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Interferon-γ regulates ClC-2 chloride channel in lung epithelial cells

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Abstract

Epithelial Cl^- channels mediate Cl^- and fluid secretion in the lung. In cystic fibrosis, aberrant Cl^- secretion is one of the major causes for lung fluid imbalance. Regulation of Cl^- channels is therefore an important issue in the lung. IFN-γ regulates Na^+ and Cl^- channels and fluid transport in the lung, but the mechanisms involved in these regulations are not clear. In expression studies, we found that IFN-γ increased ClC-2 transcripts in Calu-3 cells. Studies of the promoter identified a minimal promoter which interacts with transcription factors Sp1 and Sp3. However, reporter gene assays showed that IFN-γ did not activate the promoter. Instead, IFN-γ significantly increased ClC-2 transcript stability. Using Ussing chamber experiments, we demonstrate that IFN-γ activates a pH-regulated and Cd^{2+}-sensitive short circuit current, characteristic properties of the ClC-2 Cl^- channel. These data suggest that IFN-γ activates ClC-2 channel activity in lung epithelial cells via mRNA stabilization.

Keywords: Lung epithelium; Ion channel; Interferon-γ; Gene expression; mRNA; Promoter

In the lungs, epithelial Cl^- channels mediate *trans*-cellular Cl^- secretion, a major driving force for fluid secretion [1]. In fetal lungs, significant epithelial fluid secretion provides a positive distending pressure for appropriate lung development [2]. At birth, chloride secretion becomes significantly decreased, facilitating perinatal lung fluid clearance and preparing the lung for air breathing [3,4]. Although Cl^- secretion is reduced in adult lungs, it is essential for the maintenance of airway innate immunity [5]. In cystic fibrosis (CF) patients, lack of a functional Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) results in lung disease with characteristic airway dehydration, recurring bacterial infection, deteriorating pulmonary function, and premature death. ClC-2 is one of several Cl^- channels present in airway epithelia. Previous work has shown that ClC-2 is abundantly expressed in fetal lung epithelia and down regulated at birth [6,7]. Overexpression of ClC-2 increases pH- and $CdCl_2$-sensitive Cl^- currents in CF cells [8]. Because of its similarities to CFTR in function and location, activation of ClC-2 in lung epithelia presents a potential therapeutic strategy for cystic fibrosis. ClC-2 activation may also be beneficial in other situations where increased airway hydration is desirable.

IFN-γ is an immunoregulatory cytokine expressed predominantly by the Th1 subset of $CD4^+$ lymphocytes and the Tc1 subset of $CD8^+$ cells [9]. Its receptor is expressed on all nucleated cells [10]. IFN-γ appears to play a major role in pathogen resistance, as mice with targeted mutations in IFN-γ, IFN-γ receptor, and STAT1, the main transcription factor for IFN-γ, all show heightened susceptibility to a variety of infectious pathogens [11–14]. It has been reported that IFN-γ regulates Cl^- channels in bronchial epithelial cells [15,16]. Using primary bronchial epithelial monolayers, Galietta et al.

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[15] found that after IFN-γ treatment, the normal overnight reduction of the apical fluid was decreased by more than 50%. Although IFN-γ-induced ENaC inhibition contributes to this retention of airway surface liquid, IFN-γ-induced Cl^- channel activation might have participated as well.

In the present study, we characterized the human ClC-2 gene promoter and examined the possibility that IFN-γ-induced changes in lung chloride transport were in part through the regulation of the ClC-2 Cl^- channel. Using expression and functional assays, we present evidence that IFN-γ activates Cl^- channel activity in lung epithelial cell line Calu-3 and that this activation is at least in part due to increased ClC-2 mRNA stability.

Materials and methods

Cell culture and reagents. Calu3 cells were obtained from ATCC and grown in minimum essential medium Eagle (ATCC) supplemented with 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37 °C. Recombinant human IFN-γ was purchased from R&D Systems (Minneapolis, MN). $CdCl_2$ was purchased from Sigma (St. Louis, MO).

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR experiments were performed at the Virginia Commonwealth University DNA Core Laboratory using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) following the manufacturer's protocols. Sequences and locations of forward and reverse amplification primers and the TaqMan probe for human ClC-2 are described in Table 1 and Fig. 1C. Exon borders were localized by comparing ClC-2 mRNA sequence and corresponding genomic sequence.(GenBank Accession Nos. S77770 and AC078797) [17]. Total RNA from control and IFN-γ-treated cells was prepared using Qiagen's RNeasy Kit. On-column DNA digestion was used to reduce contaminating genomic DNA. The effect of DNA contamination was further eliminated using a TaqMan probe sitting across the junction of two exons (Fig. 1C). During the real-time PCR experiments, possible genomic DNA contamination was examined in control reactions without reverse transcriptase. No contamination was detected during the entire study.

Construction of ClC-2 promoter–luciferase reporter vectors and promoter activity assay. Restriction fragments from an original clone of the human ClC-2 promoter (Blaisdell et al., data submitted) were subcloned into the pGL3Basic plasmid (Promega) to generate ClC-2 promoter–luciferase gene fusion vectors. The promoter fragments used in this study include a 1428-bp *Kpn*I–*Xho*I fragment, a 447-bp *Sac*I–*Xho*I fragment, and a 105-bp *Sma*I–*Xho*I fragment.

