Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line

Zhenyue Tong, Beate Illek, Vikash J. Bhagwandin, George M. Verghese, and George H. Caughey.

1 Cardiovascular Research Institute and Department of Medicine, University of California at San Francisco, San Francisco 94143; and 2 Children's Hospital Oakland Research Institute, Oakland, California 94609

Submitted 5 May 2004; accepted in final form 23 June 2004


Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. Am J Physiol Lung Cell Mol Physiol 287: L928–L935, 2004. First published July 9, 2004; doi:10.1152/ajplung.00160.2004.—Prostasin is a trypsin peptidase expressed in prostate, kidney, lung, and airway. Mammalian prostasins are related to Xenopus channel-activating protease, which stimulates epithelial Na⁺ channel (ENaC) activity in frogs. In human epithelia, prostasin is one of several membrane peptidases proposed to regulate ENaC. This study tests the hypothesis that prostasin can regulate ENaC in cystic fibrosis epithelia in which excessive Na⁺ uptake contributes to salt and water imbalance. We show that prostasin mRNA and protein are strongly expressed by human airway epithelial cell lines, including immortalized JME/CF15 nasal epithelial cells homozygous for the ΔF508 cystic fibrosis mutation. Epithelial cells transfected with vectors encoding recombinant soluble prostasin secreted active, trypsin peptidase that is highly sensitive to inactivation by aprotinin. When studied as monolayers in Ussing chambers, JME/CF15 cells exhibit amiloride-sensitive, transepithelial Na⁺ currents, 9% of baseline. These data predict that prostasin is a major regulator of ENaC-mediated Na⁺ current in cystic fibrosis airway. To probe prostasin’s involvement in basal ENaC activity, we silenced expression of prostasin using short interfering RNA targeting of prostasin mRNA’s 3′-untranslated region. This drops ENaC currents to 26 ± 9% of baseline. These data predict that prostasin is a major regulator of ENaC-mediated Na⁺ current in ΔF508 cystic fibrosis epithelia and suggest that airway prostasin is a target for therapeutic inhibition to normalize ion current in cystic fibrosis airway.

epithelial sodium channel; gene silencing; short interfering RNA; ΔF508 mutation

Prostasin is so-named because it was identified initially as a secreted prostate gland product with trypsin-like activity (35). When characterization of cDNA allowed prediction of the full precursor sequence, prostasin was recognized to be synthesized initially as a transmembrane protein with a COOH-terminal peptidase anchor (34). Prostate cells can secrete and shed a fraction of prostasins as soluble enzymes with the rest remaining attached by a glycosylphosphatidylinositol anchor (6). Immunolocalization, mRNA blotting, and in situ hybridization studies led to recognition that prostasin is robustly expressed in additional human and mouse tissues, including kidney, lung, and airway (8, 26, 29, 34). Significantly, prostasin shares several similarities with a membrane-anchored frog protein, channel-activating protease, identified by expression cloning as a regulator of Na⁺ transport in Xenopus kidney cells (24, 25). Several of prostasin’s idiosyncrasies are shared by a select group of other mammalian enzymes, including the recently characterized γ-trypestases, testisins, and pancreasins (2, 5, 10). Shared features include a unique gene pattern of intron phase and placement, propeptides that are disulfide-linked to the catalytic domain, specificity for peptide substrates with arginine or lysine residues at the site of hydrolysis, and COOH-terminal hydrophobic extensions serving as membrane anchors. The COOH-terminal anchor is the defining characteristic of the type I transmembrane serine proteases, a recently recognized subset of vertebrate, trypsin-family peptidases (5, 20).

Several lines of evidence suggest that one or more serine-class peptidase in vertebrate epithelia upregulate transcellular Na⁺ current mediated by epithelial Na⁺ channel (ENaC), which is essential for airway fluid clearance (21). For example, aprotinin, which is a broad-spectrum inhibitor of serine peptidases, reduces transepithelial Na⁺ transport in frog kidney cells (24, 25). Aprotinin’s target appears to be channel-activating protease. Similar studies in cultured mammalian airway cells show that amiloride-sensitive Na⁺ current is reversibly inhibited by aprotinin or bikunin (an inhibitor of tryptic serine peptidases) and restored by trypsin (3, 4, 21). The direct mechanism of protease-mediated ENaC activation is unclear but features increased probability of channel opening (4, 24, 28). Several membrane-associated serine peptidases in addition to prostasin are candidate physiological activators of ENaC in mammalian epithelia (8, 28, 29). Evidence implicating prostasin includes activation of ENaC by prostasin when mammalian versions of these proteins are coexpressed in frog oocytes (1, 8, 29). Less direct evidence includes prostasin’s sensitivity to inhibitors of ENaC-mediated Na⁺ current, like aprotinin (35), the finding that some epithelial cells that express ENaC also express prostasin (28), and phylogenetic evidence that prostasins are relatives of frog channel-activating protease (26).

