Activation of the Epidermal Growth Factor Receptor by Respiratory Syncytial Virus Results in Increased Inflammation and Delayed Apoptosis*

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Respiratory syncytial virus (RSV) preferentially infects lung epithelial cells. Infection by RSV leads to an extended inflammatory response, characterized by the release of interleukin-8 (IL-8). Activation of ERK MAP kinase is required for both RSV-induced inflammation and the extended survival of infected cells. In this study, we analyzed the role of the epidermal growth factor receptor (EGFR) in RSV activation of ERK. We demonstrate for the first time that RSV activates EGFR in lung epithelial cells. Activation of EGFR results in increased ERK activity, contributing to both the inflammatory response (IL-8 release) and prolonging the survival of RSV-infected cells. Inhibition of EGFR with siRNA decreased both ERK activation and IL-8 production after RSV. In analyzing the effect of EGFR activation on survival of RSV-infected cells, we found that EGFR activation by RSV resulted in ERK-dependent alterations in the balance of pro- versus anti-apoptotic Bcl2 proteins. RSV altered the balance between pro- and anti-apoptotic Bcl2 proteins (increased BclXL and decreased BimEL) increasing the relative amount of pro-survival proteins. This occurred in an EGFR-dependent manner. This study supports an important role for EGFR activity in the lifespan and inflammatory potential of RSV-infected epithelial cells.

Respiratory syncytial virus (RSV) is an important pathogen in young children and immunocompromised adults (1). Worldwide, it is the most common cause of bronchiolitis-associated hospitalization of children less than 2 years of age (2). Early severe RSV infections (bronchiolitis with hospitalization) cause recurrent wheezing and asthma symptoms throughout childhood (3). RSV is found ubiquitously in the environment. It is an enveloped, non-segmented, negative-strand RNA virus, a member of the family Paramyxoviridae. Entry into the host cell (primarily the respiratory epithelium) is by cell surface fusion. Eleven genes and their encoded proteins have been identified for RSV. Infection of lung epithelial cells leads to viral replication and induction of an inflammatory response characterized by the production of chemokines and cytokines. In our previous work, we have shown that ERK signaling pathways are involved in both inflammation and epithelial cell survival in RSV-infected cells (4–10).

This study demonstrates for the first time that RSV infection activates the epidermal growth factor receptor (EGFR) and that EGFR activation is linked to ERK activity. EGFR was first described as the receptor for epidermal growth factor (EGF) and since then has been shown to respond to a diverse array of ligands leading to a diverse set of outcomes (11). EGFR is a membrane-bound glycoprotein with an extracellular ligand-binding domain and an intracellular cytoplasmic domain that following activation possesses tyrosine kinase activity (12). Though originally described as the receptor for EGF, more recent data have demonstrated EGFR activation by other extracellular ligands (TGFα, amphiregulin, epiregulin, HBEGF, beta-cellulin). These transmembrane proligands are cleaved and released into the extracellular milieu by matrix metalloproteases (a disintegrin and metalloproteinase family (ADAMs)) leading to activation of EGFR (13–16). EGFR can also be activated by a still undetermined oxidant-dependent intracellular transactivation from other receptors (17, 18). Activation of EGFR involves both Src-dependent phosphorylation and autophosphorylation of multiple tyrosines (19–21). Adaptors proteins and functional enzymes (kinases and phosphatases), including proteins involved in activation of ERK bind to phosphotyrosines on the intracellular domain of EGFR (22–25).

There have been no studies on interactions between RSV and EGFR. There are, however, other viruses that interact with EGFR. Some viruses are known to encode viral homologues of EGFR pathway genes, including a constitutively active EGFR, v-ErbB, encoded by avian erythroblastosis retrovirus (AE) (26). Other viruses, such as hepatitis B viruses and Epstein-Barr virus encode proteins that increase levels of human EGFR (27, 28). Two viruses, human cytomegalovirus (hCMV) and adenovirus have been linked to human EGFR regulation. A recent study by Wang et al. (29) found that EGFR is the cellular receptor for hCMV. They found that 5 min after exposure of human embryonic lung fibroblasts to hCMV, EGFR was activated, and this was necessary for optimal viral entry. Adenoviral proteins (Adenovirus E3–10.4k and 14.5k) have been
demonstrated to cause degradation and inhibition of the EGFR (30, 31). Our study demonstrates the novel finding that RSV activates endogenous EGFR, leading to delayed apoptosis and increased inflammatory response in infected epithelial cells.

The MAP kinases are a family of evolutionarily conserved enzymes that connect cell surface receptors to regulatory targets, including both cytoplasmic and nuclear proteins. They can be activated by divergent stimuli including hormones, growth factors, cytokines, G protein-coupled receptor agonists, and TGFβ and TLR ligands, including lipopolysaccharide (32). The three major MAP kinase families are the ERK (1, 2, 5, 7, and 8), p38 (α, β, γ, and δ) and c-Jun N-terminal kinase (1, 2, and 3) (32, 33). We have found a requirement for ERK activation in RSV-induced IL-8 production (8). More recently we have shown that ERK activation plays an important role in prolonged survival of RSV-infected airway epithelial cells (10). It is not entirely clear how RSV activates ERK. However, our previous studies supply some of the pieces. We have shown that there is differential use of protein kinase C isoforms upstream of ERK at various times after infection (7). We have also shown that at late times after infection, RSV-induced ERK requires activation of sphingosine kinase and generation of S1P (10). In the present study, we show that a proximal event in RSV-induced ERK activity is activation of EGFR. Moreover, we demonstrate that the EGFR-linked ERK activation plays an important role in extended survival of infected epithelial cells by altering the balance between pro- and anti-apoptotic Bcl2 family members.

