Molecular cloning and characterization of a novel carboxylesterase-like protein that is physiologically present at high concentrations in the urine of domestic cats (*Felis catus*)

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Normal mammals generally excrete only small amounts of protein in the urine, thus avoiding major leakage of proteins from the body. Proteinuria is the most commonly recognized abnormality in renal disease. However, healthy domestic cats (*Felis catus*) excrete proteins at high concentrations (about 0.5 mg/ml) in their urine. We investigated the possible cause of proteinuria in healthy cats, and discovered a 70 kDa glycoprotein, which was excreted as a major urinary protein in cat urine, irrespective of gender. To elucidate the biochemical functions and the excretion mechanism of this protein, we cloned the cDNA for this protein from a cat kidney cDNA library. The deduced amino acid sequence shared 47% identity with the rat liver carboxylesterase (EC 3.1.1.1), and both the serine hydrolase active site and the carboxylesterase-specific sequence were conserved. Therefore we named this protein cauxin

(carboxylesterase-like urinary excreted protein). In contrast to the mammalian carboxylesterases, most of which are localized within the cells of various organs, cauxin was expressed specifically in the epithelial cells of the distal tubules, and was secreted efficiently into the urine, probably because it lacked the endoplasmic reticulum retention sequence (HDEL). Based on our finding that cauxin is not expressed in the immature cat kidney, we conclude that cauxin is involved in physiological functions that are specific for mature cats. Recently, cauxin-like cDNAs were found from human brain and teratocarcinoma cells. These data suggest that cauxin and cauxin-like human proteins are categorized as a novel group of carboxylesterase multigene family.

Key words: cauxin, distal tubules, glycoprotein, urinary protein.

INTRODUCTION

Normal mammals physiologically excrete only small amounts of proteins into the urine to avoid major protein leakage from the body. The plasma proteins that pass through the glomerular filtration barrier are selected according to size, charge, and shape, and over 99% of the filtered proteins are reabsorbed by the proximal tubules [1,2]. Several secretory proteins, including the Tamm-Horsfall urinary glycoprotein [3], which is secreted from the distal tubules, and nephrocalcin [4], which is secreted from the proximal tubules, are physiologically excreted at low concentrations into the urine of normal mammals. In contrast increased excretion of urinary proteins is recognized as one of the indications of renal abnormality [5]. Specific urinary proteins, such as the 65 kDa albumin, the 12 kDa β 2-microglobulin, and the 21 kDa retinal-binding protein, are excreted at elevated levels from the early stages of renal disease, and proteinuria is a diagnostic marker of renal disease [6].

Domestic cats (*Felis catus*) show increased morbidity from chronic renal disease with advanced age [7]. In the course of investigating chronic renal disease in cats, we noted that healthy cats excreted high concentrations of protein in the urine. Several groups have reported the excretion levels of urinary proteins for normal cats [8,9], but these studies have not been extended. To elucidate the cause of proteinuria in healthy cats, we analysed the proteins that were excreted in healthy cat urine. Interestingly our analysis indicated that a 70 kDa protein was excreted at high

concentration as the major urinary protein in healthy cats of both genders. We analysed this protein, which was purified from cat urine, and found that the partial amino acid sequence of the 70 kDa protein showed a similarity to proteins of the serine hydrolase family, such as the mammalian carboxylesterases (EC 3.1.1.1).

In this paper, we describe the characterization and tissue distribution of a novel 70 kDa protein, which we designate as cauxin (carboxylesterase-like urinary excreted protein). Furthermore, we discuss the physiological role of cauxin, and report that a cauxin-like gene was recently found in human brain and teratocarcinoma cells [New Energy and Industrial Technology Development Organization (NEDO) Human cDNA Sequencing Project].

EXPERIMENTAL

Animals and sample collection

Urine samples were collected from 54 healthy mature cats (38 male and 16 female; aged from 8 months to 10 years) and 15 healthy immature cats (ten male and five female; aged from 1 day to 3 months) at the Veterinary Medical Center, Iwate University. Each cat was determined to be healthy, based on evaluation of results of physical examination, complete blood cell count, serum biochemical profile, and urinalysis. To obtain the tissue samples, a healthy cat (2-year-old, female) and an immature cat (1-month-

Abbreviations used: CBB, Coomassie Brilliant Blue R-250; DIG, digoxigenin; ER, endoplasmic reticulum; HRP, horseradish peroxidase; 1-NA, 1-naphthylacetate; p-NPA, p-nitrophenylacetate; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction.