Calu-3 cells at 80% confluence in 35 mm dishes were transfected using the FuGene 6 reagent (Roche, Indianapolis, IN) essentially following the protocol provided by the manufacturer. The cells were transfected in culture medium containing 2% fetal bovine serum. Full medium was added 24 h after transfection when the cells were treated with IFN-γ. For the calibration of transfection efficiency, β-galactosidase expression vector pHM6-lacZ (Roche) was used in co-transfection. The cells were harvested two days later and reactions for measurement were prepared using the Dual-Light kit from Tropix (Bedford, MA). Luciferase and β-galactosidase activities were measured simultaneously on a TopCount NXT luminescence counter (Packard, Meriden, CT). Three independent repeats were performed for each reaction and each repeat was measured two times. Student's *t* test was used for statistical analysis.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) experiments were performed as previously described [18].

RNA stability assay. Cells were untreated or treated with IFN-γ at 100 ng/ml for 24 h. Actinomycin D was then added at 10 μg/ml for 0, 6, 12, 18, and 24 h. Total RNA was isolated at the end of the treatment using the RNeasy Mini Kit (Qiagen). The total RNA, 5 μg each sample, was separated on an agarose gel containing formaldehyde and transblotted to nylon membranes.

The ^{32}P-labeled cRNA probe was synthesized using the MaxiScript kit (Ambion, Austin, TX) from a 381-bp human ClC-2 fragment (PCR cloning), from C_{934} to C_{1314} in the previously published sequence [19]. The entire length of the cloned fragment was confirmed by sequencing. Possible sequence homology with other ClC channels was examined using the Blast search, which showed alignment with only ClC-2 genes from various species. All other alignments were less than 50-bp in length and contained numerous mismatches. The probe was used within 48 h after ^{32}P labeling.

Northern hybridization was performed using ULTRAhyb solution from Ambion (Austin, TX) according to the manufacturer's protocol. After hybridization and washing, the membrane was exposed to film at −80 °C. The intensity was quantified using a Fujifilm LAS1000 CCD camera and AIDA software (raytest GmbH, Straubenhardt, Germany).

Northwestern analysis. Whole cell lysates were prepared from cells either untreated or treated with increasing concentrations of INF-γ. The lysates were separated on a conventional SDS–PAGE gel and transblotted onto nitrocellulose membrane. The proteins on the membrane were renatured by three washes, 20 min each, in an RNA binding buffer (RBB) containing (in mM) 20 Tris, pH 7.5, 60 KCl, 1 $MgCl_2$, 0.2 EDTA, 1 DTT, 10% glycerol, and 0.25% dried milk [20]. The membrane was then blocked for one hour at room temperature in RBB supplemented with 5% dried milk and 2 μg/ml yeast tRNA (Sigma, St. Louis, MO). After two rinses in RBB, the membrane was incubated at 4 °C overnight with ^{32}P-labeled RNA probe at 10^6 cpm/ml in RBB supplemented with 2 μg/ml yeast tRNA and 10 U/ml ANTI-RNase (Ambion, Austin, TX). The RNA probe was generated from a full-length human ClC-2 clone [19] using the MaxiScript kit with T7 polymerase (Ambion, Austin, TX). The probes were used within 48 h after ^{32}P labeling. Unbound probe was removed by four washes in RBB and the membrane was air-dried and exposed to film at −80 °C for 1–3 days. The intensity of the bands was quantified using the AIDA software.

Ussing chamber assay. The cells were plated at 2×10^5 cells per filter on Snapwells (Corning Costar, Cambs, MA) and grown in the same medium, feeding at 2-day intervals. The transepithelial resistance (R_{te}) was measured in an ENDOHM2.4 chamber with an EVOM meter

Table 1
Oligonucleotides used in real-time quantitative RT-PCR

Name	Sequence	Location in ClC-2 coding region
Forward primer (F)	GCAGACTCATTGGAATCGTTACTCT	938–962
Reverse primer (R)	GGGCGGCCGGACTTT	1012–1026
TaqMan probe (T)	AGGAGCT-CCGGAAGGCCATCGA[a]	965–986

[a] "-" in sequence indicates exon junction.

