CD40-mediated Activation of NF-κB in Airway Epithelial Cells*

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We have reported previously that airway epithelial cells (AEC) express CD40 and that activation of this molecule stimulates the expression of inflammatory mediators, including the chemokine RANTES (regulated on activation normal T cell expressed and secreted). Because NF-κB regulates the expression of many inflammatory mediators, such as RANTES, we utilized CD40-mediated induction of RANTES expression to investigate the mechanisms that underlie CD40-mediated activation of NF-κB in AEC. Results demonstrate that, in AEC, intact NF-κB sites were required for CD40-mediated activation of the RANTES promoter. To examine activation of NF-κB binding directly, electrophoretic mobility shift analyses were performed. These analyses revealed that CD40 ligation stimulated NF-κB binding and that the activated NF-κB complexes were composed of p65 subunits. Additional studies focused on the CD40-triggered signaling pathways that facilitate NF-κB activation. Findings show that CD40 engagement activated the IκB kinases IKK-α and IKK-β and stimulated IκBα phosphorylation. Analyses also examined the role of tumor necrosis factor-associated factor (TRAF) molecules in CD40-mediated NF-κB activation within AEC. Stable transfectants expressing wild-type or mutant forms of the cytoplasmic domain of CD40 suggested that TRAF3, but not TRAF2, binding was essential for CD40-mediated RANTES expression. Further studies indicated that exogenous expression of wild-type TRAF3 enhanced activation of the RANTES promoter, whereas exogenous expression of wild-type TRAF2 inhibited this activation; TRAF3-mediated enhancement was dependent upon NF-κB. Together, these findings suggest that, in AEC, ligation of CD40 regulates the expression of inflammatory mediators, such as RANTES, via activation of NF-κB. Moreover, these results suggest that CD40-mediated signaling in AEC differs with previously reported findings observed in other cell models, such as B lymphocytes.

Airway epithelial cells serve two important functions. First, epithelial cells serve as barrier cells that protect the lung from the external environment. To this end, airway epithelial cells respond to a variety of environmental stimuli resulting in the alteration of their cellular actions such as ion transport and movement of airway secretions. Second, airway epithelial cells function as immune effector cells in response to noxious endogenous or exogenous stimuli. Growing evidence demonstrates that airway epithelial cells express and secrete a variety of immune molecules that modulate immune responses within the lung. The array of immune molecules expressed by airway epithelial cells includes adhesion molecules and a variety of cytokines, including the chemokine RANTES1 (1). Through the production of these molecules, the epithelium is now considered important in the initiation and exacerbation of airway inflammatory diseases such as asthma and cystic fibrosis.

CD40 is a member of the TNFRI family, which includes TNFRI (p55), TNFRII (p75), CD30, and CD40. It is expressed on immune effector cells, including B lymphocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, smooth muscle cells (4), and epithelial cells (5–7). CD40 and its natural ligand, CD40L, play a central role in the regulation of humoral and cell-mediated immunity (8). Depending on the cell type and the local microenvironment, protein-protein interactions between CD40 and CD40L may modulate cell proliferation, differentiation, apoptosis, isotype switching, and inflammatory mediator production (9).

Members of the TNFRI family, including CD40, display homology in their extracellular ligand-binding domains, which are composed of tandemly repeated cysteine-rich modules. The interactions of these modules create a three-dimensional structure that provides ligand specificity (reviewed in Ref. 10). Members of the TNF ligand family trimerize, thereby allowing their cognate receptors to aggregate upon binding; this receptor aggregation, in turn, activates signal transduction cascades that facilitate the CD40-mediated actions listed above. Like other TNF family members, the cytoplasmic domain of CD40 lacks intrinsic catalytic activity; however, this domain associates with "signaling adapter proteins" termed TNFR-associated factors (TRAFs). To date, six different TRAF molecules (TRAF1–TRAF6) have been identified. Several studies have demonstrated that the cytoplasmic domain of CD40 associates with TRAF2, TRAF3, TRAF5, and TRAF6 (11–15).

Engagement of CD40 triggers multiple signaling pathways, including the kinase cascades that activate the transcription factor NF-κB. Recent reports (12, 13, 16) suggest that overexpression of TRAF2, TRAF5, and TRAF6, but not TRAF3, triggers NF-κB activation in HEK293 cells. In contrast, a separate study reported that B cells expressing a mutant form of CD40 that was able to bind TRAF2, but not TRAF6, stimulated NF-κB activation upon CD40 engagement (17). Moreover, other reports demonstrated that mice lacking functional TRAF2 molecules were able to activate NF-κB in response to CD40- and

1 The abbreviations used are: RANTES, regulated on activation normal T cell expressed and secreted; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAFs, TNFR-associated factors; RPA, ribonucleotide protection assay; EMSAs, electrophoretic mobility shift assays; ELLISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; WT, wild type; DN, dominant-negative; IL, interleukin; GST, glutathione S-transferase.
TNF-mediated signals (15, 18). Together, such conflicting results suggest that TRAF molecules exhibit cell type specificity with regard to NF-κB activation.

