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# Cell-Surface Expression of the Channel Activating Protease xCAP-1 Is Required for Activation of ENaC in the *Xenopus* Oocyte

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*Abstract.* Sodium balance, extracellular fluid volume, and ultimately BP are maintained by precise regulation of the activity of the epithelial sodium channel (ENaC). Using a functional complementation assay in the *Xenopus laevis* oocyte expression system, a channel-activating protease (CAP-1) that increases ENaC activity two to threefold in the *Xenopus* oocyte expression system is here identified. External application of trypsin mimics the effect of *Xenopus* CAP-1 (xCAP-1) on ENaC activity, which can be blocked by aprotinin, a serine protease inhibitor, suggesting the existence of a novel extracellular pathway for controlling ENaC activity. Sequence analysis predicts that CAP-1 is a secreted and/or glycosyl-phosphatidyl-inositol (GPI)–anchored protein. The aim of the present study was to determine whether cell-surface expression of xCAP-1 is required for ENaC activation. By site-directed mutagenesis of xCAP-1, the importance of the catalytic site, N-glycosylation, and the GPI anchor of xCAP-1 on ENaC activity were analyzed. Glycosylation or catalytic activity is not required for cell-surface expression of xCAP-1, whereas the deletion of the GPI anchor consensus motif at the C-terminus of xCAP-1 (G305Stop) abolishes cell-surface expression and ENaC activation. G305Stop-mutated xCAP-1 is recovered as a secreted protein in the external medium. A catalytic mutant of xCAP-1 significantly decreased ENaC activation but did not fully abolish the effect of xCAP-1. The data indicate the critical role of the GPI anchor in ENaC activation and suggest that catalytic and noncatalytic mechanisms are involved.

The epithelial amiloride-sensitive sodium channel (ENaC) is expressed at the apical membrane of the epithelial cells of aldosterone-sensitive organs, such as the kidney, the colon, and the sweat and salivary glands (1). In the kidney, ENaC mediates the aldosterone-dependent sodium reabsorption in the distal part of the nephron, thereby playing a major role in the control of sodium balance and BP. We have recently identified a *Xenopus* serine protease (xCAP-1) that increases ENaC activity when coexpressed with ENaC $\alpha$, $\beta$, and $\gamma$ subunits in the *Xenopus* oocyte (2,3). The analysis of xCAP-1 primary sequence indicates that this protein is a serine protease with its characteristic histidine-aspartate-serine catalytic triad (HDS). The sequence analysis predicts one asparagine-linked glycosylation site and a glycosyl-phosphatidyl-inositol (GPI) consensus motif, indicating that the protein may be anchored at the outer leaflet of the membrane bilayer (4). This observation suggests that xCAP-1 activates ENaC by its extracellular serine protease activity at the plasma membrane. In addition, patch clamp experiments have shown that extracellular application of trypsin inside the pipette can mimic xCAP-1 activation while trypsin outside the pipette is unable to activate ENaC (5). Overall, the data suggest that xCAP-1 is colocalized with ENaC channel protein and that the serine protease acts directly on ENaC by its catalytic activity and/or by protein-protein interaction with the extracellular domain of ENaC or an associated protein.

The aim of this study was to elucidate structural features of xCAP-1 that are important for ENaC activation in the *Xenopus* oocyte system. Site-directed mutagenesis of the catalytic triad, the N-glycosylation site, and the GPI-anchored motif demonstrate the critical role of the GPI anchor to observe ENaC activation at the plasma membrane.

## Materials and Methods

### *cDNA Constructs and cRNA Synthesis*

Cloning of xCAP-1 into pSPORT-1 vector has been performed as described (3). For this study, xCAP-1 cDNA was subcloned into pSD5 expression vector by using *Not*I-*Sal*I sites. The Flag epitope (DYKDDDDK) was inserted between serine 196 and aspartic acid 197 into xCAP-1 cDNA to generate a flagged xCAP-1 reporter construct referred to as wild type (WT) xCAP-1. Preliminary experiments showed that flagged xCAP-1 produced the same ENaC activation when compared with nonflagged wild type constructs. Three mutants were generated by introducing a single amino acid replacement into flagged xCAP-1 sequence: (*1*) a catalytic mutant (S223A) in which the codon for the predicted active serine residue (ACC) of the

