Defective Activation of c-Src in Cystic Fibrosis Airway Epithelial Cells Results in Loss of Tumor Necrosis Factor-α-induced Gap Junction Regulation

Received for publication, August 13, 2002, and in revised form, December 9, 2002
Published, JBC Papers in Press, December 27, 2002, DOI 10.1074/jbc.M208264200

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Tumor necrosis factor-α (TNF-α) signaling is central to the transmission of the innate immune response and subsequent activation of the adaptive immune system. The functioning of both systems is required for optimal clearance of pathogens from the airways. In cystic fibrosis (CF), dysfunction of the CF transmembrane conductance regulator (CFTR) is associated with recurrent pulmonary infections despite an intense inflammatory and immune response. We reported recently that TNF-α decreased gap junction connectivity in non-CF airway cells, a mechanism that was absent in CF cells expressing the ΔPhe-508 mutant of CFTR. We have now identified the tyrosine kinase c-Src as a possible pathway between the mediators of inflammation and the gap junction protein connexin43 (Cx43). Indeed, TNF-α increased the proportion of activated c-Src in non-CF airway cells. Moreover, pharmacological antagonists and expression in non-CF cells of a dominant negative construct of c-Src prevented Cx43 channel closure by TNF-α. Finally, gap junction channel closure was prevented by expression of a Cx43 mutant lacking tyrosine phosphorylation sites for c-Src. Additional experiments showed that activation of c-Src was defective in CF airway cells but rescued in CFTR-corrected CF cells. These data suggest that CFTR dysfunction is associated with altered TNF-α signaling, resulting in the persistence of gap junction connectivity in CF airway cells. We propose that altered regulation of c-Src may contribute to the dysregulated inflammatory response that is characteristic of the CF phenotype.

Airway inflammation, characterized by intense influx of neutrophils and elevated concentrations of proinflammatory mediators, is a prominent and early feature of cystic fibrosis (CF). Despite an intense inflammatory response, bacteria are not cleared efficiently from the respiratory tract of CF patients, leading to recurrent infection and progressive deterioration of lung function (1, 2). It has been proposed that chronic inflammation is maintained by increased adherence (3), decreased clearance (4, 5), or decreased killing (6, 7) of pathogens associated with recurrent airway infections in CF. Other in vitro and in vivo studies suggest that early inflammation in CF airways is associated with abnormal production of pro- and/or anti-inflammatory cytokines (8–13). Dysregulation of the inflammatory response may represent an intrinsic component of the CF phenotype because it is observed independently of the type of infectious stimulus (14, 15). Consistent with this view, an increasing number of reports have shown that CF airway epithelial cells exhibit increased translocation of the nuclear factor-κB, a transcriptional activator of immunomodulatory genes (11, 16–18). CF results from mutations of the cystic fibrosis transmembrane regulator (CFTR) gene (19). Thus, mutations in CFTR may cause alterations in intracellular signal transduction pathways, resulting in an exaggerated recruitment of neutrophils through constitutive and bacteria-induced release of inflammatory mediators that is disproportionate to the infectious stimulus (11, 20, 21). It is currently not known, however, how mutations of CFTR lead to an abnormal inflammatory response.

It is now well established that the CFTR protein, in addition to functioning as a Cl− channel, plays an important role in regulating other ion channels and transporters of the plasma membrane. Thus, CFTR influences the function and properties of Cl−, Na+, and K+ channels in epithelial cells as well as the control of electroneutral Na+ reabsorption, Cl−/HCO3− exchange and water permeability (22–24). We recently provided evidence for a role of CFTR in the control of gap junction channel connectivity (25, 26). Defects in CFTR-dependent regulation of other transport mechanisms may contribute to some of the phenotypes that are observed in the CF pathology (27). So far, the molecular mechanisms linking CFTR to other channels and transporters are unknown.