Recently, we described CD40 expression on airway epithelial cells and demonstrated that CD40 engagement on these cells stimulates the expression of inflammatory mediators, including the chemokine RANTES (5). The studies presented here extend these original findings by examining the signaling mechanisms that underlie CD40-mediated inflammatory mediator expression. Specifically, these studies examined CD40-mediated activation of the transcription factor NF-κB within airway epithelial cells. Presently, information regarding CD40 signaling is based primarily on studies performed with B cells (14, 19–22). To date, no studies have examined the CD40-mediated signaling mechanisms within an airway epithelial cell system. The results presented here suggest that CD40 ligation in airway epithelial cells triggers the activation of NF-κB through a signaling pathway that involves the IkB kinases IKKa and IKKβ, IκBα phosphorylation, and TRAF3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Experiments employed the human airway epithelial cell lines 9HTEo− (tracheal; a gift from Dr. Dieter Gruenten, University of California, San Francisco) (23) and 16HBE14o− (bronchial; a gift from Dr. Randolph Noelle, Dartmouth Medical School, Lebanon, NH) for the time periods indicated using TRIZol (Invitrogen) in accordance with the manufacturer’s protocol. A radiolabeled probe was generated using the hCK-5 RNA multiple transcripts from BD PharMingen kit components (BD PharMingen) were used to perform the RPA. Each sample RNA (30 μg) and 6 × 10^5 cpm of radiolabeled probe were mixed in hybridization buffer, heated to 90 °C for 5 min, allowed to cool to 50 °C, and incubated for 16 h. RNAse A/T1 (1:100) diluted in digestion buffer was added, and RNA digestion was allowed to proceed for 45 min. Inactivation/precipitation solution was added, and the samples were incubated for 30 min. After centrifugation, the buffer was removed, and the RNA allowed to air-dry. The RNA was resuspended in gel loading buffer and electrophoresed on a 4-mm thick 4% polyacrylamide gel in Tris borate-EDTA (TBE) buffer. After electrophoresis, the gel was transferred to filter paper and exposed to film.

**RANTES Promoter Analysis**—To analyze the role of NF-κB in CD40-mediated activation of the RANTES promoter in airway epithelial cells, constructs containing portions of the RANTES promoter ligated to a luciferase reporter gene were generated and provided by Dr. Hiro Moriuchi, National Institutes of Health, Bethesda (26). Briefly, a 1.4-kb 5′-noncoding sequence of the RANTES gene (R1.4) was cloned into pGL2-basic (Promega Corp., Madison, WI). Site-directed mutations of 5′-GCGATTG-3′ (promoter, 1:1000 in TBS containing 0.1% Tween and 5% BSA; New England Biolabs, Beverly, MA) followed by a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (diluted 1:2000 in TBS containing 1% Tween and 5% BSA). Immunoblots were developed using chemiluminescence.

**Dominant-negative Analysis of IKK Molecules**—For analysis of IKK molecule involvement in CD40-mediated NF-κB activation, 9HTEo− cells were co-transfected transiently with constructs encoding wild-type or dominant-negative forms (K44A) of IKKα or IKKβ (25–30, generous gifts from Dr. Randolph Noelle) together with constructs encoding the intact R1.4 RANTES promoter or β-galactosidase activity as described above. Briefly, using LipofectAMINE Plus (6 μg/well; Invitrogen), cells were co-transfected with the appropriate IKK construct or an empty vector control (0.5 μg/well), the R1.4 construct (2.0 μg/well), and the βSV-β-galactosidase construct (0.5 μg/well). Following transfection, cells were cultured in the presence and absence of sCD40L (400 ng/ml) for 18 h at 37 °C. Cells were then harvested, and luciferase activity was measured using the Dual Luciferase Reporter assay system (Promega Corp.). Relative transfection efficiency was determined using the β-gal Reporter Assay System (Promega Corp.) according to manufacturer’s protocols.

