Selective Transcriptional Down-Regulation of Human Rhinovirus-Induced Production of CXCL10 from Airway Epithelial Cells via the MEK1 Pathway[1]

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Human rhinovirus (HRV) infections can trigger exacerbations of lower airway diseases. Infection of airway epithelial cells induces production of a number of proinflammatory chemokines that may exacerbate airway inflammation, including CXCL10, a chemoattractant for type 1 lymphocytes and NK cells. Primary human bronchial epithelial cells and the BEAS-2B human bronchial epithelial cell line were used to examine the role of MAPK pathways in HRV-16-induced production of CXCL10. Surprisingly, PD98059 and U0126, two inhibitors of the MEK1/2-ERK MAPK pathway, significantly enhanced HRV-16-induced CXCL10 mRNA and protein. This enhancement was not seen with IFN-β-induced production of CXCL10. Studies using small interfering RNA revealed that knockdown of MEK1, but not MEK2, was associated with enhanced HRV-induced CXCL10 production. Promoter construct studies revealed that PD98059 and U0126 enhanced HRV-16-induced transcriptional activation of CXCL10. HRV-16-induced promoter activation was regulated by two NF-κB binding sites, κB1 and κB2, and by an IFN-stimulated response element. Inhibitors of the MEK1/2-ERK pathway did not alter HRV-16-induced activation of tandem repeat κB1 or κB2 constructs, nor did they alter HRV-16-induced nuclear translocation/binding of NF-κB to either κB1 or κB2 recognition sequences. Furthermore, PD98059 and U0126 did not alter phosphorylation or degradation of IκBα. In contrast, inhibitors of the MEK1/2-ERK pathway, and small interfering RNA knockdown of MEK1, enhanced nuclear translocation/binding of IFN regulatory factor (IRF)-1 to the IFN-stimulated response element recognition sequence in HRV-16 infected cells. We conclude that activation of MEK1 selectively down-regulates HRV-16-induced expression of CXCL10 via modulation of IRF-1 interactions with the gene promoter in human airway epithelial cells. *The Journal of Immunology,* **2009, 182: 4854–4864.**

Human rhinovirus (HRV)[3] infections are recognized as a major risk factor for exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (1–4). The airway epithelial cell is the dominant site of HRV infection in vivo (5, 6), and both upper and lower airway epithelial cells are infected at a similar frequency (7). HRV infections do not lead to overt changes in epithelial cell integrity or viability, but they can induce epithelial production of a number of chemokines and cytokines (8–11). Several of these molecules could contribute to the exaggerated airway inflammation, characterized by increased neutrophils and lymphocytes, that is associated with exacerbations of asthma and COPD (12–15).

CXCL10 is a chemoattractant for type 1 lymphocytes and NK cells, and it has been shown to contribute to airway hyperresponsiveness and inflammation in a murine model of asthma (16). We have previously reported that HRV-16 infection of human bronchial epithelial cells (HBE) or BEAS-2B cells in vitro induces production of CXCL10 via a mechanism that is dependent on viral replication. Moreover, experimental HRV-16 infection of normal volunteers led to increased levels of CXCL10 in airway secretions, and levels of CXCL10 correlated with symptom scores, lymphocyte numbers, and viral titers (17). It seems likely, therefore, that CXCL10 may contribute to the pathogenesis of HRV-induced exacerbations of asthma and COPD. Indeed, CXCL10 recently has been proposed as a novel biomarker for HRV-induced asthma exacerbations (18). Delineating selective signaling pathways involved in the regulation of HRV-induced production of CXCL10 may therefore have therapeutic implications.

Surprisingly little is known about the signaling pathways involved in HRV-induced production of chemokines from infected epithelial cells. We have previously shown that infection of epithelial cells with HRV-16 leads to activation of all three major MAPK pathways (19, 20). Both the p38 and MEK1/2-ERK MAPK pathways have been reported to be involved in the adenoviral induction of CXCL10 expression in murine kidney epithelial cells (21), and the MEK1/2-ERK pathway mediates CXCL10 expression in murine macrophages in response to rabies virus infection (22). In light of these findings, we evaluated the role of the p38, MEK1/2-ERK, and JNK MAPK pathways in HRV-16-induced CXCL10 expression in human airway epithelial cells. Surprisingly, we found that inhibition of the MEK1/2-ERK pathway results in a selective enhancement of HRV-16-induced CXCL10 mRNA and protein production. This effect was mediated, at least in part, at the level of transcription. Inhibition of the MEK1/2-ERK

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Received for publication July 22, 2008. Accepted for publication February 6, 2009.

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[1] This work was supported by funding from Canadian Institutes of Health Research Grant 43923. David Proud is the recipient of a Canada Research Chair in Inflammatory Airway Diseases. Raza Zaheer and Rommy Koetzler are recipients of studentship awards from the Lung Association of Alberta and Northwest Territories.

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[3] Abbreviations used in this paper: HRV, human rhinovirus; BEBM, bronchial epithelial cell basal medium; BEGM, bronchial epithelial cell growth medium; COPD, chronic obstructive pulmonary disease; HBE, human bronchial epithelial cell; IRF, IFN regulatory factor; ISGF, IFN-stimulated gene factor; ISRE, IFN-stimulated response element; MOI, multiplicity of infection; siRNA, small interfering RNA; $TCID_{50}$, 50% tissue culture-infective dose.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802401

pathway did not enhance nuclear translocation and/or binding of the NF-κB isoforms p50 and p65 in response to HRV-16 infection. In contrast, HRV-16-induced nuclear translocation and/or binding of IFN regulatory factor (IRF)-1 was enhanced. Studies using small interfering RNAs (siRNAs) indicated that knockdown of MEK1, but not selective knockdown of MEK2, was associated with enhanced HRV-16-induced production of CXCL10 and increased IRF-1 binding. These novel results suggest that MEK1 selectively down-regulates the HRV-16-induced expression of CXCL10, at least in part, via modulation of IRF-1 interactions with the CXCL10 gene promoter.

Materials and Methods

Reagents and Abs

The following reagents were purchased from the indicated suppliers: Ham's F12 medium, Eagle's minimal essential medium, HBSS, penicillin-streptomycin-amphotericin B, and L-glutamine (MediCorp Health System); TRIzol reagent, sodium pyruvate, nonessential amino acids, gentamicin, and FBS (Invitrogen); bronchial epithelial cell basal medium (BEBM) and additives to create bronchial epithelial cell growth medium (BEGM) (Lonza); PD98059, U0126, SB203580, and SP600125 (Calbiochem-Novabiochem); TaqMan Master Mix, 20× GAPDH, RNase inhibitor, and reverse transcriptase (Applied Biosystems); recombinant human IFN-β (R&D Systems); the firefly luciferase reporter plasmid pGL3 basic, the *Renilla* luciferase plasmid pRL-null, passive lysis buffer, and the dual-luciferase reporter assay system (Promega); FuGene6 and protease inhibitor tablets (Roche Diagnostics); poly(dI:dC), HRP-conjugated anti-rabbit Ig Ab, and ECL substrate reaction (GE Healthcare Bio-Sciences); anti-mouse Ig Ab (Dako); ERK1/2, ERK5, p38, and IκBα phospho-specific and total Abs (Cell Signaling Technology). Supershift Abs for p50 (sc-114), p65 (sc-109), p52 (sc-848), c-Rel (sc-70), IRF-1 (sc-497), IRF-2 (sc-498), IRF-3 (sc-9082), IRF-7 (sc-9083), and IFN-stimulated gene factor 3 (ISGF-3)γ/p48 (sc-496) were from Santa Cruz Biotechnology. All other chemicals were purchased from Sigma-Aldrich.

Viruses and cell lines

The BEAS-2B cell line was a gift from Dr. Curtis Harris (National Cancer Institute, Bethesda, MD). We have previously shown that this cell line shows similar responses to primary human bronchial epithelial cells in terms of production of CXCL10 upon HRV infection. The cell line was used in the present studies for experiments requiring large numbers of cells and for transfection experiments that could not be performed in primary cells. HRV type 16 (HRV-16) and WI-38 cells were purchased from the American Type Culture Collection. HRV-16 viral stocks used for experiments were generated by propagation in WI-38 cells and were purified by centrifugation through sucrose to remove ribosomes and soluble factors of WI-38 origin as previously described (9). Viral titers were determined using WI-38 cells grown in 96-well plates, as previously described (9).

Epithelial cell culture

Normal human lungs not used for transplantation were obtained from a tissue retrieval service (International Institute for the Advancement of Medicine). Primary HBE were obtained by protease digestion of dissected airways as described (23). Both HBE and BEAS-2B cells were grown on 6-well culture plates in serum-free epithelial growth medium (BEGM). Before stimulation, cells were cultured overnight in BEGM from which hydrocortisone had been withdrawn, and this hydrocortisone-free medium was used for all protein and mRNA experiments.