Electrophysiological studies of airway epithelia in cystic fibrosis (CF) suggest that Na⁺ uptake from the lumen is dysregulated and excessive (27, 31, 32). The leading hypothesis regarding the cause of airway disease in CF is that excessive Na⁺ absorption leads to inadequate hydration resulting in mucus stasis and recurrent infection and has prompted searches for ways to redress the postulated imbalance (15, 23). Identification and inhibition of a proteolytic regulator of airway...
ENaC could provide a pharmacological means of improving Na⁺ current in CF airway. However, disordered regulation of ENaC by mutated CF transmembrane regulator protein may be the direct and dominant cause of the defect in CF (22). The present work examines prostasin’s role in regulating ENaC in cultured CF epithelial cells. Our findings indicate that membrane-anchored prostasin is highly expressed in CF epithelial cells and that silencing of expression strongly reduces trans-epithelial Na⁺ current. This is direct evidence that prostasin is the major positive regulator of baseline ENaC activity in ΔF508 CF epithelium.

MATERIALS AND METHODS

Purification of native human prostasin. As a standard for testing antibody specificity and activity of recombinant prostanins, small amounts of native, soluble human prosasin were purified from semen as described (34). Briefly, semen diluted in 25 mM Tris-HCl (pH 7.6) was purified by sequential anion exchange (DEAE-Sepharose; Amersham Bioscience, Piscataway, NJ) and aprotinin affinity chromatography. Prostasin-containing fractions were detected by immunoblotting (see RESULTS) and activity assays of column fractions. For these and other prostasin assays, amidolytic activity was measured spectrophotometrically at 410 nm using the substrate tosyl-Gly-Ser-Pro-Arg-4-nitroanilide (Sigma, St. Louis, MO). Assays were carried out at 37°C in 25 mM Tris-HCl (pH 9.0) containing 0.3 mM substrate.

Bacterial expression of recombinant human prostasin. A 942-bp human proprostasin cDNA encompassing the protein-coding region, including the COOH-terminal transmembrane segment, was generated via the PCR using primers 5'-GAAGGGGACAGAAGCTCCTG-3' and 5'-TCAGTGCTCGCTGAGCCA-3'. Prostate cDNA served as a template. The resulting amplification product was ligated into the pCRT7/NT-TOPO (Invitrogen, Carlsbad, CA), which places an N-terminal polyhistidine-enteropeptidase tag to facilitate subsequent purification. Diagrams illustrating the design of these and other expression vectors used in this study are shown in Fig. 1. The cloned proprostasin plasmid was used to transform Escherichia coli strain BL21(DE3)pLysS (Stratagene, La Jolla, CA), which expressed recombinant enzyme upon induction by isopropylthio-β-D-galactoside. Bacterially expressed, epitope-tagged recombinant prostasin extracted into 6 M urea was loaded onto Ni-NTA His-bind chromatography columns (EMD Biosciences, Madison, WI) and eluted with 0.5 M EDTA in 6 M urea.

Generation of polyclonal antibodies against prostasin. Purified, E. coli-expressed recombinant prostasin (~0.5 mg/animal) plus adjuvant were injected into rabbits by Antibody Solutions (Palo Alto, CA). The resulting antibodies, after heat inactivation and delipidation, were purified on a protein A Hi trap column (Amersham Biosciences) and titered from a starting concentration of 1 mg/ml.

Mammalian cell expression of recombinant soluble prostasin. To determine whether prostasin needs to be expressed initially as a membrane-anchored protein for maturation and activation, we prepared vectors for expression of soluble prostasin in two epithelial cell lines: Chinese hamster ovary (CHO) and human embryonic kidney (HEK-293) epithelial cells. These lines were chosen because they natively express little prostasin (see RESULTS). A 966-bp human preproprostasin cDNA encoding Met1 through Arg322, which is the COOH terminus of secreted prostasin in seminal fluid (35), was amplified from Calu-3 cDNA using the following primers: 5'-AAGCTTGCCATGCCCAGAAAGGGGTC and 5'-CCTCAGCAAGCCTGGGGCTC, and then ligated into Invitrogen’s expression plasmid pcDNA3.1/V5-His-TOPO, which adds a COOH-terminal tag containing a V5 epitope and a hexahistidine segment. To carry out transfections, the soluble human prosasin construct (5 μg) was incubated with 2.5 × 10⁶ CHO or HEK-293 cells along with 5 μl of Lipofectamine 2000 (Invitrogen) in each well of six-well plates. For select cells expressing transcripts from the transfected cDNA, cells were incubated with 250 μg/ml of G418 (Calbiochem, San Diego, CA) for 2 days followed by 400 μg/ml for 2 wk. Cells were then cultured for 72 h in Opti-MEM 1 serum-free medium. To purify recombinant enzyme, conditioned medium was subjected to sequential anion exchange and nickel affinity chromatography. Briefly, medium clarified by centrifugation was passed over Q-Sepharose (Amersham Bioscience) preequilibrated with 75 mM NaCl in 20 mM Tris-HCl (pH 7.9). After being washed with 5 mM NaCl in the same buffer, the column was subjected to stepwise elution with concentrations of NaCl escalating to 2 M. Prostasin-rich fractions were passed over Ni-NTA agarose beads (Invitrogen) after preequilibration with 5 mM imidazole and 20 mM Tris-HCl (pH 7.9), washed with 10 and 50 mM imidazole eluted with 100 mM imidazole, and stored at −20°C. Alternatively, we used one-step purification by aprotinin-agarose (Sigma) chromatography. The recombinant prostasin was eluted with 0.1 M glycine (pH 3.0) containing 0.1 M NaCl, and eluted enzyme fractions were neutralized with 1 M Tris-HCl (pH 8.0) and stored at −20°C.

