

Tyrosine Kinase c-Src Constitutes a Bridge between Cystic Fibrosis Transmembrane Regulator Channel Failure and MUC1 Overexpression in Cystic Fibrosis*

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Cystic fibrosis (CF), a disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) chloride channel, is associated in the respiratory system with the accumulation of mucus and impaired lung function. The role of the CFTR channel in the regulation of the intracellular pathways that determine the overexpression of mucin genes is unknown. Using differential display, we have observed the differential expression of several mRNAs that may correspond to putative CFTR-dependent genes. One of these mRNAs was further characterized, and it corresponds to the tyrosine kinase c-Src. Additional results suggest that c-Src is a central element in the pathway connecting the CFTR channel with MUC1 overexpression and that the overexpression of mucins is a primary response to CFTR malfunction in cystic fibrosis, which occurs even in the absence of bacterial infection.

Although it has been clearly established that mutations in the cystic fibrosis transmembrane regulator (CFTR)¹ chloride channel are responsible for cystic fibrosis (CF) (1), the role of this channel, besides transporting chloride anions, is largely unknown (2). Therefore, the role of CFTR in the overexpression of mucins that is observed in CF patients is unclear. Within the respiratory system, the main issue has been the difficulty in establishing whether mucin overexpression is a response to subsequent infections with *Pseudomonas aeruginosa* or whether failure of the CFTR channel is indeed primarily responsible for this overexpression. To determine the mecha-

nisms involved in mucin overexpression is extremely important for therapy, since its early control may decrease the patient's susceptibility to *P. aeruginosa* infection (3). Using differential display (4, 5) and cultured tracheobronchial CFDE cells, we have identified the tyrosine kinase c-Src (6) as a bridge connecting CFTR failure with the overexpression of MUC1. These results suggest that the overexpression of mucins in the airways is a primary effect due to CFTR malfunction and that this occurs before any *P. aeruginosa* infection.

MATERIALS AND METHODS

Cell Lines—CFDE are tracheobronchial epithelial cells obtained from a CF patient of unknown genotype (7), and transformed with linearized pSVori⁻ (8), a plasmid containing a replication-deficient simian virus 40 (SV40) genome. The CFDE cells, assayed by ³⁶Cl⁻ efflux, 6-methoxy-N-(3-sulfopropyl)quinolinium (a chloride-sensitive dye), and patch clamp, are defective in cAMP-dependent chloride transport that is characteristic of CFTR (7). CFDE/6RepCFTR cells are CFDE cells in which episomal expression of wild-type (WT) CFTR corrects the defective cAMP-dependent chloride transport (7). These cells were cultured as previously described (7). HT29 cells (human colorectal adenocarcinoma cells, ATCC HTB-38) and FHC cells (epithelial cells from normal human fetal colonic mucosa, ATCC CRL-1831) were cultured under the same conditions.

Differential Display, Cloning, and Sequencing—Differential display (DD) of mRNA was carried out as described by Liang and Pardee (4, 5), with some modifications to avoid false positive results (9). For the assay, total mRNA was isolated from CFDE cells, CFDE/6RepCFTR cells, and CFDE/6RepCFTR cells treated with the Cl⁻ transport inhibitor 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 100 μM, 4 h), which causes these cells to revert to a CF phenotype. The oligonucleotide primers were 5'-GTGACATGCC-3' (random primer) and 5'-T₁₂(ACG)C-3'. The cDNA fragments isolated after DD were cloned into a pGem-T vector (Promega, Madison, WI) containing flanking EcoRI sites.

Northern Blots—The cloned plasmid was digested with restriction enzyme (*EcoRI*) to prepare ³²P-labeled probes for Northern blots, as previously described (9).

Blocking CFTR Expression by Antisense Oligodeoxynucleotide Treatment—An antisense oligodeoxynucleotide, 5'-CAGAGGCGACCTCTGCAT-3', complementary to nucleotides 1–18 of CFTR mRNA, was used to inhibit the expression of CFTR protein (10). The corresponding sense oligodeoxynucleotide was used as a control. CFDE and CFDE/6RepCFTR cells were cultured to 30–40% confluence. After growth, the medium was removed, and the oligonucleotides (10 μM) in serum-free medium, were added to the cells. After a 30-min incubation at 37 °C with the oligonucleotides, heat-inactivated serum (final concentration 10%) was added to the medium. The same procedure was used to replenish the oligodeoxynucleotides (10 μM) every 12 h for 48 h (10).

Immunoblotting of c-Src and c-Yes—Proteins from CFDE and CFDE/6RepCFTR cells, cultured for 1 day in serum-free Dulbecco's modified Eagle's medium/F-12, were isolated using the Trizol kit from Invitrogen. Western blots were then performed as previously described (11),

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¹ The abbreviations used are: CFTR, cystic fibrosis transmembrane regulator; CF, cystic fibrosis; WT, wild type; DD, differential display; PBS, phosphate-buffered saline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; FISH, fluorescence *in situ* hybridization; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

using antibodies for c-Src (rabbit polyclonal, N-terminal pp60c-Src-specific; catalog no. sc-19; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and c-Yes (rabbit polyclonal, N-terminal pp62c-Yes specific; catalog no. sc14; Santa Cruz Biotechnology).

Determination of c-Src Kinase Activity—c-Src activity was measured using a commercial kit (Upstate Biotechnology, Inc., Lake Placid, NY) that includes a synthetic peptide substrate (KVEKIGEGTYGVVYK) specific for the c-Src family of kinases (12), following the instructions of the manufacturer. The cells were lysed, and the protein was immunoprecipitated from 500 μ g of total cellular proteins, as described by Dehm *et al.* (13), using a specific rabbit anti-c-Src polyclonal IgG (rabbit polyclonal, N-terminal pp60c-Src-specific; catalog no. sc-19; Santa Cruz Biotechnology). Additional samples were immunoprecipitated with specific antibodies against pp62c-Yes (rabbit polyclonal, N-terminal pp62c-Yes-specific; catalog no. sc14; Santa Cruz Biotechnology), another member of the Src family of kinases, and the activity of c-Yes was measured with the same substrate.

