Activation of the epithelial sodium channel by the metalloprotease meprin β subunit

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Abbreviations: ENaC, epithelial sodium channel; NT, amino terminus; CT, carboxy terminus; MDCK, madin-darby canine kidney; HBE’s, human bronchial epithelial; IP, immunoprecipitate; IB, immunoblot

The Epithelial Na⁺ Channel (ENaC) is an apical heteromeric channel that mediates Na⁺ entry into epithelial cells from the luminal cell surface. ENaC is activated by proteases that interact with the channel during biosynthesis or at the extracellular surface. Meprins are cell surface and secreted metalloproteinases of the kidney and intestine. We discovered by affinity chromatography that meprins bind γ-ENaC, a subunit of the ENaC hetero-oligomer. The physical interaction involves NH₂-terminal cytoplasmic residues 37–54 of γ-ENaC, containing a critical gating domain immediately before the first transmembrane domain, and the cytoplasmic COOH-terminal tail of meprin β (residues 679–704). This potential association was confirmed by co-expression and co-immunoprecipitation studies. Functional assays revealed that meprins stimulate ENaC expressed exogenously in Xenopus oocytes and endogenously in epithelial cells. Co-expression of ENaC subunits and meprin β or α/β in Xenopus oocytes increased amiloride-sensitive Na⁺ currents approximately two-fold. This increase was blocked by preincubation with an inhibitor of meprin activity, actinonin. The meprin-mediated increase in ENaC currents in oocytes and epithelial cell monolayers required meprin β, but not the α subunit. Meprin β promoted cleavage of α and γ-ENaC subunits at sites close to the second transmembrane domain in the extracellular domain of each channel subunit. Thus, meprin β regulates the activity of ENaC in a metalloprotease-dependent fashion.

The Epithelial Na⁺ Channel (ENaC) mediates the rate-limiting step in conductive Na⁺ absorption across epithelial-lined organs including airways, kidney and intestine. ENaC is a heteromeric channel consisting of α, β and γ subunits. Each subunit contains two transmembrane domains with an amino-terminal (N-terminal) cytosolic domain, a large extracellular domain and a cytosolic carboxy-terminal (C-terminal). In the kidney and colon, ENaC helps maintain total body salt and volume homeostasis, while in the lung ENaC regulates the depth and composition of the airway surface liquid to maintain efficient mucociliary clearance. The functional importance of ENaC activity is highlighted by the fact that human mutations in ENaC and abnormalities in the regulation of ENaC, are involved in the pathogenesis of salt-sensitive hypertension, pseudo-hypoaldosteronism and cystic fibrosis.

ENaC is regulated through signaling pathways that link channel localization and activity to distinct tissue-specific stimuli. The stimuli and signaling pathways that regulate ENaC are not completely understood, but all converge on cellular processes that determine the number of active channels at the apical membrane, the single channel conductance and/or the open probability of the channels on the membrane. Recent data indicate that proteases, such as channel activating proteases (CAPs) and furin, regulate ENaC activity, however the mechanisms of activation are poorly understood. The first protease shown to increase ENaC activity was Xenopus channel-activating protein (xCAP1), which was identified in a functional screen for novel regulators of ENaC. Mouse CAPs increase amiloride-sensitive Na⁺ currents when co-expressed with ENaC in the oocyte system. However, it is unclear whether CAPs cleave ENaC directly or function in an unidentified pathway to increase Na⁺ conductance via ENaC. There is some evidence that the α and γ-ENaC subunits are substrates of the preproconvertase furin. Although furin cycles from the trans-Golgi network to the cell surface, and is known to cleave substrates in multiple cellular compartments, furin is proposed to cleave ENaC as the channel traverses the secretory pathway. On the other hand, Caldwell demonstrated that extracellular proteases markedly increase the open probability
of quiescent ENaC already present at the plasma membrane. Thus, proteases may affect ENaC activity by modulating the gating of channels trafficking in intracellular compartments or at the plasma membrane.