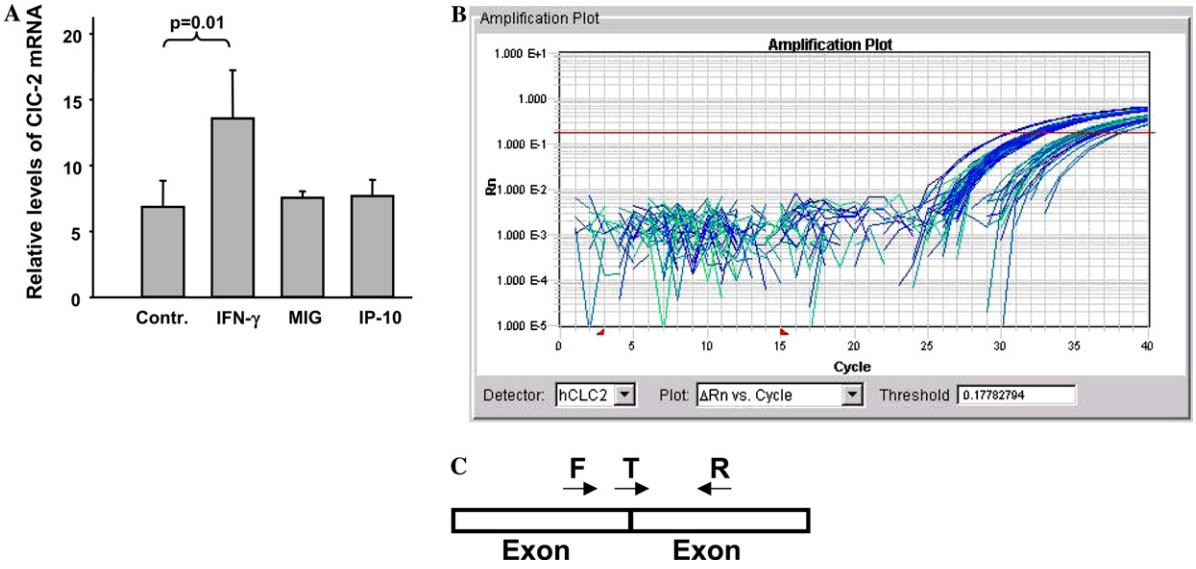

Fig. 1. IFN-γ increases ClC-2 mRNA in Calu-3 cells. (A) Calu 3 cells were treated with IFN-γ, MIG, IP-10 (all at 100 ng/ml) or vehicle (PBS for control) for 24 h. Total RNA was prepared, and ClC-2 mRNA and 18S rRNA were quantitated by real-time RT-PCR. Relative levels of ClC-2 mRNA (ClC-2 mRNA/18S rRNA) from treated and control cells are compared. Values presented are averages of 3 (control, MIG, and IP-10) or 4 (IFN-γ) independent samples. Each sample was measured 3 times and the average was used. (B) Real-time quantitative RT-PCR amplification of ClC-2 mRNA, showing increasing amounts of amplified PCR product (*Y* axis) along with increasing cycle numbers (*X* axis). (C) Schematic description of the positions of forward primer (F), reverse primer (R), and the TaqMan probe (T) on ClC-2 mRNA used in real-time RT-PCR.

(World Precision Instruments, Sarasota FL). When R_{te} reached $\geqslant 480\ \Omega\ cm^2$, cells on Snapwells were treated with medium (control) or medium supplemented with human IFN-γ at 100 ng/ml on both apical and basolateral surfaces. Cells on Snapwells were used for short circuit current (I_{sc}) measurements after 48 h of IFN-γ treatment.

Snapwells were mounted in Ussing chambers (Navicyte/Costar,) bathed in bicarbonate-free Ringer solution of (in mM) 142 NaCl, 2 $CaCl_2$, 4 KCl, and 5 Hepes, pH 7.4. The chambers were maintained at 37 °C with a circulating water bath (Julabo, Allentown, PA). Low chloride solutions of varying pH were, in mM, 142 sodium gluconate, 2 calcium gluconate, 4 potassium gluconate, and 5 Hepes. The pH was adjusted to 7.4, 6.4, and 5.4 using acetic acid. $CdCl_2$ at final concentration of 1 mM was added to inhibit pH sensitive changes in I_{sc} as shown previously [21]. Silver–silver chloride electrodes in saturated KCl in glass barrels terminating in ceramic tips (Navicyte, San Diego, CA) were used to clamp the voltage and measure the current. Clamping and measurement were controlled by a Physiologic Instruments VCC MC6 with Acquire and Analyze software (Physiologic Instruments, San Diego, CA) allowing 4 chambers to be tested simultaneously at a frequency of 3 readings a minute. Data are expressed as means with SEM. Graphs and *t* tests were prepared using Microsoft Excel.

Results

IFN-γ treatment increases the level of ClC-2 mRNA in Calu-3 cells

To test the hypothesis that IFN-γ activates ClC-2 Cl^- channels in lung epithelial cells, we first asked whether ClC-2 mRNA is increased by IFN-γ in Calu-3 cells. We used real-time quantitative RT-PCR to determine the level of ClC-2 mRNA in IFN-γ-treated and control cells (Figs. 1B and C, Table 1). Calibration of the quantities of total RNA in each sample was accomplished using amplification of 18S rRNA. Amplified ClC-2 product was detected/quantified using a fluorescent-labeled sequence-specific TaqMan probe (Table 1). Detection of amplicons derived from contaminating genomic DNA was eliminated by placing the TaqMan probe at the junction of two exons (Fig. 1C) and using no-reverse transcriptase controls. The relative amounts of ClC-2 mRNA (ClC-2 mRNA/18S rRNA) in IFN-γ-treated cells were significantly increased compared to those in control cells (Fig. 1A, $p = 0.01$), supporting the hypothesis that IFN-γ activates ClC-2 at the mRNA level. This activation by IFN-γ could be a direct or indirect regulation. Because IFN-γ activates chemokines such as monokine induced by IFN-γ (MIG) and IFN-γ-inducible protein-10 (IP-10) in lung epithelial cells [22], we examined the possibility that these chemokines mediate IFN-γ-induced increase of ClC-2 mRNA. Direct treatment of Calu-3 cells with MIG and IP-10 did not result in significant difference in the level of ClC-2 mRNA (Fig. 1A), suggesting that MIG and IP-10 are not likely involved in the activation.