Immunocytochemistry and Immunohistochemistry—CFDE and CFDE/6RepCFTR cells were cultured on coverslips (Nalge Nunc International, Naperville, IL) coated with a fibronectin-collagen solution, as described previously (14). After culture, the cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS) at room temperature and fixed with a 4% paraformaldehyde solution containing 4% sucrose in PBS for 20 min at room temperature. Nonspecific binding sites were blocked with PBS, 5% bovine serum albumin for 1 h, and the cells were exposed to polyclonal primary antibodies (1:30; sc-19; Santa Cruz Biotechnology) overnight at 4 °C in PBS with 1% bovine serum albumin. Control and CF human lung slices (corresponding to seven patients with CF) were obtained as paraffin tissue sections (5 μ m). The sections were stained with Giemsa (1:10 diluted Giemsa staining solution from Merck (Darmstadt, Germany)). Microwave pretreatment was performed following the technical protocols from Pharmingen (San Diego, CA). Nonspecific binding sites were blocked with PBS, 5% bovine serum albumin for 1 h, and the slices were exposed to polyclonal primary antibodies (1:30; sc-19; Santa Cruz Biotechnology) overnight at 4 °C in PBS with 1% bovine serum albumin. The slices or cells were then rinsed with PBS, incubated with secondary antibody (1:100) for 1 h, rinsed again with PBS, and developed with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Invitrogen). The primary antibodies directed against c-Src (sc-19) and MUC1 (a goat polyclonal IgG that is specific for the MUC1 C-terminal and does not react with MUC2 or MUC3; catalog no. sc-6827) and the secondary antibody (donkey anti-goat IgG) were obtained from Santa Cruz Biotechnology, and the other secondary antibody, horseradish peroxidase-linked goat anti-rabbit IgG, was from Sigma. To test the effect of the c-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; 10 μ M, 48 h) on MUC1 expression in CFDE cells, the same procedure was used. To test the effects of NPPB (100 μ M, 24 h), glibenclamide (50 μ M, 24 h), and CFTR antisense oligonucleotide (10 μ M, 48 h) on MUC1 expression in CFDE and CFDE/6RepCFTR cells, the same primary antibody against MUC1 was used (MUC1 C-terminal-specific goat polyclonal IgG), followed by labeling with fluorescein isothiocyanate-linked secondary donkey anti-goat IgG (catalog no. sc-2024; Santa Cruz Biotechnology), and cells were observed under confocal fluorescence microscopy (Zeiss LSM510 microscope).

Transfection of CFDE Cells with c-Src Mutants—CFDE cells grown as described above were transfected with different concentrations of a plasmid encoding a c-Src dominant negative mutant (0, 0.5, 1, and 3 μ g/ml) and with a plasmid containing WT c-Src (6 μ g/ml) to overexpress the c-Src protein (15). As a control, the empty plasmid was transfected. For transfection, the ProFection Mammalian Transfection System-Calcium Phosphate kit was used (Promega), following the protocols included in the kit. Forty-eight hours after transfection, the cells were washed twice with PBS at room temperature and fixed with a 4% paraformaldehyde solution containing 4% sucrose in PBS for 20 min at room temperature. Immunocytochemical analysis for MUC1 protein was performed. Confocal fluorescence *in situ* hybridization (FISH) was used, as described below, to determine MUC1 mRNA levels, with slides prepared and treated as described above.

Confocal Fluorescence in Situ Hybridization for MUC1 mRNA—To determine MUC1 mRNA levels, confocal FISH was performed as described by other authors (16), with the following modifications. CFDE and CFDE/6RepCFTR cells were cultured on coverslips as described above and treated with plasmids encoding WT c-Src (6 μ g/ml) or the dominant negative mutant of c-Src (6 μ g/ml). The probe, biotin-TCATGGTGGT-GGTGAAATGGGTGGGGGAGGGGCGAGAACAGATTCAAGCAGCCAGGGAATTC (61 bp), was designed using the GCG program (Accelrys, Madison, WI) to hybridize to MUC1 mRNA. After *in situ* hybridization,

the cells were visualized using streptavidin-Cy3 and observed using a Zeiss LSM510 confocal microscope.

Quantification and Statistical Analysis—Northern blots, immunocytochemical preparations, and Western blots were scanned with an HP4C scanner and quantified using NIH Image software (available on the World Wide Web at www.scioncorp.com). Densitometric analysis for immunocytochemical preparations was performed as described previously (17). Sample loading for Northern blots was quantified using methylene blue staining. A linear dose response to staining was observed up to 40 μ g of total RNA (11). The figures are representative of at least four different results performed in duplicate. Analysis of variance and Tukey tests were used for statistical analysis.

RESULTS

Differential Display Applied to CFDE and CFDE/6RepCFTR Cells—We used differential display to test the hypothesis that CFTR is involved in other complex functions besides chloride transport, such as indirect gene regulation. Several previously described strategies were applied to avoid false positive/negative results (9). As a model system, we used a cultured cell line derived from a CF patient (CFDE cells) and the same cell line transfected with WT CFTR (CFDE/6RepCFTR cells) (14). In addition, CFDE/6RepCFTR cells were treated with the chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (18, 19). Using this model system, we were able to show that several genes might be under CFTR control. As shown in Fig. 1A, several differentially expressed mRNAs were detected in CFDE and CFDE/6RepCFTR cells. As expected, after NPPB treatment, the differential display pattern of some mRNAs, probably regulated by CFTR activity, reverted in the CFDE/6RepCFTR cells, becoming similar to the pattern found in CFDE cells. The characterization of these putative CFTR-dependent genes will open the way to a better understanding of CF pathology and the mechanisms involved and help identify new possible targets for therapy.

Identification of c-Src as a CFTR-dependent Gene—One gene that was overexpressed in CFDE cells compared with CFDE/6RepCFTR cells and that reverted to CFDE levels with NPPB treatment of CFDE/6RepCFTR cells was selected for further characterization. The corresponding cDNA fragment (Fig. 1A, arrow) was isolated from the differential display gel, PCR-amplified, purified by agarose gel electrophoresis, cloned, and sequenced (9). The sequence (Fig. 1B) was identical to the sequence encoding the human tyrosine kinase c-Src (pp60c-Src, GenBank™ AF077754). Northern blots were probed with the ³²P-radiolabeled cDNA fragment isolated by DD and confirmed the differential expression of c-Src mRNA in CFDE cells (Fig. 1C). Since NPPB is not CFTR-specific, these results were further confirmed by treatment of CFDE/6RepCFTR cells with a CFTR antisense oligonucleotide that inhibits CFTR protein expression and the cAMP-activated chloride current but does not affect the calcium-activated chloride currents (10). The CFTR antisense oligonucleotide produced a reversion to c-Src mRNA levels similar to those obtained with NPPB (Fig. 1C). These results are in agreement with the concept that c-Src mRNA is modulated by CFTR.

