A disintegrin and metalloprotease 10 activity sheds the ectodomain of the amyloid precursor-like protein 2 and regulates protein expression in proximal tubule cells

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Am J Physiol Cell Physiol 300: C1366–C1374, 2011. First published February 16, 2011; doi:10.1152/ajpcell.00451.2010.—A disintegrin and metalloprotease 10 (ADAM10) is a zinc protease that mediates ectodomain shedding of numerous receptors including Notch and members of the amyloid precursor protein family (APP, APLP1, and APLP2). Ectodomain shedding frequently activates a process called regulated intramembrane proteolysis (RIP) that links cellular events with gene regulation. To characterize ADAM10 in kidney and in opossum kidney proximal tubule (OKP) cells, we performed indirect immunofluorescence microscopy and immunoblotting of renal membrane fractions using specific antibodies. These studies show that ADAM10 and APLP2 are coexpressed in the proximal tubule and in OKP cells. To study the role of ADAM10 activity in the proximal tubule, we stably overexpressed wild-type ADAM10 or an inactive mutant ADAM10 in OKP cells. We found a direct correlation between the amount of active ADAM10 expressed and 1) the amount of APLP2 ectodomain shed into the culture supernatant and 2) the amount of Na+/K+ exchanger 3 (NHE3) and megalin mRNA and protein expressed compared with control proteins. To establish a link between ADAM10-mediated shedding of APLP2 and the effect on NHE3 and megalin mRNA expression we performed RNA interference experiments using APLP2-specific short hairpin RNA (shRNA) in OKP cells. Cells expressing the APLP2 shRNA showed >80% knock down of APLP2 protein and mRNA as well as 60–70% reduction in NHE3 protein and mRNA. Levels of megalin and Na-K-ATPase protein and mRNA were not changed. These studies show 1) ADAM10 and APLP2 are expressed in proximal tubule cells and, 2) ADAM10 activity has a pronounced effect on expression of specific brush-border proteins. We postulate that ADAM10 and APLP2 may represent elements of a here-to-fore unknown signaling pathway in proximal tubule that link events at the brush border with control of gene expression.

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to regulate expression of specific brush-border proteins (21). Together these data suggest a possible molecular link between protein absorption at the brush border and gene regulation in proximal tubule cells. One of our goals is the identification of brush-border metalloproteases that might be involved in RIP-mediated signaling. Within the family of ADAM proteases, ADAM10 (Kuzbanian) has been extensively studied due to its role in Notch signaling and processing of the members of amyloid precursor protein family. This study is the first to describe ADAM10 expression in the renal brush border and provides evidence for the existence of a heretofore unknown signaling pathway in proximal tubule that includes ADAM10 and the APLP2.

MATERIALS AND METHODS

Materials. The phorbol 12-myristate 13-acetate (PMA) and 4α-PMA were purchased from Sigma (St. Louis, MO). DMEM (high glucose), fetal bovine serum, penicillin-streptomycin, l-glutamine, sodium pyruvate, Lipofectamine 2000, and Genetecin (G418) were from Invitrogen (Carlsbad, CA). Sterile cloning disks were purchased from Bel-Alt Products (Pequannock, NJ). Amicon Ultra-4 and PVDF filter (immobilon P) were from Millipore (Billerica, MA). ECL, secondary antibody ECL donkey anti-rabbit IgG, and Protein G-Sepharose were from GE Healthcare (Buckinghamshire, UK). Horse-radish peroxidase (HRP)-goat anti-mouse IgG was from ZYMED (San Francisco, CA).

Experimental animals. BALB/c mice (Charles River, Raleigh, NC) were immunized and used to make hybridomas. Animals were euthanized by injection of pentobarbital sodium (Butler, Columbus, OH). All experiments were carried out in accordance with a protocol approved by the Yale Institutional Animal Care and Use Committee.

