NF-κB mediates up-regulation of CFTR gene expression in Calu-3 cells by interleukin-1β

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Inflammation of the airways is a major feature of the inherited disease cystic fibrosis. Previous studies have shown that the pro-inflammatory cytokines TNFα and INFγ reduce the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in HT-29 and T84 cells by acting post-transcriptionally. We have investigated the effect of the pro-inflammatory peptide IL-1β on the expression of the CFTR gene in Calu-3 cells. IL-1β increased the production of CFTR mRNA in a dose- and time-dependent manner. Its action was inhibited by inhibitors of the NF-κB pathway, including N-acetyl-L-cysteine (NAC), pyrrolidine dithiocarbamate (PDTC) and a synthetic cell-permeable peptide containing the NF-κB NLS sequence. Gel shift analysis showed that IL-1β activated NF-κB in Calu-3 cells, and transfection experiments using p50 and RelA expressing vectors showed that exogenous transfected NF-κB subunits increased the concentration of CFTR mRNA. Gel shift analysis with antibody supershifting also showed that IL-1β caused the binding of NF-κB to a κB-like response element at position (-1103/-1093) in the CFTR 5’ flanking region. Transfection experiments using (-2150/+52) CFTR reporter gene constructs showed that the activity of the CFTR promoter is enhanced by exogenous transfected NF-κB and IL-1β and that this enhancement is due, at least in part, to the (-1103/-1093) κB site. We conclude that the intracellular signaling that leads to increased CFTR mRNA in response to IL-1β in Calu-3 cells includes the binding of NF-κB to the (-1103) κB element and a subsequent increase in CFTR promoter activity.
Cystic fibrosis (CF) is an inherited multisystem disease caused by a number of mutations that affect the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (1-3), an integral membrane protein that functions as a cAMP-regulated chloride channel (4). Despite our increased understanding of the structure and function of CFTR, the mechanisms by which the absence or dysfunction of CFTR causes numerous disorders is less well documented. One of these puzzling disorders is the destruction of the lung by infective-inflammatory injury, which is the most common cause of morbidity in cystic fibrosis. Several clinical studies of the airways of young patients with cystic fibrosis have found excessive amounts of pro-inflammatory cytokines, even in the absence of any clinical lung disease or detectable infection (5). At least two of these pro-inflammatory peptides, TNFα and INFγ, modulate the expression of the CFTR gene by acting post-transcriptionally (6, 7). However, the effects of other pro-inflammatory cytokines, such as interleukin-1β (IL-1β), on CFTR gene expression are poorly documented (8). TNFα and IL-1β, which have overlapping and synergistic effects on cell function, should be considered to be proximal or primary cytokines, in many respects, as they are produced early in the response to infection, and determine the pattern of later cytokine production and secretion in the inflammatory response. The signaling pathways mediated by the receptors for both IL-1β and TNFα are complex and involve multiple coordinated kinases, including JNK, SAP/p38 and ERK2 MAP kinases. These activate transcription factors such as AP-1 and NF-IL6 (C/EBPβ). The receptor signaling pathways ultimately converge upon the NF-κB-inducing kinase (NIK), which activates the IKK (IκB kinase) complex. Phosphorylation of the NF-κB cytoplasmic inhibitory binding protein, IκB, by IKK triggers its ubiquination/degradation and allows the release of the active form of the NF-κB factor.
Thus, we need to know whether IL-1β, like TNFα, modulates CFTR gene expression, and whether this effect depends on transcription factors such as NF-κB.

NF-κB is clearly involved in the inducible regulation of genes in the immune system and the inflammatory response. NF-κB consists of dimeric complexes of Rel/NF-κB proteins sequestered in the cytosol by the inhibitory proteins IκB. The phosphorylation and degradation of IκB leads to the translocation of NF-κB to the nucleus, where it binds to specific cis-regulatory elements located in transcriptional regulatory regions of its target genes.