Immunoblotting of c-Src—We next examined whether the c-Src protein levels correlate with mRNA levels and are modulated by CFTR. Western blot analysis applied to CFDE and CFDE/6RepCFTR cells indicated that this is the case (Fig. 2A). Levels of c-Src protein (pp60c-Src) increased in CFDE cells (cystic fibrosis cells) compared with CFDE/6RepCFTR (expressing normal CFTR protein). Treatment with glibenclamide (50 μ M, 48 h), a CFTR chloride channel inhibitor (20), restored the c-Src protein levels in CFDE/6RepCFTR cells to levels similar to those found in CFDE cells (Fig. 2, A and B). In contrast, c-Yes, another member of the Src-like family of kinases and very closely related to Src, showed no change in

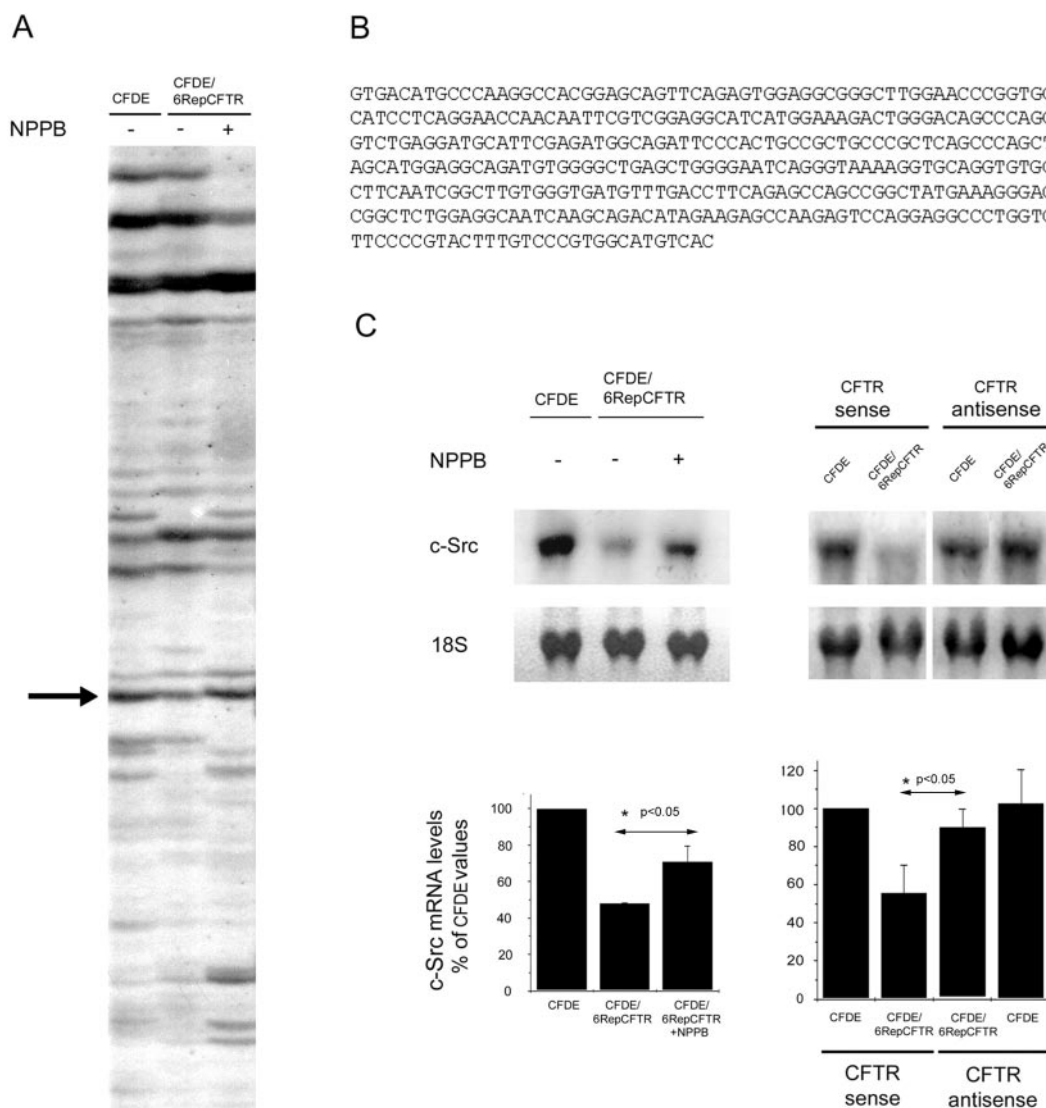


FIG. 1. Differential display of CFDE and CFDE/6RepCFTR airway epithelial cells. A, DD of CFDE cells (derived from a cystic fibrosis patient) and CFDE/6RepCFTR cells (CFDE cells transfected with WT CFTR). *NPPB*, CFDE/6RepCFTR cells treated with the Cl⁻ channel inhibitor NPPB (100 μ M, 4 h). B, sequence corresponding to the cDNA fragment indicated with an *arrow* in A. PCR amplification, cloning, and sequencing showed that the fragment has 100% sequence identity with tyrosine kinase c-Src. C, Northern blots using the c-Src fragment cloned from the DD as probe. Both NPPB (100 μ M, for 4 h) and the CFTR antisense oligodeoxynucleotide (10 μ M, 48 h) increased the levels of c-Src mRNA in CFDE/6RepCFTR cells. Values are means \pm S.E. expressed as percentages of levels in CFDE cells. An *asterisk* indicates a significant difference between *bars* connected by *arrows* ($p < 0.05$).

protein levels between CFDE and CFDE/6RepCFTR cells (Fig. 2A) or with glibenclamide treatment (Fig. 2B).

Since CF also affects the intestinal tract, we also studied c-Src protein levels in the HT29 colon cell line (transformed colon cells) and the FHC cell line (nontransformed colon cells), blocking CFTR activity with glibenclamide (50 μ M, 48 h) (20). The levels of c-Src protein increased in FHC, HT29, and CFDE/6RepCFTR cells after glibenclamide treatment (Fig. 2B), suggesting that the regulation of c-Src protein by CFTR occurs not only in airway CFDE cells but also in other cell types expressing WT CFTR. The c-Src-like kinase, c-Yes, did not show changes in FHC cells with or without glibenclamide (50 μ M, 48 h) (Fig. 2B), indicating again that the levels of c-Yes are not dependent on CFTR activity.

Determination of c-Src Kinase Activity—Src-like tyrosine kinases contain N-terminal Src homology 2 and 3 domains, the kinase domain, and the regulatory domain that contains a tyrosine residue (Tyr-527 for c-Src). An additional regulatory tyrosine residue (Tyr-192 for c-Src) is present in the Src homol-

ogy 2 domain. The activity of Src kinases is generally tightly regulated by phosphorylation of the C-terminal tyrosine residue through C-terminal Src kinase (Csk kinase) (21). Dephosphorylation of this residue induces a conformational change that activates the kinase domain, inducing the autophosphorylation of a stimulatory tyrosine in the kinase domain (Tyr-416 for Src) (for a review of Src, see Ref. 22). For this reason, increased expression of c-Src does not necessarily imply that c-Src kinase activity also increases. To determine whether the elevated levels of c-Src mRNA and protein observed in CF cells were reflected in c-Src activity, c-Src activity was measured using a peptide substrate (KVEKIGEGTYGVVYK) specific for the Src family of kinases (12). To assure specificity, c-Src was first immunoprecipitated using a specific polyclonal antibody. In agreement with elevated mRNA and protein levels, a significant ($p < 0.001$) 5-fold increase in c-Src kinase activity was observed in CFDE cells compared with CFDE/6RepCFTR cells (Fig. 2C). In contrast, under the same assay conditions, immunoprecipitated c-Yes displayed low activity, similar to basal

