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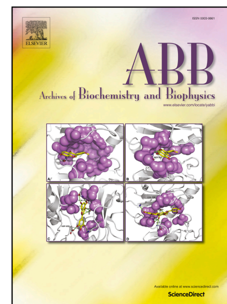

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CFTR Impairment Upregulates c-Src Activity through IL-1β Autocrine Signaling

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Keywords: Autocrine loop; CFTR; cystic fibrosis; DCFH-DA; c-Src; IL-1β; IL1RN; mitochondria; mitochondrial Complex I; MitoSOX; NOX; PP2; reactive oxygen species (ROS).

Abbreviations:

CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; DCF, 2´,7´-dichlorofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; IL1RN, IL-1 receptor antagonist; mCx-I, Mitochondrial Complex I; PP2, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; NOX1/4, NOX1 or NOX4; RQ, Relative Quantification; SDS, sodium dodecyl sulfate; WBs, Western blots; Wild-type CFTR, wt-CFTR.

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Abstract

Cystic Fibrosis (CF) is a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Previously, we found several genes showing a differential expression in CFDE cells (epithelial cells derived from a CF patient). One corresponded to c-Src; its expression and activity was found increased in CFDE cells, acting as a signaling molecule between the CFTR activity and MUC1 overexpression. Here we report that bronchial IB3-1 cells (CF cells) also showed increased c-Src activity compared to 'CFTR-corrected' S9 cells. In addition, three different Caco-2 cell lines, each stably transfected with a different CFTR-specific shRNAs, displayed increased c-Src activity. The IL-1β receptor antagonist IL1RN reduced the c-Src activity of Caco-2/pRS26 cells (expressing a CFTR-specific shRNA). In addition, increased mitochondrial and cellular ROS levels were detected in Caco-2/pRS26 cells. ROS levels were partially reduced by incubation with PP2 (c-Src inhibitor) or IL1RN, and further reduced by using the NOX1/4 inhibitor GKT137831. Thus, IL-1β→c-Src and IL-1β→NOX signaling pathways appear to be responsible for the production of cellular and mitochondrial ROS in CFTR-KD cells. In conclusion, IL-1β constitutes a new step in the CFTR signaling pathway, located upstream of c-Src, which is stimulated in cells with impaired CFTR activity.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease [1] caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [2-4]. CFTR is a cAMP regulated and ATP gated-chloride channel [5]. Protein kinase A (PKA) [6], protein kinase C (PKC) [7], c-Src (SRC) [8], and phosphatase PP2A regulate its chloride transport activity [9, 10].

Previously, applying differential display to cultured CFDE cells (tracheobronchial epithelial cells of unknown genotype, derived from a CF patient), we found that several genes had altered expression due to the CFTR failure of these cells [11-15]. Other laboratories found similar results by using microarrays and different CF cellular models [16-18]. One of these differentially expressed mRNAs was further characterized and corresponded to c-Src; it was up-regulated in CFDE cells (CF cells) or in CFDE/6RepCFTR cells (CFTR-corrected CFDE cells) treated with the CFTR inhibitors NPPB or glibenclamide [11]. In addition, incubation of HT-29 cells (a human colorectal adenocarcinoma cell line possessing wt-CFTR) with glibenclamide resulted in the up-regulation of c-Src [11]. These results suggested that c-Src expression and activity are

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under CFTR regulation [11]. Two additional differentially expressed genes corresponded to the mitochondrial proteins CISD1 [13] and MTND4 [14]. The last protein was reported to be essential for the assembly and activity of the mitochondrial Complex I (mCx-I) [19]. Accordingly, we found later that the activity of this complex was reduced in cells with impaired CFTR function [15], an effect originally observed by Burton Shapiro [12, 20-23]. Noteworthy, the Shapiro's "mitochondrial theory" for cystic fibrosis was erroneously disregarded after the CFTR was cloned and found to be a Cl^- channel. Many years later, due to the differential display results showing a reduced expression of mitochondrial MTND4 in CF cells, we further explored the issue and confirmed Shapiro's observations of a reduced mitochondrial Complex I (mCx-I) activity in cells with impaired CFTR activity [12-15, 24]. Recently, mitochondrial effects in CF cells were also confirmed by Atlante et al. [25]. In addition, we found that IL-1β was able to modulate the expression of CFTR [26, 27]. We later found that an autocrine IL-1β loop was responsible for this effect on mCx-I and for the increased ROS levels observed in cells with impaired CFTR activity [24].

The aim of the present work was to explore whether or not the autocrine IL-1β loop present in cells with impaired CFTR activity was responsible for the increased c-Src activity observed, identifying in this way a new effector in the CFTR signaling pathway, upstream of c-Src. The results obtained, using different cell models and strategies, indicate that the impairment of the CFTR activity in IB3-1 or Caco-2/pRS26 cells determines increased c-Src activity levels compared to S9 cells (CFTR-rescue IB3-1 cells) and Caco-2/control cells, respectively. In addition, the increased c-Src activity observed in Caco-2/pRS26 cells (cells with KD-CFTR by shRNA transfections) was reduced by incubation with the IL-1 receptor antagonist IL1RN, implying that IL-1β is upstream of c-Src in the CFTR signaling pathway. Total cellular and mitochondrial ROS levels were also found elevated in Caco-2/pRS26 cells, and these effects were significantly reduced by incubation with IL1RN, the c-Src inhibitor PP2 or the NOX1/4 inhibitor GKT137831, suggesting that both, c-Src and NOX1/4, contribute to the increased cROS and mitoROS levels in these Caco-2/pRS26 cells with impaired CFTR activity.

2. Materials and Methods

2.1 Reagents – Dimethyl sulfoxide (DMSO) culture grade, luminol, p-coumaric acid, protease inhibitor cocktail (cat. No. P2714) and IL-1 receptor antagonist (ILRN) were purchased from

Sigma-Aldrich (St. Louis, MO). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Promega (Madison, WI), and Coomassie Brilliant Blue G-250 was from Bio-Rad Laboratories (Hercules, CA). 4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2) were from Calbiochem (San Diego, CA). MitoSOX (Molecular Probes, Cat. No. M36008), 2´,7´-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Cat. No. D399) and ROX (glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester, Invitrogen) were from Life Technologies Corporation (Carlsbad, CA). The NOX1/4 inhibitor GKT137831 was from MedChem Express (MCE, Monmouth Junction, NJ). All other reagents were analytical grade. Antibodies: goat anti-rabbit antibody coupled to alkaline phosphatase (goat polyclonal, sc-2007) and rabbit anti-Src antibody (rabbit polyclonal, sc-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-rabbit antibody coupled to horseradish peroxidase (polyclonal, W401B) was from Promega (Madison, WI); rabbit anti-phospho-Tyr-418-Src antibody (polyclonal, S1940) and rabbit anti-actin antibody (polyclonal, A2066)) were from Sigma-Aldrich.