Viral infection and IFN-β stimulation of epithelial cells

BEAS-2B cells were infected with $10^{4.5}$ 50% tissue culture-infective dose ($TCID_{50}$) U/ml (multiplicity of infection (MOI) of ~0.1) HRV-16, while HBE were infected with $10^{5.5}$ $TCID_{50}$ U/ml (MOI of ~1.0) HRV-16. The higher dose used in HBE is needed to ensure robust responses, as it has been reported that, even at high virus doses, no more than 10% of primary cells are infected (7). To determine whether MAPK inhibitors have any effect on mRNA and protein expression, subconfluent cell monolayers of BEAS-2B or primary airway epithelial cells were washed with HBSS and preincubated with PD98059, U0126, SB203580, SP600126, or DMSO 0.1% (v/v) for 1 h at 37°C. Cells were then exposed to HRV-16 and incubated at 34°C. Alternatively, cells were preincubated with PD98059, U0126, or DMSO 0.1% (v/v) for 1 h at 37°C followed by exposure to IFN-β (3 ng/ml) for 24 h at 37°C. At 24 h, supernatants were collected and total cellular RNA was harvested using TRIzol.

Assessment of chemokine protein and mRNA

Protein levels for CXCL10 and CCL5 were assayed by ELISA using matched Ab pairs according to the manufacturer's protocol (R&D Systems). CXCL10 and CCL5 mRNA expression analysis was performed by real-time RT-PCR using the Applied Biosystems model 7900 sequence detector. To ensure there was no contribution of genomic DNA to amplification, samples were treated with DNase I (Ambion) before use. Input RNA (400 ng) was reverse transcribed into cDNA, followed by PCR amplification in the presence of specific primers and probe (Applied Biosystems). Primer and probe sequences for CXCL10 have been described previously (17). To permit absolute quantification, a first-strand cDNA standard was synthesized (University of Calgary DNA Services, Calgary, Alberta, Canada). Expression of the housekeeping gene GAPDH was also assayed using reagents obtained from Applied Biosystems. Data were expressed as attograms calculated from the standard curve after correction for variations in housekeeping gene levels. Expression of CCL5 mRNA was assessed using a primer and probe gene expression kit available from Applied Biosystems. We confirmed that efficiency of amplification was comparable to that of GAPGH (not shown), permitting us to express data as fold induction as previously described (19).

Western blotting for MAPK activation

Cell extraction and SDS-PAGE were performed as previously described (19). Membranes were blocked with 5% skim milk for 1 h and probed overnight at 4°C with specific Abs to phospho-ERK1/2 (1/750 dilution), phospho-p38 (1/750), or phospho-ERK5 (1/200). Membranes were washed and incubated for 1 h with 1/7500 dilution (ERK1/2 or p38) or 1/2000 (ERK5) of HRP-conjugated anti-rabbit Ig Ab, and proteins were visualized with ECL substrate reagent. Membranes were stripped and reprobed with total ERK1/2, p38, or ERK5 Abs to confirm equal loading.

CXCL10 promoter constructs

The 972-bp CXCL10 promoter-luciferase construct, corresponding to the sequence from −875 to +97 (relative to the transcriptional start site) of the 5′ flanking region of the human CXCL10 gene, and the 376-bp truncated construct (sequence from −279 to +97) have been described (17). Point mutations in the putative AP-1, more proximal NF-κB1 (κB1), more distal NF-κB2 (κB2), and IFN-stimulated response element (ISRE) promoter sites were generated in the truncated construct using standard site-directed mutagenesis techniques. Boldface, lowercase letters in the following sequences denote mutation sites in forward primers. For AP-1, 5′-CCAGC AGGTTTTGCTAAG**at**AACTGTAATGC-3′; for κB1, 5′-GCAACATG**t** GACTTC**a**CCAGG-3′; for κB2, 5′-GCAGAG**t**GAAATT**a**CGTAACT TGG-3′; and for ISRE, 5′-GTTTTGGA**c**AGTGA**c**ACCTAATTC-3′. Constructs with five copies of the κB1 recognition sequence (TGGGACT TCCCCA), κB2 sequence (GGGAAATTCCGT), or the ISRE sequence (GGAAAGTGAAACCTA) were synthesized. Successful generation of all constructs was confirmed by sequencing (University of Calgary DNA Services).

Transient transfection and luciferase assay

Subconfluent (40–50%) monolayers of BEAS-2B cells were transiently transfected with 1 μg of each CXCL10 promoter construct as well as 0.1 μg of pRL-null vector for 5 h at 37°C using FuGene6 in basal medium as per the manufacturer's protocol. After 5 h, medium was aspirated and cells were allowed to recover overnight in BEGM (without hydrocortisone) containing 5% FBS. The next day, cells were preincubated for 1 h at 37°C with DMSO 0.1% (v/v) or the MEK1/2-ERK pathway inhibitors PD98059 (10 μM) or U0126 (3 μM), followed by the addition of HRV-16 (1×10^5 $TCID_{50}$ U/ml) for 24 h at 34°C. Cells were then lysed in passive lysis buffer followed by scraping, sonication, and centrifugation. Firefly and *Renilla* luciferase activity were measured with the dual luciferase reporter assay system. Firefly luciferase activity was normalized to *Renilla* luciferase activity and data were expressed as fold increase of stimulated cells over control. Within each experiment, results were averaged from triplicate wells.

Western blotting for IκBα activation

Subconfluent monolayers of BEAS-2B cells were preincubated in BEBM overnight and then preincubated with PD98059 (10 μM) or U0126 (3 μM) for 1 h followed by HRV-16 in BEBM for the designated time points. At each time point, supernatants were removed and cells were washed with HBSS and lysed in ice-cold lysis buffer (1% Triton X-100 in 1× MES

buffered saline, 5 mM EDTA (pH 7.4), antiprotease tablets, 1 mM PMSF, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, and 50 mM sodium fluoride). Cells were scraped, incubated on ice for 10 min, sonicated, and centrifuged. Triton-soluble samples were separated using 10% SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% skim milk for 1 h and probed with either 1/1000 dilution of phospho-specific or total anti-IκBα overnight at 4°C with gentle shaking. Membranes were washed and then incubated for 1 h with 1/2000 dilution of HRP-conjugated anti-rabbit Ig Ab or anti-mouse HRP-conjugated Ab. Equal loading was determined by stripping each membrane and reprobing with Ab to GAPDH.

EMSA

Nuclear extracts were performed using a modification of a previously described method (24). In brief, cells were scraped and centrifuged at 5000 × *g* for 5 min. Supernatant was removed, and the pellet was resuspended in 50 μl Gough buffer (0.01 M of Tris-HCl, 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% Nonidet P-40, 0.5 mM PMSF, and 0.01 M DTT), vortexed for 15 s, left on ice for 10 min, and centrifuged at 12,000 × *g* for 2 min. Supernatant was then saved (cytoplasmic fraction), while pellet was resuspended in 15 μl of buffer C (0.02 M HEPES (pH 7.9), 25% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 0.01 M DTT), subjected to agitation every 15 min for 2 h, and centrifuged at 12,000 × *g* for 10 min. Samples were kept on ice during the whole procedure. The supernatant (nuclear fraction) was transferred to a fresh tube containing 35 μl of buffer D (0.02 M HEPES (pH 7.9), 20% glycerol, 0.05 M KCl, 0.2 mM EDTA (pH 8.0), 0.5 mM PMSF, and 0.01 M DTT) and kept at −80°C.

Oligonucleotides were end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (PerkinElmer). For binding reactions, 5 μg of nuclear extracts were incubated with 4 μl of binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 1 μg of poly(dI:dC), 250 mM NaCl, 50 mM Tris-HCl, and 0.01 M DTT), and buffer D was used to bring the mixture to 14 μl. The samples were then incubated for 20 min, labeled probe was added, and samples were incubated for 1 h. For supershift experiments, samples were incubated for 2 h with appropriate Abs before addition of the probe. Samples were fractionated on 6% acrylamide gels before vacuum drying and autoradiography. The forward oligonucleotide sequences used were as follows: for NF-κB1, 5′-TGCAACATGGGACT TCCCCAGGAAC-3′; for NF-κB2, 5′-GGAGCAGAGGGAAATTCC GTAACTT-3′; and for ISRE, 5′-TGTTTTGGAAAGTGAAACCTAA TTC-3′.

siRNA knockdown of MEK1 and MEK2

Subconfluent (40–50%) monolayers of BEAS-2B cells were transfected with 30 nM MEK1, MEK2, or control siRNA (Invitrogen) for 6 h at 37°C using Lipofectamine RNAiMAX transfection reagent (Invitrogen) in BEGM without antibiotics. After 6 h, medium was aspirated and cells were allowed to recover for 72 h, with media changed to BEGM without hydrocortisone after 48 h. Cells were infected with HRV-16 for 24 h at 34°C. Supernatants were assayed for IP-10 protein. Whole cell lysates were separated using SDS-PAGE and immunoblotted for MEK1 or MEK2 using 1/1000 dilution of selective Abs overnight at 4°C (Cell Signaling Technology), and proteins were visualized with ECL substrate reagent. Membranes were stripped and reprobed with GAPDH to confirm equal loading. The specific siRNA sequences used were as follows: MEK1 duplex A, 5′-GCCUUGAGGCCUUUCUUACCCAGAA-3′; MEK1 duplex B, 5′-CCCGCAAUCCGGAACCAGAUCAUAA-3′; MEK2 duplex A, 5′-CCAUCUUUGAACUCCUGGACUAUAU-3′; MEK2 duplex B, 5′-GAACUCAAAGACGAUGACUUCGAAA-3′.