RSV induces apoptosis of infected cells. We have shown previously that activation of ERK delays the normal viral-induced apoptotic response (10). In examining the role of EGFR in RSV-induced apoptosis, this study demonstrates novel ERK-dependent effects on Bcl2 family members. Mitochondrial homeostasis is regulated via the balance between pro- and anti-apoptotic members of the Bcl2 family, which are divided into three classes based on their Bcl2 homology domains (B1H-1—4). Anti-apoptotic members (Bcl2 and BclXL) contain four BH domains. The two classes of pro-apoptotic Bcl2 proteins are divided by function and number of BH domains. Bax-like proteins (including Bax and Bak) contain multiple BH domains and are physically responsible for increased mitochondrial membrane permeability. BH3-only Bcl2 proteins include Bad, Bim, and Bid. The BH3-only proteins play a role in facilitating the increase in mitochondrial membrane permeability caused by Bax and Bak (34). We found that RSV infection results in a decrease in the amounts of the BH3-only protein, BimEL. The role of Bim regulation in virally infected epithelial cells has not previously been studied. Bim is regulated transcriptionally, but more importantly it is actively degraded by the proteosome following phosphorylation by ERK (35–37). There have been no previous studies demonstrating viral subversion of the normal apoptotic process via an ERK-dependent effect on BimEL.

In summary, we evaluated the role of EGFR in RSV-infected lung epithelial cells. We found that infection with RSV caused activation and internalization of EGFR. Our data suggest that the EGFR activation is via RSV-dependent cleavage of a membrane bound EGFR ligand. We found that RSV induced membrane localized ADAM protease activity, release of amphiregulin, and MMP-dependent IL-8 production. EGFR activity was required for RSV-induced ERK activity, which is necessary for both optimal RSV-induced inflammation and prolonged cell survival. Inhibition of EGFR, by a chemical inhibitor, blocking antibody or siRNA, resulted in a decreased inflammatory response (IL-8 release) and premature death of virally infected cells. The anti-apoptotic effect of EGFR in RSV-infected cells was at least partially caused by an ERK-dependent effect on the balance of pro- versus anti-apoptotic Bcl2 proteins. This is the first time that RSV has been shown to activate EGFR (contributing to the inflammatory response), and the first time that ERK-dependent modulation of Bcl-2 proteins (downstream of EGFR) has been shown to play a role in sustained survival of RSV-infected epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were obtained from Sigma and Calbiochem, La Jolla, CA. Protease inhibitors were obtained from Roche Applied Science. Antibodies to Akt1 (sc-1618), ERK2 (sc-154), GFP (sc-9996), TNFR1 (sc-7895), and Bcl-XL (sc-7195) were obtained from Santa Cruz Biotechnology. Antibodies to phosphorylated ERK (Thr202/Tyr204) and EGFR (Tyr1068) were obtained from Cell Signaling (Beverly, MA), 9101, 9271, and 2234, respectively. Also from Cell Signaling were antibodies to EGFR (2232) and cleaved PARP (9541). Antibody to Bim (AAP-330) was obtained from Stressgen (Victoria, BC, Canada). Antibody to β-actin (A-5316) was obtained from Sigma. Antibody to RSV (B65860G) was obtained from Biosciences International (Sacramento, CA). Nitrocellulose, ECL, and ECL Plus were obtained from Amersham Biosciences. Horseradish peroxidase-conjugated developing antibodies were from Santa Cruz Biotechnology.

**Epithelial Cell Culture and Viral Infection**—A549 lung epithelial cells were obtained from American Type Culture Collection. Cells were maintained in 75-cm² tissue culture flasks (Corning, Corning, NY) in minimal essential medium (Invitrogen, Grand Island, NY) with 10% fetal calf serum and gentamicin. For infection, cells at ~80% confluence were treated with human RSV, strain A-2 (moi of 5). Viral stocks were obtained from Advanced Biotechnologies Inc (Columbia, MD). Because of a report of possible adenovirus contamination in some RSV stocks (38), we tested our stock for adenovirus by PCR and found it to be completely free of adenoviral contamination. The initial stock (1 × 10⁶ TCID₅₀) was aliquoted and kept frozen at −135 °C. A fresh aliquot was thawed for each experiment. The virus was never refrozen.

**Primary Tracheobronchial Epithelial Cells (hTECs)**—All protocols were approved by the University of Iowa Institutional Review Board. Primary tracheobronchial epithelial cells were obtained from a minimum of four donors. Bcl2-like apoptosis members were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in laboratory of carcinogenesis (LHC)-8e medium on plates coated with collagen/albumin for study up to passage 10. For infection, cells at ~80% confluence were treated with human RSV, strain A-2 (moi of 5).