Identification of the human ClC-2 minimal promoter

The increase in ClC-2 mRNA in IFN-γ-treated cells could be due to an activation of ClC-2 transcription by IFN-γ. To investigate this possibility, we first examined the upstream regulatory sequence of the human ClC-2 gene. A 2 kb upstream fragment was initially cloned by PCR (Blaisdell et al. unpublished data). The

genomic organization in this region is similar to that found in rat, i.e., promoters for ClC-2 and hRPB17 (an RNA polymerase II subunit gene) share a 2 kb region between the two genes residing on opposite strands [18]. The intervening region in human between the two genes is 2013 bp (It is 1930 bp in rat). In a region proximal to the ClC-2 coding sequence (from −240 to −100), a particularly high GC content (84%) and a high concentration of GC boxes are present (Figs. 2A and 3). To investigate the functional importance of the proximal region with high GC content and multiple predicted GC boxes, ClC-2 promoter fragments were subcloned into the pGL3 luciferase vector (Fig. 2A). Luciferase assays showed significant promoter activity from a 447 bp fragment (Fig. 2B). In contrast, elimination of the GC-rich region significantly reduced luciferase activity, suggesting the presence of a core promoter in the GC-rich region.

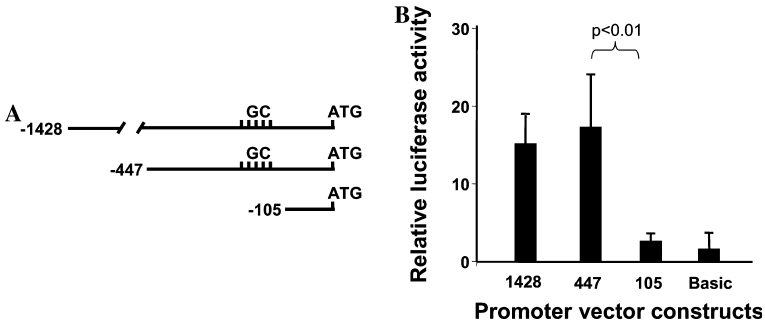

Fig. 2. Characterization of the human ClC-2 promoter. (A) Schematic description of the ClC-2 promoter-luciferase constructs used in promoter–reporter gene assays. ATG at right is the first Met codon in the ClC-2 coding region. Numbers at left are the lengths of promoter inserts in the clones, counting from the first Met codon. Short vertical lines labeled with "GC" depict the region with a number of predicted GC boxes (also see Fig. 3). (B) Luciferase assays testing promoter activities of the promoter-luciferase clones. Transfection efficiency was calibrated by co-transfection with a β-galactosidase expression vector. Relative luciferase activity was calculated as luciferase activity/β-galactosidase activity. Numbers at the bottom are the lengths (bp) of promoter inserts in the clones. "Basic" is the cloning vector without promoter insert. Each column is the average of four independent samples.

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ggcgcgggct tgcgccttcc cagcgcccct tgccgcctct -280
cgggaggccc cgcgcggacc ccagcctggc tgggccgtcc -240

                  14                    131
cctccggatg ggccGGCGGG CGGagcagcg cagagggcaC -200

               132
CGCCCtcggc CCGCCCgccg gggaggggac gcgagcggga -160

                              12
ggctgcgcca gcggcgCCCG CCgggggCCC GCCgcactct -120

                  ▼
gctctcggcc tcccgggctg cggggacggg acggctgccg -80
gcgcggactt tgcgggccgg gagccgagtc caggacagag -40
ccggaaccgc cgagggaggc gagagggcag tgcgcggagA +1
TG

Oligo 14:  gatgggccGGCGGGCGGagcagcg
Oligo 131: gagggcaCCGCCCtcggcc
Oligo 132: ctcggCCGCCCgcggggag
Oligo 12:  agcggcgCCCGCCggggggCCCGCCgcactct
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Fig. 3. DNA sequence of the GC-rich region in the human ClC-2 promoter. The first Met codon ATG is at the end of the sequence and in bold capital letters. Predicted GC box motifs are in bold capital letters. Possible transcription initiation sites are marked with "▼" [17]. The positions of oligonucleotides used in EMSA are underlined and numbered above the sequence. These oligonucleotides are also listed below.

