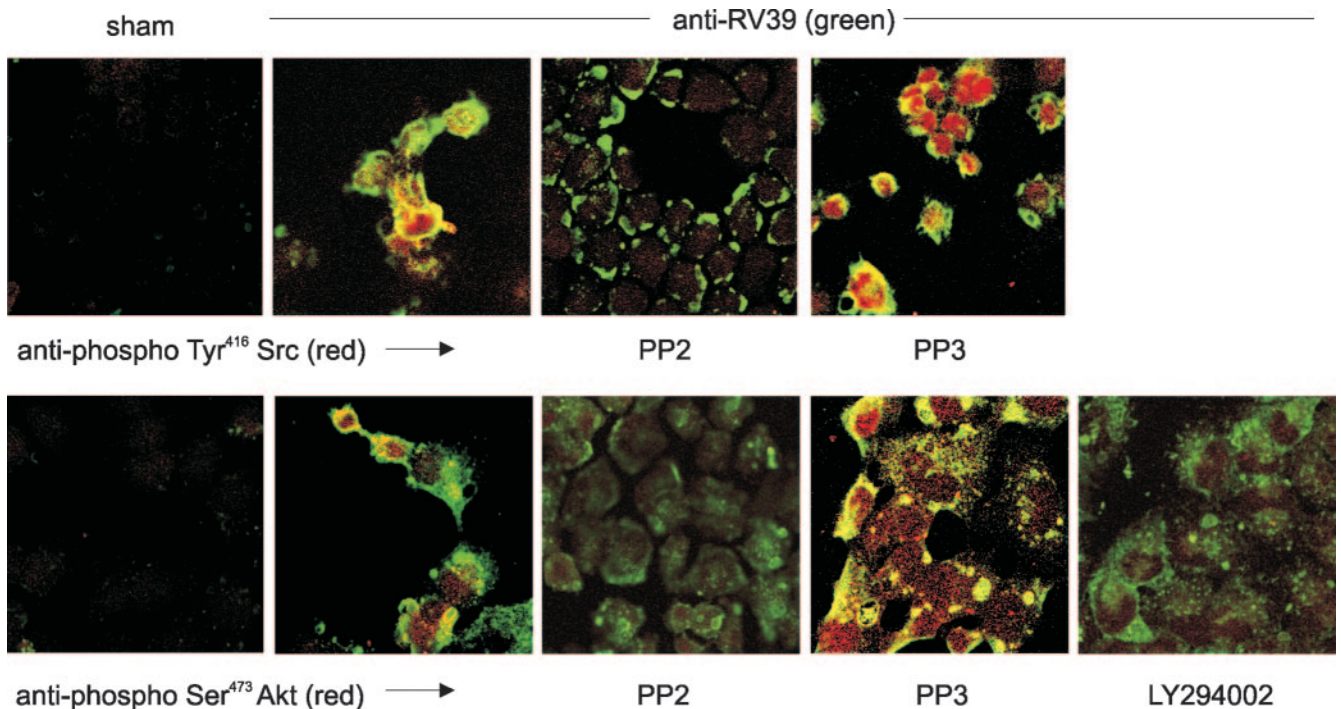


FIG. 2. RV39 internalization and colocalization with phospho-Tyr⁴¹⁶ Src (upper panel) and phospho-Ser⁴⁷³ Akt (lower panel) are blocked by chemical Src inhibitors. 16HBE14o⁻ cells were incubated with sham or RV39 at an MOI of 100. RV39 (shown in green) induced Tyr⁴¹⁶ phosphorylation of Src (red, upper panel) and colocalized with Src (yellow). The high-affinity Src inhibitor PP2 blocked pTyr⁴¹⁶ Src colocalization of RV but did not block virus binding. The low-affinity inhibitor PP3 did not block the association of RV and Src. RV39 also induced Ser⁴⁷³ phosphorylation of Akt (red, lower panel) and colocalized with an Akt fraction (yellow). PP2 appeared to block Ser⁴⁷³ Akt phosphorylation and the colocalization of RV and Akt. The low-affinity inhibitor PP3 did not block the association of RV and Akt. LY294002 also appeared to block virus internalization and colocalization with phospho-Ser⁴⁷³ Akt.

Transfection of cells and measurement of IL-8 promoter activity. 16HBE14o⁻ cells grown in six-well plates were transiently transfected with the -162/+44 fragment of the human IL-8 promoter (3) and *Renilla* luciferase by use of Lipofectamine (Invitrogen, Carlsbad, CA). Selected cultures were cotransfected with cDNA encoding dominant-negative K296R/Y528F Src (20). Other cells were cotransfected with 100 nM of Src small interfering RNA (siRNA), p110 β PI 3-kinase, or nontargeting RNA control (Dharmacon, Lafayette, CO). The following day, the cells were shifted to serum-free media and infected with RV at a multiplicity of infection (MOI) of 1.0 for 1 h. Inoculum was replaced with fresh serum-free medium and incubated at 33°C for 24 h, and the cells were harvested for analysis. Luciferase activity was measured with a luminometer. Promoter activity was normalized for transfection efficiency by dividing luciferase light units by *Renilla* luciferase light units. Results were reported as increases (*n*-fold) over values for the empty vector/untreated control.