Cell culture and plasmid transfection. An OKP cell line (9, 18) was used for in vitro studies except where noted. OKP cells were grown in media containing DMEM, 10% fetal bovine serum, 1% penicillin-streptomycin, 1% l-glutamine, and 1% sodium pyruvate. Lipolectamine 2000 was used for the transfection of plasmids into OKP cells. pcDNA3.1 plasmids representing bovine ADAM10 or the inactive (E384A) ADAM10 were a gift from Drs. Rolf Postina and Falk Fahrenholtz (Johannes Gutenberg-University Mainz, Germany) and have been described previously (12). For stable expression, both were subcloned into the pLNCX2 vector (CLONTECH) using BamHI. The GST-fused ADAM10 fusion protein was digested out with BamHI and XhoI. OKP cells stably expressing wild-type ADAM10, the E384A mutant ADAM10, and empty vector as control were cultured in OKP media with 1.4 mg/ml G418. Multiple clones of each cell line were selected and analyzed. Cells were solubilized in ice-cold solubilization buffer containing Tris (30 mM), MES (20 mM), and NaCl (100 mM), pH 7.4, with 1% Triton X100 and Complete Protease Inhibitor EDTA-free tablet (Roche Applied Science) (one tablet/25 ml of buffer).

Mouse kidney proximal tubule (mPT) cells were provided by Dr. Stefan Somlo (Dept. of Internal Medicine/Nephrology, Yale University, New Haven, CT). The protocol used to obtain mPT cells and their growth medium has been described previously (15).

Anti-ADAM10 monoclonal antibodies. Monoclonal antibodies were prepared from mice immunized with fusion protein representing ADAM10. The fusion protein was prepared as follows. A 3′-end 156-bp fragment of the bovine ADAM10 gene encoding the entire carboxyl terminus was PCR amplified using forward primer 5′-GGATCCAAGATATGCAGTGTACACACTC-3′ and reverse primer 5′-CTCGAGTAAACGTCATGTTGCCCATC-3′. The PCR product was ligated into TOPO-TA vector (Invitrogen) and digested out with BamHI and XhoI. The GST-fused ADAM10 fusion protein was expressed in bacteria and purified using Glutathione-Sepharose 4B according to the manufacturer’s protocol (Amersham Biosciences). The purified fusion protein was dialyzed into phosphate-buffered saline and stored at −70°C. BALB/c mice were immunized with the ADAM10 COOH terminus-GST fusion protein (100 µg/injection) using the pertussis-alum method (4). Splenocytes from an immunized mouse were fused with Ag8 cells, and hybridomas were prepared by routine methods using Cloning Factor (IGEN International, Gaithersburg, MD). Hybridomas secreting IgG with specificity for the COOH-terminal domain of ADAM10 were selected by enzyme-linked immunoabsorbent assay (ELISA) using equal amounts the GST-ADAM10 COOH terminus and GST control fusion proteins as antigen. Hybridomas shown to be positive to the ADAM10 fusion protein and not the control were screened by Western blot using fusion proteins as antigen. Monoclonal antibodies (mAbs) 12F9 and 14C2 were selected and purified IgG was prepared by Protein G affinity chromatography. mAbs were used either as purified IgG or as hybridoma supernatant. The specificity of the mAbs is shown in Fig. 1.

Primary antibodies. The following antibodies were used for these studies. D2II (cat. no. 171617), a rabbit polyclonal antibody raised to the NH2-terminus of APLP2, and CT12 (cat. no. 171616), a rabbit polyclonal antibody against the COOH-terminus of APLP2, were purchased from Calbiochem (San Diego, CA). A polyclonal Ab (anti-MC220) raised to the COOH-terminus of megalin (32) was used. A mAb to NHE3 (3H13) was used as described previously (17). A polyclonal antibody to the COOH-terminus of human ADAM10 was purchased from Abcam (Cambridge, MA). A mAb raised to the α-subunit of Na-K-ATPase (27) was a gift from Dr. Michael Caplan, Yale University. An antibody raised to a β-tubulin was purchased from Sigma (St. Louis, MO).

Preparation of renal membrane fractions. Renal cortex dissected from mouse kidney was used to prepare microsomes (14) and brush-border membrane vesicles using the divalent cation precipitation method (1). Animals were euthanized by injection of pentobarbital sodium (Butler).