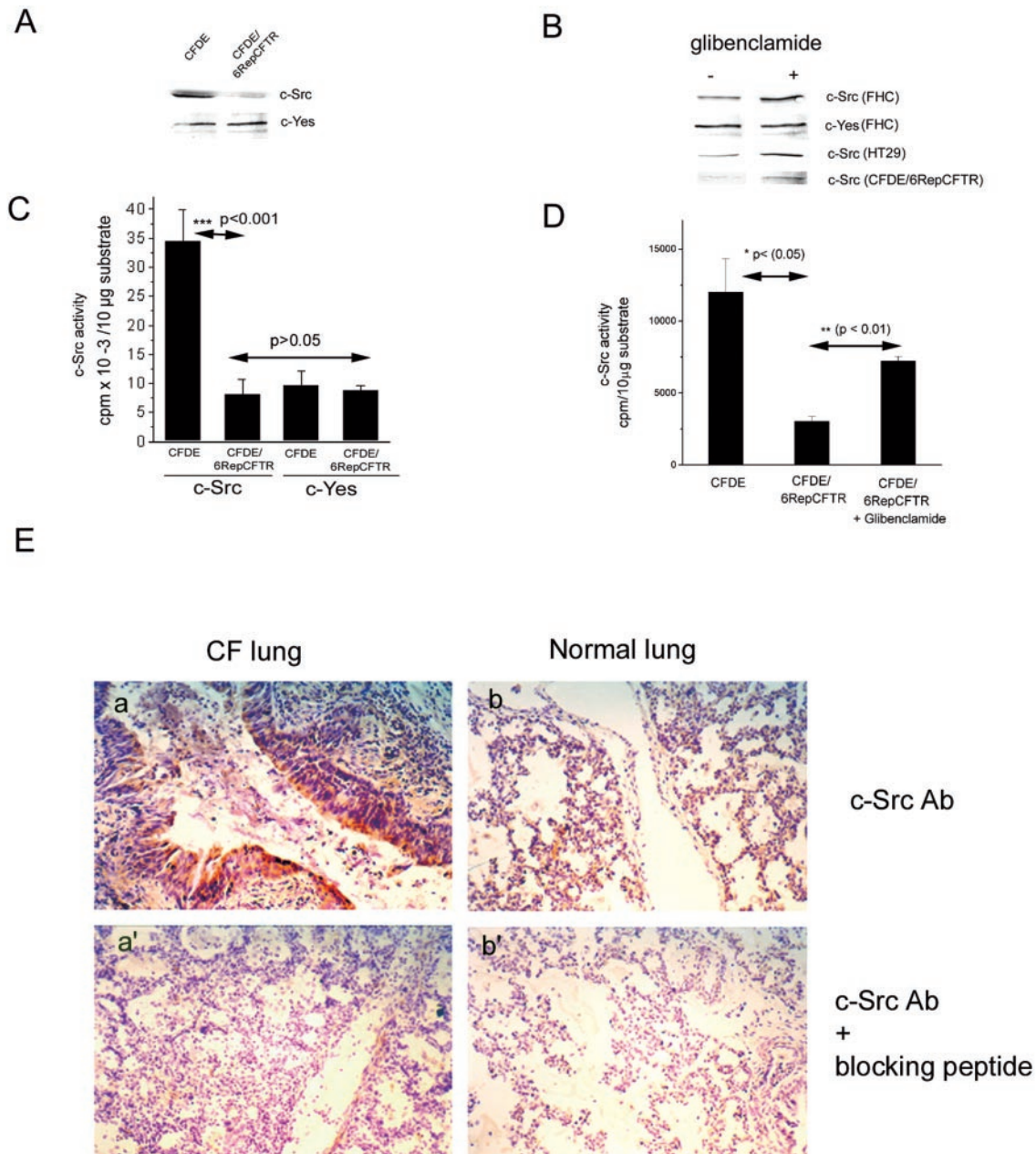


FIG. 2. Western blotting, immunohistochemistry, and activity of c-Src protein. *A*, immunoblot for c-Src and c-Yes in CFDE and CFDE/6RepCFTR cells. Although c-Src levels were diminished in CFDE/6RepCFTR cells, levels of the c-Src-like kinase, c-Yes, remained constant. *B*, glibenclamide (50 μ M, 48 h) treatment of CFDE/6RepCFTR or colon cells (FHC, HT29) increased c-Src but not c-Yes protein levels. *C*, after specific c-Src immunoprecipitation from cell extracts, the activity of c-Src was measured by 32 P incorporation into the Tyr of a specific synthetic peptide substrate of c-Src. A 5-fold increase in c-Src activity was observed in CFDE cells. Specifically immunoprecipitated c-Yes was also measured as a control, and no changes in its activity were observed. *D*, a partial reversion (50–100%) of c-Src activity in CFDE/6RepCFTR cells was observed with 50 μ M glibenclamide treatment for 48 h. *E*, the expression of c-Src in normal and CF human lungs was also determined (only one sample is shown of those of seven CF patients studied). CF (*a* and *a'*) and non-CF (*b* and *b'*) human lung tissues were studied by immunohistochemistry of paraffin sections (c-Src immunoperoxidase-3,3'-diaminobenzidine tetrahydrochloride dihydrate plus Giemsa staining). *a* and *b*, using an antibody specific for the c-Src protein. *a'* and *b'*, negative controls, in which the primary antibody was treated with a blocking peptide for the c-Src antibody (0.5 μ g of peptide/0.2 μ g of antibody). The c-Src protein was highly expressed in CF lung tissue (*a*) compared with non-CF lung tissue (*b*).

c-Src, with no difference in activity between CFDE and CFDE/6RepCFTR cells (Fig. 2C), implying that c-Yes activity and protein levels (as shown previously by immunoblotting) are not up-regulated by CFTR in these cells. Nevertheless, other members of the Src-like family of kinases might still be under CFTR regulation (particularly c-Fyn; see “Discussion”) (23). c-Src activity was also measured in CFDE cells and CFDE/6RepCFTR cells treated with glibenclamide (50 μ M, 48 h). As shown in Fig. 2D, the low activity observed in CFDE/6RepCFTR cells was partially restored to CFDE values by glibenclamide treatment

(in some experiments, restoration was 100%, but only the average is shown).

Immunohistochemistry of c-Src in Human CF and Normal Lungs—To determine whether the overexpression of c-Src observed in CFDE cells is actually reflected in the CF human airway, the expression of c-Src protein was studied by immunohistochemistry. As shown in Fig. 2E, overexpression of c-Src was observed in human lung tissue derived from CF patients (six other patients were studied with similar results, not shown), suggesting that c-Src is also overexpressed *in vivo*.