Measurement of IL-8 production. IL-8 production following RV39 infection was measured using Duoset enzyme-linked immunosorbent assay development kits purchased from R&D Systems (Minneapolis, MN). In selected cultures, cells were pretreated with dimethyl sulfoxide, PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine} (16), or PP3 {4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine} (36) for 1 h prior to viral infection. PP2 and PP3 were obtained from Calbiochem (San Diego, CA). 16HBE14o⁻ cells were incubated with virus (MOI of 1.0) for 1 h, the virus-containing media removed, and fresh media with serum and the indicated concentrations of inhibitors added for a further 48 h before IL-8 release was assessed. Primary mucociliary differentiated cells were apically infected with virus (50% tissue culture infectivity dose [TCID₅₀] of 5 × 10⁶), and fresh medium and inhibitors added for a further 24 h before IL-8 release into the basolateral medium was assessed.

Western blot analysis of cell lysates and immunoprecipitates. In selected experiments, 16HBE14o⁻ cells were washed briefly in cold phosphate-buffered saline and incubated with homogenization buffer consisting of 50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 40 mM β -glycerophosphate, 2 mM EDTA, 200 μ M Na₃VO₄, and 1% Triton X-100 containing complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). Cells were homogenized by passing

through a 28-gauge needle and centrifuged. Supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins transferred to nitrocellulose, and membranes probed with either rabbit C-terminal anti-Src recognizing amino acids 400 to 422 (Upstate Biotechnology, Charlottesville, VA), rabbit anti-phospho-Ser⁴⁷³, rabbit anti-Akt (both from Cell Signaling, Beverly, MA), or rabbit anti-p110 β PI 3-kinase (Epitomics, Burlingame, CA). Bound antibody was detected by secondary antibody conjugated to horseradish peroxidase, and the signal detected by chemiluminescence. In other experiments, cell lysates were immunoprecipitated with mouse monoclonal 4G10 anti-phosphotyrosine (2 μ g; Upstate Biotechnology). Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either rabbit anti-phospho-Tyr⁴¹⁶ Src (Cell Signaling) or mouse anti-Src (clone GD11 recognizing amino acids 82 to 169; Upstate Biotechnology). No bands were detected when isotype control antibodies were substituted for primary antibodies (not shown).

Immunofluorescent staining. To visualize RV39 internalization into 16HBE14o⁻ and submerged primary airway epithelial cells, cells were plated on collagen-coated slides (Becton Dickinson Labware, Bedford, MA) and infected with virus at an MOI of between 10 and 100, or an equal volume of cell lysate from uninfected HeLa cells, for 10 min at 33°C. The cells were then washed extensively and fixed in 1% paraformaldehyde. Cells were permeabilized with 1% (vol/vol) Triton X-100 in phosphate-buffered saline, blocked, and incubated with either guinea pig anti-RV39 (American Type Culture Collection), rabbit anti-phospho-Tyr⁴¹⁶ Src, mouse anti-Src (monoclonal GD11), rabbit anti-phospho-Ser⁴⁷³ Akt, rabbit anti-Akt, mouse or sheep anti-digoxigenin (Roche Diagnostics), rabbit anti-p85 α PI 3-kinase, rabbit anti-p110 β PI 3-kinase, or mouse anti-ceramide immunoglobulin M (Sigma Chemical, St. Louis, MO). After being incubated with the appropriate Alexa-Fluor-conjugated secondary antibody (Molecular Probes, Portland, OR), cells were mounted with ProLong Antifade reagent (Molecular Probes) and visualized by confocal fluorescent microscopy with a Zeiss LSM 510 confocal microscope mounted on a Zeiss Axiovert 100 M inverted microscope. Some experiments were performed with 16HBE14o⁻ cells stably transfected with Cit-Akt-PH, a cDNA encoding a fusion protein of Citro-