2.2 Cell Cultures – IB3-1 cells (a bronchial epithelial CF cell line with ΔF508/W1282X CFTR genotype) [28] and S9 cells (IB3-1 cells transduced with an adeno-associated viral vector to stably express wild type (wt) CFTR) [29] were purchased from ATCC (www.atcc.org; the IB3-1 and S9 cells were then discontinued from ATCC and are now provided by the John Hopkins University). Caco-2 cells (ATCC; human colon carcinoma epithelial cells) expressing wt-CFTR [30, 31] were previously selected and cloned after transfections with four short hairpin RNA interference (shRNA) directed against different regions of CFTR [15]. The plasmid were constructed by OriGene Technologies, Inc. (Rockville, USA) and the four sense sequences were pRS25: AAGAAATATGGAAAGTTGCAGATGAGGTT; pRS26: AAATATCATCTTTGGT GTTTCCTATGATG; pRS27: ACAACTGGAATCTGAAGGCAGGAGTCCAA; pRS28: CT TACTTTGAAACTCTGTTCCACAAAGCT. The sequence corresponding to pRS-shGFP was used as a control (pRSctrl), corresponding to a non-effective shRNA plasmid against GFP, provided by OriGene. These cells were cultured adding 1 μg/ml puromycin to the culture medium for cells expansion, and without puromycin during the experiments. All cells were cultured in DMEM/F12 (Life Technologies, GIBCO BRL products, Rockville, MD) supplemented with 5% FBS (Internegocios S.A., Mercedes, Buenos Aires, Argentina), 100

units/ml penicillin, 100 µg/ml streptomycin (Life Technologies, GIBCO BRL, Rockville, MD). Cells were seeded at a density of 20.000 cells/cm^2 in p100 dishes (~ 60 cm^2, CellStar dishes, Greiner Bio-One) and cultured for 24 h in 10 ml (0.167 ml/cm^2) DMEM-F12 plus 5% FBS, at 37°C in a humidified air atmosphere containing 5% CO_2. Before treatments, cells were cultured 24 h in serum-free medium. The different treatments (PP2, GKT137831 and IL-1 receptor antagonist IL1RN) were performed in this second 24 h period, in serum free medium. The experiments were performed in the absence of serum (for a period of 24-48 h) to avoid the possible effects of serum components that might be present in excess in the fetal bovine serum compared to the human serum accessible to epithelial cells in the intact human tissue (growth factors, etc.). The relevance of these effects was pointed-out by Ye and Lotan [32].

2.3 Protein extraction – Cells were incubated as indicated above, washed twice with cold PBS, scraped with cold extraction buffer (10 mM Tris pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol) containing the protease inhibitor cocktail (5 ml of cocktail/20 g of cell extract) plus phosphatase inhibitors (2 mM Na_3VO_4, 1 mM NaF and 10 mM Na_2PO_7), and centrifuged at 14000 x g for 20 min at 4°C. The supernatant was stored at -80 °C until use. The protein concentration was measured using the method of Lowry et al. [33].

2.4 Western Blots– Western blots to quantify c-Src, p-c-Src and actin, were performed as previously described for c-Src and p-P38 [11, 24, 27]. Briefly, cytosolic extracts (30-50 µg of proteins) were separated on a denaturing SDS-PAGE (11% acrylamide/bis-acrylamide) and transferred to nitrocellulose membranes using a transfer buffer containing 20% methanol (39 mM glycine, 48 mM Tris-base, 0.037% SDS, pH 8.3) for 2 h at 100 V (constant voltage). The membranes were blocked with 5% BSA 1 h in TBST buffer (TBS pH 7 plus Tween-20 0.1% v/v) and incubated with a polyclonal antibody raised against phospho-Tyr-418-Src (polyclonal, S1940, from Sigma Aldrich, dilution 1:1000 in TBST buffer). Results were visualized by using an ImageQuant LAS 4000 system (GE Healthcare Life Sciences, Piscataway, NJ). After development by using the phospho-Tyr-418-Src antibody, the membranes were incubated with a stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 20 min at 50°C and then washed 10 min using distilled water and 30 min with TBST buffer (changing the buffer every 10 minutes), in order to remove the remaining β-mercaptoethanol. Stripped membranes

were blocked again as described and incubated with a polyclonal anti-Src antibody (N-16; dilution 1:1000 in TBST buffer) for 1 h. The membranes were then washed three times with TBST buffer, incubated with the secondary antibody and developed as before. Finally, as internal control, the membranes were incubated with a polyclonal anti-actin antibody (Sigma-Aldrich, A2066, dilution 1:1000 in TBST buffer) for 1 h, washed three times with TBST buffer, and then incubated with the secondary antibody, and developed as above. The band intensities were quantified by using the Image J software (http://rsbweb.nih.gov). c-Src activity is expressed as p-c-Src/c-Src and p-c-Src/actin ratios and c-Src expression as c-Src/actin ratio. WBs for CFTR were performed as previously described by Mailhot et al. [34].

2.5 Reverse Transcription and Quantitative Real-time PCR (qRT-PCR) for CFTR- Real-time PCRs (RT-PCR) were performed as previously described [15]. qRT-PCR reactions were carried out in triplicates (intra-assay and inter-assay triplicates). The final quantification values are expressed as the mean of the Relative Quantification (RQ) for each biological triplicate.

2.6 Reactive oxygen species (ROS)- Mitochondrial (mitoROS) and cellular ROS (cROS) levels were measured by using the fluorescent probes MitoSOX and DCFH-DA, respectively, in 96 well black plates (Greiner Bio-One, Germany; 655090) as previously reported [24]. The cells were cultured 24 h in serum-free medium as indicated above for 24 h in the presence of different concentrations of PP2, a specific Src family kinase inhibitor (0, 1, 5, 10 and 20 μM), of GKT137831, a NOX1/4 inhibitor (inhibits both NOX1 and NOX4; used at 0, 2.5, 5,10 and 20 μM) or IL1RN (0, 2.5, 5, 15, 30 ng/ml). To measure mitochondrial ROS, at the end of the incubation, DMEM-F12 medium was changed to Hanks´s medium containing 5 μM of MitoSOX and incubated at 37ºC in a 5% CO_2/air incubator for 10 min. For cROS, cells were incubated in Hank´s medium containing 10 μM of DCFH-DA and incubated at 37ºC in a 5% CO_2/air incubator for 40 min. Then, cells were washed with 0.2 ml of Hank´s buffer three times and the fluorescence was measured in a fluorescence plate reader (NOVOstar BMG LABTECH GmbH Ortenberg, Germany) at 37 ºC. Filters were Ex = 510 ± 10 nm, Em = 580 ± 10 nm for MitoSOX and Ex = 510 ± 10 nm, Em = 540 ± 10 nm for DCF, and readings were performed by using 10 cycles (3 flashes per well and cycle; excitation and measurements were done from the bottom of

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the plate). DCFH-DA is de-esterified intracellularly and, upon oxidation, turns into highly fluorescent 2',7'-dichlorofluorescein (DCF).