Previous studies on CFTR gene transcription have shown that the cell-specificity and the low concentration of CFTR are at least partly dictated by the genomic sequences 5’ upstream of the transcription initiation region (9, 10). Analysis of the CFTR 5’ region revealed that the CFTR promoter is a TATA-less promoter, which probably explains why transcription is initiated at multiple start points (9-11), and has a high G/C content. Studies on the CFTR promoter have provided differing results on the location of the major transcription initiation site and the minimal promoter length (9, 11). This proximal region of the CFTR promoter contains many GC boxes that could be SP1 binding sites (9) and a cAMP-response element (CRE) at -48 (with respect to the initiation site by Yoshimura et al. adjacent to an inverted CCAAT element (Y box) at -60 (12, 13). Li et al. (14) recently suggested that the transcription of CFTR is regulated in part by factors directing modifications of chromatin and interacting with the Y box element. However, other more distal regulatory regions within the CFTR 5’ region may also be responsible for basal and PKA-mediated gene expression (13). These regions include putative AP-1 elements (at positions -976 and -1058) close to a DNase I hypersensitive region specific to cells expressing the CFTR gene (around -950) (10).
We have therefore investigated the effect of IL-1β on the expression of the CFTR gene in Calu-3 cells. Northern blot analysis showed that IL-1β stimulated the production of CFTR mRNA via a NF-κB-dependent mechanism. Electrophoretic mobility shift assays and reporter transfection assays indicated that a κB element at position (-1103) of the CFTR 5’ flanking region was involved in this regulation.
MATERIALS AND METHODS

Cell culture and reagents: Calu-3, HT-29 and T84 cells were obtained from the ATCC (American Type Culture Collection, Rockeville, MD); Calu-3 and HT-29 cells were cultured at 37 °C, 5%CO₂ in DMEM and T84 cells in DMEM/F-12 medium (Life Technologies, France). The medium for Calu-3 cells was supplemented with non-essentials amino acids, pyruvate and Hepes. All media contained antibiotics (penicillin, streptomycin, 50 µg/ml each) and 10% fetal calf serum (Life Technologies). IL-1β and active and inactive forms of NF-κB NS 50 peptides were purchased from Calbiochem, N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) from Sigma-Aldrich.

Northern blot analysis: Aliquots (15 µg) of total RNA isolated from Calu-3 or T84 cells using the Trizol reagent (Life Technologies) were size-fractionated by agarose gel electrophoresis. The RNA was then transferred to a nylon membrane (Promega, Charbonnières, France) by capillary blotting, fixed by heating and hybridized under standard conditions with the Quick Hyb protocol provided by Stratagene (Ozyme, Les Ulis, France). The ³²P-labeled CFTR probe consisted of the 1.5 kb EcoRI-EcoRI fragment of human CFTR cDNA labeled by random priming. The membrane was also hybridized with a human β-actin cDNA probe from Oncogene Science (France Biochem, Meudon, France). The mRNAs were quantified by densitometric scanning of the autoradiograms using an ImageMaster VSD (Pharmacia-Biotech-Amersham, Orsay, France)

Nuclear extracts: Cells were rinsed twice with cold phosphate-buffered saline pH 7.4 (PBS), scraped off into cold PBS and centrifuged at 600 g for 3 min at 4 °C. The resulting cell pellet was suspended in lysis buffer A (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-NaOH (pH7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM...
EDTA, and supplemented with an antiprotease cocktail (Boehringer). IGEPAL CA 630 (Sigma) detergent was then added (0.05% (V/V)) and the cells were left on ice for 10 min. The crude nuclei released by lysis were pelleted by centrifugation at 1100 g for 10 min at 4°C, washed in lysis buffer A and suspended in buffer C (20 mM HEPES (pH 7.9), 25% (V/V) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA and antiprotease cocktail (Boehringer) by vigorous pipeting. The lysis of nuclei was checked under a phase-contrast microscope. The nuclei were left on ice for 15 min, vortexed and clarified by centrifugation at 15000 g for 5 min at 4 °C. The protein concentration (approximately 5mg/ml) was determined by the Lowry method and the nuclear extracts were rapidly frozen and stored at -80 °C.