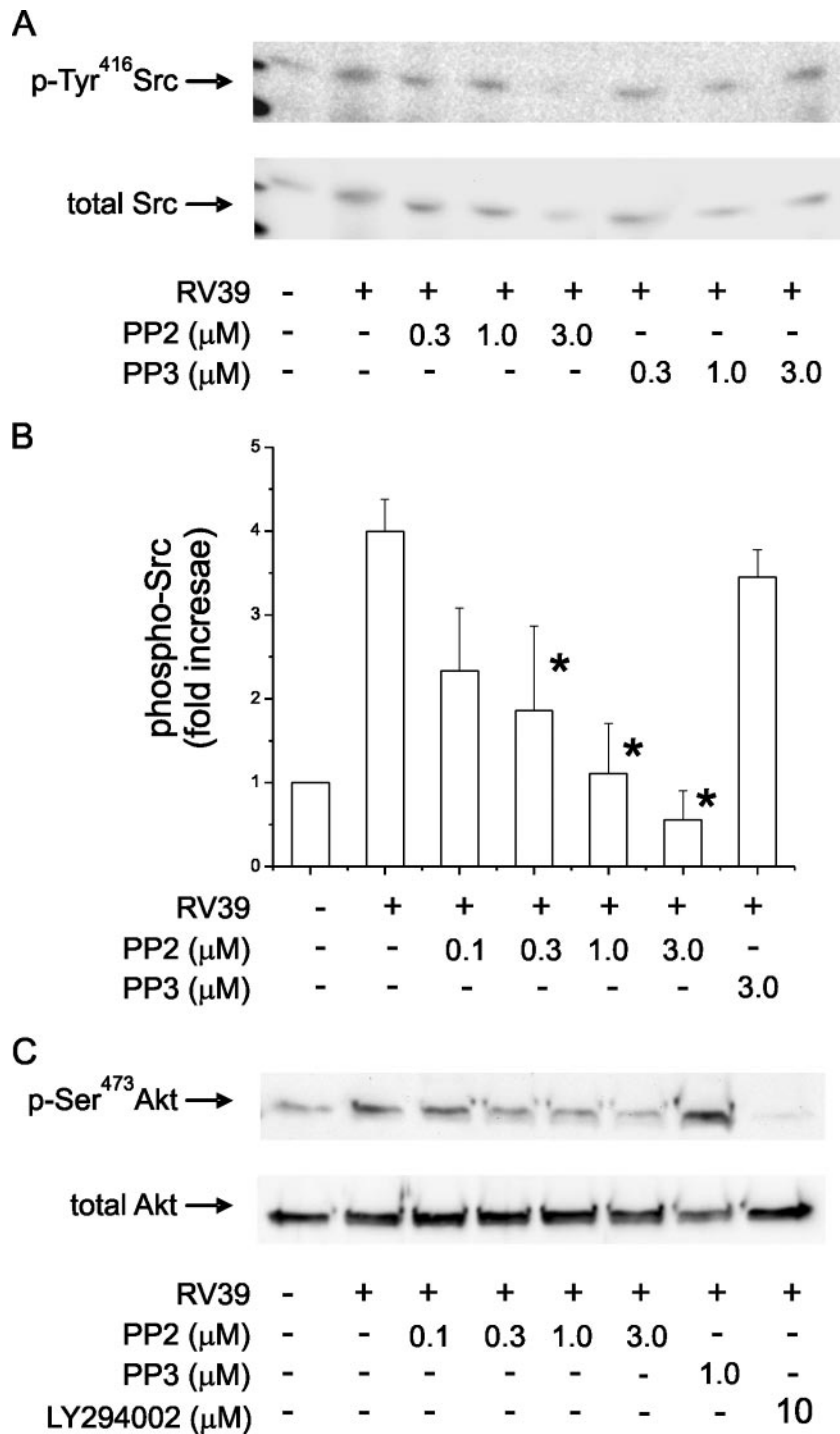


FIG. 3. RV39 increases phosphorylation of Ser⁴⁷³Akt in a Src- and PI 3-kinase-dependent manner. (A) 16HBE14o- cells without or with inhibitor (1 h at 37°C) were treated with sham or RV39 infection (MOI of 1, 10 min at 33°C). Detergent-soluble proteins (500 μg) were immunoprecipitated with mouse monoclonal 4G10 anti-phosphotyrosine (2 μg). Immunoprecipitates were immunoblotted for phospho-Tyr⁴¹⁶ (p-Tyr⁴¹⁶) Src (top panel) or total Src. (B) Group mean data for three experiments (means ± standard errors of the means; asterisk indicates a value different from that for RV alone; *P* < 0.05 by ANOVA). (C) Whole-cell lysates were immunoblotted for phospho-Ser⁴⁷³ Akt (20 μg protein/lane). The PP2 Src family kinase inhibitor blocked phosphorylation of both Src and Akt more effectively than PP3 did. These results are representative of three experiments.

gen and the PI(3,4,5)P₃-binding Akt pleckstrin homology domain (21). Minimal staining was detected when isotype control antibodies were substituted for primary antibodies (not shown).