SDS-PAGE and immunoblotting. Protein samples were solubilized in SDS-PAGE sample buffer and separated by SDS-PAGE using 3.5%-8% gradient or 7.5% polyacrylamide gels according to Laemmli method (1). Animals were euthanized by injection of pentobarbital sodium (Butler).
(19). For immunoblotting, proteins were transferred to PVDF (Millipore Immobilon-P) at 400 mA for 4–6 h at 4°C with a Transphor transfer electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA) and stained with Ponceau S in 0.5% trichloroacetic acid. Immunodetection was performed as follows: PVDF membranes containing transferred protein from entire gels were incubated first in Blotto (5% nonfat dry milk in PBS, pH 7.4) for 1 to 3 h to block nonspecific binding of antibody, followed by overnight incubation in primary antibody. Primary antibodies, diluted in Blotto, were used at dilutions ranging from 1:10 to 1:5,000. The membranes were then washed in Blotto and incubated for 1 h with an appropriate HRP-conjugated secondary antibody diluted 1:2,000 in Blotto. After being washed 3× in Blotto, 1× in PBS (pH 7.4), and 1× in distilled water, bound antibody was detected with the ECL chemiluminescence system (Amersham, Arlington Heights, IL) according to manufacturer’s protocols. In some experiments PVDF blots were stripped in 50 mM Tris-HCl (pH 6.9), 2% SDS, and 100 mM β-mercaptoethanol for 60 min at 70°C and reprobed with additional antibodies.

**Immunocytochemistry.** Mice were anesthetized with pentobarbital sodium injected intravenously, and the kidneys were perfusion-fixed with paraformaldehyde-lysine-periodate fixative (PLP) (26) as described previously (2). Indirect immunofluorescence microscopy was performed using either semithin cryosections of fixed tissue or Epon sections of the same tissue that was further subjected to antigen retrieval. Cryosections were prepared and stained exactly as described previously (4). For antigen retrieval, fixed tissue was embedded in Epon 812 as described previously (2). However, the tissue was not subjected to osmium tetroxide or uranyl acetate steps. After embedding, 0.5-μm sections were cut with glass knives and the sections mounted on glass slides. The sections were then etched by incubating for 5 min in a solution containing 10 ml of 100% methanol, 5 ml propylene oxide, and 2 g KOH. The slides were then washed 2× with 5 min in 100% methanol and once in Tris-buffered saline (TBS) (50 mM Tris, 100 mM NaCl, pH 7.4). For antigen retrieval a 10 mM Na citrate buffer (pH 6.0) was used. Briefly, 500 ml of buffer in a 2-liter glass beaker were heated to boiling in a microwave oven. The slides were then removed from the hot buffer and heated in the oven for 20 min at ~40°C power. After being cooled, the sections were washed 3× 5 min in TBS, quenched for 15 min in 0.5 M ammonium chloride, and washed again in TBS. After an additional 5-min wash in 1% SDS, the sections were stained as described above.

**Quantitative reverse transcriptase PCR.** Total RNA was isolated from transfected OKP cells with RNeasy mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Total RNA was treated with DNase (Ambion, Austin, TX) to digest and remove DNA. Reverse transcription reactions were performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to standard protocols. Quantitative PCR was carried out using the Power SYBR Green PCR Master Mix (Applied Biosystems), and the product was detected using a 7300 Real-time PCR System (Applied Biosystems). PCR primers were designed using Primer Express 3.0 (Applied Biosystems). The primers were the following: megalin forward primer 5′-TGGTTTGGTTTATTCCAAGTTTGGTAGA-3′ and reverse primer 5′-ATCAAACGAAGATGAAAAACAAGACAAAGA-3′; tyrosine aminotransferase (TAT) forward primer 5′-CTCAACAAAACGAGGTATTTTTTTT-3′ and reverse primer 5′-AGGAACTGGCGCCATTCCAGGCC-3′; cyclophilin (cyclo) forward primer 5′-TGCCCCACCCGTTATC-3′ and reverse primer 5′-TCTGTCTTTCTGTTCAGCACG-3′; and reverse primer 5′-TCTGTCTTTCTGTTCAGCACG-3′. Measuring the shed ectodomain APLP2. OKP cells, mPT, or ADAM10-transfected OKP cells were grown to confluence in 12-well culture plates. All cells were serum-starved overnight. For constitutive shedding, serum-free media were collected after 24 h, concentrated to 50 μl in a Amicon Ultra-4 (Millipore, Billerica, MA), and the entire sample was prepared for SDS-PAGE and Western blot analysis. To study PKC-activated shedding, cells were serum starved overnight, and in the morning the media were replaced with serum-free media containing either PMA (1 μM) or 4α-PMA (1 μM). After 4 h, the media were collected and prepared for SDS-PAGE as described above. For all studies the cells were solubilized in 200 μl of lysis buffer, and 50 μl were used for SDS-PAGE. Blots containing shed ectodomains were probed with an antibody to the NH2-terminus of APLP2. Blots containing cell lysates were probed sequentially with antibodies to the COOH-terminus of APLP2 and to α-tubulin (as loading control).

