Activation of Epithelial Sodium Channels by Mouse Channel Activating Proteases (mCAP) Expressed in Xenopus Oocytes Requires Catalytic Activity of mCAP3 and mCAP2 but not mCAP1

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Mouse channel activating proteases 1, 2, and 3 (mCAP1, mCAP2, and mCAP3) were described recently as regulators of the epithelial sodium channel (ENaC). The mCAP are membrane-bound serine proteases that are synthesized as inactive proenzymes. To mature into active proteases, they undergo intramolecular cleavage by auto- and/or heterocatalytic processing. Specific antibodies against each mCAP were developed to distinguish between proenzyme and active protease by Western blot analysis. Various point mutations were introduced in the catalytic or protein–protein interacting domains of mCAP and wild-type and mutant enzymes were expressed in the Xenopus oocyte expression system to test for ability to activate ENaC. In mCAP3, an intact catalytic triad was necessary for activation of ENaC but not for intramolecular cleavage of the protease. This suggests a heterocatalytic mechanism. Mutating the catalytic triad of mCAP2 not only abolished ENaC activation completely but also impeded cleavage of the protease. Processing of mCAP2 therefore seems to be autocatalytic. Furthermore, mutations in conserved residues of mCAP2 located in two protein–protein interacting domains significantly modulated ENaC activation. Surprisingly, mCAP1 catalytically inactive mutants were still able to fully activate ENaC, and no evidence of mCAP1 intramolecular cleavage was seen. The presence of an intact glycosylphosphatidylinositol anchor, however, was required. It is concluded that auto- and heterocatalytic requirements are specific for each CAP and that endogenous partners are a necessity for activation of ENaC by mCAP in the Xenopus oocyte expression system.


Serine proteases (1) belong to a large gene family (approximately 2% of identified genes in vertebrate) (2). The catalytic triad of the three amino acids (aa) HDS (or DHS) characterizes the catalytic domain. Two classes of membrane-bound serine proteases have been delineated: (1) Type II transmembrane serine proteases, a rapidly expanding family of serine proteases (3), and (2) glycosylphosphatidylinositol (GPI)-anchored proteins (e.g., channel activating protease 1 [CAP1]/prostasin), which are inserted in the external leaflet of the membrane bilayer (4,5). Type II transmembrane serine proteases share common structural features: A short N-terminal cytoplasmic tail; a single transmembrane domain, a linker region that consists of protein interaction domains (e.g., complement factor IR–urchin embryonic growth factor–bone morphogenetic protein [CUB], group A scavenger receptor [SRCR], LDL receptor A [LDLRA]), suggesting association with other proteins at the cell surface; and a C-terminal serine protease domain that faces the extracellular milieu (3).
A tight control of serine protease activity in the intracellular compartments and extracellular milieu obviously is necessary. Three levels of control have been described: (1) By serine protease inhibitors (14), (2) by transcriptional/translational/post-translational regulation of the enzymes (15–17), and (3) by proteolytic conversion from an inactive proenzyme into an active enzyme (18). Serine proteases are translated as inactive proenzymes that need to be activated by intramolecular cleavage at a specific consensus site situated between two cysteines to form a disulfide bridge and link the pro- and catalytic domains. This process can be auto- or heterocatalytic (3). The latter case necessitates catalysis by another serine protease, therefore suggesting a serine protease cascade.

For the Xenopus CAP1 (xCAP1), approximately 10% of the ENaC activation remained in catalytic mutants (5), but the very small amplitude of the noncatalytic activation raised the question of its physiologic relevance. Our aim, therefore, was to define for each mCAP whether activation processing is auto- and/or heterocatalytic and to examine whether catalytic activity is necessary for ENaC activation. We generated specific antibodies against each mCAP to allow the detection of the full-length proenzyme and the cleaved, active enzyme. Using the oocyte expression system, we correlated the enzyme activation assessed by Western blotting with the ability of the enzyme to increase ENaC activity, measured as the amiloride-sensitive sodium current.

Materials and Methods

cDNA Constructs

Cloning of mCAP1, mCAP2, and mCAP3 cDNA was described by Vuagniaux et al. (6). Point mutations were introduced into the mCAP1, mCAP2, and mCAP3 cDNA using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. For functional expression studies in Xenopus laevis oocytes, wild-type (wt) and mutant protease cDNA were subcloned into pSD5 expression vector and in vitro transcribed (19).