**Electrophorectic Mobility Shift Assays (EMSA)**—To examine CD40-mediated effects on NF-κB binding directly, electrophoretic mobility shift assays (EMSAs) were performed as described previously (27). Briefly, nuclear extracts from cells stimulated with and without sCD40L (400 ng/ml) for the time periods indicated were prepared. Cells were grown in 100-mm dishes and then were stimulated with or without sCD40L (400 ng/ml) as indicated. Cells were washed with cold phosphate-buffered saline, harvested by scraping, and pelleted. Cells were resuspended in 1 ml of buffer A (10 mM KCl, 20 mM HEPS, 1 mM MgCl2, 1 mM DTT, 0.4 mM PMSF, 1 mM NaN3, 15% glycerol, 1 mM NaN3, and 1 mM Na2VO4), and nuclear extracts were cleared by centrifugation at 14,000 × g for 15 min at 4 °C. EMSA was performed using the following oligonucleotides as probes and/or competitors: the oligonucleotides 5′-att gta gag act cta tta gg-3′ (Invitrogen) and 5′-tgg agg gga tgg cca ttc gcgg-3′ (Santa Cruz Biotechnology). The gel shift reaction was then prepared by incubating the P-labeled oligonucleotide (250,000 cpm/reaction) with 10 μg of nuclear extract in a volume of 20 μl containing 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-Cl, pH 7.5, 5% glycerol, and 1 μg of poly(dI-dC) for 20 min at room temperature. For competition assay, molar excess (as indicated) of the respective unlabeled DNA was included in the initial gel shift reaction mix. For supershift analysis, 1 μl of antibody (directed against the NF-κB family members, Santa Cruz Biotechnology) directed against the NF-κB subunits p50, p52, p65, c-Rel, and RelB) via immunoblotting. Specifically, equivalent amounts of protein (25 μg/lane; determined via Bio-Rad DC Protein Assay, Bio-Rad) for each sample were electrophoresed and transferred to a polyvinylidene difluoride membrane. Nonspecific sites were blocked with Tris-buffered saline (TBS; 20 mM Tris-HCl, 140 mM NaCl, pH 7.6) and incubated for an additional 45 min at room temperature. Bound and free DNA were resolved by electrophoresis through a 4% polyacrylamide gel at 190 V in 1× TGE buffer (50 mM Tris-Cl, 380 mM glycine, and 2 mM EDTA). Dried gels were processed via autoradiography.

**Immunoblotting for NF-κB Subunits**—Cells were lysed with lysis buffer (10 μg Tris, 0.15 mM NaCl, 0.5% Triton X, and the protease inhibitor cocktail, proteinant (1 μg/ml each), and 1 μg/ml PMSF) and then examined for the presence of the NF-κB subunits p50, p52, p65, c-Rel, and RelB via immunoblotting. Specifically, equivalent amounts of protein (25 μg/lane; determined via Bio-Rad DC Protein Assay, Bio-Rad) for each sample were electrophoresed and transferred to a polyvinylidene difluoride membrane. Nonspecific sites were blocked with Tris-buffered saline (TBS; 20 mM Tris-Cl, 140 mM NaCl, pH 7.6) and incubated for an additional 45 min at room temperature. Bound and free DNA were resolved by electrophoresis through a 4% polyacrylamide gel at 190 V in 1× TGE buffer (50 mM Tris-Cl, 380 mM glycine, and 2 mM EDTA). Dried gels were processed via autoradiography.
were lysed with lysis as described above. Equivalent amounts of protein (25 μg/lane) for each sample were electrophoresed and transferred to a polyvinylidene difluoride membrane. Blots were then immunoblotted, as described above, with a polyclonal rabbit antibody directed against InBe-Ser-32 (diluted 1:1000 in TBS containing 0.1% Tween and 5% BSA) followed by a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (diluted 1:2000 in TBS containing 0.1% Tween and 5% BSA). Immunoblots were developed using chemiluminescence. Blots were then stripped (0.2 N NaOH for 5 min at room temperature) and reprobed with a polyclonal rabbit antibody against IBe (diluted 1:1000 in TBS containing 0.1% Tween and 5% BSA) in order to verify equivalent IBe protein expression in each sample.

Detection of TRAF Molecule Expression—To examine TRAF molecule expression in airway epithelial cells, 9HTEo− and 16HBE14o− cells were cultured and lysed in lysis buffer as described above. Whole cell lysates (5 × 106 cells/sample) were precleared with glutathione-agarose (Sigma) for 2 h at 4 °C and then immunoprecipitated with GST or GST-CD40ct (a GST-CD40 fusion protein containing the cytoplasmic tail of CD40; a gift from Dr. Randolph Noelle) each at 20 μg/ml and glutathione-agarose for 18 h at 4 °C. Precipitated proteins were washed three times with cold lysis buffer, eluted, electrophoresed, and immunoblotted with rabbit polyclonal antibodies specific for TRAF2 or TRAF3 (each at 1 μg/ml; gifts from Dr. Randolph Noelle) followed by a goat anti-rabbit-horseradish peroxidase secondary antibody (1:2000 dilution in lysis buffer, Sigma), as described above, and developed via ECL chemiluminescence (Amersham Biosciences).

Stable CD40 Transfectants—HT-29 cells, which do not express endogenous CD40 (5), were transfected stably with constructs encoding wild-type or mutant forms of human CD40. Specifically, cells were transfected in 100-mm dishes using LipofectAMINE Plus, as described above, and developed via ECL chemiluminescence (Amersham Biosciences).