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old, female) were killed with pentobarbital (30 mg/kg, intravenous injection) after anaesthetizing with ketamine (50 mg/kg, intramuscular injection). All procedures were approved by the Animal Research Committee, Faculty of Agriculture, Iwate University, and they were conducted under the Guidelines for Animal Experiment in Iwate University.

Analyses of urinary proteins

A sample of urine was taken by direct manual pressure of the cat bladder. The urine samples were sedimented for 5 min at 500 *g*, to discard occasional cell debris, and were stored at -20°C for later analysis. The urinary protein concentrations were measured by the Bradford method using BSA as the standard. Aliquots of urine (20 μl) were resolved by SDS/PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue R-250 (CBB).

Isolation of a full-length cDNA clone for cauxin

Cauxin was purified from a CBB-stained polyacrylamide gel band using an electro-eluter (Electro-Eluter 422, Bio-Rad, Hercules, CA, U.S.A.). Cauxin was transferred to PVDF membranes (Immobilon; Millipore, Canton, MA, U.S.A.) by semi-dry blotting, and its N-terminal amino acid sequence was determined using a protein sequencer (PPSQ-21, Shimadzu, Kyoto, Japan). The internal peptide products, which were produced by digestion of cauxin with lysyl endopeptidase (Wako Chemicals, Osaka, Japan), were purified by reversed-phase HPLC (SMART system, Amersham Pharmacia, Piscataway, NJ, U.S.A.) using a $\mu\text{RPC C2/C18}$ column (Amersham Pharmacia). The amino acid sequence of the purified internal peptide products were determined using a protein sequencer. Based on the amino acid sequences, pairs of degenerate PCR primers were designed as follows: the forward primer, 5'-TYCCNGAYGARCCNG-NGTNGC-3' (where Y = C or T; N = C, G, A, or T; R = G or A) was based on the internal peptide sequence STVPVNMF; and the reverse primer, 5'-CATRTTNACNGGCATNGTNG-3' was based on the internal peptide sequence SIPVIDD. In order to determine the tissue distribution of cauxin, we produced an anti-cauxin rabbit serum by immunization with gel-purified cauxin. Subsequently, we used this antibody to perform Western blotting and immunohistochemistry. On the basis of these results, RT-PCR was performed with 1 μg of total RNA from cat kidney with the degenerate PCR primers using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions. After PCR amplification, a partial fragment of cauxin cDNA was sequenced and labelled using the Alkphos Direct Labeling Kit (Amersham Pharmacia). Using this labelled fragment, the full-length cauxin cDNA clone was isolated by plaque hybridization from a cat kidney cDNA library, which was constructed using the SMARTTM cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, CA, U.S.A.), according to the manufacturer's instructions. All sequencing reactions were performed using the BigDye terminator method and the ABI Prism 310 DNA Sequencer (Applied Biosystems).

The amino acid sequence of the cauxin cDNA was compared with the amino acid sequences in the GenBank database using the FASTA homology search program [10]. The sequences were aligned using the CLUSTAL W program [11]. A pair-wise distance matrix was constructed by calculating the incidence of different amino acids. The phylogenetic tree was calculated by the neighbour-joining method [12]. Bootstrap analysis was used to assess the reliability of branching patterns. One thousand bootstrap replications were performed for each tree.

Deglycosylation

Approximately 20 μl of healthy cat urine was incubated at 37°C for 18 h with endoglycosidase F (Takara, Osaka, Japan) in 10 mM Tris/HCl (pH 8.6) plus 0.01 % SDS. The samples were subjected to SDS/PAGE (12 % gels) under reducing conditions and stained with CBB.