To assess the relative amount of ectodomain shed for each condition, the density of shed ectodomain, cellular APLP2 protein, and α-tubulin was measured using densitometry. The density of APLP2 cellular protein was first determined as a ratio to that of α-tubulin. The density of the shed ectodomains was then expressed relative to the cellular protein density.

**Knockdown of APLP2 expression in vitro and overexpression of APLP2 intracellular domain in the APLP2 knockdown cells.** Short hairpin RNA (shRNA) constructs based upon the mouse APLP2 gene were purchased from Open Biosystems (Huntsville, AL). The shRNA constructs (cat. no. RHS3979-9575613 and RHS4430-98895684) were designed to include a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop. The same hairpin sequence was cloned into the different lentiviral vectors (pKO.1 and pGIPZ) and sequence verified. The mouse APLP2 shRNA sequence used in this study has 100% identity to the opossum gene (GenBank accession no. 1240742). The APLP2 shRNAs were cotransfected with packaging plasmids pCMVΔ R8.91 and pMD.G into HEK293T cells using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The virus-containing supernatant was collected 30–72 h after transfection and filtered through a 0.45-μm filter and used for infection. OKP cells were infected for 6 h and allowed to recover for 24 h with fresh medium. The virus-infected cells were selected with puromycin. Cells were collected 72 h after infection and analyzed by qRT-PCR and immunoblot.

Plasmid representing the APLP2 intracellular domain (APLP2-ICD) was constructed based on the mouse APLP2 mRNA sequence. The 150-bp fragment of mouse APLP2 gene encoding the entire intracellular domain was PCR amplified using forward primer 5′-AGATTTGGATC

**AJP-Cell Physiol • VOL 300 • JUNE 2011 • www.ajpcell.org**
CAGATGTCTCATCATCATCATCATCAT GTGATGCTGAGGAA-
GAGGCAGTA-3 and reverse primer 5'-AGATACGTACG AGCGTA-
ATCTGGAACATCGTATGGGTAAATCTGCATCTGCTCCAG-
GTA-3' (His and HA tags incorporated into sense and antisense primers, respectively, are underlined). The PCR product was cloned into the pIREShyg3 vector (Clonetech, Mountain View, CA), and the plasmid sequence was verified by sequencing. For transient transfection, the OKP cells stably expressing APLP2 shRNA were grown in 12-well culture plates, and the cells were transfected with control (empty vector) or the APLP2-ICD plasmid. Cells were harvested 6 or 24 h after transfection, and NHE3 mRNA expression was measured using quantitative RT-PCR.

Statistical analysis. All results are expressed as arithmetic means ± SD. Statistical significance was assessed by t-test using SigmaStat 3.1 (Systat software, Chicago, IL). P < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

ADAM10 is expressed in the renal brush border. In an effort to assess its level of expression and to localize ADAM10 in the kidney, we combined indirect immunofluorescence microscopy on fixed kidney sections with immunoblotting studies using membrane fractions prepared from specific regions of mouse kidney. As shown in Fig. 2A, anti-ADAM10 antibody stained the brush border of proximal tubules. Supporting the specificity of this observation, when we performed immunoblotting studies, we found ADAM10 protein enriched in brush-border membrane vesicles compared with cortical whole homogenate or cortical microsomes. In fact, the pattern of expression for ADAM10 in the membrane fractions was the same as that seen for the brush-border NHE3 (3) (Fig. 2B).