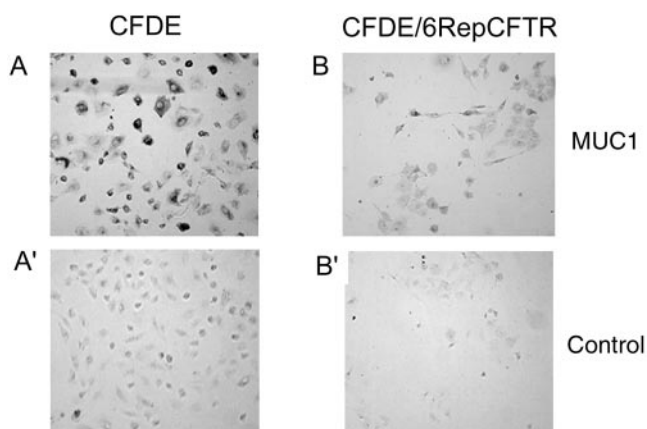


FIG. 3. **Expression of MUC1 in CF cells.** Immunohistochemistry of MUC1 protein in CFDE cells (A) and CFDE/6RepCFTR cells (B). A' and B', negative controls, in which the primary antibody was omitted. Increased expression of MUC1 was observed in the CFDE cells (A) compared with the CFDE/6RepCFTR cells. Results are representative of four independent experiments.

However, it is clearly still possible that the secondary effects of infection with *P. aeruginosa* contribute to the observed high levels of c-Src in these patients (23, 24).

Effect of c-Src Overexpression/Inhibition on MUC1 Protein Levels—Several lines of evidence suggested to us that the mucin MUC1 might be elevated as a consequence of the up-regulation of c-Src (see “Discussion”). To test this hypothesis, the levels of MUC1 protein in CFDE and CFDE/6RepCFTR cells were determined using immunocytochemistry. As expected, MUC1 was overexpressed in CFDE cells compared with CFDE/6RepCFTR cells (Fig. 3). Most importantly, CFDE cells treated with the c-Src inhibitor PP2 (25) showed decreased MUC1 expression (Fig. 4A), suggesting that this phenomenon was not only a correlation but also a cause-effect relationship. To confirm this, CFDE cells were transfected with a plasmid expressing a dominant negative mutant of c-Src, and again, MUC1 was inhibited (Fig. 4B), with the inhibition following a clear dose-response curve (Fig. 4C). Because simultaneous inhibition of other members of the c-Src family by the c-Src dominant negative mutant was a possibility, the up-regulation of MUC1 by c-Src was further supported by transfection of CFDE/6RepCFTR cells with a plasmid containing WT c-Src. Again, increased MUC1 protein expression was observed (Fig. 5). These results strongly suggest that MUC1 expression is under the control of c-Src in these CF cells.

Confocal FISH of MUC1 mRNA—To determine whether c-Src also regulates MUC1 mRNA levels, confocal FISH was applied to CFDE (CF cells) and CFDE/6RepCFTR cells (CFTR-restored CF cells). As shown in Fig. 6, CFDE cells showed high levels of MUC1 mRNA compared with CFDE/6RepCFTR cells. As expected from the results obtained for MUC1 protein expression, the c-Src dominant negative mutant caused down-regulation of MUC1 mRNA in CFDE cells. Furthermore, transfection of CFDE/6RepCFTR with WT c-Src restored these cells to a CF phenotype, with increased MUC1 mRNA expression (Fig. 6). These results imply that MUC1 mRNA levels are also under c-Src control.

Effects of the Chloride Transport Inhibitors NPPB and Glibenclamide on MUC1 Protein Expression—We have shown in the results described above that CFTR is associated with c-Src modulation and that c-Src in turn modulates MUC1 levels. Therefore, to test the hypothesis that c-Src constitutes a bridge between CFTR failure and MUC1 overexpression (CFTR → c-Src → MUC1), the chloride transport inhibitors NPPB (100 μ M, 24 h) and glibenclamide (50 μ M, 24 h) were added to CFDE

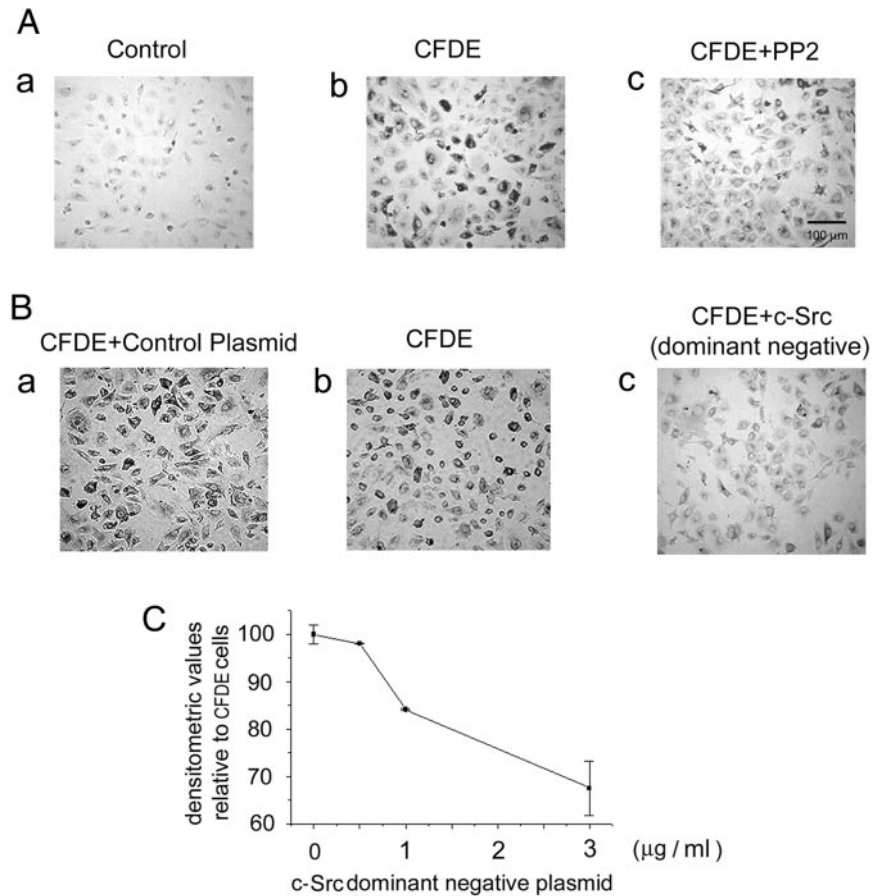
and CFDE/6RepCFTR cells, and the MUC1 protein levels were assessed after 24 h using confocal fluorescence microscopy. As expected, overexpression of MUC1 was observed in CFDE/6RepCFTR cells after treatment with either NPPB or glibenclamide (Fig. 7). The levels of MUC1 protein attained after the inhibition of chloride transport in CFDE/6RepCFTR cells were similar to those found in CFDE cells. It should be pointed out here that, although glibenclamide is a more specific CFTR inhibitor than the nonspecific Cl⁻ channel inhibitor, NPPB (26), it may also affect other sulfonylurea-sensitive ion channels, such as the ATP-dependent potassium channel (27). Therefore, to confirm these results, CFDE/6RepCFTR cells were treated with 10 μ M CFTR antisense oligonucleotide for 48 h, to inhibit the expression of CFTR protein. The results (Fig. 7) were in agreement with those obtained with NPPB and glibenclamide, further supporting the CFTR → c-Src → MUC1 link. These cell cultures, however, lack polarization. Therefore, the results should be accepted with caution because the expression of CFTR and MUC1 may be different in culture-polarized cells.