RV39 labeling. In selected experiments, virus was labeled for immunoprecipitation and fluorescent microscopy using an *N*-hydroxysuccinimide deriv-

ative of digoxigenin, an amine-reactive form of this epitope tag (Roche Diagnostics). In a modification of described methods previously used to label RV with Alexa-Fluor dye (21), 0.4 ml of 0.1 M NaHCO₃ was added to a 0.1-ml aliquot of purified, concentrated virus to raise the pH to 8.5. The virus was then incubated in the dark for 1 h with 250 μg of probe. This virus maintained

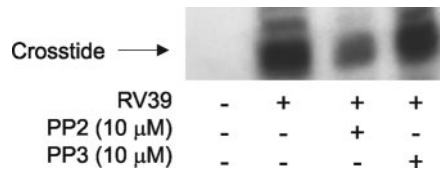


FIG. 4. Activated Akt coimmunoprecipitates with labeled RV39 after infection, and its activity is inhibited by a chemical Src inhibitor. Cells were treated with 3 nM digoxigenin-labeled sham or digoxigenin-labeled RV39 (MOI of 1 for 10 min). Selected cultures were pre-treated with PP2 or PP3 (10 μ M). Cell lysates were immunoprecipitated for digoxigenin. Immunoprecipitates were incubated with [γ - 32 P]ATP and Crosstide. Digoxigenin immunoprecipitates contained Crosstide kinase activity, which was reduced by PP2. PP2 also reduced the abundance of phosphorylated Akt in the immunoprecipitates. This experiment was repeated twice.

infectivity, with no reduction in TCID₅₀ compared with unlabeled virus. The incorporation of digoxigenin into labeled virus or sham protein was quantified on an fmole digoxigenin-per- μ g protein basis by methods described by the manufacturer.

Crosstide kinase assay. After serum deprivation for 24 h, cells were incubated with digoxigenin-labeled sham protein or digoxigenin-labeled RV39 at an MOI

of 1.0 for 10 min. Cell homogenates were immunoprecipitated with mouse anti-digoxigenin antibody (Roche Diagnostics) and precipitates incubated with Crosstide (Cell Signaling) and [γ - 32 P]ATP. Crosstide is a glycogen synthase kinase α/β fusion protein sequence (GRPTSSFAEG) which is a substrate for Akt (6). Samples were processed for autoradiography and immunoblotting using rabbit anti-phospho-Tyr⁴¹⁶ Src, mouse anti-Src (clone GD11), rabbit anti-phospho-Ser⁴⁷³, or rabbit anti-Akt.

Reverse transcriptase PCR (RT-PCR). cDNA was generated using 0.2 to 0.5 μ g of RNA using Superscript reverse transcriptase (Invitrogen) and specific antisense first-strand primers for PI 3-kinase class IA α (5'CT TTT CAG TTC AAT GCA TGC), IA β (5'TTA AGA TCT GTA GTC TTT CC), or IB γ (5'TTA GGC TGA ATG TTT CTC TGG) cDNA. *Taq* DNA polymerase (Invitrogen) and 2.5 μ M of specific primer were used.

To detect class I PI 3-kinases, we used 0.4 μ M of each primer and 10 ng of cDNA template in a reaction volume of 100 μ l for a total of 30 cycles. Specific PI 3-kinase primer sequences were as follows. For IA α (accession no. NM_006218), forward primer 5' TGGGATGTATTGAAGCACC 3' and nested reverse primer 5' TTTCGCACCACCTCAATAAG 3' was used to produce a 474-bp product. For IA β (accession no. NM_006219), forward primer 5' GTTGGCGCTTGATGGATTTACT 3' and nested reverse primer 5' TCACAA CACTGGCGGAACC 3' were expected to produce a 506-bp product. Finally, for IB γ (accession no. NM_002649), forward primer 5' ATGCTGCACGACTT TACCC 3' and nested reverse primer 5' TGGGGCTTGGGGTCTTCTG 3' were expected to produce a 490-bp product.

Data analysis. All experiments were performed a minimum of three times. Statistical significance was assessed by repeated-measures analysis of variance