Gap junctions, the only channels that allow direct exchange of ions and small (<1 kDa) metabolites between cells, are composed of proteins called connexins (Cx) in vertebrates. Gap junctions contribute to tissue homeostasis and have been involved in the regulation of diverse biological functions (28–31). Critical roles for gap junctions have been elucidated by the discovery of disease-causing mutations in human connexin43; PDZ, PSD95/Dlg/ZO-1 protein; EBP50, ezrin-binding phosphoprotein 50.
genes and the observation that mice with targeted deletions of connexins develop distinct phenotypes (32–34). These recent findings confirm the view that perturbation of gap junction connectivity contributes to disease initiation and/or progression. We recently reported that the proinflammatory cytokine TNF-α differentially regulates gap junctional communication in airway epithelial cell lines. In non-CF airway cells, gap junction channels rapidly close in response to TNF-α. In contrast, this mechanism is defective in CF airway cells but can be rescued by expression of wild-type CFTR (26). These observations, together with other reports (18, 35–38), suggest that CFTR may interfere with some of the signal transduction pathways initiated through receptor-ligand interactions. In this context, differential regulation of channel activity may represent a model to search for the signaling pathways that are defective in CF cells. Elucidating the mechanisms linking genotype to disease may be of critical importance to understand the pathogenesis of exaggerated airway inflammation in the CF lung.

Here we have attempted to identify the signal transduction pathways involved in the down-regulation of gap junction connectivity by TNF-α in non-CF airway cells. We provide evidence that TNF-α signaling initiates the activation of the tyrosine kinase c-Src in airway epithelial cells. We further show that inhibition of c-Src tyrosine kinase activity abolished TNF-α-induced closure of gap junction channels in non-CF airway cells. Moreover, we show that activation of c-Src is defective in CF airway cells but rescued in CFTR-corrected CF cells. These results indicate that c-Src signaling pathway links the mediators of inflammation to gap junction channels. They also suggest that mutations in the CFTR protein alter the c-Src tyrosine kinase transduction pathway. The defective activity of c-Src in CF cells may contribute to some dysfunction of the CF airway epithelium, including ion channel activity, cytokine production, mucus secretion, and epithelial cell differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—The normal human bronchial epithelial Beas2B cell line was purchased from the American Type Culture Collection (Manassas, VA); the human nasal epithelial CF15 cell line, which was derived from a patient homozygous for the F508/W128X mutation, or a constitutively active c-Src (SrcKPA) and processed using Adobe Photoshop 5.5.

Expression Vectors—pMT2-plasmids encoding either for a kinase-dead version of c-Src (SrcK'), which acts as a dominant negative (K295M mutation), or a constitutively active c-Src (SrcK'), in which the inhibitory intramolecular interaction between phosphotyrosine 527 and the SH2 domain is disrupted (Y827A mutation), were used (42, 43). A chimeric version of SrcK was also constructed using PCR by destroying the stop codon and appending the cDNA for the enhanced green fluorescent protein (EGFP). Full-length cDNAs of wild-type human Cx43 (WT-Cx43) and of Cx43 where tyrosines 247 and 265 were replaced by alanines (Y247A,Y265A-Cx43) were constructed by conventional two-step PCR mutagenesis and sub-cloned into pIRESS2-EGFP (Clontech, Palo Alto, CA). Point mutations were verified by sequencing.

Cell Transfection—About 50% confluent cells were transfected either with the Effectene transfection reagent (Qiagen AG, Basel, Switzerland) or the FuGENE transfection reagent (Roche Diagnostics), according to manufacturers’ instructions. The efficiency of cell transfection when using pMT2 plasmids encoding for SrcK’ or SrcK’ was evaluated by co-transfecting the cells with pMT2 encoding for EGFP. Thirty-four h later, small colonies of EGFP-positive cells among EGFP-negative cells were observed and subjected to dye coupling.

Dye Coupling—Dye coupling studies were performed on subconfluent monolayers of cells incubated in a solution (external solution) containing (in mM): 136 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, and 2.5 glucose and was bubbled with 5% CO2/95% O2 at pH 7.4 with 10% HEPES NaOH. Simultaneously, cells were observed and subjected to dye coupling.

Electrical Coupling—For Western blots, subconfluent monolayers of cells were washed with PBS and scraped into an ice-cold solubilization buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Roche Molecular Biochemicals). After a 30-min incubation, the samples were centrifuged at 4 °C for 10 min at 50,000 × g. Supernatants were recovered, and total amounts of protein were determined by a bichinchoninic acid quantification assay (Sigma).