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catalytic triad (S223) was mutated to an alanine codon (GCC); (*2*) a glycosylation mutant (N288Q) in which the asparagine codon (AAC) for the predicted N-glycosylation site (N288) was mutated into a glutamine codon (CAG); and (*3*) a GPI-anchor mutant (G305Stop) in which the C-terminal hydrophobic tail of xCAP-1 was deleted by introducing a STOP codon (TGA) instead of the glycin (GGA) G305 residue. The introduction of the Flag epitope or the point mutations was done by PCR. Accuracy of each construct was confirmed by DNA sequencing. Plasmids were linearized by using *Fsp*I restriction enzyme, and complementary cRNAs were transcribed by using SP6 RNA polymerase. *Xenopus* ENaC (ENaC) subunits were cloned into pSD5 expression vector, as described previously (6). Plasmids were linearized by using *Afl*III enzyme for $\alpha$ and $\gamma$ ENaC subunits and *Bgl*II for $\beta$ subunit.

### *Expression in Xenopus Oocytes*

Stage V to VI oocytes of *Xenopus laevis* were injected with 100 nl containing 1 ng of each $\alpha$, $\beta$ and $\gamma$ ENaC cRNA subunit (7), together with 2 ng of wild type or mutant xCAP-1 cRNA. Oocytes were either kept in modified Barth saline (MBS: 88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO3, 0.41 mM CaCl2, 0.33 mM CaNO3, 0.82 mM $MgSO_4$, 10 mM HEPES, pH 7.2) for electrophysiologic measurements and immunoprecipitation experiments or in low-sodium MBS (10 mM NaCl, 90 mM NMDG-Cl, 5 mM KCl, 0.41 mM CaCl2, 0.33 mM CaNO3, 0.82 mM $MgSO_4$) for binding experiments. To inhibit N-glycosylation, oocytes were preinjected with 100 nl of tunicamycin (40 ng/$\mu$l in 4% DMSO) 24 h before cRNA injection. The other inhibitors were coinjected with cRNA by dissolving cRNA in 100 $\mu$M castanospermine, 100 $\mu$M 1-deoxymannojirimycin, or 100 $\mu$M swainsonine. Each oocyte was injected with 100 nl inhibitor containing cRNA.

### *Electrophysiologic Measurements*

Standard electrophysiologic measurements were performed 16 to 20 h after injection as described previously. The amiloride-sensitive sodium current was determined by using two-electrode voltage-clamp as described previously (7). Macroscopic amiloride-sensitive sodium current (Iam) is defined as the difference between the inward current obtained in the absence and in the presence (5 $\mu$M) of amiloride in the bath at a holding potential of $-100$ mV. For each oocyte, Iam was measured before and after perfusion of 2 $\mu$g/ml trypsin during 2 min in the bath. Results are reported as mean $\pm$ SE.

### *Cell-Surface Expression of xCAP-1*

Anti-Flag (M2Ab) monoclonal antibody (Eastman Kodak, Rochester, NY) was iodinated by using the IODO-BEADS Iodination reagent (Pierce, Rockford, IL) and carrier-free Na (125)I (Amersham Pharmacia, Uppsala, Sweden), according to the Pierce protocol. The cell-surface expression of wild type or mutant xCAP-1 was determined by specific binding of iodinated M2Ab antibody 16 to 20 h after the cRNA injection to oocytes expressing flagged xCAP-1 constructs as described previously (8). Briefly, oocytes were incubated with iodinated anti-Flag antibody, washed eight times with low-sodium MBS, and then transferred individually into tubes for $\gamma$ counting.

### *Immunoprecipitation Experiments*

Injected oocytes were incubated 16 h in MBS containing 1 mCi/ml [$^{35}$S]methionine (NEN Life Science Products, Boston, MA). Microsomal membranes were prepared as described (9), and xCAP-1 flagged protein was immunoprecipitated by using the anti-Flag M2Ab antibody under nondenaturing conditions. Microsomal proteins solubilized in a triton washing buffer (TWB: 20 mM Tris [pH 7.6], 100 mM NaCl, 1% TX-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin, antipain, pepstatin A [Sigma, St. Louis, MO] [LAP]) were incubated for 16 h at 4°C with M2Ab antibody, and immunoprecipitates were recovered with protein G-agarose–conjugated beads and washed three times with TWB and twice with TWB devoid of TX-100. Deglycosylation of the proteins was performed by incubating the beads for 1 h at 37°C in 100 $\mu$l of 50 mM sodium acetate containing either 1000 units of endoglycosidase H or 500 units N-glycosidase F. Immunoprecipitates were separated on a 5 to 13% gradient sodium dodecyl sulfate–polyacrylamide gel, and the gel was exposed for 4 to 7 d on Kodak XS film.