**Electrophoretic mobility shift assay (EMSA):** Calu-3 cells were placed in medium without serum for 16-18 hours, and IL-1β (Calbiochem, France) (2 ng/ml) was then added to the medium. Oligonucleotides, synthesized by Eurobio (France), were annealed, end-labeled with γ³²P dATP (50 µCi at 300 Ci/mmol, Amersham) using the T4 polynucleotide kinase (Life Technologies) and purified on micro spin purification columns (Qiagen, France). Binding reactions were done by mixing approximately 40 fmol double stranded end-labeled oligonucleotides with 10 µg nuclear extract proteins for 20 min at room temperature in a final volume of 20 µl. The mix contained EMSA binding buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.5mM DTT, 0.5 mM EDTA, 5% glycerol and 0.2-1 µg poly(dI-dC)). Unlabeled double-stranded competitors were added to the binding reaction mixture 10 min prior to adding labeled probe. Supershift analyses were done by incubating nuclear extracts with 1.5 ng the appropriate antibody (anti p50, RelA or cRel from Santa Cruz Biotecnology, Inc. Tebu, France) for 30 min on ice prior to adding³²P-labeled probe. Samples were loaded onto a 5% non-denaturing polyacrylamide gel in 0.5x Tris-borate EDTA, without any loading dye, and electrophoresed at 125 V for 2.5 hours. The separated DNA-protein complexes
were visualized on gels by autoradiography at -80 °C for 4-16 hours. All experiments were done at least 3 times.

**Plasmid constructions:** The (-2150/+52)pGL3 construct was obtained by subcloning the SacI/HindIII2.2 kb fragment of the (-2150/+52)-luc construct, generously provided by Dr G. S. McKnight (University of Washington, Seattle, WA), into the pGL3 basic vector Promega, Charbonnières, France). In vitro site-directed mutagenesis was performed on this plasmid using the Stratagene mutagenesis system following the manufacturer’s instructions. The sequences of the (-2150/+52)pGL3 constructs were confirmed by DNA sequencing (Genome Express, France). Two independent clones were tested in transfection experiments and gave similar luciferase activities. CMV-driven expression plasmids for p50 and RelA were kindly provided by Dr Bauerle (Tularik, San Francisco, CA). The β-galactosidase encoding vector (CMV-βgal) was purchased from Clontech.

**Transfections and assays of reporter gene constructs:** Plasmids for transfection were purified with the Qiagen endo-free plasmid Mega kit. To avoid any possible influence of the quality of plasmid preparation on transfection efficiency, each series of transfection experiments was performed using products from at least 2 different amplifications. Calu-3 and T84 cells were transiently transfected in six-well dishes with LipofectAmine Plus reagent (Life Technologies) and 2μg/well of DNA in opti-MEM medium (Life Technologies) for 20 hours, according to the manufacturer's directions. The transfected cells were washed three times with cold PBS and scraped off into 240 μl lysis buffer (Promega). Each lysate was mixed vigorously and clarified by centrifugation at 12000 g for 3 min at 4 °C. Supernatants were used for reporter assays. The luciferase activity in 20 or 30 μl extract was evaluated with the Luciferase Reporter Assay System (Promega) and a Berthold Biolumat LB9500 luminometer.
**RESULTS**

*Effect of IL-1β on CFTR mRNA in Calu-3 cells*— Two pro-inflammatory cytokines, TNFα and INFγ, have been reported to down-regulate CFTR gene expression in colonic cell lines (6, 7). We determined whether this response to pro-inflammatory mediators was a general phenomenon by testing the ability of TNFα and IL-1β to modulate CFTR gene expression in colonic (HT-29) and pulmonary (Calu-3) cell lines. Cultures of HT-29 and Calu-3 cells were stimulated for 24 hours with TNFα or IL-1β, and CFTR mRNA was measured by northern hybridization analysis. TNFα reduced the amount of CFTR mRNA in HT-29, as it has already been described (6, 7), but increased it slightly in Calu-3 cells (Fig. 1A). These results contrast with those obtained with IL-1β, which significantly increased the CFTR mRNA in both cell lines. The increase in CFTR mRNA in Calu-3 cells induced by IL-1β treatment was always greater than that induced by TNFα, under our experimental conditions.