Our finding that ADAM10 localizes to the brush border of proximal tubule seems to contradict previous studies in other cell types that describe ADAM10 on the basolateral membrane in some epithelia. For example, Maretzky and coworkers (24) demonstrated that ADAM10 activity sheds E-cadherin at the basolateral membrane and regulates cell-cell adhesion in the developing mouse (24). Consistent with this study, others have shown ADAM10 traffics to adherens junctions at the basolateral plasma membrane in MDCK cells (31). We speculated that polarized trafficking of ADAM10 is cell-type specific and thus examined its polarized expression in the cultured proximal tubule cell, OKP. We grew OKP cells stably expressing ADAM10 on permeable supports and selectively biotinylated either the apical or basolateral surfaces. Figure 2C shows that, while endogenous NHE3 (an apical protein) was biotinylated only on the apical plasma membrane and Na-K-ATPase only on the basolateral membrane, ADAM10 was biotinylated on both the apical and basolateral membranes. These data show transfected ADAM10 targets to both apical and basolateral

Fig. 2. Apical expression of ADAM10 in proximal tubule. A: fixed kidney sections were stained for ADAM10 using a polyclonal antibody as described in MATERIALS AND METHODS. Arrows indicate staining of the brush border of proximal tubules. Bar = 10 μm. B: Western blot analyses for ADAM10 and control proteins in membrane fractions from mouse kidney cortex. Equal amounts (25 μg) of whole homogenate, microsomes, or brush-border membrane vesicles (BBMV) separated by SDS-PAGE using 7.5% gels and prepared for Western blot analysis. Blots were probed with antibodies to ADAM10 (polyclonal), Na⁺/H⁺ exchanger 3 (NHE3), and the α-subunit of the Na-K-ATPase. C: polarized biotinylation of surface plasma membrane proteins in ADAM10-transfected OKP cells. Cells were biotinylated from either the apical or basolateral surfaces as indicated. Each condition is shown in triplicate. An aliquot (50 μl from 1 ml) of the total lysate is also shown. The resulting blots were probed with antibodies to ADAM10 (mAb 12F9), NHE3, and Na-K-ATPase (α-subunit). Note that the NHE3 and Na-K-ATPase shown here represent the endogenous proteins.
membrane domains in OKP cells. We should note that differences in polarized protein trafficking in proximal tubule cells compared with distal tubule cells have been well documented (6). The reported differences in polarized trafficking of ADAM10 with respect to OKP and MDCK cells likely reflect the presence of cell-type specific signals or adaptor proteins (13). We also acknowledge the possibility that overexpressing exogenous protein might overwhelm the OKP cell sorting machinery and cause mislocalization.

The fact that ADAM10 is expressed in the renal brush border combined with its well-described role as a sheddase and activator of RIP predicts a similar role in the proximal tubule. To test this hypothesis we studied the effect of ADAM10 activity on protein shedding and on the expression of specific proximal tubule proteins in OKP cells.

**ADAM10 activity affects expression of specific proteins in OKP cells.** To study the effects of ADAM10 activity, we stably overexpressed wild-type ADAM10 (ADAM10wtt) or an inactive mutant (E384A) ADAM10 (ADAM10dn) in OKP cells. The E384A mutation resides in the zinc-binding pocket (12), blocks zinc binding, and renders ADAM10 proteolytically inactive. Overexpression of the ADAM10 E384A mutant has been shown to suppress endogenous ADAM10 activity in HEK293 cells probably by a dominant/negative mechanism (20). Cells expressing the empty vector served as control. We studied multiple clonal cell lines expressing each plasmid with consistent results.

Expressed ADAM10 protein is shown in Fig. 1. ADAM10 is synthesized as a 90- to 95-kDa inactive proenzyme seen as band a. The proenzyme is posttranslationally modified by proteolytic removal of the prodomain yielding the active enzyme seen as the 75–80 kDa band b (Fig. 1). For details of ADAM processing see a review by Seals and Courtneidge (26). We consistently found when we overexpressed wild-type ADAM10 most was seen as the larger, inactive form. In contrast, expression of inactive ADAM10 E384A showed high levels of both the proenzyme and the cleaved protease. We speculate that this result reflects the cellular need to regulate levels of active protease, although details of the mechanisms of this regulation are largely unknown.