### *Immunohistochemical Detection of xCAP-1 in Xenopus Oocytes*

Forty-eight hours after cRNA injection, oocytes were fixed with 3% paraformaldehyde in PBS for 4 h at 4°C. Tyramide signal amplification kit (TSA-Direct, NEN) was used for immunofluorescence according to the manufacturer's instructions. Cryosections of the oocytes (6-$\mu$m thick) were placed on chrome alum gelatin-coated glass slides and incubated with a 1:100 dilution of an anti-Flag M2Ab antibody for 1 h at room temperature. After repeated washings, sections were incubated for 1 h at room temperature with a 1:100 dilution of a horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Pharmacia) and subsequently revealed by incubation for 10 min with a 1:75 dilution of the provided fluorescein-tyramide conjugates. To stain F actin, a 1:50 dilution of rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR) was added to the amplification buffer. Sections were then washed, mounted in DAKO-glycergel (DAKO, Glostrup, Denmark) containing 2.5% 1,4-diazabicyclo(2,2,2)octane to retard fading, and were studied by epifluorescence with a Polyvar microscope (Reichert-Jung, Vienna, Austria). Micrographs were acquired with a charge-coupled device camera (Visicam 1280, Visitron Systems, Puchheim, Germany) and processed by Image-Pro Plus software (version 3.0; Media Cybernetics, Silver Spring, MD).

## Results

Primary sequence of xCAP-1 predicts the following important features (Figure 1). The N-terminus contains a signal sequence (SS) formed by 17 hydrophobic residues. Three amino acids expected to form the classical catalytic triad (HDS) of serine proteases are observed: histidine 70 (H70), aspartate 119 (D119), and serine 223 (S223). A putative N-glycosylation site is present at position 288 (N288). The C-terminus contains a 23-residue long hydrophobic tail, which is consistent with a GPI-anchored signal (4). The aim of this study was to determine if any of these structural features of xCAP-1 are important for ENaC activation. For this purpose, the catalytic activity of xCAP-1 was inactivated by mutating the serine 223 of the catalytic triad into an alanine (S223A mutant); a nonglycosylated mutant was generated by mutating the asparagine 288 site into a glutamine (N288Q mutant); the C-terminal hydrophobic tail was deleted by mutating the glycine 305 into a STOP codon to prevent the GPI-anchoring of the protein (G305Stop mutant). Each mutant was expressed in *Xenopus* oocytes and analyzed for its glycosylation status, its subcellular localization, and its effect on ENaC activity.

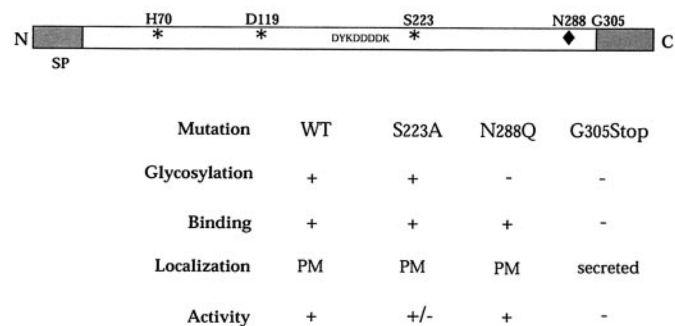


*Figure 1.* Linear model and functional properties of *Xenopus* channel-activating protease (xCAP-1) mutants and schematic structure of xCAP-1 protein. The amino acids of the catalytic triad (*), the N-glycosylation site (◆), and the position of the Flag insertion (DYK-DDDDK) are indicated. Gray boxes represent hydrophobic sequences; SS, signal sequence; PM, plasma membrane. Results of the experiments performed in this article are summarized for each mutant. Activity means the ability of xCAP-1 protein to increase ENaC activity by coexpression in *Xenopus* oocytes.