Statistical analysis

For normally distributed data, between group comparisons were made by one-way ANOVA, with appropriate post hoc analysis using Fisher's least significant difference test. For nonparametric data, Kruskal-Wallis ANOVA was used, followed by Wilcoxon matched-pairs signed-rank test. Alternatively, Mann-Whitney *U* test was performed. For all statistical tests, a p value of ≤ 0.05 was assumed to be significant.

Results

Inhibition of the MEK1/2-ERK pathway enhances HRV-16-induced CXCL10 expression

We have previously shown that infection of epithelial cells with HRV-16 activates the p38, MEK1/2-ERK, and JNK MAPK pathways (19, 20). To define the role of each of these pathways in viral induction of CXCL10, we initially used selective pharmacologic inhibitors. In BEAS-2B cells, inhibition of the p38 pathway with SB203580 (10 μM) significantly ($p < 0.05$) inhibited both HRV-16-induced CXCL10 mRNA and protein (Fig. 1, *A* and *B*). Similar data were also obtained in HBE (Fig. 1, *E* and *F*). Inhibition of JNK MAPK with SP600125 (10 μM) did not significantly affect HRV-16-induced CXCL10 mRNA or protein expression in BEAS-2B cells (Fig. 1, *A* and *B*). In HBE, inhibition of the JNK pathway resulted in significant inhibition of CXCL10 protein but had no significant effect on HRV-16-induced mRNA levels (Fig. 1, *E* and *F*).

Surprisingly, inhibition of the MEK1/2-ERK pathway with either PD98059 (1–10 μM) or U0126 (0.3–3 μM) significantly ($p < 0.001$ in all cases by appropriate ANOVA) enhanced both HRV-16-induced CXCL10 mRNA and protein expression from BEAS-2B cells in a concentration-dependent manner (Fig. 1, *C* and *D*). Using the optimal dose determined in BEAS-2B cells, similar data were obtained in HBE, with significant enhancement of HRV-16-induced production of CXCL10 mRNA ($p < 0.05$) and protein ($p < 0.05$) using PD98059 (10 μM). With U0126 (3 μM), CXCL10 mRNA expression also was significantly enhanced ($p < 0.05$), but enhancement of protein production did not achieve statistical significance (Fig. 1, *E* and *F*). This likely reflects the wide variability of the absolute magnitude of responses using cells from different donor lungs. Taken together, these data suggest that activation of the MEK1/2-ERK pathway down-regulates HRV-16-induced CXCL10 expression.

None of the MAPK pathway inhibitors, at the concentrations used, had any effect on cell viability, as assessed using lactate dehydrogenase release, nor did any of the drugs alter HRV-16 replication, as assessed by viral titers measured in cell supernatants (data not shown).

Enhancement of HRV-16-induced CXCL10 expression occurs through the MEK1 pathway

Our earlier studies on HRV-16-induced activation of the p38, JNK, and MEK1/2-ERK MAPK pathways in human airway epithelial cells focused on short-term activation (19, 20). We have extended these studies to show that, consistent with an earlier report (25), HRV-16 induces chronic activation of ERK1/2 in BEAS-2B cells, with phosphorylation detected up to 24 h postinfection (data not shown).

PD98059 and U0126 have been reported to inhibit activation of both the ERK1/2 and ERK5 pathways in other cell types via effects on the upstream activating kinases MEK1/2 and MEK5, respectively (26, 27). To evaluate the role of these two pathways in the enhancement of HRV-induced CXCL10 production, we first confirmed by Western blotting that ERK5 was activated in HRV-16-infected cells. Activation was observed within 15 min after viral exposure, peaked at 1 h, and returned to baseline within 8 h (data not shown). Next, we compared the susceptibility of these two pathways to inhibition by varying doses of PD98059 and U0126. As anticipated, U0126, which inhibits both MEK1 and MEK2 (28), inhibited phosphorylation of both ERK1 and ERK 2 in a concentration-dependent manner (Fig. 2*A*). In contrast, PD98059, which is more selective for MEK1 (28), primarily caused a concentration-dependent inhibition of phosphorylation of the upper, ERK1, band (Fig. 2*A*). At the concentrations shown to be optimal for enhancement of HRV-16-induced production of CXCL10 in these cells, U0126 (3 μM) almost completely inhibited phosphorylation of ERK1 and ERK2, while PD98059 (10 μM) markedly inhibited ERK1 phosphorylation. In contrast, at the same concentrations, PD98059 caused modest enhancement of ERK5 phosphorylation, while U0126 (3 μM) did not exert any marked effects (Fig. 2*B*). Given that both drugs enhance CXCL10 production in response to HRV infection to a similar degree, but have differing effects on ERK5 phosphorylation, it seems unlikely that the MEK5-ERK5 pathway mediates this enhancement. Because there is also ample literature precedent for crosstalk between ERK and p38 MAPK pathways (29), and

