

Activation of the Amiloride-Sensitive Epithelial Sodium Channel by the Serine Protease mCAP1 Expressed in a Mouse Cortical Collecting Duct Cell Line

GRÉGOIRE VUAGNIAUX,* VÉRONIQUE VALLET,* NICOLE FOWLER JAEGER,*
CORINNE PFISTER,* MARCELLE BENS,[†] NICOLETTE FARMAN,[†]
NATHALIE COURTOIS-COUDRY,[†] ALAIN VANDEWALLE,[†]
BERNARD C. ROSSIER,* and EDITH HUMMLER*

**Institut de Pharmacologie et de Toxicologie, Université de Lausanne, Switzerland; and* [†]*Institut Nationale de la Santé et de la Recherche Médicale U478, Institut Fédératif de Recherche, Paris Cedex, France.*

Abstract. This study examines whether serine proteases can activate the amiloride-sensitive sodium channel (ENaC) in mammalian kidney epithelial cells. The transepithelial sodium transport assessed by amiloride-sensitive short-circuit current appears to be sensitive to aprotinin, a protease inhibitor in a mouse cortical collecting duct cell line (mpkCCD_{c14}). This result indicated that serine proteases may be implicated in the regulation of ENaC-mediated sodium transport. Using degenerated oligonucleotides to a previously isolated serine protease from *Xenopus*, xCAP1 (channel activating protease), a novel full-length serine protease (mCAP1), has been isolated and characterized. RNA analysis showed a broad pattern of expres-

sion in tissues (kidney, lung, colon, and salivary glands) expressing ENaC. Reverse transcription-PCR experiments also showed that mCAP1 was abundantly expressed in proximal tubule cells and was also expressed in intact and cultured collecting duct cells. Coexpression of the *Xenopus*, rat, or human α -, β -, and γ -ENaC subunits in *Xenopus* oocytes also showed that mCAP1 induces a significant increase in ENaC-mediated current accompanied by a decrease of channel molecules at the cell surface. It is proposed that this novel mouse channel activating protease may act as a regulator of ENaC within the kidney.

Sodium balance, extracellular volume, and BP are maintained by precise control of the activity of the epithelial sodium channel ENaC (1,2). The activity of ENaC is tightly controlled by hormones such as aldosterone and vasopressin (1). The molecular mechanisms are still poorly defined and the signaling pathway is not known. Cellular proteins can associate directly or indirectly with cytoplasmic amino or carboxy terminus of ENaC to regulate its membrane expression and/or activity. Components of this intracellular signaling pathway comprise α spectrin (3), Nedd-4 (4), and proteins such as K-Ras2 and sgk kinase (reviewed in reference (5)). We have previously identified an epithelial serine protease (xCAP1) that activates the amiloride-sensitive sodium channel ENaC in a

Xenopus kidney epithelial A6 cell line (6,7). xCAP1 is a 329-residue protein belonging to the serine protease family. The coexpression of xCAP1 with ENaC in the *Xenopus* oocyte increases the activity of the sodium channel by two- to threefold. This effect of xCAP1 is inhibited by aprotinin, a serine protease inhibitor (6). According to its sequence analysis, xCAP1 is thought to be a secreted and/or a glycosyl-phosphatidylinositol (GPI)-anchored protein. We proposed that ENaC activity could be regulated by the activity of an extracellular protease expressed at the apical surface of the kidney cell. This defined a novel extracellular signaling pathway for ion channels that has not yet been studied in the mammalian kidney. The most closely related mammalian protein to xCAP1 is a human serine protease, prostasin, which has been identified in semen (8) and which shares 53% homology with the amphibian gene product xCAP1. Tissue-specific expression of prostasin mRNA revealed the presence of transcripts in prostate, liver, testes, salivary glands, kidney, lung, pancreas, and colon and in a proximal tubule kidney cell line (8). The specific distribution of prostasin mRNA along the human nephron was not reported and, specifically, it was not shown whether prostasin is expressed in the distal part of the nephron. This is an important issue, since sodium balance is achieved by the regulation of ENaC in the distal nephron, namely, in the cells of distal convoluted tubules and collecting ducts, which is the main target for aldosterone response (1). The amphibian A6 cells, derived from the principal cells of the collecting duct of the

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Bernard C. Rossier and Edith Hummler contributed equally to this work.

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Correspondence to Dr. Bernard C. Rossier, Institut de Pharmacologie et de Toxicologie, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland. Phone: +41 21 6925351; Fax: +41 21 6925355; E-mail: Bernard.Rossier@ipharm.unil.ch or Dr. Edith Hummler, Institut de Pharmacologie et de Toxicologie, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland. Phone: +41 21 6925357; Fax: +41 21 6925355; E-mail: Edith.HummlerBeermann@ipharm.unil.ch

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Xenopus kidney, express ENaC, which is controlled by aldosterone (9) and activated by xCAP1 (6). This raises the question of whether the mammalian prostasin is the gene product corresponding to the *Xenopus* CAP1.