In a proteomic screen of mouse kidney to find ENaC-interacting proteins we identified the meprin α subunit among proteins that bind to the cytoplasmic N-terminus of γ-ENaC. Meprin α is one of two evolutionarily-related proteins (α and β) that form homo- and hetero-oligomeric complexes of meprin metalloproteinase (α2, β2 or αβ; Fig. 1). Meprins are abundantly expressed in epithelial cells of the kidney and intestine, and also found in skin, leukocytes and lung. They are multidomain zinc-containing proteinases that are members of the ‘astacin family’ and ‘metzincin superfamily’ of metalloproteinases. The meprin α and β subunits both have proteolytic activity, but are distinct in their substrate specificities. For example, meprin α has a preference for small or hydrophobic residues flanking the cleavage site, whereas meprin β has a preference for negatively charged residues. The meprin subunits are also processed differentially during biosynthesis. Thus, both are synthesized as type 1 membrane spanning proteins, however, the meprin α protein, but not β, is proteolytically cleaved in the endoplasmic reticulum consequently loosing its transmembrane and cytoplasmic domain. Thus, meprin β remains a transmembrane protein in the secretory pathway and at the cell surface, whereas the meprin α subunit is extracellular. The meprin subunits form disulfide-linked homo- and heterodimers. Thus, meprin β dimers (called meprin B) and meprin α/β dimers (heteromeric meprin A) are membrane-bound proteins, whereas meprin α homodimers (that tend to self-associate into larger oligomers of over 10 subunits) are secreted: meprin α is only at the cell surface when it is covalently (through disulfide bridges) associated with meprin β.

The aim of the studies herein was to determine: (1) molecular details on how ENaC and meprin subunits interact and (2) whether meprins affect the function of ENaC through proteolytic activity.

Results

Interactions of ENaC and meprin subunits. The short (70–90 residues) cytosolic N-terminal tails of ENaC subunits contain a highly conserved domain essential for channel opening and multiple positively charged residues that have been implicated in regulation of the channel by lipid binding. We reasoned that proteins capable of interacting with ENaC N-termini could affect ENaC activity. Therefore, affinity chromatography and mass spectrometry were used to identify proteins that associate with the N-terminus of γ-ENaC. A biotinylated peptide encompassing residues 1–54 of rat γ-ENaC was immobilized on streptavidin beads and incubated with solubilized membrane proteins prepared from C57BL/6 mouse kidney. Several proteins were identified as γ-ENaC-interacting proteins using MALDI-TOF and electrospray tandem MS/MS including the α subunit of the metalloprotease meprin A. Immunoprecipitation techniques using biotinylated peptides derived from α, β and γ-ENaC with antibodies directed against meprin α were used to further characterize the ENaC-meprin interaction. Meprin α robustly associated with the N-terminus of γ-ENaC but did not associate with the N-terminus of α-ENaC (Fig. 2A). The meprin α migration pattern on a denatured SDS-gel (~100 kDa) corresponded to that of the glycosylated latent precursor (14). Minor association of meprin α with the N-terminus of
latent and active highly glycosylated forms of meprin (βγ). However, western blots confirmed that meprin α would not be present in membrane fractions without meprin β. 

Figure 2. Meprins interact with the γ-ENaC N-terminal (NT) peptide. Affinity purification assays were done using the following biotinylated peptides; α-ENaC(42–193), β-ENaC(149) and γ-ENaC(126). Kidney membrane preparations (500 μg) from C57BL/6 mice were incubated with each peptide (5 μg) and streptavidin beads for 2 hr at 4°C. The beads were subsequently washed three times with BB150 buffer and twice with PBS buffer. Laemmli sample buffer was added, the samples were boiled for 7 min and elutes were subjected to SDS-PAGE analysis on 4–20% gradient acrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (PVDF) and western blot analysis was performed using a monoclonal antibody to meprin α (A) and a polyclonal antibody to meprin β (B). Representative immunoblots are shown (n = 3).