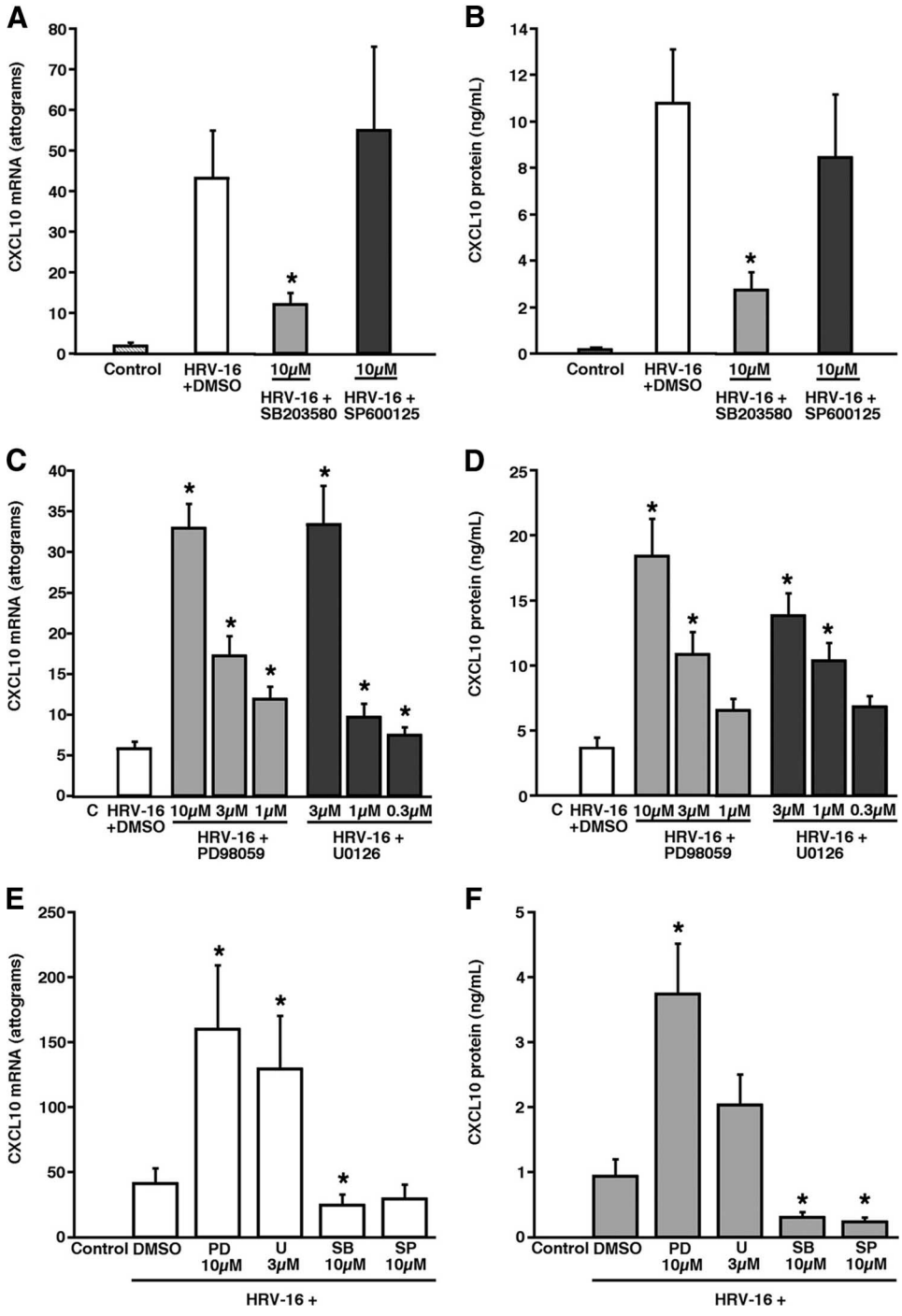

FIGURE 1. Effects of MAPK pathway inhibitors on HRV-16-induced CXCL10 mRNA and protein in human airway epithelial cells. Effects of inhibitors of the p38 (SB203580) and JNK (SP600125) MAPK pathways on HRV-16-induced expression of CXCL10 mRNA (*A*) and protein (*B*) in BEAS-2B cells ($n = 7$). Concentration-dependent enhancement of HRV-16-induced expression of CXCL10 mRNA (*C*) and protein (*D*) by inhibitors (PD98059 and U0126) of the MEK1/2-ERK pathway in BEAS-2B cells ($n = 6$). Effects of MAPK pathway inhibitors on HRV-16-induced expression of CXCL10 mRNA (*E*) and protein (*F*) in HBE ($n = 9$). Asterisks indicate significant differences compared with HRV-16 plus DMSO.

p38 inhibition reduces HRV-16-induced CXCL10 production, we also evaluated if PD98059 and U0126 may modulate CXCL10 production via indirect effects on the p38 pathway. Although modest enhancement of p38 phosphorylation was observed using U0126, no change in p38 phosphorylation was seen with PD98059 (Fig. 2*C*). Again, given these variable effects of the two drugs on p38 phosphorylation, it seems unlikely that this pathway can account for the enhancement of CXCL10 expression that is commonly induced by both drugs. Taken together, the data are consistent with a dominant role of MEK1/2 inhibition in the enhancement of HRV-16-induced production of CXCL10 by these drugs.

Although PD98059 and U0126 have been shown to be among the more selective kinase inhibitors available (30), concerns about specificity always remain. To further evaluate the role of MEK1 and MEK2 in HRV-induced production of CXCL10 from epithelial cells, therefore, we also utilized siRNA knockdown experiments. Two siRNA duplex sequences designed to target MEK1 or MEK2 were used alone or in combination. Both MEK1 siRNAs, used individually or in combination, successfully knocked down expression of MEK1, while the lipid tranfection reagent alone or a control siRNA had no effect. Neither of the siRNAs directed against MEK2 had any effect on MEK1 expression (Fig. 3*A*). Knock down of MEK1 using siRNAs either individually or in combination induced significant enhancement of HRV-16-induced CXCL10 production, reproducing effects seen with the MEK inhibitors (Fig. 3*B*). Both MEK2 siRNAs, used individually or in combination, successfully knocked down expression of MEK2. Interestingly, both MEK1 siRNAs also affected MEK2 expression. MEK1 duplex A modestly

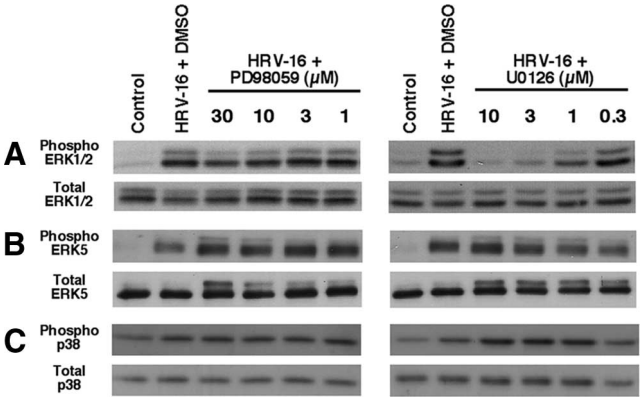

FIGURE 2. The actions of PD98059 and U0126 are mediated via effects on the ERK1/2 and not the ERK5 pathway. HRV-16 induction of phospho-ERK1 and ERK2 is inhibited in a concentration-dependent manner by U0126 (*A*, *right*), while PD98059 shows a predominant concentration-dependent inhibition of the induction of phospho-ERK1 (*A*, *left*). It appeared that PD98059, but not U0126, enhanced HRV-16-induced ERK5 phosphorylation, but the effects of PD98059 were not concentration-dependent (*B*). In contrast, U0126, but not PD98059, modestly enhanced HRV-16-induced phosphorylation of p38, although, again, the effects of U0126 did not appear to be concentration-dependent (*C*). Experiments were performed 1 h after exposure to medium (Control) or HRV-16, and data are representative of $n = 3$.

reduced MEK2 expression, while MEK1 duplex B caused marked knockdown of MEK2 (Fig. 3*C*). The siRNAs to MEK2, either alone or in combination, did not result in any enhancement of HRV-16-induced production of CXCL10. MEK2 duplex B did not significantly alter viral induction of CXCL10, while MEK2 duplex A, or the combination of the two siRNAs, caused significant inhibition of HRV-induced epithelial production of CXCL10 (Fig. 3*D*). Thus, MEK2 knockdown alone is not sufficient to induce enhancement of HRV-induced CXCL10 production. Knockdown of MEK1 appears to be essential to reproduce the enhancement seen with inhibitors, but given the partial knockdown of MEK2 by siRNAs to MEK1, we cannot rule out that some crosstalk between MEK1 and MEK2 contributes to these effects.

MEK1/2 pathway inhibition does not alter IFN-β-induced CXCL10 expression

To determine whether the MEK1/2 pathway inhibitor-mediated enhancement of HRV-16-induced CXCL10 expression was selective, we examined the effects of these inhibitors on IFN-β-induced production of CXCL10. The concentration of IFN-β used (3 ng/ml) was selected to give comparable production of CXCL10 to that seen with HRV-16 infection. Neither PD98059 (10 μM) nor U0126 (3 μM) significantly altered CXCL10 mRNA or protein expression in either BEAS-2B cells (Fig. 4, *A* and *B*) or HBE (Fig. 4, *C* and *D*). Consistent with this, IFN-β did not induce the phosphorylation of ERK1/2 (data not shown).

MEK1/2 pathway inhibitors enhance HRV-16-induced CXCL10 transcription

We have previously shown that HRV-16-induced CXCL10 production is transcriptionally regulated (17). To determine whether inhibition of the MEK1/2 pathway enhanced HRV-16-induced transcriptional activation, we initially examined the effects of PD98059 (10 μM) and U0126 (3 μM) on HRV-16-induced activation of a 972-bp CXCL10 promoter-luciferase construct and of a truncated 376-bp construct (Fig. 5*A*). As we previously reported (17), while HRV-16 induced responses with both promoter constructs, slightly, but significantly, greater activation was seen with

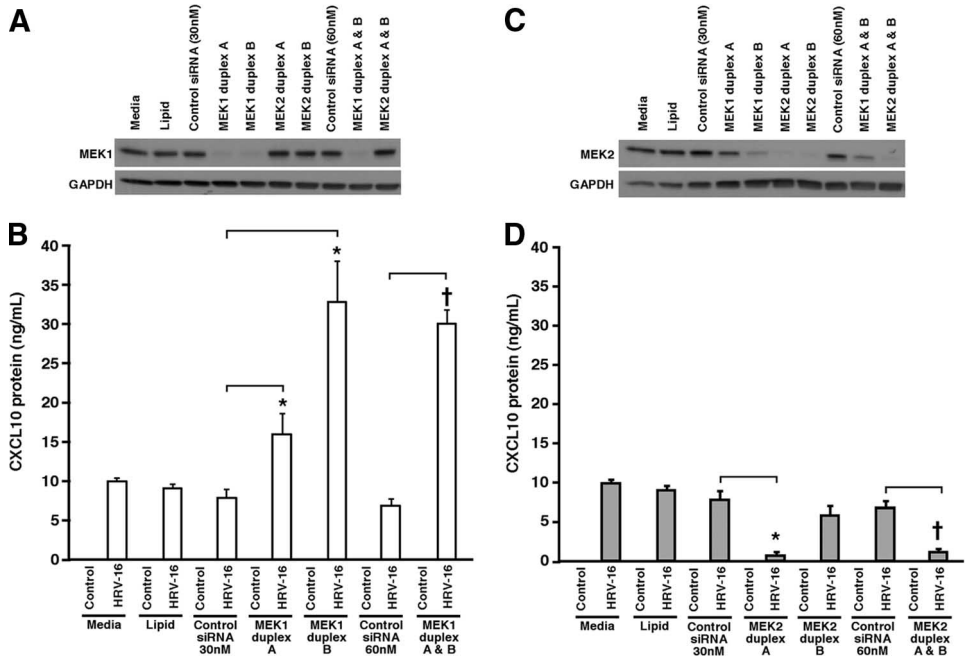

FIGURE 3. Effects of siRNA knockdown of MEK1 and MEK2 on HRV-16-induced epithelial production of CXCL10. *A*, Each of two siRNAs targeted to MEK1, as well as the combination of the two, led to marked knockdown of MEK1 expression, while neither control siRNA nor two siRNAs against MEK2, used either individually or in combination, affected MEK1 expression. *B*, Knockdown of MEK1 with either siRNA used individually or in combination significantly enhanced HRV-16-induced production of CXCL10 from BEAS-2B cells ($p < 0.05$ in each case). Data are means ± SEM from four experiments. *C*, Each of two siRNAs targeted to MEK2, as well as the combination of the two, led to marked knockdown of MEK2 expression, while control siRNA had no effect. Two siRNAs against MEK1, used either individually or in combination, also affected MEK2 expression. *D*, Knockdown of MEK2 with siRNA duplex A significantly inhibited HRV-16-induced production of CXCL10 from BEAS-2B cells, while siRNA duplex B had no effect. Neither siRNA nor the combination of the two caused enhancement of CXCL10 production. Data are means ± SEM from four experiments.