Electrophysiologic Measurements in Xenopus Oocytes

Expression studies were performed in stage V/VI oocytes that were isolated from Xenopus laevis (Noordhoek, South Africa), as described previously (20). A total of 0.25 ng of each cRNA encoding the rat α-, β-, and γ-ENaC (rENaC) subunits in the presence or absence of 4 ng of mCAP1, 2 ng of mCAP2, or 2 ng of mCAP3 wt or mutant cRNA in a total volume of 100 nl was injected into oocytes. Oocytes were incubated in modified Barth saline solution. Twenty-four hours after cRNA injection, electrophysiologic measurements were performed using the two-electrode voltage clamp technique. The amiloride-sensitive current (INa) was measured in the presence of 120 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl (pH 7.6), 1 mM PMSF, protease inhibitor cocktail [LAP], and 10 mM methionine) after electrophysiologic measurements. Total protein extracts were obtained basically as described previously (20). The oocytes were lysed by triturating with a Pasteur pipette 10 times followed by vortexing 3 × 15 s, and yolk granules were removed by centrifugation at 12,000 rpm for 10 min. In vitro translated mCAP1, mCAP2, and mCAP3 was obtained by using the TNT SP6 Quick Coupled Transcription/Translation System (Promega, Madison, WI) according to the manufacturer's instructions. Protein extract corresponding to 0.25 oocyte or 1 μl of in vitro translated protein was loaded in each lane for SDS-PAGE using 10% acrylamide gels, and proteins were transferred by tank-blotting to a polyvinylidene difluoride membrane (Millipore, Velpetswil, Switzerland). Primary antibodies were affinity-purified from sera of rabbits that were immunized with one of the following glutathion-S-transferase (GST)-fused epitopes: mCAP1-2 (aa 286 to 312) (12), mCAP2-1 (aa 1 to 28), or mCAP3-1 (aa 1 to 54). Preimmune and immune sera were used at a dilution of 1:1,000, and affinity-purified antibodies were used at 1:200 (mCAP1 and mCAP2) or 1:100 (mCAP3) dilutions. The antibodies were preabsorbed with 1 μg of the respective GST-peptide fusion protein as controls. A Multiscreen Apparatus (Bio-Rad, Hercules, CA) was used for antibody characterization. The secondary antibody was horseradish peroxidase–linked anti-rabbit IgG (Amersham, Munich, Germany), and SuperSignal West Dura Extended Duration chemiluminescence (Pierce, Rockford, IL) was used for detection.

Protein Size, Structure, and Cleavage Predictions

Protein domains were predicted using the Motif Scan tool at http://myhits.isb-sib.ch/cgi-bin/motif_scan. On the basis of aa sequence, theoretical protein and fragment sizes that did not comprise glycosylations were calculated using the PeptideMass tool on the ExPASy server (http://www.expasy.org/tools/peptide-mass.html).

Statistical Analyses

All electrophysiology results are reported as means ± SEM. Differences within the series were determined by Kruskal-Wallis test, and differences between individual conditions were determined by Dunn multicomparison. P < 0.05 was considered to be significant; n represents the number of measurements performed.

Results

Characterization of mCAP3 Antibody

On the basis of the aa sequence of the mCAP3, the full-length protein was predicted to be 95 kD, plus up to six glycosylations. Two cleavage sites were also predicted within the protein: Between R614 and V615 for the conversion from inactive proenzyme to active protease and an N-terminal cleavage product proposed by Cho et al. (21), at G149. To be able to detect and distinguish from among these three forms of the protein, we raised an antibody against a peptide at the N-terminus of mCAP3 (mCAP3-1; Figure 1A). This antibody was expected to recognize the full-length protease, the activated form (predicted as 68 kD plus up to five glycosylations), and the N-terminal product proposed by Cho et al. (theoretical size 17 kD plus one possible glycosylation site); theoretically predicted sizes, without N-glycosylation, are indicated in Figure 1A.