Dominant-negative Analysis of TRAF Molecules—For analysis of TRAF molecule involvement in CD40-mediated NF-κB activation, 9HTEo− cells were transfected transiently with constructs encoding wild-type (WT) or dominant-negative (DN) forms of the molecules TRAF2 (11) and TRAF3 (33) as described above. Briefly, using LipofectAMINE Plus (6 μg/well), cells were co-transfected with the appropriate TRAF construct or empty vector control (0.5 μg/well), the R14 or ΔB construct (2.0 μg/well), and the pSV-β-galactosidase construct (0.5 μg/well) in 6-well plates for 6 h at 37 °C. WT and DN forms of TRAF molecules were utilized in these studies and were gifts from Dr. Randolph Noelle; each construct contained a peptide tag, either c-Myc or FLAG. Following transfection, cells were stimulated with or without sCD40L as described above. Cells were then harvested, lysed, and analyzed for luciferase activity via the Dual Luciferase Reporter assay system (Promega Corp.).

Statistical Analysis—Data are expressed as the mean ± S.D. of replicate determinations as indicated. Statistical significance was determined by analysis of variance. A p ≤ 0.05 was considered significant.

RESULTS

CD40 Engagement Stimulates RANTES mRNA Expression—We have demonstrated previously (5) that engagement of CD40 expressed on airway epithelial cells increased the protein production of several inflammatory mediators, including the chemokine RANTES. To determine whether engagement of epithelial CD40 also modulated RANTES expression at the steady-state mRNA level, RPA was performed. To this end, 9HTEo− airway epithelial cells were cultured in the presence and absence of soluble CD40L (sCD40L) for various time points and prepared for analysis. The 9HTEo− cell line was utilized because it expresses CD40 on its surface constitutively and is responsive to stimulation by sCD40L (5). Results presented in Fig. 1A indicate that CD40 ligation up-regulated RANTES mRNA expression and that this expression increased over time. Importantly, CD40 ligation also up-regulated the mRNA expression of other chemokines, including MCP-1 and IL-8; we have shown previously (5) that the protein expression of MCP-1 and IL-8 is modulated upon CD40 engagement.
Previous studies (34) have reported that, in airway epithelial cells, RANTES mRNA expression is delayed in comparison with the mRNA expression of other chemokines, including IL-8. Because RANTES mRNA expression did not reach maximal levels until 18 h post-treatment with sCD40L, we determined whether de novo protein synthesis was required for CD40-mediated increases in RANTES mRNA expression in airway epithelial cells. For this analysis, 9HTE0—cells were cultured in the presence and absence of sCD40L and/or cycloheximide, a protein synthesis inhibitor that can cause super-induction of some genes through the prevention of mRNA degradation. Results presented in Fig. 1B demonstrate that cycloheximide treatment alone induced RANTES mRNA expression suggesting that newly synthesized proteins degrade RANTES mRNAs transcripts in the absence of stimuli. In cells stimulated with cycloheximide and sCD40L simultaneously, interestingly, RANTES mRNA expression was not significantly increased over that level observed with cycloheximide or sCD40L alone (Fig. 1B). In contrast, mRNA expression of MCP-1 and IL-8 was superinduced in the presence of cycloheximide and sCD40L (Fig. 1B). Together, these results indicate that engagement of CD40 on airway epithelial cells induces the mRNA expression of RANTES, but not MCP-1 or IL-8, in a manner that is not regulated by de novo protein synthesis.

CD40-mediated RANTES Gene Activation Is Dependent upon NF-κB—NF-κB regulates the expression of many genes that encode inflammatory mediators, including RANTES (35) (reviewed in Ref. 36). Because previous studies indicate that CD40 engagement activates NF-κB in HEK293 cells (12, 13, 16) and B lymphocytes (17), we determined whether CD40 ligation in airway epithelial cells stimulates the RANTES gene via activation of NF-κB. For this analysis, 9HTE0—cells were transfected with reporter constructs that contained the RANTES promoter with either intact or mutated NF-κB sites. Two putative NF-κB sites, κB1 (−44 relative to the transcription start site) and κB2 (−30), have been shown to contribute positively to RANTES promoter activity in various cell types, including T lymphocytes (26). In parallel, cells were transfected with a construct encoding tandem NF-κB sites fused to a luciferase reporter gene (NF-κB-luc) as a positive control. Transfected cells were then cultured in the presence and absence of sCD40L and analyzed for changes in reporter activity. As shown in Fig. 2, sCD40L induced approximately a 3-fold increase in activation of the NF-κB-luc reporter construct as well as the intact RANTES promoter construct. Importantly, mutations within either the κB1 or κB2 NF-κB sites rendered the RANTES promoter construct non-responsive to the effects of sCD40L (Fig. 2). These results suggest that ligation of CD40 expressed on airway epithelial cells activates the RANTES promoter via a mechanism that is dependent upon NF-κB.