DISCUSSION

Applying differential display to CFDE (human CF cells) and CFDE/6RepCFTR (human CF cells transfected with WT CFTR) cells, we found differential expression of different genes that may be under CFTR regulation, as the lower or higher levels of several of the genes products observed in CFDE/6RepCFTR cells reverted to CFDE levels after treatment with the chloride channel inhibitor, NPPB. Among these gene products, we selected one for further characterization; it proved to encode the tyrosine kinase c-Src. Overexpression of c-Src mRNA in CFDE cells was confirmed by Northern blots, and the low levels of c-Src mRNA in CFDE/6RepCFTR cells reverted to the levels seen in CFDE cells when either antisense CFTR or NPPB was applied. This last result suggests an association between the levels of c-Src mRNA and CFTR transport activity, although NPPB might also affect the interaction of CFTR with other CFTR-interacting proteins. We further demonstrated that c-Src protein levels correlate with c-Src mRNA levels in CFDE and CFDE/6RepCFTR cells. Moreover, c-Src protein and c-Src activity were also modulated by CFTR transport inhibition with glibenclamide. Similar results were obtained using the intestinal HT29 cell line (derived from a colon tumor) and FHC cells (derived from normal colon tissue), suggesting that this regulation may operate in the intestine and perhaps in other tissues as well as in the respiratory system. When human lungs from CF patients were analyzed, overexpression of c-Src was also observed. However, some contribution to c-Src overexpression resulting from a secondary bacterial infection (23) in these patients cannot be completely ruled out.

How might CFTR control gene expression? When we first initiated this work, it was difficult to envision how a chloride channel might regulate specific genes. One possibility was a direct connection between the CFTR protein and other membrane proteins that might serve as transducers for CFTR signaling after putative conformational changes induced by the activation of chloride transport or by CFTR phosphorylation that allows such transport. Another possibility was the presence of proteins or pathways sensitive to changes in membrane potential, due to the impairment in chloride transport in CF, which might indirectly regulate several genes. Similar mechanisms could also explain the reversion of mRNA levels observed with NPPB treatment. However, while this work was in progress, a PSD95/Dlg/ZO-1 protein (PDZ)-domain binding C-terminal consensus (-T(K/R)L) sequence was identified in CFTR, and several other CFTR-associated proteins have been found (28, 29). Therefore, the presence of transducer proteins for

FIG. 4. c-Src activity modulates MUC1 expression. A, immunocytochemistry of MUC1. *a*, control, with primary antibody omitted; *b*, MUC1 expression in CFDE cells; *c*, MUC1 expression in CFDE cells plus the c-Src inhibitor PP2 (10 μ M, 48 h). B, immunocytochemistry of MUC1 in Src-transfected cells. *a*, CFDE cells treated with 1 μ g of control plasmid for 48 h; *b*, CFDE cells; *c*, CFDE cells treated with 1 μ g of plasmid encoding dominant negative mutant c-Src for 48 h. C, quantification of MUC1 protein expression in CFDE cells treated with increasing amounts of a plasmid encoding a c-Src dominant negative mutant. Results are representative of four independent experiments performed in duplicate. Values are means \pm S.E. ($n = 2$) expressed as a percentage of values for untreated CFDE cells.



CFTR has now emerged as a clear possibility. Nevertheless, this possibility does not exclude the others.

The finding that c-Src modulates CFTR activity (30) suggests that the effect might operate in both directions, as occurs with other channels regulated by members of the c-Src family of kinases (including K^+ channels, the inositol 1,4,5-trisphosphate receptor, other Ca^{2+} channels, and glutamate, NMDA, and *N*-acetylcholine receptors) (22), and that CFTR plays a direct role in the regulation of c-Src activity. However, in the experiments reported here, CFTR negatively regulated c-Src activity, and therefore, the mechanism of activation may be indirect. On the other hand, inhibition of CFTR transport activity in CFDE/6RepCFTR cells induced c-Src stimulation, and this effect may involve a conformational change affecting proteins sensitive to cell depolarization (membrane potential) rather than the CFTR-linked proteins specifically. Therefore, it is also possible that other proteins sensitive to changes in membrane potential are involved, in addition to anchor and transducer proteins. It is clear that further work is required to identify the mechanisms involved in CFTR transduction, which may be of various different types.

After establishing an association between CFTR modulation and c-Src mRNA, protein, and activity, the next step was to attempt to identify the possible c-Src target in CF. Several lines of evidence directed us toward the mucins. In CF, the major pathological problem results from the accumulation of mucins within the respiratory and digestive systems. The airway can then become susceptible to subsequent infections with *P. aeruginosa* (3), because mucus constitutes a favorable niche for bacterial growth (31). Airway mucins are produced mainly by goblet cells in the surface epithelium and by glands in the submucosal tissue. Among the better studied mucins (MUC1–4, MUC5AC, MUC5B, MUC6, MUC7, and MUC8), all

but MUC6 appear to be produced by the epithelial goblet cells. It has been suggested that the monomeric mucins, such as MUC1, play an important role in the pathogenesis of CF (32). Mice lacking functional CFTR suffer from intestinal obstruction due to large amounts of mucus in the lumen, an effect that is abrogated in double-knockout mice that also lack MUC1 (33). Therefore, MUC1 may have a major role in intestinal obstruction. It has also been postulated that early CF pathology may involve MUC1 in the respiratory system and both MUC1 and MUC2 mucins in the intestine (34). Therefore, MUC1 seemed to be relevant as a possible c-Src-responsive protein in CF cells. c-Src is a kinase involved in the control of MUC2 transcription by the mammalian respiratory mucosa in response to diverse challenges, including *P. aeruginosa* infection (31). The finding by Li *et al.* (24), that activation of NF- κ B via a Src-dependent Ras-MAPK-pp90^{rsk} pathway is required for *P. aeruginosa*-induced MUC2 overproduction in epithelial cells, also suggests that MUC2 is under CFTR regulation via c-Src. In fact, expression of all of the genes for gel-forming mucins that are clustered on chromosome 11p15, including those for MUC2, MUC5AC, MUC5B, and MUC6, are under c-Src control (35). Since no data were available regarding the regulation of MUC1 expression by c-Src, we decided to determine whether the increased expression and activity of c-Src in CFDE cells, due to CFTR failure, leads to the overexpression of MUC1.