One hundred to 250 μg of protein were electrophoresed on 10% SDS-PAGE and electrotransferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore AG, Volketswil, Switzerland). Membranes were then soaked overnight at 4 °C in a 2% defatted milk saturation buffer containing 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 133 mM NaCl, 0.05% Triton X-100, and 0.2% sodium azide. Blotted proteins were then incubated for 1 h at room temperature with either a mouse monoclonal (1:500 dilution) antibody (Chemicon International Inc., Temecula, CA), a monoclonal v-Src (1:250 dilution) antibody (Oncogene Research Products, La Jolla, CA), or a polyclonal c-Src(Tyr(P)-418) (1:200 dilution) phosphospecific antibody (BioSource International, Palo Alto, CA). This step was followed by a 1-h incubation with goat anti-mouse or anti-rabbit IgG secondary antibodies conjugated to peroxidase (The Jackson Laboratories, West Grove, PA). Immunoreactivity was detected through the Super Signal West Pico kit (Pierce).

Localization of Cx43—For immunofluorescence labeling, non-CF and CF airway cell lines were cultured on glass coverslips and fixed for 2–3 min with methanol at −20 °C. The coverslips were rinsed and incubated for 1 h with a 0.1% Triton X-100 in PBS supplemented with 2% bovine serum albumum for another 30 min. The cells were rinsed and incubated overnight with a polyclonal antibody (1:30 dilution) raised against Cx43 (Alpha Diagnostics, San Antonio, TX). After washing in PBS, the coverslips were incubated with a secondary antibody conjugated to fluorescein isothiocyanate for 3 h, counter-stained with Evans Blue, and then examined using fluorescent microscopy. Images were acquired with a high sensitivity black and white CCD VisiCam (VisiTron systems GmbH, Puchheim, Germany) camera connected to a personal computer. Images were captured using the software Metafluor 4.01 (Universal Imaging Corp., Downingtown, PA) and processed using Adobe Photoshop 5.5.

Electrical Coupling—For Western blots, subconfluent monolayers of cells incubated in a solution (external solution) containing (in mM): 136 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, and 2.5 glucose and was bubbled with 5% CO2/95% O2 at pH 7.4 with 10% HEPES NaOH. Simultaneously, cells were observed and subjected to dye coupling.
Results

Effects of TNF-α on Gap Junction Channel Activity in Airway Cells—We have reported previously that 100 units/ml TNF-α, a concentration that maximally stimulates the release of IL-8, decreased gap junction connectivity in non-CF but not CF airway cells within 15 min by a yet undefined mechanism (26). Plausible candidates for transduction of cell uncoupling could include direct closure of the channels by cytoplasmic factors like pH or Ca2+ or decreased expression of Cx43, the gap junction protein that is expressed in these airway cell lines (26). To address these possibilities, immunolabeling and Western blot analysis were performed to evaluate the expression of Cx43, and the dual patch clamp approach was used to monitor Cx43 channel activity. As shown in Fig. 1a (left panel), Cx43 was detected in intracellular compartments as well as in cell-cell contact areas as revealed by indirect immunofluorescence. No change in the distribution of Cx43 was observed in cells exposed to TNF-α (right panel). Similar observations were made for Cx43 in non-CF and rescued CF airway cells exposed either to TNF-α or the proinflammatory endotoxin LPS for 30 min (not shown). The total amount of Cx43 protein detected by Western blot did not change after exposure of non-CF cells for 30 min with TNF-α or LPS. In addition, no changes in the relative isoforms of Cx43 were detected for both mediators (Fig. 1b). Longer exposure (1, 3, 6, and 12 h) of the cells to TNF-α yielded similar results (not shown).

Gap junction channel activity was studied under conditions of strong pH and Ca2+ buffering. Under these conditions, exposure of non-CF cells to TNF-α for 30 min decreased \( p < 0.001 \) junctional conductance \( \left( g_j \right) \) from 11.2 ± 3 \( \text{mean} \pm \text{S.E.} \), \( n = 6 \) to 2.8 ± 0.7 nS \( n = 8 \). To investigate the effects of TNF-α on single gap junction channel activity, large driving forces were applied to cell pairs in which \( g_j \) was reduced with halothane. The single channel conductance \( \left( \gamma_j \right) \) was measured before and after 30 min of treatment with TNF-α, and frequency histograms were constructed. As shown in Fig. 2, TNF-α did not change the distribution of \( \gamma_j \) values measured. These results suggest that one mechanism by which TNF-α modulates gap junction connectivity in non-CF cells is by de-
increasing the open probability of Cx43 channels, likely via kinase activation of a signal transduction cascade.

