Activity and Inhibition of Prostasin and Matriptase on Apical and Basolateral Surfaces of Human Airway Epithelial Cells

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Prostasin is a membrane-anchored protease expressed in airway epithelium, where it stimulates salt and water uptake by cleaving the epithelial Na⁺ channel, ENaC. Prostasin is activated by another trans-membrane tryptic protease, matriptase. Because ENaC-mediated dehydration contributes to cystic fibrosis (CF), prostasin and matriptase are potential therapeutic targets, but their catalytic competence on airway epithelial surfaces has been unclear. Seeking tools for exploring sites and modulation of activity, we used recombinant prostasin and matriptase to identify substrate t-butyloxy carbonyl-L-Gln-Ala-Arg-4-nitroanilide (QAR-4NA), which allowed direct assay of proteases in living cells. Comparisons of bronchial epithelial cells (CFBE41o-) with and without functioning CFTR revealed similar levels of apical and basolateral aprotinin-inhibitable activity. Although recombinant matriptase was more active than prostasin in hydrolyzing QAR-4NA, cell surface activity resisted matriptase-selective inhibition, suggesting that prostasin dominates. Surface biotinylation revealed similar expression of matriptase and prostasin in epithelial cells expressing wild type versus ΔF508-mutated CFTR. However, the ratio of mature to inactive pro-prostasin suggested surface enrichment of active enzyme. Although small amounts of matriptase and prostasin were shed spontaneously, prostasin anchored to the cell surface by glycosylphosphatidylinositol was the major contributor to observed QAR-4NA-hydrolyzing activity. For example, the apical surface of wild type CFBE41o- epithelial cells express 22% of total, extractable, aprotinin-inhibitable, QAR-4NA-hydrolyzing activity and 16% of prostasin immunoreactivity. In conclusion, prostasin is present,
mature and active on the apical surface of wild type and CF bronchial epithelial cells, where it can be targeted for inhibition via the airway lumen.

Keywords: Channel-activating protease; cystic fibrosis, epithelial sodium channel, aprotinin, glycosylphosphatidylinositol
INTRODUCTION

Prostasin is a tryptic, type I transmembrane serine protease encoded by the PRSS8 gene. In mammals, including mice and humans, prostasin is widely expressed, in epithelial cells, where it supports barrier integrity (25, 35) and regulates open probability of the epithelial Na⁺ channel (ENaC). Prostasin was discovered as a tryptic peptidase of seminal fluid (39). Immunohistochemical studies revealed its presence in epithelial cells of the prostate gland, which inspired naming the enzyme prostasin. Subsequent studies revealed expression by epithelial cells of many organs (36, 38). Sequencing of cDNA revealed sequence predicting initial synthesis as a membrane-anchored, zymogen with an N-terminal prepro-segment and a C-terminal transmembrane peptide anchor (38). These predictions have been borne out in several types of epithelia. In the prostate gland, some prostasin is shed via peptide anchor hydrolysis to yield the soluble enzyme (38). However, the peptide anchor is exchanged for a lipid (glycosylphosphatidylinositol, GPI) in some other epithelial cells (9). The catalytic domain of cell surface-expressed, lipid-anchored prostasin can be shed by bacterial lipase or by endogenous GPI-specific PLD, which mediates a proposed mechanism of down-regulation (35).

Major clues regarding prostasin’s biological functions in mammals arose from studies of ENaC function in frogs. Expression cloning strategies identified a channel-activating protease (CAP) (34). Data-mining and phylogenetic analysis identified mammalian prostasin as a likely CAP ortholog (8, 34, 36) alternatively termed CAP1. Among several mammalian epithelial serine proteases with potential to activate ENaC, prostasin/CAP1 is a leading candidate as an endogenous regulator of Na⁺ transport, as
reviewed in (25). Co-expression of prostatins and ENaC in *Xenopus* oocytes augments Na⁺ absorption via ENaC by augmenting channel open probability (1, 37). In cultured mammalian epithelial cells, prostatin inhibitors (like aprotinin and bikunin), reduce amiloride-sensitive (ENaC-mediated) Na⁺ transport (4, 13). Additionally, siRNA-mediated knockdown of prostatin in wild type and CF cells reduces ENaC activity to a degree similar to that produced by non-selective protease inhibitors applied to the cell surface (33). Biochemical studies suggest that hydrolysis of ENaC itself is the basis for augmentation of Na⁺ transport by prostatin and identify prostatin-sensitive sites in ENaC's γ subunit (5). Mutagenesis studies suggest that catalytically active prostatin in its GPI-anchored form is required for effects on ENaC (35).

