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Syk Is Downstream of Intercellular Adhesion Molecule-1 and Mediates Human Rhinovirus Activation of p38 MAPK in Airway Epithelial Cells

Xiaomin Wang,* Christine Lau,* Shahina Wiehler,§ André Pow,* Tony Mazzulli,† Carlos Gutierrez,* David Proud,§ and Chung-Wai Chow2*‡

The airway epithelium is the primary target of inhaled pathogens such as human rhinovirus (HRV). Airway epithelial cells express ICAM-1, the major receptor for HRV. HRV binding to ICAM-1 mediates not only viral entry and replication but also a signaling cascade that leads to enhanced inflammatory mediator production. The specific signaling molecules and pathways activated by HRV-ICAM-1 interactions are not well characterized, although studies in human airway epithelia implicate a role for the p38 MAPK in HRV-induced cytokine production. In the current study, we report that Syk, an important immunoregulatory protein tyrosine kinase, is highly expressed by primary and cultured human airway epithelial cells and is activated in response to infection with HRV16. Biochemical studies revealed that ICAM-1 engagement by HRV and cross-linking Abs enhanced the coassociation of Syk with ICAM-1 and ezrin, a cytoskeletal linker protein. In polarized airway epithelial cells, Syk is diffusely distributed in the cytosol under basal conditions but, following engagement of ICAM-1 by cross-linking Abs, is recruited to the plasma membrane. The enhanced Syk-ICAM-1 association following HRV exposure is accompanied by Syk phosphorylation. ICAM-1 engagement by HRV and cross-linking Abs also induced phosphorylation of p38 in a Syk-dependent manner, and conversely, knockdown of Syk by short interfering (si)RNA substantially diminished p38 activation and IL-8 gene expression. Taken together, these observations identify Syk as an important mediator of the airway epithelial cell inflammatory response by modulating p38 phosphorylation and IL-8 gene expression following ICAM-1 engagement by HRV. The Journal of Immunology, 2006, 177: 6859–6870.

The airway epithelium plays an important role in regulating the host immune response and provides the first line of defense against inhaled pathogens, particulate matter, and allergens. Epithelial cells line >95% of the surface of the respiratory tract and produce a diverse array of antimicrobial compounds, including surfactants, collectins, defensins, lysozymes, lactoferrin, reactive oxygen, and nitrogen species. Furthermore, they express pattern recognition receptors that include the TLR, lactoferrin, reactive oxygen, and nitrogen species. The intracellular signaling pathways that regulate epithelial cell activation and replication. Binding of HRV to ICAM-1 appears to be crucial in this response. Studies have shown that inhibition of the HRV-ICAM-1 interaction by blocking Abs abrogates not only viral entry and replication (4, 5) but also cytokine production (5, 6). The intracellular signaling pathways that regulate epithelial cell activation have not been fully characterized. Recent studies have shown that NF-κB (7, 8) and p38 MAPK (6, 9) are important for up-regulation of cytokine production following HRV infection of airway epithelial cells.

Syk is an important proximal immunoregulatory protein tyrosine kinase that has been identified recently in bronchial epithelium (10). Originally described in hemopoietic cells where it is abundantly expressed, Syk is a crucial regulator of several components of the immune response, including humoral, allergic, and cytotoxic immunity (11). Studies with Syk-deficient (Syk−/−) mice and Syk−/− bone marrow chimeric mice have revealed that the kinase is crucial for the maturation and proliferation of B lymphocytes, as well as γδ T lymphocytes (12, 13). Moreover, Syk also plays a crucial early role in signaling following engagement of immunoreceptors such as Fce and Fcγ receptors. Syk−/− leukocytes exhibit failure of IgE-mediated degranulation (14) and FcγR-mediated phagocytosis, as well as inflammatory mediator production (15, 16). Syk also plays a role regulating signal transduction following integrin engagement. In neutrophils, Syk has been shown to be necessary for the β2 integrin-mediated respiratory burst, spreading, and site-directed migration (17–20). In B lymphocytes, Syk is required for β1 integrin-mediated cell polarization in response to CXCL12 (21).
In primary human bronchial epithelial cells and the HS-24 lung epithelial tumor cell line, the function of Syk is not well understood, although it has been implicated in TNF-α-induced production of IL-6 and ICAM-1 (10). Together, these early findings suggest a role for Syk in mediating the inflammatory response of the airway epithelium.

In the current report, we identify expression of two Syk isoforms, Syk and its splice variant Sykβ, in primary small airway epithelial cells and several human pulmonary epithelial cell lines and determine that it is downstream of ICAM-1 signaling. We demonstrate that Syk is recruited to the plasma membrane following ICAM-1 engagement where it associates with ICAM-1 and ezrin, a cytoskeletal linker protein that had previously been shown to bind directly to ICAM-1 in vivo (22) and Syk in vitro (23, 24).

Using short interfering RNA (siRNA) to down-regulate expression of Syk in BEAS-2B cells, we found that Syk is necessary for the activation of p38 MAPK following HRV infection.

Materials and Methods

Cell lines

Primary human small airway epithelial cells (hSAEC; Cambrex) were grown in small airway cell basal medium supplemented with growth factors and antibiotics according to the manufacturer’s instructions. BEAS-2B, a human bronchial epithelial cell line, was provided by Dr. C. Harris (National Cancer Institute, National Institutes of Health, Bethesda, MD). Cells were grown in a 1/1 mix of high glucose-DMEM and Ham’s F-12 Nutrient Mix (DMEM/F12) (Invitrogen Life Technologies) supplemented with the following: 10 μg/ml human recombinant insulin, 25 ng/ml recombinant human epidermal growth factor, 5 μg/ml transferrin, 2% (v/v) penicillin/streptomycin (all from Invitrogen Life Technologies), and 0.1 μM hydrocortisone (Sigma-Aldrich). The human airway epithelial cell line, Calu-3, and the human lung cancer-derived A549 cells were obtained from American Type Culture Collection (ATCC). Calu-3 cells were cultured in MEM-nonessential amino acids solution medium supplemented with 1.5 μL bicitarone and 20% FBS. A549 cells were grown in HG-DMEM with 8% FBS. TS20IMH cells (gift from Dr. S. Grinstein, Hospital for Sick Children, Toronto, Ontario, Canada) are Chinese hamster lung fibroblasts. RAW264.7 is a murine macrophage cell line obtained from ATCC. Both are grown in DMEM with 10% FBS. All media were supplemented with 2 mM l-glutamine (Sigma-Aldrich). All cell types were cultured at 37°C, 5% CO2, in a humidified environment.