2.7 CFTR transport activity in shRNA-transfected cells - The fluorescent probe SPQ (6-methoxy-N-[3-sulfopropyl]quinolinium) was used to measure the CFTR chloride transport activity, as SPQ fluorescence is quenched by chloride. This method was used previously by our laboratory [15, 35]. Briefly, a Hitachi's slice holder was adapted to form a perfusion chamber, allowing to measure CFTR activity by using fluorescence spectrophotometry. Caco-2 cells were grown in p60 plates containing at least 4 rectangular coverslips (22×8 mm, from Hitachi) in DMEM-F12 medium plus 5% FBS. The coverslips were pre-treated with a coating solution (10 µg/ml fibronectin, 4.4 µg/ml collagen, 1.5 µg/ml BSA, in DMEM/F12). Then, cells were cultured for 24 h in serum-free DMEM/F12 medium and incubated ON in 5 mM SPQ (dissolved in serum-free DMEM/F12), washed three times with PBS and NaI buffer (135 mM NaI, 10 mM Glucose, 1 mM $CaSO_4$, 1 mM $MgSO_4$, 10 mM HEPES, 2.4 mM K_2HPO_4, and 0.6 mM KH_2PO_4, pH 7.4) and maintained at 37 °C for 30 min. Each coverslip was then placed in a separated culture dish and maintained under light-protecting conditions. For measurements, the coverslips containing confluent monolayer cells were inserted in a holder specially designed by Hitachi for the F2000 spectrophotometer, and immersed into a quartz cuvette containing NaI buffer, inside the fluorescence spectrophotometer. The coverslip holder was previously modified with a drill to allow the insertion of two cannulas of different diameter and length. These cannulas, coupled to a peristaltic pump, were used to perfuse the quartz chamber. All measurements were carried out at 37 °C, under perfusion and stirring. The selected wavelengths for SPQ were Ex =344 nm and Em =443 nm. To measure the baseline fluorescence (Fb), cells were perfused with NaI buffer for 100 s. Then, the cells were sequentially perfused with the $NaNO_3$ buffer (135 mM $NaNO_3$, 10 mM Glucose, 1 mM $CaSO_4$, 1 mM $MgSO_4$, 10 mM HEPES, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, pH 7.4), a cocktail containing CFTR activators (buffer NaNO3 containing 200 µM dibutyryl cAMP, 200 µM IBMX and 20 µM isoproterenol) and the quenching buffer (5 µM valinomycin in NaI buffer). Perfusion times were 200 s, 200 s and 100 s, respectively. The stock solutions of valinomycin, IBMX, dibutyryl cAMP and isoproterenol were prepared at 1000 X in culture-grade DMSO (Sigma-Aldrich). The collected data were plotted as F-Fq vs. time (F: fluorescence;

Fq: fluorescence value obtained after quenching the SPQ fluorescence by adding NaI plus valinomycin, at the end of SPQ fluorescence quenching).

2.8 Confocal Microscopy – Caco-2 cells were seeded on cell chambered coverglasses (4-chamber, Nunc, Cat. No. 155383, Lab-Tek, Thermo Fisher Scientific, Rochester, NY) and cultured in DMEM F12 containing 5% FBS. Before ROS measurements, cells were cultured 24 h in serum free media. To measure mitochondrial ROS, at the end of the incubation, the DMEM-F12 medium was changed to Hanks´s medium containing 5 µM of MitoSOX and incubated at 37 ºC in a 5% CO_2/air incubator for 10 min. For cROS, cells were incubated in Hank´s medium containing 10 µM of DCFH-DA and incubated at 37 ºC in a 5% CO_2/air incubator for 40 min. Then, cells were washed with 0.4 ml of Hank´s buffer three times and cell images were obtained by using a LSM510 Zeiss confocal microscope. To detect DCF and MitoSOX, laser lines of 488 and 543 nm were used, with filters LP505 and LP560 nm, respectively. Images were taken with a 20X objective. The adjustments of the confocal parameters were maintained between the different line cells to compare the intensity of the signal. For quantification we used Live Histogram from the ImageJ software (http://imagej.nih.gov/). Ten fields were averaged in three independent experiments (n=3, inter-assay).

2.9 Statistics – Unless otherwise indicated, the assays were performed at least by duplicates (intra-assay duplicates) and the experiments were repeated at least three times (inter-assay replicates, n=3), as specified in each figure legend. The results were expressed as mean ± SEM (n), obtained from inter-assay calculations. One-way ANOVA and the Tukey's test were applied to calculate significant differences among samples ($\alpha = 0.05$) and the Student's (William Gosset) t-test was used to obtain the significance level for R^2. * indicate significant differences ($p<0.05$). ED_{50} for IL1RN and PP2 were calculated using a sigmoidal dose-response curve (Hill plot).

3. Results

3.1 c-Src protein expression and activity in bronchial epithelial S9 and IB3-1 cells- First, we measured the total c-Src protein expression (total c-Src/actin) and activity (p-c-Src/actin) in bronchial epithelial IB3-1 (FQ) and S9 cells (IB3-1 "CFTR-corrected" cells). As shown in Figure

1A (WB) and 1B (quantification), IB3-1 cells incubated in serum-free medium for 48 h, had increased levels ($p<0.05$) of c-Src activity (p-c-Src/actin) (175 ± 27 % (n=3)) compared S9 cells (100 ± 15 % (n=3)). On the other hand, the total c-Src protein levels of S9 and IB3-1 cells, expressed as c-Src/actin (Fig. 1A and 1C), did not show significant differences. When the results were expressed as p-c-Src/c-Src activity, a significant difference ($p<0.05$) was also observed between IB3-1 and S9 cells (Fig. 1A and 1D). Taken together, these results suggest that the basal c-Src activity is increased in IB3-1 cells, which have impaired CFTR activity [28, 29].

3.2 Levels of c-Src activity in Caco-2 cells transfected with shRNAs specifics for CFTR- To further support the results showing that cells with impaired CFTR expression/activity have increased c-Src activity, we then used Caco-2 cells transfected with a control plasmid (pRSctrl) or with four different plasmids (pRS25, pRS26, pRS27, pRS28), containing each a CFTR-specific shRNA sequence. These transfected cells were selected and cloned from single cells to obtain stable CFTR knock-downs.

As shown in Figure 2A, a significant ($p<0.05$) reduction in the CFTR mRNA levels was observed in Caco-2/pRS25, pRS26 and pRS27 cells (Caco-2/pRS25 41 ± 6 % (n=3); Caco-2/pRS26 59 ± 3 % (n=3); CacCo-2/pRS27 49 ± 6 % (n=3)), transfected with CFTR-shRNA, compared to Caco-2/pRSctrl cells (100 ± 9 % (n=3)) (the last transfected with a control plasmid). Cells transfected with pRS28 shRNA did not have reduced CFTR mRNA levels. As shown in Figure 2B (WB) and 2C (quantification), CFTR protein levels were also significantly ($p<0.05$) reduced in Caco-2/pRS25, pRS26 and pRS27 cells. The CFTR shRNAs induced a significant increase of p-c-Src/actin ($p<0.05$) (Fig. 3A and 3B) in Caco-2/pRS25 (135 ± 4 % (n=3)), Caco-2/pRS26 (144 ± 7 % (n=3)) and Caco-2/pRS27 (140 ± 7 % (n=3)) cells compared to Caco-2/pRSctrl control (100 ± 5 % (n=3)) cells. The total c-Src levels (c-Src/actin) did not show significant differences after 24 h in serum-free media (Fig. 3A and 3C). On the other hand, Caco-2/pRS28, which did not show a decreased CFTR expression compared to Caco-2/pRSctrl, also did not show increased c-Src activity. Noteworthy, as shown in Fig. 3A, there is an inverse relationship between the levels of total c-Src and the levels of p-c-Src (most evident in pRS27 cells); it seems that the cells try to compensate the low levels of total c-Src increasing the active c-Src (p-c-Src). When the results were expressed as p-c-Src/c-Src activity, significant differences ($p<0.05$) were observed between shRNA cells and control cells (Fig. 3A and 3D). In addition, a

significant correlation ($p<0.05$) between p-c-Src/actin vs. mRNA CFTR (Fig. 3E) and p-c-Src/c-Src vs. mRNA CFTR (Fig. 3F) was observed between these cell lines. Therefore, this model system, in which the mRNA expression of CFTR was reduced, also showed increased c-Src activity, as it was observed for IB3-1 and S9 cells (Fig. 1). Taken together, the results obtained with IB3-1/S9 and Caco-2/Caco-2-shRNA cells, and the previous results obtained with CFDE and CFDE/6RepCFTR cells [11], we can conclude that the down-regulation of CFTR expression (Caco-2/pRS25-27 CFTR-shRNA cells), the CFTR mutations (IB3-1 and CFDE cells [11]) or the CFTR activity inhibition (CFDE cells plus glibenclamide [11]), induce increased levels of c-Src activity.