Figure 3. Increase in amiloride-sensitive Na⁺ currents by co-expression of αβγ-ENaC with meprin α and β subunits in Xenopus oocytes. Wild-type α, β and γ-ENaC cRNA and meprin α and β cRNA (0.5 ng) subunits were injected into oocytes. Twenty-four hr post-injection, two-electrode voltage clamp assays were conducted. Currents measured in the presence and absence of amiloride (10 μM), while clamping the membrane voltage to -100 mV, were digitized and recorded. Batches of oocytes were extracted from 3–5 different frogs. Results are expressed as the means ± S.E. Statistical significance was determined using an unpaired Student’s t test. ENaC + Meprin α/β, *Significant p < 0.0001.

β-ENaC was evident in some experiments but it was not reproducible. Meprin β was not detected by mass spectrometry in the initial precipitation assays, even though meprin α would not be present in membrane fractions without meprin β. However, western blots confirmed that meprin β was also present in γ-ENaC precipitates (Fig. 2B). The antibody to meprin β identified two bands with molecular masses equivalent to the latent and active highly glycosylated forms of meprin β (~110 and ~75 kDa, respectively). These results demonstrate that meprin α and β subunits associate, directly or indirectly, with the N-terminus of γ-ENaC in vitro.

To determine whether meprins can affect the function of ENaC, amiloride-sensitive Na⁺ currents were measured in Xenopus oocytes expressing rat ENaC subunits alone or co-injected with human meprin α and β (Fig. 3). A significant increase in amiloride-sensitive Na⁺ currents was observed when ENaC was co-expressed with meprin α and β (8,413 ± 529 nA (N = 33)) compared to control oocytes expressing ENaC alone (4,259 ± 390 nA (N = 32)). Taken together, our data indicate that hetero-oligomeric meprin can associate with ENaC and can modulate ENaC function in a heterologous system.

For membrane-associated forms of meprins, meprin β spans the plasma membrane, with its C-terminus located within the cell. To determine if the cytosolic N-terminus of γ-ENaC binds the C-terminus of meprin β, peptides derived from the meprin α and β cytosolic domains were incubated with membranes prepared from MDCK cells expressing HA-tagged γ-ENaC together with untagged α and β-ENaC subunits. MDCK cells express exogenous amiloride-sensitive Na⁺ channels on the cell surface and provide an excellent model system to study the regulation of ENaC by specific protein-protein interactions. The C-terminal peptide from the nascent meprin α subunit is cleaved from the subunit during biosynthesis in the endoplasmic reticulum, therefore there is no meprin α cytoplasmic tail in the cell. The biotinylated C-terminus of meprin β (Ct), but not meprin α (αCt), was found to bind γ-ENaC in vitro (Fig. 4A). These data indicate that the ENaC-meprin interaction involves the N-terminus of γ-ENaC and the C-terminus of meprin β. Thus meprin A (containing meprin α and β subunits) or meprin B (consisting exclusively of meprin β subunits) could associate with ENaC in cells.

To test the prediction that full-length γ-ENaC and meprin β interact, C-terminal HA epitope tagged γ-ENaC was co-expressed with wild type α and β-ENaC and meprin β in Xenopus oocytes. HA antibodies were used to immunoprecipitate γ-ENaC/C-HA from solubilized membranes and antisera directed against meprin β subunits were used to probe the immunoprecipitates for meprin. The data showed that γ-ENaC associates with meprin β, but not with the IgG controls, in oocytes (Fig. 4B). These results are consistent with previous findings using MDCK-ENaC cells and meprin C-termini peptides (Fig. 4A). Taken together these data indicate that the γ-ENaC N-terminus and meprin β C-terminus can interact to form an ENaC-meprin multimeric complex.