Generation of a vector to overexpress recombinant, membrane-anchored prostasin in CF epithelial cells. A 1,032-bp cDNA encoding the full native sequence of human preproprostasin, including the COOH-terminal membrane anchor, was amplified from Calu-3 cDNA by PCR using primers introducing HindIII/NotI sites as follows: 5'-AAGCTTGCCATGCCCAGAAAGGGGTC and 5'-GCGGC-
CGCTC AGTGCTGCT GAGCCA. The resulting amplimer was cloned into the polylinker of plasmid pcDNA3.1.

Airway epithelial cell culture, transfection, and measurement of transepithelial current. CF airway epithelial (JME/CF15) cells were grown in DMEM/F-12 culture medium containing 10% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin as described (14). These cells were selected for study because they are homozygous for the most common CF allele, ∆F508, and form high-resistance monolayers suited to electrophysiological studies. The medium was supplemented with 10 μg/ml growth factor, 1 μM hydrocortisone, 5 mg/ml insulin, 5 μg/ml transferrin, 30 mM tridiothyronine, 180 μM adenine, and 5.5 μM epinephrine. For transfections, medium was changed to reduced-serum Opti-MEM I, followed in 30 min by coinubcation with 1 μg of pcDNA3.1-prostasin vector construct [or 1 μg of pcDNA3.1-green fluorescent protein (GFP) as a negative control, kindly provided by Dr. Guo-Ping Shi] plus 3 μl of Lipofectamine 2000 for 16–18 h, after which medium was replaced with fresh DMEM/F-12/fetal bovine serum as above. For transepithelial measurements, JME/CF15 cells were seeded onto permeable filter inserts (Snapwell; Corning Costar, Kennebunk, ME) at a density of ~10⁶ cells/cm². After seeding, transepithelial resistance (Rₑ) was monitored with an epithelial voltohmeter (World Precision Instruments, Sarasota, FL). Epithelial monolayers were used for transepithelial experiments 2–8 days after seeding.

Short-circuit current measurement. Filter-grown JME/CF15 epithelial monolayers were placed into Ussing chambers designed for use with Snapwell inserts (World Precision Instruments). Each side of the monolayer was bathed in Krebs-Henseleit solution containing (in mM): 120 NaCl, 20 NaHCO₃, 5 KHCO₃, 1.2 NaH₂PO₄, 5.6 glucose, 2.5 CaCl₂, and 1.2 MgCl₂ and was gassed with 95% O₂-5% CO₂ at 37°C. Transepithelial voltage was clamped to 0 mV using a standard four-electrode voltage clamp (Physiologic Instruments, San Diego, CA), and short-circuit current (Iₛₑ) was recorded to a computer through an analog-to-digital board (DataQ Instruments, Akron, OH) as described (13). At 50-s intervals, transepithelial voltage was clamped to 2 mV for 1 s to monitor and calculate Rₑ. To determine the magnitude of ENaC-mediated Na⁺ transport across the apical membrane, an inhibitor of active Na⁺ absorption (10 μM amiloride) was added to the mucosal Ussing chamber compartment. In some experiments, trypsin (3 μM) or aprotinin (10 μM) was added to the apical Ussing chamber fluid.