**Cell Survival Analysis**—For analysis of cell survival, A549 epithelial cells (seeded in 24-well tissue culture plates and infected with RSV (moi of 5)) were cultured alone or with pathway inhibitors (ERK, U0126 at 10 μM) for the described times. Triplicate cultures were performed on all experiments. After the incubation period, the cells were stained with ethidium homodimer (EthD-1, Molecular Probes, Eugene, OR) at 8 μM and images obtained of both bright field and fluorescence using a DMRB microscope equipped with a Qimaging RETICA 1300 digital camera and imaging system. After obtaining images, the percentage of EthD-1-positive cells was determined. Quantification was by direct cell count. 200 cells were counted from a minimum of four different fields. Average viability was determined and statistics performed using Graphpad software.

**Whole Cell Protein Isolation**—Whole cell protein was obtained by lysing the cells on ice for 20 min, in 300 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 μ NaCl, 1% Nonidet P-40, with added protease and phosphatase inhibitors: 1 protease minitab (Roche Applied Science)/10 ml and 100 μM of 100× phosphatase inhibitor mixture (524625, Calbiochem)/10 ml). The lysates were sonicated for 20 s, kept at 4 °C for 30 min, spun at 15,000 g at 4 °C for 30 min, spun at 15,000 × g for 10 min, and the supernatant saved. Protein determinations were made using a protein measurement kit (Protein Assay, 500-0006) from Bio-Rad. Cell lysates were stored at −70 °C until use.

**Biotin Labeling of Cell Surface Proteins**—A549 cells were treated by incubating the cells on ice for 20 min, in 300 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 μ NaCl, 1% Nonidet P-40, with added protease and phosphatase inhibitors: 1 protease minitab (Roche Applied Science)/10 ml and 100 μM of 100× phosphatase inhibitor mixture (524625, Calbiochem)/10 ml). The lysates were sonicated for 20 s, kept at 4 °C for 30 min, spun at 15,000 × g for 10 min, and the supernatant saved. Protein determinations were made using a protein measurement kit (Protein Assay, 500-0006) from Bio-Rad. Cell lysates were stored at −70 °C until use.

**Immunoprecipitation of Cellular Proteins and Biotin-labeled Proteins**—For immunoprecipitation using a specific antibody, lysates were incubated with the antibody of interest (BclXL) overnight at 4 °C.
tein A-agarose beads (Santa Cruz Biotechnology) were then added for 2 h. Beads were washed three times with lysis buffer, and then Western sample buffer added directly to the pellet (see below). To isolate biotin-labeled membrane proteins, 1 mg of protein was incubated with 100 μl of streptavidin-agarose beads (Novagen, 69203). The beads were then washed three times with 500 μl of lysis buffer, and the supernatant removed after final wash. The beads were suspended in 50 μl of 2× sample buffer and heated for 5 min at 95 °C. The beads were spun down and proteins analyzed by SDS-PAGE. Western blot protocol was followed as described below. 

Western Analysis—Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed as described previously (5). Briefly, 40 μg of protein was mixed with 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, and 1.25 μM Tris, pH 6.8) and loaded onto a 10% SDS-PAGE gel and run at 110 V for 2 h. Cell proteins were transferred to nitrocellulose with a Bio-Rad semidyed transfer system, according to the manufacturer’s instructions. Equal loading of the protein groups on the gels was evaluated using Ponceaus S (Sigma), a staining solution designed for staining proteins on nitrocellulose membranes. The nitrocellulose was then blocked with 5% milk in TTBS (Tris-buffered saline with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary antibody at dilutions of 1:500 to 1:2000 overnight. The blots were washed with TTBS and incubated for 1 h with horse-radish peroxidase-conjugated anti-IgG antibody (1:5000 to 1:20,000). Immuno-reactive bands were developed using a chemiluminescent substrate, ECL Plus, or ECL (Amersham Biosciences). An autoradiogram was obtained with exposure times of 10 s to 2 min. Protein levels were quantified using a FluorImager scanner and Quantity One software for analysis (Bio-Rad). The data were analyzed and statistics performed using Graphpad statistical analysis software. Western analysis is expressed as fold increase (experimental value/control value).

Transfection Protocol—A549 cells were plated in regular medium. The next day the medium was changed to 10% Opti-MEM (Invitrogen) without antibiotic. The cells were allowed to equilibrate overnight and transfected the next day with the plasmid EGFR-GFP using the transfection reagent FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. A vector for the expression of the C-terminal enhanced green fluorescent protein (GFP) tag was constructed by recombinant PCR amplifications of the human EGFR cDNA sequence and the GFP cassette in the pEGFP-N1 vector (Clontech). The EGFR and GFP cDNA sequences were ligated in-frame and cloned into the pcDNA3 expression vector (Invitrogen). All amplified sequence elements were validated by DNA sequencing. The next day, cells were infected with RSV and at appropriate time points assayed by confocal microscopy or by biotinylation of membrane proteins and Western analysis using an anti-GFP antibody.

Confocal Microscopy—Confocal images of A549 cells in LabTek® II chamber slides were transfected with EGFR-GFP and then infected with RSV. For EGFR-GFP subcellular localization analysis, cells were fixed at various times with phosphate-buffered saline containing 4% paraformaldehyde and 0.15 M NaCl. Cell nuclei were counterstained with DAPI. Confocal microscopy or by biotinylation of membrane proteins and Western analysis using an anti-GFP antibody.