The aim of this study was to identify a mammalian CAP1 homologue in cells of the cortical collecting duct. To achieve this goal, we selected a novel established clonal mouse collecting duct cell line, mpkCCD_{c14}, which faithfully reproduces the expected properties of collecting duct cells (10). As in A6 cells, this cell line has a high transepithelial electrical resistance and expresses an amiloride-sensitive transepithelial sodium transport that is responsive to physiologic concentrations of aldosterone. In the present report, we describe the identification of a mouse serine protease homologous to xCAP1, termed mCAP1, which has properties similar to the *Xenopus* counterpart. We propose that this homologue is responsible for regulating ENaC activity in the mammalian kidney.

Materials and Methods

Culture of mpkCCD_{c14} Cells and Short-Circuit Current Measurements

Cells were grown in a hormonally defined Dulbecco's modified Eagle's medium:Ham's F12 (1:1) medium supplemented with 2% fetal calf serum as described by Bens *et al.* (10) in an atmosphere of humidified air/5% CO₂ at 37°C. To ensure high rates of basal transport, cells (between passages 24 and 35) were cultured for 5 consecutive days on collagen-coated Transwell permeable filters (Costar) with complete medium followed by a 4- to 8-d culturing in minimal medium lacking fetal calf serum, epidermal growth factor, and transferrin (10). Short-circuit current (I_{sc}) measurements were performed using a modified Ussing chamber (9). The mpkCCD_{c14} cells were incubated with increasing concentrations (5, 50, 500, and 5000 μ g/ml) of aprotinin in the luminal bath. After 18 h of incubation, I_{sc} was measured, then 5 μ M amiloride was added to the apical side of the filter and I_{sc} was measured again after 30 min incubation. The amiloride-sensitive short circuit current was deduced using these two values.

Identification and Isolation of a Novel Full-Length Murine Serine Protease cDNA

Reverse transcription (RT)-PCR was performed on total RNA from mpkCCD_{c14} cells. Briefly, 3 μ g of total RNA was treated with DNase I (Boehringer Mannheim, Germany) followed by reverse transcription using Superscript II (Life Technologies) and random primers (Pharmacia). PCR was performed using 1/10 of the cDNA digested with RNase H (Life Technologies) at 55°C for 15 min. PCR was done in 50- μ l reactions containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 150 μ M of each deoxynucleoside 5'-triphosphate, 5% DMSO, 0.5 μ M of each primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). Forty cycles were run using primers D1 and D2 each consisting of 30 s at 94°C, 30 s at 48°C, and 1 min at 72°C. After the last cycle, elongation was allowed to proceed for 7 min at 72°C. A second PCR was performed using 1/20 of the first PCR reaction with nested primers D1 and D3 under the same conditions. The 512-bp amplified PCR product was separated on a 1.2% agarose gel, extracted, and purified (Qiagen, Chatsworth, CA), subcloned into pT7Blue Vector (Blunt Cloning Kit, Novagen, Madison, WI), and sequenced. The primers used were as follows: D1 (sense) 5'-AA(AG)TT(CT)CCITGGCA(AG)GT-3', nt +118 to +134; D2

(antisense) 5'-CC(AG)CA(CT)TC(AG)TCICCCCA-3', nt +743 to +727; D3 (antisense) 5'-CCIGC(AG)CA(AGT)ATCAT(AG)TC-3', nt +629 to +613; according to xCAP1 (5' and 3').

Rapid Amplification of cDNA Ends

For 5' rapid amplification of cDNA ends (5'-RACE), after reverse transcription of mpkCCD_{c14} total mRNA (as described above), mCAP1-specific reverse primers RC1 (5'-CTTCACCTCATACGCT-TCC-3', nt position +297 to +279) and RC2 (5'-GTTGCCATCG-TAGGTGATG-3', nt position +201 to +183) were used sequentially with adapter-specific primer dC-5R (5'-GCATGCTCGAGCGGCCG-CAACCCCCCCCCCCCCCCCC-3') and 5R (5'-GCATGCTC-GAGCGGCCGCAAC-3'), according to the manufacturer's instructions (Life Technologies). For 3'-RACE, reverse transcription was performed using an oligo(dT) adapter primer (5'-CGAGATCTAT-GCGGCCGCTTTTTTTTTTTTTTTTTTTT-3'). mCAP1-specific primers RC3 (5'-TCAGTGAGCCTCCAGACC-3', nt position +535 to +552) and RC4 (5'-CTGTAGCTGCCTGTACAA-3', nt position +600 to +617) were used. To amplify the full-length cDNA from mpkCCD_{c14} cells, primer 1 (sense 5'-TGCCTTCAAACCAGC-CTTC-3', nt position -73 to -54) and primer 2 (antisense 5'-TGTCTTGGGTGTGCTGTG-3', nt position +1083 to +1065; mCAP1; accession no. AF 188613) covering the open reading frame of mCAP1 were used according to the manufacturer's instructions (High Fidelity Expand PCR System, Boehringer, Germany). The 1156-bp PCR product was purified by agarose gel electrophoresis, cloned into pT7Blue Vector (Blunt Cloning Kit, Novagen), and confirmed by sequencing.

Northern Blot Analysis

Total RNA (20 μ g) extracted from various mouse tissues were run on a 0.8% denaturing glyoxal agarose gel and blotted onto nylon membranes (Hybond-N, Amersham). Membranes were hybridized with random-primed ³²P-labeled probes for mCAP1 (512 bp: nt +163 to +674), α rENaC, and rat GAPDH.