When MUC1 expression was studied by immunocytochemistry and confocal FISH, overexpression of MUC1 protein and mRNA levels were indeed observed when CFDE cells were compared with CFDE/6RepCFTR cells. Furthermore, transfection of CFDE/6RepCFTR cells with a plasmid expressing WT c-Src produced up-regulation of MUC1 mRNA and protein expression. The levels of MUC1 mRNA and protein were also decreased in CFDE cells transfected with a dominant negative

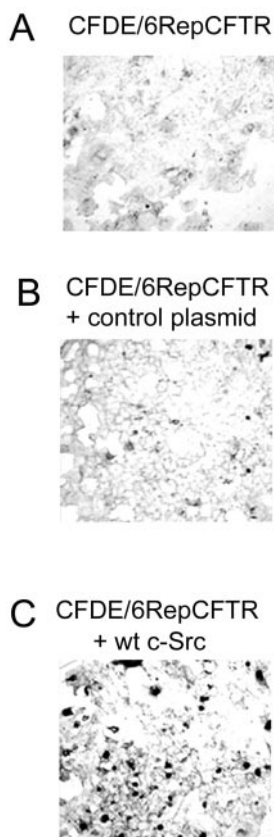


FIG. 5. Overexpression of c-Src up-regulates MUC1 protein in CFDE/6RepCFTR cells. CFDE/6RepCFTR cells were transfected with a plasmid expressing WT c-Src, and the levels of MUC1 were determined. *A*, CFDE/6RepCFTR cells, which express low levels of MUC1 protein. *B*, CFDE/6RepCFTR cells transfected with control plasmid. *C*, CFDE/6RepCFTR cells transfected with a plasmid expressing WT c-Src. The overexpression of WT c-Src increased MUC1 levels.

c-Src mutant. Finally, a rise in MUC1 protein levels could be induced by the inhibition of CFTR with NPPB, glibenclamide, or CFTR antisense oligonucleotides in CFDE/6RepCFTR cells. These results strongly suggest that the increased activity of c-Src observed in CFDE cells is responsible for the overexpression of MUC1. They also suggest a CFTR \rightarrow c-Src \rightarrow MUC1 link and suggest that c-Src might constitute a bridge between CFTR failure and mucin overexpression in CF. However, MUC1 levels have not yet been determined in CF lungs; nor were the levels of other mucins measured in our experiments. Therefore, the potential for tissue specificity and functional differences should be taken into account.

The genes for the transmembrane mucin MUC1 (36, 37) and the four gel-forming mucins, MUC2, MUC5AC, MUC5B, and MUC6, are clustered on the p15 arm of chromosome 11, and their expression is regulated by c-Src (35). Therefore, a CFTR \rightarrow c-Src \rightarrow MUCX link may also operate in different CF-affected tissues, through the elevation of c-Src or c-Src-like kinase activities. In this context, the protein kinase c-Src or other members of this family of kinases may be possible new targets for CF therapy. However, it is important to note that lymphocytes, for example, regulate the outwardly rectifying chloride channels through Lck (p56-Lck) (38), another member of the c-Src family of kinases, which is abundant in immune cells and the brain (22). Although elevated Lck may compensate CFTR failure to some degree, its inhibition via an Src-like inhibitor could be detrimental for the immune system.

Another interesting observation is that MUC1 constitutes a receptor for *P. aeruginosa* (39) and that CF cells have a clear

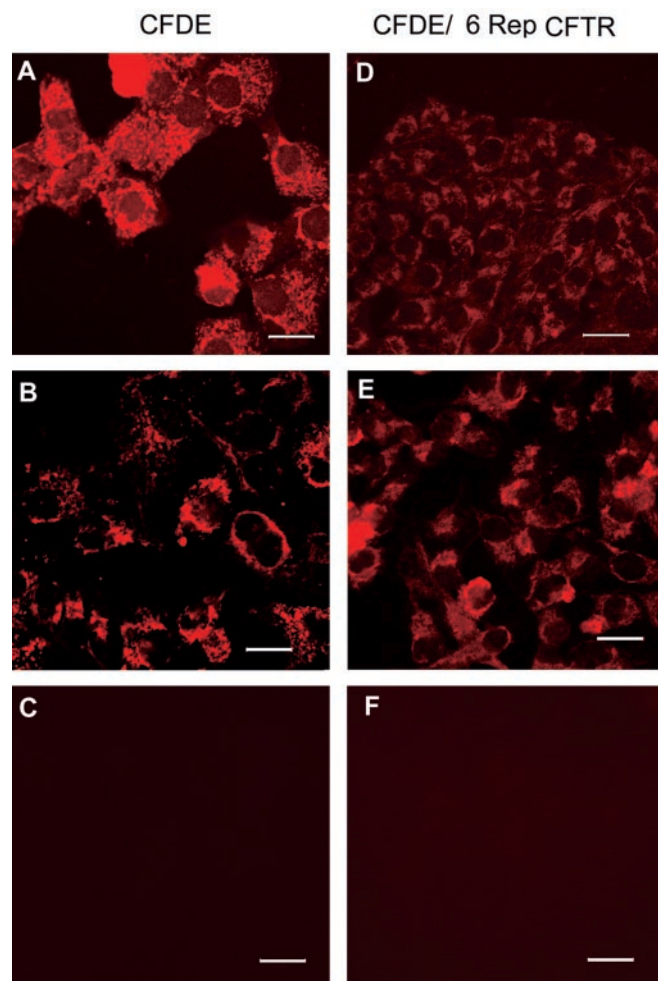
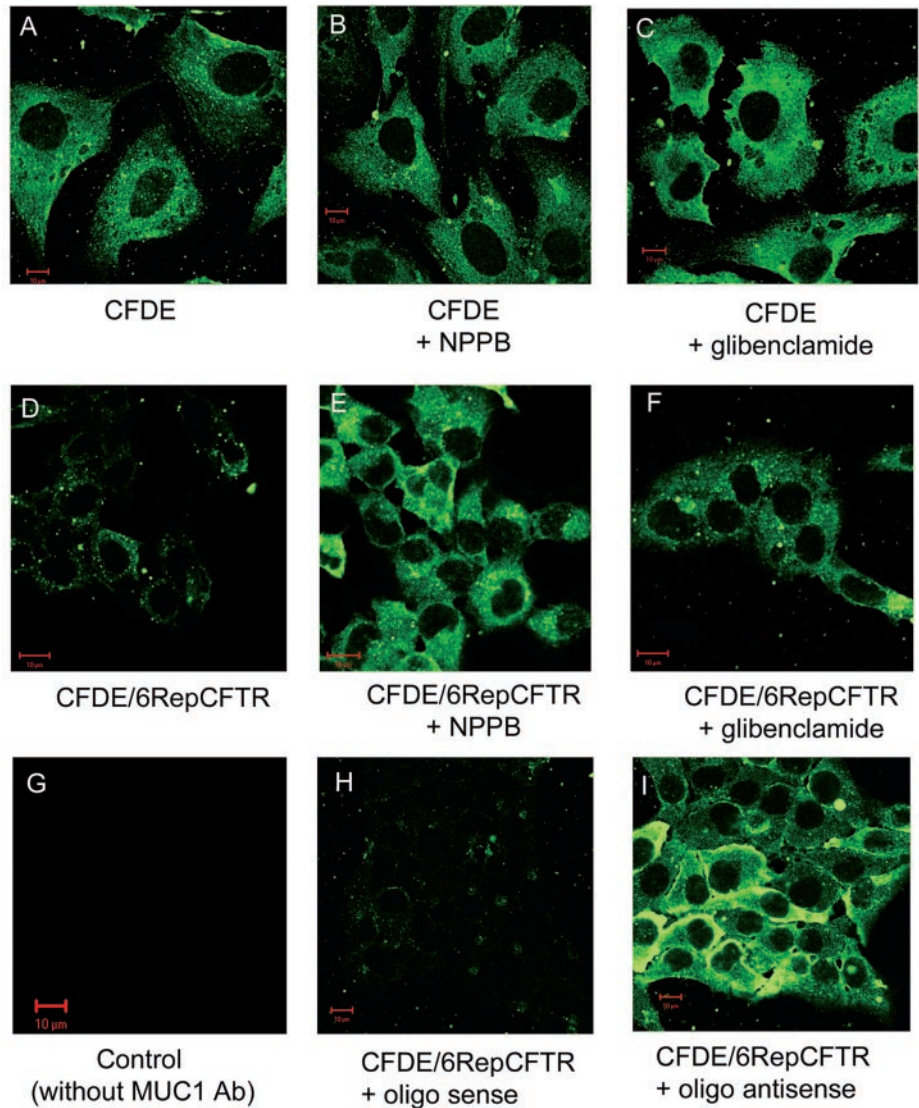