We tested the preimmune and immune sera, the affinity-purified antibody, and preabsorption with the GST-mCAP3-1 fusion protein against a total protein extract from oocytes that received co-injected of all three ENaC subunits and wt mCAP3. Probing with both the preimmune and immune sera gave a
smear of bands (Figure 1B). After affinity purification, only a faint band at approximately 95 kD and a fuzzy lower molecular band at approximately 22 kD were detected, neither of which had been present in the preimmune serum (Figure 1B). These likely correspond to the proenzyme and N-terminal cleavage forms of the protein. No band corresponding to the cleaved, activated form of the protease was seen in this experiment. Preabsorbing the antibody with the GST–mCAP3-1 fusion protein left no bands.

We also tested the glycosylation state of the protein by performing deglycosylation with PNGase F. This led to a shift in protein size from the 22-kD band to a sharper band at approximately 17 kD. It is curious that the 95-kD band that was interpreted as full-length protein did not shift in size upon deglycosylation. An in vitro translated protein run in parallel on the same gel confirmed a full-length, nonglycosylated size of approximately 95 kD (Figure 1C).

Functional Analysis of mCAP3 in Xenopus Oocytes

To determine the importance of catalytic activity in mCAP3, we mutated the serine predicted as part of the catalytic triad to an alanine (S805A; Figure 1A). When mCAP3 cRNA was co-expressed with rENaC α, β, and γ subunits in the Xenopus laevis oocyte expression system, we observed a 10-fold increase in INa (Figure 1D, top). As expected, the S805A mutation yielded a protein with no effect on ENaC activity (Figure 1D, top).

Biochemical Analysis of mCAP3 in Xenopus Oocytes

Western blots on total protein that was extracted from oocytes that were used for the electrophysiologic measurements were performed using the affinity-purified antibody (Figure 1D, bottom). Routinely, the band that corresponded to full-length mCAP3 at approximately 95 kD was faint or undetectable. Instead, mCAP3 showed up as an approximately 25-kD cleaved form. There was no change in band sizes in the S805A mutant, although the approximately 25-kD band always seemed weaker in the mutant compared with the wt samples. Two nonspecific bands, also detected in the negative (noninjected) controls, were sometimes seen at approximately 80 and approximately 90 kD (Figure 1D, bottom).
Characterization of the mCAP2 Antibody

mCAP2 was predicted to be a 47-kD protein with up to two glycosylations added. It was predicted to be converted from inactive proenzyme to active protease by proteolytic cleavage between R202 and V203.

An antibody was raised against an N-terminal epitope (mCAP2-1). This antibody was expected to recognize the proenzyme as well as a cleavage product of 22 kD plus two possible glycosylations, corresponding to the active enzyme (theoretical predictions are depicted in Figure 2A).

An antibody test was performed on protein extracts from ENaC and mCAP2 co-injected oocytes. No bands were detected with the preimmune serum, whereas the anti-mCAP2 immune serum showed three bands (Figure 2B). After affinity purification, two bands at approximately 50 and approximately 30 kD remained, and these could well correspond to the glycosylated full-length proenzyme and the cleaved, activated form of the protein as depicted in Figure 2A. Both of these bands completely disappeared upon preabsorption with the GST–mCAP2-1 fusion protein.

To test the glycosylation state of mCAP2 and compare this with the expected sizes, we again performed a deglycosylation of an oocyte extract and compared this with in vitro transcribed protein. We indeed saw that the band that was interpreted as full-length proenzyme shifted from 50 kD to the theoretically predicted 47 kD, exactly at the same size as the nonglycosylated in vitro translated protein. The double band at 30 to 35 kD that was interpreted as the cleaved, activated form of the protease shifted to a single band of 22 kD upon deglycosylation. This corresponds well with the prediction and suggests the presence of two glycosylation states (Figure 2C).

Functional Analysis of mCAP2 in Xenopus Oocytes

To test the role of the catalytic domain of mCAP2 on ENaC activation, we introduced a mutation in the serine that was predicted to be part of the catalytic triad (S385A; Figure 2A). Co-expression of the wt protease resulted in an eight-fold increase in \( I_{\text{Na}} \) whereas the S385A mutation completely abolished ENaC activation by the protease (Figure 2D, top).

Seven mutations have been identified in TMPRSS3 in patients with nonsyndromic autosomal recessive deafness (DFNB8/10) (22,23). An alignment between mTMPRSS3 and mCAP2 revealed that all except one of the aa that were found to be mutated in TMPRSS3 and to confer deafness were identical between the two proteases (the remaining aa was a conservative substitution). Because of this high conservation, we
decided to introduce the corresponding mutations into mCAP2 to determine whether they had similar effects on functionality. The mutants D86G, R103W, C181L, R202L, W237C, P388C, and Y391R (Figure 2A) correspond to the TMPRSS3 mutants D103G, R109W, C194F, R216L, W251C, P404L, and C407R (22,23).