To validate this ENaC-meprin protein interaction in a more physiological context, we next asked if γ-ENaC and meprin β could interact in mouse kidney tissue. γ-ENaC immunoprecipitates from wild-type and meprin β knockout mice kidney lysates were probed with a meprin β antibody. As expected, meprin β was found to coimmunoprecipitate with γ-ENaC subunits only from wild-type but not from β knockout kidney lysates (Fig. 4C). Thus γ-ENaC and the metalloprotease meprin β interact in the nephron where both proteins are known to be expressed abundantly.24,25
The N-terminus of γ-ENaC is highly conserved (Fig. 5A) and contains residues that are important for channel gating.26 Biotinylated peptides corresponding to amino acids 1–18, 19–36 and 37–54 of rat γ-ENaC were immobilized on streptavidin beads and incubated with membranes prepared from mouse kidney. After extensive washing, bound proteins were separated by SDS-PAGE and analyzed by western blot with meprin α or meprin β antibodies. Endogenous meprin did not bind γ-ENaCγ1-18, γ-ENaCγ19-36 or a peptide from the C-terminus of γ-ENaC640-650 used as an additional specificity control (Fig. 5B). In contrast, robust association of meprin α and β to γ-ENaCγ37-54 as well as the γ-ENaC1-54 peptide was detected. The association of meprin with the γ-ENaC1-54 peptide is stronger than that with a shorter γ-ENaCγ37-54 peptide, suggesting that other regions of the γ-ENaC N-terminus contribute to this interaction. Thus, meprin subunits associate with γ-ENaC between Asnγ26 and the beginning of the first transmembrane domain. Interestingly, this segment contains an absolutely conserved gating domain centered at His-Glyγ39-40,26 as well as multiple arginines that have been implicated in channel gating and in lipid binding.26,27

Functional analysis of meprin’s action. To determine whether the catalytic activity of hetero-oligomeric meprin is required to increase ENaC currents in Xenopus oocytes, meprin activity was inhibited with actinonin, a selective inhibitor that interacts with zinc in the active site of both meprin subunits (IC50 values for mouse meprin A and B; 100 and 400 nM, respectively; reviewed in ref. 19). As before, amiloride-sensitive Na+ currents were significantly increased by ~1.8 fold in vehicle-treated oocytes expressing ENaC + meprin α/β (Fig. 6A). In contrast, amiloride-sensitive Na+ currents in actinonin-treated oocytes co-expressing ENaC + meprin did not differ from Na+ currents recorded from oocytes expressing ENaC subunits alone (110.6 ± 13.06% of basal (n = 44)). Because actinonin had no effect when applied to oocytes expressing ENaC alone, it is concluded that catalytic activity of meprin is involved in the stimulation of ENaC-mediated Na+ currents in Xenopus oocytes.
To further investigate the role of each meprin subunit, changes in amiloride-sensitive Na⁺ currents from oocytes co-expressing ENaC with meprin α or β, were measured. When expressed alone, meprin α had no appreciable effect on amiloride-sensitive Na⁺ currents (meprin α: 4,154 ± 261 nA (N = 41) vs. control: 3,644 ± 274 nA (N = 42)). In contrast, meprin β significantly increased amiloride-sensitive current (7,027 ± 518 nA; (N = 44)) (Fig. 6B). The meprin β-induced increase in amiloride-sensitive Na⁺ currents was near the full expected increase if compared with ENaC currents recorded from oocytes expressing ENaC and meprin α + β subunits (Fig. 3). The data presented in Figure 6 indicate that the catalytic activity of meprin is required for meprin’s effect on ENaC and that meprin β is responsible for activating ENaC in the Xenopus oocyte expression system.