RESULTS

RSV Activates the EGF Receptor

Lung epithelial cells were infected with RSV at an moi of 5, cultured for 24 h with and without the EGFR inhibitor, AG1478 (control and RSV-infected cells were treated with an amount of Me2SO equal to that in the AG1478-treated cells). Fig. 1A demonstrates that inhibition of EGFR activity blocks induction of IL-8 by RSV in A549 cells. In other experiments A549 cells, infected with RSV, with and without the EGFR inhibitor AG1478. Supernatants were harvested and IL-8 release evaluated by ELISA. As with the A549 cells, RSV infection of primary hTECs induced IL-8 release that was dependent on EGFR activity (Fig. 1A). We addressed the question of inhibitor specificity two ways. Cells were treated with a neutralizing antibody to EGFR. This also decreased the production of IL-8 after RSV infection (Fig. 1B). In other experiments A549 cells were transfected with either control siRNA or siRNA specific for EGFR. The blot in Fig. 1C demonstrates that at 72-h post-transfection, we have significantly decreased the amount of EGFR. At that time point, there was no difference in the cell viability between control siRNA-treated cells and EGFR siRNA-treated cells. The cells were then infected with RSV for 24 h, and supernatants harvested. As with the blocking antibody, decreasing EGFR with siRNA decreased the production of IL-8 by RSV-infected cells. Neither the blocking antibody nor the siRNA experiments decreased IL-8 to the same extent as the AG1478. One distinct possibility is that AG1478 has actions beyond EGFR inhibition. Another possibility is that there are experimental limitations with the use of both blocking antibodies and siRNA that prohibit a total block. We do not
FIG. 1. Inhibition of EGFR activity decreases RSV-induced IL-8 production without altering viral replication. A, A549 cells or hTBE cells were infected with RSV (moi of 5) for 24 h. In some groups, the cells were treated with an EGFR inhibitor (AG1478, 30 μM) beginning 30 min before RSV infection. After a 24-h incubation, the supernatants were harvested and IL-8 release evaluated. *p values were determined for inhibitor + RSV versus RSV samples (*p < 0.01, RSV versus RSV+AG1478). B, A549 cells were infected with RSV (moi of 5) with and without the neutralizing antibody. C, EGFR siRNA was used to inhibit EGFR expression, and IL-8 production was measured. **p values were determined for Control siRNA versus EGFR siRNA samples (**p < 0.01). D, Western blot analysis of G protein, M1+2, and NS1+2 expression in the indicated groups.
believe that EGFR is the only pathway contributing to IL-8 production by RSV. Rather, we believe that activation of EGFR by RSV augments and builds on inflammatory responses that begin with viral binding to the epithelial cells (40). Because of the identical findings in primary lung epithelial cells compared with A549 cells, we chose to carry out the remainder of our

![RSV Activates the EGF Receptor](http://www.jbc.org/)

**FIG. 2. RSV activates and internalizes EGFR.** A, A549 cells were infected with RSV (moi of 5) for 1, 6, 18, and 24 h. Whole cell lysates were obtained and Western analysis performed for tyrosine-phosphorylated EGFR tyrosine (Tyr$^{1068}$) and for total EGFR. B, A549 cells were transfected with EGFR-GFP or a control vector. 18 h later, cells were either infected with RSV (moi of 5) for 24 h or exposed to EGF (10 ng/ml) for 2 h. At the end of the exposure time, cells were fixed and stained for nuclei and Golgi (see under "Experimental Procedures") and EGFR-GFP localization evaluated on a Zeiss 510 confocal microscope. Ten random fields ($512 \times 512$ pixels each) were collected for each experimental group. C, A549 cells were transfected with EGFR-GFP or a control vector, 18 h later the cell were infected with RSV for 24 h. Outer membrane proteins were then biotin-labeled and isolated using streptavidin immunoisolation. The biotin-labeled proteins were run on an SDS-PAGE gel, and Western analysis performed using an antibody to GFP. The band in the top panel represents membrane localized EGFR-GFP. The blot was reprobed for TNFR1 as a control for the biotin plasma membrane localization.

addition of a neutralizing antibody to EGFR antibody (25 μg/ml). Cells were cultured for 24 h, supernatants harvested, and IL-8 release analyzed by ELISA. $p$ values were determined for antibody + RSV versus RSV samples ($p < 0.05$, RSV versus RSV + neutralizing antibody). C, A549 cells were infected with RSV (moi of 5) 72 h after transfection of the cells with either a control siRNA or EGFR siRNA. Cells were cultured for 24 h, supernatants harvested and IL-8 release analyzed by ELISA. $p$ values were determined for control siRNA RSV versus EGFR siRNA RSV samples ($p < 0.01$). Shown also is a Western blot for EGFR using cell lysates from 72-h post-transfection with either control siRNA or EGFR siRNA. Equal loading is demonstrated by reprobing the blot for β-actin. D, before infection with RSV, cells were treated with one of several doses of AG1478 (300 nM, 3 μM, 30 μM). Whole cell lysates were obtained after 24 h of RSV infection (moi of 5) and a Western blot probed using a polyclonal antibody to RSV (see under "Experimental Procedures"). Individual RSV proteins are identified by molecular weight analysis.
experiments using A549 cells as a model of lung epithelial cells. RSV does not require EGFR activity for cell entry. One possible reason for the decreased IL-8 with EGFR blockade was that EGFR is required for viral entry and replication. In Fig. 1D, we show that inhibition of EGFR has no affect on RSV replication. This is demonstrated by Western analysis using a polyclonal anti-RSV antibody. As a composite these data suggest that RSV activates EGFR in airway epithelial cells contributing the magnitude of the inflammatory response.