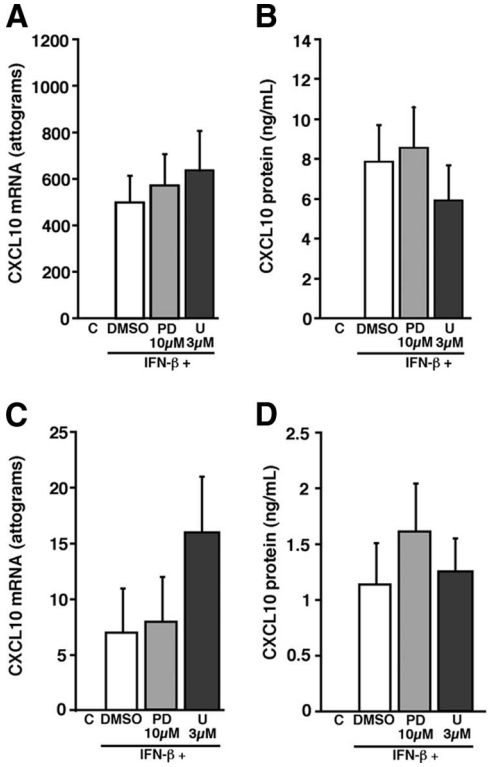

FIGURE 4. Inhibitors of the MEK1/2 pathway do not enhance CXCL10 expression induced by IFN-β. Neither PD98059 (PD) nor U0126 (U) significantly enhanced IFN-β-induced expression of CXCL10 mRNA (*A*) or protein (*B*) in BEAS-2B cells ($n = 6$). Additionally, neither drug enhanced IFN-β-induced expression of CXCL10 mRNA (*C*) or protein (*D*) in HBE ($n = 6$).

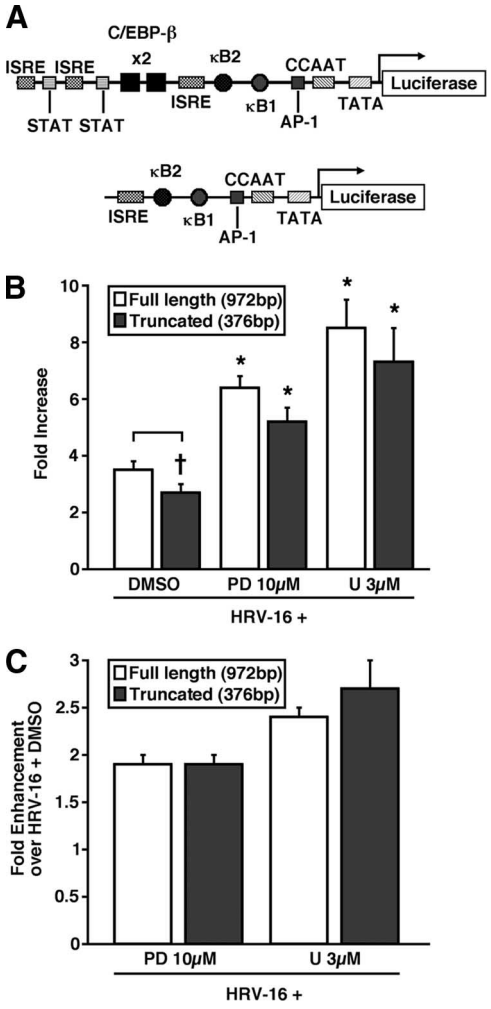

FIGURE 5. Inhibitors of the MEK1/2 pathway enhance HRV-16-induced CXCL10 transcription. *A*, Schematic diagrams showing putative transcription factor binding sites in the full-length (972 bp) and truncated (376 bp) human CXCL10 promoter-luciferase constructs. *B*, Both PD98059 (PD) and U0126 (U) significantly enhance HRV-16-induced activation of both the full-length and truncated promoter constructs ($n = 6$). Asterisks indicate statistical significance compared with HRV-16 plus DMSO (DMSO) alone. Dagger indicates significant difference between HRV-16-induced activation of the full-length vs truncated promoter. *C*, No difference in the degree of enhancement induced by PD98059 (PD) and U0126 (U) was seen when data were expressed as fold enhancement above HRV-16 plus DMSO for each of the two promoter constructs.

the 972-bp promoter (Fig. 5*B*). Interestingly, however, significant enhancement of HRV-16-induced activation of both the full-length and truncated promoters were seen in the presence of PD98059 and U0126 compared with virus in the presence of DMSO vehicle (Fig. 5*B*). When expressed as fold-enhancement over the virus alone response (Fig. 5*C*), an almost identical degree of enhancement was seen for both the full-length and truncated promoters. This indicates that all of the essential elements needed for enhancement of HRV-16-induced CXCL10 promoter activation upon MEK1/2 pathway inhibition are contained within the 376-bp promoter, so this was used as the template for additional studies of point mutations to identify potentially important transcription factor binding sites mediating the actions of the MEK1/2 pathway.

Mutation of the AP-1 site did not alter HRV-16-induced promoter activity or the enhancement of CXCL10 promoter activity seen in the presence of PD98059 or U0126 (Fig. 6). We have previously reported that the two proximal NF-κB recognition sequences, κB1 and κB2, are involved in HRV-16-induced drive of the CXCL10 promoter, with κB1 playing a more prominent role (17). This was confirmed in our present experiments. Mutation of the κB1 site led to almost complete abrogation of HRV-16-induced promoter activation. In the absence of significant promoter drive, neither PD98059 nor U0126 enhanced or restored promoter activity with this mutant (Fig. 6). In contrast, mutation of the κB2 site led to significantly reduced, but not absent, promoter activation in response to HRV-16, and significant enhancement of HRV-16-induced promoter activation was seen in the presence of either PD98059 or U0126. Interestingly, mutation of the ISRE site in the promoter also led to almost complete inhibition of HRV-16-induced promoter activation, and neither PD98059 nor U0126 had any significant effect on this lack of response (Fig. 6). Control studies were also performed with a pGL3-basic promoter containing only a TATA box. HRV-16 did not induce activation of this promoter, and neither PD98059 nor U0126 had any effect on the basal activity of this construct (data not shown). Taken together, these data suggest that the NF-κB and ISRE recognition sequences essential for HRV-16-induced promoter activation are also necessary for the MEK1/2-ERK-mediated modulation of HRV-16-induced promoter responses.

Evaluation of the role of NF-κB in MEK1/2-mediated modulation of HRV-16-induced CXCL10 expression

To delineate the role of NF-κB in the effects of MEK1/2 inhibition, we first generated tandem repeat constructs containing five copies each of the κB1 or κB2 recognition sequences from the CXCL10

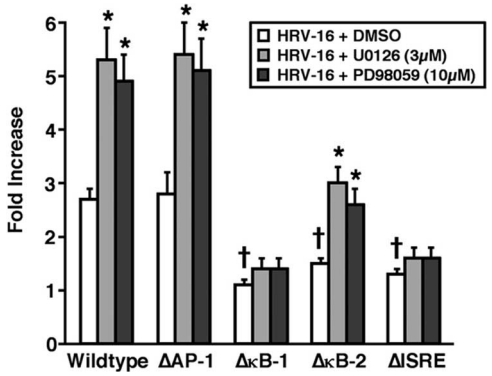

FIGURE 6. Effects of inhibitors of the MEK1/2 pathway on HRV-16-induced activation of wild-type and point-mutant versions of the truncated CXCL10 promoter. Asterisks indicate significant enhancement by PD98059 or U0126 compared with HRV-16 plus DMSO. Daggers indicate significant reduction of HRV-16-induced promoter activation compared with wild-type ($n = 6$). Each point-mutant is indicated as shown. Thus, ΔAP-1 represents the point-mutant of the AP-1 site, and so forth.