Cleavage of ENaC by meprin β. When Xenopus oocytes co-expressing ENaC + meprin (α + β) were pre-incubated with 1µM actinonin, meprin failed to increase ENaC-mediated Na⁺ currents, suggesting that the proteolysis of ENaC or an ENaC-associated regulatory protein is involved in the activation process (Fig. 6A). To distinguish between these two possibilities, we asked whether the application of soluble meprin α or meprin β was able to cleave ENaC subunits at the cell surface of intact Xenopus oocytes. Soluble meprins were activated with trypsin (1:20 µg/ml, trypsin:meprin) and excess soybean trypsin inhibitor (STI) was used to inactivate trypsin prior to the experiments. Activated meprin α or β was applied to oocytes expressing a combination of tagged and untagged ENaC subunits; proteolytic cleavage of individual ENaC subunits was assessed by SDS-PAGE and western blot analysis using monoclonal antibodies against the N-terminal HA or C-terminal V5 epitope tags. When oocytes expressing ENaC were treated with activated soluble homomeric meprin A (α/α) no differences in the migration pattern of any full-length ENaC subunit were detected (data not shown). These data indicate that meprin α does not cleave ENaC and are consistent with our previous results demonstrating that meprin α alone cannot activates ENaC-mediated currents in Xenopus oocytes (Fig. 6B). In contrast, proteolysis of α and γ, but not β-ENaC, was consistently observed when activated soluble meprin β (β/β) was added to intact oocytes (Fig. 7). In oocytes expressing untagged β and γ-ENaC together with double tagged α-ENaC C/HA + N/V5, fragments of ~80 and ~17 kDa were observed with antibodies directed against HA or V5 (Fig. 7A and B), respectively. The proteolysis of α-ENaC was abolished when 1 µM actinonin was included in the bath solution (Fig. 7C). These data indicate that meprin β (or a protease activated by the addition of meprin β) cleaves the extracellular domain of α-ENaC near the second transmembrane domain (TMII). γ-ENaC fragments of ~76 and ~17 kDa were detected when oocytes expressing γ-ENaC containing N-terminal V5 and C-terminal HA tags were treated with soluble activated meprin β (Fig. 7), indicating that γ-ENaC was also cleaved near the second transmembrane domain. Thus α and γ-ENaC are substrates for meprin β.

Meprin regulates ENaC in human bronchial epithelial cell monolayers. To extend our studies from oocytes to epithelial cells, the effects of soluble recombinant meprin on ENaC currents were tested in16HBE14o- cells, a cultured human airway epithelial cell line endogenously expressing α-ENaC. These cells were stably transfected with cDNAs encoding human β and γ-ENaC to generate a cell line with 5–10 μAmps of amiloride-sensitive Na⁺ currents, when studied in Ussing chambers. Meprin expression is not detected in native respiratory epithelium. Soluble recombinant meprin β (10 µg/ml) applied in the luminal bath at steady state increased amiloride-sensitive current by 1.0 ± 0.2 µA (N = 6) (Fig. 8). This response required meprin β activity because it was blocked by actinonin (1 µM) and because inactive meprin β was ineffective. This effect was specific to meprin β because active meprin α did not affect ENaC currents in this human bronchial epithelial cell line (data not shown).
shown). These data show that the proteolytic activity of soluble meprin β stimulates Na⁺ transport in an epithelial monolayer.

**Discussion**

The studies herein demonstrate that the metalloprotease meprin β and γ-ENaC associate directly through cytoplasmic domains. In addition, meprin β is capable of cleaving the α and γ-ENaC subunits in extracellular portions of these proteins and activating the ion channel when the channel proteins and protease are co-expressed in Xenopus oocytes. Channel proteins were activated by membrane-bound meprin β (the homodimer meprin B or the hetero-oligomer meprin αβ) or by activated soluble meprin B added extracellularly to ENaC expressing cells. This activation was inhibited by the meprin inhibitor actinonin, indicating that meprin catalytic activity mediates the effect. Indeed, actinonin-sensitive cleavage of α and γ-ENaC was observed when activated soluble meprin B (β/β) (but not homeric meprin A (α/α)) was applied to ENaC-expressing cells. Thus, the meprin β protease is capable of regulating ENaC activity in cells when the channel and protease are both expressed as membrane proteins or in channel expressing cells that are exposed to extracellular soluble meprin B (β/β). These findings are particularly relevant to kidney and intestinal tissues where ion channels and meprins are highly expressed on apical membranes and where ENaC functions critically in salt and volume homeostasis. 24, 25

Because the meprins are also secreted or shed into the lumen of these organs, 31 they are capable of cleaving and activating channel proteins distal to their site of synthesis (e.g., in the kidney distal tubules) where there is a high concentration of ion channel proteins. In addition, meprins are expressed in leukocytes under certain conditions (e.g., in human and experimental models of inflammatory bowel diseases) and thus have the potential to affect cell surface proteins in inflammation. 32