RSV Activates EGFR—The IL-8 data strongly suggest that RSV activates EGFR. To investigate this, we infected lung epithelial cells with RSV and evaluated EGFR activation and membrane localization. Fig. 2A demonstrates that infection with RSV causes tyrosine phosphorylation of EGFR (more specifically of tyrosine 1068 in the intracellular domain of EGFR). This particular tyrosine residue on the intracellular domain of EGFR is linked to activation of ERK (41, 42). Activation of EGFR leads to internalization of the receptor and ultimately degradation by fusion of endosomal and lysosomal intracellular vesicles (21, 43). Fig. 2A also shows that total EGFR is significantly decreased by 24 h of infection, a time point when there is increased activity of the receptor as demonstrated by tyrosine phosphorylation. We evaluated EGFR internalization two ways. First, we transfected epithelial cells with a construct expressing EGFR tagged with enhanced green fluorescent protein (EGFR-GFP). 24 h after transfection, the cells were serum starved to cause optimum membrane localization of EGFR and infected with RSV. Localization of the EGFR fused to GFP was determined by confocal microscopy. Fig. 2B demonstrates that RSV causes internalization of EGFR, some of which is shown to colocalize with the Golgi. The effects of EGF on internalization are shown as a positive control. It is interesting that it takes longer to see internalization of EGFR in the RSV-infected cells than in the EGF-exposed cells. This is consistent with a need for matrix metalloproteinase induction leading to cleavage of an extracellular ligand and EGFR activation. We also examined EGFR internalization using co-immunoprecipitation techniques. Because of the difficulty immunoprecipitating endogenous EGFR proteins, we used an overexpression system to evaluate membrane localization of EGFR. In the experiment shown in Fig. 2C, epithelial cells were transfected with the EGFR-GFP construct. This was followed by infection with RSV and biotin labeling of the extracellular membrane proteins at the end of the infection period. Whole cell lysates were exposed to streptavidin beads, and the biotin-linked plasma membrane proteins were isolated. Western analysis for biotin-labeled tagged EGFR was done using an antibody to GFP. The blot demonstrates that at baseline in the EGFR-GFP-transfected
cells, EGFR is present on the plasma membrane. There is significant loss of EGFR from the membrane following RSV infection. The TNFR1 data are shown to confirm that the isolation of biotin-labeled proteins brought down equal amounts of an unrelated protein. As a composite, these experiments demonstrate for the first time that RSV infection of lung epithelial cells leads to activation and internalization of EGFR.

**RSV Induces Metalloproteinase (ADAM) Activity and Release of Amphiregulin—**

EGFR activation can occur by binding of exogenous EGF to the receptor. EGFR activation can also occur after cleavage of membrane-bound ligands (TGFα, amphiregulin, epiregulin, hhEGF, betacellulin) by matrix metalloproteinases (ADAMs) (15, 25, 44). These ligands then activate EGFR via autocrine binding to the receptor. We first examined the effect of RSV on ADAM activity. We found that in an *in vitro* assay, membrane proteins from RSV-infected cells cleaved significantly more of a substrate specific for ADAM 9, 10, and 17 (Thr202/Tyr204). Densitometry, shown as arbitrary units, is shown beside the blot. We examined a possible link between RSV-induced metalloprotease activity, amphiregulin release and IL-8 production by treating cells with the compound GM6001, an inhibitor of MMP activity. We found that MMP inhibition decreased the amount of IL-8 released after RSV infection (Fig. 3B). The increased

**FIG. 4. Inhibition of EGFR decreases RSV-induced ERK, which is required for optimal IL-8 induction.**

A. A549 cells were infected with RSV (moi of 5) for 24 h. In some groups, the cells were treated with ERK inhibitor (U0126, 10 μM) beginning 30 min before RSV infection. After a 24-h incubation, the supernatant was harvested and IL-8 release evaluated. *p* values were determined for inhibitor+RSV versus RSV samples (*p* < 0.01, RSV versus RSV+U0126). B. A549 cells were infected with RSV (moi of 5) for various times. In some cases, cells were pretreated with the EGFR inhibitor, AG1478 (30 μM). At the end of the culture period, Western analysis was performed for active ERK using an antibody specific for phosphorylated ERK (Thr202/Tyr204). Densitometry, shown as arbitrary units, is shown beside the blot. C. A549 cells were infected with RSV (moi of 5) for 6 h starting 72 h after transfection with either a control siRNA or EGFR siRNA. At the end of the culture period, Western analysis was performed for active ERK using an antibody specific for phosphorylated ERK (Thr202/Tyr204). Densitometry, shown as arbitrary units, is shown beside the blot.

Effect of RSV on ADAM activity. We found that in an *in vitro* assay, membrane proteins from RSV-infected cells cleaved significantly more of a substrate specific for ADAM 9, 10, and 17 (Fig. 3A). One of the substrates that can be cleaved by these ADAMs (9, 10, and 17) is amphiregulin. We examined release of amphiregulin after RSV infection by collecting supernatants of infected cells and evaluating amphiregulin release by ELISA. In Fig. 3A, we show that RSV infection of lung epithelial cells causes a significant increase in amphiregulin release.
release of amphiregulin suggests that one of the ligands contributing to EGFR activation is amphiregulin. It is beyond the scope of this study to determine if this is the only released ligand or whether the increase in ADAM activity leads to release of multiple EGFR ligands. Those studies are ongoing. What the data does show is that in a time frame consistent with RSV-induced EGFR activation, RSV induces metalloproteinase activity and causes the release of at least one EGFR ligand. As a composite, these data demonstrate that RSV-induced inflammation requires both MMP activity and EGFR activity.