Sp1 and Sp3 transcription factors interact with the ClC-2 core promoter

The 447 bp fragment contains several predicted Sp1 binding sites, which are absent in the 105 bp fragment. The luciferase assay data support a role of the Sp1 and Sp3 transcription factors in human ClC-2 expression [23]. We then set out to investigate whether these predicted GC boxes interact with transcription factors Sp1 and Sp3. Oligonucleotides were synthesized containing the predicted GC boxes as shown in Fig. 3, and EMSA was used to determine whether these GC boxes interact with protein factors in Calu-3 cell nuclear extract. We found that all these oligonucleotides interact with protein factors. But the protein profiles involved in the interactions are different (Fig. 4). To examine specifically the involvement of Sp1 and Sp3, antibody super-shift was used. Our results show that oligo probes 131 and 14 interact with both Sp1 and Sp3 (Fig. 4). The identities of other protein factors interacting with the oligo probes are not clear. These results suggest that two of the five predicted GC boxes interact with Sp1 and Sp3.

IFN-γ does not activate the ClC-2 promoter

With the cloned human ClC-2 promoter fragments, we then examined the possibility that IFN-γ activates the ClC-2 promoter. Two ClC-2 promoter clones, with 1428 and 477 bp upstream sequence, were used in transfection experiments in Calu-3 cells. Unexpectedly, 24-h IFN-γ treatment did not lead to any significant changes in the luciferase activities from the promoter–reporter gene constructs (Fig. 5). This indicates that IFN-γ induced increase of ClC-2 mRNA is not likely due to an increase in transcription initiation.

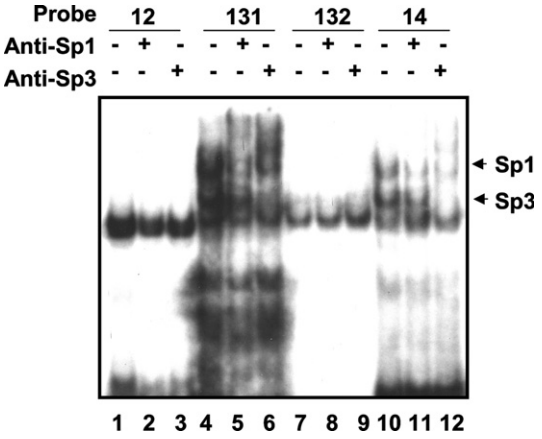

Fig. 4. Some GC boxes interact with Sp1 and Sp3 transcription factors. EMSA was performed to examine interactions between promoter sequences and transcription factors. Nuclear extract was prepared from Calu-3 cells. Double-stranded oligo probes are as described in Fig. 3. Antibodies were added to some of the reactions to super-shift Sp1 or Sp3. Oligo probes and antibodies used in each reaction are labeled on top. Original positions of Sp1- and Sp3-containing protein–DNA complexes are marked at right.

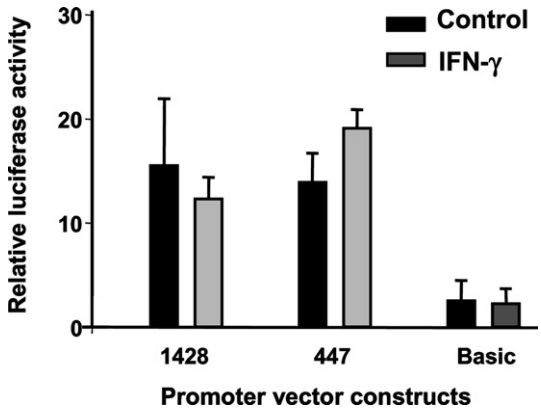

Fig. 5. IFN-γ does not activate the ClC-2 promoter. Calu-3 cells were transfected with two ClC-2 promoter–luciferase gene constructs containing promoter sequences of 1428 and 447 bp. "Basic" is the pGL3Basic plasmid without insert. Co-transfection with a β-galactosidase expression vector was used as an internal control. Relative luciferase activity was calculated as luciferase activity/β-galactosidase activity. In the treatment group, the cells were treated with 100 ng/ml IFN-γ for 24 h the second day after transfection. Each reaction is the average of four independent samples.

IFN-γ increases ClC-2 transcript stability

The other possibility is that INF-γ increases the stability of the ClC-2 transcripts. We therefore examined ClC-2 mRNA stability in Calu-3 cells using Northern hybridization. The cells were treated with IFN-γ for 24 h. Actinomycin D was added to halt transcription for various lengths of time. The quantity of ClC-2 mRNA was calibrated with 18S and 28S rRNA. Fig. 6A shows one representative Northern blot. At the end of 24 h IFN-γ treatment, the average level of ClC-2 mRNA in the cells was significantly higher than that in the control cells (Fig. 6B). These data suggest that the increase of mRNA transcripts is due to IFN-γ-induced ClC-2 transcript stabilization.