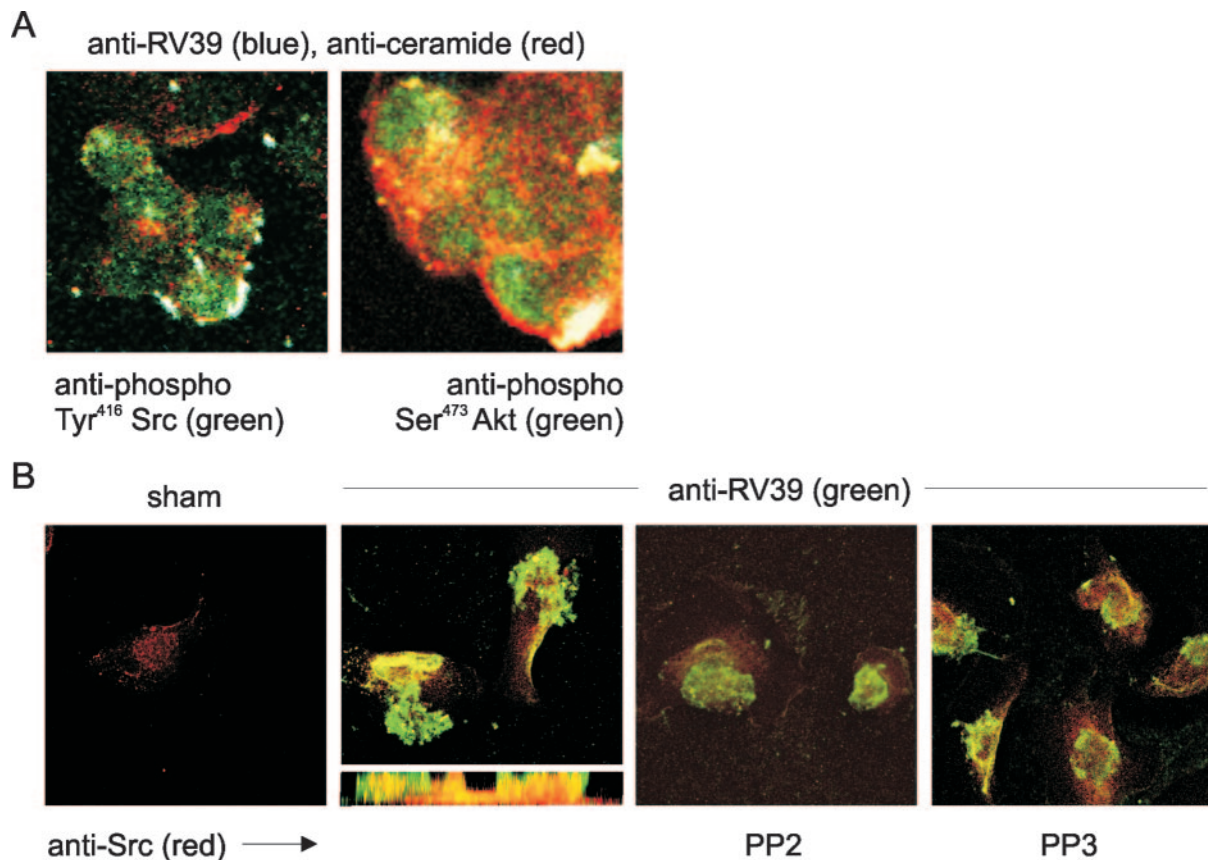


FIG. 5. Digoxigenin-labeled RV39 colocalizes with phosphorylated Src and Akt in ceramide-containing lipid rafts. (A) RV39 immunoreactivity (blue) colocalized with ceramide (red), phospho-Tyr⁴¹⁶ Src (green, left panel), and phospho-Ser⁴⁷³ Akt (green, right panel) in a white merged image. The colocalization of both Src and Akt with ceramide in punctate structures at the cell surface suggests that these protein kinases are associated with raft complexes where bound RV is aggregated. (B) Digoxigenin-labeled RV39 also colocalized with Src in primary mucociliary differentiated airway epithelial cells. The apical surface of primary airway epithelial cells grown at an air-liquid interface were incubated with sham or digoxigenin-labeled RV39 at an MOI of 100. Digoxigenin-labeled RV39 (shown in green) localized to the airway epithelial cell surface. Src was visualized using the clone GD11 antibody (shown in red). RV39 appeared to colocalize with Src just under the plasma membrane (see z-axis section). The high-affinity Src inhibitor PP2 appeared to block the colocalization of RV and Src. The low-affinity inhibitor PP3 did not block the association of RV and Src.