From these four cell lines, for the following assays, we selected the pRS26 clone since these cells showed the highest p-c-Src/actin levels, although the differences between pRS25, pRS26 and pRS27 were minimal and non-significant (Fig. 3B). To corroborate that the CFTR knock down in the pRS26 cells was effective, the CFTR activity was measured by using the chloride sensitive probe SPQ (Figure 3G). As shown in Figure 3H, the halide efflux (area under the curves) was significantly ($p<0.05$) reduced in Caco-2/pRS26 cells (73 ± 3 % (n=3)) compared to control cells (100 ± 5 % (n=3)).

3.3 Effects of the IL1R antagonist (IL1RN) on the c-Src activity of Caco-2/pRS26 cells- Taken into account previous results in which the IL-1β mRNA and protein secretion were increased in Caco-2/pRS26 cells compared to Caco-2/pRSctrl cells [24], we hypothesized that secreted IL-1β could be involved in the increased activity of c-Src observed in cell lines with impaired CFTR, through an autocrine effect. As shown in Figure 4A (WB) and 4B (quantification), incubation of Caco-2/pRS26 cells with increasing concentration of IL-1β receptor antagonist (IL1RN) [36-38] significantly decreased ($p<0.05$) p-c-Src/actin levels in Caco-2/pRS26 cells, almost reaching the values of control cells. On the other hand, although there was also a tendency to a reduced total c-Src expression in Caco-2/pRS26 cells treated with increased IL1RN concentrations, the differences did not reached significance (Fig. 4C). However, under these conditions (48 h incubation in serum free media), a significant difference was observed between the c-Src basal levels of Caco-2/pRS26 cells and Caco-2/pRSctrl cells (Fig. 4C), which were not observed when 24 h of incubation were used (Fig. 3C). The dose-response curve obtained for IL1RN as p-c-

Src/actin in Caco-2/pRS26 cells is shown in Figure 4D (ED_{50}= 3.2 ± 1.2 ng/ml, n=3, R^2=0.83, ~ 0.17 nM). These results are in consonance with our previous results regarding an IL-1β loop responsible for ROS overproduction in cells with impaired CFTR activity [24], and with the results reported by Verhaeghe et al. in CFT-2 and 16-HBE (cells with mutated CFTR), regarding the presence of an autocrine IL-1β loop [39]. The results suggest that cells with impaired CFTR expression or activity increase the p-c-Src levels due to an autocrine/paracrine effect of IL-1β. These results also imply that IL-1β is upstream of c-Src in the CFTR signaling pathway connecting CFTR and c-Src: impaired-CFTR→ IL-1β → c-Src.

The role of IL-1β on c-Src stimulation was further supported by the results obtained on p-c-Src using exogenous IL-1β (Fig. 4E and 4F). In Caco-2/pRSctrl cells, IL-1β induced a significant activation of p-c-Src (2 fold). However, in pRS26 cells the effect was less obvious and did not reach significance, since the p-c-Src basal values are already high. As a control, the mRNA expression of IL1R1 was measured; not significant differences were observed between pRS26 and pRSctrl cells (Fig. 4G). On the other hand, pRS25 and pRS27, but not pRS26 cells, showed a significant rise in IL1R1 mRNA levels (Figure S1). IL1RN also significantly affected the c-Src activity (p-c-Src levels) in Caco-2/pRS25 and Caco-2/pRS27 cells, compared to pRSctrl cells (Figure S2), further supporting the equivalent results obtained with pRS26 cells (Fig. 4B).

3.4 Reactive oxygen species (ROS) levels in Caco-2/pRS26 CFTR-shRNA cells. To evaluate the possible significance of the increased c-Src levels observed in Caco-2 shRNA cells, we measured the levels of reactive oxygen species (ROS), which are known to be increased in CF cells [40-42] and have a profound impact in the cellular physiopathology [43]. Even though the reduction in the CFTR mRNA (Fig. 2A) and transport activity (Fig. 3 G, H) of Caco-2/pRS26 were relatively small (~ 50% and ~30% respectively), a significant rise in ROS levels was observed for these cells. As shown in Figure 5, we observed by confocal microscopy that cellular ROS (cROS) (Fig. 5A and 5B) and mitochondrial ROS (mitoROS) (Fig 5C and 5D) were significantly increased ($p<0.05$) in Caco-2/pRS26 compared to Caco-2/pRSctrl cells. In addition, after 24 h of incubation in the presence of PP2, a significant reduction of the cROS and mitoROS levels of Caco-2/pRS26 cells was obtained (Fig. 5E and 5F). The ED_{50} obtained for PP2 were 1.1 ± 0.5 μM for DCF fluorescence and 1.3 ± 0.4 μM for MitoSOX fluorescence, which are similar to the value obtained by Ma et al. [44] in pancreatic cancer cells, in which the cleavage of Notch-1

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stimulated by TNF was reduced by PP2 incubation [44]. As is shown in Fig. 5G and 5H, increased concentrations of IL1RN (0 to 30 ng/ml) also reduced cROS and mitoROS in Caco-2/pRS26 cells, reaching the mitoROS levels similar values to those of pRSctrl cells when cells were incubated in the presence of IL1RN at 30 ng/ml. The effect on cROS was less pronounced (at IL1RN 30/ng/ml did not reach control values). On the other hand, the possible effect of NOX1/4 on the IL-1β induced ROS generation was explored. The incubation of pRS26 cells with increased concentrations of GKT137831, a NOX1 and NOX4 selective inhibitor, produced a clear inhibitory effect on both cROS and mitoROS (Figure S3). These results suggest that NOX1/4 have a key role in the generation of ROS species in pRS26 cells. Whether the c-Src and the NOX effects represent effects derived from parallel or consecutive actions in these cells remain to be determined.

It should be pointed-out here that even small changes in the CFTR mRNA levels and activity in KD cells produced significant changes in c-Src activity (24 h in serum-free medium) and expression (48 h in serum-free medium), and strong changes in the levels of cROS and mitoROS. Taken together, as occurs with MUC1 in CFDE cells [11], these results indicate that c-Src constitutes an intermediate molecule in the CFTR-signaling mechanism, connecting here the CFTR failure with increased cellular and mitochondrial ROS levels. On the other hand, IL-1β acts as a bridge connecting the CFTR failure and the increased c-Src activity.