Treatment of Calu-3 cells with various doses of IL-1β (0.5, 1.0 and 2.5 ng/ml) increased the amount of CFTR mRNA in a dose-dependent manner (Fig. 1B, upper bands), while the mRNA of the constitutively-expressed control, β-actin, was stable under all conditions (Fig. 1B, lower bands). We investigated the time course of this effect by incubating Calu-3 cells with IL-1β (2 ng/ml) for 0, 3, 6, 9, 15 and 24 hours and analyzing the CFTR mRNA by northern blotting (Fig. 1C). Figure 1C, right panel, shows the kinetic of the CFTR mRNA increase in response to IL-1β. IL-1β caused a rapid increase in steady state levels of CFTR mRNA and continued to exert its effect for up to 24 hours thereafter. Thus, these data indicated that the effect of IL-1β on CFTR mRNA production is dose and time-dependent.
Influence of IL-1β on CFTR gene expression: role of a NF-κB dependent pathway- Because IL-1β is known to activate NF-κB in several cell types, we determined whether the IL-1β-induced expression of CFTR mRNA was mediated by a NF-κB-dependent mechanism. In the first series of experiments, the cells were incubated with N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), two radical scavengers that inhibit IκB phosphorylation/degradation, and consequently NF-κB activation. In the second series of experiments the cells were pre-treated with a synthetic peptide containing the NF-κB nuclear localization signal (NLS) fused to a membrane-permeable hydrophobic region, that specifically prevents NF-κB nuclear translocation, or with its inactive analogue (15, 16). The cells were then stimulated with IL-1β. NAC and PDTC did not modulate the amount of constitutive CFTR mRNA, but markedly reduced the IL-1β-mediated increase in CFTR mRNA (Fig. 1B). Similar results were obtained with cells pre-treated with the NF-κB NLS, but not those pre-treated with its inactive form. These results suggest that IL-1β acts on CFTR gene expression, at least in part, via a NF-κB-dependent pathway. We investigated the link between IL-1β, NF-κB and the expression of the CFTR gene using two experimental strategies. We first measured the NF-κB activity by EMSA using nuclear extracts from Calu-3 cells treated with IL-1β and a labeled consensus κB probe (mouse immunoglobulin kappa light chain enhancer, κB element). IL-1β activated NF-κB, as indicated by the appearance of DNA-NF-κB complexes (Fig. 2). We also transiently transfected Calu-3 and T84 cells (a human intestinal cell line that also expresses the CFTR gene constitutively) with CMV-driven RelA (p65) and p50 expressing vectors, or the empty vector (mock control) and then subjected them to northern blot analysis. We checked that this transfection procedure mimicked the endogenous activation of the NF-κB factor by looking for specific factors binding the κB element in the nucleus of the p50/p65-transfected cells (Fig. 2). The relative amounts of CFTR mRNA in NF-κB-expressing cells were
greater than those of the control cells (Fig. 3). Hence, IL-1β activates NF-κB in Calu-3 cells and the generation of active NF-κB can lead to the up-regulation of CFTR gene expression.