## *xCAP-1 Is N-Glycosylated*

When expressed in oocytes, wild type xCAP-1 (WT) migrates with an apparent Mr of 40 kD. Digestion with endoglycosidase H (EndoH) or with N-glycosidase F (PNGase or EndoF) reduced the apparent Mr to 38 kD (Figure 2). Using a pulse-chase experiment (16 h pulse and 24 h chase), we did not observe the appearance of an EndoH-resistant, fully-glycosylated, form of xCAP-1 (not shown). These results indicate that xCAP-1 expresses only one N-glycosylation site, which is core-glycosylated. The S223A mutant exhibited the same apparent molecular mass as WT protein and is sensitive to EndoH and PNGase. Thus, the S223A mutant is N-glycosylated, and a mutation in the catalytic triad does not interfere with N-glycosylation of the protein. The N288Q mutant migrates with an apparent molecular mass of 38 kD and was not sensitive to EndoH or PNGase (EndoF) (Figure 2), indicating that this mutant is not N-glycosylated, identifying asparagine 288 residue as the unique N-glycosylation site of xCAP-1. The G305Stop mutant exhibited the same glycosylation profile as the N288Q mutant. Thus, deletion of the C-terminal hydrophobic tail of xCAP-1 impairs the glycosylation at the position 288 of the G305Stop-truncated protein.

xCAP-1 can be deglycosylated by EndoH, indicating that N288 glycosylation site is occupied by mannose-rich oligosaccharides. To test the N-glycosylation pathway of xCAP-1, we used different inhibitors of N-glycosylation: tunicamycin (TUN), which blocks the N-acetyl-glucosaminyltransferase involved in the first step on N-glycosylation in the endoplasmic reticulum (core-glycosylation), castanospermine (CST), 1-deoxymannojirimycin (DMJ), and swainsonine (SW), which inhibit glucosidase I and $\alpha$-mannosidase I and II, respectively. These three enzymes participate in the processing of core-glycosylated proteins into fully-glycosylated proteins in the endoplasmic reticulum and the Golgi apparatus. As shown in Figure 3, we observed that tunicamycin prevents glycosylation of xCAP-1. However, when castanospermine, deoxymannojirimycin, or swainsonine were injected, xCAP-1 was still glycosylated. The three inhibitors are, however, active, because they prevent full glycosylation of the $\beta$ subunit of the Na,K-ATPase used as a positive control (not shown). xCAP-1 is monoglycosylated; therefore, gel resolution is not sensitive enough to determine whether the glycosylated form of xCAP-1 has been processed in the presence of castanospermine, deoxymannojirimycin, or swainsonine. However these forms are EndoH-sensitive, confirming that they are mannose-rich core-glycosylated forms and have not been processed through the usual Golgi pathway.

## *Cell-Surface Expression of xCAP-1*

Analysis of primary sequence indicates that xCAP-1 possesses a N-terminal hydrophobic signal peptide, which would allow the secretion of the protease in the external medium. A C-terminal hydrophobic tail precedes a consensus motif, predicting a GPI-anchor to the outer leaflet of the plasma membrane (2,4). We used an immunofluorescence assay and a binding assay to test the localization of xCAP-1 in *Xenopus* oocytes. Oocytes injected with WT xCAP-1 exhibited a bright immunofluorescent signal at the plasma membrane, which colocalizes with actin staining (Figure 4A). This indicates that the majority of the the Flag-tagged protein is expressed at the cell surface. A diffuse staining under the plasma membrane is

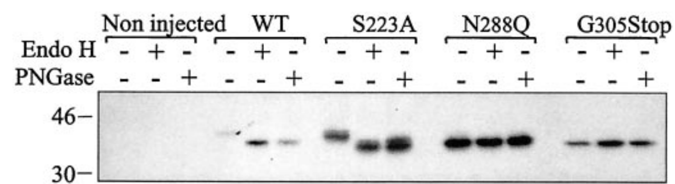


*Figure 2.* Effects of endoglycosidase H (EndoH) and N-glycosidase F (PNGase [EndoF]) treatment on the electrophoretic mobility of xCAP-1. Injected oocytes were pulse-labeled for 16 h with modified Barth saline (MBS) containing 100 $\mu$Ci/ml [$^{35}$S]methionine. Microsomal membranes were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were digested with EndoH or PNGase before gel loading. Migration positions of the size markers are indicated on the left.