CD40 Ligation Induces NF-κB Binding—To examine directly the effects of CD40 engagement on the binding activity of NF-κB in airway epithelial cells, EMSAs were performed. The EMSA stabilizes DNA-protein interactions, facilitates the measurement of protein DNA-binding affinity, and through the use of specific antibodies, permits identification of the transcription factor subunits participating in the DNA-protein complex. For these experiments, airway epithelial cells were cultured in the presence and absence of sCD40L for various time points and then prepared for EMSA analysis with oligonucleotides representing the RANTES promoter NF-κB sites κB1 and κB2 described above. As shown in Fig. 3A, CD40 ligation induced NF-κB binding to the κB1 site within 30 min post-CD40L treatment; binding was maximal at 30 min and decayed thereafter. In contrast, NF-κB binding to the κB2 site appeared to be maximal at 4 h following CD40 engagement (Fig. 3A). Importantly, CD40-mediated NF-κB binding to both κB1 and κB2 sites was inhibited with the addition of increasing amounts of the respective cold oligonucleotide competitor (data not shown). Interestingly, κB1 cold competitor also out-competed NF-κB binding to the κB2 oligonucleotide and vice versa (Fig. 3B). These findings indicate that, in airway epithelial cells, CD40 engagement activated the binding of multiple NF-κB complexes to the RANTES promoter directly.

At present, five members of the NF-κB subunit family have been characterized; these members include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (reviewed in Ref. 37). To identify the subunits participating in the CD40-mediated NF-κB complexes detected above, antibodies directed against each of the NF-κB subunits were included in the EMSA analyses. Fig. 3C demonstrates that only an anti-p65 antibody shifted the NF-κB complexes bound to κB1; antibodies directed against other NF-κB subunits failed to do so. Similar results were observed for NF-κB complexes bound to κB2 (data not shown). Western blot analysis for the protein expression of each of the five NF-κB subunits described above revealed that 9HTE0—cells expressed all of these subunits (data not shown). These results suggest that CD40 engagement triggers the binding of an NF-κB complexes composed of p65 subunits to the RANTES promoter in airway epithelial cells.

Ligation of Epithelial CD40 Triggers Phosphorylation of IκBα—NF-κB is retained in the cytoplasm of unactivated cells through interaction with members of the IκB inhibitor family, including IκBα (38). Phosphorylation (at serines 32 and 36) and subsequent degradation of IκBα releases NF-κB and allows NF-κB to translocate to the nucleus and activate transcription (38). Because phosphorylation at Ser-32 is required for the release of NF-κB, phosphorylation at this site is a reliable marker of NF-κB activation. To further support the role of CD40 engagement in the activation of NF-κB within airway epithelial cells, the ability of sCD40L to trigger IκBα phosphorylation in these cells was examined. For this analysis, airway epithelial cells were stimulated with and without sCD40L for varying time points, lysed, and then examined for the presence of phosphorylated IκBα via immunoblotting with an antibody that recognizes IκBα-Ser-32 specifically. Blots were then stripped and reprobed with an antibody against IκBα in order to monitor total IκBα levels in each lane. As shown in Fig. 4, sCD40L induced an increase (~4-fold over basal levels) in the phosphorylation of IκBα.
phosphorylation of IκBα at serine 32 within 5 min post-sCD40L treatment; the sCD40L-induced increase in phospho-IκB-α (Ser-32) decreased over time. These results support the conclusion that CD40 engagement on airway epithelial cells activates NF-κB, which may stimulate the gene expression of immune molecules such as the chemokine RANTES.

IKK-α and IKK-β Play a Role in CD40-mediated NF-κB Activation—The serine/threonine kinases IKK-α (28) and IKK-β (29) associate directly with IκB proteins and phosphorylate the requisite residues that promote IκB degradation. Because CD40 ligation stimulated IκBα phosphorylation in airway epithelial cells (Fig. 4), the role of IKK-α and/or IKK-β in CD40-mediated NF-κB activation was examined. For these studies, airway epithelial cells were transfected with constructs encoding dominant-negative (DN) forms of IKK-α and IKK-β together with the R1.4 RANTES promoter-reporter construct. The DN-IKK-α and DN-IKK-β constructs each contained alanine substitutions of conserved lysine residues within the kinase domain, thereby rendering each kinase catalytically inactive (28–30). As shown in Fig. 5, expression of DN-IKK-α or DN-IKK-β blocked activation of the RANTES promoter either in the presence or absence of sCD40L (Fig. 5A). These results suggest that IKK-α and IKK-β are required for CD40-mediated activation of an exogenous RANTES promoter in airway epithelial cells.