Role of c-Src in Mediating TNF-α-induced Airway Cell Uncoupling—To identify other potent pro-inflammatory mediators on gap junction connectivity, we have studied the effects of LPS, LPA, and IL-8 on dye coupling of non-CF and CF cells. As shown in Table I, LPS and LPA markedly decreased dye coupling within minutes in non-CF cells, whereas IL-8 had no effect. LPS and LPA did not change the strength of gap junctional communication in CF cells (Table I).

LPA has emerged as a ligand for G-coupled membrane receptors on various cell types. MAPK and the tyrosine kinase c-Src have been proposed to mediate the closure of Cx43 channels by LPA (44, 45). To investigate whether these signaling pathways are involved in TNF-α-induced dye uncoupling of non-CF cells, pharmacological inhibitors of MAPK (PD98059) and of tyrosine kinases (PP1 and tyrphostin 47) were used. As shown in Fig. 3, pretreatment of non-CF cells for 30–120 min with MAPK or tyrosine kinase inhibitors did not affect their normal extent of intercellular communication. However, the uncoupling effect of TNF-α was abrogated in the presence of PP1 or tyrphostin 47 but not PD98059 (Fig. 3). Similarly, the extent of dye coupling reached by LPS in the presence of PP1 (5.1 ± 0.8 cells, n = 7) was not different from that observed under control conditions (Table I). Pervanadate, an inhibitor of tyrosine phosphatases, is known to inhibit gap junctional communication by increased tyrosine phosphorylation activity (44–46). Indeed, treatment with pervanadate for 30 min markedly decreased dye coupling in both non-CF (2.7 ± 0.5 cells, n = 7) and CF cells (1.5 ± 0.3 cells, n = 6).

To examine whether c-Src is involved in the regulation of gap junction channel connectivity by TNF-α, non-CF cells were transiently transfected with a plasmid encoding a dominant negative (SrcK−) version of the tyrosine kinase. Under these conditions, the uncoupling effect of TNF-α and LPS was fully prevented (Fig. 4). Conversely, the expression of an active mutant of c-Src (SrcK+) abolished gap junctional communication in CF cells. Under these conditions, the extent of dye coupling was reduced to 2.5 ± 0.3 cells (n = 14). These results suggest that tyrosine kinase activity is stimulated by proinflammatory mediators and mediates gap junction closure in CF cells. ND, not determined.

**TABLE I**

| Airway cells | Basal | TNF-α | LPS | LPA | IL-8 |
|--------------|-------|-------|-----|-----|------|
| Non-CF       | 5.3 ± 0.6 (15) | 2.2 ± 0.4 (18) | 2.1 ± 0.3 (20) | 1.7 ± 0.2 (16) | 5.1 ± 0.9 (12) |
| CF           | 6.0 ± 0.6 (18) | 6.3 ± 0.4 (18) | 6.1 ± 0.8 (13) | 7.2 ± 1.0 (13) | ND               |

**Fig. 3.** Quantitative evaluation of dye coupling in non-CF airway cells exposed to inhibitors of MAPK and tyrosine kinases. Pre-treatment of the cells with PD98059, PP1, or tyrphostin 47 (Tyr47) did not change the basal extent of intercellular communication, as revealed by intracellular microinjection of Lucifer Yellow (LY). Exposure of the cells for 30 min to TNF-α decreased the extent of dye coupling. Although inhibitors of p42-MAPK (PD98059) had no effect, tyrosine kinase inhibitors abrogated TNF-α-induced cell uncoupling. Numbers indicate the dye injections performed. Asterisks indicate significance at p < 0.01.

**Fig. 4.** Dye coupling in non-CF cells transfected with a dominant negative version of c-Src. Under control conditions (Control), TNF-α and LPS markedly reduced basal dye coupling in non-CF cells. In contrast, transient expression of a dominant negative form of c-Src (SrcK−) prevented the uncoupling effect by both proinflammatory mediators. Numbers indicate the dye injections performed. Asterisks indicate significance at p < 0.01. The expression of SrcK− was confirmed by Western blot using an antibody directed against v-Src. As shown in the inset, increased expression of the tyrosine kinase was detected in cells transfected with SrcK− cDNA.