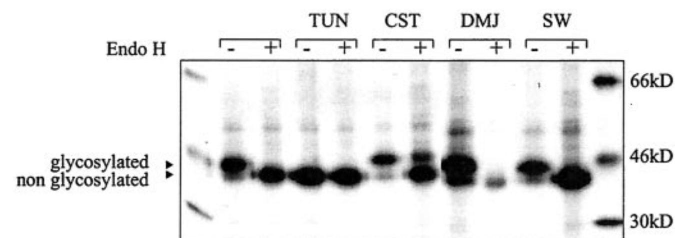


*Figure 3.* Effects of inhibitors of N-glycosylation on wild type (WT) xCAP-1 N-glycosylation. Oocytes were injected with WT xCAP-1 cRNA diluted in either tunicamycine (TUN), castanospermine (CST), 1-deoxymannojirimycin (DMJ), or swainsonine (SW). After immunoprecipitation, proteins were either treated with EndoH or not to identify glycosylated and nonglycosylated forms of xCAP-1.

sometimes observed (Figure 4B) but not seen reproducibly in other experiments using different batches of oocytes. Oocytes expressing the S223A (Figure 4B) and N288Q (Figure 4B) mutants exhibited a similar signal at the plasma membrane. G305Stop-injected oocytes (Figure 4B) showed no immunofluorescence signal for xCAP-1 and were identical to noninjected oocytes. This indicates that the G305Stop mutant is not anchored into the plasma membrane and/or is not accumulated in intracellular compartments. Binding experiments, using an iodinated anti-Flag antibody, allow for the quantification of cell-surface, membrane-bound expression of xCAP-1–flagged protein. As shown in Figure 5, oocytes injected with WT xCAP-1 exhibited a strong binding of the anti-Flag antibody (0.31 ± 0.03 fmol/oocyte *versus* noninjected oocytes, 0.07 ± 0.005 fmol/oocyte; $P < 0.0001$). Similarly, S223A and N288Q mutants exhibited a highly significant binding of the anti-Flag antibody (0.40 ± 0.04 fmol/oocyte and 0.41 ± 0.03 fmol/oocyte, respectively). Thus, this indicates that WT, S223A, and N288Q xCAP-1 proteins are expressed at the cell surface and are bound to the membrane. On the other hand, the G305Stop mutant is not detectable at the cell surface (0.07 ± 0.003 fmol/oocyte, NS *versus* noninjected oocytes). This confirms the immunofluorescence observations indicating that the G305Stop mutant is not present at the plasma membrane. Immunofluorescence studies and binding assays were also performed on oocytes injected with xCAP-1 alone as well as with xCAP-1 plus ENaC. Whether ENaC is coexpressed with xCAP-1 or not, no difference in immunostaining of xCAP-1 was observed (data not shown). Thus ENaC does not interfere with the targeting and/or cell surface expression of xCAP-1.

To determine whether G305Stop xCAP-1 is secreted, immunoprecipitation of the incubation medium was carried out. As shown in Figure 6, we observed an accumulation of xCAP-1 protein in the incubation medium of G305Stop-injected oocytes, whereas no protein is detected in WT-, S223A- or N288Q-injected oocytes. This indicates that G305Stop xCAP-1 protein is a secreted protein. Taken together, these results showed that xCAP-1 is an extracellular membrane–bound protein and that its membrane anchoring needs the presence of its C-terminal hydrophobic tail. On the other hand, the extracellular membrane localization of xCAP-1 does not require serine protease catalytic activity or N-glycosylation of the protein. The mutant lacking the GPI-anchor domain (G305Stop mutant) is not glycosylated, is not detected at the cell surface, and has no effect on ENaC activity (see below). The reason that the G305Stop mutant is not glycosylated is unknown. The STOP codon has been inserted close to the N-glycosylation site (17 amino acids), and the N-glycosylation site is probably hidden in the conformation of the truncated C-terminal tail. Whatever the mechanism might be, the lack of N-glycosylation of the G305Stop mutant cannot explain its loss of activity because the nonglycosylated N288Q mutant is as active as wild type (see below).