4. Discussion

Using different cell lines (IB3-1 and Caco-2/pRS26) and strategies to inhibit the CFTR activity (ΔF508-CFTR, or shRNA), we found here that the c-Src activity, measured as p-Src/actin, is increased in cells with impaired CFTR activity. First, using IB3-1 cells (ΔF508 mutation in CFTR), we found a significant increase in the c-Src basal activity compared to S9 cells (IB3-1 cells ectopically expressing wt-CFTR). Then, to rule out the possibility of differences due to clonal selections or some other epiphenomena in the IB3-1 transduced cells, we used a different cell model and strategy to reduce the CFTR activity: Caco-2 cells (colon carcinoma cells expressing wt-CFTR) stably transfected with four different shRNA specific for CFTR. In agreement with the results obtained using IB3-1 and S9 cells, these Caco-2 KD-CFTR cells also showed a significant rise in c-Src activity. These results strongly suggest that cells with impaired CFTR activity have an increased c-Src activity, in agreement with previous results were we used CFDE CF cells (of unknown genotype) and the same cells treated with CFTR inhibitors

[11]. Interestingly, Huang et al. [45] found defective c-Src activation in IB3-1 cells compared to C38 cells (IB3-1 CFTR-corrected cells) and in CF_{15} (human nasal epithelial cell line) compared to Beas 2B cells (normal, non-CF, human bronchial epithelial cell line). However, these cells were cultured and stimulated adding serum or serum supplements containing an undefined composition of growth factors and other components, with unknown effects over CFTR expression and activity (or over other cells responses, including c-Src activation). For this reason, as pointed-out by Lotan et al. [32], we preferred to use serum-free medium. However, serum-free medium conditions are not free of consequences, since serum starvation can trigger apoptosis [46] and increased ROS production [47]. Therefore, the results obtained here should be taken with care, since these cells could be under exacerbated oxidative stress. Nevertheless, both control and CF impaired cells were cultured here under the same conditions, and the differences should reflect the influence of the CFTR activity.

We have shown previously that cells with impaired CFTR activity overexpress IL-1β, which in turn is responsible for the increased ROS levels and the reduced mitochondrial Complex I (mCx-I) activity of Caco-2/pRS26cells, a process mediated by NF-κB and p38, and not influenced by JNK or ERK1/2 [24]. This autocrine IL-1β loop, which can be disrupted by using the IL-1 receptor (IL1R) antagonist IL1RN (IL-1Ra or anakinra) [24], explains the elevated NF-κB found in cells with impaired CFTR activity [48], which may also have a role in tumor progression [49], as occurs with c-Src [50]. The relevance of IL-1β signaling in CF and its disruption by using IL1RN [24], has been recently corroborated *in vivo* by Iannitti et al., using a murine model and human genetic epidemiology, in which the pathogenic NLRP3 activity in CF could be negatively regulated by anakinra (IL1RN) [51]. Therefore, we hypothesized that the increased c-Src levels found in CF cells could be also due to the autocrine IL-1β signaling. The results obtained in the presence of IL1RN are in agreement with this idea: increased concentrations of IL1RN resulted in a reduction of p-c-Src in Caco-2/pRS26cells to values comparable to Caco-2/pRS26cells controls cells (Fig 4A and B). Also, increased concentrations of IL1RN significantly decreased both cytoplasmic and mitochondrial ROS (Fig. 5G and 5H), reaching a complete reversal of mitoROS induction at the higher IL1RN concentration (30 ng/ml).

The possible consequences of a persistent high basal value of c-Src activity in CF cells on the cellular physiopathology are numerous and complex. In this regard, we have shown previously

that the increased c-Src activity of CFDE cells induce a rise in MUC1 mRNA and protein expression [11]. On the other hand, Fiorotto et al. [52] have shown that the TLR4 phosphorylation by c-Src was significantly increased in cholangiocytes from Cftr-KO mice [52]. c-Src is also involved in the mechanism that determines *P. aeruginosa* invasion to epithelial cells [53], and in regulating the fast gate of the CFTR in airway epithelial Calu-3 cells [8]. In addition, many cellular functions affected in cystic fibrosis, such as apoptosis [54-58], cytokine secretion and signaling [59-61], and the levels of reactive oxygen species [12, 24, 42], were also known to be under c-Src modulation in other cell systems. However, a direct link between these cellular functions and c-Src has not been extensively explored for CF cells [62]. In these regard, we show here increased cellular and mitochondrial ROS levels in Caco-2/pRS26 cells (expressing shRNA against CFTR) compared to control cells (Caco-2/pRSctrl), and that the cellular and mitochondrial ROS levels of Caco-2/pRS26 and Caco-2/pRSctrl cells can be significantly reduced by incubation with PP2, an inhibitor of the Src family of protein tyrosine kinases. However, even in the presence of PP2, some difference between the ROS levels of Caco-2/pRS26 and Caco-2/pRSctrl control cells remains. On the other hand, an IL-1β/NOX1/4 pathway [63] seem to be present, since the incubation of Caco-2/pRS26 cells with the NOX1/4 selective inhibitor GKT137831, intriguingly reduced both cytoplasmic (DCF fluorescence) and mitochondrial (MitoSOX fluorescence) ROS (Supplementary Figure S3), and with a stronger inhibition compared to the effects of PP2 (c-Src inhibitor). Thus, the PP2 and GKT137831 results suggest that two pathways contribute to ROS generation in CFTR-KD cells: CFTR --| IL-1β → c-Src → ROS and CFTR --| IL-1β → NOX1/4 → ROS. c-Src may actually stimulate NOX, in turn producing a rise in the cRos and mitoROS, as occurs with HT29 colon carcinoma cells [64]. In addition, the cytoplasmic/RE ROS might be transported into mitochondria and vice versa, affecting both the MitoSOX and the DCF fluorescence. Alternatively, a mitochondrial NOX induced by c-Src [65] could account for the effects of GKT137831 over mitoROS. These possible alternatives are illustrated in Figure 6. Further studies are needed to understand the mechanisms involved in the overproduction of ROS in CFTR-impaired cells.

5. Conclusions

Figure 6 summarizes the results obtained here. The wt-CFTR channel activity, using a yet unknown mechanism, probably involving Cl^- as a second messenger [66], produces a signal that keeps the pathway IL-1β → c-Src at low levels of activity. By the contrary, the impairment of the

CFTR activity or expression, determines a rise in the c-Src activity and in the cellular and mitochondrial ROS levels, mediated through an autocrine effect of IL-1β. This autocrine effect of IL-1β, affecting c-Src expression/activity, might have profound effects on cells, including increased MUC1 expression [11], NF-κB and ROS levels [24], decreased mitochondrial Complex I activity [12, 14, 15], increased inflammasome activity [51], and angiogenesis [67]. Thus, IL-1β constitutes an additional element in the CFTR signaling pathway, located upstream of c-Src. In addition, two pathways (parallel or consecutive) seem to contribute to total ROS, one involving c-Src and the second involving the NOX activities inhibited by GKT137831.