Y247A,Y265A mutation did interfere with the effect of TNF-α on gap junctional communication in non-CF airway cells, suggesting further that Cx43 is a target of the c-Src tyrosine kinase.

**c-Src Activity in Non-CF and CF Airway Cells**—The expression of c-Src in non-CF airway cells was confirmed by Western blot using antibodies against v-Src (Fig. 6a). To ensure that TNF-α activates c-Src, Western blots were performed using specific antibodies against the phosphorylated Tyr-418, which is indicative of tyrosine kinase activation (42, 49). As shown in...
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Fig. 5. Dye coupling in non-CF cells expressing a mutant of Cx43 lacking Tyr-247 and Tyr-265 (Y247A,Y265A-Cx43) or wild-type Cx43 (WT-Cx43). The expression vector pIRES2-EGFP was used to visualize transfected cells by the fluorescence of EGFP. Small EGFP-fluorescent clusters appear in red for cells expressing Y247A,Y265A-Cx43 (a) or WT-Cx43 (d). EGFP-positive clusters were subjected for dye coupling in the presence or absence of pro-inflammatory mediators. In the presence of TNF-α, Lucifer Yellow diffused from the injected cells to several neighboring cells expressing Y247A,Y265A-Cx43; in contrast, microinjection of Lucifer Yellow in an EGFP-negative cell (arrow in a–c) showed no dye diffusion (b). c, phase-contrast view of the injected field. Extensive diffusion of Lucifer Yellow was also detected in EGFP-positive cells expressing WT-Cx43 (e). In the presence of TNF-α, however, the diffusion of Lucifer Yellow was strongly reduced; red and green images were merged to visualize Lucifer Yellow labeling in EGFP-positive cells expressing WT-Cx43 (f). Bar, 10 μm.

Fig. 6. Expression and activity of c-Src in non-CF airway cells exposed to TNF-α. Western blot analysis was performed on cellular lysates using either a v-Src antibody (a, top panel) or an antibody directed against the activated c-Src (a, bottom panel). Although the amount of c-Src remained stable during TNF-α treatment, the expression of activated c-Src increased with time as compared with basal conditions. Quantitation of the effect of TNF-α was measured by normalizing the activated c-Src signal with that of v-Src (b). TNF-α increased (p < 0.05) the amount of activated c-Src by 52.4 ± 6.7% during 10 min of treatment. Activated c-Src decreased with longer incubation times, reaching basal levels within 30 min. The number of measurements, which were performed in at least 4 independent experiments, is indicated.

Fig. 6a, endogenous c-Src activity could be detected in these cells under control conditions. The proportion of activated c-Src increased with time of treatment with TNF-α. Quantitative analysis revealed that TNF-α increased (p < 0.05) c-Src activity by 52.4 ± 6.7% (n = 4) after 10 min of treatment (Fig. 6b). The increased activity of c-Src was transient and reached basal levels within 30 min of treatment with TNF-α (not shown).

The activity of c-Src was also found to be strongly stimulated by refreshing non-CF cells with culture medium after 48 h of serum starvation (Fig. 7, a and c). By using similar experimental approaches, however, no changes in c-Src activation could be detected in CF cells. This was observed for both CF15 (Fig. 7, a and c) and IB3-1 (Fig. 7, b and c) cells. Interestingly, increased c-Src activity was detected in the CFTR-corrected CF cell line C38, suggesting that expression of CFTR rescued the activation of the tyrosine kinase signal transduction cascade (Fig. 7, b and c). Additional experiments using inhibitors of CFTR Cl− channels (200 μM diphenylamine carboxylic acid) or scavengers of extracellular ATP (0.5 units/ml hexokinase) failed to affect the activation of c-Src in non-CF cells (not shown). The expression of the total c-Src protein remained stable during the stimulation of each cell line, as revealed by Western blots using antibodies against v-Src (Fig. 7). These results indicate that activation of c-Src is defective in CF airway epithelial cells.

DISCUSSION

Our results describe one possible mechanism that regulates gap junction connectivity in non-CF and CF airway cell lines exposed to the proinflammatory mediator TNF-α. We identified the c-Src tyrosine kinase as a link between the mediators of inflammation and Cx43-composed gap junction channels. Importantly, we provide evidence that activation of c-Src is defective in CF cells.