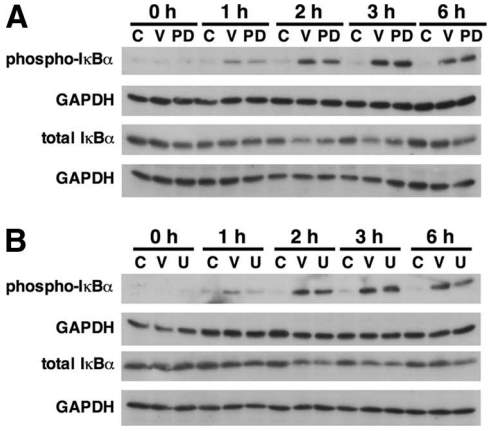

FIGURE 8. Inhibitors of the MEK1/2 pathway do not affect HRV-16-induced phosphorylation or degradation of IκBα in airway epithelial cells. *A*, PD98059 (PD) did not alter phospho-IκBα or degradation of total IκBα. *B*, U0126 (U) also failed to affect IκBα phosphorylation or degradation. C represents medium control; V, rhinovirus alone. Data are representative of $n = 3$.

promoter upstream of a luciferase reporter. HRV-16 infection induced activation of both the 5× κB1 (Fig. 7*A*) and 5× κB2 constructs (Fig. 7*B*), but neither PD98059 nor U0126 caused enhancement of the drive of either construct. Next, EMSA analysis was performed to examine the effects of MEK1/2 pathway inhibition on nuclear translocation and/or binding of NF-κB isoforms to labeled oligonucleotides containing the κB1 or κB2 recognition sequences from the CXCL10 promoter. Initial time course studies showed rapid HRV-16-induced formation of two specific complexes with both the κB1 and κB2 oligonucleotides, with strong binding evident at 3 h postinfection (data not shown). Accordingly, this time point was used to examine effects of preincubation with PD98059 or U0126 on complex formation. The addition of U0126 alone without HRV-16 infection did not alter the intensity of binding compared with media control, but PD98059 increased the intensity of the lower complex (band 2). However, neither drug led to any enhancement of HRV-induced complex formation (band 1 or band 2) with the κB1 oligonucleotide (Fig. 7*C*). Very similar results were obtained using the κB2 oligonucleotide (data not shown).

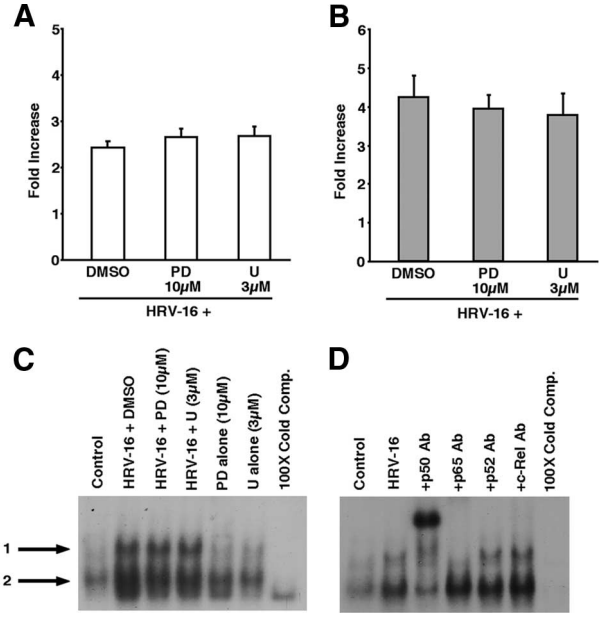

FIGURE 7. Evaluation of the role of NF-κB recognition sequences in the actions of MEK1/2 pathway inhibitors. Neither PD98059 (PD) nor U0126 (U) significantly enhanced HRV-16-induced activation of constructs containing five copies each of the κB1 ($n = 6$) (*A*) or of the κB2 ($n = 4$) (*B*) recognition sequences. *C*, Neither PD98059 nor U0126 enhanced the intensity of the two specific bands (arrows) produced when nuclear extracts from HRV-16-infected cells were incubated with a labeled oligonucleotide containing the κB1 recognition sequence. Data are representative of $n = 3$. *D*, Supershift experiments suggest that the upper of the specific bands contains p65-p50 heterodimers, while the lower band contains p50 homodimers. Data are representative of $n = 3$.

To determine the protein components comprising the two HRV-16-induced complexes on κB1 and κB2, supershift experiments were performed using Abs to individual Rel family proteins. This analysis showed that the upper band was shifted by Abs to both p65 and p50, suggesting it represents the canonical p65-p50 heterodimer, which we have previously reported to be induced in HRV-16-infected cells (31). In contrast, the lower band was shifted only by Abs to p50, suggesting it may represent p50 homodimers. Abs to cRel and p52 were without effect (Fig. 7*D*). Identical supershift results were seen using the κB2 oligonucleotide (not shown).

Activation of NF-κB classically occurs due to phosphorylation, ubiquitination, and subsequent degradation of the inhibitor IκBα via the actions of inhibitory κB kinase β (IKKβ) (32). We have shown that HRV-16 induction of CXCL10 expression is dependent on IKKβ using selective small molecule inhibitors of this enzyme (our unpublished observations). To further determine whether the MEK1/2 pathway modulates NF-κB signaling, we determined if either PD98059 or U0126 altered phosphorylation or degradation of IκBα. HRV-16 infection of BEAS-2B cells resulted in a time-dependent phosphorylation of IκBα, peaking at 3 h with a decrease by 6 h (Fig. 8). Degradation of IκBα was apparent at 2 h postinfection, with a decrease in degradation by 6 h. Neither PD98059 (Fig. 8*A*) nor U0126 (Fig. 8*B*) altered the HRV-16-induced phosphorylation or degradation of IκBα.

Evaluation of the role of interactions with the ISRE site in MEK1/2-mediated modulation of HRV-16-induced CXCL10 expression

To determine the role of the ISRE promoter site in the MEK1/2-inhibitor-mediated enhancement of HRV-16-induced CXCL10 expression, we generated a luciferase reporter construct containing five copies of the ISRE site. Interestingly, HRV-16 infection did

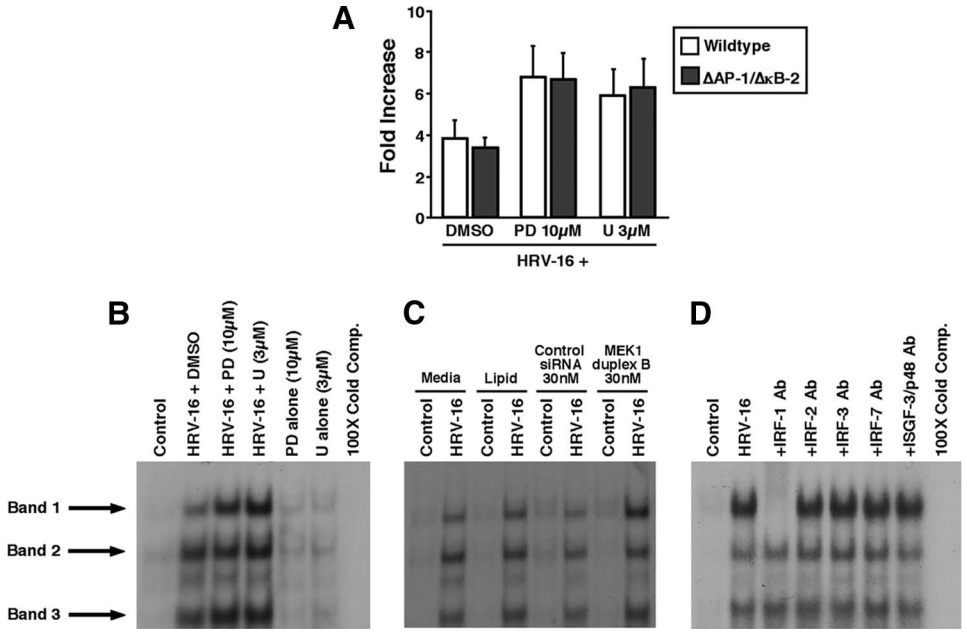

FIGURE 9. Evaluation of the role of ISRE recognition sequences in the actions of MEK1/2 pathway inhibitors. *A*, Both PD98059 (PD) and U0126 (U) induced comparable enhancement of HRV-16-induced activation of both the wild-type and the AP-1/κB2 double mutant promoter constructs ($n = 4$). *B*, Both PD98059 and U0126 enhanced the intensity of the uppermost (band 1) of three specific bands (arrows) produced when nuclear extracts from HRV-16-infected cells were incubated with a labeled oligonucleotide containing the ISRE recognition sequence. Data are representative of $n = 4$. *C*, In cells treated with siRNA to MEK1, the intensity of band 1 was also enhanced. Data are representative of $n = 4$. *D*, Supershift experiments suggest that the uppermost of the specific bands contains IRF-1. None of the Abs tested shifted the lower two bands. Data are representative of $n = 3$.