**RSV Activation of EGFR Contributes to IL-8 Production via an Effect on ERK Activity**—Fig. 4A shows that inhibition of either ERK (U0126) decreases the production of IL-8 by RSV-infected cells. We then used an EGFR inhibitor (AG1478) and analyzed the role of EGFR in activation of ERK. RSV causes extended activation of ERK (5, 7, 10). Inhibition of EGFR blocked ERK activation at multiple time points (Fig. 4B). To confirm the results from the AG1478 experiments, we also used siRNA to decrease EGFR in A549 cells (see Western blot demonstrating EGFR knockdown in Fig. 1). As with AG1478, EGFR siRNA caused a decrease in ERK activation by RSV (Fig. 4C). These data demonstrate that within the first day of RSV infection, activation of EGFR leads to increased and sustained ERK activity resulting in increased inflammation as demonstrated by an increase in IL-8. Inflammation after RSV infection is possible because the infected cells live long enough for the induction of the inflammatory response. We next evaluated if EGFR contributed to inflammation by prolonging survival of infected cells.

**EGFR Activity Contributes to Sustained Survival of RSV-infected Epithelial Cells**—To evaluate the effect of EGFR on survival of RSV-infected epithelial cells, we pretreated epithelial cells with an EGFR activity inhibitor, AG1478. In cells infected with RSV at an moi of 5, cell death begins to occur at 48 h, and significant cell death has occurred by 72–96 h (data not shown). When we evaluated the effect of EGFR inhibition on survival at 24 h there was no increase in the number of dead cells (data not shown). However, after 36 h of infection with RSV there was significant cell death only in the AG1478-treated cells. Fig. 5A shows by fluorescent microscopy that AG1478 shortens survival times in RSV-infected cells. A, A549 cells were infected with RSV (moi of 5) for 24 h. In some groups, the cells were treated with an EGFR inhibitor (AG1478, 30 μM) beginning 30 min before RSV infection. Cell survival at 36 h of infection was analyzed by EthD-1 cell entry. The figure shows bright field photomicrographs of the cell monolayer and fluorescent photomicrographs of the EthD-1 positive cells. The images are representative of three separate experiments. The graph depicts the percentage of dead cells. The data are from three separate experiments. B, A549 cells were infected with RSV (moi of 5) for various times. Whole cell lysates were obtained, and Western analysis was performed with an antibody that is specific for the cleaved form of PARP. Equal loading is shown by restaining the blot with an antibody specific for β-actin.
RSV Increases Amounts of BclxL and Decreases Amounts of BimEL in Infected Cells. A549 cells were infected with RSV (moi of 5) for various times. Whole cell lysates were obtained and Western analysis performed for BclxL (32 kDa) and BimEL (22.1 kDa). Primary and secondary antibody dilutions for BclxL were of 1:1000 and 1:10,000, respectively. Primary and secondary antibody dilutions for BimEL were 1:500 and 1:5,000, respectively. Shown are blots for BclxL, BimEL, and β-actin. Also shown is densitometry for both BclxL and BimEL.

Inhibition of ERK or EGFR Results in Binding of BimEL to BclxL—BimEL exerts its pro-apoptotic effect by binding the anti-apoptotic Bcl2 proteins, Bcl2, and BclxL. Lung epithelial cells were infected with RSV with and without an MEK inhibitor (Fig. 8A) or an EGFR inhibitor (Fig. 8B). We then precipitated BclxL from the whole cell lysates and evaluated BimEL binding to BclxL. BclxL levels were measured as a control for the immunoprecipitations. Inhibition of ERK (U0126) resulted in binding of BimEL to BclxL. This was increased in the RSV-infected cells. The increased binding would block the anti-apoptotic effect of BclxL. Inhibition of EGFR had a similar effect. In this experiment, baseline binding of BimEL to BclxL can be seen. The baseline binding was decreased by RSV infection. If EGFR activity was blocked (AG1478), this was reversed and the RSV-infected cells showed increased binding of BimEL to BclxL. In both of these experiments, inhibition of a pathway leading to ERK activity in RSV-infected cells led to increased binding of BimEL to BclxL. This would inhibit the anti-apoptotic function of BclxL and sensitize the cells to viral-induced apoptosis. As a composite, these data demonstrate that RSV activates EGFR leading to ERK activation. Activation of this kinase contributes to RSV-induced inflammation. Our previous data have demonstrated a pro-survival effect of RSV-induced ERK activity. In this report, we expand that observation by showing that EGFR is upstream of ERK and that alterations of the Bcl2 protein balance downstream of ERK create conditions that favor cell survival. We conclude that RSV activation of EGFR plays an important role in inflammation and the prolonged survival of infected cells, contributing to viral replication and inflammation.
DISCUSSION