Further probing of prostatin regulation in mammalian cells predicts activation from its zymogen form by another transmembrane protease, matriptase (22). Although global deletion of prostatin in mouse cells is lethal during embryogenesis, tissue-selective knockouts yield less severe phenotypes. For example, deletion of *Prss8* expression in epidermal cells generates mice with skin barrier defects dying within 60 hours of birth (19). Skin-specific reduction of matriptase generates a similar phenotype (20, 21), consistent with biochemical evidence that prostatin is activated by matriptase and that prostatin's mature form is required for ENaC stimulation (24). On the other hand, prostatin may activate matriptase in addition to the converse (7), reinforcing the concept that fates and activity of the two enzymes are intertwined. More recently, prostatin catalytic domain mutations were linked to defects in hair and skin development in established strains of mice and rats (28), and skin over-expression caused inflammation and ichthyosis (15). Selective deletion of mouse *Prss8* in distal
airway epithelial cells reduces alveolar fluid clearance and ENaC-mediated Na⁺ absorption (26), which is consistent with mounting in vitro evidence that prostasin regulates epithelial Na⁺ transport.

Although no known genetic defects directly involve prostasin in humans, an inactivating mutation in the catalytic domain of matriptase is associated with autosomal recessive ichthyosis with hypotrichosis (2, 12, 20). Both enzymes are potential targets for therapeutic inhibition in diseases such as CF and systemic hypertension (40). In CF, Na⁺ hyper-absorption by ENaC is thought to contribute to excessive drying of secretions, ciliary dysfunction, and susceptibility to infection. Work with cultured CF cells suggests that prostasin regulates ENaC (33) and may be over-expressed in CF (23, 32).

Furthermore, inhaled, non-specific inhibitors of prostasin improve mucociliary clearance in sheep (11). In hypertension, retention of Na⁺ increases blood volume and pressure. Recent evidence suggests that expression of matriptase and prostasin is polarized and that the enzymes are transported vectorially across epithelium (16).

Although tryptic activity has been detected on the surface of frog oocytes and cultured epithelial cells (18), little is known concerning relative surface expression of active matriptase and prostasin. The present work tests the hypothesis that prostasin is active on the apical/lumenal surface of airway epithelial cells and over-active in cells with defective CFTR.

MATERIALS AND METHODS

Comparison of substrate preferences. Recombinant, soluble catalytic domains of human prostasin (R&D Systems; Minneapolis, MN) and matriptase (Enzo Life Sciences;
Plymouth Meeting, PA) were used to screen potential peptidyl 4-nitroanilide (4NA) substrates of the two enzymes. Screened substrates included D-Val-L-Leu-Lys-4NA, N-benzoyl-L-Lys-Gly-Arg-4NA, N-benzoyl-L-Val-Gly-Arg-4NA, N-benzoyl-L-Arg-4NA, β-Ala-Gly-L-Arg-4NA, and N-(p-tosyl)-Gly-L-Pro-Lys-4NA from Sigma (St. Louis, MO), and carboxbenzoxyl-L-Arg-Arg-4NA and t-butyloxycarbonyl-L-Gln-Ala-Arg (QAR)-4NA from Bachem Americas (Torrance, CA). Substrates (1 mM) were incubated with 1.45 μg/ml of human prostasin or 1 ng/ml of human matriptase in PBS at 37°C. Generation of free 4-nitroaniline by enzymatic hydrolysis was monitored at 410 nm in cuvettes using a Genesys 5 spectrophotometer (Thermo Fisher Scientific; Waltham, MA).

*Comparison of inhibitor susceptibilities.* Recombinant, soluble human prostasin and matriptase were pre-incubated in PBS at room temperature for 15 min with aprotinin (100 μM; Sigma) or with 4 μM cell surface-active, human single-chain (sc) Fv human matriptase-inactivating MAb (3, 30), then assayed at 37°C for residual amidolytic activity by addition of QAR-4NA (1 mM) and detection of 4-nitroaniline release as above.

*Culture of human bronchial epithelial cell lines at an air-liquid interface.* This study used established sub-clones of the stable ΔF508-homozygous line CFBE41o−, which originated from bronchial epithelial cells from a subject with CF and was immortalized using origin of replication-deficient SV40 (6, 10). The CFBE41o− sub-clones were complemented by transfection with an episomal plasmid vector expressing cDNA encoding full length (6.2 kb) wild type CFTR (CFBE41o-WT) or transfected with the same vector expressing non-functional, truncated (4.7 kb) CFTR (CFBE41o-ΔF508) to control for CFTR over-expression, as described (17). Retention of both plasmids, which contain a gene conferring resistance to toxic effects of hygromycin, was maintained by
culturing cells with hygromycin. CFBE41o-WT and CFBE41o-∆F508 cells were seeded to
confluence at 3x10^5 cells/well onto polyester membrane Transwell supports (0.33 cm²,
0.4 µm pores; Corning; Lowell, MA) coated with a mixture of BSA (1 mg/ml; Invitrogen,
Carlsbad, CA), human fibronectin (30 µg/ml; BD Biosciences; San Jose, CA), and bovine
collagen type II (10 µg/ml; BD Biosciences) in LHC basal medium (Invitrogen) and
grown in minimal essential medium with Earle’s salt supplemented with 10% FBS, 4 mM
L-glutamine, 100 U/ml penicillin G, and 300 µg/ml hygromycin at 37°C in a 5% CO₂
incubator. Apical medium was removed 48 h after seeding to initiate air-interface
culture. Basolateral medium was changed every 48 h for 12 days. Except as specified,
culture media and supplements were from Invitrogen.