The D86G mutant is present in the LDLRA domain, and, in contrast to TMPRSS3, this mutant showed unaltered activation of ENaC as compared with wt mCAP2. The next two mutants, R103W and C181F, were introduced in the SRCR domain. These two mutants showed a reduced ability to activate ENaC, by approximately four-fold as opposed to the eight-fold for wt mCAP2. The remaining R202L, W237C, P388C, and Y391R all were in the catalytic domain. Except for Y391R, these mutations yielded complete abolishment of ENaC activation. Y391R gave only a partial, statistically insignificant three-fold activation of the ENaC-mediated currents (Figure 2D, top).

**Biochemical Analysis of mCAP2 in Xenopus Oocytes**

Using the affinity-purified mCAP2 antibody, we performed Western blots on protein extracts from the oocytes that were used in the electrophysiology experiments (Figure 2D, bottom). In wt as well as all mutants, mCAP2 showed up as an approximately 50-kD band that corresponded to the proenzyme. The D86G mutant (which had fully activated the amiloride-sensitive currents) consistently showed a fainter band than the other mutants. In the wt sample, we furthermore detected two approximately 30-kD bands that likely corresponded to the cleaved form, possibly with different glycosylation levels. These bands were not detected in any of the mutants, except for R103W (one of the partially activating SRCR domain mutants). A band of slightly different size was detected in the W237C mutant.

**Characterization of the mCAP1 Antibody**

For mCAP1, we expected a full-length proenzyme of 34 kD with two potential glycosylations after removal of the N-terminal signal peptide. Proteolytic cleavage between R44 and I45 was predicted to transform the proenzyme into the catalytically active protease. We used the C-terminal antibody previously described by Planes et al. (12) using a peptide epitope (mCAP1-2) located between the catalytic domain and the hydrophobic C-terminus that contained the GPI anchor site (Figure 3A). This antibody therefore was expected to recognize both the full-length and the activated, cleaved forms (predicted at 32 kD plus two glycosylations), as well as the S314stop truncation (predicted as 31 and 29 kD plus two glycosylations, depending on cleavage or not), as indicated in Figure 3A.

A characterization of the antibody against proteins that were purified from oocytes that received co-injections of ENaC and mCAP1 was performed. The immune serum gave several bands, one of which was detected faintly even in the preimmune serum. A single approximately 40-kD band remained after affinity purification, and this band could be preabsorbed completely with the GST–mCAP1-2 fusion protein (Figure 3B). To determine whether this band corresponded to the glycosylated form of the proenzyme or the active, cleaved protease, we performed a deglycosylation and compared this with *in vitro* translated protein (Figure 3C). The deglycosylated protein had a molecular weight slightly lower than the *in vitro* translated, which includes the signal peptide. This specific band likely corresponds to a glycosylated form of the full-length size of the protein minus the signal peptide, as proposed in Figure 3A.

**Function of mCAP1 in Xenopus Oocytes**

We then studied the importance of the catalytic triad of mCAP1 for ENaC activation. Co-expression of mCAP1 wt cRNA with ENaC led to an almost 10-fold significant (*P < 0.01*) increase in *I*_Na_, compared with oocytes that received injections of rENaC alone (Figure 3C, top). We initially mutated the predicted serine of the catalytic triad (S238A). Unexpectedly, this mutation had no effect on ENaC activation. Mutation of the predicted catalytic histidine (H85A) and aspartate (D134A) gave the same result. Even when we mutated all three catalytic triad aa at the same time (HDS), we observed an activation of ENaC that was not different from the wt (Figure 3D, top).

We further examined the function of the potential GPI anchor by using a truncated mCAP1 mutant with a premature stop codon immediately before the C-terminal hydrophobic domain (S314stop). The S314stop truncation prevents the GPI anchor addition and generates a secreted protein (5). This mutant did not modulate the channel activity (Figure 3D, top).