Several proteases have now been linked to the biosynthesis and activity of ENaC, 4-9, 33, 34 but definitive mechanisms for the actions of proteases underlying these observations are not yet agreed upon. Most studies on protease regulation of ENaC have detected stimulation of ENaC by co-expressed proteases (CAPs, furin). In these studies, proteases are envisioned acting on ENaC either during biosynthesis (e.g., furin) or at the cell surface (e.g., CAPs). Recently, exogenously applied soluble proteases, including trypsin and elastase, have been shown to acutely increase the open probability of ENaC. 13, 28 Overall, these observations are consistent with a model in which proteases can (1) act during ENaC biosynthesis to increase the activity of channels prior to insertion into the membrane; or (2) act in the extracellular space on “near silent” ENaC channels that have been inserted to the membrane without the action of a protease during biosynthesis. Although some studies clearly demonstrate that ENaC is a
protease substrate,8-10 proteases could regulate ENaC by indirect mechanisms that do not include cleavage of channel subunits.14
Our study is the first to show a biochemical interaction between an ENaC subunit and a protease that stimulates ENaC activity and the first to show a metalloproteinase affects activity.

The biochemical association of meprins with ENaC and cleavage and regulation of ENaC by meprins add to an increasingly complex model of regulation of ENaC by proteases. The essential feature of the model is that ENaC responds to proteases with increased activity. However, major unresolved questions include the number and identities of ENaC-regulating proteases in different tissues, the cellular vs extracellular site of protease-ENaC interaction and the substrates targeted by proteases that regulate ENaC. In addition, proteases commonly exist as inactive zymogens and the conversion to active protease provides an additional layer of complexity to ENaC regulation. For example, it was recently proposed that CAP1-3 could form part of a proteolytic layer of complexity to ENaC regulation. For example, it was recently proposed that CAP1-3 could form part of a proteolytic cascade at the cell surface that regulated ENaC.7 Previous studies in mouse kidney,36 and the present studies indicate that meprin β is only partially activated in mouse kidney, as demonstrated by the immunoblots of Figure 2B, implying that activation of this protease could also be part of a regulated cascade of events at the cell surface.

We observed actinonin-sensitive cleavage of α and γ-ENaC by meprin β. Within the region of α and γ-ENaC where meprin β cleaves there are several glutamate and aspartate residues and meprin β prefers to cleave peptide bonds containing these residues. Interestingly, a recently constructed database that predicts substrates for meprin β coded for by the mouse and human genomes identified several other ion channel proteins as excellent substrates, including for example, the δ subunit of the amiloride-sensitive sodium channel, amiloride-sensitive cation channel 4 isoform 1; acid-sensing ion channel 4, voltage-gated type I–IV sodium channel and voltage-dependent α1F and α1H calcium channel, (http://vbc.med.monash.edu.au/~sboyd/Meprins/). Thus, ENaC may be a model for a group of proteins that interact, are cleaved and functionally altered by meprins at the cell surface.

The cytoplasmic tail of meprin β is also known to interact with OS-9, a ubiquitously expressed cytoplasmic protein that assembles efficiently with the transfected δ and γ subunits to form functional channels.28 Rat ENaC subunits containing C-terminal HA tags (ENaC-C/HA) were generated by PCR and cloned into pcDNA3.1(+) using AscI and EcoRI restriction sites. These cDNAs were used to transiently transfect MDCK-ENaC cells using Lipofectamine 2000 (Invitrogen) and transfected cells were used for biochemical assays. For biochemical analyses of ENaC subunits proteolysis, cDNAs encoding rat β and γ-ENaC with N-terminal HA and C-terminal V5 epitope tags (N/HA + C/V5) were generated by PCR and cloned into pCR-BluntII-TOPO (Invitrogen), linearized (HindIII) and in vitro transcribed using T7 RNA polymerase. A PolyA tail was added after transcription (Ambion). Full-length cDNAs encoding human meprin α and β were excised from pcDNA3.1(+) and cloned into pcDNA3.1(+) and cloned into pSDE using BamHI/NotI or XbaI/NotI, respectively. The sequence of all plasmids was verified at the University of North Carolina sequencing facility.