_Growth of cells in liquid culture._ Alternatively, to obtain larger numbers of cells
and to serve as a basis of comparison with cells subjected to air-interface culture,
CFBE41o- bronchial epithelial cells were cultured to confluence on polystyrene 6- or 96-
well cell culture plates (Corning, Inc.; Corning, NY) coated as above with BSA, fibronectin
and collagen and cultured by immersion of the apical surface in the same medium as
described above in connection with air-interface culture.

_Measurement of transepithelial electrical resistance and passage of phenol red._ To
assess integrity of cell monolayers cultured at an air-liquid interface, ∆F508 and wild-
type CFBE41o- cell transepithelial electrical resistance was measured with an epithelial
voltmeter (World Precision Instruments; Sarasota, FL). Monolayer integrity also was
assessed spectrophotometrically by tracking passage of the small molecule phenol red
from apical to basal medium. Basolateral and apical media were replaced, respectively,
with 0.8 ml of PBS and 0.2 ml of minimal essential medium containing phenol red (27
µM). After 6 h of incubation at 37°C, Absorbance at 559 nm of medium was measured to detect transepithelial passage of phenol red, as reflected by Absorbance ratios in basolateral versus apical medium.

Confocal microscopic imaging of air interface-cultured cells. After 12 days of culture, cells on inserts were fixed with 4% paraformaldehyde in PBS at 4°C for 10 min, washed with PBS, exposed to 1% Triton X-100 (Sigma) on ice for 10 min, rewashed with PBS, then stained with Alexa Fluor 568 phaloidin (1:1000; Invitrogen) and mounted onto glass slides using ProLong Gold Antifade Reagent with DAPI (4’6-diamidino-2-phenylindole; Invitrogen). Cells on inserts were imaged using a LSM 510 META confocal laser scanning module (Carl Zeiss Microscopy, Thornwood, NY) mounted onto an inverted Axio Observer (R&D in collaboration with EMBL Heidelberg, Germany). Images were acquired using a 40x EC Plan-NEOFLUAR (NA 1.3) oil objective. Excitation wavelengths were 405 nm for DAPI and 543 nm for Alexa Fluor 568 phalloidin. A gallery of 0.4-µm optical sections was collected and processed using LSM 510 software (Carl Zeiss).

Assay of apical and basolateral surface-associated tryptic activity in living epithelial cells. After 12 days of air-interface culture, CFBE41o-WT and CFBE41o-ΔF508 cells were washed in PBS. In preparation for measuring cell surface tryptic activity, the apical or basolateral side of the monolayer cultured on inserts was immersed in 0.2 ml (apical) or 0.8 ml (basolateral) of PBS containing QAR-4NA (1 mM) alone as control, QAR-4NA plus aprotinin (100 μM), or QAR-4NA plus anti-matriptase scFv MAb (4 μM). For measurements of apical activity, the basolateral surface was bathed in PBS alone, whereas for measurements of basolateral activity, the apical surface was bathed in PBS.
alone. Tryptic activity in aliquots of apical or basolateral conditioned medium was monitored hourly for 4 h at 37°C by spectrophotometry using a Synergy 2 SL Microplate Reader (BioTek Instruments; Winooski, VT). To allow comparison of surface QAR-4NA-hydrolyzing activity with total cellular activity, apical and basolateral activity measurements in cells cultured under liquid culture or air-interface conditions were compared with that of the same number of cells lysed by repeated freeze-thaws or by vigorous pipetting.

**Solubilization, separation and blotting of matriptase, pro-prostasin and mature prostatin in whole cell extracts.** Cells were solubilized in lysis buffer (10 mM Tris-HCl, 50 mM EGTA, 0.4% Na+ deoxycholate, 1% NP-40, and 1% Triton X-100, pH 7.4). After centrifugation for 7.5 min at 10,000xg, supernatants were prepared for SDS-PAGE by adding lithium dodecylsulfate sample buffer and reducing agent (50 mM DTT). Samples were denatured at 70°C for 10 min before loading onto NuPAGE Novex 10% bis-Tris gels (1-mm thick; Invitrogen) and resolved with MOPS SDS running buffer (Invitrogen). Proteins then were transferred to a polyvinylidene difluoride membrane (Perkin Elmer; Shelton, CT), which was incubated with anti-human prostasin MAb (diluted 1:360; BD Biosciences), anti-human matriptase rabbit polyclonal IgG (1:2000; EMD Chemicals, Gibbstown, NJ) or β-tubulin rabbit polyclonal IgG (1:500 in 5% BSA; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology and then with SuperSignal West Dura chemiluminescence substrate from Thermo Fisher Scientific (Waltham, MA).

Densitometry was performed using ImageJ software.
Biotinylation, purification and blotting of apical and basolateral surface proteases in polarized bronchial epithelial cells. For cells cultured on porous polyester membranes at an air-liquid interface, inserts were washed 3 times with cold PBS to remove residual serum proteins. Apical surfaces of the cells then were incubated for 20 min at room temperature in PBS containing N-hydroxysuccinamide-SS-biotin (1 mg/ml; Thermo Fisher Scientific). Biotinylation was quenched by flooding cells with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Cells then were lysed and proteins solubilized as described above for un-biotinylated cells. Resulting supernatants, after centrifugation, were incubated with streptavidin-agarose resin (Thermo Fisher Scientific), which were rotated gently overnight at 4˚C. Biotinylated proteins captured in this manner were released into solution and de-biotinylated by incubating PBS-washed resin for 20 min at room temperature with sample buffer containing 50 mM DTT, followed by heating for 3 min at 95˚C.