It is well established that the products of the Rous sarcoma virus oncogene pp60v-src or of the proto-oncogene c-src can abolish gap junctional communication of Cx43-expressing cells (44, 45, 50). There is increasing evidence that v-Src or c-Src tyrosine kinases physically associate with Cx43 (43, 47, 51, 52), the SH3 and SH2 domains of Src being necessary for binding and phosphorylation of Cx43. Thus, mutations introduced in the proline-rich region (which binds to the SH3 region of Src) or on Tyr-265 (which binds to the SH2 region of Src) of Cx43 COOH terminus markedly reduced the interaction between v-Src or c-Src and Cx43 in vitro and in vivo (43, 51). Active Src phosphorylates tyrosine residues in target proteins. Accordingly, mutation of Tyr-265 and Tyr-247 completely prevented...
Cx43 channel closure by Src, suggesting that phosphorylation of both tyrosine residues is involved in the disruption of gap junction connectivity (43, 47, 48). Recently, a correlation between increased phosphoxygen content of Cx43 and c-Src activity was observed in the heart of Syrian B6 14.6 hamsters, which systematically develop heart dysfunction at a late stage in their life (53).

Growth factors, tumor promoters, and mediators of inflammation have been shown to inhibit gap junctional communication in various cells expressing Cx43 (54). Transduction mechanisms of cell uncoupling have been difficult to identify because of the multiple intracellular signaling pathways that are activated by these agents (44). In non-CF airway cells, the uncoupling effect of TNF-α occurs within 15 min by a mechanism that does not involve cytoplasmic factors like pH or Ca2+ or decreased expression of Cx43. We present several lines of evidence that indicate that TNF-α, as well as LPS, close Cx43 channels in non-CF airway cells by activation of Src-like tyrosine kinases. First, the uncoupling effects of TNF-α and LPS were both abrogated by broad spectrum and Src family inhibitors of tyrosine kinases. Second, a dominant negative version of c-Src prevented the uncoupling effects of TNF-α and LPS. Third, the uncoupling effect of TNF-α was prevented in cells expressing a mutant of Cx43 lacking Tyr-247 and Tyr-265. Finally, LPA, a lipid messenger that closes Cx43 gap junction channels by activation of c-Src tyrosine kinase (43, 46), also abrogated intercellular communication in non-CF airway cells. However, we could not detect Cx43 tyrosine phosphorylation in non-CF cells where Cx43 and c-Src are endogenously expressed. The stoichiometry of tyrosine phosphorylation on the 12 connexins that constitute a gap junction channel to induce its closure is unknown. Because most Cx43 detected by Western blots are not at the plasma membrane, tyrosine phosphorylation of functional gap junction channels may not be detectable biochemically in our cell model. Consistent with previous observations (48, 55, 56), we did not detect changes in the distribution of Cx43 isoforms and Cx43 single-channel unitary conductances, suggesting that activation of c-Src alters the gating properties of Cx43 channels in non-CF airway cells exposed to TNF-α. In agreement with these results, TNF-α was found to activate c-Src in non-CF cells with a time course that parallels the closure of gap junction channels. Thus, activated TNF receptors may recruit adaptor proteins that in turn bind and activate additional key pathway-specific tyrosine and serine/threonine kinases, including c-Src and MAPK (57–59). Similarly, c-Src has been involved in linking LPA and LPS membrane receptors to MAPK signaling pathways (60–62).