Enzyme assay

Approximately 5 ml of cat urine was dialysed against 3 litres of 10 mM Tris/HCl (pH 7.4) for 12 h at 4°C . The dialysed urine was applied to a 6 ml anion-exchange column (Resource Q; Amersham Pharmacia), which was equilibrated with the buffer. The adsorbed proteins were eluted with a gradient of 0–400 mM NaCl in the buffer at a flow rate of 3 ml/min, and 35 fractions were collected at 30 s intervals. Aliquots (10 μl) of each fraction (1.5 ml) were assayed for esterase activity against *p*-nitrophenylacetate (*p*-NPA). The hydrolysis of *p*-NPA was determined in 50 mM Tris/HCl (pH 8.0), according to the method of Krusch [13]. The K_m and maximal velocity (V_{max}) values for *p*-NPA were calculated from the linear region of the Lineweaver–Burk plots. Aliquots (20 μl) of each fraction were resolved on native-PAGE (12 % gel) at 4°C . After electrophoresis, the polyacrylamide gel was washed for 15 min in 0.1 M potassium phosphate buffer (pH 6.5), and incubated in the same buffer containing 1-naphthylacetate (1-NA, 5 mM) and Fast Blue RR (0.4 mg/ml) for 15 min at 25°C . The esterase staining method was based on the formation of a black, insoluble complex consisting of 1-naphthol (the product of 1-NA hydrolysis) and diazotized Fast Blue RR [14].

Northern blotting

Aliquots (20 μg) of total RNA were prepared from major cat tissues using the acid guanidium thiocyanate phenol-chloroform extraction method. The RNA samples were electrophoresed on a 1.0 % agarose gel containing formaldehyde, and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia). The membrane was hybridized at 65°C for 12 h in the presence of 50 % formamide, together with the full-length open reading frame (ORF) of cauxin cDNA, which was labelled using the Alkphos Direct Labeling Kit (Amersham Pharmacia). Following hybridization, the membrane was washed twice for 15 min at 65°C with $1 \times \text{SSC}$ (0.15 M NaCl/0.015 M sodium citrate) plus 0.1 % SDS, and twice for 15 min at 65°C with $0.1 \times \text{SSC}$ plus 0.1 % SDS. The bands were detected using CDP-Star (Amersham Pharmacia) and exposed to an X-ray film (50 NIF, Fuji Photo Film, Tokyo, Japan).

Production of the anti-cauxin peptide antibody

The peptide SVGQKLKEQEVEFWMTIVP, which corresponds to the C-terminal region of cauxin, was synthesized using an automatic peptide synthesizer (PSSM-8, Shimadzu). The synthetic peptide was conjugated with keyhole limpet haemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide, and was injected into a rabbit to raise a polyclonal anti-cauxin peptide antibody [15].

Western blotting

Approximately 1.5 g of frozen major cat tissues were homogenized in 5 ml of 10 mM Tris/HCl (pH 7.4) containing 5 mM EDTA. The homogenate was centrifuged at 15000 *g* for 30 min at 4°C . The supernatant fraction was filtered through DISMIC-25cs (Advantec, Tokyo, Japan) to remove fat. For Western-blot

Figure 1 Analysis of urinary proteins by SDS/PAGE and CBB staining

Aliquots (20 μ l) of the urine samples and 10 μ g of cat serum protein were resolved by SDS/PAGE (12% gel) under non-reducing conditions with CBB staining. **(A)** Analysis of urinary proteins from healthy male and female cats, immature cat, and of cat serum proteins. **(B)** Analysis of urinary proteins from healthy cat, human, dog, and cow. The location of the cauxin protein, albumin (Alb), and Tamm–Horsfall urinary glycoprotein (THP) are indicated on the left. The positions of the molecular mass markers (kDa) are shown on the right. **(C)** Aliquots (20 μ l) of cat urine were incubated for 18 h in the absence (–) or presence (+) of endoglycosidase F (endo F), and subjected to SDS/PAGE under reducing conditions, followed by CBB staining.

analysis, the 1 μ g samples of protein from cat urine, major cat tissues, and serum were resolved by SDS/PAGE (12% gel) under reducing conditions, and then transferred to PVDF membranes by semi-dry blotting. The membranes were blocked with 1% polyvinyl-pyrrolidone in TBS-T [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20], and incubated with a 1:10000 dilution of the anti-cauxin peptide antibody. After incubation with a 1:8000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), the membranes were washed with TBS-T, developed using ECL[®] (Amersham Pharmacia), and exposed to X-ray film (50 NIF, Fuji Film).