Detection of apically shed matriptase and prostasin. The apical surface of wild-type and ΔF508-homozygous CFBE41o- cells grown to confluent monolayers on porous membrane inserts or tissue culture plastic as above were exposed for 1 h at 37˚C to 1 U/ml Bacillus cereus phosphatidylinositol-specific PLC (PI-PLC; Molecular Probes, Eugene, OR) in PBS (with ambient 5% CO₂ in a tissue culture incubator). PI-PLC-conditioned medium was assayed at 37˚C for amidolytic activity by addition of QAR-4NA (1 mM) alone or with aprotinin followed by detection of released 4-nitroaniline as above. Conditioned medium then was concentrated 10-fold using a centrifugal filter (5K NMWL; Millipore, Billerica, MA) and subjected to SDS-PAGE and immunoblotting for prostatic and matriptase as above.
Statistical analysis. Results are reported as means ± SEM. Except where otherwise specified, differences between groups were analyzed for significance using two-tailed Student t tests. For data not normally distributed, differences were compared using Mann-Whitney U tests. P values of <0.05 were considered significant.

RESULTS

Identification of a peptidic chromogenic substrate preferred by matriptase and prostasin. As shown in Fig. 1, the screen of peptidic tryptic substrates identified several substrates hydrolyzed by prostasin and matriptase. Of the substrates screened, QAR-4NA was the substrate preferred by both enzymes and on this basis was selected to use in assays of living cells for prostasin and matriptase activity. Based on specific activity, matriptase was intrinsically more active than prostasin towards all substrates, including QAR-4NA.

Selective inhibition of matriptase. As shown in Fig. 2, QAR-4NA-hydolyzing activity of soluble, recombinant human matriptase is completely blocked by scFv anti-matriptase at a concentration with no effect on soluble prostasin activity. Both proteases are highly sensitive to inhibition by the Kunitz-class inhibitor aprotinin. Thus, scFv anti-matriptase distinguishes matriptase activity from prostasin activity.

Bronchial epithelial cells expressing ΔF508 and wild type CFTR form electrically resistant monolayers in air interface culture. CFBE41o-ΔF508 and CFBE41o-WT cells grown under air interface conditions both achieved confluence within 2 days of plating. As shown in Fig. 3a, transepithelial electrical resistance of ΔF508-homozygous CFBE41o-cells is significantly higher than that of inserts without cells but remains less than 250 Ω.
cm² without significantly increasing between days 2 and 12 of culture. However, measured resistance of wild-type CFBE41o- cells increases with time in culture and is higher at day 12 than in ΔF508-homozygous CFBE41o- cells. As shown in Fig. 3b, both types of monolayers largely prevented equilibration of phenol red dye from apical to basolateral medium between culture day 2 and 12, during a 6-hour incubation mimicking conditions used in the tryptic protease assay of living cells. At confluence, both cell types formed monolayers of similar appearance, as assessed by confocal fluorescence microscopy (see Fig. 3c). Thus, ΔF508-homozygous and wild type CFBE41o- cells both develop electrically resistant monolayers that hinder passage of small ions, though resistance developing in the latter cells is higher with sustained culture.

Tryptic serine peptidases are active and inhibitable on both surfaces of polarized, living epithelial cells. As shown in Fig. 4a, apical tryptic serine protease activity, as reflected by hydrolysis of QAR-4NA, is detected on the surface of ΔF508-homozygous CFBE41o- cells as well as of wild type CFBE41o- cells. Aprotinin at a concentration 100-fold and 2000-fold higher than the $K_i$ for prostasin and matriptase, respectively (14, 27), significantly reduced apical tryptic activity compared to activity of cells incubated with substrate alone, indicating that observed activity is due to one or more trypsin-like serine peptidases. A concentration of scFv anti-matriptase 100-fold above the $K_i$ for human matriptase (14) produced either no change or statistically insignificant reduction in surface activity, suggesting that little if any apical activity is due to matriptase. Fig. 4a reveals that hydrolysis is roughly constant over the interval of observation, suggesting that the apical tryptic enzymes are in a steady state, which is inconsistent with activity
being produced by accumulation of enzyme shed into medium, which would produce increasing activity over time. Compared to wild type CFBE41o- cells, ΔF508-homozygous CFBE41o- cells had higher QAR-4NA-hydrolyzing activity remaining in cells incubated with aprotinin, suggesting higher levels of tryptic cysteine proteases or aprotinin-resistant serine proteases in the ΔF508-homozygous cells. Similar results (not shown) were obtained in assays of basolateral activity. As depicted by Fig. 3b, QAR-4NA-hydrolyzing activity was much higher in lysates of wild type CFBE41o- cells than detected on the apical surface, consistent with the assay detecting surface activity rather than activity from intracellular sources. Similar results (not shown) were obtained in comparisons of apical and lysates activity in ΔF508-homozygous CFBE41o- cells, and in comparisons of basolateral versus lysate activity. To facilitate comparisons between the two cell types and both surfaces, activity expressed as substrate turnover is compared in Figs. 4c and 4d. These results show that wild type and ΔF508-homozygous CFBE41o- cells had little if any detectable matriptase activity on the apical or the basolateral surface, and that both had prostasin-like activity (defined as activity that is aprotinin-sensitive and anti-matriptase-resistant) that was similar for both cell types on both surfaces.