Abs and reagents

Abs were purchased from the following sources: mouse monoclonal ezrin from Zymed Laboratories; rabbit polyclonal ezrin from Upstate Cell Signaling; Syk antibody, mouse monoclonal 2B10 and rabbit polyclonal C-20 from Santa Cruz Biotechnology; rabbit polyclonal Syk-phospho-Y525,526, rabbit polyclonal phospho-p38, rabbit polyclonal phospho-p38, rabbit polyclonal phospho ERK1/2 (Thr202/Tyr204), rabbit polyclonal ERK1/2, rabbit polyclonal phospho-stress-activated protein kinase/Jnk (Thr183/Tyr185), rabbit polyclonal stress-activated protein kinase/Jnk, rabbit polyclonal phospho-MAP kinase (MKK) 3/MKK6, and rabbit polyclonal MKK3 from Cell Signaling Technology; and mouse monoclonal anti-ICAM-1 clone BBIG-11, used for cross-linking experiments, was from R&D Systems; mouse monoclonal anti-ICAM-1 clone 15.2, used for Western blot analysis, from Research Diagnostic and rabbit polyclonal ICAM-1, used for Western blot analysis, from Santa Cruz Biotechnology. Goat anti-mouse F(ab)2 was from Jackson ImmunoResearch Laboratories. HRP-labeled anti-mouse and anti-rabbit secondary Abs, FITC-labeled goat-anti-rabbit, and Texas Red-labeled goat anti-mouse secondary Abs were from Jackson ImmunoResearch Laboratories. TNF-α was purchased from BioSource International. The isotype control Ab for the anti-SykD10, used for the immunoprecipitation experiments, was mouse IgG2a,k, and the isotype control for the anti-ICAM-1 Ab used for the cross-linking studies was mouse IgG1,k. Both isotype control Abs were purchased from Sigma-Aldrich.

RNA extraction, RT-PCR, and real-time PCR

Total RNA was isolated from cultured lung epithelial cells using TRIzol reagent (TRI Reagent; Sigma-Aldrich), following the manufacturer’s protocol. Purity was checked by the A260/A280 ratio. Total RNA was reverse transcribed using random hexamer priming and SuperScript II Rnaase H-Reverse Transcripts (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen Life Technologies). cDNA was prepared from 5 μg of RNA in a 20-μl volume, adding 50 U of SuperScript II Reverse Transcriptase, 2 μl of 10X reverse transcriptase buffer, 40 U of RNaseOut Recombinant RNase Inhibitor, 5 mM MgCl2, 10 mM DTT, 0.5 mM dNTP mix, and 7.5 ng/μl random hexamers. A negative control reaction lacking reverse transcriptase was also performed for each RNA sample. The random hexamer primer was annealed for 10 min at 25°C. CDNA synthesis was performed for 50 min at 42°C, followed by 15 min at 70°C, to terminate the reaction. One microliter of cDNA was added to each tube and incubated for 20 min at 37°C, and cDNA was stored at −20°C until used. PCR was performed in 100 μl of reaction solution, containing 0.2 mM dNTPs, 1.5 mM MgCl2, 2.5 U/100 μl Taq polymerase (TaqDNA polymerase; Fermentas), and 0.5 μM oligonucleotide primers. After an initial denaturation step (3 min at 94°C), PCR cycling was performed for 20 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). The final extension period was of 7 min at 72°C. The PCR products were analyzed by electrophoresis using a 2% agarose gel with ethidium bromide.

The primer pairs used are as follows: 1) human GAPDH, accession no. M33197 (GenBank) sense, 5’-TCAACGACCTTGTGACAGTC3’- and antisense, 5’-GCTGTTGTTCAAGGCTTACT3’-; PCR product 120 bp and first strand cDNA products; 2) primer set 1, human Syk, accession no. GI:21361552, sense 5’-GGAAGTGAAGTCCACGCTTATG-3’ and antisense, 5’-GGGAGCGGTTAGTTCACCAC-3’; PCR product 184 bp; and 3) primer set 2, human Syk, accession no. GI:21361552, sense 5’-ACTGTCCTCAATGTTAAAAATCGGC-3’ and antisense, 5’-GGGCCCTCTGTCACCT-3’; PCR product 268 bp for wild type-Syk and 219 bp for Syk B.

For real-time PCR experiments, total RNA were extracted from BEAS-2B cells following HRV exposure (described below) using the RNeasy Mini kit from Qiagen, according to the manufacturer’s directions. The average RNA yield is 5 μg of RNA per 106 cells. IL-8 gene expression analysis was performed using the Applied Biosystems model 7900 Sequence Detector. Input RNA (400 ng) was reverse transcribed into cDNA, followed by PCR amplification in the presence of specific forward 5’-CTGGGCGCTGTGCCTCTTTG-3’ and reverse 5’-TTCAGCCTACTGGAATAAACGT-3’ primers and probe 5’-FAM-CTCTCAGGCAATTTCTCCACGAGCGTTGTGGA-TAMRA-3’ (Applied Biosystems). Expression of the housekeeping gene GAPDH was also assessed using reagents obtained from Applied Biosystems. Efficiency curves were performed for each gene of interest, relative to the housekeeping gene, and data were calculated as fold increase over control.

Transfection and siRNA

Pulmonary epithelial cells were transfected using the Amaxa Nucleofector system, according to the manufacturer’s instructions, using 2 × 106 cells and 0.75 μg of Cy3-Luciferase Control SMARTpool siRNA reagent or Syk SMARTpool siRNA (Upstate Cell Signaling). The cells were plated in normal culture medium at 37°C in 5% CO2 and used for the experiments at 36–48 h posttransfection.