In situ hybridization

Kidney samples from both mature and immature cats were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 12 h. For the paraffin sections, the samples were dehydrated through a graded series of ethanol solutions at 4 °C, embedded in paraffin, and sectioned at 4 μ m. For experiments with riboprobes, digoxigenin (DIG)-labelled antisense and sense RNA probes were prepared from the full-length ORF of cauxin cDNA using a DIG RNA Labeling Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization was performed at 42 °C on the deparaffinized sections. Hybridized riboprobes were detected with an alkaline

phosphatase-conjugated anti-DIG antibody (Roche), and visualized with Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

Immunohistochemistry

After the endogenous peroxidase was blocked with 0.3% H_2O_2 -methanol, the deparaffinized sections were incubated with normal porcine serum (Dako, Glostrup, Denmark). The sections were then incubated with the anti-cauxin peptide antibody (1:500 dilution) for 12 h at 4 °C. After treatment with a 1:100 dilution of porcine biotin-conjugated anti-rabbit immunoglobulin, the sections were incubated to permit formation of the streptavidin–biotin–horseradish peroxidase (HRP) complexes. The sections were treated with 0.02% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 to enable visualization of the antigen–antibody reaction. Finally the sections were counterstained with haematoxylin. For the cryostat sections, the kidneys of mature and immature cats were immersed in 0.1 M sodium phosphate buffer, pH 7.4, with 30% sucrose, snap frozen in OCT compound (Miles Scientific, Naperville, IL, U.S.A.) at –80 °C, and sectioned at 6 μ m. The cryostat sections were treated with goat serum, incubated with a 1:500 dilution of the anti-cauxin peptide antibody for 12 h at 4 °C, and incubated with a 1:100 dilution of mouse FITC-conjugated anti-rabbit IgG antibody

cauxin	MSGVWHPGRTLIWLVLAAPVKGPAADAEVRSRLGWVRGKOTVVLGSVVPVNVFLCI	60
h-teratocarcinoma	MSGVWHPGQILWLVLAAPVKGPSAEAGEORNLRLGWTQSKOVTVLGSVVPVNVLELV	60
h-brain	-----	
cauxin	PYAAPPLGFLRFKQPEALPGNDPRNATSYEKLCFDLEWTVSYQHVLKVRYPKLEASD	120
h-teratocarcinoma	PYAAPPLGSLRFETNEQASEWDLNLCATSYENLCLNSEWILLDOHMLKVHYPKFGVSED	120
h-brain	-----MLKVHYPKFGVSED	14
cauxin	CLYLNLYAPAHADNGSNLPVWVFPFGGAFKGSASSFDGSALAAYEDVLIIVTQYRLGIF	180
h-teratocarcinoma	CLYLNLYAPAHADNGSKLPVWVFPFGGAFKGSASSFDGSALAAYEDVLIIVVQYRLGIF	180
h-brain	CLYLNLYAPAHADNGSKLPVWVFPFGGAFKGSASSFDGSALAAYEDVLIIVVQYRLGIF	74
cauxin	GFFDTCDDHAFGNWALDQVAALTWVRDNIIEFFGGDPRSVTIFGESAGAISSVSLILSP	240
h-teratocarcinoma	GFFFTWDCHAFGNWAFDQVAALSWVQRNIEFFGGDPRSVTIFGESAGAISSVSLILSP	240
h-brain	GFFFTWDCHAFGNWAFDQVAALSWVQRNIEFFGGDPRSVTIFGESAGAISSVSLILSP	134
cauxin	AKGLFHKAIMESGVATLPLDMRPPGDERKKDQVVARICCCASDSALLOCLRAKPSSE	300
h-teratocarcinoma	AKGLFHKAIMESGVATLPLDLEADYE-KSEDLQVVAHFCGNASDSALLCLRAKPSSE	299
h-brain	AKGLFHKAIMESGVATLPLDLEADYE-KSEDLQVVAHFCGNASDSALLCLRAKPSSE	193
cauxin	LDLISKILTFSTIPYIDFFPDEPVALLTOKAFNSVPSIIGVNNHECAFLLSTEPS-EPI	360
h-teratocarcinoma	LDLTSQRTKSFTRVVGAFPPNEPLDLLSQAKFAIPSIIGVNNHECGFLPMKEAPEPI	359
h-brain	LDLTSQRTKSFTRVVGAFPPNEPLDLLSQAKFAIPSIIGVNSHECGFLPMKEAPEPI	253
cauxin	SGSNRSLALYFVHTFNIPIFOYLHLVADHYEYKHSFVEIRDSFLDLLGDVLEVVFGVVA	420
h-teratocarcinoma	SGSNKSLALHIIQNIHPIFOYLHLVANEYEDKHSLTEIRDSFLDLLGDVLEVVFGVVA	419
h-brain	SGSNKSLALHIIQNIHPIFOYLHLVANEYEDKHSLTEIRDSFLDLLGDVLEVVFGVVA	313
cauxin	ARYHRDAGAPVYFYEFOHPQCLNDTRPAFVKADHSDENRFVFGGAFLKGDIVMFEGATE	480
h-teratocarcinoma	ARYHREG-----ATE	429
h-brain	ARYHRDAGAPVYFYEFRHRPQCFEDTKPAFVKADHSDENRFVFGGAFLKGDIVMFEGATE	373
cauxin	EEKLLSRKMMRYWANFARTCPNGEGVPLWPAYTQSEOYLKDLISVSVQKLEKBOEVEFW	540
h-teratocarcinoma	EEKLLSRKMMRYWANFARTCPNPGNDLSLWPAYNLTEOYLQDLNMSLQORLEKBPVDEFW	489
h-brain	EEKLLSRKMMRYWANFARTCPNPGNDLSLWPAYNLTEOYLQDLNMSLQORLEKBPVDEFW	433
cauxin	MNTIYP-----	545
h-teratocarcinoma	TSIIPLILSASDMLHSLSLTFLSLLQFFFFFCAP	525
h-brain	TSIIPLILSASDMLHSLSLTFLSLLQFFFFFCAP	469