Affinity precipitation of ENaC-interacting proteins. Membranes from C57BL/6 mouse kidney were prepared by ultracentrifugation at 100,000x g for 30 min in 50 mM Tris pH 7.5, 150 mM NaCl containing 2 μg/ml leupeptin, 16 μg/ml benzimidne, 2 μg/ml PMSF (phenylmethanesulfonyl fluoride) and 2μg/ml trypsin inhibitor.42 Membrane proteins were solubilized in buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA and 10 mM EGTA and protease inhibitors. Soluble proteins were collected by centrifugation at 16,100 g for 10 min. Supernatant fractions were pre-cleared by incubation with streptavidin beads (Sigma St. Louis, MO) and then incubated for 2 hr at 4°C with a rat γ-ENaC1,54 peptide covalently linked to α C-terminal biotin (Genemed synthesis Inc., San Francisco, CA). γ-ENaC-interacting proteins were collected by incubation with streptavidin-agarose beads for 1 hr at 4°C and, after extensive washing, bound proteins were analyzed by SDS-PAGE and visualized by silver staining (Invitrogen). Visible bands were excised and samples analyzed by MALDI/TOF-MS (Bruker Instruments Co., Bremen, Germany) and Nano-ESI-MS/MS on an API QSTAR-Pulsar (QSTAR, Applied Biosystems Div., Perkin-Elmer Corp., Foster City, CA) as described.43 Alternatively small-scale binding assays, using 500 μg of solubilized membrane proteins from MDCK-ENaC or mouse kidney fractions and 5 μg biotinylated peptide, were analyzed by western blot.
Affinity precipitation of meprin subunit interacting proteins. The COOH-terminal peptides of mouse meprin α (amino acids 750–760) and β (amino acids 679–704) were synthesized and covalently linked to biotin at the C-terminus (Genemed synthesis Inc., San Francisco, CA). Kidney membrane preparations were incubated with the peptides and streptavidin beads as described for ENaC peptides.

Functional studies of Na+ channels in Xenopus oocytes. Xenopus oocytes were harvested as described previously44 and maintained in modified Barth’s solution at 18°C. cRNAs encoding ENaC peptides were incubated with the peptides and streptavidin beads as described previously46 on a mixed background (C57BL/6 x 129 x 1Sv) and then backcrossed for 13 generations on a C57BL/6 strain; C57BL/6 mice were used as the WT controls.

Treatment of intact oocytes with activated recombinant meprin proteins. Various combinations of tagged and untagged rat ENaC subunits were expressed in Xenopus oocytes for twenty-four hr. Batches of twenty oocytes were incubated with 10 μg/ml of rat meprin α or β at 25°C with gentle rocking for 30–120 min in 20 mM Tris, 150 mM NaCl, pH 7.5. Oocytes were transferred to the same buffer containing 50 mM NaF, 10 mM β-glycerophosphate, 1 mM EDTA, 2 μg/ml PMSF (phenylmethanesulfonyl fluoride), 2 μg/ml leupeptin, 16 μg/ml benzimidine, 2 μg/ml trypsin inhibitor and 10 μM actinomycin. Yolk-depleted lysates and membrane fractions were prepared as described above and proteins were fractionated by SDS-PAGE and analyzed by western blot using monoclonal antibodies directed against HA or V5 epitope tags.44

Ussing chamber experiments. 16HBE14o-βγ cells were grown in Minimal Essential Medium with 10 μM amiloride and seeded onto Costar Snapwell culture inserts (Corning) for study, as reported previously. After 6–10 days when R >200 Ohm.cm and PD >1 mV (lumen negative), cells were induced for twenty-four hr with 1 mM Na-butyrate and 10−6 M dexamethasone. Induced preparations were mounted in Ussing chambers in Krebs-Bicarbonate-Ringer Buffer (KBR); basal I, K and equivalent PD were recorded continuously for 30 min. In these preparations, I, represents active Na+ absorption, a process for which ENaC activity at the apical membrane is limiting. At 30 min active rat meprin α or β (10 μg/ml) were applied to the luminal bath in the presence or absence of 1 μM actinonin (Sigma St. Louis, MO); I, was recorded for 45 min and 10 μM amiloride was applied. Some tissues were treated with 2 μg/ml trypsin (Sigma St. Louis, MO) before amiloride to assess the maximum proteolytic responsiveness.

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