RT-PCR Analysis

Total RNA was prepared from whole mouse kidney, confluent mpkCCD_{c14} cells, and microdissected tubules. Thin kidney slices from 1-mo-old mice ($n = 3$) fed with a standard diet were incubated in Dulbecco's modified Eagle's medium:Ham's F12 (1:1) supplemented with 0.1% collagenase (Boehringer Mannheim) for 1 h at 37°C. S2 (proximal convoluted tubule [PCT]) and S3 (pars recta [PR]) segments from proximal convoluted tubules, medullary and cortical ascending limbs of Henle's loop, distal convoluted tubules, and cortical (CCD) and inner medullary collecting ducts were microdissected as described (11). Single microdissected tubules (0.3 to 0.5 mm long) from each segment were pooled (five to eight single tubules) and stored at -80°C before use. Total RNA was extracted from whole kidney, cultured cells, and microdissected tubules using the RNA-PLUS extraction kit (Bioprobe Systems, Montreuil-sous-bois, France). The RNA were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Eragny, France) at 42°C for 45 min. Two hundred fifty nanograms of whole kidney and mpkCCD_{c14} cell cDNA and non-reverse-transcribed RNA were amplified for 32 cycles in 100 μ l of total volume containing 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 40 μ M dNTP, 1 U *Taq* polymerase, 30 pmol of mCAP1 primer 1 and RC1. Microdissected tubule cDNA were amplified for 38 cycles using the same buffer containing 4 pmol of mCAP1 primers and 0.45 pmol of β -actin primers (5'-CGTGGGCCGCCCTAGGCACCA-3' and 5'-TTGGC-

CTTAGGGTTCAGGGGGG' as described previously) (10). The thermal cycling program was as follows: 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. Amplification products were run on 3.5% agarose gel, stained with ethidium bromide, and photographed, or were run on 4% polyacrylamide gel and autoradiographed. The identity of the amplified products was controlled by digestion with *NciI* (Life Technologies).

Electrophysiologic Measurements in *Xenopus* Oocytes

For functional expression studies, mCAP1 cDNA was subcloned into pSD5 expression vector and *in vitro*-transcribed (7). Expression studies were performed in stage V/VI oocytes isolated from *Xenopus laevis* (Noerdhoek, South Africa). Routinely, oocytes were injected with 0.33 ng of each cRNA coding for the rat α -, β -, and γ -ENaC subunits in the presence or absence of 2 ng of cRNA coding for mCAP1 in a total volume of 100 nl. Oocytes were incubated overnight in modified Barth saline solution in the presence or absence of 100 μ g/ml aprotinin. Electrophysiologic measurements were performed 24 to 48 h after cRNA injection using the two-electrode voltage-clamp technique, and the amiloride-sensitive current (I_{Na}) was measured in the presence of 120 mM of Na^+ in frog Ringer's solution with 5 μ M amiloride at a holding potential of -100 mV. The oocytes were perfused with 2 μ g/ml trypsin during 2 to 3 min and I_{Na} was remeasured. Amiloride sensitivity of injected sodium channel subunits has been measured with 0.001, 0.01, 0.1, 1, and 10 μ M amiloride in the presence of 120 mM Na^+ . Cell surface expression has been performed as described (12). Briefly, 1 ng of tagged rat α -, β -, and γ -ENaC cRNA were expressed in oocytes either coinjected with 2 ng of mCAP1 cRNA or water-injected. The density of the channel was then estimated by binding of iodinated anti-FLAG (M2) monoclonal antibody (Sigma, Buchs, Switzerland).

Statistical Analyses

All results are reported as means \pm SEM. Comparing independent sets of data, unpaired *t* tests were used to determine significance. In experiments in which oocytes were perfused with trypsin, paired *t* tests were used. $P < 0.01$ was considered significant. *n* represents the number of experiments performed.

Results

Aprotinin Inhibits Amiloride-Sensitive Short Circuit Current (I_{sc}) in mpkCCD_{c14} Cells

The mouse mpkCCD_{c14} cell line has been derived from the mouse CCD (10). In these cells, the sodium transport (measured as a short circuit current [I_{sc}]) is 77 ± 12 μ A/cm² ($n = 20$). When 5 μ M amiloride was added to the luminal bathing solution, the I_{sc} dropped to 8 ± 2 μ A/cm² ($n = 20$; $P < 0.01$), suggesting that most of the measured I_{sc} is due to an electrogenic ENaC-mediated Na^+ transport. Overnight exposure of the apical side of mpkCCD_{c14} cells with increasing concentrations of aprotinin, an inhibitor of serine proteases, reduced the amiloride-sensitive I_{sc} by about 50% (Figure 1). These results indicated that ENaC-mediated sodium transport in this mouse cortical collecting duct cell line might be controlled in part by serine proteases.