To determine whether IKK-α and IKK-β were also required for CD40-mediated activation of the endogenous RANTES promoter, airway epithelial cells transfected with DN forms of IKK-α and IKK-β were stimulated with and without sCD40L and then examined for secreted protein expression of RANTES via ELISA. As shown in Fig. 5B, expression of DN-IKK-α or DN-IKK-β blocked RANTES protein expression in airway epithelial cells. These results suggest that IKK-α and IKK-β are required for activation of the endogenous RANTES.

TRAF Molecule Expression in Airway Epithelial Cells—CD40 engagement triggers the activation of signaling pathways that involve a variety of molecules, including TRAFs. To examine TRAF molecule expression in airway epithelial cells, lysates from these cells were immunoprecipitated with a GST-CD40 fusion protein containing the cytoplasmic tail of CD40 (GST-CD40cyt). Precipitated proteins were then immunoblotted with antibodies specific for TRAF2 and TRAF3 as described under “Experimental Procedures.” As shown in Fig. 6, GST-CD40cyt immunoprecipitated TRAF2 and TRAF3 proteins from airway epithelial cells; GST control immunoprecipitations showed little or no cross-reactivity for TRAF2 and TRAF3 detection.

TRAF Molecule Involvement in CD40-mediated RANTES Expression—TRAF2 and TRAF3 have been shown recently (32) to have significantly higher binding affinities to the cytoplasmic tail of CD40 than the molecules TRAF5 or TRAF6. Therefore, the involvement of TRAF2 and TRAF3 in CD40-mediated RANTES expression. To examine the role of TRAF2 and TRAF3 in RANTES expression, HT-29 cells, a colon carcinoma epithelial cell line that does not express CD40 (5), were transfected stably with constructs that encoded wild-type or mutant forms of human CD40. The mutant forms of human CD40 that were significantly different from the wild type were CRD and TRAF5; the doublet observed in Fig. 6B is composed of both TRAF3 and TRAF2 proteins as the anti-TRAF3 antibody cross-reacts with TRAF2. The TRAF molecules TRAF5 and TRAF6 were also detected via this approach (data not shown). The presence of TRAF molecules in airway epithelial cells confirms their availability for participation in CD40 signaling events.

TRAF Molecule Involvement in CD40-mediated RANTES Expression—TRAF2 and TRAF3 have been shown recently (32) to have significantly higher binding affinities to the cytoplasmic tail of CD40 than the molecules TRAF5 or TRAF6. Therefore, the involvement of TRAF2 and TRAF3 in CD40-mediated RANTES expression. To examine the role of TRAF2 and TRAF3 in RANTES expression, HT-29 cells, a colon carcinoma epithelial cell line that does not express CD40 (5), were transfected stably with constructs that encoded wild-type or mutant forms of human CD40. The mutant forms of human CD40 that were analyzed in these experiments included a CD40 molecule that contained either an alanine substitution for threonine at posi-
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As shown in Fig. 8, engagement of CD40 on WT-CD40 clones enhanced RANTES production ~2-fold over basal levels. In contrast, neither T254A, Q263A, nor Δ201 clones were responsive to the effects of CD40 ligation (Fig. 8). Significantly, Q263A and Δ201 clones expressed little or no detectable RANTES protein in the presence and absence of CD40 engagement as compared with WT-CD40 or T254A clones (Fig. 8). Together, these findings suggest that binding of TRAF3, but not TRAF2, is critical for CD40-mediated RANTES expression.

TRAF Molecule Involvement in CD40-mediated RANTES Expression—Previous studies (11) have utilized the exogenous expression of TRAF molecules, either wild-type or dominant-negative forms, to elucidate signaling mechanisms initiated via receptors that associate with these molecules. To further examine the role TRAF2 and TRAF3 in CD40-mediated activation of the RANTES promoter, airway epithelial cells were transfected with plasmid constructs containing either wild-type or dominant-negative forms of TRAF2 and TRAF3 molecules together with the R1.4 RANTES promoter-reporter construct. The TRAF2 and TRAF3 constructs that were utilized for these studies contained TRAF molecule coding regions that were either wild-type or truncated in the ring and zinc finger domains. Truncations of these domains rendered the TRAF molecules as dominant-negative mutants, thereby allowing these molecules to bind the cytoplasmic tail of CD40 but eliminating their ability to signal downstream events (11, 33). In addition, each TRAF construct contained a peptide tag so that its expression could be confirmed independently via immunoblotting. Following transfection, cells were cultured in the presence and absence of sCD40L and then analyzed for changes in reporter activity and the relative expression of each epitope-tagged TRAF molecule.