**Biochemical Analysis of mCAP1 in Xenopus Oocytes**

Western blotting on total protein extracts from the oocytes that were used in the electrophysiologic experiments for mCAP1 were performed with the purified antibody. These showed that the wt mCAP1 migrated with an apparent molecular mass of approximately 40 kD, corresponding to the calculated size of the full-length protease minus signal peptide (predicted to be 34 kD) plus two possible N-glycosylations (Figure 3D, bottom). There was no evidence for the presence of a cleaved, activated form, which would be predicted to have a mass of approximately 32 kD (minus signal peptide) plus two possible N-glycosylations. The S238A, H85A, D134A, and HDS mutants exhibited the same apparent molecular mass as wt mCAP1. The mCAP1 truncated mutant (S314stop) showed a fainter band at the lower molecular weight of approximately 35 kD (Figure 3D, bottom). This corresponds well to the predicted size of 31 kD for the truncated proenzyme minus signal peptide, plus the two possible N-glycosylation sites and therefore verified the loss of the GPI anchor.

**Discussion**

The necessity of tightly regulating ENaC becomes evident in light of its implicit role in Na⁺ homeostasis and thereby extracellular volume and BP control (11). The evidence suggesting regulation of ENaC activity through proteolytic cleavage is by now persuasive (4–8,10,12,22–29). In this study we investigated some of the requirements for mCAP3, mCAP2, and mCAP1 activation.
Antibody Characterizations

Our findings of a fuzzy approximately 22-kD mCAP3 band that shifted to approximately 17 kD upon deglycosylation corresponds well to the findings of Cho et al. (21). They used both C- and N-terminal antibodies as well as an antibody that recognizes the full protein in a study of mouse epithin (m/H11005 CAP3) and found only the full-length protein of 95 kD and an N-terminally cleaved form. That we did not find the band at the exact same size as Cho et al. (18 kD) may be explained easily by differences in glycosylations between the expression systems (mammalian cell lines versus Xenopus oocytes). As did Cho et al., we found a full-length band at 95 kD, but this band is of the theoretically expected size before addition of glycosylations, exactly at the same size as the in vitro translated protein, and does not shift size upon deglycosylation. It may be nonspecific in our case, because it sometimes occurred even in noninjected oocytes (data not shown), or it may be nonglycosylated endogenous CAP3. We did attempt to raise and use an additional C-terminal antibody, which should be able to recognize the activational cleavage. Unfortunately, we were unsuccessful in making this second antibody work.

For CAP2, our findings corresponded very well to theoretical predictions; both the bands that were interpreted as full-length and cleaved forms shifted to exactly the predicted sizes upon deglycosylation, and the deglycosylated full-length band was at exactly the same size as the in vitro translated protein.

For mCAP1, we used a previously published antibody (12). Planès et al. (12) used two different mCAP1 antibodies on Xenopus oocytes as well as various mouse lung preparations. Similar to our findings, they routinely saw a band of 36 to 38 kD.
in extracts of mCAP1-expressing *Xenopus* oocytes, which upon deglycosylation shifted to 32 to 34 kD. We were curious about whether this band corresponded to full-length proenzyme (34 kD without glycosylation) or the cleaved, proteolytically active form (32 kD). Because the size difference of the two bands is only 2 kD, it can be difficult to distinguish between the two just by comparing with the size marker. However, when comparing with full-length, nonglycosylated *in vitro* translated mCAP1, we saw that the deglycosylated mCAP1 was of only slightly lower molecular weight than the *in vitro* translated protein, corresponding well to the 3-kD size difference expected from signal peptide sequence. We therefore conclude that the specific mCAP1 band corresponds to uncleaved proenzyme.

**Requirement of Catalytic Activity for mCAP3 and mCAP2 Activation of ENaC**

The loss of ability to activate ENaC that we detected upon introduction of the catalytic S805A and S385A mutations in mCAP3 and mCAP2, respectively, indicates that these proteases do indeed work their effects on ENaC through cleavage, either directly on one or more ENaC subunits or indirectly.