ICAM-1 cross-linking, TNF-α, and HRV stimulation experiments

Cells were grown to subconfluence and deprived of growth factors (BEAS-2B cells) or serum (Calu-3 cells) overnight before the experimental procedure. ICAM-1 cross-linking was performed by incubating the cells with 15 μg/ml anti-ICAM-1 Ab (clone BBIG-11; R&D Systems) for 30 min. Unbound Ab was washed with PBS, followed by cross-linking with 50 μg/ml F(ab)2 goat anti-mouse secondary Ab for 10 min at 37°C. Cross-linking of the isotype control for anti-ICAM-1 Ab was performed in the same manner using 15 μg/ml IgG1 (Sigma-Aldrich) for the initial incubation, followed by cross-linking with 50 μg/ml F(ab)2 goat anti-mouse secondary Ab for 10 min at 37°C. TNF-α stimulation experiments were performed by exposing the cells to 10 ng/ml TNF-α for 10 min at 37°C. HRV16 viral stocks used for experiments were generated by propagation in WI-38 cells and were purified by centrifugation through sucrose gradient at 72°C. The average RNA yield is 5 μg of RNA per 106 cells. IL-8 gene expression analysis was performed using the Applied Biosystems model 7900 Sequence Detector. Input RNA (400 ng) was reverse transcribed into cDNA, followed by PCR amplification in the presence of specific forward 5’-CTGCGCCGCTGTGCCTCTTTG-3’ and reverse 5’-TTCAGCCTACTGGAATAAACGT-3’ primers and probe 5’-FAM-CTCTCAGGCAATTTCTCCACGAGCGTTGTGGA-TAMRA-3’ (Applied Biosystems). Expression of the housekeeping gene GAPDH was also assessed using reagents obtained from Applied Biosystems. Efficiency curves were performed for each gene of interest, relative to the housekeeping gene, and data were calculated as fold increase over control.

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Syk MEDIATES HRV-ICAM-1 SIGNALING TO p38 MAPK
P-40, 10 mM sodium pyrophosphate, 25 mM NaF, 120 mM NaCl, 200 μM Na3VO4, 1% aprotinin, 1 mM PMSE, and 5 mM DTT) and then boiled in 5% Laemmli sample buffer. The samples were then subjected to protein electrophoresis and Western blot analysis as described below.

**Immunoprecipitation, SDS-PAGE, and Western blot analysis**

Cells were washed with PBS and harvested with lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 1% Triton X-100 supplemented with 1 tablet/10 ml Complete-Mini protease inhibitor mixture; Roche). Protein assay was performed using the Bradford method. For Western blot analysis of whole cell lysates, 25–30 μg of protein was loaded per lane and separated by SDS-PAGE using 7.5–10% polyacrylamide gel as described previously (26).

For immunoprecipitation, the cell lysates were precleared with 20 μl of protein A/G-Sepharose (Santa Cruz Biotechnology) for 60 min, then incubated with 1 μg of anti-Syk4D10 Ab or the isotype control IgG2a for 60 min before the addition of 30 μl of protein A/G-Sepharose for another 60 min in an end-over-end rotor. The reaction was washed three times with radioimmunoprecipitation assay buffer (120 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS). The entire procedure was done at 4°C. After the final wash, the pellet was resuspended in 40 μl of 2X Laemmli sample buffer and boiled for 10 min. The samples were separated by SDS-PAGE, and Western blot analysis was performed as described previously (26). Densitometry was analyzed using ImageJ and expressed as mean ± SE. ANOVA was used for factorial analysis. Two and three factor interactions were tested and then removed when not significant. Post hoc tests were conducted when ANOVA reached \( p < 0.05 \), using Tukey’s adjustment method. Data were log transformed when data were not normally distributed. Statistical analysis software was used for analysis (SAS for Windows, version 8.2; SAS Institute).

**Confocal microscopy**

Calu-3 cells were cultured on Transwell filters (3.0-μm pore; Costar) to confluence and transepithelial resistance was measured with a dual-voltage Ohmometer clamp (World Precision Instruments) (27). When the transepithelial resistance reached ~800 Ohm cm², the cells were stimulated by cross-linking ICAM-1 as described above and then fixed with 4% paraformaldehyde. Control cells were not stimulated before fixation. The cells were quenched for 15 min with 100 mM glycine, permeabilized with 0.1% Triton X-100 for 15 min, blocked with 10% goat serum plus 1% BSA in PBS for 1 h, then incubated with 1/100 anti-Syk Ab or anti-ezrin Ab for 1 h. After washing with PBS, the cells were counterstained with 1/300 of FITC- or Cy3-labeled goat anti-mouse or anti-rabbit IgG (Jackson Immuno-Research Laboratories) for 1 h. The membranes were cut out and mounted using Dako fluorescent mounting medium onto a glass slide. Images were obtained using a Zeiss LSM510 confocal microscope and the LSM5 Image software.

**Results**

Syk is expressed in primary small airway epithelial cells and human pulmonary epithelial cell lines

We assessed Syk mRNA expression by RT-PCR. We extracted total RNA from hSAEC and human pulmonary epithelial cell lines and performed RT-PCR using two different sets of primers (Fig. 1A). Primer set 1 flanks a region in the carboxy terminus and correspond to nt 1888–1908 and 2072–2055, respectively, of the human Syk gene sequence (GenBank accession no. GI:21361552). The expected PCR product of this primer set is 184 bp and does not differentiate between Syk and SykB. Primer set 2, corresponding to nt 726–750 and 1178–1202 of human Syk, flanks a 23-aa sequence within the linker region of the molecule that is deleted in the SykB splice variant. This primer set yields a product of 288 bp for WT Syk and 219 bp for SykB. As shown in Fig. 1B, hSAEC and the human pulmonary epithelial cell lines, BEAS-2B and Calu-3 cells, revealed a single PCR product that correspond to Syk/SykB mRNA expression was an-alyzed by RT-PCR using two different primer sets. The location of the primer pairs with respect to the amino-terminal Src homology (SH2) domains, the carboxy-terminal kinase domain, and the 23-aa linker insert that is absent in the SykB splice variant is shown. Primer set 1 does not differentiate between Syk and SykB. Primer set 2 yields a PCR product of 219 bp for Syk B and 288 bp for Syk B. RT-PCR revealed Syk and SykB mRNA expression in BEAS-2B, Calu-3, and hSAEC cells. A549 cells express a single band that corresponds to Syk (middle panel, primer set 2). The bottom panel represents GAPDH mRNA expression and used as a control of expression of a housekeeping gene. Representative of three experiments. C, Syk protein expression was evaluated by Western blot analysis. BEAS-2B, Calu-3, and hSAEC cells have two immuno-reactive bands that correspond to Syk and SykB. A faint band corresponding to Syk was seen in A549 cells after prolonged exposure. The murine macrophage RAW267.4 cell line, which expresses Syk, was used as a positive control. TS200MH, a Chinese hamster lung fibroblast cell line that does not express Syk, was used as a negative control. Syk 4D10 Ab (top panel) is human specific. Syk C20 Ab (middle panel) is species cross-reactive. The bottom panel is blotted with a housekeeping protein, α-actin, to indicate equal protein loading per lane. Representative of four experiments.