*The (-1103/-1093) region as a putative NF-κB response element*— Previous studies on the CFTR promoter have shown that the CFTR 5’ flanking region is sufficient to trigger the tissue-specific, low activity of the CFTR gene (9, 10). If the NF-κB factor acts on CFTR gene transcription there must be a NF-κB response element in a region regulating the transcription of the CFTR gene, and in particular, in the 5’ flanking region. Analysis of the sequence of this region revealed a putative NF-κB response element between -1103 and -1093 (5’-GGGAATGCCC-3’) that differed by one bp from the consensus NF-κB response element (GGGNNTYYCC) (17). We next investigated whether the action of NF-κB on CFTR mRNA production involved the binding of NF-κB to this putative cis-regulatory sequence. We used an electrophoretic mobility shift assay (EMSA) to determine the capacity of the (-1103/-1093) sequence to bind NF-κB proteins using the (-1111/-1090) region of CFTR as probe and nuclear extracts from T84 cells treated with IL-1β (2 ng/ml) (Fig. 4). Cells incubated with IL-1β formed a single shifted complex. This complex was displaced by excess unlabeled consensus κB element from the mouse immunoglobulin kappa light chain enhancer (B site), whereas the same excess of unlabeled consensus AP-2 element did not change the binding of the labeled probe (Fig. 4, compare lanes 4 and 5). Thus, a specific IL-1β-induced factor that has a high affinity for the consensus κB element binds to the (-1111/-1090) probe. We attempted to identify the proteins that formed the shifted complex in a supershift analysis using antibodies to 3 members of the NF-κB/Rel family (p50, RelA (p65) and cRel). The formation of the shifted complex was prevented by anti-p50 or anti-RelA, but not by anti-cRel antibodies (Fig. 4, lanes 7, 8 and 9).
Enhanced CFTR promoter activity by NF-κB- NF-κB (probably the p50/RelA heterodimer) bound the (-1103) κB element in vitro. Thus, this sequence could constitute a κB regulatory element in the CFTR 5’ flanking region. We investigated this and determined whether NF-κB acted on the transcription of the CFTR gene by constructing a human CFTR promoter-luciferase plasmid, (-2150/+52)pGL3, and transfecting Calu-3 cells with it (Fig. 5A). Co-transfection experiments using this construct plus the CMV-p50 and RelA expressing vectors, or the mock plasmids as control, showed a 2.0 fold increase in the promoter activity when the cells were cotransfected with the CMV-p50 and RelA plasmids. Thus the CFTR promoter activity was stimulated by NF-κB subunits. We then generated a site-specific mutation of the (-1103) κB element in the (-2150/+52)pGL3 construct by changing two nucleotides within the (-1103/-1093) sequence. This base transition was sufficient to destroy the binding capacity of the NF-κB proteins (data not shown). The activity of mutated CFTR promoter was less strongly stimulated (1.4 fold induction) than was the wild type one when this reporter construct was co-transfected with p50 and RelA expressing vectors (Fig. 5A). Hence, the (-1103/-1093) κB element of the CFTR 5’ flanking region may take part in the up-regulation of the CFTR gene by exogenous transfected NF-κB factor. Finally, we performed transfection experiments with the (-2150/+52)pGL3 reporter gene constructs and stimulated cells with IL-1β to determine whether NF-κB activation mediated by IL-1β involved this κB element. IL-1β produced more luciferase activity in cells transfected with the wild type constructs than in cells transfected with the mutated one (Fig. 5B). These results indicated that IL-1β stimulation reproduced the results obtained with exogenous NF-κB factor. The reproducible 1.4 - 2-fold induction of CFTR promoter activity after co-transfection with p50/p65 expressing vectors or after IL-1β treatment is within the range of induction reported for the same promoter after stimulation with forskolin (12, 13). The modest increase caused by IL-1β
was abolished when the (-1103) κB element was mutated. Thus, the transfection data support the
northern blot analyses presented in Fig 2 and 3. Therefore we believe that this inducibility of the
CFTR promoter could be biologically important and conclude that the (-1103) κB element of the
CFTR gene takes part in the induction of CFTR mRNA production in response to IL-1β and in the
subsequent NF-κB activation in Calu-3 cells.
The development of an inflammatory response is a complex biological process that involves many changes in gene expression in populations of interacting cells, all with different time courses. In the microenvironment of the inflammation site, this complex cascade of events produces extracellular signals, such as IL-1β and TNFα, that tightly control the changes in gene expression. This response helps to restore the equilibrium disturbed by the initial injury (bacterial, viral or parasitic infection), but excessive production of inflammatory mediators may have negative effects and can lead to the destruction of the damaged tissue. Such a failure to modulate the inflammatory response occurs in cystic fibrosis. The abundance of pro-inflammatory cytokines in the airways of CF patients reflects the dramatic lung inflammatory injury that is often lethal in cystic fibrosis. Several studies have focused on the effect of cytokines in the inflamed CF airways on the expression of the CFTR gene (6, 7). These studies have shown that CFTR gene expression is modulated by inflammatory signals, suggesting that CFTR contributes to the change in cell functions caused by the inflammatory stress. We have now obtained evidence that IL-1β stimulates the production of CFTR mRNA in a dose-, time-, and NF-κB-dependent manner in a pulmonary cell line derived from the serous cells of submucosal glands. The EMSA and promoter-reporter gene transfection assays indicate that IL-1β causes NF-κB to bind to the CFTR 5’ flanking region, leading to increased CFTR gene transcription. This is of particular interest, since molecules, such as forskolin, that increase intracellular cAMP were the only extracellular stimuli that increased CFTR gene transcription until now. The cells were incubated with IL-1β for close to 20 hours in most experiments, so that the observed effect on CFTR gene expression reflects primary and possibly secondary IL-1β-induced transduction pathways.
IL-1β and TNFα have broadly overlapping, often synergistic, effects on cell functions. Not surprisingly, these cytokines also share signaling pathways, such as those that activate the NF-κB transcription factor and the p38, JNK and ERK MAP kinases. Our data for Calu-3 cells show that TNFα and IL-1β stimulate the expression of CFTR mRNA, but to different extents. Surprisingly, the two cytokines have opposing effects in HT-29 cells, TNFα decreases CFTR mRNA expression, whereas IL-1β increases it. This finding suggests that IL-1β involves signaling events that are not shared with TNFα. One possible explanation is that TNFα and IL-1β signaling pathways stimulate different signals and/or activate the same signal but confer on it different properties under some conditions.