not induce activation of this construct (data not shown). It has previously been reported that there is strong cooperativity between NF-κB and ISRE sites in the RANTES promoter (33). Thus, it was possible that the ISRE site contributes to activation of the CXCL10 promoter only when present in conjunction with an NF-κB site. We therefore mutated both the κB2 and AP-1 sites in the 376-bp CXCL10 truncated promoter (Fig. 5*A*), leaving only functional ISRE and κB1 sites. HRV-16-induced promoter activity of both the wild-type and double mutant constructs equally, and both PD98059 and U0126 enhanced HRV-16-induced activation of both constructs to a comparable degree (Fig. 9*A*). EMSA studies using a labeled oligonucleotide containing the ISRE recognition sequence from the CXCL10 promoter showed that nuclear extracts from HRV-infected cells formed three major specific complexes with this oligonucleotide. Based on initial time-course data (not shown), the effects of MEK1/2 pathway inhibitors were examined using extracts from cells 6 h postinfection. As shown (Fig. 9*B*), both PD98059 and U0126 enhanced the intensity of the uppermost complex (band 1), with no significant or consistent effects on the other complexes (bands 2 and 3). Cells treated with siRNA to MEK1 also showed enhanced intensity of band 1 (Fig. 9*C*). Densitometric analysis of data from all four experiments revealed the PD98059 increased the intensity of band 1 by 190 ± 20% (mean ± SEM) compared with virus alone ($p < 0.02$) while U0126 increased band intensity to 190 ± 30% compared with virus alone ($p < 0.05$). Treatment with siRNA to MEK1 increased the intensity of band 1 to 210 ± 50% compared with virus alone. An increase was seen in each of four experiments. Supershift experiments revealed that the upper band was completely shifted using an Ab to IRF-1 (Fig. 9*D*). The protein components of the two lower bands remain to be identified, as they were unaffected by Abs to IRF-1, IRF-2, IRF-3, IRF-7, or ISGF-3/p48 (Fig. 9*D*).

Inhibitors of the MEK1/2 pathway also enhance HRV-induced production of CCL5

Given our data indicating that inhibitors of the MEK1/2 pathway increase HRV-induced production of CXCL10 via enhancement of IRF-1 nuclear translocation and binding, one may anticipate that this should also occur with other genes that are transcriptionally regulated by IRF-1. The chemokine CCL5 has a promoter se-

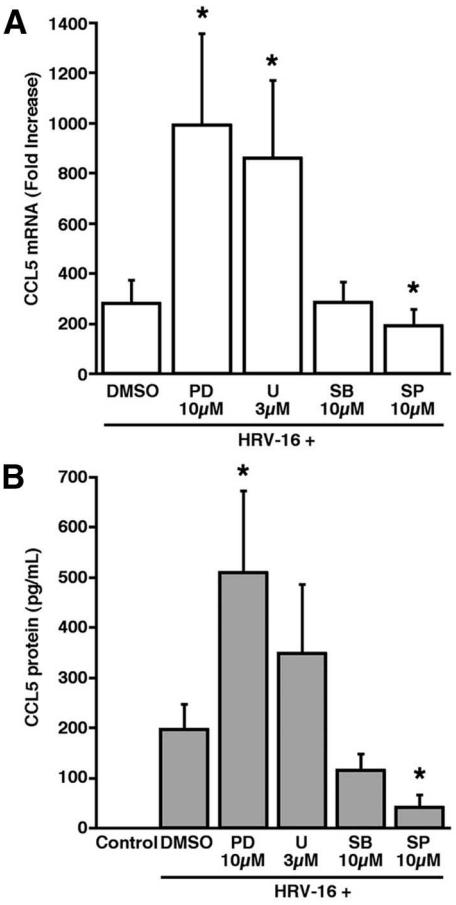

FIGURE 10. Inhibitors of the MEK1/2 pathway enhance HRV-16-induced CCL5 mRNA and protein expression in HBE. *A*, Both PD98059 (PD) and U0126 (U) significantly ($p < 0.05$) enhanced HRV-16-induced epithelial expression of CCL5 mRNA. *B*, PD98059 significantly ($p < 0.05$) increased HRV-16-induced CCL5 protein production. U0126 also enhanced CCL5 protein, but this just failed to achieve statistical significance. Data are means ± SEM from seven experiments.

quence that also contains both NF-κB and ISRE recognition sites and is transcriptionally regulated via interaction of IRF-1 with the ISRE (34). We, therefore, examined the effect of MAPK pathway inhibitors on HRV-16-induced production of CCL5 from HBE. In contrast to CXCL10, inhibition of the p38 pathway did not significantly affect CCL5 production. As observed for CXCL10, however, both PD98059 and U0126 enhanced HRV-16-induced CCL5 mRNA expression and protein production (Fig. 10), supporting the concept that IRF-1-regulated genes are susceptible to this effect.

Discussion

Production of proinflammatory chemokines from HRV-infected airway epithelial cells may contribute to the exaggerated host inflammatory response associated with HRV-induced exacerbations of asthma and COPD. The ability to inhibit HRV-induced production of selected chemokines, such as CXCL10, may provide a novel therapeutic approach to reduce exacerbations. Despite this potential, our understanding of the signaling pathways regulating HRV-16-induced chemokine production remains limited. Recent studies, by us and others, have implicated the spleen tyrosine kinase, Syk, as well as PI3K and MAPK pathways in early, replication-independent viral signaling leading to production of CXCL8 (19, 25, 35). In contrast, few studies have focused on signaling involved in HRV-16-induced production of chemokines, such as CXCL10, that absolutely require rhinovirus replication for induction. In the present studies, we evaluated the role of the p38, MEK1/2-ERK, and JNK MAPK pathways in HRV-16-induced CXCL10 expression in human airway epithelial cells.

Initial studies used selective inhibitors of each MAPK pathway. We confirmed that none of the drugs used had any effect either on cell viability or on viral replication within epithelial cells. Inhibition of the JNK MAPK pathway had no significant effect on either CXCL10 mRNA or protein levels in BEAS-2B cells, nor did it significantly alter mRNA expression in HBE. The significant inhibition of CXCL10 protein release from HBE is therefore difficult to explain, unless the JNK pathway exerts effects at the translational or posttranslational level in primary cells, but not in the BEAS-2B cell line. In contrast, blockade of the p38 MAPK pathway with SB203580 significantly inhibited HRV-16-induced CXCL10 mRNA and protein expression in both BEAS-2B and HBE. This is consistent with an earlier report of the involvement of the p38 MAPK pathway in adenoviral induction of this chemokine (21).

In marked contrast, inhibition of the MEK1/2 pathway by either PD98059 or U0126 significantly enhanced HRV-16-induced CXCL10 mRNA and protein expression in BEAS-2B cells in a concentration-dependent manner. Similar data were seen when the optimal concentration of each drug was used in HBE. These data clearly indicate that activation of the MEK1/2 pathway exerts a down-regulatory effect on HRV-16-induced CXCL10 production. This was somewhat surprising, given that the MEK1/2 pathway has been reported to be involved in both adenoviral induction of CXCL10 expression in murine kidney epithelial cells (21) and in rabies virus induction CXCL10 in murine macrophages (22). Precedent does also exist, however, for the MEK1/2 pathway to negatively regulate CXCL10 production. In keratinocytes stimulated with TNF-α, inhibition of the MEK1/2-ERK pathway increased CXCL10 mRNA expression via alteration of mRNA stability (36).

The ERK MAPK pathway is composed of two major kinases, ERK1/2 and ERK5, which have separate upstream kinases, MEK1/2 and MEK5, respectively. Additionally, both kinases share a similar phosphorylation consensus site (TEY) and a similar kinase domain (37). As such, both PD98059 and U0126 compounds have been reported to inhibit the ERK1/2 and ERK5 MAPK pathways (26, 27). To evaluate which of these pathways was important in regulating HRV-16-induced CXCL10 expression, we first determined if both pathways were activated in HRV-16-infected cells. Because CXCL10 is not induced until several hours after infection, we extended our time-course studies to determine whether each pathway was chronically activated. We confirmed that ERK1/2 phosphorylation occurred in virally infected cells and determined that activation was maintained out to 24 h after initial virus exposure. We also provided the first demonstration of ERK5 activation in HRV-infected epithelial cells. Although activation occurred within 15 min after viral exposure, it was not chronically maintained as for ERK1/2. Although modest enhancement of ERK5 phosphorylation was observed with PD98059, this effect was not concentration-dependent and was not seen with U0126. Since both drugs were equally effective in enhancing HRV-16-induced CXCL10 production, this implies that the MEK5-ERK5 pathway does not mediate the enhancement. In contrast, both drugs inhibited ERK1/2 activation in a concentration-dependent manner. Consistent with their reported specificities for MEK1 and MEK2, U0126 inhibited phosphorylation of both ERK1 and ERK2 at the concentration (3 μM) used for our experiments. In contrast, PD98059 (10 μM) was effective at inhibiting ERK1 phosphorylation, while having little effect on ERK2. Since both PD98059 and U0126 enhanced HRV-16-induced CXCL10 expression to similar levels, this suggested that inhibition of ERK1 is the main mechanism of the observed enhancement of CXCL10. To further evaluate this, and to confirm the specificity of the effects seen with MEK1/2 inhibitors, siRNA knockdown was used. Selective knockdown of MEK2 did not reproduce the enhancement of HRV-16-induced CXCL10 production seen with inhibitors, indicating that MEK2 alone does not mediate enhancement. In contrast, siRNAs targeting MEK1 led to enhanced HRV-16-induced CXCL10 production. This, together with the inhibitor data above, indicates that MEK1 plays an essential role in enhancement of CXCL10 production. Because siRNAs to MEK1 also had variable effects on MEK2, however, we cannot rule out the possibility of some crosstalk between MEK1 and MEK2.