**FIGURE 1.** Syk expression in epithelial cells. A, Syk mRNA expression was analyzed by RT-PCR using two different primer sets. The location of the primer pairs with respect to the amino-terminal Src homology (SH2) domains, the carboxy-terminal kinase domain, and the 23-aa linker insert that is absent in the SykB splice variant is shown. Primer set 1 does not differentiate between Syk and SykB. Primer set 2 yields a PCR product of 219 bp for Syk B and 288 bp for Syk B. RT-PCR revealed Syk and SykB mRNA expression in BEAS-2B, Calu-3, and hSAEC cells. A549 cells express a single band that corresponds to Syk (middle panel, primer set 2). The bottom panel represents GAPDH mRNA expression and used as a control of expression of a housekeeping gene. Representative of three experiments. B, Syk protein expression was evaluated by Western blot analysis. BEAS-2B, Calu-3, and hSAEC cells have two immuno-reactive bands that correspond to Syk and SykB. A faint band corresponding to Syk was seen in A549 cells after prolonged exposure. The murine macrophage RAW267.4 cell line, which expresses Syk, was used as a positive control. TS200MH, a Chinese hamster lung fibroblast cell line that does not express Syk, was used as a negative control. Syk 4D10 Ab (top panel) is human specific. Syk C20 Ab (middle panel) is species cross-reactive. The bottom panel is blotted with a housekeeping protein, α-actin, to indicate equal protein loading per lane. Representative of four experiments.
Syk is a cytosolic protein that is associated with ICAM-1 and is recruited to the membrane following ICAM-1 engagement

In leukocytes, activation of Syk by immunoreceptors such as the Fcγ (15, 29) and Fce receptor (30), and by β integrin (20, 31), is dependent on recruitment of the cytosolic kinase to the receptor at discrete regions of the plasma membrane. To evaluate the association of Syk with the HRV receptor, ICAM-1, we performed co-precipitation experiments in BEAS-2B cells under basal conditions and following ICAM-1 engagement with HRV and cross-linking Abs. As shown in Fig. 2A, Western blot analysis of the Syk immunoprecipitates revealed the presence of ICAM-1 under basal conditions (time 0). HRV16 enhanced the amount of ICAM-1 present in the Syk immunoprecipitates as early as 15 min following exposure. UV-HRV16 is HRV16 that has been subjected to irradiation to render it replication deficient and was used to eliminate potential confounding effects of viral replication from receptor binding on downstream signaling. As shown in Fig. 2A, UV-HRV16 also increased Syk-ICAM-1 coassociation at 15 and 30 min. When we engaged ICAM-1 by use of cross-linking Abs as described in Materials and Methods, we observed a similar enhancement of Syk-ICAM-1 coassociation (data not shown). Densitometry of ICAM-1 band revealed a 1.42 ± 0.05-fold increase in the cross-linked cells when compared with control (p < 0.05, n = 3).

In a second approach, we determined the intracellular distribution of Syk in Calu-3 cells on Transwell filters (Costar) to promote polarization. Using transepithelial resistance as a functional marker of tight junction formation, we cultured Calu-3 cells until they reached a transepithelial resistance of >800 Ωcm² before evaluating Syk localization by confocal microscopy. The cells were assessed under basal control conditions and following ICAM-1 engagement with cross-linking Abs as described in Materials and Methods. Under basal conditions, Syk is diffusely distributed in the cytosol of Calu-3 cells (Fig. 2B, left panel). Following ICAM-1 engagement with cross-linking Abs, enhancement of Syk at the apical and lateral membranes is observed (Fig. 2B, right panel). Similar experiments were not performed with BEAS-2B cells as this cell line does not form tight junctions.
We tested this hypothesis first by evaluating the association of Syk with ezrin with coimmunoprecipitation experiments. The cell lysates of control and ICAM-1-engaged cells were immunoprecipitated with anti-Syk 4D10 Ab and then subject to protein electrophoresis and Western blot analysis. We identified ezrin in the Syk-immunoprecipitates (Fig. 3A, second panel). Following HRV16 and UV-HRV16 stimulation, enhanced ezrin was detected in the Syk immunoprecipitates at both time points evaluated. UV-HRV16 infection also increased the amount of ezrin pulled down at 15 and 30 min. We also used an in vitro model of ICAM-1 engagement with cross-linking Abs to assess Syk-ezrin association. Cross-linking of ICAM-1 (ICAM-1 XL) also enhanced ezrin pulldown in the Syk immunoprecipitates, albeit with a lower response (Fig. 3B, lane 3). As a control, we incubated the cells with IgG1, an isotype control for anti-ICAM-1 Ab used in these studies. Following cross-linking with goat anti-mouse F(ab’)2, no enhancement of ezrin-Syk interaction was observed (Fig. 3B, lane 4). The isotype control for the immunoprecipitating Ab, IgG2a, did not pull down Syk or ezrin (Fig. 3B, lane 1). Therefore, the association of Syk and ezrin and the enhancement of this coassociation following ICAM-1 engagement is specific.