Studies on the IL-1β signaling pathway have often focused on the complex cascade of intracellular molecular events that converge ultimately on the activation of others transcription factors including AP-1 (18) and C/EBP (19). However, the IL-1 receptor-mediated signaling pathway also activate the NF-κB transcription factor (20, 21) which is of central importance to immune and inflammatory responses. Our findings provide evidence that the IL-1β-induced expression of the CFTR gene is mediated by NF-κB activation and binding to the CFTR gene 5’ flanking region. Nonetheless, activation of NF-κB does not appear to be sufficient, by itself, to activate CFTR gene transcription, as indicated by the effects of TNFα in HT-29 and Calu-3 cells. Also, TNFα is a strong activator of NF-κB in both cell lines (data not shown), it inhibits CFTR mRNA expression in HT-29 cells and only slightly stimulates CFTR gene expression in Calu-3 cells. These findings suggest that regulation of CFTR gene transcription by IL-1β that we unmasked in Calu-3 cells involves different IL-1β-induced transcription factors in addition to NF-κB. This is supported by the presence of several putative binding sequences for IL-1-induced transcription factors in the CFTR 5’ flanking region,
including two AP-1 response elements in the vicinity of the (-1103) κB element (13). There is strong evidence for interactions between NF-κB and bZIP transcription factors, such AP-1 and C/EBP, influencing the ability of NF-κB to regulate gene expression in a selective manner (22-26). Thus, interactions between these factors could lead to a synergistic activation of the CFTR transcription.

The subtle difference between the (-1103) κB element and the consensus κB motif probably explains the slight difference in NF-κB binding affinity, between the (-1103) κB element and the canonical κB element from the immunoglobulin kappa light chain enhancer (in vitro assay, data not shown). Nevertheless such variant NF-κB binding sites have been found to be important in gene transcription (27), because protein-protein interactions with others transcription factors are believed to stabilize their binding to DNA. Thus, interactions between NF-κB and other IL-1β-induced transcription factors could stabilize NF-κB binding to the CFTR gene 5’ flanking region.

The increased CFTR gene expression in response to NF-κB activation could be of pathophysiological significance in cystic fibrosis, since it was recently demonstrated that NF-κB is endogenously activated in CF cells. The endogenous activation of transcription factor NF-κB has been correlated with the constitutive inflammatory response in CF cells (28). It is suggested that the presence of the F508del- mutated CFTR proteins in the cells produces an endogenous stress that results in increased NF-κB activation and thus an increased production of endogenous pro-inflammatory cytokines. The present study shows that the activation of NF-κB can also lead to increased CFTR gene activity in airway epithelial cells. Thus, the F508del- protein could exert a positive influence on its own synthesis in some conditions by activating the NF-κB system.
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FIGURE LEGENDS

Fig. 1. Northern blot analysis of CFTR mRNA in Calu-3 and HT-29 cells following stimulation with IL-1β and/or TNFα

A) HT-29 and Calu-3 cells were incubated with or without 15ng/ml TNFα or 2.5ng/ml IL-1β for 24 hours. Total RNA was then prepared, fractionated on agarose gel, transferred onto nylon membranes, and hybridized with the appropriate cDNA probe (CFTR β-actin). A Northern blot autoradiograph representative of three independent experiments is shown.

B) Calu-3 cells were incubated for 30 minutes with 30 mM N-acetyl-L-cysteine (NAC) or 100µM pyrrolidine dithiocarbamate (PDTC) and subsequently stimulated with increasing concentrations of IL-1β (0.5, 1.0 and 2.5 ng/ml) for 24 hours (left panel); with 18mM NF-κB-NS50 (NF-κB NLS peptide) and with its inactive form (control peptide) for 24 hours (right panel). The total RNA were then subjected to Northern blot analysis as in A.

C) The IL-1β-induced increase in CFTR mRNA. Right panel, a typical Northern blot obtained after incubating the cells with IL-1β (2 ng/ml) for the indicated time. Left panel, the mRNAs were quantified by densitometry using an ImageMaster VSD (Pharmacia-Biotech-Amersham, Orsay, France), and the amounts of CFTR mRNA were normalized to those of β-actin. The results are expressed as ratios of arbitrary units.