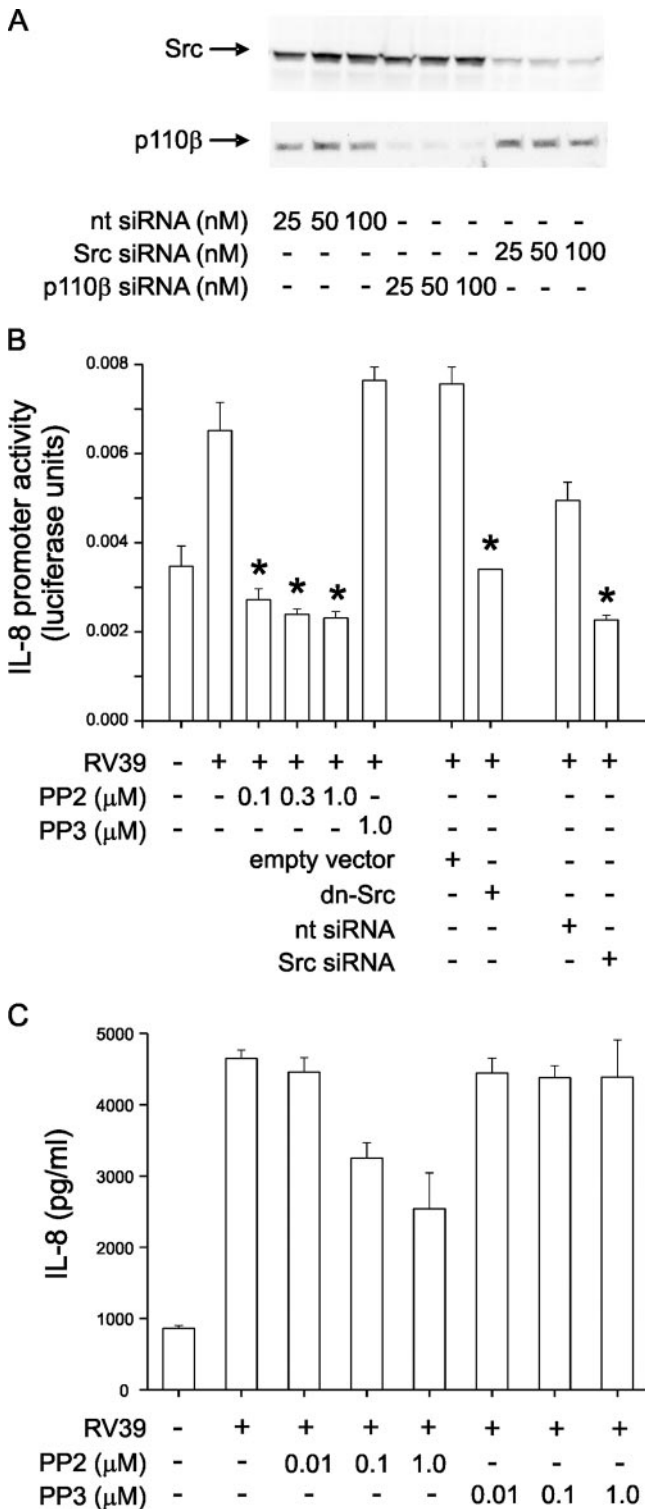


FIG. 6. Inhibition of Src kinase activity blocks the IL-8 response to RV39. (A) Immunoblots showing knockdown of Src and p110β PI 3-kinase with specific siRNAs. (B) 16HBE140– cells were transiently transfected with an IL-8 luciferase reporter and either 100 ng empty vector, dominant-negative K297R Src (dnSrc), 100 nM nontargeting RNA (nt siRNA), or Src siRNA. Dominant-negative Src inhibited RV39-induced IL-8 promoter activity. Selected cells were also treated either with dimethyl sulfoxide carrier, with the indicated concentration of Src family tyrosine kinase inhibitor PP2, or with its less potent and

(ANOVA). Differences identified by ANOVA were pinpointed by the Student Newman-Keuls multiple range text.

RESULTS

Src is activated by RV infection and required for RV39-induced Akt activation. We have recently shown that the infection of human bronchial epithelial cells with RV39 induces the rapid phosphorylation of the p85 regulatory subunit of PI 3-kinase (21). To determine whether Src is involved in RV-induced PI 3-kinase activation, we examined the localization of RV39, Src, and PI 3-kinase in RV39-infected 16HBE140– human bronchial epithelial cells by confocal fluorescence microscopy. We found a close association of RV, Src, and p85 PI 3-kinase (Fig. 1, upper panel). There was also colocalization of RV, Src, and stably expressed Cit-Akt-PH, a marker of PI(3,4,5)P₃-containing membranes (middle panel). Finally, RV and Cit-Akt-PH colocalized with p110β, a catalytic subunit of PI 3-kinase (lower panel).

Compared to sham-infected cells, 16HBE140– cells infected with RV39 also demonstrated colocalization of RV39 and phospho-Tyr⁴¹⁶ Src (Fig. 2, upper panel). Colocalization was blocked when cells were preincubated with the Src family kinase inhibitor PP2 but not with its less potent analogue PP3. A similar pattern of colocalization was observed with RV and phospho-Ser⁴⁷³ Akt (Fig. 2, lower panel). Again, colocalization was demonstrated by preincubation with PP2 but not with PP3. The PI 3-kinase inhibitor LY294002 blocked virus internalization and colocalization of RV39 and phospho-Akt but did not block virus binding.

Infection of 16HBE140– cells with RV39 (MOI of 1.0 for 10 min at 33°C) was also sufficient to increase Tyr⁴¹⁶ phosphorylation of a 60-kDa Src family kinase (Fig. 3A and B). Immunoblots of cell extracts with the C-terminal anti-Src antibody showed the presence of Src. We were unable to detect Fyn, another Src family kinase with a molecular mass of approximately 60 kDa (not shown). Src phosphorylation was efficiently blocked by PP2 but not by PP3. RV39 infection also induced phosphorylation of Ser⁴⁷³ Akt (Fig. 3C). Akt phosphorylation was blocked by PP2 as well as the PI 3-kinase inhibitor LY294002.

Digoxigenin-labeled RV39 associates with an active complex of signaling molecules, including Src and Akt. To better study such RV39-protein complexes, an immunoprecipitable RV39 was developed using a chemically modified form of the virus similar to that used in our previous work (21). An *N*-hydroxy-succinimide derivative of digoxigenin was used to tag the capsid surface, thereby providing a specific epitope for immunochemistry. The chemically modified form of RV39 retained

specific analog PP3. Cells were infected with RV39 (MOI of 1 for 1 h), incubated for 24 h, and harvested for assessment of luciferase activity. (C) Primary mucociliary differentiated human airway epithelial cells were infected with 5 × 10⁶ TCID₅₀/ml RV39 for 1 h in the presence of either PP2 or PP3 and incubated for an additional 48 h. IL-8 protein abundance was measured by enzyme-linked immunosorbent assay for 48 h. Protein abundance was inhibited by PP2 but not by a comparable concentration of PP3. For each panel, results from three experiments are shown, data shown are means ± standard errors of the means, and asterisks indicate *P* values of <0.05 versus those for RV39, RV39 plus empty vector, or RV39 plus nontargeting siRNA by ANOVA.