Epithelial cell death is an integral feature of airway inflammation. We have shown that RSV infection of lung epithelial cells results in cell death, but only after the cells have survived long enough to allow for the release of inflammatory mediators and support viral replication (4, 5, 7, 40). In this study we evaluated the role of EGFR in both RSV-induced inflammation and in sustained survival of infected epithelial cells. We found that RSV activated endogenous EGFR, leading to internalization and later degradation of the receptor. Consistent with the known link between EGFR and activation of the ERK pathway, we found that RSV-induced sustained activation of ERK required EGFR activity. In addition, both EGFR and ERK activity was necessary for optimal IL-8 production. Inhibiting EGFR by a number of means (chemical inhibition, blocking antibody, siRNA) led to a decrease in RSV-induced inflammatory response. We then directly evaluated activation EGFR. We found that RSV infection resulted in phosphorylation of tyrosines on the intracellular portion of the molecule, internalization and degradation of EGFR. This is the first description of RSV activating EGFR and the first time that activation of ERK by RSV has been shown to occur via EGFR. In examining a mechanism of EGFR activation by RSV, we found that RSV activated membrane-localized metalloprotease activity (ADAM-specific) and induced the release of the EGFR ligand, amphiregulin. We found that inhibition of matrix metalloproteinases decreased IL-8 production by RSV-infected epithelial cells. This suggests that RSV activates EGFR by activating a MMP resulting in cleavage of one (amphiregulin) or more of possible membrane bound EGFR proligands.

ERK is not only involved in inflammatory responses but also contributes to the sustained survival of RSV-infected cells (5, 47). When we evaluated the role of EGFR in cell survival, we found that inhibition of EGFR resulted in shortened survival of infected cells. The death of infected cells did not explain the effect of EGFR on inflammation as even with EGFR inhibition

Fig. 7. RSV-dependent increases in BclxL and decreases in BimEL require ERK and EGFR activity. A, A549 cells were infected with RSV ( moi of 5) for 24 h with and without the ERK inhibitor, U0126 (10 μM). Whole cell lysates were obtained and Western analysis performed for BclxL (32 kDa) and BimEL (22.1 kDa). Primary and secondary antibody dilutions for BclxL were of 1:1000 and 1:10,000, respectively. Primary and secondary antibody dilutions for BimEL were 1:500 and 1:5000, respectively. Shown are blots for BclxL, BimEL, and β-actin. Also shown is densitometry for both BclxL and BimEL. B, identical experiments were performed using the EGFR inhibitor, AG1478 (30 μM).
cell death occurred after the inflammatory response. Studies analyzing the mechanism of survival modulation by EGFR-linked ERK activity in RSV-infected cells, found that inhibition of ERK (or EGFR which is upstream of ERK activity) altered the balance between pro- and anti-inflammatory Bcl2 proteins in favor of apoptosis. The data demonstrate both viral-induced increases in BclXL and degradation of BimEL to be dependent on both ERK and EGFR. This is the first time that a virus has been shown to increase survival of infected cells by EGFR-dependent alterations in Bcl2 protein balance. The summary of the data presented here: RSV activates EGFR, but as a mechanism it is consistent with the literature on epithelial cells. A study by Prenzel et al. (14) demonstrated that G protein-coupled receptor transactivation of EGFR required processing of proHB-EGF by an undetermined MMP. Sahin et al. (46) have demonstrated distinct role for the MMPs, ADAM10 and ADAM17, in ectodomain shedding of six EGFR ligands. In bronchial epithelial cells, shedding of amphiregulin after exposure to particulates leads to GM-CSF release and further inflammation (49). In human mammary epithelial cells, TNF has been shown to augment its signals by activating a MMP leading to the shedding of the EGFR ligand, TGFα (50). One interesting hypothesis suggested by the literature is that EGFR is normally segregated to the basal lateral side of the epithelial monolayer and that RSV infection disrupts the monolayer enough to expose the receptor to apical ligands generated by MMP activity. The study by Vermeer et al. (15) elegantly demonstrates that in primary bronchial epithelial cells, the EGFR family members erbB2, erbB3, and erbB4 are found in airway epithelial cells as is the ligand heregulin. They show in well differentiated cells that erbB1–4 segregate to the basolateral side of the cells, while the ligand heregulin-α segregates to the apical side. In the Vermeer study, mechanical disruption of the monolayer results in exposure of the receptors to heregulin-α, resulting in increased growth to restore epithelial integrity. The lack of tight junctions in the cells we used does not allow us to directly ask if disruption of the monolayer by RSV contributes to the RSV-induced EGFR signaling. However, in the confocal analysis we performed, the majority of the EGFR was found at the basal side of the cell (data not shown), and infection with RSV caused a relocalization of the receptor to intracellular vesicles (see Fig. 2).

The link between ERK activity and BimEL degradation (and prevention of cell death) has been established in a number of recent studies in other systems. The study by Regnato et al. (36) demonstrated that detachment of epithelial cell-induced death (anoikis) involved up-regulation of BimEL. The up-regulation of BimEL required no β1-integrin engagement, down-regulation of EGFR expression, and inhibition of ERK signal-
RSV has been linked to asthma by epidemiological studies (51–56). Interestingly, increased expression of EGFR has also been found in asthmatic airways (57–59). Though not directly addressed by this study, it is interesting to speculate that increased EGFR expression in asthmatics would lead to increased inflammation in response to RSV in young asthmatics. In this study, we have shown that RSV activates EGFR during a productive epithelial infection. More specifically, we show that EGFR activation by RSV is linked to a downstream pathway involved in inflammation and cell survival (ERK). The ERK activation directly impacts inflammation (via IL-8 release) and mitochondrial homeostasis by altering the pro- versus anti-apoptotic Bcl2 family balance in favor of cell survival. In conclusion, this study demonstrates an important role for EGFR activity in the lifespan and inflammatory potential of RSV-infected epithelial cells.