Fig. 2. NF-κB DNA binding activity in Calu-3 cells. Electrophoretic mobility shift assay using a consensus κB element as probe and nuclear extracts from control (lanes 1-3), IL-1β stimulated (lanes 4-8), mock transfected (lane 9) and p50/RelA transfected cells (lane 10). The specificity of the labeled complex is illustrated by the ability of a 40-fold molar excess of unlabeled probe to prevent its formation (lanes 2, 5), and by the incapacity of the same molar excess of an unrelated
oligomer (AP-2 response element) to produce the same effect (lanes 3, 6). The specificity of the labeled complexes is also shown by supershift analysis using an anti-IκB antibody as control (lane 7) and an anti-RelA antibody (lane 8) which prevents its formation. Finally, the nuclear extracts from p50/RelA transfected cells (lane 10) produced the same shifted complex, further indicating that this complex corresponds to NF-κB. The arrow denotes the NF-κB specific complex.

**Fig. 3. Northern blot analysis of the CFTR mRNA in T84 and Calu-3 cells expressing exogenous transfected NF-κB p50 and RelA subunits.** T84 and Calu-3 cells were transfected with 1.4 μg and 0.6 μg/well of CMV-P50 and CMV-RelA expressing vectors or with 2 μg of CMV empty vector in 35 mm dishes for 24 hours. Total RNA was extracted and subjected to Northern blot analysis to detect CFTR (top) and β-actin (bottom) mRNAs. There was more CFTR mRNA in Calu-3 cells (left) and T84 cells (right) transfected with NF-κB-expressing vectors (p50/RelA) than in cells transfected with the control vector (mock).

**Fig. 4. DNA binding activity of the (-1111/-1090) region.** Gel retardation analysis of the (-1111/-1090) region. 32P-labeled oligomers corresponding to the (-1111/-1090) region of the CFTR promoter were incubated with nuclear extracts from control (lane 2) and IL-1β-stimulated (lanes 3-9) Calu-3 cells. The DNA/protein complex indicated by arrow was present only in IL-1β-stimulated cells. Competition analysis of the shifted complex (lanes 4, 5). The formation of the IL-1β-induced complex was specifically inhibited by a 40-fold molar excess of a consensus κB element (lane 4, oligo κB), but not by the same excess of an unrelated oligomer corresponding to the binding sequence for AP-2 factor (lane 5, oligo.AP2). Supershift analysis of the shifted complex
anti-p50 (lane 7, α p50), RelA (lane 8, α RelA) and cRel (lanes 9, α cRel) were added to the nuclear extracts prior to 32P-labeled probe. No nuclear extracts were added in lane 1.

**Fig. 5. Activities of control and mutated CFTR promoter in Calu-3 cells.** A) Calu-3 cells were transiently transfected using 1.4 µg (-2150/+52)pGL3 or mutated (-2150/+52)pGL3, or the pGL3 basic vector (control) in combination with 0.6 µg/well CMV-driven expression vectors for p50 (0.2 µg/well) and for RelA (0.4 µg/well), or with 0.6 µg/well of the empty CMV vector alone to keep the concentration of transfected DNA constant. The results are expressed as the ratio of the luciferase activities in the (-2150/+52)pGL3 constructs to the activities in the control constructs. Promoter activity is given as the mean of triplicate assays for one of two experiments. The parentheses show the -fold increase in luciferase activity in cells transfected with p50/RelA NF-κB plasmids compared to the luciferase activity in the cells transfected with the reporter constructs without p50 and RelA expressing vectors. B) Calu-3 cells were transiently transfected as previously with the same pGL3 vectors in combination with a CMV-β gal vector to normalise for transfection efficiency. 36 hours post-transfection cells were stimulated with IL-1β for 16 hours in a medium deprived in serum. Promoter activity is expressed as the ratio of the luciferase/β-galactosidase activities in the (-2150/+52) pGL3 constructs to the activities in the control constructs. Data are expressed as the means +/- SE of three independent experiments, each in triplicate. ** P<0.005, using Student’s *t*-test for unpaired values.
NF-κB mediates up-regulation of the CFTR gene expression by interleukin-1β in Calu-3 cells
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