Generation and application of prostasin short interfering RNAs. Three 23-nucleotide double-stranded short interfering (si)RNAs were designed to target prostasin mRNA encoding portions of propeptide, catalytic domain, and 3’-untranslated region (UTR), respectively. In prostasin cDNA, the targets are as follows: 5'-CAGAAAGCTCC CT-GGCGGTGG G (propeptide), 5’-CTCCAATCTCA GCAGACCCAT C (catalytic domain), and 5’-CTCTTCCAG ATGGATCAGC C (3’- UTR). As a negative control, we used a nonsilencing sequence, 5’-AAAATTTCCGA ACGTGTCACG T (scramble, which does not correspond to any known human transcript). Each of the prostasin targets was specific for prostasin by database screening. The siRNAs were generated by in vitro transcription. Briefly, four DNA oligonucleotide primers were designed for each construct. First, double-stranded DNA oligonucleotides encoding the sense and antisense target sequences attached to a T7 promoter sequence were combined and transcribed using the RibomAX in vitro transcription system (Promega, Madison, WI). Resulting duplex RNA was incubated with DNase I, RNaseT1 (Ambion, Austin, TX), and RNase A (Roche Applied Science, Indianapolis, IN) to generate siRNA with 3’-UU overhangs. SiRNA corresponding to each of the three targets was purified by phenol/chloroform extraction and stored at ~20°C. JME/CF15 airways epithelial cells were cultured as above offered on six-well permeable filter supports. Medium was changed to Opti-MEM I 30 min before transfection, which was carried out by apical incubation of cells with 40 pmol of siRNA plus 3 μl of Lipofectamine (Invitrogen) per well for 16–18 h, after which Opti-MEM was replaced with fresh serum-containing medium. To allow recovery from transfection, cells were incubated for 2 or more days with daily changes of medium, followed by Ussing chamber studies.

Immunoblotting. A549 and Calu-3 cells were harvested, washed with cold PBS, and lysed in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P (NP)-40 with Complete mini protease inhibitors (Roche Applied Science). Extracted proteins were resolved by electrophoresis on 4–20% Tris-glycine polyacrylamide gels (Invitrogen), transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), and then incubated with 1:1,000 dilution of affinity-purified polyclonal rabbit anti-human prostasin followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Alternatively, to confirm the specificity of the polyclonal antibodies and to control for potential differences in protein loading, blots were incubated with 1:1,000 anti-human prostasin monoclonal antibody (BD Biosciences, San Jose, CA) and -β-actin monoclonal antibodies (Santa Cruz Biotechnology), followed by incubation with 1:5,000 horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence using LumiGlo Reagents (Cell Signaling Technology, Beverly, MA). JME/CF15 immunoblots were similar, except that cells were lysed in 50 mM Tris-HCl at pH 8 containing 2 mM EDTA, 250 mM NaCl, 1% NP-40, and 0.25% deoxycholate plus protease inhibitor cocktail. Blots were blocked with 5% fat-free milk in Tris-buffered saline with 0.05% Tween 20 and then incubated with anti-prostasin, anti-actin, and secondary antibodies as above.

Data analysis and statistical methods. Current measurements in JME/CF15 cells transfected with GFP-expressing vector alone versus cells transfected with prostasin-expressing vector were compared via two-tailed Student’s t-tests. Amiloride-sensitive Iₛₑ in cells incubated first in saline buffer and then in buffer plus trypsin were compared using paired, two-tailed t-tests. Basal Iₛₑ in cells transfected with (scramble) siRNA was compared with that of cells transfected with prostasin siRNA via one-tailed t-tests.

RESULTS

Bacterial expression of recombinant human proprostasin. The integrity of proprostasin cDNA ligated into pCRT7/NT-TOPO was confirmed by sequencing. E. coli-expressed proprostasin retaining the native COOH-terminal hydrophobic sequence and containing NH₂-terminal modifications (as shown in Fig. 1A) was nearly pure by electrophoresis after immobilized metal-chelate affinity chromatography (not shown), yielding a major ~40-kDa band, which was recognized by monoclonal and polyclonal anti-human prostasin antisera by immunoblotting (Fig. 2). This recombinant proprostasin serves as a positive control for immunoblotting experiments, as in Fig. 2. As expected of the proenzyme form of a peptidase, the purified product was catalytically inactive and thus suited for injection into animals for antibody generation.

Generation of polyclonal antisera recognizing human prostasin. Affinity-purified, polyclonal rabbit antiserum raised against purified, E. coli-expressed recombinant human proprostasin recognized electrophoresed native and recombinant soluble and membrane-anchored prostasins in immunoblots (as in the examples in Fig. 3) and also in immunohistochemical applications (not shown).

Expression and activity of recombinant, soluble, epitope-tagged human prostasin in mammalian cells. The nucleotide sequence of modified preproprostasin cDNA with a deleted COOH-terminal transmembrane domain ligated into pcDNA3.1/
human epithelial cell lines, including Calu-3, A549, and JME/CF15, but little if any is detected in CHO and HEK-293. Most prostasin in Calu-3, A549, and JME/CF15 cells appears to be membrane anchored, based on extraction by detergents, the paucity of immunoreactive material in medium conditioned by these cells (not shown), and the somewhat larger size of the principal immunoreactive bands compared with recombinant soluble prostasin (Fig. 3). More rapidly migrating bands in A549 cell extracts may be degradation products of less glycosylated protein.