*Mature prostasin is enriched on the apical surface of bronchial epithelial cells in air-liquid interface culture.* As shown in Fig. 5a by immunoblots of streptavidin-purified, biotin-labeled surface proteins, apical matriptase and prostasin are readily detected in detergent extracts of ΔF508-homozygous and wild-type CFBE41o- cells cultured at an air-liquid interface to mimic *in vivo* conditions faced by bronchial epithelial cells. Bands corresponding to both proteins also were detected in extracts of corresponding whole-
cell lysates. Apical prostasin is a small fraction of total prostasin in cell lysates. However, apical prostasin is enriched in the 37-kDa mature form relative to the 41 kDa pro-form. Sequential immuno-screening of the same membranes using antibodies recognizing tubulin, which is an intracellular protein, provides a negative control for selective biotinylation of surface proteins as well as a normalization control for protein loading, thereby allowing comparison of levels of matriptase and prostasin expression in ΔF508-homozygous versus wild-type CFBE41o- cells. Reduced matriptase in the apical preparations appears almost exclusively as a narrow immunoreactive band of ~85 kDa, consistent with partially processed membrane-bound zymogen, as previously observed in transfected mammalian cells (12). However, prostasin appears as broader bands with ~38 and ~41 kDa components (attributed to mature and inactive pro-prostasin, respectively (16)). As shown in Fig. 5b, the results of immunoblotting of basolaterally biotinylated proteases were similar, with the exception that a ~27 kDa band likely corresponding to activated matriptase was more apparent, although minor in intensity compared to the band at 85 kDa attributed to unactivated zymogen. The densitometric comparisons of matriptase and total (mature plus pro) prostasin band density in Fig. 5c show no significant differences in expression between ΔF508-homozygous and wild type CFBE41o- cells or in apical versus basolateral expression. In extracts of both cell types, however, the ratio of mature to pro-prostasin was much higher in biotinylated surface preparations than in whole cell extracts, suggesting enrichment of cleaved, active prostasin at the apical surface.

* Matriptase and prostasin are shed slowly from the apical surface of cultured bronchial epithelial cells. As shown in Fig. 6a, immunoreactive bands corresponding to...
matriptase and prostasin appear in apical medium conditioned by cultured ∆F508-
homozygous or wild-type CFBE41o- cells, with band intensity increasing over time and
most evident after 24 h. However, release is scant overall and nearly undetectable
during the first 4 h, which brackets the interval during which the cells were assayed for
surface activity, consistent with most of the measured activity being derived from
surface-bound rather than shed enzyme. The mechanism of surface attachment and
release was further explored and compared between liquid-cultured ∆F508-
homozygous and wild type CFBE41o- cells by incubating with PI-PLC, which sheds lipid-
anchored proteins attached via GPI (9, 35). As revealed by the examples in Fig. 6b,
prostasin band intensity in apical medium markedly increases following incubation of
either type of cell with PI-PLC, with concomitant marked decrease in surface prostasin
immunoreactivity derived from apically biotinylated cells. These results suggest that GPI
anchors most apical prostasin to the plasma membrane and that relatively little
prostasin sheds spontaneously during short-term culture. The results suggest that
matriptase is similar to prostasin in that little surface enzyme sheds; however,
matriptase differs in not being GPI-anchored or releasable by PI-PLC. Shedding of
prostasin was further compared in ∆F508-homozygous and wild type CFBE41o- cells
cultured for 10 days at an air-liquid interface. As shown in Fig. 6c, neither cell type
secretes detectable prostasin during short-term (1 h) culture. Marginally more prostasin
is released by PI-PLC from ∆F508-homozygous cells than from wild type CFBE41o- cells.
Thus, spontaneous release of prostasin and matriptase is slow from the surface of these
bronchial epithelial cell lines grown and matured in liquid culture or air-interface
conditions, and the amounts released are low compared to protein anchored to the
apical surface, from which most of the observed apical activity originates. Furthermore, because nearly all of the biotinylated (apical surface) prostasin is released into medium by PI-PLC (as revealed in Fig. 6d), almost all of the apical membrane-associated prostasin in these cells is lipid-anchored via GPI. This is in contrast to apical matriptase (Fig. 6b and data not shown), which is not released into medium by PI-PLC. Spontaneous and GPI-induced shedding of prostasin from apical membrane is similar in ΔF508-homozygous and wild-type CFBE41o- cells, and is also similar in cells matured with either liquid or air interfacing with the apical surface of the polarized monolayers (Fig. 6b and data not shown).

**DISCUSSION**

This work is the first report of a substrate and assay enabling detection of tryptic proteolytic activity on the apical surface of living airway epithelial cells. By screening recombinant, soluble versions of the dominant tryptic, membrane-associated epithelial proteases—prostasin and matriptase—this work identified tripeptide-based, colorimetric substrates hydrolyzed by both enzymes. The best of these substrates, QAR-4NA, seeded development of a sensitive, microplate-based method to detect and quantify matriptase and prostasin activity on the lumen-facing side of polarized bronchial epithelial cell monolayers. The findings reveal that recombinant matriptase catalytic domain is much more active than recombinant prostasin in hydrolyzing QAR-4NA, and that tryptic serine peptidase activity is present and readily detected on the apical as well as basolateral surfaces of cultured human bronchial epithelial cells. Somewhat unexpectedly, the great majority of QAR-4NA-cleaving activity on the cell surfaces was
prostasin-like rather than matriptase-like, given that the activity is sensitive to aprotinin, but mostly insensitive to matriptase-selective, inhibitory antibodies. However, the suggestion by these data that comparatively little apical matriptase is active fits well with the immunoblots of electrophoresed, membrane-associated matriptase, which is detected principally as an ~85 kDa band consistent with pro-matriptase but not with the proteolytically active catalytic domain, which is released as a ~27 kDa protein in the presence of reducing agents (12). These findings establish that there is much more active prostasin than active matriptase on the apical cell surface, and that prostasin is the more obvious target for potential therapeutic inhibition by agents inhaled or otherwise introduced via the airway lumen.

We compared matriptase and prostasin-like expression and activity in a line of cultured human airway cells homozygous for the ΔF508 CFTR mutation with cells from the same line in which the CFTR defect was complemented by expression of normal CFTR. The ΔF508 and complemented lines both expressed immunoreactive matriptase and prostasin and both exhibited apical surface matriptase- and prostasin-like peptidolytic activity in both liquid and air-liquid interface culture. Although a prior study found expression of prostasin protein to be upregulated in response to increases in airway surface liquid volume in normal bronchial epithelial cells and to be upregulated in CF cells despite Na+ hyperabsorption (23), the present study detected little difference in apical prostasin immunoreactivity and activity between ΔF508-homozygous CF cells and CF cells complemented with wild type CFTR. Nonetheless, the past and present studies involving cells with and without functioning CFTR suggest that prostasin is expressed, active in regulating ENaC, and inhibitable on the apical surface (4, 23, 33),
even though some of the electrophysiological defect in CF airway epithelial cells may be
due to failure of CFTR to regulate ENaC via pathways independent of prostanin (29).

The observed preference of matriptase for QAR-4NA among the peptidic
substrates surveyed is consistent with observed peptide cleavage preferences as
determined by profiling of matriptase catalytic domain substrate preferences using a
combinatorial fluorogenic peptide library or multiple rounds of phage display screening
(31), which identified glutamine as one of the residues preferred at P3, alanine as a
residue tolerated at P2, and arginine or lysine as preferred at P1. For human prostanin,
the QAR-4NA’s peptide sequence is optimal only with respect to arginine, which is
strongly preferred at the P1 position in a combinatorial peptide library (27), with
alanine at P2 and glutamine at P3 being tolerated but not preferred. Nonetheless, our
data with the recombinant enzyme establish that prostanin prefers QAR-4NA to several
potential tryptic substrates with P1 basic residues, and that it also can hydrolyze QAR-
4NA while attached to the apical and basolateral surfaces of airway epithelial cells. As a
substrate that is fully optimized for neither matriptase nor prostanin but is readily
cleaved by both enzymes, QAR-4NA is useful when used in conjunction with selective
inhibitors for assaying active forms of both enzymes on epithelial surfaces.

Although endogenous pathways for shedding of prostanin mediated by a tryptic
protease (38) or by GPI-specific PLD (35) have been identified in some tissues and cells,
little spontaneous shedding was observed in the human bronchial epithelial cells
examined in the present study, given that prostanin was detected in conditioned medium
only after prolonged incubation, but was readily detectable after hydrolysis of the GPI
anchor with bacterial PI-PLC. Thus, the present study suggests that there are no intrinsic
differences in use of endogenous pathways of prostasin shedding between airway epithelial cells with and without functioning CFTR. This does not preclude the possibility that lumenal proteases present in vivo, such as neutrophil and bacterial proteases in infected CF airway, shed prostasin and thereby alter function, including ability to activate ENaC. The results also suggest that little matriptase is shed spontaneously. Further, PI-PLC does not release matriptase from the apical surface, in contrast to PI-PLC-mediated release of prostasin. This difference is expected, in that matriptase, in contrast to prostasin, is a type II transmembrane protease that is anchored to the plasma membrane via an N-terminal membrane-spanning peptide segment that is incapable of being exchanged for a GPI lipid anchor (31).

The finding in the present study that most of the tryptic activity on the apical/lumenal surface of airway epithelial cells is prostasin-like is consistent with recent findings in colonic epithelial cells suggesting that matriptase is activated on the basolateral surface (16). However, the present studies, which differ not only in the type of cell studied but in culture conditions (air-liquid interface), suggest that matriptase was present on the apical surface, although largely inactive given lack of inhibition by scFv, anti-matriptase, which is capable of inactivating membrane-bound matriptase in its active form (14, 30). Alternatively, a fraction of apical bronchial epithelial cell matriptase is active, but is present in small amounts compared to active prostasin.

An additional intriguing finding, shown in Fig. 5c, is that prostasin labeled on the apical or basolateral surface and captured on streptavidin beads is enriched in the lower molecular weight mature form of the enzyme, in contrast to prostasin in whole cell lysates, which contain primarily prostasin's larger pro-enzyme (zymogen) form.
cell lysates contain prostasin originating from both polarized surfaces and from
membrane-bound intracellular organelles, like endoplasmic reticulum and transport
vesicles. This finding is consistent with prostasin being activated on the cell surface or
being transported promptly to the surface after activation, and is also consistent with
the recent suggestion that prostasin can be activated by basolateral matriptase and
conveyed to the apical surface in transcytotic vesicles (16). These observations
regarding surface localization of mature prostasin, in combination with comparisons of
surface and whole cell lysate prostasin-like activity, suggest that a substantial pool of
activated prostasin lies on each of the membrane surfaces. According to the
densitometric results summarized in Fig. 5c, 22% and 17% of total cellular prostasin
resides on the surfaces of wild type and ΔF508 cells, respectively, with no significant
difference in apical versus basolateral distribution. The findings further invite
speculation that proteolytic activation of ENaC occurs at the apical surface, a possibility
that also fits with the observed loss of ENaC-mediated absorption of Na⁺ in cultured
epithelial monolayers apically exposed to aprotinin or bikunin (4, 13). A less
straightforward possibility is that hydrolytic activation of ENaC occurs in a subsurface
compartment via mature prostasin taken up into vesicles from the cell surface. The
finding in this work that a sizable pool of active prostasin localizes to the basolateral
surface, which is thought to lack ENaC, raises the possibility of targets and roles for
prostasin apart from activating ENaC. Roles for prostasin in epithelial monolayer
preservation and function not clearly related to regulation of ENaC have been predicted
by several studies of engineered and naturally occurring prostasin variants in cultured
cells and rodent models of epithelial function (15, 19, 28, 35).
In conclusion, this report reveals for the first time that there is much more active prostasin than active matriptase on the apical and basolateral surfaces of airway epithelial cells, that cells with and without functioning CFTR are similar in this regard, that apical prostasin is an airway-accessible target for potential therapeutic inhibition by inhaled antagonists, and that the identification of active prostasin on basolateral surfaces suggests the possibility of important targets for prostasin in addition to ENaC.
FOOTNOTES

1Abbreviations: Channel-activating protease, CAP; epithelial Na⁺ channel, ENaC; cystic fibrosis, CF; boc-L-Gln-Ala-Arg-4-nitroanilide, QAR-4NA; glycosylphosphatidylinositol, GPI; phosphatidylinositol-specific phospholipase C, PI-PLC; single chain variable fragment, scFv.
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CONFLICTS OF INTEREST

None.
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Fig. 1. Comparison of peptidic substrate preferences of human matriptase and prostasin. Amidolytic activity was tested against soluble, recombinant proteases using 8 tryptic 4-nitroanilide (4NA) peptidic substrates, including D-Val-L-Leu-Lys-4NA (VLK), carboxbenzoxyl-L-Arg-Arg-4NA (RR), N-benzoyl-L-Lys-Gly-Arg-4NA (KGR), N-benzoyl-L-Val-Gly-Arg-4NA (VGR), t-butyloxycarbonyl-L-Gln-Ala-Arg-4NA (QAR), N-benzoyl-L-Arg-4NA (R), β-Ala-Gly-L-Arg-4NA (AGR) and N-(p-tosyl)-Gly-L-Pro-Arg-4NA (GPK). For each enzyme, data obtained with each substrate were normalized to specific activity (Absorbance (Abs) min⁻¹ mg⁻¹ of protease) towards QAR-4NA, which was the best substrate identified for both matriptase and prostasin (N = 3).

Fig. 2. Comparison of inhibitor susceptibilities of matriptase and prostasin. Amidolytic activity towards colorimetric substrate QAR-4NA was tested in preparations of recombinant soluble human matriptase and prostasin with and without incubation with anti-matriptase single chain MAb or aprotinin. N = 3; ***P ≤ 0.001.