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Figure Legends

Fig. 1. c-Src activity in S9 and IB3-1 cells. S9 and IB3-1 cells were incubated 24 h in 5% FBS and 48 h in serum-free DMEM/F12 medium. After incubation, cells were collected and proteins extracted to determine c-Src levels by WBs, as indicated in M&M. (A) Representative WB of phospho-Tyr-418-c-Src (p-c-Src) and total c-Src of whole cellular lysates. (B) Densitometric quantification of p-c-Src/actin. (C) Densitometric quantification of c-Src/actin. (D) Densitometric quantification of p-c-Src/c-Src. The results were expressed as percentage (%) relative to S9 values (mean ± SE, n=3, inter-assay values of three independent experiments). * indicates $p<0.05$.

Fig. 2. CFTR expression in Caco-2 cells transfected with shRNA for CFTR. Cells were incubated 24 h in 5% FBS and 24 h in serum-free medium. After incubation, total RNA or total proteins were extracted and the CFTR RNA and protein levels determined by using real-time PCR or WBs. (A) mRNA CFTR levels in Caco-2/pRSctrl, Caco-2/pRS25, Caco-2/pRS26, Caco-2/pRS27 and Caco-2/pRS28 cells. (B) Representative WB of CFTR of whole cellular lysates. (C) Densitometric quantification of CFTR/actin. The results were expressed as percentage (%) relative to Caco-2/pRSctrl values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRSctrl cells.

Fig. 3. c-Src activity in Caco-2 cells transfected with shRNA for CFTR. Caco-2/pRSctrl and Caco-2/pRS26 cells were incubated 24 h in 5% FBS and 24 h in serum-free media. Then, levels or c-Src and p-c-Src were measured by WB. (A) Representative WB of phospho-Tyr-418-Src (p-c-Src) and total c-Src of whole cellular lysates. (B) Densitometric quantification of p-c-Src/actin. (C) Densitometric quantification of c-Src/actin. (D) Densitometric quantification of p-c-Src/c-Src. (E) Correlation between p-c-Src/actin vs. CFTR mRNA, $R^2 = 0.90$, $p<0.05$. (F) Correlation between p-c-Src/c-Src vs. CFTR mRNA, $R^2 = 0.98$, $p<0.01$. (G) CFTR channel halide transport activity of Caco-2/pRSctrl (black) and Caco-2/pRS26 cells (grey). Arrows indicate the points of buffers addition. F, indicates fluorescence values; Fq, are the fluorescence values after SPQ quenching by adding NaI plus valinomycin. (H) Changes in the halide efflux between Caco-

2/pRSctrl and Caco-2/pRS26 cells were represented as the areas under the curve (total halide efflux). The results were expressed as percentage (%) relative to Caco-2/pRSctrl values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRSctrl cells.

Fig. 4. c-Src activity in Caco-2 cells transfected with shRNA for CFTR treated with IL-1 receptor antagonist (IL1RN). Caco-2/pRSctrl and Caco-2/pRS26 cells were cultured 24 h in 5% FBS and 24 h in serum-free medium before treatments for another 24 h, as indicated below. Cells were incubated with different concentrations of IL1RN (0, 2.5, 5, 15 and 30 ng/ml) (A) Representative WB of phospho-Tyr-418-Src (p-c-Src) and total c-Src of whole cellular lysates. (B) Densitometric quantification of p-c-Src/actin. (C) Densitometric quantification of c-Src/actin. The results were expressed as percentage (%) relative to Caco-2/pRSctrl values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRS26 untreated cells. (D) Dose-response curve for IL1RN in Caco-2/pRS26 cells (ED_{50}= 3.2 ± 1.2 ng/ml or ~ 0.17 nM; R^2= 0.83). Cells were incubated with 5 ng/ml IL-1β. (E) Representative WB of phospho-Tyr-418-Src (p-c-Src) of whole cellular lysates. (F) Densitometric quantification of p-c-Src/actin. (G) mRNA IL1R1 levels in Caco-2/pRSctrl and Caco-2/pRS26.

Fig. 5. Cellular ROS (cROS) and Mitochondrial ROS (mitoROS) in Caco-2/pRSctrl and Caco-2/pRS26 cells are modulated by the c-Src inhibitor PP2 and the IL1R1 antagonist IL1RN. Caco-2/pRSctrl and Caco-2/pRS26 cells were cultured 24 h in 5% FBS and 24 h in serum-free medium before treatments for another 24 h (A,B) cROS were measured by using the fluorescent probe DCFH-DA by confocal microscopy. (C,D) mitoROS levels were measured by using the fluorescent probe MitoSOX by confocal microscopy. Ten fields were averaged in each case. (E,F) Cells were incubated 24 h in serum-free medium and 24 h in the presence of different concentrations of the c-Src inhibitor PP2 (0, 1, 5, 10 and 20 μM). (E) cROS levels in Caco-2/pRSctrl and Caco-2/pRS26 cells. (F) mitoROS levels in Caco-2/pRSctrl and Caco-2/pRS26 cells.. (G-H) Cells were incubated 24 h in serum-free medium and 24 h in the presence of different concentrations of IL1RN (0, 2.5, 5, 15 and 30 ng/ml). (G) cROS levels in Caco-2/pRSctrl and Caco-2/pRS26 cells.. (H) mitoROS levels in Caco-2/pRSctrl and Caco-2/pRS26 cells. * indicates $p<0.05$ compared to Caco-2/pRS26 untreated cells, (n=3, inter-assay).

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Fig. 6. The graphic summarizes the results obtained. The down-modulation of the CFTR activity, either by using shRNA or CFTR ΔF508 mutation, upregulates c-Src activity. The increased c-Src activity in Caco-2/pRS26 cells is due to an IL-1β autocrine loop, inhibited by IL1RN. IL-1β is upstream of c-Src in the CFTR signaling pathway CFTR --| IL-1β → c-Src → ROS. A parallel pathway of CFTR --| IL-1β → NOX1/4→ROS signaling is also involved in the response an impaired CFTR activity. NOX1 is mostly a cellular membrane protein; however, depending on the cell type, NOX4 can be located in the plasma membrane, focal adhesions, the nucleus, or the endoplasmic reticulum. The intermediate molecule connecting CFTR and IL-1β expression/secretion is yet unknown. (--| red, inhibition; → blue, stimulation; ? unknown signaling mechanism).

Fig. S1. IL1R1 mRNA expression in Caco-2 cells transfected with shRNA for CFTR (Caco-2/pRSctrl, Caco-2/pRS25, Caco-2/pRS26 and Caco-2/pRS27). Cells were cultured 24 h in 5% FBS and 24 h in serum-free medium before RNA extraction. The results were expressed as percentage (%) relative to Caco-2/pRSctrl values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRSctrl cells.

Fig. S2. IL-1β receptor antagonist (IL1RN) effect on c-Src activity. Caco-2 cells transfected with shRNA for CFTR were incubated 24 h in serum-free medium and treated for 24 h with 30 ng/ml IL1RN. (A) Representative WB corresponding to phospho-Tyr-418-Src (p-c-Src) from whole cellular lysates of Caco-2/pRSctrl cells. (B) Densitometric quantification of p-c-Src/actin. (C) Representative WB corresponding to p-c-Src from Caco-2/pRS25 cells. (D) Densitometric quantification of p-c-Src/actin. The results were expressed as percentage (%) relative to Caco-2/pRS25 values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRS25 untreated cells (E) Representative WB corresponding to p-c-Src of Caco-2/pRS27 cells. (F) Densitometric quantification of p-c-Src/actin. The results were expressed as percentage (%) relative to Caco-2/pRS27 values (mean ± SE, n=3, inter-assay). * indicates $p<0.05$ compared to Caco-2/pRS27 untreated cells.