**Auto- versus Heterocatalytic Cleavage in mCAP2 and mCAP3**

Although the mCAP2 S385A and mCAP3 S805A mutants seemed to have similar effects on ENaC activation, there were clear differences in the processing of the proteases themselves. The finding that mCAP2 was present both as uncleaved proenzyme and as two lower molecular weight bands that corresponded to the activated protease whereas the catalytically dead mutant S385A was found only in the proenzyme form suggests that the protease is auto-activated; *i.e.*, the wt protease is activated by cleaving itself. This function evidently would become idle upon abolishing the catalytic activity. Alternatively, this mutation could have resulted in a conformational change that would impede the activation cleavage. The two lower molecular bands found in the wt could be two different glycosylation states, which is confirmed by the deglycosylation by PNGase F, resulting in a single band of approximately 22 kD.

In contrast, we detected both wt and S805A mCAP3 mainly as a short cleavage product of approximately 22 kD, corresponding to the N-terminal cleavage proposed by Cho *et al.* (21). They presented evidence that this cleavage is necessary for secretion of mCAP3 (epithin) but that the cleaved form also is found on the cell surface. Whether the cleavage that is needed for activation of mCAP3 takes place in the wt *versus* S805A mutant cannot be ascertained, because we did not detect the band that corresponded to this cleavage. However, it seems that the mCAP3 N-terminal cleavage is heterocatalytic; *i.e.*, the work of a separate protease. In relation to ENaC activation, this question also might be of less importance than that of mCAP1 and mCAP2, because we have indications that mCAP3 is normally expressed in the basolateral membrane of cortical collecting duct cells (Andreasen, unpublished data).

**Requirement for Protein–Protein Interacting Domains for mCAP2 versus TMPRSS3: mCAP2 Activates ENaC by Similar but not Identical Mechanisms**

Seven mutations were identified in TMPRSS3, a homolog of mCAP2 (TMPRSS4) (38% identity, 53% homology at the protein level), in patients with nonsyndromic autosomal recessive deafness (DFNB8/10) (22,23). In the *Xenopus* oocyte expression system, TMPRSS3 was cleaved and able to increase ENaC-mediated currents. In contrast, the mutants that cause deafness all failed to undergo proteolytic cleavage and activate ENaC. These data suggested that TMPRSS3 is autocatalytically activated and that ENaC that is expressed in the inner ear is a potential substrate (22,23). We introduced the corresponding mutations in mCAP2 to determine whether the effects were comparable.

The main difference from TMPRSS3 was lack of effect of the D86G mutation in the LDLRA domain on ENaC activation. The reduced amount of expressed protein suggests that degradation of the mutated protease was faster than that of the wt protease. Indeed, lower molecular bands of approximately 10 kD were seen in some blots (data not shown), corresponding to degradation. The mutant then must activate ENaC before it is degraded or is perhaps even more efficient than wt, arriving at the same activation level with fewer molecules present.

The reduced but not abolished activation of ENaC by R103W and C181L, both in the SRCR domain, also differed from TMPRSS3. These intermediary currents suggest that protein–protein interaction involving the SRCR domain is of some importance for proteolytic function but not vital. It is interesting that R103W is the only mutant in which we detected the cleaved, activated form on Western blots. This corresponds well with its being at least partially active.

The complete lack of cleavage of the R202L mutant, in the predicted activation cleavage site, was not surprising. The same goes for the W237C and P388C mutants that were situated close to the histidine and serine of the catalytic triad. It is possible that these mutations cause conformational changes in the catalytic domain. The decreased activation of ENaC by the final mutant, Y391R close to the C-terminus of the catalytic domain, was NS. This might not be surprising, because this was the one targeted aa that was not completely conserved between human TMPRSS3 and mCAP2, indicating a less important role. The low molecular weight band that appeared for W237C mutant, as the only catalytic triad mutant, was not at exactly the same size as was seen in the wt. This could be a degradation product.