**Overexpression of native, membrane-anchored prostasin in ΔF508 CF cells.** The nucleotide sequence of unmodified preproprostasin cDNA with an intact COOH-terminal transmembrane domain ligated into pcDNA3.1 expression vector (as shown in Fig. 1C) was confirmed by DNA sequencing. As shown by the immunoblots in Fig. 4A, JME/CF15 cells transfected with this vector express more detergent-extractable prostasin immunoreactivity (compared with actin) than cells transfected with GFP control vector alone. The migration position and appearance of the bands are otherwise similar, suggesting that native and recombinant transcripts are translated and posttranslationally modified similarly or identically. Filter-grown transfected cells developed RO of >400 Ω·cm2 after ~2 days in culture. Figure 4B summarizes and compares basal Na+ currents in control and JME/CF15 cells overexpressing transmembrane prostasin. I\textsubscript{sc} is slightly, but not significantly, higher, suggesting that any stimulation of trans-epithelial Na+ current in these cells by prostasin is nearly maximal.

**Effect of trypsin and aprotinin on I\textsubscript{sc} in ΔF508 CF cells.** Figure 4B summarizes the stimulatory effect of pancreatic trypsin (3 μM) on basal ENaC currents. Trypsin stimulated ENaC currents from 27.6 ± 0.9 to 35.5 ± 1.2 μA/cm² (n = 5). This modest increase (18%) occurs within 5 min of adding trypsin to the apical bathing medium. The inhibition of ENaC currents upon exposure to aprotinin (not shown), amiloride-sensitive current diminished to ~20% of the level before addition of aprotinin. Figure 4D reveals that exposure to trypsin after

---

**Fig. 2. Expression and purification of recombinant, soluble prostasin.** A truncated prostasin with the COOH-terminal membrane anchor removed was expressed in HEK-293 and CHO cells. **A:** results of immunoblotting of electrophoresed proteins using antibodies raised against human prostasin before and after Q-Sepharose chromatography. Lane 1 contains *Escherichia coli*-expressed recombinant human prostasin as a positive control. Lane 2 contains medium conditioned by HEK-293 cells transfected with a soluble prostasin expression construct. Subsequent lanes contain aliquots of column fractions, including flow through, wash, and step-elutions with escalating concentrations of NaCl in elution buffer. **B:** Coomassie blue-stained SDS-PAGE gel showing further purification of His-tagged soluble recombinant prostasin from the 0.25 M NaCl Q-Sepharose eluate via nickel affinity chromatography. Aliquots of medium and column fractions are as indicated. In the last lane, the major band in the imidazole eluate is ~40 kDa (arrow), corresponding to prostasin. C: immunoblot comparing Chinese hamster ovary (CHO)-expressed recombinant soluble prostasin with *E. coli*-expressed full-length prostasin and native soluble prostasin purified from human semen. The slightly larger size of recombinant prostasin compared with native prostasin can be attributed to the COOH-terminal polyhistidine tag in the recombinant protein.

V5-His-TOPO (as shown in Fig. 1B) was confirmed by DNA sequencing. CHO and HEK-293 cells, which natively express little if any soluble or membrane-anchored prostasin (as reflected by the lack of immunoreactivity in extracts screened with polyclonal anti-prostasin in the type of studies shown in Fig. 3), secrete immunoreactive prostasin into conditioned medium after transfection with pcDNA3.1/prostasin-V5-His-TOPO vector (see Fig. 2, A and C). A highly enriched preparation of HEK-293-expressed prostasin was obtained by Q-Sepharose and Ni-NTA chromatography, as shown by the Coomassie blue-stained gel in Fig. 2B. This material was peptidolytically active and aprotinin sensitive, although limited amounts prevented extensive characterization. As shown by the immunoblots in Fig. 2C, the recombinant, soluble enzyme migrates at a slightly higher apparent molecular weight compared with native, soluble prostasin purified from semen. The soluble, recombinant enzyme had a specific activity of 0.13 absorbance units·min\textsuperscript{-1}·mg\textsuperscript{-1} compared with 1.4 for the more highly purified native enzyme from semen. Both preparations of soluble enzyme could be completely inhibited by aprotinin and benzamidine, with estimated 50% inhibitory concentrations of 0.7 nM and 90 nM, respectively, when tested against recombinant prostasin.