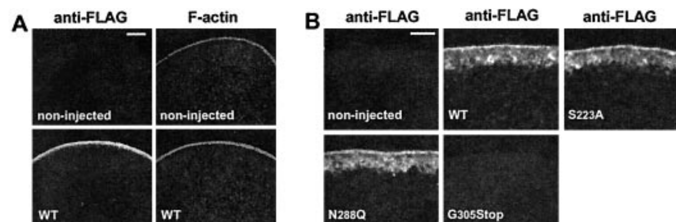


*Figure 4.* Cell surface expression of xCAP-1 by immunofluorescence. (A) Immunofluorescence on cryosections of noninjected *versus* flagged xCAP-1 injected oocytes. Detection of FLAG-tagged xCAP-1 with an anti-FLAG monoclonal antibody and of microvillar F actin with rhodamin-conjugated phalloidin. (B) Immunocytochemical detection of FLAG-tagged xCAP-1 in WT, S223A, N288Q, or G305Stop xCAP-1–injected oocytes. Scale bars, 40 $\mu$m.

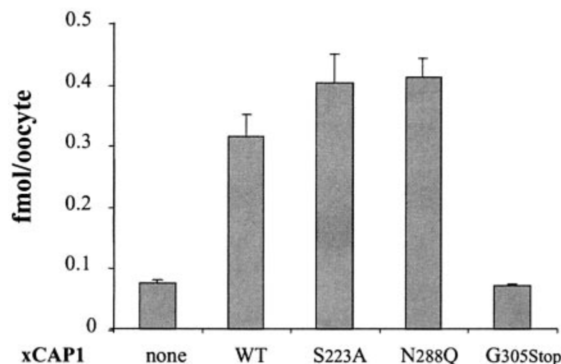


*Figure 5.* Cell-surface expression by binding to flagged xCAP-1. Iodinated anti-Flag–binding assay on flagged xCAP-1 constructs–injected oocytes. Cell-surface expression of xCAP-1 was determined by using the $^{125}$I-labeled anti-Flag M2Ab antibody and expressed as femtomol per oocyte. Each bar represents the mean of two experiments in which the radioactivity bound to 6 to 10 individual oocytes have been counted for each condition.

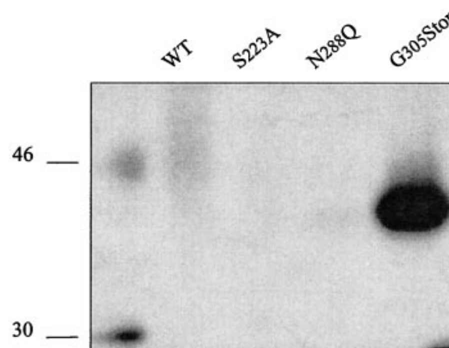


*Figure 6.* Immunoprecipitation on MBS-conditioned medium. Injected oocytes were incubated for 16 h in 100 $\mu$l of MBS medium containing [$^{35}$S]methionine. MBS was then recovered, clarified by 10,000-rpm centrifugation, and submitted to immunoprecipitation by using anti-Flag antibody.

### *Activation of ENaC by xCAP-1 Requires the Presence of the GPI Anchor*

Finally, we have analyzed the functional properties of WT xCAP-1 and mutants by testing their ability to increase ENaC activity. ENaC activity was measured by two-electrode voltage

clamp as an amiloride-sensitive sodium current (Iam). The results are shown in Figure 7. When ENaC is expressed alone, the oocytes exhibit a low Iam, which is greatly increased (10.6-fold) after trypsin perfusion (control, 61.3 ± 5.4 nA *versus* trypsin, 651.3 ± 51.6 nA; $P < 0.001$) as described previously (2,5). When WT xCAP-1 is coexpressed together with ENaC, the basal Iam is 8.5-fold higher than in the absence of xCAP-1 (524 ± 79.3 nA with xCAP-1 *versus* 61.3 ± 5.4 nA; $P < 0.001$). Perfusion of trypsin induces a small (1.9-fold) increase of Iam (524 ± 79.3 nA *versus* 1000.7 ± 142.7 nA; $P < 0.01$), indicating that the presence of xCAP-1 achieves most of the activating effect of trypsin. These results are similar to those previously observed (2). They also indicate that insertion of the Flag epitope into the xCAP-1 sequence does not impair the activating effect of xCAP-1 on ENaC. We then tested the activity of the different xCAP-1 mutants. We observed that the N288Q mutant behaves like the WT protein by increasing basal ENaC activity and competing for the action of trypsin (control, 578.2 ± 110.2 nA *versus* trypsin, 980.9 ± 216.6 nA; 1.7-fold). This result indicates that N-glycosylation of xCAP-1 is not necessary for activation of ENaC. The catalytic mutant, S223A, greatly diminishes the ability to activate ENaC but surprisingly did not abolish it completely (61.3 ± 5.4 *versus* 147.7 ± 23.0 nA; $P < 0.01$). Trypsin induced a 7.7-fold increase in ENaC activity (1339.3 ± 162.5 nA *versus* 147.7 ± 23.0; $P < 0.001$), indicating that the S223A mutant is greatly impaired in its ability to activate ENaC. Similar results were obtained when the two other residues of the catalytic triad were mutated (H70A or D119A) (data not shown). Finally, the G305Stop mutant was unable to significantly increase (+30%) basal ENaC activity (89.2 ± 16.0 nA *versus* 61.3 ± 5.4; NS), whereas trypsin was still able to increase ENaC activity 8.4-fold (746.7 ± 117.4 nA *versus* 89.2 ± 16 nA; $P < 0.001$). This result indicates that the C-terminal tail of xCAP-1 is necessary for channel activation. The G305Stop mutant is not properly expressed at the cell surface but rather secreted in the medium; therefore, our data suggest that a colocalization of xCAP-1 with ENaC at the plasma membrane is critical for ENaC activation.