As an independent method, we assessed colocalization of Syk and ezrin using confocal microscopy. Polarized Calu-3 cells were fixed, permeabilized, and then doubly stained with anti-Syk 4D10 Ab (secondary Ab = Texas-Red anti-mouse IgG), followed by anti-ezrin Ab (secondary Ab = FITC anti-rabbit IgG). The intracellular localization of Syk (Fig. 3C, left panel) and ezrin (Fig. 3C, middle panel) were assessed by confocal microscopy. As shown in the merged image (in Fig. 3C, right panel), there is considerable colocalization of Syk with ezrin, as indicated by the yellow color.

Considered together, these results indicate that Syk associates with ezrin in airway epithelial cells under basal conditions with enhancement following HRV exposure and ICAM-1 engagement by cross-linking Abs, suggesting that ezrin is likely the adaptor protein that links ICAM-1 signaling to Syk. However, the role of tyrosine phosphorylation in regulating these interactions is not clear and was evaluated next.

Syk undergoes phosphorylation on Y525/526 in response to infection with HRV16 and UV-HRV16

In leukocytes, Syk is not phosphorylated under basal (resting) conditions but undergoes tyrosine phosphorylation following engagement of the immunoreceptors (32–34). Phosphorylation of Y525/Y526 within the activation loop of the molecule is necessary for activation of the kinase activity of Syk (33, 34). To assess whether pulmonary epithelial Syk undergoes tyrosine phosphorylation following ICAM-1 engagement, we evaluated Syk phosphorylation by Western blot analysis with the phospho-Syk-Y525/526-specific Ab following immunoprecipitation with anti-Syk4D10 Ab. Growth factor-starved BEAS-2B cells were exposed to HRV16 and UV-HRV16 for 15 and 30 min. As shown in Fig. 4, Syk undergoes tyrosine phosphorylation at Y525/526 in response to both HRV16 and UV-HRV16.

**FIGURE 3.** Syk is associated with ezrin. BEAS-2B cells were cultured, stimulated, and harvested as described in Fig. 2A. The Syk-immunoprecipitates reveal coprecipitation of Syk and ezrin under basal conditions (time 0). Following stimulation with HRV16 and UV-HRV16, enhanced ezrin was detected. Densitometry of the ezrin band is normalized to Syk and represents the mean ± SE fold change in comparison with time 0 (defined as 1). HRV16 (15 min) = 8.75 ± 4.83; HRV16 (30 min) = 6.64 ± 2.68; UV-HRV16 (15 min) = 3.93 ± 1.77; UV-HRV16 (30 min) = 5.67 ± 2.13; p > 0.05 for all conditions, n = 4. B, The coprecipitation of Syk with ezrin and its enhancement following ICAM-1 engagement are specific. BEAS-2B cells were growth factor deprived overnight and then incubated with anti-ICAM-1 Ab (lane 2) or its isotype control IgG1 (lane 4), followed by cross-linking with F(ab’)2 as described in Materials and Methods. Controls include nonstimulated cells immunoprecipitated with the isotype control IgG2a for the Syk4D10 Ab (lane 1) or nonstimulated cells immunoprecipitated with anti-Syk4D10 Ab (lane 2). Densitometry normalized to Syk revealed a 1.46 ± 0.15-fold increase when compared with control (n = 3, p < 0.05). Representative of three experiments. C, Calu-3 cells were grown on Transwell filters and fixed as in Fig. 2. The cells were then doubly labeled with anti-Syk Ab (right panel) and anti-ezrin Ab (middle panel), followed by the appropriately Cy-3-labeled and FITC-labeled secondary Abs. The merged image (left panel) shows significant colocalization of the two proteins and is indicated in yellow. Images were obtained with the LSM5 confocal microscope. Representative of three separate experiments.

**FIGURE 4.** Syk undergoes tyrosine phosphorylation on Y525/526 in BEAS-2B cells following infection with HRV16 and UV-HRV16. Growth factor-deprived BEAS-2B cells were grown to subconfluence and then exposed to the indicated stimuli and harvested as described in Fig. 2. The Syk immunoprecipitate was then analyzed by Western blotting with the phospho-specific SykY525/526 Ab. HRV16 and UV-HRV16 induces tyrosine phosphorylation of Syk. Densitometry of the phospho-Syk band, normalized to total Syk, is expressed as mean ± SE fold change in comparison with time 0 (defined as 1). HRV16 (15 min) = 4.18 ± 1.73; HRV16 (30 min) = 2.55 ± 1.75; UV-HRV16 (15 min) = 1.85 ± 0.57; UV-HRV16 (30 min) = 3.29 ± 1.56; p > 0.05 for all conditions, n = 4.
HRV induces p38 phosphorylation in BEAS-2B cells in a Syk-dependent manner

Activation of p38 MAPK following HRV inoculation depends on the binding of HRV to ICAM-1 (6). Syk is known to regulate p38 activation in lymphocytes in response to diverse extracellular stimuli such as TNF-α (35), oxidative stress (36, 37), and BCR engagement (38). Therefore, we postulated that Syk could mediate the activation of p38 following ICAM-1 engagement in airway epithelial cells.
We exposed growth factor-deprived BEAS-2B cells to HRV16 and UV-HRV16 for 15 and 30 min. As shown in Fig. 5A, BEAS-2B cells that had not undergone transfection (non-transfected) responded to HRV16 and UV-HRV16 by increasing p38 phosphorylation. To evaluate the role of Syk in HRV-mediated p38 phosphorylation, we down-regulated Syk expression using siRNA (Syk SMARTpool siRNA; Upstate Cell Signaling) and subsequently exposed the cells to HRV16 and UV-HRV16. As shown in Fig. 5A (Syk siRNA), knockdown of Syk expression significantly diminished the phosphorylation of p38 following exposure to both HRV16 and UV-HRV16 at 15 and 30 min. To ensure that transfection of siRNA does not affect p38 phosphorylation in a nonspecific manner, we also transfected BEAS-2B cells with a control siRNA (control SMARTpool siRNA; Upstate Cell Signaling). As shown in Fig. 5A (control siRNA), transfection with nonspecific control siRNA had no effect on Syk expression or on p38 phosphorylation in response to HRV16 and UV-HRV16. We performed densitometry on the phospho-p38 bands, normalized to total p38, for each of experimental conditions for five separate experiments. Statistical analysis using ANOVA, followed by Tukey’s post hoc analysis, revealed that p38 phosphorylation in response to HRV16 and UV-HRV16 in the untransfected and control siRNA cells was not significantly different from one another and that the response to HRV16 was also not statistically different from the response to UV-HRV16. However, in the Syk siRNA-transfected cells, p38 phosphorylation was significantly decreased when compared with the untransfected BEAS-2B cells following exposure to HRV16 and UV-HRV16 (p < 0.05, n = 5).