**Native expression of prostasin by epithelial cell lines.** As shown in Fig. 3, prostasin is expressed natively by several epithelial cell lines. Electrophoresed proteins from detergent extracts of various cell types were blotted and incubated with a 1:1,000 dilution of polyclonal antisera raised against human prostasin (Fig. 3). The nucleotide sequence of unmodified preproprostasin cDNA with an intact COOH-terminal transmembrane domain ligated into pcDNA3.1 expression vector (as shown in Fig. 1C) was confirmed by DNA sequencing. As shown by the immunoblots in Fig. 4A, JME/CF15 cells transfected with this vector express more detergent-extractable prostasin immunoreactivity (compared with actin) than cells transfected with GFP control vector alone. The migration position and appearance of the bands are otherwise similar, suggesting that native and recombinant transcripts are translated and posttranslationally modified similarly or identically. Filter-grown transfected cells developed RO of >400 Ω·cm² after ~2 days in culture. Figure 4B summarizes and compares basal Na+ currents in control and JME/CF15 cells overexpressing transmembrane prostasin. I\textsubscript{sc} is slightly, but not significantly, higher, suggesting that any stimulation of trans-epithelial Na+ current in these cells by prostasin is nearly maximal.

Effect of trypsin and aprotinin on I\textsubscript{sc} in ΔF508 CF cells. Figure 4B summarizes the stimulatory effect of pancreatic trypsin (3 μM) on basal ENaC currents. Trypsin stimulated ENaC currents from 27.6 ± 0.9 to 35.5 ± 1.2 μA/cm² (n = 5). This modest increase (18%) occurs within 5 min of adding trypsin to the apical bathing medium. The inhibition of ENaC currents upon exposure to aprotinin (not shown), amiloride-sensitive current diminished to ~20% of the level before addition of aprotinin. Figure 4D reveals that exposure to trypsin after
prolonged incubation with aprotinin (followed by washout of inhibitor) restores much amiloride-sensitive $I_{\text{sc}}$.

**Effect of prostasin gene silencing on $I_{\text{sc}}$ in ΔF508 CF cells.**

As shown in the representative immunoblot in Fig. 5A, siRNA directed against prostasin’s 3′-UTR is more effective in reducing prostasin protein expression than was siRNA targeting prostasin’s catalytic domain or propeptide region. Silencing of prostasin expression was selective for prostasin as suggested by a relative increase in prostasin expression in cells transfected with pcDNA3.1-prostasin vector encoding full-length, membrane-anchored prostasin.

DISCUSSION

By examining properties and manipulating expression of native and recombinant prostasins, this work directly tests our hypothesis that prostasin is the major positive regulator of basal ENaC-mediated Na+ current in CF airway epithelium. Prostasin has been implicated by several groups of investigators, including our own, in regulation of Na+ transport in mammalian epithelia (3, 8, 19, 26, 29, 30). These suspicions are based on prostasin’s phylogenetic similarity to a channel-activating peptidase in frog kidney cells (26, 28), coexistence of prostasin and ENaC in several types of epithelia (29), identification of serine peptidase inhibitor-sensitive Na+ transport pathways in mammalian airway (3) and kidney (18, 19, 29) epithelial cells, stimulation of Na+ transport when mammalian prostasins and ENaC are expressed together in frog oocytes (8, 28), and stimulation of Na+ uptake by incubation of mouse kidney cells with soluble prostasin (19). In vivo support for a physiologically significant role in Na+ homeostasis comes from studies in rats, which become hypertensive and increase urinary Na+ excretion after exposure to adenosine vectors expressing human prostasin (30). Human studies correlating prostasin with Na+ excretion in subjects with primary aldosteronism (19) provide additional support. Despite this evidence of a role for prostasin, other membrane-associated tryptic serine proteases are expressed in epithelia and are candidates for regulation of ENaC in CF and normal cells in and outside of the airway. Three of these enzymes, the type II transmembrane serine peptidases Tmprss2, Tmprss3, and St14/matriptase/MT-SP1, are aprotinin inhibited and can stimulate Na+ transport when coexpressed with ENaC in oocytes (9, 28). Inactivating mutations of Tmprss3 cause deafness (9, 16), which is hypothesized to be due to loss of ENaC stimulation by Tmprss3 in Na+-reabsorbing tissues of the inner ear. Another type II candidate is airway trypsin-like protease (33). Additional untested possibilities are closer relatives of prostasin, such as the membrane-anchored type I trypsin peptidase testisin, pancreasin, and $\gamma$-trypsin (2, 5, 10). Recent studies demonstrate that the intracellular proprotein convertase furin, a serine peptidase unrelated to trypsin family peptidases, is involved in proteolytic activation of ENaC by a mechanism distinct from that of extracellularly applied trypsin (11, 12). Nonproteolytic regulators of Na+ transport (and channels other than ENaC) are also involved in regulating salt and water balance in ENaC-expressing epithelia. One of these is the CF transmembrane conductance regulator (CFTR), a chloride channel that is the target of known genetic defects in CF. Loss of inhibition of ENaC function by defective CFTR is proposed to contribute to overactive Na+ absorption in CF (22) by as yet undefined mechanisms. Nonetheless, and more in keeping with
our hypothesis, a role for peptidases in regulating Na\(^+\) current in CF airway has been suggested by studies using placental bikunin, an aprotinin-related peptidase inhibitor that suppresses Na\(^+\) transport in cells from CF bronchi (3).