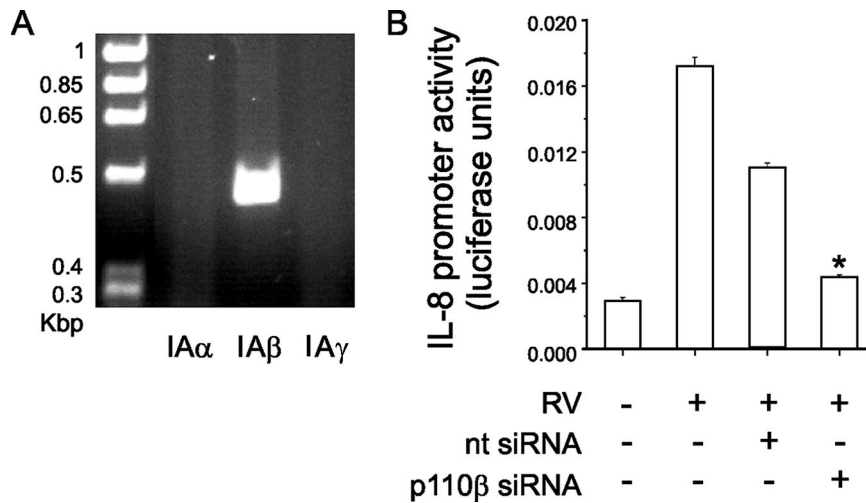


FIG. 7. p110 β PI 3-kinase is required for RV39-induced IL-8 expression. (A) RT-PCR showing presence of p110 β (IA α) but not p110 α (IA β) or p110 γ (IA γ), in airway epithelial cells. (B) Transient transfection of 16HBE14o– human bronchial epithelial cells with p110 β siRNA, but not with nontargeting (nt) siRNA, inhibited RV39-induced transcription from the IL-8 promoter ($n = 3$; *, $P < 0.05$ by ANOVA).

infectivity identical to that of unmodified virus. We reasoned that immunoprecipitation with anti-digoxigenin would obtain a Triton-insoluble complex of RV39 bound to its receptor and associated signaling molecules.

16HBE14o– cells were infected with digoxigenin-labeled RV39 (MOI of 1.0) for 10 min. Cells were lysed in 1% Triton X-100 and centrifuged ($10,000 \times g$), and the supernatant was collected for immunoprecipitation with anti-digoxigenin. Immunoprecipitates were incubated with [γ - ^{32}P]ATP and Cross-tyde, a glycogen synthase kinase α/β fusion protein sequence which is a substrate for Akt (6). Cells exposed to RV39 demonstrated a Cross-tyde kinase activity in vitro (Fig. 4). This kinase activity was not found when cells were exposed to an equimolar (based on digoxigenin) amount of labeled sham protein. Serine phosphorylation of Cross-tyde was confirmed by immunoblotting (not shown), and phosphorylation was blocked by PP2 but not by PP3. These data suggest that RV infection activates Akt in a Src-dependent manner.

Colocalization of RV, Src, and Akt with ceramide, a constituent of lipid rafts. It has recently been shown that RVs infect human epithelial cells via ceramide-enriched membrane platforms (11). While nonionic detergents like Triton X-100 are sufficient to dissociate many membrane-associated complexes, initial experiments to biochemically enrich virus-associated rafts by sucrose density flotation met with unsatisfactory results because the virus tends to sediment in sucrose density gradients. We therefore colocalized RV and relevant signaling intermediates with ceramide. In this set of experiments, digoxigenin-labeled RV (MOI of 10) was used. Composite projection views show colocalization of RV39, ceramide, phospho-Tyr⁴¹⁶ Src, and phospho-Ser⁴⁷³ Akt (Fig. 5A). The association of RV39 with Src and Akt in ceramide-enriched lipid domains strongly suggests the formation of raft protein aggregates.

Digoxigenin-labeled RV39 colocalizes with Src in primary airway epithelial cells. We tested whether the colocalization of digoxigenin-labeled RV39 and Src occurs in primary human airway epithelial cells. Compared to sham-infected cells, cells infected with RV39 showed colocalization of RV and Src, as

recognized by the GD11 antibody (Fig. 5B). A z-axis section more clearly demonstrates the three-dimensional arrangement of RV and Src, with colocalization occurring just below the cell surface. Colocalization of RV39 and Src was blocked by PP2 but not by PP3.