In studying the phenotype of these cells we noticed a correlation between ADAM10 activity and expression of specific apical membrane proteins (Fig. 3). More specifically, the levels of megalin and NHE3 expression increased in cells over expressing wild-type ADAM10 and decreased, relative to controls, when the inactive ADAM10 mutant was expressed (Fig. 3). We found no change in overall protein expression (Fig. 3A) or in expression of other specific proteins such as clathrin heavy chain (Fig. 3B), the α-subunit of the Na-K-ATPase, or α-tubulin (data not shown). These data suggest ADAM10 activity, possibly by activating RIP of an unknown membrane protein, can regulate NHE3 and megalin gene expression in OKP cells.

Although ADAM10 activity seems to regulate megalin and NHE3 expression, it seems unlikely that this results from RIP of megalin. First, we found no effect of ADAM10 activity on ectodomain shedding of megalin in vitro (data not shown). In addition, our previous study showed that the COOH-terminus of megalin produced by RIP inhibits expression of NHE3 and megalin (21). Therefore, the proteolytic activity that sheds the ectodomain of megalin appears to have the opposite effect (inhibits NHE3 and megalin) than that of ADAM10 activity, which increases expression of NHE3 and megalin. Together, these data allow us to speculate that ADAM10 participates in...
shedding and thus the activation of RIP of brush-border protein(s) other than megalin (21).

APLP2 is expressed in kidney and in OKP cells. In neurons, APLP2 is subjected to RIP and includes ADAM10-mediated ectodomain shedding (12). Moreover, Northern blotting studies show that APLP2 mRNA is highly expressed in adult mouse kidney (23). Although the localization of APLP2 has not been established, we postulate that APLP2 might represent
the ADAM10 substrate in proximal tubule. To test this, we analyzed APLP2 mRNA and protein expression in kidney and OKP cells. We easily detected the predicted PCR products of APLP2 in both kidney and OKP cells (data not shown). Although available antibodies have not been useful for immunocytochemical studies, we did perform immunoblotting studies using microsomes prepared from specific regions of kidney. As shown in Fig. 4A, APLP2 is found in all regions of the kidney. We also found APLP2 expression increased in brush-border membrane vesicles (BBMV) compared with cortical microsomes in a manner similar to that seen for ADAM10 and NHE3. These data show that APLP2 is expressed in cells of proximal tubule origin and suggest that APLP2 is present in the brush border.

RIP is initiated by PKC-regulated metalloprotease activity (7, 8, 12, 20). To determine whether APLP2 is subjected to RIP in cultured proximal tubule cells, we used the phorbol ester PMA to activate PKC and monitored ectodomain shedding of APLP2 in OKP cells and in a mouse proximal tubule cell called Pkd1floxtargeted to knockdown APLP2 expression in OKP cells and then to monitor the effect on megalin and NHE3 expression. As seen in Fig. 6, ectodomain shedding of APLP2 is increased in both proximal tubule cell lines following PMA-activation of PKC. These results are consistent with data derived from neuronal cells (12) and demonstrate that APLP2 is subjected to RIP in proximal tubule.

**ADAM10 sheds the ectodomain of APLP2.** We next sought to determine whether ADAM10 participates in ectodomain shedding of endogenous APLP2 in OKP cells. We examined constitutive shedding of APLP2 ectodomain in ADAM10-transfected OKP cells. As shown in Fig. 5, the ectodomain of APLP2 was increased in the supernatant of cells overexpressing wild-type ADAM10. In cells overexpressing inactive ADAM10 only a small amount of shed ectodomain of APLP2 was detected in the supernatant compared with controls. We consistently observed that the molecular weight of the small amount of APLP2 ectodomain shed in the inactive ADAM10 cells was greater than that in controls or in cells expressing wild-type ADAM10 (see Fig. 5). We also noted that the amount of total APLP2 protein detected in inactive ADAM10-transfected OKP cells was less than that found in the other cells. Combined, our data indicate that ADAM10 activity participates in shedding of the ectodomain of APLP2 and regulates APLP2 expression in cultured proximal tubule cells.