We also assessed ICAM-1 and ezrin expression following transfection with control siRNA and Syk siRNA. Expression of these two proteins was unaffected in both conditions (Fig. 5A, bottom two panels), indicating that siRNA transfection did not down-regulate protein expression in a nonspecific manner.

In a second method to engage ICAM-1, we used ICAM-1-crosslinking Abs as described in Materials and Methods. As shown in Fig. 5B, cross-linking of ICAM-1 (ICAM-1 XL) induced phosphorylation of p38 in untransfected (non-trans) BEAS-2B cells. Densitometry of the phospho-p38 band in ICAM-1 cross-linked cells (ICAM-1 XL), normalized to total p38, revealed a 4.63 ± 1.97-fold increase (n = 4) when compared with control cells (control). Following knockdown of Syk expression (Syk siRNA; Fig. 5B), there was substantial abrogation of p38 phosphorylation in response to ICAM-1 cross-linking. Densitometry of the phospho-p38 band following ICAM-1 cross-linking in the siRNA-transfected cells, when normalized to total p38, revealed only a 1.89 ± 0.44-fold increase when compared with cells not subjected to stimulation (n = 4, p = 0.09 when compared with the nontransfected group). Syk knockdown, however, did not affect p38 phosphorylation in response to stimulation with TNF-α (Fig. 5B), indicating that Syk regulation of p38 is selective for ICAM-1 signaling.

To evaluate the specificity of Syk as a downstream regulator of ICAM-1 signaling to p38, we assessed p38 phosphorylation in BEAS-2B cells following cross-linking of IgG1, the isotype control for the anti-ICAM-1 Ab, and following exposure to HRV1A, a minor HRV serotype that does not bind to ICAM-1. As shown in Fig. 3C, there is no p38 phosphorylation under these two conditions. Taken together, these experiments reveal that HRV-induced p38 activation is modulated by Syk and indicate that this occurs as a result of ICAM-1 engagement.

Syk selectively regulates p38 MAP kinase activation following HRV inoculation

In leukocytes, Syk has been shown to mediate the activation of the other two MAPK, ERK and Jnk, in response to different stimuli (39, 40). To assess the role of Syk in the regulation of ERK and Jnk activation following HRV infection, we performed Western blot analysis of the whole cell lysates using phospho-specific ERK1/2 and phospho-specific Jnk Abs. As shown in Fig. 6 (bottom four panels), HRV16 induced the phosphorylation of ERK1/2 and Jnk. However, no differences were detected in the nontransfected, control siRNA, and Syk siRNA-transfected BEAS-2B cells, indicating that these activation pathways were regulated independently of Syk. Furthermore, time course studies revealed that HRV-induced ERK and Jnk phosphorylation was sustained for up to 17 h following HRV exposure. On the other hand, while p38 phosphorylation was still present at 60 min following HRV16 infection, it returned to baseline by 3 h (Fig. 6). Taken together, these observations indicate that HRV signaling to p38 is different from the pathway(s) that activate ERK and Jnk and that Syk selectively regulates the activation of the p38 following HRV16 infection.

Syk-mediated p38 activation following ICAM-1 engagement is dependent on MKK3/MKK6

Because our results indicate that Syk involvement in p38 activation in BEAS-2B cells is selective for HRV-ICAM-1 downstream signaling, it is important to identify intermediary molecules in this signaling pathway. In other cell populations such as pulmonary vascular endothelium, activation of p38 is dependent on its upstream kinases, MKK3 and MKK6 (41). Therefore, we sought to identify the role of MKK3 and MKK6 in regulating Syk-mediated p38 phosphorylation following ICAM-1 engagement in BEAS-2B cells by assessing the phosphorylation state of MKK3/MKK6 using Western blot analysis. As shown in the Fig. 7A, HRV16 and

FIGURE 6. Syk induces p38 phosphorylation early following HRV16 infection and is selective for the p38 MAPK. Non-transfected, control siRNA-transfected (control siRNA), and Syk siRNA-transfected (Syk siRNA) BEAS-2B cells were treated using the same protocol as in Fig. 5. One hour after infection with HRV16, the virus-containing medium was replaced with fresh medium, and the cells were harvested at the indicated time points. The cell lysates were harvested and subjected to Western blot analysis with the indicated Abs. Phosphorlation of p38 was evident at 60 min in the nontransfected and control siRNA-transfected cells but was abrogated in the Syk siRNA-transfected cells, indicating Syk dependence. Prolonged p38 activation was not observation. On the other hand, phosphorylation of ERK and Jnk was sustained to 17 h post-HRV infection and was similar in the three different groups of BEAS-2B cells, indicating that the activation of these two MAPK is independent of Syk.
UV-HRV16 induces phosphorylation of MKK3/6 in untransfected BEAS-2B cells (non-transfected) and in cells transfected with control siRNA (control siRNA). Following knockdown of Syk expression by Syk siRNA, phosphorylation of MKK3/6 in response to HRV16 and UV-HRV16 is diminished. These results indicate that HRV-induced MKK3/6 phosphorylation is Syk dependent and results from HRV engagement of ICAM-1 rather than viral replication.

To further evaluate the role of ICAM-1 engagement, rather than viral replication, in regulating MKK3/6 phosphorylation, we used cross-linking Abs to engage ICAM-1. As shown in Fig. 6B, ICAM-1 cross-linking (ICAM-1 XL) induced MKK3/6 phosphorylation in untransfected cells (non-trans). Knockdown of Syk expression with siRNA (Syk siRNA) abrogated phosphorylation of MKK3/6 in response to ICAM-1 cross-linking. Phosphorylation of MKK3/6 in response to TNF-α was not different in untransfected and Syk siRNA-transfected cells (Fig. 7B, middle two lanes), indicating that Syk does not play a role in the TNF-α signaling pathway. Taken together, these results indicate that Syk mediates p38 activation following ICAM-1 engagement in the classical MKK3/MKK6 phosphorylation pathway and that the role of Syk is selective for ICAM-1 downstream signaling.