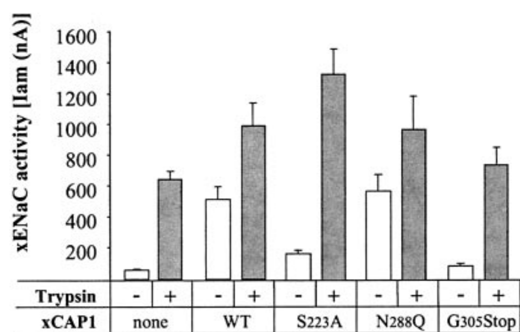


*Figure 7.* Functional properties of xCAP-1 on ENaC activity. Oocytes were coinjected with cRNA encoding the three subunits of ENaC and one of the xCAP-1 mutant. Absolute values of amiloride-sensitive sodium current (Iam) measured before (−) and after (+) 2-min trypsin perfusion (2 μg/ml) of the oocyte. Each bar represents the mean of two experiments (two different batches of oocytes) in which 8 to 10 oocytes have been measured for each condition.

## Discussion

CAP-1 is a serine protease that increases ENaC activity when coexpressed in *Xenopus* oocytes. In this article, we have analyzed some features of xCAP-1 (proteolytic activity, glycosylation, membrane-anchoring) and their influence on the effect of xCAP-1 on ENaC activity. The data are summarized in Figure 1.

### *ENaC Activation: Importance of the Catalytic Site*

Members of the serine protease family are characterized by a typical HDS catalytic triad containing a histidine, an aspartate, and a serine. Mutation of any of these residues abolishes the proteolytic activity of the protease (10). When the proteolytic activity of xCAP-1 is abolished by mutating the serine residue of the catalytic triad (S223A mutant), the protein is still glycosylated and expressed at the cell surface, but it loses about 90% of its effect on ENaC activity. This confirms our previous results from experiments using the inhibitor aprotinin and suggesting that xCAP-1 acts through its proteolytic activity to increase ENaC activity (2). Similar conclusions were drawn from experiments performed on a kidney cell line (2,5). It appears, however, that a small (approximately 10%), but statistically significant, fraction of ENaC activation by xCAP-1 cannot be explained by its catalytic activity. The mechanism is not known, but it could involve protein-protein interaction. Serine proteases can interact with serine protease inhibitors, such as aprotinine expressing a Kunitz domain or other serine protease inhibitors expressing WAP-type 4 disulfide core domain (11). The mouse orthologue (mCAP-1) of xCAP-1 has been identified in a cortical collecting duct cell line (12). A rat orthologue (*i.e.*, rat prostasin) (13) has also been recently reported. Mouse CAP-1 shares many biologic properties with xCAP-1. The corresponding catalytic mouse mutant, however, diminishes ENaC activation by only 40 to 50%. Interestingly, in the same cell line, we have been able to identify two other membrane anchored serine proteases (mCAP2 and mCAP3) that require an intact catalytic triad to activate ENaC. This cell line also expresses high levels of various serine protease inhibitors (Vuagniaux G and Rossier B; unpublished observations). This suggests the existence of a complex cascade involving at least three distinct serine proteases that can act catalytically and/or by protein-protein interaction with serine protease inhibitors. This may represent a novel extracellular signaling cascade that controls ENaC activity in the extracellular environment. The molecular mechanisms involved are unknown and could involve a direct cleavage of ENaC (the $\gamma$ subunit has been proposed in reference 14) or by an indirect mechanism involving associated protein of the extracellular environment.