**Knockdown of APLP2 inhibits expression of NHE3 in OKP cells.** We speculated that the effect of increasing ADAM10 expression to increase both megalin and NHE3 abundance in OKP cells might be mediated through ectodomain shedding and thus the activation of RIP of APLP2. If this idea is true then knocking down APLP2 should inhibit megalin and NHE3 expression. To test this idea we carried out RNAi experiments designed to knockdown APLP2 expression in OKP cells and then to monitor the effect on megalin and NHE3 expression. As seen in Fig. 6 expressing APLP2 gene-specific shRNA allowed us to achieve >80% knockdown of APLP2 as evidenced by analysis of both protein and mRNA expression. Consistent with our model, we found NHE3 expression was significantly reduced (60–70%) in APLP2-deficient cells. In contrast, reduction of APLP2 expression appeared to have no effect on the expression of megalin, APP, or the Na-K-ATPase (α-subunit). In...
further support of this model, we carried out experiments designed to “rescue” NHE3 expression by transfecting the soluble intracellular domain of APLP2 into the cells in which endogenous APLP2 had been knocked down. As shown in Fig. 6C, we observed a significant recovery of NHE3 expression in these cells. These data support a model predicting that ADAM10 activity, possibly by its proteolytic action on the ectodomain of APLP2, can regulate NHE3 expression in vitro.

This study is the first to document expression of ADAM10 and APLP2 in the proximal tubule. Our data showing that the ectodomain of APLP2 is shed into the luminal supernatant of cultured proximal tubule cells by PKC-activated proteases combined with the fact that ADAM10 activity seems to modulate NHE3 and megalin expression in OKP cells supports a model linking RIP of receptors at the plasma membrane with regulation of specific genes. However, our understanding of the molecular mechanisms involved in RIP in the brush border is still very incomplete. Based on the data presented here, the effects of ADAM10 activity on protein expression are only partly explained by action on APLP2. Although we found a direct relationship among ADAM10 activity, cellular APLP2 levels, and NHE3 expression, knocking down APLP2 had no effect on megalin protein or mRNA levels. This finding implies that either the relationship between ADAM10 activity and megalin expression is mediated through cleavage of APLP2, is indirect, or that ADAM10 activity cleaves an additional, and as yet unidentified, receptor.

In addition to ADAM10, our study provides evidence that other metalloproteases involved in ectodomain shedding are also expressed in the renal brush border. Since ADAM10 does not appear to shed the ectodomain of megalin in OKP cells, the protease(s) that mediates megalin shedding remains unknown. Our data also suggest that APLP2 may be shed by additional proteases since blocking ADAM10 activity yielded a small amount of higher molecular weight APLP2 ectodomain. Certainly, studies of RIP of other receptors, including APP and APLP2 in the central nervous system, implicate other ADAMs as important sheddases (8, 12, 20). Further studies will be required that will identify the metalloproteases and match them with specific substrates in proximal tubule.

Our studies of ADAM10 and APLP2 combined with our previous studies of megalin (21, 32), identify elements of what appear to be multiple signaling pathways in proximal tubule that use RIP. The importance of RIP for proximal tubule function is also highlighted by the fact that high levels of γ-secretase are found in the endocytic pathway of the renal brush border (32). Modulating levels of ADAM10 and APLP2 have profound effects on NHE3 and megalin expression in vitro and suggest these proteins represent elements of signaling pathways capable of regulating brush-border Na⁺/H⁺ exchange and protein absorption in vivo. Our studies presented here, combined with knowledge of RIP in general, allow us to propose a model shown in Fig. 7. It is important to note that the function of APLP2 is not well defined making it difficult to understand how RIP of APLP2 is activated and how its processing might contribute to known tubular processes. Further studies, especially loss of function experiments in mammalian kidney, will be required to completely characterize these protease-dependent pathways and to understand how they function in the cell biology of the proximal tubule.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES