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

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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 secrete 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 that are markedly diminished by aprotinin, suggesting regulation by serine-class peptidases. Overproduction of membrane-anchored prostasin in transfected JME/CF15 cells does not augment Na+ currents, and trypsin-induced increases are small, suggesting that baseline serine peptidase-dependent ENaC activation is maximal in these cells. 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 peptide 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-trypsins, 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 peptidases, 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 trypsin 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 prostasins, small amounts of native, soluble human transmembrane prostasin 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-L-Pro-L-Arg-EDTA in 6 M urea.

37\(^\circ\)C in 25 mM Tris-\(\times\)20-Tris-HCl (pH 9.0) containing 0.3 mM substrate.

Bacterial expression of recombinant human prostasin. A 942-bp human prostasin cDNA encompassing the protein-coding region, including the COOH-terminal transmembrane segment, was generated via the PCR using primers 5'-GAAAGGGGAC AAGCTCTCGT-3' and 5'-TCAGTGCCTG CTGAGCCA-3'. Prostate cDNA served as a template. The resulting amplimer was ligated into pCR2 plasmid vector (Invitrogen, Carlsbad, CA), which places an NH\(_2\)-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 prostasin plasmid was used to transform Escherichia coli strain BL21(DE3)pLysS (Stratagene, La Jolla, CA), which expressed recombinant enzyme upon induction by isopropylthio-\(\beta\)-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 (\(\sim\)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'-AAGCTTGCAC TTGCCCAGAA GGGGTGTG C and 5'-CCCTAGCAAG CCCTGGGCTG G, 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 prostasin construct (5 μg) was incubated with 2.5 × 10\(^5\) CHO or HEK-293 cells along with 5 μl of Lipofectamine 2000 (Invitrogen) in each well of six-well plates. For select for 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 I 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'-AAGCTTGCAC TTGCCCAGAA GGGGTGTG C and 5'-CCCTAGCAAG CCCTGGGCTG G, 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 prostasin construct (5 μg) was incubated with 2.5 × 10\(^5\) CHO or HEK-293 cells along with 5 μl of Lipofectamine 2000 (Invitrogen) in each well of six-well plates. For select for 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 I 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.

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PROSTATIN REGULATES ENaC

Fig. 1. Design of recombinant prostasin expression vectors. A: pCRT7/NT-TOPO-based vector for bacterial expression of full-length prostasin, with the native signal peptide replaced by a segment encoding polyhistidine and an enteropeptidase (EK) cleavage site. B: modified cDNA encoding soluble prostasin ligated into plasmid vector pcDNA3.1/V5-His-TOPO, with the COOH-terminal transmembrane segment retained by a segment (V5-His) containing a V5 antibody-binding epitope and a polyhistidine sequence for purification by nickel affinity chromatography. C: design of a cDNA encoding full-length native prostasin, including signal peptide (Pre), propeptide (Pro), and COOH-terminal transmembrane segment (TM). This cDNA was ligated into plasmid vector pcDNA3.1 for overexpression of prostasin in cystic fibrosis (CF) epithelial cells.
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 μg/ml streptomycin as described (14). These cells were selected for study because they are homoygous for the most common CF allele, ΔF508, and form high-resistance monolayers suited to electrophysiological studies. The medium was supplemented with 10 μg/ml epithelial growth factor, 1 μM hydrocortisone, 5 mg/ml insulin, 3 μg/ml transferrin, 30 nM triiodothyronine, 180 μM adenine, and 5.5 μM epinephrine. For transfections, medium was changed to reduced-serum Opti-MEM I, followed in 30 min with 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-12/10% 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 (Rt) was monitored with an epithelial voltmeter (World Precision Instruments, Sarasota, FL). Epithelial monolayers were placed into Ussing chambers designed for use in Ussing chamber studies. 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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 a 50% inhibitory concentration compared with wild-type cells (not shown) as indicated.

**Effect of trypsin and aprotinin on Isc in ΔF508 CF cells.** Figure 4B summarizes and compares basal Na⁺ currents in control and JME/CF15 cells overexpressing transmembrane prostasin. Isc is slightly, but not significantly, higher, suggesting that any stimulation of transepithelial Na⁺ current in these cells by prostasin is nearly maximal.

**Native expression of prostasin protein by mammalian 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 E. coli-expressed recombinant human prostasin (rhProstasin), which serves as a positive control in the first lane. The other lanes contain extracts of hamster CHO cells (as a negative control), and various human epithelial lines, including adenocarcinoma-derived Calu-3 and A549, embryonic kidney-derived HEK-293, and JME/CF15 (a homozygous ΔF508 CF line), as indicated. The size of the rhProstasin band, as determined by marker proteins, is indicated in kDa.
prolonged incubation with aprotinin (followed by washout of inhibitor) restores much amiloride-sensitive \( I_{sc} \).

**Effect of prostasin gene silencing on \( I_{sc} \) in \( \Delta F508 \) 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 unchanged expression of \( \beta \)-actin in cells with reduced levels of prostasin. The effect of the 3’-UTR siRNA was a specific effect of prostasin-targeted siRNA as suggested by the lack of suppression of prostasin expression in cells transfected with control (scramble) siRNA. On the basis of these results, the 3’-UTR siRNA was selected to test effects of prostasin gene silencing on transepithelial movement of \( Na^+ \). Results of pilot experiments established 3 days after transfection as the optimum time for studying \( Na^+ \) current in JME/CF15 cells. At earlier time points, cells were recovering from transfection and were less likely to form high-\( R_0 \) monolayers and were more likely to harbor residual prostasin (not shown). At later time points, cells were more likely to have escaped from the silencing by siRNA as suggested by a relative increase in prostasin in immunoblots. As shown in Fig. 5, B and C, basal amiloride-sensitive \( Na^+ \) currents were downregulated after transfection with prostasin 3’-UTR siRNA. On average, \( I_{sc} \) decreased 74% compared with cells transfected with control (scramble) siRNA.

**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 adenoviral 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 trypsic peptides testisin, pancreasin, and \( \gamma \)-trypsin (2, 5, 10). Recent studies demonstrate that the intracellular proteinase 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\textsuperscript{+} current in CF airway has been suggested by studies using placental bikunin, an aprotinin-related peptidase inhibitor that suppresses Na\textsuperscript{+} 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\textsuperscript{+} current, which increases promptly (but modestly) upon exposure to trypsin and decreases strikingly upon prolonged incubation with aprotinin, an inhibitor of tryptic 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\textsuperscript{+} across JME/CF15 monolayers. On the other hand, silencing of prostasin expression reduces amiloride-sensitive, transepithelial Na\textsuperscript{+} transport to just 26 ± 9% of baseline, which is similar to the observed ~30% reduction of amiloride-sensitive Na\textsuperscript{+} transport in CF cells by the peptidase inhibitor bikunin (3). Considered as a whole, these evidence suggests that prostasin plays a major role in stimulating ENaC-mediated Na\textsuperscript{+} 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 antisera 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 proprostasin, 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\textsubscript{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...
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 peptidase inhibitor (3, 8, 17, 18). We noted a slight and gradual decline in Rₑ 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. Vergheze).

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