Identification of the Murine Homologue for xCAP1

RT-PCR analysis of mpkCCD_{c14} cells using degenerated oligonucleotides to xCAP1 revealed a 512-bp PCR product. A

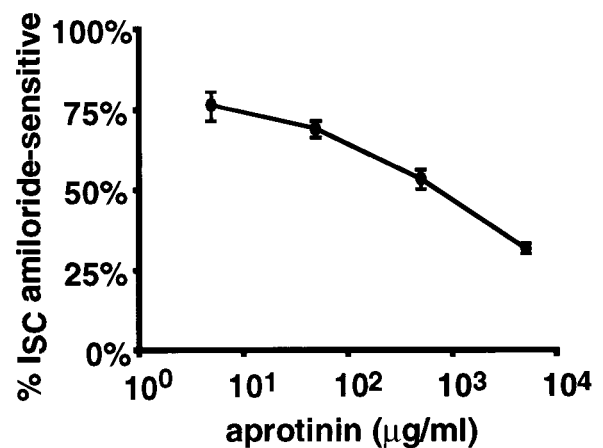


Figure 1. Aprotinin decreases I_{sc} of amiloride in mpkCCD_{c14} cells. Measurements of the proportional amiloride-sensitive I_{sc} after overnight exposure of the apical side of mpkCCD_{c14} cells with gradual concentrations of aprotinin (0 μ g/ml, $n = 20$; 5 μ g/ml, $n = 8$; 50 μ g/ml, $n = 14$; 500 μ g/ml, $n = 14$; 5000 μ g/ml, $n = 4$; 5 μ M amiloride, $n = 20$).

1768-bp cDNA (without polyA⁺) was obtained by 5'- and 3'-RACE (Figure 2). This clone contained an open reading frame of 339 amino acids. Alignment of the predicted amino acid sequence (Genetic Computer Group, Inc., Madison, WI) revealed that the deduced protein belongs to the serine protease family. It shows high homology with the trypsin-like protease family. It shared 50% homology with the channel activating protease xCAP1, 46% with the mouse trypsinogen precursor, and 80% with the human prostaticin (Figure 2). The N terminus exhibits a putative cleavable signal peptide and the C terminus a hydrophobic domain with a presumed GPI membrane-anchoring site known to act as a dominant apical target signal in polarized epithelial cells (6). Therefore, this new mouse serine protease was named mCAP1 for mouse channel activating protease 1.

Tissue and Intrarenal mCAP1 mRNA Expression

Northern blot analysis revealed strong hybridization signals (about 1.8 kb) in kidney, lung, and salivary glands and a weak signal in distal colon, as well as in mpkCCD_{c14} cells (Figure 3), and in skin, stomach, duodenum, small intestine, colon, bladder, and prostate (data not shown). There was no detectable hybridization signal in liver, heart, and brain (Figure 3), or in spleen, testis, ovary, pancreas, and muscle (data not shown). Hybridization with mouse α -ENaC subunit (lower band) revealed coexpression with mCAP1 in the relevant tissues (kidney, mpkCCD_{c14}, colon, lung, salivary gland). The upper band corresponds to a cross-hybridization to a 28S RNA (Figure 3).

To map precisely the distribution of mCAP1 along the nephron, we used RT-PCR on microdissected nephron segments. The sets of mCAP1 primers used permitted detection of amplified products in both whole mouse kidney and cultured mpkCCD_{c14} cells. The specificity of the amplified products was confirmed by their digestion with the *NciI* restriction enzyme (Figure 4A). Because the intrarenal distribution of