**Catalytic versus Noncatalytic Requirement: An Unexpected Mechanism for mCAP1**

When we introduced the S238A, H85A, and D134A mutations in mCAP1, alone or in concert, we expected to abolish or at least reduce ENaC activation. To our surprise, we found that these mutants were still fully able to activate ENaC. On the protein level, we detected only one band in wt as well as catalytic mutants that corresponded to the full-length protease after deletion of the signal peptide. We did not detect the cleaved, activated form, which may explain why there does not seem to be an activation of ENaC depending on the catalytic
activity. Vallet et al. (5) previously found that the xCAP1 catalytic mutant S223A retained slight ability to activate co-expressed ENaC, whereas Vuagniaux found that catalytically dead human prostasin mutants fully stimulated ENaC (Vuagniaux, unpublished data). The lack of an absolute requirement of catalytic activity indicates the existence of an alternative mechanism or mechanisms by which CAP1/prostasin activates ENaC. The differential efficacy of catalytically dead xCAP1 and mCAP1/human prostasin may reflect the relative importance of such mechanisms. One of the factors that control serine protease activity is pH. Yu et al. (30) previously showed that human prostanin preferred basic pH for proteolysis. Similarly, mCAP1 might have little catalytic activity when expressed in Xenopus oocytes at pH 7.4 (approximately 12% of the maximal activity for prostasin [30]) and could be catalytically inactive in the acidic environment of the cortical collecting duct. This raises the question of whether mCAP1 normally acts as a protease at all, a notion supported by lack of effect of catalytic inactivation.

Because we were not able to demonstrate any importance of the catalytic activity of mCAP1 on ENaC inactivation, we propose a direct protein–protein interaction between mCAP1/prostasin and an ENaC subunit or an indirect interaction between mCAP1/prostasin and a channel-associated extracellular protein. One possible interaction mechanism is through the catalytic domain. It is plausible that mCAP1 interacts with protease inhibitors, thereby squelching their activity on other proteases that are needed in the catalytic pathway that leads to ENaC activation. It also is possible that mCAP1 could interact with proteins with inhibitor-like domains. It is notable that an extracellular domain matrix protein, mec-9, which contains protease inhibitor–like domains, is involved in the mechanotransduction signaling cascade in Caenorhabditis elegans, which implicates an ENaC-like channel (31,32). Furthermore, noncatalytic biologic function in serine proteases has been illustrated by the hepatocyte growth factor and macrophage stimulatory protein hormones, both of which have lost their catalytic function but interact with transmembrane tyrosine kinase receptors to induce intercellular signaling cascades (reviewed in [33]).

The stop codon immediately before the GPI anchor sequence (S314stop), theoretically resulting in a secreted form of the protease, was introduced to test whether the membrane association was important for ENaC activation, which seemed to be the case. The shift in molecular weight of the S314stop compared with wt detected in Western blots confirms the truncation of the protein, whereas the decreased intensity suggests that we detected only the cytosolic fraction. Our findings correlate well with those of Vallet et al. (5), who demonstrated that removal of the GPI anchor led to secretion of xCAP1 to the medium in Xenopus oocytes. A band of approximately 23 kD did appear in all samples, but we interpreted this as a degradation product rather than the active form of the protease, because the molecular weight was too low and there was no shift in size in the stop mutant.

Under salt restriction and high plasma aldosterone, a low molecular cleavage form (70 kD) of the γ ENaC subunit has been observed, and in vivo proteolysis has been proposed (34). More recently, it was proposed that direct cleavage of α and γ (but not β) ENaC subunits by furin, a ubiquitously expressed membrane-bound serine protease, plays a critical role in activating ENaC that is expressed in heterologous systems (27,28). We speculate that the channel-activating proteases could act in a cascade to activate ENaC, whereby one protease activates the next, finally leading to cleavage and activation of ENaC. The data presented here would be consistent with such a hypothesis. As discussed above, the lack of direct catalytic activity for mCAP1 would concur with this protease’s acting as a competitor for protease inhibitors, thereby allowing catalysis by other proteases. One such possible protease is mCAP2, which like mCAP1 is inhibited by aprotinin (6). Our data would agree with mCAP2’s being activated by autocatalysis. mCAP3, in contrast, is not a target for aprotinin inhibition, and our data suggest cleavage by a separate protease, possibly mCAP2. We surely have not found all players, but our results may help in the extensive work that remains to identify the substrates and cleavage sites for each of the involved proteases.

Conclusion

From the data presented here, we conclude that mCAP1 seems to be involved in ENaC activation via a mechanism that does not involve catalytic activity of the protease. In contrast, the mechanisms that lead to ENaC activation via mCAP2 and mCAP3 do require proteolytic activity, either directly on an ENaC subunit or indirectly on a protein or proteins that are involved in a cascade that leads to ENaC activation. To establish the physiologic relevance of these observations, it will be necessary to study CAP and ENaC activation in vivo in native cells (e.g., the mCCD cell line [35]) and even more important in vivo by conditional gene targeting of various mCAP and furin in the kidney, colon, lung, and skin.

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