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REFERENCES
1. McBride, T. J. (1999) J. Pediatr. 135, 28–32
2. Shay, D. K., Holman, R. C., Newman, R. D., Liu, L. L., Stout, J. W., and Anderson, L. J. (1999) J. Am. Med. Assoc. 282, 1440–1446
3. Stein, R. T., Sherrill, D., Morgan, W. J., Holberg, C. J., Halonen, M., Taussig, L. M., Wright, A. L., and Martinez, F. D. (1999) J. Pediatr. 135, 262–267
4. Monick, M. M., Staber, J. M., Thomas, K. W., and Hunninghake, G. W. (2004) J. Infect. Dis. 189, 1775–1781
5. Stein, R. T., Sherrill, D., Morgan, W. J., Holberg, C. J., Halonen, M., Taussig, L. M., Wright, A. L., and Martinez, F. D. (1999) J. Pediatr. 135, 262–267
6. Mastronarde, J. G., Monick, M. M., Mukaida, N., Matsushima, K., and Hunninghake, G. W. (1996) J. Immunol. 156, 2681–2687
7. Chen, W., Monick, M. M., Carter, A. B., and Hunninghake, G. W. (1999) Exp. Lung Res. 25, 13–26
8. Monick, M. M., Yarovskyi, T. O., Powers, L. S., Butler, N. S., Carter, A. B., Gudmunsdottir, G., and Hunninghake, G. W. (2003) J. Biol. Chem. 278, 53035–53044
9. Monick, M. M., Cameron, K., Powers, L. S., Butler, N. S., McCoy, D., Mallampani, R. K., and Hunninghake, G. W. (2004) Am. J. Respir. Cell Mol. Biol. 30, 769–779
10. Monick, M. M., Staber, J. M., Thomas, K. W., and Hunninghake, G. W. (2001) J. Immunol. 166, 2681–2687
11. Chen, W., Monick, M. M., Carter, A. B., and Hunninghake, G. W. (1999) Exp. Lung Res. 25, 13–26
12. Miller, W. E., and Raab-Traub, N. (1999) J. Biol. Chem. 274, 25025–25032
13. Miller, W. E., Earp, H. S., and Raab-Traub, N. (1995) J. Virol. 69, 5603–5609
14. Buchanan, F. G., Wang, D., Bargiacchi, F., and DuBois, R. N. (2003) J. Biol. Chem. 278, 34530–34536
15. Ullrich, A. (1999) J. Biol. Chem. 274, 322–326
16. Miller, W. E., Torres-Lozano, C., Puddicombe, S. M., Richter, A., Kimber, I., Dearman, R. J., Vrugt, B., Aalbers, R., Holgate, S. T., Djukanovic, R., Dearman, R. J., Vrugt, B., Aalbers, R., Holgate, S. T., Djukanovic, R., and Drazen, J. M. (2004) Nature 429, 769–777
17. Blachet, S., Rangolam, K., Baulig, A., Marano, F., and Baeza-Squiban, A. (2003) Am. J. Respir. Cell Mol. Biol. 29, 18488–18496
18. Chen, W. N., Woodbury, R. L., Kloth, G., and Hunninghake, G. W. (2003) J. Biol. Chem. 278, 18488–18496
19. Eberle, S., Mennett, R., and Nilsson, A. (2000) Pediatr. Allergy Immunol. 11, 193–197
20. Lewis, S. J., and Housley, P. H. (1998) J. Pediatr. 132, 439–445
21. Merchant, S., and Housley, P. H. (1997) J. Pediatr. 131, 333–337
22. Kinsella, K. A., and Housley, P. H. (1996) J. Pediatr. 129, 822–828
23. Kinsella, K. A., and Housley, P. H. (1995) J. Pediatr. 127, 757–764
24. Kinsella, K. A., and Housley, P. H. (1994) J. Pediatr. 125, 542–546
25. Kinsella, K. A., and Housley, P. H. (1993) J. Pediatr. 122, 308–314
26. Lemen, R. J. (1995) J. Fam. Pract. 40, 77–81
27. Levy, B. T., and Graber, M. A. (1997) J. Fam. Pract. 45, 473–481
28. Sigurs, N., Bjarnason, R., Sigurbergsson, F., Bjelland, M., and Bjaerkjenn, B. (1995) Pediatr. Pulmonol. 15, 309–314
29. Hamilton, L. M., Torres-Lozano, C., Puddicombe, S. M., Richter, A., Kimber, I., Dearman, R. J., Vrugt, B., Aalbers, R., Holgate, S. T., Davies, D. E., Wilson, S. J., and Davies, D. E. (2003) Clin. Exp. Allergy 33, 233–240
30. Polosa, R., Puddicombe, S. M., Krishna, M. T., Howarth, P. H., Holgate, S. T., and Davies, D. E. (2002) J. Allergy Clin. Immunol. 109, 75–81
31. Polosa, R., Puddicombe, S. M., Richter, A., Krishna, M. T., Howarth, P. H., Holgate, S. T., and Davies, D. E. (2000) PASEB J. 14, 1362–1374