As shown in Fig. 9, sCD40L induced approximately a 3-fold increase in the RANTES reporter activity of mock control transfectants; this increase, however, was not observed in cells transfected with either WT- or DN-TRAF2 constructs. In contrast, exogenous expression of WT-TRAF3 enhanced the basal activation of the RANTES promoter relative to mock controls; this activation was further increased in the presence of sCD40L. Surprisingly, expression of DN-TRAF3 also resulted in elevated basal activation of the RANTES promoter relative to mock controls; this level of activation, however, was not responsive to the effects of sCD40L. Exogenous expression of
TRAF-WT and TRAF-DN molecules was detected in each respective sample (Fig. 9). Together, these results indicate that TRAF3, but not TRAF2, regulates CD40-triggered signaling pathways positively.

To determine whether exogenous expression of TRAF3 activated the RANTES promoter via an NF-κB-dependent mechanism, cells were transfected with TRAF3-WT and the ΔκB1 mutant RANTES promoter-reporter construct, stimulated with sCD40L, and then analyzed as described above. Interestingly, mutations within the κB1 site decreased the ability of TRAF3 to constitutively activate the RANTES promoter (Fig. 10); similar results were observed with κB2 (data not shown). These
results suggest that TRAF3 constitutively activates the RANTES promoter via an NF-κB-dependent pathway.

**DISCUSSION**

To date, the literature describing CD40-activated signaling pathways has been performed largely in B lymphocytes. Although this literature is extensive, it is contradictory (even within B cells) suggesting that CD40-mediated events may be cell-specific. Because of this possibility, we examined signaling pathways that were activated as a consequence of CD40 ligation in airway epithelial cells. To this end, our studies focused on the signaling pathways that lead to NF-κB activation. NF-κB regulates the expression of many genes, including the gene that encodes the chemokine RANTES. We have shown previously that engagement of CD40 stimulates RANTES protein expression in airway epithelial cells (5). Results presented in this study indicate that CD40 engagement regulates RANTES gene expression by means of NF-κB activation.

Results presented herein demonstrate that ligation of CD40 expressed on airway epithelial cells stimulates signaling events that culminate in the activation of NF-κB. These results were generated through an integrated analysis of the effects of CD40 ligation on NF-κB binding in vitro and in vivo as well as on specific signaling events that promote NF-κB activation. Specifically, the data show that CD40 engagement triggered NF-κB binding to the κB1 and κB2 sites within the RANTES promoter. NF-κB binding to the κB1 and κB2 sites occurred with varying kinetics indicating that NF-κB complexes may bind to these sites in a cooperative fashion. Competition and supershift electrophoretic mobility shift analyses revealed that the composition of the CD40-activated NF-κB complexes was similar and consisted of p65 subunits; the further characterization of these NF-κB complexes is in progress currently.

These results contrast sharply with previously published reports describing CD40-activated NF-κB complexes in B cells. Warren and co-workers (38) reported that, upon stimulation with CD40L, NF-κB complexes composed mainly of p50 and RelB were observed in the BCL1-3B3 Lymphaoma cell line. An earlier report by Lapointe and co-workers (39) demonstrated that CD40-mediated activation of fresh Epstein-Barr virus-negative lymphocytes stimulated the formation of NF-κB complexes composed of p65 and c-Rel subunits. The consequence of such differences in NF-κB complex composition between airway epithelial cells and B lymphocytes is not clear at present; however, these results do underscore the cell-specific nature of CD40-mediated signaling.

With regard to the triggering of specific signaling events that promote NF-κB activation in airway epithelial cells, the data demonstrate that CD40 ligation rapidly stimulates IκBα phosphorylation at serine 32. Phosphorylation at this position occurred within 5 min post-sCD40L treatment and decayed significantly thereafter. Such kinetics are consistent with inhibitor-induced IκBα phosphorylation observed in a number of cell model systems (reviewed in Ref. 36). Because the targeted phosphorylation of IκBα is mediated by a multisubunit kinase complex that contains IKK-α and IKK-β (36), additional experiments examined the role of these kinases in CD40-mediated RANTES promoter activation. For these studies, constructs encoding wild-type (WT) or dominant-negative (DN) forms of each of these kinases were employed. Results presented herein demonstrated that expression of DN-IKK-α or -IKK-β blocked activation of both exogenous and endogenous...
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RANTES promoters in cells stimulated with or without sCD40L. These results further support the conclusion that ligation of CD40 activates NF-κB. Moreover, these findings corroborate previous studies (28, 29, 32, 40) that have shown that overexpression of DN-IKK-α or -IKK-β fail to activate NF-κB reporter genes and inhibit TNFα-induced NF-κB activation.