The antibodies raised against bacterially expressed recombinant human prostasin in the current study reveal strong expression of detergent-extractable prostasin in a variety of epithelial lines, including the ΔF508 CF airway line, JME/CF15. When cultured on permeable supports, JME/CF15 cells form high-resistance monolayers exhibiting strong, baseline amiloride-sensitive transepithelial Na\(^+\) current, which increases promptly (but modestly) upon exposure to trypsin and decreases strikingly upon prolonged incubation with aprotinin, an inhibitor of trypsin and serine peptidases. Aprotinin is a potent inactivator of our recombinant epithelial prostasin, as it is of prostatic prostasin purified from semen (35). Because aprotinin inhibits not only prostasin but a variety of serine peptidases, the results of our prostasin overexpression and siRNA-mediated silencing studies are critical for testing the hypothesis that prostasin specifically is important in regulating ENaC function in cells carrying the classic homozygous ΔF508 CF mutation. Results of these studies indicate that prostasin overexpression has little if any effect on transport of Na\(^+\) across JME/CF15 monolayers. On the other hand, silencing of prostasin expression reduces amiloride-sensitive, transepithelial Na\(^+\) transport to just 26 ± 9% of baseline, which is similar to the observed ∼30% reduction of amiloride-sensitive Na\(^+\) transport in CF cells by the peptidase inhibitor bikunin (3). Considered as a whole, this evidence suggests that prostasin plays a major role in stimulating ENaC-mediated Na\(^+\) current in ΔF508 cell monolayers and that this stimulation is nearly maximal at baseline.

Production of recombinant proprostasin in E. coli allowed generation of polyclonal antibody, which was similar to commercially available monoclonal anti-prostasin in specific detection of human prostasin as suggested by immunoblots of purified native and recombinant prostasins and of various cell extracts. The polyclonal antiserum may have the additional advantage of detection of prostasin from a broader range of mammals. For example, it detects native, low-level expression of prostasin-like protein in hamster (CHO) cells. By design, the recombinant bacterially expressed prostasin is inactive to facilitate use as an antigen injected into living rabbits. The small quantities of soluble prostasin purified from seminal fluid and secreted from transfected HEK-293 cells were useful as positive controls for electrophoresis, immunoblotting, and peptidase activity assays. In the immunoblots shown in Fig. 2, E. coli-derived recombinant human prostasin, although unglycosylated, migrates on SDS-polyacrylamide gels in a position equivalent to that of epithelial cell-expressed soluble prostasin (at ∼40 kDa) because it contains an engineered NH\(_2\)-terminal epitope tag and uncleaved propeptide as well as the native COOH-terminal hydrophobic peptide. Native, soluble prostasin purified from seminal fluid appears smaller by electrophoresis than the E. coli- and HEK-293-expressed recombinant prostasins because it has no engineered tags, no propeptide, and no COOH-terminal hydrophobic peptide. As expected, the immunoreactive prostasin natively expressed by Calu-3, A549, and JME/CF15 cells in Fig. 3 appears slightly larger (∼45 kDa) than HEK-293-expressed recombinant soluble prostasin because it retains the COOH-terminal hydrophobic peptide. The heterogeneity and smaller bands of immunoreactivity seen principally in A549 extracts may represent partially degraded protein or material that is less N-glycosylated. In JME/CF15

![Fig. 5. Na\(^+\) current in CF cells after silencing of prostasin expression with short interfering (si)RNA. A: representative immunoblots of prostasin and actin extracted from JME/CF15 cells 72 h after incubation with prostasin siRNA corresponding to portions of prostasin propeptide, mature catalytic domain, and 3′-untranslated region (UTR), as indicated. The first 2 lanes, respectively, show protein expression in cells not exposed to siRNA and in cells incubated with scramble (nonsilencing) siRNA. These blots are representative of results in 5 experiments. B: representative traces of \(I_{sc}\) in JME/CF15 cell monolayers mounted in Ussing chambers and exposed to control (scramble) siRNA or prostasin 3′-UTR-targeted siRNA. Amiloride (20 μM) was added to determine and compare magnitudes of epithelial Na\(^+\) current (ENaC)-mediated \(I_{sc}\). The portion of \(I_{sc}\) collapsed by amiloride is due to activity of the ENaC. C: inhibitory effect of prostasin 3′-UTR siRNA on basal \(I_{sc}\) (means ± SE; n = 6; *P < 0.05). On average, silencing of prostasin expression reduces \(I_{sc}\) by 74%.