Figure 3 Amino acid sequence alignment of cauxin, the BRAWH2002191 protein, and the NT2RI2001010 protein

Amino acid residues that are shared with the cauxin protein are shown in black boxes. Hyphens represent gaps that were introduced for maximal alignment. The conserved active site GXSGXG motif of serine hydrolases is underlined. The conserved specific site EDCLY motif of carboxylesterases is double underlined. BRAWH2002191 protein (h-brain), T2RI2001010 protein (h-teratocarcinoma).

Figure 4 Analysis of the esterase activity of cauxin

Cat urine (5 ml) was dialysed against 10 mM Tris/HCl (pH 7.4) and applied to an anion-exchange column (Resource Q, Amersham Pharmacia). The adsorbed proteins were eluted with a gradient of 0–400 mM NaCl at a flow rate of 3 ml/min, and 35 fractions were collected at 30 s intervals. The fractions were assayed for esterase activity against *p*-NPA (A, solid line with circles), and subjected to native PAGE with staining for esterase activity against 1-NA (B). Elution was monitored by spectrophotometry at A_{280} (A, dotted line). (C) Western blots of the same fractions, in which the anti-cauxin peptide antibody was used.

Isolation and characterization of a cauxin-encoding cDNA clone

The partial amino acid sequence of the N-terminus and eight internal peptides of cauxin were determined using a protein

Figure 5 Tissue distribution analysis of cauxin by Northern and Western blotting

(A) Northern blotting of cauxin mRNA. Aliquots (20 μ g) of total RNA from various tissues of normal cats were analysed as described in the Experimental section. (B) Western blotting using the anti-cauxin peptide antibody. Samples of protein (1 μ g) from cat urine, kidney, bladder, and serum were analysed. The tissue sources are indicated at the top of the figure.

sequencer, and degenerate PCR primers were designed. The tissue distribution of cauxin was also analysed by Western blotting and immunohistochemistry using anti-cauxin rabbit serum, which was produced by immunization with cauxin that was purified from urine, and it was found that cauxin was

Figure 6 Intracellular localization of cauxin in cat kidney

In situ hybridization and immunohistochemical analyses were performed on tissue sections of normal cat kidney. (A), (C), and (D) show images from the *in situ* hybridization experiments. (B) and (E) show images from immunohistochemical analysis using HRP detection. (A) and (B) show images of the kidney between the cortex and medulla. (C), (D), and (E) show images of the renal tubules of the outer medulla of the kidney. (A) and (B), original magnification $\times 50$; (C), original magnification $\times 100$; (D) and (E), original magnification $\times 200$.

expressed in the renal tubules of cat kidney (results not shown). Based on these results, a 742 bp fragment of cauxin cDNA was amplified from total RNA of cat kidney using the degenerate PCR primers. The amino acid sequence encoded by this fragment included five internal peptides of cauxin. Using the labelled 742 bp fragment, a full-length cauxin cDNA clone was isolated from the cat kidney cDNA library. The nucleotide sequence of the cauxin cDNA and the deduced amino acid sequence of cauxin are shown in Figure 2. The cDNA clone carried 2139 bp of DNA and contained an ORF that encoded a putative polypeptide of 545 amino acid residues with a molecular mass of 60.2 kDa. The putative cauxin protein contained a signal peptide of 28 amino acid residues and four potential *N*-linked Asn-X-

Ser/Thr glycosylation sites. Cauxin was treated with endoglycosidase F to examine the extent of glycosylation, and the relative molecular mass of the deglycosylated product was compared with that of the untreated protein. A reduction in molecular mass of approx. 10 kDa was observed, which suggests that cauxin is a glycoprotein (Figure 1C).