Mechanisms involved in the regulation of mRNA stability are not fully understood. However, cellular proteins that interact with mRNA might play a role. We then used Northwestern assays to determine whether cellular proteins interact with ClC-2 mRNA and whether INF-γ treatment affect the protein–mRNA interaction. Using an in vitro synthesized ClC-2 cRNA as the probe, we detected a number of RNA binding proteins. Two protein species of 170 and 135 kDa, respectively, appeared to be down-regulated by IFN-γ in a dose-dependent fashion; a 100 kDa protein was unaffected; and a 90 kDa protein appeared to be up-regulated (Fig. 6C). The 170 kDa protein was significantly down regulated when the cells were treated with 10 ng/ml IFN-γ whereas the 135 kDa protein was more sensitive to IFN-γ at 100 ng/ml. The 90 kDa protein could be

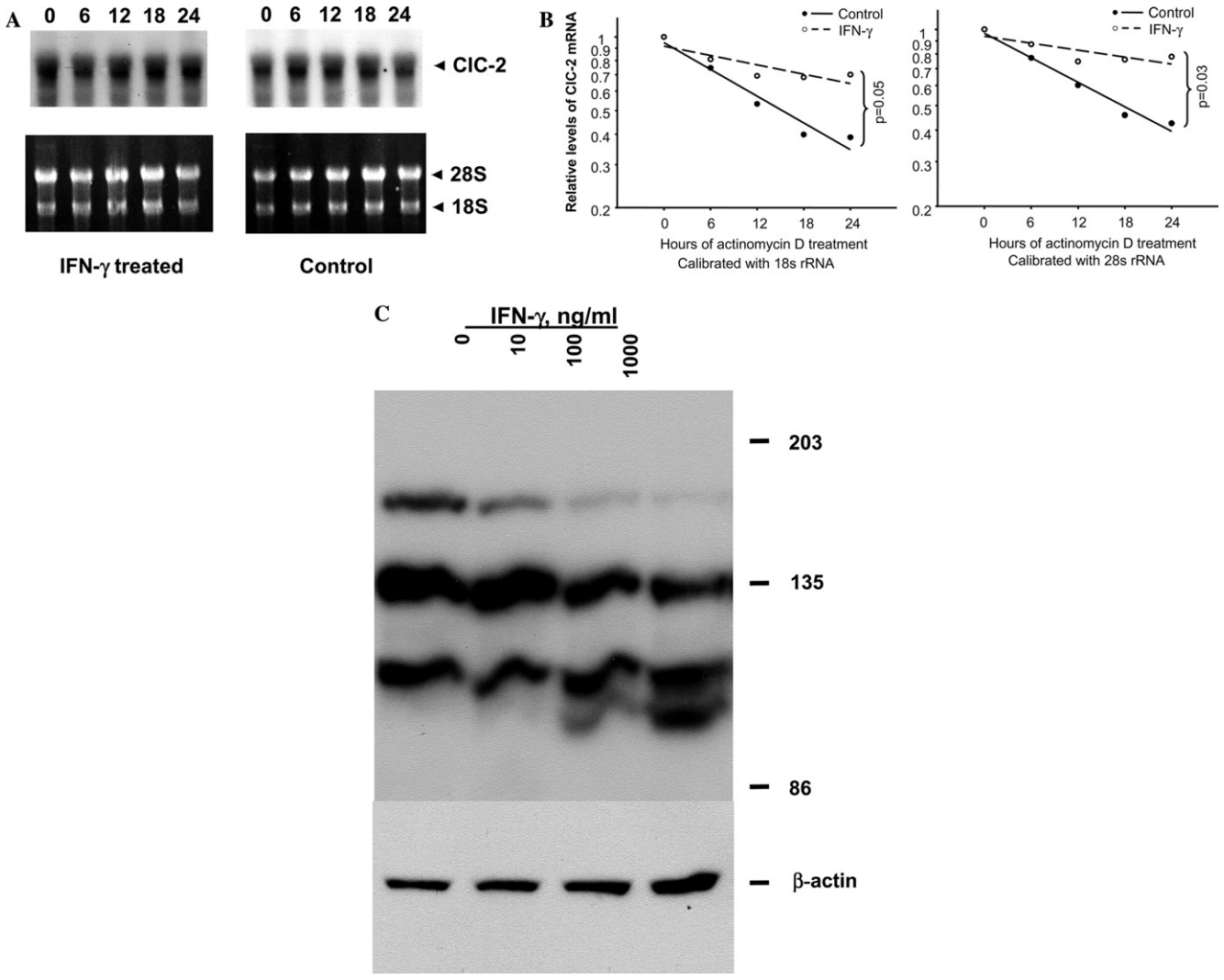

Fig. 6. IFN-γ increases ClC-2 transcript stability. (A) Calu-3 cells were treated with INF-γ for 24 h. Actinomycin D was added for 0, 6, 12, 18, and 24 h. Total RNA was isolated and resolved in agarose gel containing formaldehyde. Before transblotting, the gel was stained with ethidium bromide and photographed. Images of autoradiograph (ClC-2 mRNA, upper panel) and ethidium bromide-stained gel (18S and 28S rRNA, lower panel) were analyzed. Cell treatments are labeled on top of the lanes. (B) Relative quantity of ClC-2 mRNA was calculated as intensity ratios of ClC-2 mRNA/18S rRNA (left) and ClC-2 mRNA/28S rRNA (right). Averages of 4 experiments were plotted and regression lines were generated using SigmaPlot software. Student's *t* test was used for statistical analysis of values at 24-h actinomycin D treatment. (C) Northwestern analysis was used to examine RNA-binding proteins interacting with ClC-2 mRNA. The cells were treated with INF-γ for 24 h as labeled on top. Cell lysates were then prepared and resolved in protein mini gels. After transblotting, the membrane was incubated with an RNA probe. The amounts of bound probe were revealed by autoradiography. Molecular weights are marked on right. The lower panel shows a Western blot using the same cell lysate samples for protein quantification. The amounts of β-actin indicate the amounts of lysates used in the experiment.

a different protein or a degradation product of a larger protein. The identity and function of these proteins remain to be investigated in future studies. However, our data suggest that proteins do interact with ClC-2 mRNA and the interactions are affected by IFN-γ.