### *Role of N-Glycosylation*

The present data suggest that xCAP-1 undergoes the first step of core glycosylation in the endoplasmic reticulum but that full glycosylation is not required for targeting to the plasma

membrane. We can clearly demonstrate that the nonglycosylated N288Q mutant is expressed at the cell surface as well as the wild type by both immunofluorescence and binding assays. N288Q remains able to fully activate ENaC. Therefore, N-glycosylation of xCAP-1 is not necessary for its trafficking to the plasma membrane or for its biologic activity.

### *Importance of the GPI Anchor*

Many extracellular biologic processes are mediated by proteases. Some of these proteases are secreted, and others are bound to the cell surface through transmembrane domains or GPI-anchoring. Examples of GPI-anchored proteases include the membrane type 4 matrix metalloproteinase (15), the carboxypeptidase M (16), or the $\alpha$-secretase activity (17). xCAP-1 possesses an N-terminal signal peptide and a C-terminal hydrophobic tail, a typical structure of GPI-anchored proteins. Analysis of its primary sequence predicts that the C-terminal hydrophobic tail is cleaved between residues N304 and G305 and that the novel C-terminal tail is then linked to GPI moiety by a transamidase. Insertion of a STOP codon at position 305 will impair membrane-anchorage of xCAP-1 and generate a secreted protein. Immunofluorescence experiments and binding assay results indicate that WT xCAP-1 is a cell surface–attached protein, but the G305Stop mutant was not detected at the cell surface and does not appear to accumulate in some intracellular vesicles, indicating that it is effectively secreted. The C-terminal hydrophobic tail of xCAP-1 is then necessary for its membrane-anchoring, strongly suggesting that WT xCAP-1 should be a GPI-anchored protein. In the present study, we have not been able to directly measure serine protease activity of the GPI-anchored, membrane-bound form of the protein. It has recently been biochemically demonstrated that prostasin, the human ortholog of CAP-1, is a GPI-anchored active serine protease (18). It was also shown that both the secreted and the GPI-anchored form of prostasin were able to covalently bind a 82-kD prostasin–binding protein, an interaction that could be inhibited by aprotinin or other serine proteases inhibitors (18). The function of prostasin in the prostate is not yet defined, but a complex extracellular signaling cascade involving protein-protein interaction and catalytic activity could operate in the prostate. This is consistent with the data and the working hypothesis presented here for the kidney.

### *Physiologic and Pathophysiologic Relevance*

In the kidney, the control of sodium balance by the fine regulation of the amiloride-sensitive electrogenic sodium reabsorption mediated by ENaC in the distal nephron is critical for the control of blood volume and BP (1). Gain-of-function mutations in ENaC (19) or in the mineralocorticoid receptor (with a constitutive activation of the aldosterone-dependent signaling cascade) (20) lead to severe forms of salt-sensitive hypertension. Loss-of-function mutation in 11 $\beta$-HSD-2, the enzyme protecting MR from illicit occupation by cortisol, is also associated with early and severe hypertension in newborn babies (21). From these observations, one can deduce that any gain (or loss) of function mutations in genes leading to the activation (or repression) of the amiloride-sensitive electrogenic sodium tranport can contribute to a hypertensive phenotype. Genes involved in this novel extracellular signaling cascade are obviously candidate genes for such a role.

In the lung and airways, ENaC plays a critical role in controlling lung fluid clearance at birth (22) and air surface liquid, thereby determining the rate of mucociliary clearance (23). Amiloride has been tested as an inhalation therapy in cystic fibrosis to decrease sodium reabsorption and increase mucociliary clearance (24). It has recently been shown that bicunin, a serine protease inhibitor (Kunitz type), can block up to 60% of the electrogenic amiloride-sensitive sodium transport in primary cultures of human airway epithelia (25). CAP-1 is highly expressed in human airways and thus a potential drug target to increase mucociliary clearance in cystic fibrosis or related pathophysiologic disturbances.

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