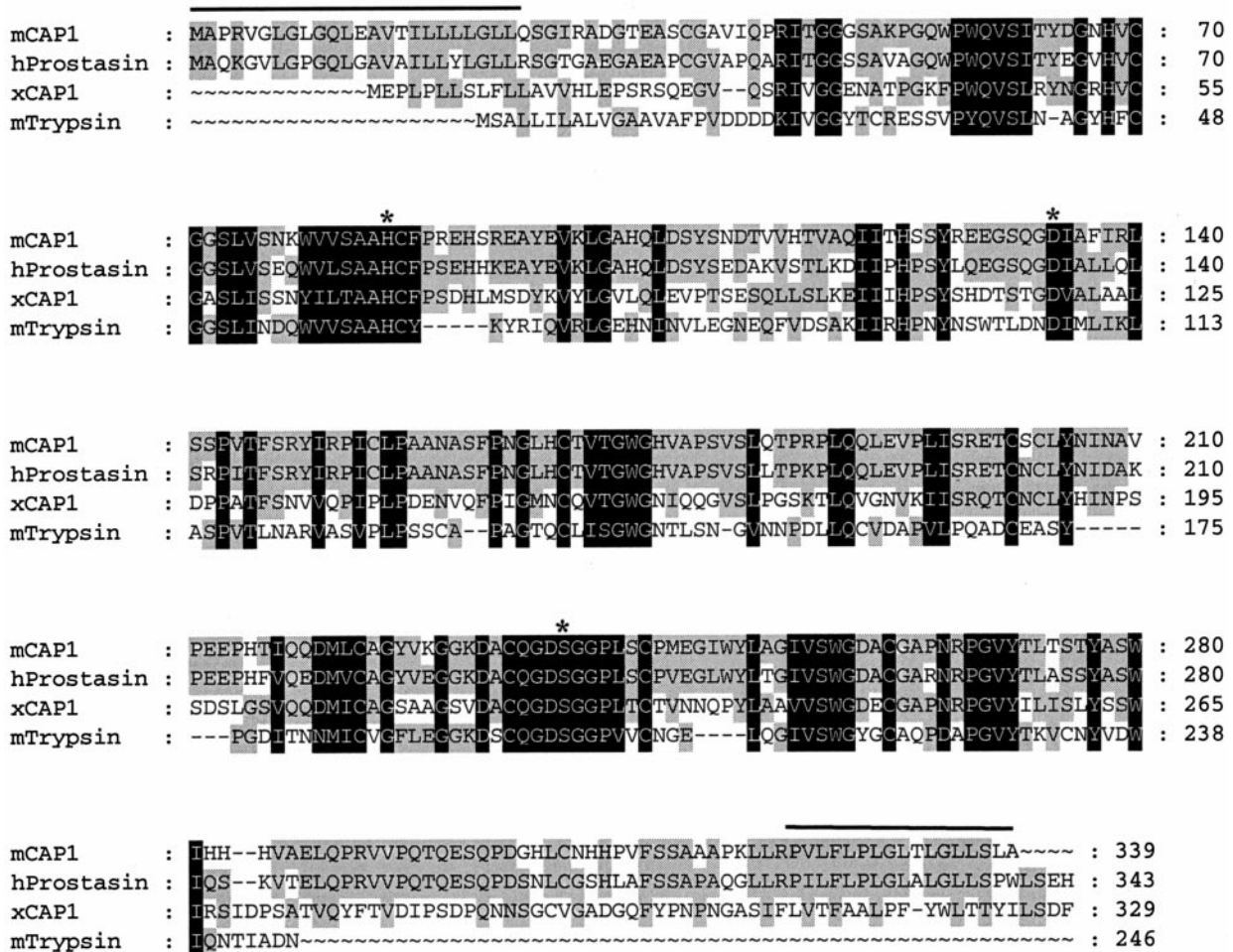


Figure 2. Mouse CAP1 (mCAP1) exhibits high homology with the trypsin-like protease family. Alignment of the amino acid sequence of mCAP1 with other serine proteases: human prostatic trypsin (1 to 343), *Xenopus* xCAP1 (1 to 329), and mouse trypsinogen precursor (1 to 246). Gray and white boxes indicate identical and similar residues, respectively. The catalytic triad (H₈₅, D₁₃₄, and S₂₃₈) is indicated by asterisks. Signal sequences in the N terminus and hydrophobic domain in C terminus of the protein are shown by black lines.

proteases is not known, we have analyzed the expression of mCAP1 along the mouse nephron (Figure 4B). The convoluted (PCT) and straight terminal portion (PR) of the proximal tubule exhibited the highest mCAP1 transcript abundance when compared with the levels of β -actin transcripts. Substantial amounts of mCAP1 transcripts were also detected in cortical ascending limbs of Henle’s loop and CCD (Figure 4B). In contrast, very low levels of mCAP1 expression were detected in medullary ascending limbs of Henle’s loop and inner medullary collecting duct as well as in distal convoluted tubules.

Functional Analysis of mCAP1 in *Xenopus* Oocytes

Coexpression of mCAP1 with each of the ENaC subunits from *Xenopus* (xENaC), rat (rENaC), and human (hENaC) led to an approximately sixfold significant ($P < 0.01$, unpaired t test) increase in the macroscopic amiloride-sensitive current (xENaC I_{Na} : $0.2 \pm 0.02 \mu A$ [$n = 21$] versus $1.01 \pm 0.04 \mu A$ [$n = 20$]; rENaC I_{Na} : $0.75 \pm 0.04 \mu A$ [$n = 19$] versus $5.03 \pm 0.16 \mu A$ [$n = 21$]; hENaC I_{Na} : $2.41 \pm 0.07 \mu A$ [$n = 21$] versus $12.12 \pm 0.26 \mu A$ [$n = 21$]) (Figure 5A). In the absence of α -,

β -, and γ -ENaC subunits, mCAP1 injected into oocytes did not generate measurable amiloride-sensitive current (data not shown).

We then compared the amiloride sensitivity of ENaC channel activity consisting of rat α -, β -, and γ -ENaC subunits coinjected with mCAP1 cRNA into *Xenopus* oocytes. The $K_{i\text{amil}}$ of ENaC activity alone ($K_{i\text{amil}}$: $0.106 \pm 0.008 \mu M$; $n = 18$) did not significantly differ ($P > 0.1$, unpaired t test) from the $K_{i\text{amil}}$ in oocytes coexpressing all ENaC subunits and mCAP1 ($K_{i\text{amil}}$: $0.91 \pm 0.005 \mu M$; $n = 17$). We conclude that the concentration of amiloride used in our assay is sufficient to block effectively ENaC-dependent sodium transport (over 95%) and that the effect of mCAP1 is therefore not underestimated.