The CD40 cytoplasmic domain, which lacks intrinsic kinase activity, interacts with TRAF molecules to trigger downstream signaling events. Studies presented here focused on the role of TRAF2 and TRAF3 in the CD40-mediated activation of the RANTES promoter. Although several TRAF molecules have been shown to associate with the cytoplasmic tail of CD40, TRAF2 and TRAF3 were highlighted in these studies because recent reports (32) indicate that TRAF2 and TRAF3 exhibit higher binding affinities for CD40 than other TRAF molecules. Moreover, the importance of TRAF2 and TRAF3 as ubiquitous signaling molecules has been well documented in several TRAF-knockout and transgenic-related studies. For example, TRAF2-deficient mice suffer from atrophy of the thymus and the spleen as a result of increased sensitivity to TNF-induced apoptosis and are defective in TNF-mediated stress-activated protein kinase/c-Jun N-terminal kinase activation (14, 41). Loss of the Traf3 gene in mice causes impaired T cell-dependent immunity and results in early postnatal lethality (41).

Data presented here suggest that TRAF3, but not TRAF2, positively regulates CD40-mediated events in airway epithelial cells. Moreover, these data also suggest that TRAF3 regulation of CD40-mediated events is dependent upon NF-κB activation. These data may be explained by several hypotheses. First, endogenous TRAF3 may be sequestered and not available for immediate CD40 receptor association. Ling and Goeddel (42) have reported that CD40 engagement may release sequestered TRAF3 and, thereby, make it available for receptor association. This mechanism would explain the ability of exogenous (“free”) WT-TRAF3 to activate the RANTES promoter in the absence of sCD40L as well as the observed enhancement of promoter activation in the presence of sCD40L. Second, TRAF3 may associate with other signaling molecules, such as TRAF5 (43) or epithelial cell-specific factors, via its C-terminal domain in order to activate downstream signaling events that are not CD40-specific. Third, TRAF3 may compete with another protein for a shared binding site on the cytoplasmic tail of CD40 and “push” it off to initiate a signaling cascade that is not CD40-responsive. Both of these mechanisms could account for the observed DN-TRAF3-mediated activation of the RANTES promoter in the absence of CD40 ligation; CD40 ligation had no affect on this response. Fourth, TRAF2 is a negative regulator of CD40-mediated events in airway epithelial cells. TRAF2 may interact with signaling molecules, such as TRAF1, to negatively regulate CD40-activated signaling events. Alternatively, TRAF2 may compete with TRAF3 for binding to the CD40 cytoplasmic tail. It has been postulated that TRAF2 and TRAF3 bind to the CD40 cytoplasmic domain at overlapping sites (11). Such mechanisms would explain the observation that both WT- and DN-TRAF2 inhibited CD40-mediated activation of the RANTES promoter.

The observation that TRAF3 and not TRAF2 positively regulates CD40-mediated activation of the RANTES promoter is in sharp contrast with previously published reports examining the role of TRAF molecules in CD40-mediated signaling. Studies have shown that co-transfection of HEK293 cells with plasmids encoding CD40 and full-length TRAF2, TRAF5, and TRAF6 molecules induces a significant increase in NF-κB activation (12, 13, 16). Importantly, co-transfection of HEK293 cells with plasmids encoding CD40 and either a mutant form of TRAF2 or a full-length TRAF3 inhibited NF-κB activation, suggesting that TRAF3 disrupts TRAF2-CD40 interactions (11). Interestingly, CD40 ligation on B lymphocytes has been shown to recruit both TRAF2 and TRAF3 to the CD40 receptor complex (14). Moreover, studies examining TRAF2-deficient or TRAF2-dysfunctional mice revealed a mild effect on NF-κB activation, suggesting that TRAF2-independent pathways exist (15, 18). Despite the fact that TRAF3 appears to interact with CD40, the role of TRAF3 in CD40-mediated signaling is unclear. A recent study (44) demonstrates that expression of a dominant-negative form of TRAF3 in Ramos B cells results in the abrogation of p38 and partial blockage of JNK activation, indicating that TRAF3 initiates independent signaling pathways via p38 and JNK. In contrast, other studies (11) have shown that TRAF3 does not play a role in the JNK pathway. It should be noted that, recently, van Eyndhoven et al. (45) have cloned isoforms of TRAF3 resulting from splice-deletion variants capable of activating NF-κB in HEK293 cells. The findings presented herein together with results reported previously, as described above, support the hypothesis that TRAF molecules exhibit cell type specificity with regard to NF-κB activation.

Engagement of CD40 triggers multiple signaling pathways, including the kinase cascades that activate NF-κB. CD40, as borne out by the data presented here, may signal differently in airway epithelial cells versus other cell models, including B cells. Future studies in our laboratory will examine the proteins that bind to CD40 endogenously in order to address these possibilities. It is important to analyze further the specificity of TRAF signaling because the pleiotropic nature of receptors such as CD40 is dictated by finely regulated differences in adapter protein associations.

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