**IL-8 expression following HRV infection is regulated by Syk**

Previous studies have shown that IL-8 expression is up-regulated early following HRV infection and is dependent on p38 activation (9). Because we observed p38 activation early following HRV infection and found it to be dependent on Syk, we next assessed the role of Syk in HRV-induced IL-8 expression. We used real-time PCR to assess IL-8 mRNA expression 90 min following HRV16 infection. As shown in Fig. 8, HRV16 induced a 141.8 ± 28.8-fold increase in IL-8 expression at 90 min in nontransfected BEAS-2B cells and a 152.5 ± 57.7-fold increase in the control siRNA-transfected group (mean ± SE, n = 4 for both groups). In contrast, the Syk siRNA-transfected cells exhibited only a 67.7 ± 26.5-fold increase (mean ± SE, n = 4) in mRNA expression at 90 min when compared with time 0. Therefore, Syk knockdown using Syk siRNA significantly (p < 0.05) decreased HRV16-induced IL-8 mRNA expression to 47% of that observed in the nontransfected cells and to 44% of the control siRNA-transfected cells, indicating a role for Syk in regulating IL-8 gene expression following HRV infection.

**Discussion**

In this report, we identify expression of the tyrosine kinase Syk and the SykB splice variant in primary hSAEC and in the BEAS-2B and Calu-3 human pulmonary epithelial cell lines and provide evidence that Syk is important for p38 activation following engagement of ICAM-1. More importantly, in studies with HRV16, a physiologically relevant ligand for airway epithelial ICAM-1, we identified Syk to be important for p38 activation. Previous studies in different cell populations that include airway epithelial cells (9), airway smooth muscle cells (42), alveolar macrophages, and human monocytes (6) have shown that HRV-induced p38 activation is dependent on HRV-ICAM-1 binding and that p38 activation is required for induction of inflammatory cytokine expression. Our observations confirm and enhance these studies, identifying a role for Syk in mediating ICAM-1 activation of p38 and IL-8 gene expression, and thus strongly suggest a role...
for the kinase in modulating the airway epithelial inflammatory response following HRV infection.

Syk was described originally in hemopoietic cells where it plays a central role in regulation of the innate immune response (reviewed in Ref. 28). It is crucial for humoral immunity by regulating lymphocyte maturation and proliferation. Via its role in regulating Fcγ and Fcε receptor signal transduction, Syk also plays an important role in the phagocytic and allergic immune responses. Recently, a potential role for Syk in the inflammatory response has been identified in airway epithelium. In studies with primary human bronchial epithelial cells and the HS-24 human bronchial squamous carcinoma cell line, Syk is tyrosine phosphorylated following engagement of the β1 integrin, an adhesion molecule that mediates leukocyte adhesion and transepithelial migration (10). Moreover, down-regulation of Syk expression using siRNA significantly abrogated the induction of IL-6 and ICAM-1 expression following exposure to TNF-α in HS-24 cells (10). In this report, Ulanova et al. (10) reported no Syk expression in Calu-3 cells when assessed by Western blot analysis. This observation is in sharp contrast to ours, which revealed robust Syk expression at both the mRNA and protein levels. While there are technical differences in our detection methodology, it is possible that variations in endogenous protein expression and function occur due to differences between different strains or passages of Calu-3 cells. For example, it has been reported that Calu-3 cells of higher passage numbers exhibit significantly lower chloride secretion in response to a pharmacological stimuli when compared with those of lower passage numbers (43). This phenomenon was attributed to lower endogenous cAMP concentrations in the higher passages cells, indicating alterations in cell physiology and function due to repeated passages in culture. Despite the differences in the observed Syk expression in the Calu-3 cells, both our observations and those of Ulanova et al. (10) indicate that Syk is an important molecule in the epithelial response during inflammation.

Translocation of Syk from the cytosol to the plasma membrane where it binds directly to its upstream signaling molecule plays a critical role in Syk-mediated signaling in leukocytes. Indeed, Syk has been shown to be recruited to plasma membrane microdomains following engagement of FcεR1 (44–46) and the BCR (47). Similarly, β1 and β2 integrin activation of Syk in airway epithelium (10) and in neutrophils (48) also occurs in conjunction with recruitment to Syk to specific membrane regions. We have not only identified Syk expression at the mRNA and protein levels but also made observations of Syk recruitment to the plasma membrane following ICAM-1 engagement. These observations strongly suggested a functional role for Syk in mediating ICAM-1 signaling in airway epithelial cells. Our subsequent studies using a physiological model with HRV16 and an in vitro model system with cross-linking Abs to engage ICAM-1 revealed Syk to be an intermediary molecule involved in the regulation of p38 MAPK activation.

In airway epithelial cells, ICAM-1 is the receptor for the majority of HRV serotypes (2). While HRV infections are self-limited, they can lead to more serious complications in susceptible populations, particularly in children and patients with asthma. In children, HRV infection is one of the most common causes of wheezing that requires hospitalization (49, 50). In patients with documented asthma, HRV infections are associated with >80% and 45% of asthma exacerbations in children aged 9–11 years and adults, respectively (51, 52). The associated airflow obstruction and airway hyperresponsiveness (53–59) appear to be related to the ability of HRV to up-regulate airway epithelial expression of proinflammatory cytokines and chemokines that include IL-1α, IL-1β, IL-6, IL-8, eosinophil cationic protein, β defensin 2, and IFN-γ-inducible protein 10 (CXCL10) (56).