Src kinase activity is required for the IL-8 response to RV39. We have recently shown that infection of human bronchial epithelial cells with RV39 induces rapid activation of PI 3-kinase and phosphorylation of the PI 3-kinase p85 α regulatory subunit (21). Since Src is associated with focal adhesion complexes and phosphorylates p85 (15), we tested the dependence of IL-8 production induced by RV39 on Src kinase activation. Src siRNA specifically reduced Src protein expression (Fig. 6A). In 16HBE14o– human bronchial epithelial cells, expression of the dominant-negative K296R/Y528F Src or Src siRNA or treatment with the Src family-specific kinase inhibitor PP2 (0.1 to 1 μ M) significantly inhibited RV39-induced transcription from the IL-8 promoter (Fig. 6B). PP2 also attenuated IL-8 release from primary mucociliary differentiated airway epithelial cells infected with RV39 (Fig. 6C). PP3 (1 μ M), an inactive analogue of PP2, did not significantly inhibit IL-8 release. Together, these findings suggest that Src is required for RV-stimulated IL-8 expression.

p110 β PI 3-kinase is required for RV39-induced IL-8 expression. Using RT-PCR, we confirmed that p110 β is present in airway epithelial cells (Fig. 7A). Transient transfection of 16HBE14o– human bronchial epithelial cells with siRNA against p110 β significantly inhibited p110 β protein expression (Fig. 7A) and RV39-induced transcription from the IL-8 promoter (Fig. 7B), demonstrating that p110 β is required for the response.

DISCUSSION

We have recently shown that infection of human airway epithelial cells with RV39 induces rapid activation of PI 3-kinase and phosphorylation of Akt. (21). PI 3-kinase activation was accompanied by phosphorylation of the PI 3-kinase p85

regulatory subunit. RV39 also colocalized with Cit-Akt-PH, a fluorescent fusion protein containing the pleckstrin homology domain of Akt, indicating that 3-phosphorylated PI accumulates at the site of RV infection. We now show that, following the engagement of ICAM-1 by RV39, Src functions as an upstream activator of Akt and that RV colocalizes with Src, PI 3-kinase p110 β , and Akt in lipid rafts. These data are consistent with previous work with endothelial cells showing that antibody cross-linking of ICAM-1 causes colocalization with Src in lipid rafts (35) as well as Src phosphorylation and activation (7, 37).

Src/PI 3-kinase signaling has been noted to be activated in the context of viral infection twice previously. Engagement of the B lymphocyte Epstein-Barr virus receptor activates PI 3-kinase via Src (2). Expression of human herpesvirus 8 envelope glycoprotein gB induces Src/PI 3-kinase signaling in human foreskin fibroblasts (33). We now extend this mode of entry to RV-infected human bronchial epithelial cells. The data shown here demonstrate that activation of a Src/p110 β PI 3-kinase/Akt pathway occurs coincident with the binding of RV into ceramide-enriched lipid domains as a prelude to internalization.

Expression of dominant-negative K296R/Y528F Src and Src siRNA, as well as pretreatment with a Src family kinase chemical inhibitor, each significantly attenuated RV39-induced IL-8 expression, demonstrating that Src is required for maximal RV-induced IL-8 expression. However, IL-8 expression was not completely eliminated, suggesting that other pathways are also required. Consistent with this, we have found that Toll-like receptor-3, a receptor for double-stranded RNA, is also required for RV-induced chemokine expression (31), as it is for respiratory syncytial virus and influenza virus, two other positive-strand RNA viruses (14, 29).

Confocal micrographs showed that while RV39 infection induces Tyr⁴¹⁶ phosphorylation of Src, only a fraction of phosphoprotein colocalizes with RV. Lipid-lipid interactions involving amino-terminal acyl groups on Src family kinases, for example, palmitoylation, are the primary mechanism for membrane localization, particularly localization to membrane microdomains or lipid rafts (18). The exclusion of a Src fraction from rafts implies that it may not encounter substrates needed for localization to lipid rafts and that this Src fraction may be performing functions distinct from those of raft-localized Src. Similarly, Ser⁴⁷³ Akt phosphorylation was not confined to areas of colocalization with RV, suggesting diffusion of the class IA PI 3-kinase product PI(3,4,5)P₃ outside the raft area. These data are consistent with our previous data indicating membrane and cytoplasmic localization of the pleckstrin homology domain of Akt following RV infection (21).

As in a previous study (11), we did not biochemically enrich RV-associated rafts by sucrose density flotation, as the virus tended to sediment in sucrose density gradients. Interestingly, rafts not associated with RV contained inactive/closed Tyr⁵²⁷-phosphorylated Src (19) (not shown). However, the association of RV, Src, Akt, and ceramide, which brings about the coalescence of microscopic rafts into large-membrane macrodomains, strongly suggests that RV initiates the formation of Src- and Akt-containing membrane platforms. Src may therefore represent a cellular target for intervention against RV-associated respiratory disease.

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