Fig. S3. Effect of the NOX1/4 inhibitor GKT137831 on cellular and mitochondrial ROS (cROS and mitoROS). Caco-2/pRSctrl and Caco-2/pRS26 cells were incubated 24 h in serum-free

medium and 24 h in the presence of different concentrations of the NOX1/4 inhibitor GKT137831 (0, 2.5, 5, 10 and 20 μM). (A) Cellular ROS levels (DCF fluorescence) in Caco-2/pRSctrl and Caco-2/pRS26 cells. * indicates $p<0.05$ compared to Caco-2/pRS26 untreated cells, (n=3, inter-assay). (B) Mitochondrial ROS levels (MitoSOX fluorescence) in Caco-2/pRSctrl and Caco-2/pRS26 cells. * indicates $p<0.05$ compared to Caco-2/pRS26 untreated cells, (n=3, inter-assay).

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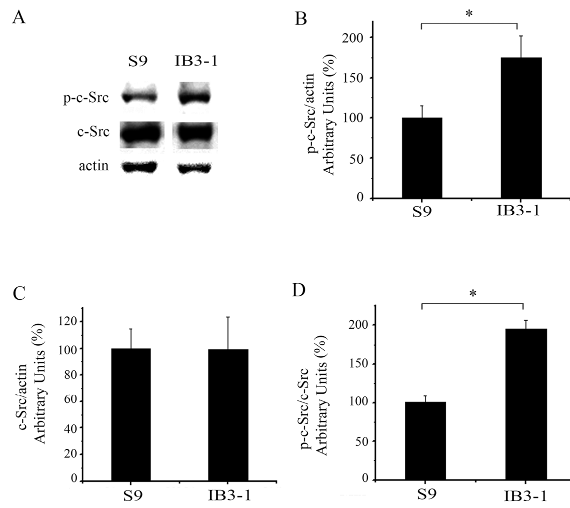

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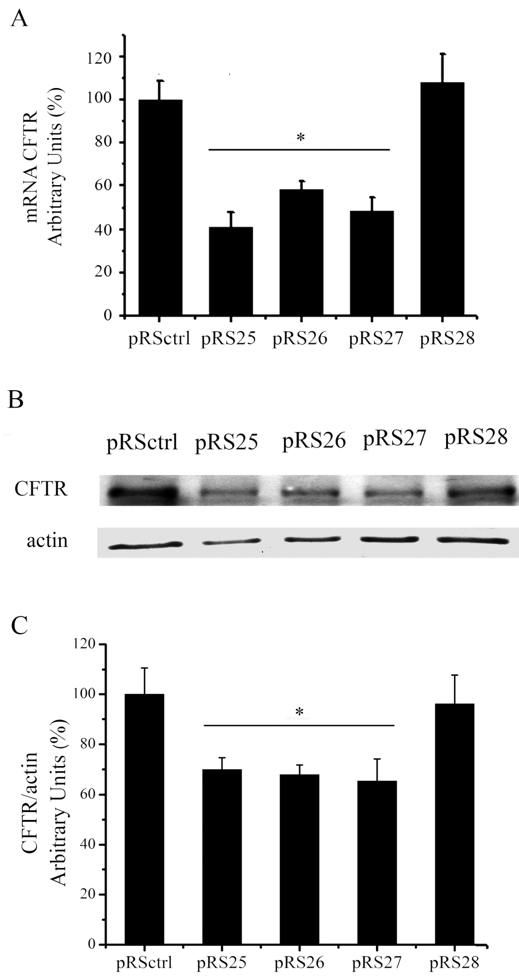