The intracellular signaling pathways that are activated following engagement of ICAM-1 by HRV are incompletely characterized. Studies with BEAS-2B cells (9), human alveolar macrophages, peripheral blood monocytes, and the human promonocytic THP-1 cell line (6) have identified p38 to be a downstream signal that is important for up-regulating cytokine production in vitro. Griego et al. (9) showed that p38 activation in BEAS-2B was both time and dose dependent, occurring within 5–15 min and peaking at 30 min in following HRV exposure. Treatment of BEAS-2B cells with p38-specific inhibitors, SB203580 and SB239063, resulted in substantial reduction of cytokine production following HRV exposure (9). Pharmacologic inhibition of p38 did not affect viral replication or cell viability, indicating that HRV induction of cytokine production was a result of virally induced p38 signal transduction rather than viral infection (9). In conjunction with observations in alveolar macrophages and peripheral blood monocytes which showed that inhibiting the interaction of HRV with its receptor by use of ICAM-1-blocking Abs abrogated p38 activation and cytokine expression (6), these observations indicate that HRV induces its proinflammatory effects by initiating ICAM-1 downstream signal transduction.

In our studies with BEAS-2B cells, HRV16 induced phosphorylation of p38 within 15 min of exposure. p38 activation was abrogated when Syk expression was down-regulated by Syk siRNA.
but not by control siRNA, indicating a role for Syk in the HRV-induced p38 phosphorylation pathway. Similar observations were made in response to infection with the replication-deficient UV-HRV16, indicating that these cellular response occur as a result of HRV engagement of its receptor, ICAM-1, rather than via replication-dependent mechanisms. Furthermore, our time course studies revealed p38 phosphorylation within 60 min of HRV infection with a return to baseline by 3 h, a period of time when viral replication has not yet occurred (5, 9, 60, 61). We also demonstrated that p38 activation does not occur following infection with HRV-1A, a minor group of HRV that does not recognize ICAM-1 but that replicates in epithelial cells. Lastly, in studies with cross-linking Abs to ICAM-1, an in vitro model of ICAM-1 engagement, we demonstrated activation of the MKK3/6-p38 signaling pathway occurs in a Syk-dependent manner, providing independent evidence that Syk-mediated p38 activation occurs as a result of ICAM-1 engagement rather than viral replication.

While Syk is known to mediate the activation of the other two MAPK, ERK1/2 and Jnk, in response to various stimuli (15, 35, 40, 62), our studies revealed that following engagement of ICAM-1, Syk selectively regulates p38 activation in BEAS-2B cells. The role of Syk in mediating different MAPK activation pathways may be individualized for both the cell type and the stimulus. For example, while TNF-α-induced p38 phosphorylation was found to be independent of Syk in BEAS-2B cells (our data, Fig. 5B) and human synoviocytes (62), this is in contrast to the observations in Jurkat cells, a human T cell line (35). Among leukocyte subpopulations, differences in Syk-mediated signaling are also apparent with LPS-induced Jnk activation being Syk dependent in neutrophils (40) but Syk independent in macrophages (15).

Of the multiple inflammatory mediators induced by HRV, IL-8 has been shown to be up-regulated early and dependent on p38 activation (9). Our observations that Syk knockdown by siRNA diminished IL-8 mRNA expression to 47 and 44% of nontransfected and control siRNA-transfected cells, respectively, provide novel evidence that Syk plays a physiologically relevant role in regulating airway epithelial cell activation following HRV infection. Residual Syk expression is evident in the Syk-siRNA-transfected cells and could account for the persistent albeit diminished IL-8 expression in these BEAS-2B cells.

Unlike immunoreceptors (63, 64) and β integrins (31, 65), which bind Syk directly, ICAM-1 does not possess putative binding sites for Syk. Therefore, we sought potential molecules that could couple ICAM-1 to Syk. A third protein was identified in the immunoprecipitates, which was revealed by immunoblot analysis to be ezrin, a member of the ezrin-radixin-moesin family of cytoskeletal proteins. Ezrin is highly expressed in epithelial cells where it plays an important role in maintaining polarization and differentiation (66, 67). Ezrin possesses an ITAM-like motif at its amino terminus, which has been shown to bind directly to Syk in vitro (23, 24). We identified association of the Syk and ezrin in airway epithelial cells using two independent methods; first, by confocal microscopy, and second, by protein biochemistry with immunoprecipitation studies. Although both techniques have some limitations, with confocal analysis performed in fixed cells and protein biochemistry in cell lysates, the observations derived from these two methods complement one another. The confocal analyses reveal physical proximity of the two proteins in their intracellular localization, and the biochemical data reveal physical association of the proteins. Moreover, we also identified ICAM-1, which is known to bind directly to ezrin (22), in the Syk immunoprecipitates. The coprecipitation studies revealed that both ezrin and ICAM-1 are enhanced in the Syk immunoprecipitates following HRV exposure and ICAM-1 cross-linking. Considered together, these observations indicate that Syk, ezrin, and ICAM-1 form a complex in airway epithelial cells and that ezrin is the probable adaptor molecule that couples ICAM-1 to Syk following HRV infection.

Syk and ezrin likely interact with one another via binding of Syk to the ITAM of ezrin. A functional role for ezrin-radixin-moesin proteins in mediating Syk signaling has been shown in studies with U937 and HL-60 myeloid cells where moesin was identified as the adaptor molecule that recruits and activates Syk following engagement of the adhesion molecule PSGL-1 (24). In these studies, mutations of both Y191 and Y205 to phenylalanine in the ITAM of moesin abrogated Syk recruitment, activation and subsequent downstream signaling to the SRE (serum response element) following P-selectin glycoprotein ligand 1 engagement (24). We are currently undertaking studies to evaluate the role of the ezrin-ITAM in mediating Syk recruitment and downstream signaling using specific ezrin and Syk mutants.

In conclusion, our findings identify several novel functions of Syk in regulating airway epithelial cells activation. Following engagement of ICAM-1, Syk is translocated to the plasma membrane where it associates with ICAM-1 and ezrin and undergoes tyrosine phosphorylation. Syk then regulates p38 activation and IL-8 expression. Taken together, these observations strongly implicate a role for Syk in regulating the airway epithelial inflammatory response to HRV infections. The accessibility of the airway epithelial to aerosolized agents offers an opportunity to target Syk when developing novel therapies for management of complications of HRV infections such as asthma exacerbations.

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Disclosures
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