Next, we tested the effects of trypsin and aprotinin on amiloride-sensitive ENaC-mediated sodium current in *Xenopus* oocytes injected with all three subunits of the rat (α -, β -, and γ -ENaC) in the presence (Figure 5B, lanes 5 to 8) or absence of mCAP1 cRNA (Figure 5B, lanes 1 to 4). In the

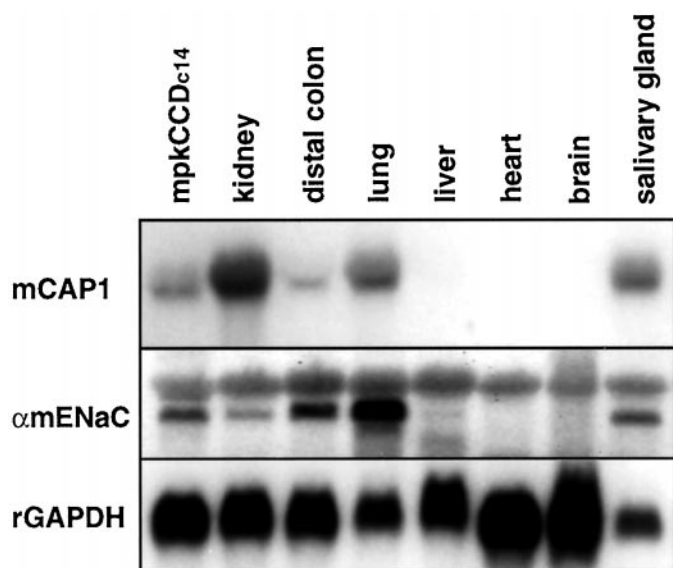


Figure 3. Northern blot analysis of mCAP1 (1.8 kb) and α ENaC (3.5 kb) mRNA expression in mpkCCD_{c14} cells and various mouse organs revealed coexpression in the same tissues. RNA integrity was controlled with a rat GAPDH cDNA probe. After hybridization with α mENaC, a nonspecific band (upper band) appeared in all tissues.

absence of aprotinin, mCAP1 induced a five- to sixfold increase of the amiloride-sensitive sodium current (I_{Na}) (Figure 5B, lane 1 *versus* lane 5). Trypsin (2 μ g/ml) was able to activate ENaC in the absence of mCAP1 (Figure 5B, lane 1 *versus* lane 2) but did not further increase the I_{Na} stimulated by mCAP1 (Figure 5B, lane 5 *versus* lane 6). When oocytes were incubated overnight with 100 μ g/ml aprotinin, the increase of I_{Na} by mCAP1 was inhibited (Figure 5B, lane 5 *versus* lane 7). The inhibitory effect of aprotinin was only effective in the presence of mCAP1, as it was not observed in its absence (Figure 5B, lane 1 *versus* lane 3). This finding suggests that the oocyte has very little endogenous proteolytic activity able to activate ENaC. The effect of aprotinin on I_{Na} could be reversed by trypsin in noninjected or injected mCAP1 oocytes (Figure 5B, lane 3 *versus* lane 4 and lane 7 *versus* lane 8). The lack of additive effect of trypsin and mCAP1 and the inhibition of mCAP1 activation by aprotinin and its reversal by trypsin strongly suggested that mCAP1 and trypsin acted through a common proteolytic pathway. mCAP1 could increase the amiloride-sensitive sodium current mediated by ENaC by two mechanisms: either by changing the electrophysiologic properties of the channel (conductance, ion selectivity, gating kinetics) or by increasing its cell-surface expression.

Coexpression of FLAG-tagged α -, β -, and γ -ENaC subunits and mCAP1 cRNA in oocytes produced a fourfold increase (4.4 ± 0.7 ; $n = 25$) in I_{Na} (Figure 5C, left panel). This increase of I_{Na} using the tagged ENaC subunits was lower than the increase using nontagged subunits. Higher basal currents measured under those conditions might be responsible for this difference in decreasing the effect of the protease (13). We also observed a twofold decrease (1.8 ± 0.1 ; $n = 25$) in expression of the channel molecules at the cell surface (Figure 5C, right