IFN-γ activates a pH-sensitive I_{sc} in Calu-3 cells

As a result of increased expression, we postulated that IFN-γ activates ClC-2 channel activity in Calu-3 cells. To determine changes in ClC-2 channel activity, we examined short circuit current (I_{sc}) in control and IFN-γ-treated (100 ng/ml, 48 h) Calu-3 monolayers. Because ClC-2 channel activity is activated by low pH, we used three different pH solutions (7.4, 6.4, and 5.4) to examine changes in I_{sc}. Cell monolayers were mounted in Ussing chambers. The transepithelial voltage (V_{te}) was -1.4 to -3.0 mV. To enhance our ability to measure active chloride transport, Ringer solution was replaced with chloride-free (gluconate) solution in the apical bath. In the presence of gluconate-replaced apical solutions at pH 7.4, IFN-γ doubled the I_{sc} compared to the control (46 and 23 μA/cm^2, respectively, $p = 0.002$). IFN-γ treated cells also had a greater stimulation of I_{sc}

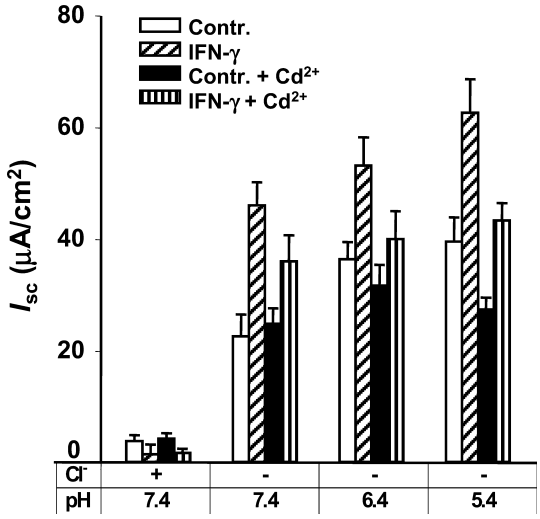

Fig. 7. IFN-γ increases a pH-activated and Cd^{2+}-sensitive I_{sc} in Calu-3 cells. Confluent Calu-3 monolayers grown on Snapwell membranes were tested in Ussing chambers for I_{sc}. Presence of Cl^- in the apical solution and pH in both apical and basal solutions are indicated at the bottom. Acidic pH stimulated I_{sc} in both control and IFN-γ-treated cells. IFN-γ significantly enhanced I_{sc} at neutral and acidic pHs compared to control. This pH-activated I_{sc} was $CdCl_2$-sensitive especially in IFN-γ-treated cells. Cd^{2+} also inhibited I_{sc} in untreated cells but only at pH 5.4 (see text for statistics).

at other pH's. At pH 6.4, I_{sc} were 53 and 36 μA/cm^2 for IFN-γ and control cells, respectively ($p = 0.02$). At pH 5.4, I_{sc} were 63 μA/cm^2 vs. 39 μA/cm^2 ($p = 0.01$) (Fig. 7). As ClC-2 current is inhibited by $CdCl_2$ [24], we examined the contribution of ClC-2 using 1 mM $CdCl_2$, applied to the apical bath. Cadmium inhibited IFN-γ-induced I_{sc} stimulation at pH 7.4, 6.4, and 5.4 (p values are 0.008, 0.015, and 0.004, respectively), whereas in control cells, cadmium inhibited I_{sc} only at pH 5.4 ($I_{sc} = 27$ μA/cm^2, $p = 0.03$). These results demonstrate that INF-γ activates a Cl^- I_{sc} in Calu-3 cells and this I_{sc} is partially inhibited by Cd^{2+}.

Discussion

IFN-γ regulates Cl^- channels (CFTR, CaCC, and ORCC) and the sodium channel ENaC in bronchial epithelial cells [15,16]. Except for the electrophysiological characterizations of these channels, the mechanisms involved in the regulation have not been elucidated. Furthermore, other ion channels in addition to the ones mentioned above could be regulated by IFN-γ. We therefore examined the possibility that IFN-γ regulates ClC-2 in lung epithelial cells.

Our initial approach was to examine ClC-2 expression in lung epithelial cells. We used Calu-3 cells because they express ClC-2 and form tight junctions [25,26], the latter being a prerequisite for Ussing chamber experiments in functional studies. Using real-time quantitative PCR, we found a marked increase of ClC-2 transcript, resulting from IFN-γ treatment of the cells (Fig. 1A). This increase could be due to either increased transcription initiation (activation of the promoter) or increased transcript stability or both.