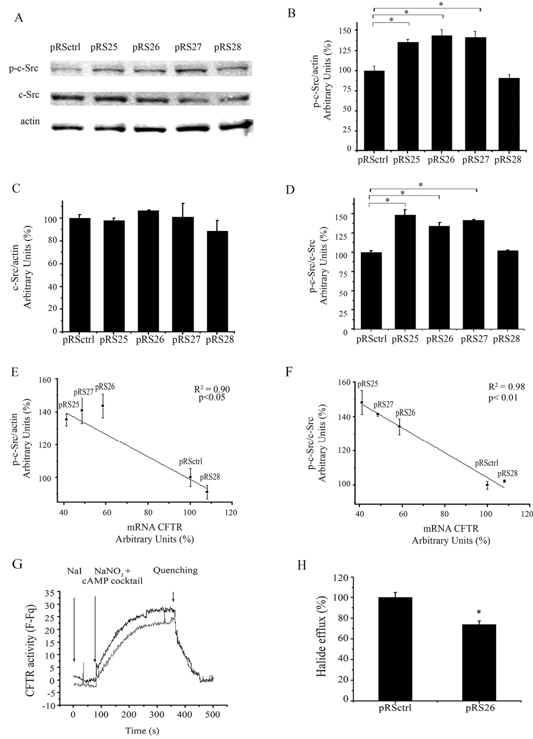

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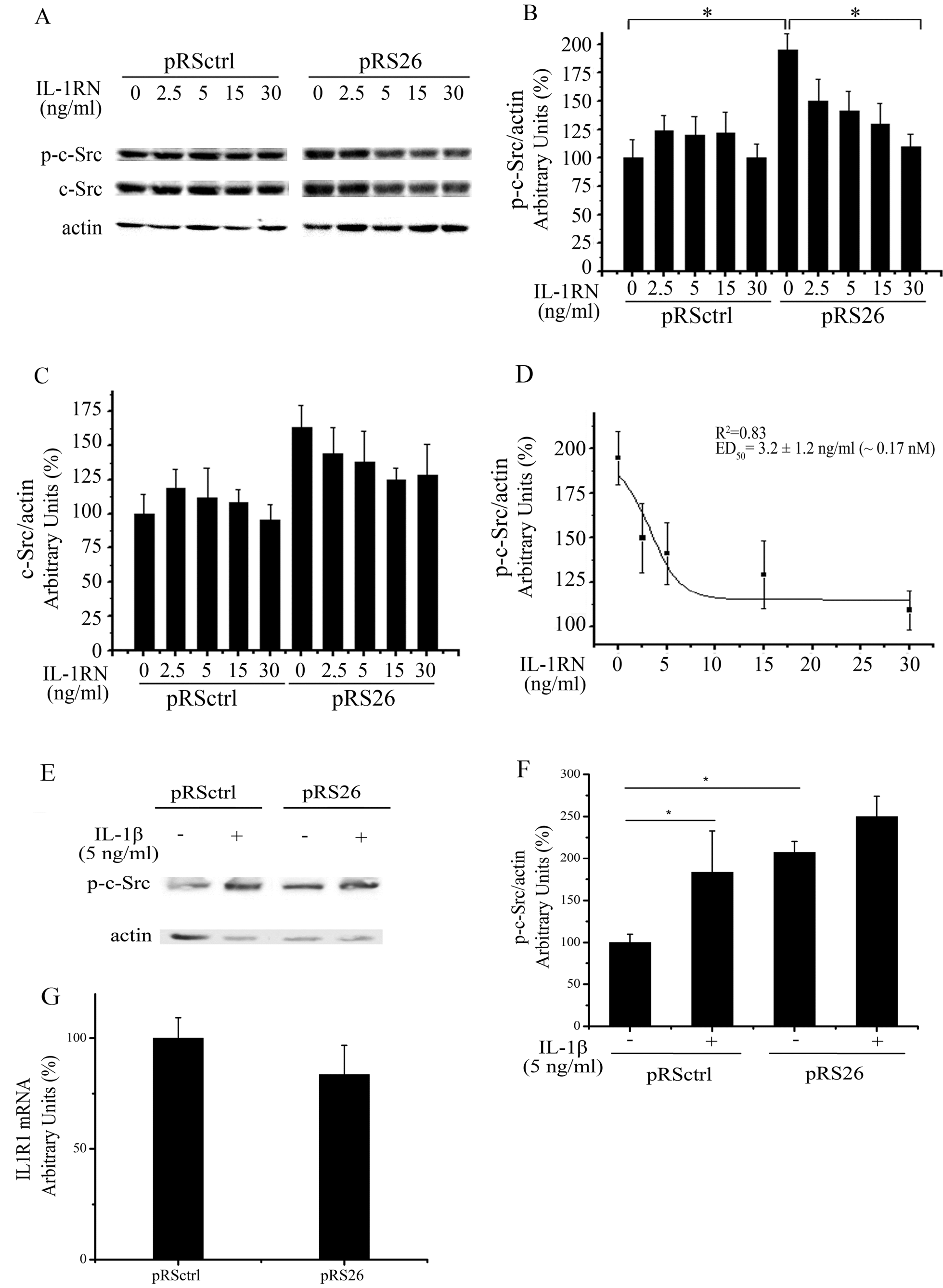
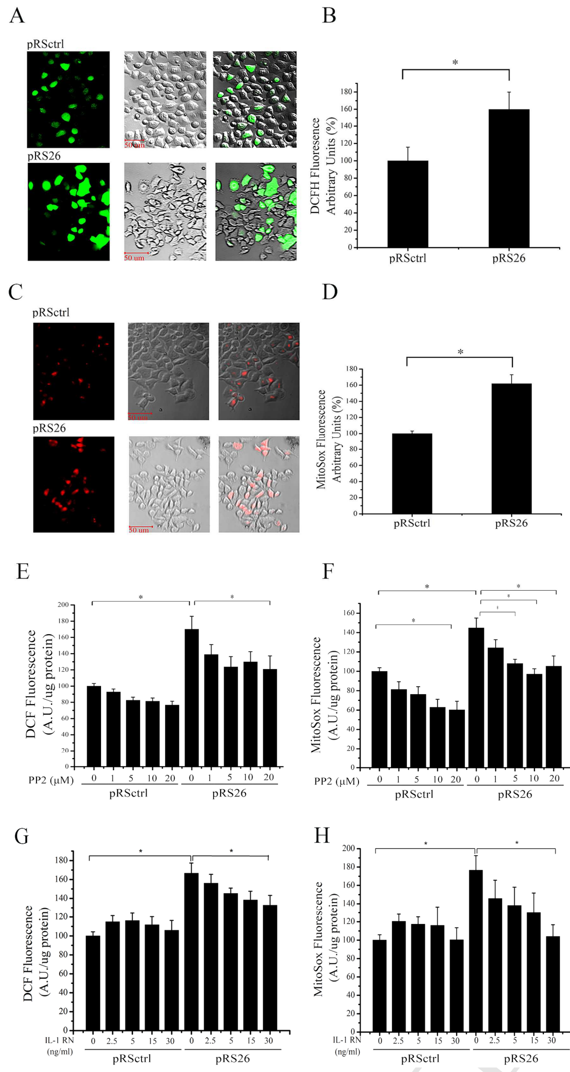


A
pRSctrl
pRS26
IL-1RN (ng/ml)
0 2.5 5 15 30 0 2.5 5 15 30
p-c-Src
c-Src
actin
B
p-c-Src/actin Arbitrary Units (%)
C
c-Src/actin Arbitrary Units (%)
D
R²=0.83
ED50= 3.2 ± 1.2 ng/ml (~ 0.17 nM)
p-c-Src/actin Arbitrary Units (%)
E
IL-1β (5 ng/ml)
F
G
IL1R1 mRNA Arbitrary Units (%)



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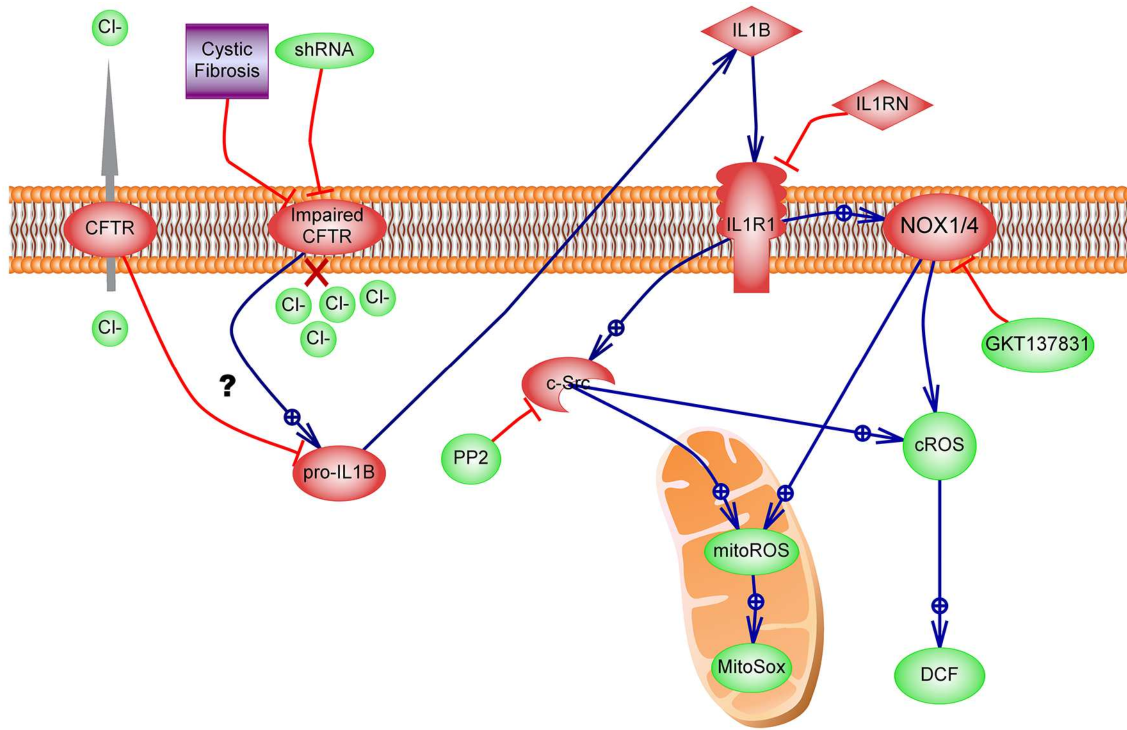

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Highlights

- c-Src activity is increased in cells with impaired CFTR activity
- The IL1R antagonist IL1RN (anakinra) restores normal c-Src levels
- An IL-1β loop is involved in this effect over c-Src
- IL-1β/c-Src and NOX1/4 pathways increase ROS levels in CFTR-KD cells

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