FIG. 6. MUC1 mRNA steady-state levels in CFDE and CFDE/6RepCFTR cells transfected with the c-Src dominant negative mutant or WT c-Src. CFDE and CFDE/6RepCFTR cells were transfected with a plasmid expressing WT c-Src or a plasmid expressing the dominant negative c-Src mutant. MUC1 mRNA levels were determined by hybridization with a biotinylated oligonucleotide probe complementary to MUC1 mRNA, using streptavidin-Cy3 as the fluorescent stain with which the probe was visualized. *A*, MUC1 mRNA expression in CFDE cells, which express high levels of MUC1 mRNA. *B*, CFDE cells transfected with a plasmid expressing the dominant negative mutant of c-Src. Lower MUC1 mRNA expression was observed (compared with *A*). *C*, CFDE control, in which the probe is omitted. *D*, CFDE/6RepCFTR cells, which normally express lower MUC1 levels compared with CFDE cells (*A*). *E*, CFDE/6RepCFTR cells, transfected with a plasmid expressing WT c-Src. Overexpression of MUC1 was observed in some cells (compared with untransfected cells in *D*). *F*, CFDE/6RepCFTR control, in which the probe is omitted. White lines indicate scale (20 μ m). These results suggest that the expression of MUC1 mRNA is also under c-Src control in these CF cells.

impairment in their ability to phagocytose this bacterium. Together, these variables may contribute to the high susceptibility of CF patients to *P. aeruginosa* infection. However, the problem appears to be far more complex. We have observed that interleukin-1 β modulates CFTR synthesis in a biphasic manner (11), partially through NF- κ B (40), with inhibition observed at doses of interleukin-1 β similar to the levels found in CF patients. In consequence, the chronic inflammation that occurs with elevated interleukin-1 β in CF patients (41, 42) might contribute to the further reduction of the already low levels of CFTR, with a consequent rise in c-Src activity, mucin overproduction, and exacerbation of the disease. In this context, the susceptibility to *P. aeruginosa* infection in individuals with CF seems to be a multifactorial and complex system.

It is also important to note that the inhibition of c-Src, using

FIG. 7. Effects of NPPB, glibenclamide, and CFTR antisense oligonucleotide on MUC1 protein levels in CFDE and CFDE/6RepCFTR cells. CFDE (CF cells; A–C) and CFDE/6RepCFTR cells (expressing WT CFTR; D–I) were incubated for 24 h in the presence or absence of the chloride channel inhibitors NPPB (100 μ M; B and E) or glibenclamide (50 μ M; C and F). After incubation, confocal immunofluorescence detection was used with an MUC1-specific goat antibody and a secondary antibody labeled with fluorescein isothiocyanate. Overexpression of MUC1 protein was observed in CFDE cells (A), and very low levels of expression were observed in CFDE/6RepCFTR cells (D). Treatment with NPPB or glibenclamide had no appreciable effect on CFDE cells (B and C, respectively). In contrast, CFDE/6RepCFTR cells showed very low MUC1 protein levels in untreated cells (D) that were up-regulated by either NPPB or glibenclamide treatments (E and F, respectively). G, control for CFDE/6RepCFTR cells with the primary antibody omitted. No fluorescein isothiocyanate signal was observed in control CFDE cells (not shown). H, CFDE/6RepCFTR cells expressing low levels of MUC1 in the presence of CFTR sense oligonucleotide. I, CFDE/6RepCFTR cells showing high MUC1 expression in the presence of CFTR antisense oligonucleotide. The scale in red indicates 10 μ m (magnification, \times 1000).



PP2 or the dominant negative mutant, was enough to restore a normal (CFDE/6RepCFTR), low level mucin phenotype in CF cells, suggesting that c-Src is a key component in the regulation of MUC1. Moreover, Li *et al.* (36, 37) recently found that the MUC1 cytoplasmic domain interacts with c-Src tyrosine kinase in human ZR-75-1 breast carcinoma cells, thereby increasing the binding of MUC1 to β -catenin, leading to the translocation of β -catenin into the nucleus for signaling (43). Because c-Src is overexpressed in CF cells, its effects on the wingless \rightarrow β -catenin \rightarrow Tcf/LEF-1 pathway may have a role in determining the CF phenotype, which may include increased cell motility, increased proliferation, gap-junction disruption, and other effects typical of the activated Wnt/wingless signaling pathway (44).

In conclusion, c-Src appears to constitute a bridge between CFTR failure and the overproduction of MUC1 in CFDE cells. Our results also suggest that c-Src overexpression in cultured CF cells is a primary effect due to CFTR malfunction, occurring in the absence of *P. aeruginosa* or any other bacterium. Because c-Src modulates the expression of not only MUC1 but also all of the mucins encoded in the p15 arm of chromosome 11 (MUC2, MUC5AC, MUC5B, MUC6, and MUC5AC) (35), c-Src may be responsible for the overexpression of several mucins in CF tissues, and this may occur before any bacterial infection. This does not preclude the possibility that in subsequent CF stages, after an infection is established, *P. aeruginosa* or other bacteria contribute further to the overproduction of mucins.

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Tyrosine Kinase c-Src Constitutes a Bridge between Cystic Fibrosis Transmembrane Regulator Channel Failure and MUC1 Overexpression in Cystic Fibrosis

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