Because the human ClC-2 promoter had not been characterized, we studied the structure and function of the promoter using sequence analysis, reporter gene assays, and EMSA. A minimal promoter is identified in a proximal region between −477 and −105, which has a high GC content, contains multiple predicted GC boxes, interacts with Sp1 and Sp3 transcription factors and other proteins, and mediates a significant portion of promoter activity as shown in reporter gene assays (Figs. 2–4). Based on these data, Sp1 and Sp3 likely contribute to the basal activity of the ClC-2 promoter. This is similar to our previous observation in the rat ClC-2 promoter where Sp1 and Sp3 play an important role in the activation of the promoter [18]. However, the human ClC-2 promoter appears irresponsive to IFN-γ treatment. Treatment of the cells with IFN-γ did not change luciferase activities driven by two promoter fragments, both containing the minimal promoter region (Fig. 5). These data suggest that the increase of ClC-2 mRNA by IFN-γ is not due to the activation of the ClC-2 promoter.

The other possible mechanism of increased ClC-2 transcript would be the stabilization of the transcript. Our studies show that IFN-γ increases the stability of ClC-2 transcript in Calu-3 cells (Figs. 6A and B). mRNA stability has been increasingly recognized as an important mechanism in the regulation of gene expression [27]. KGF has previously been shown to stabilize ClC-2 transcripts [28]. IFN-γ enhances the expression of many mammalian genes, such as B7-2, IL-6, and ICAM-1, through mRNA stabilization [29–31]. So far, little is known about IFN-γ-regulated RNA-binding proteins that may modulate mRNA stability. Dose-dependent reduction of ClC-2 cRNA binding to the two proteins in the Northwestern experiment may suggest either down regulation of these proteins or simply a reduction in protein–RNA binding (Fig. 6C). These two protein species could be related to ClC-2 mRNA degradation in Calu-3 cells. In this scenario, the IFN-γ-induced changes might result in a decrease in the rate of ClC-2 mRNA degradation and thus an increase in the stability of ClC-2 mRNA. The nature of these proteins is yet to be investigated.

The above expression data support an increase in ClC-2 expression in IFN-γ-treated Calu-3 cells. Because ClC-2 is expressed in Calu-3 cells, IFN-γ-activated ClC-2 expression might result in an increase of I_{sc}. In a number of cell systems ClC-2 currents are increased at low pH [8,21]. In this study, acidic pH increased the magnitude of I_{sc} in both control and IFN-γ-treated cells. To further investigate the possibility that ClC-2 is involved in the pH-activated I_{sc} in Calu-3 cells, we chose to use $CdCl_2$,

an inhibitor of CLC-2 [8,32]. As shown in Fig. 7, $CdCl_2$ consistently suppressed IFN-γ-activated I_{sc} at all pH's whereas in control cells, $CdCl_2$ only inhibited I_{sc} at pH 5.4. These data strongly support the hypothesis that $CdCl_2$-sensitive ClC-2-mediated current is more predominant in IFN-γ-activated cells and in low pH solutions.

We cannot exclude however contributions of other Cl^- channels in the IFN-γ-activated Cd^{2+}-sensitive I_{sc} in Calu-3 cells. $CdCl_2$ did not completely block IFN-γ-activated I_{sc} at low pH. Although one would not expect any channel blockers to completely inhibit ion channels, the incomplete inhibition could be due to activation of other pH-sensitive and $CdCl_2$-resistant Cl^- channels as seen in fetal lung epithelial cells [21]. The IFN-γ-activated pH-sensitive I_{sc} is not likely a Na^+-related current because the pH changes would likely have resulted in a decrease of I_{sc} [33]. Although we did not specifically examine the possible involvement of CFTR, CFTR is less likely a contributing channel because it has been shown that IFN-γ suppresses CFTR expression in two cell systems including bronchial cells [15,34].

The expression studies reported here, i.e., mRNA quantification by real-time PCR, promoter–reporter gene assay, and transcript stability experiments, suggest that IFN-γ treatment increases ClC-2 expression in Calu-3 cells through increased transcript stability. Combined with the functional data that IFN-γ-activates Cd^{2+}-sensitive I_{sc}, we propose that ClC-2 contributes to the IFN-γ-activated Cl^- transport in Calu-3 lung epithelial cells.

Consistent with the role in pathogen resistance, IFN-γ likely plays a beneficial role in CF lungs. The presence of IFN-γ in CF bronchial mucosal biopsies is associated with milder lung disease and less frequent pulmonary exacerbations [35]. IFN-γ production is significantly reduced in peripheral monocytes from CF patients with *Pseudomonas aeruginosa* infection compared to those without *P. aeruginosa* infection. These observations led to the proposal that Th1 cytokines may improve the prognosis of CF patients [36]. Indeed, IFN-γ treatment of *P. aeruginosa*-infected rats resulted in marked reduction in lung inflammation and a shift from PMN infiltration to monocyte infiltration [37].

Because of the protective effect of IFN-γ in CF lungs [35–37], its regulation of lung fluid movement through the inhibition of Na^+ transport and the activation of Cl^- transport [15,16], and because that ClC-2 Cl^- channel is an alternative Cl^- channel to CFTR in CF lungs [8,38], our current finding provides a potential new strategy for the therapy for CF.

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