To determine whether the enhancement phenomenon occurred with all stimuli for epithelial CXCL10 production, we used IFN-β as an additional stimulus. Neither PD98059 nor U0126 enhanced IFN-β-induced CXCL10 mRNA and protein expression in BEAS-2B cells or HBE. Moreover, IFN-β did not induce activation of ERK1/2. Thus, enhancement of HRV-16-induced CXCL10 expression shows some selectivity. Although both HRV-16 and IFN-β readily induce CXCL10 expression, they clearly do so via different signaling pathways.

We have previously shown that HRV-16-induced production of CXCL10 is regulated at the level of transcriptional activation, in part via the actions of NF-κB (17). We now establish that the enhancement effects of inhibitors of the MEK1/2 pathway are mediated, at least in part, by modulating this transcriptional activation. Moreover, the truncated (376 bp) version of the promoter appeared to contain all of the essential elements necessary for the MEK1/2 pathway inhibitor-mediated enhancement of HRV-16-induced promoter activity. Analysis of point mutants indicated that the AP-1 site does not appear to be involved either in HRV-16-induced promoter activation or in enhancement seen with PD98059 or U0126. Consistent with our earlier data (17), mutation of either the κB1 or κB2 recognition sites for NF-κB reduced HRV-16-induced promoter activation, although to variable degrees. Mutation of the κB1 site led to complete abrogation of promoter activation, while mutation of κB2 reduced, but did not eliminate, virally induced promoter activation. The differential effects on promoter activation of mutation of the κB1 vs κB2 site could reflect

some residual affinity of binding at the mutated κB2 site, but additional studies will be needed to assess this. Importantly, we showed that the ISRE site is also critical for HRV-16-induced CXCL10 promoter activation, as mutation of this site again led to complete inhibition of viral activation of the promoter, which could not be reversed by addition of the MEK1/2 pathway inhibitors. Although it has been reported that constitutively active ERK1/2 prevents the phosphorylation of TATA-binding protein, which is important for TATA box binding and also interaction with the p65 subunit of NF-κB (38), we found that enhancement of CXCL10 promoter activity was not due to effects on basal transcription, as a pGL3-basic-TATA construct was not driven by HRV-16, nor was activity enhanced with PD98059 and U0126. Taken together, these data suggest that the ability of the MEK1/2 inhibitors to enhance virally induced activation of the CXCL10 promoter must be mediated via effects on one or both of the two promoter sites critical for viral drive.

We used several approaches to show that the NF-κB recognition sequences in the promoter were not responsible for the effects of MEK1/2 inhibition on virally induced transcription. First, while HRV-16 infection induced activation of constructs containing multiple copies of either the κB1 or κB2 recognition sequences, neither PD98059 nor U0126 caused any enhancement of these responses. Second, neither PD98059 nor U0126 modulated HRV-16-induced phosphorylation or degradation of IκBα, a key process in the HRV-16-induced activation of NF-κB-dependent transcription. Finally, neither PD98059 nor U0126 enhanced nuclear translocation and/or binding of p65-p50 heterodimers to oligonucleotides containing either the κB1 or κB2 recognition sequences from the CXCL10 promoter.

The ISRE sequence in the CXCL10 promoter has previously been shown to play a role in promoter activation induced by IFN-γ, synthetic dsRNA, and measles virus (39–41). Our data demonstrate that the ISRE site is also important in HRV-16-induced promoter activation. Interestingly, while mutation of the ISRE site markedly abrogated HRV-16-induced promoter activation, a synthetic construct containing five copies of just this site was not responsive to HRV-16 infection. This implied that the ISRE contribution to promoter activation may be dependent upon its context. There is precedent for cooperativity between ISRE and NF-κB sites for binding of their respective ligands in the RANTES promoter (33). Indeed, when we mutated both AP-1 and κB2 sites in the endogenous promoter, leaving only functional ISRE and κB1 sites in their original context, HRV-induced activation of the promoter and the extent of enhancement by PD98059 and U0126 were indistinguishable from those seen with the wild-type truncated promoter. EMSA analysis demonstrated that nuclear extracts from HRV-16-infected cells generated three major specific complexes when incubated with the ISRE recognition sequence from the CXCL10 promoter. Treatment with either PD98059 or U0126 resulted in a selective marked enhancement in the intensity of the uppermost band. This enhancement was also seen upon knockdown of MEK1 using siRNA. In supershift experiments, this band was completely shifted using an Ab to IRF-1.

The identity of the two lower bands is not clear at this time, as they were not affected by Abs to IRF-1, IRF-2, IRF-3, IRF-7, or ISGF-3γ p48. Although IRF-3 previously has been linked to the induction of CXCL10 by dsRNA in BEAS-2B cells (42), the failure to see any effect of IRF-3 Abs in supershift experiments is consistent with a recent report that HRV does not induce IRF-3 in epithelial cells (43). Similarly, despite the fact that ISGF-3, a complex of IRF-9 (also known as p48), with STAT1 and STAT2 has been implicated in mouse and human CXCL10 promoter induction in response to some stimuli (39, 44), Abs to ISGF-3γ/p48 did not affect any of the bands in our present experiments. Fortunately, identification of the protein components of the two lower bands was not crucial for our present work given the effects of MEK1 inhibition exclusively on the uppermost band containing IRF-1.

Although IRF-1 has been found to be a weak transcriptional activator (45, 46), it has been shown to be involved in the induction of CXCL10 in response to other stimuli (47). This suggests that cooperation with other transcription factors, such as NF-κB, may indeed be required for optimal promoter activation. In support of this, IRF-1 and NF-κB have been shown to physically interact on the long terminal repeat enhancer of HIV (48). Moreover, cooperation between IRF-1 and NF-κB has been described in the transcriptional activation of CCL5 and inducible NO synthase (34, 49), genes that can also be induced by HRV (10, 50). Our demonstration that inhibitors of the MEK1/2 pathway also enhance HRV-16-induced production of CCL5 provides evidence that other IRF-1 responsive genes can be regulated via the MEK1/2 pathway.

Regulation of IRF-1 activity via the MEK1/2-ERK pathway could occur either at the level of transcription or at the level of posttranslational modification. In other systems, it has been shown that IRF-1 gene induction is dependent on STAT1α, which can be phosphorylated by ERK1/2, targeting it for ubiquitination and subsequent degradation (51). Thus, inhibition of MEK1/2 may decrease STAT1α degradation, potentially leading to increased IRF-1 transcription (51). In terms of posttranslational modification, it has been shown that IRF-1 can be a substrate for the small ubiquitin-related modifier (SUMO). SUMO conjugation has been linked to effects on protein stability, localization, and repression of transcriptional activity (52), and SUMOylation has been shown to decrease IRF-1 transcriptional activity (53). Given that ERK1/2 promotes the SUMOylation of STAT1α (54), it is feasible that a similar pathway may also modulate IRF-1 in our system. In addition to ubiquitination and SUMOylation, IRF-1 can be phosphorylated by casein kinase II (CKII), a serine/threonine protein kinase, which is essential for IRF-1 binding and p65 synergistic induction of IFN-β promoter expression (55). Further studies will be needed to delineate which, if any, of these mechanisms underlie the effects of the MEK1/2 pathway on HRV-16-induced CXCL10 production in human airway epithelial cells.

In summary, our data demonstrate that activation of the MEK1 pathway in airway epithelial cells selectively negatively regulates the HRV-16-induced expression of CXCL10 through effects on transcription. These transcriptional regulatory mechanisms appear to involve modification of the interaction of IRF-1 with the ISRE site in the CXCL10 promoter. To our knowledge, this is the first description of the negative regulation of HRV-induced epithelial cell chemokine induction via the MEK1 pathway, and indicates that this pathway has anti-inflammatory and protective roles during HRV infections.

Disclosures

The authors have no financial conflicts of interest.

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