![Protein expression and actin blots](image-url)
cells, the immunoreactive prostasin in extracts of transfected cells is of higher intensity but is superimposed on prostasin natively expressed by these cells, indicating that the transcripts originating from the expression vector and the resulting translation products and posttranslational processing are indistinguishable from those originating from the native gene.

Both forms of soluble prostasin (native and recombinant) are active as peptidases and are inactivated by aprotinin. For recombinant enzyme, the 50% inhibitory concentration of aprotinin is comparable to that for prostasin purified from seminal fluid (35). Both manifest essentially stoichiometric sensitivity to aprotinin. Like many tryptic serine peptidases, native and recombinant soluble prostasins are orders of magnitude less sensitive to benzamidine than to aprotinin. The sensitivity to aprotinin is consistent with prostasin being an important target in mediating aprotinin suppression of amiloride-sensitive Na\(^+\) current in CF epithelial cells. Recombinant prostasin’s specific activity is lower than that of the seminal fluid enzyme due at least in part to lower purity but perhaps also due to an effect of the COOH-terminal epitope tag on catalytic efficiency. Nonetheless, the ability of an epithelial cell (i.e., HEK-293) to secrete an engineered form of active, soluble prostasin suggests that membrane tethering, although an obligatory step in the biogenesis of the natively expressed enzyme (6), is not required for folding and activation. Furthermore, it suggests that these cells (even those like CHO and HEK-293, natively expressing little or no prostasin) possess the machinery for activating prostasin, which requires hydrolysis of the zymogen at a tryptic cleavage site (26, 34). Because prostasin itself is tryptic in specificity, this activation may be carried out by one prostasin cleaving another, although the present data provide no direct evidence of this. It should be noted that the native, immunoreactive prostasin detected in Calu-3, A549, and JME/CF15 cells was obtained by detergent extraction. Very little was seen in cytosolic extracts or in conditioned medium (not shown), consistent with the great majority of prostasin in these cells being membrane associated, most likely in glycosylphosphatidylinositol-anchored form, as described in prostate cancer cell lines (6). Although some studies have demonstrated formation of detergent-stable inhibitory complexes between prostasin and serpins (notably protease nexin-1) (6, 7), we did not detect such complexes in our immunoblots of natively or recombinantly expressed prostasin, probably because of the low levels of the relevant serpins in our cell culture and conditioned media.

The failure of transepithelial Na\(^+\) current to increase in JME/CF15 cells overexpressing prostasin indicates that prostasin is not limiting in this regard and that it is able to exert maximal stimulation at concentrations at or lower than those reached by untransfected cells. This conclusion is also consistent with the small stimulation produced by trypsin exposure in cells not pretreated with aprotinin. The small responses to prostasin and trypsin by JME/CF15 cells at baseline stand in contrast to reported responses of mouse kidney (M1) cells, which can increase Na\(^+\) uptake in response to incubation with trypsin or prostasin without prior incubation with aprotinin (19); thus in these cells, prostasin levels were limiting. This is not a generalizable difference between CF and non-CF cells, however, because several cell types, including non-CF airway cells and even M1 kidney cells cultured under different conditions respond to trypsin only after preincubation with pep tidase inhibitor (3, 8, 17, 18). We noted a slight and gradual decline in \(R\) of JME/CF15 cells incubated with aprotinin, and this observation is consistent with reported effects of aprotinin and other serine peptidase inhibitors on resistance across monolayers of cultured mouse kidney cortical collecting duct cells (17). This could mean that epithelial serine peptidases, possibly including prostasin, could regulate resistance in addition to promoting Na\(^+\) transport.

The gene silencing experiments reported in this work, by achieving a 74% reduction in ENaC-mediated Na\(^+\) current in conjunction with a profound decrease in prostasin expression, offer the most direct evidence that prostasin is a major positive regulator in ΔF508 CF cells, notwithstanding any loss of the basal inhibition of transport contributed by normally functioning CFTR. Overall, this study suggests that prostasin may be an appropriate target for inhibition in airway epithelium with the therapeutic aim of reducing excessive epithelial uptake of Na\(^+\) and maintaining a more normal state of airway hydration.

ACKNOWLEDGMENTS

We thank Dr. Jonathan Widdicombe for encouragement and suggestions.

GRANTS

This work was supported in part by grants from Cystic Fibrosis Research, Inc. (Z. Tong), the Cystic Fibrosis Foundation (B. Illek and G. H. Caughey), and National Heart, Lung, and Blood Institute Grants HL-024136 (G. H. Caughey) and HL-67920 (G. M. Verghese).

REFERENCES