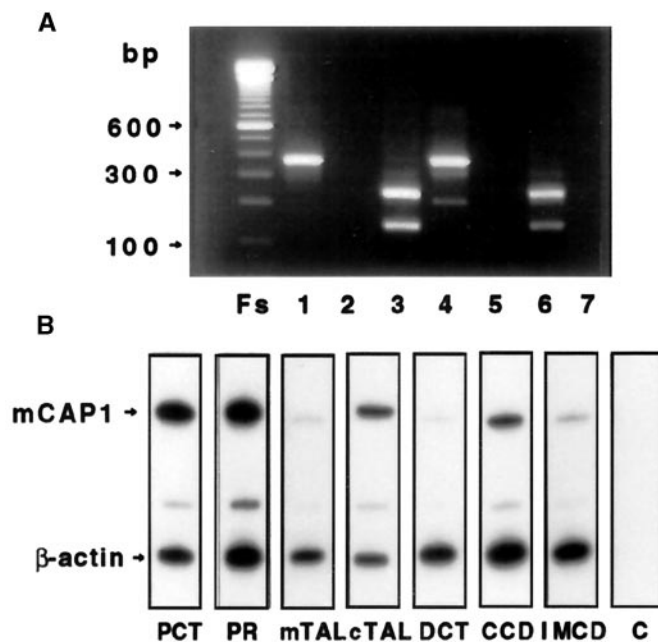


Figure 4. mCAP1 expression in whole mouse kidney, mpkCCD_{c14} cells, and along the renal tubule. (A) cDNA and non-reverse-transcribed RNA extracted from whole kidney (lanes 1 to 3) and cultured mpkCCD_{c14} cells (lanes 4 to 6). Amplified products for mCAP1 (372 bp) were obtained in mouse kidney (lane 1) and cultured cells (lane 4). Digestion of the amplified products with *NciI* yielded two bands (231 bp and 141 bp) in both mouse kidney (lane 3) and cultured cells (lane 6). No amplified products were detected with non-reverse-transcribed RNA (lanes 2 and 5) or by omitting cDNA (lane 7). Fs, molecular weight marker. (B) Representative illustrations of three independent autoradiograms of ³²P-labeled amplified products from proximal tubules (PCT and PR), medullary ascending limb (mTAL) and cortical ascending limb (cTAL) of Henle's loop, distal convoluted tubule (DCT), cortical collecting duct (CCD), and inner medullary collecting duct (IMCD), using mCAP1 and β -actin primers. No labeled bands were detected by omitting cDNA (C).

panel). These results suggested that the intrinsic channel activity was increased in the presence of mCAP1.

Discussion

mCAP1 Has the Expected Properties for a Mammalian Serine Protease Regulating ENaC Activity in CCD Cells

mCAP1 shares 50% homology with *Xenopus* CAP1 (6) and 80% with prostaticin (8). These three serine proteases share the same predicted structure, *i.e.*, a cleavable signal sequence at the amino terminus and a consensus sequence for GPI membrane anchoring site, known to target proteins at the apical membrane of epithelial kidney cells (6). mCAP1 shares the same functional properties as xCAP1, *i.e.*, its ability to activate ENaC from three different species (*Xenopus*, rat, and human). The activation by mCAP1 can be inhibited by aprotinin. Recently, Nakhoul *et al.* (14) have found that aprotinin added to the luminal side of cultured mouse collecting duct M-1 cells induces a 50% decrease of I_{sc} , similar to that found in the cultured mpkCCD_{c14} cells (Figure 1). As aprotinin fully inhib-

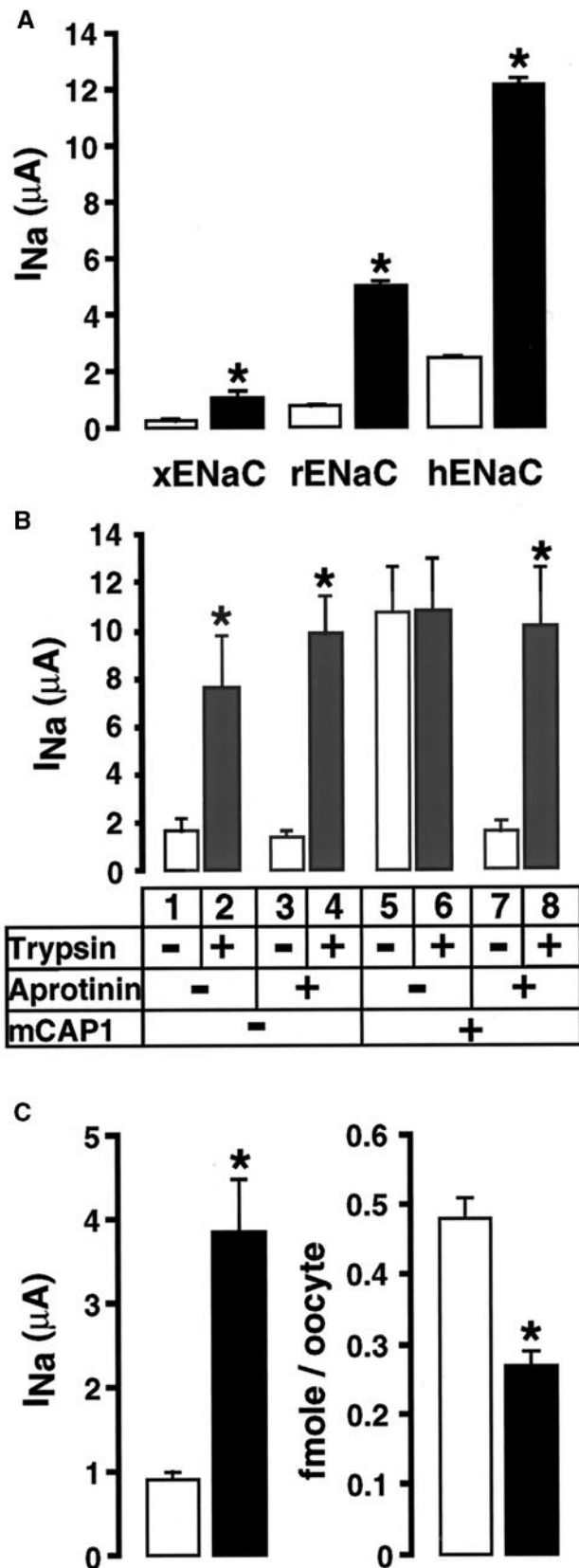


Figure 5. Functional analysis in *Xenopus* oocytes. (A) Effect of mCAP on I_{Na} in oocytes. Oocytes were injected with *Xenopus* (left), rat (middle), and human (right) ENaC subunits in the presence of mCAP1 (filled bars) or water (open bars). (B) Effect of perfusion of trypsin (closed bars, lanes 2, 4, 6, and

its the I_{sc} measured in *Xenopus* A6 cells (6), mCAP1 appears to be less sensitive to aprotinin than its amphibian homologue, suggesting that the binding site for the serine protease inhibitor may be somewhat different. Alternatively, mpkCCD_{c14} cells may express other proteolytic enzymes that would be resistant to aprotinin. Additional pharmacologic studies should clarify this point.