Fig. 3. Characteristics of epithelial monolayers in air-interface culture. Panel a shows transepithelial electrical resistance in CFBE41o- cell monolayers in air-interface culture. Resistance normalized for monolayer surface area (Ω x cm²) was assessed at intervals during air-interface culture in ΔF508 CFTR-homozygous CFBE41o- cells or in the same line of cells complemented with wild type CFTR. N = 5-6 for Wild type and ΔF508 cells; *P <0.05 and ***P <0.001 (Wild type versus ΔF508). Panel b compares permeability of
wild type and ΔF508 monolayers to phenol red after 2 to 12 days in air-interface culture, as assessed by apical/basolateral ratios of Absorbance (Abs) at 559 nm 6 h after addition of phenol red to basolateral medium. Control permeability was assessed in coated inserts without cells. N = 3-6; no significant difference between wild type and ΔF508 at days 6-12; P < 0.05 between wild type and ΔF508 at day 2; P = 0.01 for all monolayers versus Control. Panel c contains representative confocal images of wild type and ΔF508 monolayers cultured on inserts at an air interface. Cells were stained with DAPI to visualize nuclei and with Alexa Fluor 568 phalloidin to detect F-actin. The main image is a plane in the z-stack; rectangular images are orthogonal reconstructions with the air interface facing outward.

Fig. 4. Bronchial epithelial cell surface activity of matriptase and prostasin. Panel a shows time course of peptidase activity measured in living cells. Tryptic activity on the apical surface of bronchial epithelial cells (ΔF508 CFTR-homozygous CFBE41o- cells or the same line of cells complemented with wild type CFTR) was assayed in polarized monolayers of cells that had been cultured at an air interface. Cells were incubated in PBS with substrate QAR-4NA (“Cells, no inhibitor”) or with substrate plus inhibitor (“+ Anti-matriptase” or “+ Aprotinin”). As an additional control, substrate was incubated in PBS alone (“Substrate, no cells”). Panel b compares QAR-4NA-hydrolyzing activity at the apical surface of wild type cells with total activity in the same number of cells subjected to lysis by repeated freeze thawing. In all conditions in which substrate was present, QAR-4NA-hydrolyzing activity was higher in cell lysates than at the surface. QAR-4NA was omitted from the “Cells only” negative control condition. Panel c compares activity
curves derived from apical surface data shown in Panel a, as well as from data obtained similarly from the basolateral surface of cells expressing ΔF508 and wild type CFTR.

Panel d compares apical and basolateral prostasin-like activity (substrate hydrolysis in the presence of anti-matriptase minus activity in the presence of aprotinin). N = 4-6; *P <0.05; **P <0.01; ***P <0.001; N.S. = not significant.

Fig. 5. Immunodetection of bronchial epithelial cell matriptase and pro- and mature prostasin. Surface proteins were labeled by biotinylation of polarized epithelial cells, purified from cell lysates using streptavidin beads, and subjected to SDS-PAGE and sequential immunoblotting with antibodies specific for matriptase and prostasin. Results were compared with those of unpurified whole cell lysates. Extracts were also immunoblotted to detect tubulin as a loading control for cell lysates and as a negative control for purified biotinylated surface proteins. 1.5x10^5 cells were plated for each cell type. Panels a and b, respectively, show representative immunoblots obtained after apical and basolateral biotinylation of wild type and ΔF508 epithelial cells maintained in air-interface culture. Panel c shows results of densitometric analysis of repeated blots (N = 5-9) assessing total prostasin (mature + pro) expression on apical and basolateral surfaces relative to expression in whole cell lysates; no significant difference in ratios were found. Panel d shows results of densitometric comparison of the ratio of bands corresponding to mature (37 kDa) versus pro-prostasin (41 kDa); N = 5-10; *P <0.05; **P <0.01..

Fig. 6. Spontaneous and induced shedding of matriptase and prostasin from CFBE41o-
bronchial epithelial cells. Panel a shows results of immunoblotting to detect
spontaneous shedding into apical medium by polarized monolayers of ΔF508 CFTR-homozygous ("ΔF508") CFBE41o- cells or the same line of cells complemented with wild-type CFTR ("Wild type"). Cells were incubated with medium bathing apical and the basolateral surfaces to mimic conditions under which apical protease activity was assayed in living cells (see Fig. 4). Medium harvested at the intervals noted was electrophoresed and immunoblotted using protease-selective antibodies. Immunoblots in Panel b explore apical expression and modes of membrane attachment by proteases in cells cultured under liquid conditions. ΔF508 and wild-type CFBE41o- cells were incubated with PBS or with PI-PLC to release GPI-anchored proteins. Apical protease expression with or without exposure to PI-PLC was detected in purified preparations of apically biotinylated surface proteins and compared with expression in un-biotinylated whole cell extracts. The immunoblot in Panel c reveals PI-PLC-induced shedding of prostasin in un-biotinylated ΔF508 and wild-type cells cultured for 10 days at an air interface followed by 1-h exposure to PI-PLC in liquid medium. The graph compares prostasin band density in ΔF508 and wild-type cells with and without exposure to PI-PLC. *P <0.05, PI-PLC-exposed ΔF508 versus wild type; ***P <0.001, PI-PLC-exposed versus unexposed ΔF508 cells. Panel d compares apical surface prostasin to prostasin shed into medium by wild-type cells in liquid culture exposed apically to PBS or PI-PLC. Band density is normalized to density of recombinant prostasin band (equal amounts loaded for each gel). **P = 0.01, apical surface prostasin in PBS- versus PI-PLC-exposed cells; #P = 0.07, shed prostasin in PBS- versus PI-PLC-exposed cells.