TNF-α, LPS, and LPA did not affect the strength of intercellular communication in the CF airway cell lines used in this study. It is known that all these cell lines, irrespective of their CF or non-CF origin, express Cx43 (26). The possibility that the association of c-Src with Cx43 is altered in CF airway cells is unlikely. Indeed, disruption of gap junction connectivity could be achieved in CF cells treated with pervanadate, an inhibitor of tyrosine phosphatases, or transiently transfected with a plasmid encoding for a constitutively active version of c-Src. In contrast, we report that activation of c-Src is defective in CF airway cells but can be rescued in CFTR-corrected CF cells. Little is known about the involvement of c-Src tyrosine kinase in CF pathogenesis. The stimulation of production of mucin by TNF-α and growth factors has been shown to be prevented in part by tyrosine kinase inhibitors (63, 64). Importantly for CF, the induction of mucin production by normal airway epithelial cells infected with *P. aeruginosa* involves an Src-dependent Ras-MAPK-pp90**α** signaling pathway that activates nuclear factor-κB (61). The Src-dependent Ras-MAPK-pp90**α** pathway is the only downstream cascade known to activate mucin gene transcription to date (65). Recently, increased expression of c-Src mRNA was detected by differential display in the tracheobronchial epithelial CFDE cells as compared with the same cells transfected with CFTR (66). This increased expression was confirmed at the protein level and was shown to be associated with increased endogenous tyrosine kinase activity and mucin overproduction. An inverse relationship between the presence of functional CFTR and c-Src expression was not observed in our study. Instead, the expression level of c-Src appeared variable and dependent on the cell line. CFDE is a non-ΔF508 CF cell line, which was derived from a CF patient of unknown genotype. Possibly, different genotypes may have diverse impacts on c-Src expression. On the other hand, c-Src tyrosine kinase activity is extremely sensitive to the extracellular environment and stimuli. In this context, we observed that rapid activation of c-Src was altered in CF15 and IB3-1 cells, which express CFTR with class II mutations, whereas it could be readily detected in non-CF cells and in CF cells corrected with CFTR. Of note, invasion of human epithelial cells by *P. aeruginosa* has been shown to involve Src-like tyrosine kinases (67). Possibly, the defective activation of c-Src may prevent the internalization and thereby the clearance of *P. aeruginosa* by CF airway cells, contributing to the recurrent airway infection (5). Taken together, our results point to the existence of a link between CFTR and c-Src-dependent signaling pathways. Furthermore, activation of c-Src appears as a central element in the signaling pathway connecting CFTR with gap junction channels in airway epithelial cells.

So far, the mechanisms that link CFTR to c-Src signaling are not known. Blocking the Cl− activity of CFTR with diphenylamine carboxylic acid or interfering with the possible release of ATP by the addition of extracellular hexokinase did not affect the activation of c-Src in non-CF airway cells. Recent data demonstrated that the last amino acids at the COOH-terminal end of CFTR form a PSD95/Dlg/ZO-1 protein (PDZ)-binding domain. PDZ-binding domains are involved in the clustering of transmembrane ion channels and in connecting intracellular signaling pathways. Interestingly, CFTR binds to the PDZ1 domain of the ezrin-binding phosphoprotein 50 (EBP50) (38, 68). Thus, EBP50 and other scaffolding proteins have not only the ability to target CFTR to membrane compartments via ezrin and the actin cytoskeleton but may also mediate the regulation by CFTR of other ion channels via protein-protein or signaling interactions (34, 38, 69, 70). Indeed, EBP50 has been involved in the interaction with tyrosine kinases of the Src family, which are known to regulate ion channel activity, such as the amiloride-sensitive Na+ channels. It is tempting, therefore, to hypothesize that complexes containing CFTR and c-Src tyrosine kinase may transduce signals from membrane receptors to ion channels, including gap junction channels. In CF epithelial cells, the absence of CFTR from the plasma membrane may compromise the regulation and/or the stability of these complexes, resulting in altered regulation of ion channels and gap junction connectivity.

The tyrosine kinase c-Src plays a central role in linking membrane receptors to various signaling pathways. NF-κB signaling is an essential component of inflammation by regulating the transcriptional activity of inflammatory and mucin genes. Thus, defects in upstream transduction signals that lead to nuclear factor-κB translocation may contribute to the exaggerated inflammatory response that is characteristic of the CF phenotype. In addition, defects in c-Src-dependent tyrosine phosphorylation of protein substrates may have yet unsuspected consequences on the response of airway epithelial cells to the environment. Interestingly, gap junctions have been
involved in most of the functions fulfilled by c-Src, including membrane trafficking, cell proliferation, cell adhesion, cell migration, and cell differentiation (28–31, 42). Future studies will be necessary to unravel the contribution of gap junctional communication in the CF pathogenesis.

Acknowledgments—We thank Brenda Kwak, Ludovic Wissienwsky, and Wouter H. Moolenaar for critical reading of the manuscript.

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J. Biol. Chem. 2003, 278:8326-8332.
doi: 10.1074/jbc.M208264200 originally published online December 27, 2002

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