In a previous study (6), we showed that xCAP1 increased the amiloride-sensitive sodium current but induced a small but nonsignificant decrease (–20%) in the binding of anti-FLAG antibodies. Because the increase in I_{Na} could not be explained by a change in the single channel conductance or in the cell surface expression of ENaC, we suggest that the increase in I_{Na} was due to an increase in the overall open probability of the channel molecules present at the oocyte surface (12,13,15).

Like xCAP1, mCAP1 appears to activate preexisting channels located in the plasma membrane, because the number of molecules expressed at the surface is not increased by mCAP1 but is significantly decreased (by 50%). According to this assay, we propose that mCAP1 has a major effect on the overall open probability of the channel if one assumes that mCAP1 has no effect on ion selectivity and/or single channel conductance.

mCAP1 Is Probably the Mouse Counterpart of Human Proximin

According to the data presented in this study, mCAP1 mRNA transcripts are expressed at high levels in epithelial tissues that are known to be the site of an amiloride-sensitive transepithelial sodium transport (CCD, colon, lung, salivary gland, skin, bladder). mCAP1 is also expressed at low levels in other epithelial tissues, such as the stomach, duodenum, and small intestine, where ENaC might be expressed under some special pathophysiologic conditions. It is not yet known whether human proximin can activate ENaC in the kidney, the colon, or the lung. However, preliminary experiments have shown that it is the case in the *Xenopus* oocyte system (unpublished observation). On the basis of tissue-specific expression and functional studies, we would therefore like to propose

8) and preincubation with aprotinin (lanes 3, 4, 7, and 8) of oocytes injected with rat ENaC in the presence (lanes 5 to 8) or absence of mCAP1 (lane 1 to 4). The amiloride-sensitive current I_{Na} increases in the presence of mCAP1 in oocytes incubated in modified Barth saline solution (MBS) (lane 1 versus lane 5). Preincubation of the oocytes in MBS containing 100 µg/ml aprotinin prevented the effect of mCAP1 on I_{Na} (lanes 3 and 7), and perfusion of 2 µg/ml trypsin during the recording had no additive effect on the increase of the I_{Na} due to mCAP1 expression (lane 5 versus lane 6). Trypsin induced a large increase with maximum intensity after 2 to 3 min of perfusion in the absence of mCAP1 (lane 2), and this effect was not repressed by preincubation of the oocytes with aprotinin in the absence (lane 4) or presence (lane 8) of mCAP1. (C) I_{Na} (left) and cell surface expression of ENaC (right panel) in the presence (filled bars, $n = 25$) or absence (open bars, $n = 29$) of mCAP1.

that mCAP1, xCAP1, and prostaticin are the corresponding genes for the three species studied so far.

Regulation of ENaC Activity and Sodium Transport

Using isolated microdissected tubules, the results from RT-PCR experiments clearly indicate that mCAP1 is expressed at high levels in the proximal tubule and to a lesser extent in the distal part of the nephron and the CCD (Figure 4). Although we have not analyzed the expression of mCAP1 at the protein level, the high expression of mCAP1 in the proximal tubule could indicate a local mode of action. In the rat, ENaC transcripts and ENaC activity are specifically found in the S3 segment of the proximal tubule, and the presence of an amiloride-sensitive sodium transport supports this finding (16). In the present study, mCAP1 appears to be expressed all along the proximal tubule (from S1 to S3). Another interesting and more intriguing possibility is that mCAP1 is secreted into the tubular fluid of the proximal tubule to activate ENaC expression in cells of the distal part of the nephron. Such a distant mode of action has also been proposed for other secreted proteases such as kallikrein (17). The second possibility predicts that mCAP1 is synthesized in cells of the CCD, then targeted to the apical membrane to control *in situ* the activity of ENaC. This is supported by: (1) the detection of mCAP1 mRNA transcripts in the collecting duct and in a highly differentiated cell line deriving from mouse CCD; (2) the presence of serine protease activity in the apical membrane of these cells, which can be inhibited by aprotinin; and (3) the regulation of ENaC activity in oocytes. Both modes of action are not mutually exclusive. The regulation of each of these two pathways could be different in function of various physiologic or pathophysiologic stimuli. Although it is not excluded that other serine protease may activate ENaC *in vivo*, the cloning of mCAP1 will allow us to study the physiologic role of this serine protease *in vivo* by gene targeting and to assess its physiologic relevance in the control of sodium balance, blood volume, and BP.

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