A FASTA homology search of the GenBank® database revealed that the amino acid sequence of the putative cauxin protein shared 73% identity with a protein encoded by the human cDNA clone BRAWH2002191 (GenBank® accession no. AK090997), 64% identity with a protein encoded by the human cDNA clone NT2RI2001010 (GenBank® accession no. AK056109), 48% identity with the domestic duck thioesterase B,

Figure 7 Comparison of tissue localization of esterase activity with that of cauxin

(A, B), and (C, D) show images of cryostat sections from similar locations in the renal cortexes of mature and immature cats, respectively. (A) and (C) show sections that were stained for 1-NA esterase activity. (B) and (D) show immunofluorescent images of sections that were treated with anti-cauxin peptide antibody and detected using FITC.

and 47% identity with the rat carboxylesterase (EC 3.1.1.1). The amino acid sequence comparisons are shown in Figure 3. BRAWH2002191 was cloned from a human brain cDNA library (NEDO Human cDNA Sequencing Project), encoding 469 amino acids. NT2RI2001010 was cloned from a cDNA library of the human teratocarcinoma NT2 cell line (NEDO Human cDNA Sequencing Project), encoding a protein of 525 amino acids. BRAWH2002191 and NT2RI2001010 are reported to be slightly similar to the fatty acyl-CoA hydrolase (thioesterase B). Thioesterase B and the carboxylesterases belong to the serine hydrolase family. Thioesterase B is thought to be a member of the carboxylesterase multigene family, whose members have acyl specificity for medium-chain-length fatty acids [17]. These serine hydrolase family proteins have a conserved GX SXG motif as the active site of the enzyme. Furthermore, the carboxylesterase family proteins have in common the EDCLY sequence, which is associated with a disulphide bridge that forms a variable lid-like loop over the active site in serine esterases. We found that cauxin contained these two motifs, in the form of GESAG and EDCLY.

Esterase activity of cauxin

Based on the results of the homology search, we analysed the esterase activity of the purified cauxin. Cat urine was dialysed against 10 mM Tris/HCl (pH 7.4) and applied to a 6 ml anion-exchange column (Resource Q; Amersham Pharmacia). The adsorbed proteins were eluted with a gradient of 0–400 mM NaCl, and 35 fractions were assayed for esterase activity. The activity of *p*-NPA esterase was detected in fraction numbers 16–21 as a single peak (Figure 4A). The fractions were also analysed by native PAGE with 1-NA esterase activity staining, as shown in Figure 4(B). The major esterase activity bands were detected in fractions 16–21. The native PAGE samples were also subjected to Western blotting using the anti-cauxin peptide antibody. Western blotting showed that the bands that had esterase activity in native PAGE (fractions 16–21) corresponded to cauxin (Figure 4C). These results indicate that cauxin hydrolyses *p*-NPA and 1-NA. Furthermore, we determined that

the K_m of cauxin for *p*-NPA hydrolysis was 506 μ M, and that the V_{max} value of cauxin was 7.61 μ mol/mg/min, which are about 10–40-fold lower than the corresponding values for carboxylesterase isozymes reported previously [18–20].

Tissue distribution of cauxin

Northern blotting showed that cauxin mRNA was present only in the kidney, and not in any of the other tissues examined (Figure 5A). Western blotting of urine, whole tissue homogenates, and plasma proteins, with anti-cauxin peptide antibody, showed that cauxin was present in cat urine and kidney homogenates (Figure 5B). *In situ* hybridization with the antisense and sense RNA probes, which were prepared from the ORF of cauxin cDNA, demonstrated that cauxin mRNA was expressed on the epithelial cells of the distal tubules, between the outer and inner medullae of cat kidneys (Figures 6A, 6C, and 6D), but not in immature cat kidneys (results not shown). No hybridization signal was observed with the sense probes (results not shown). Immunohistochemistry with the anti-cauxin peptide antibody demonstrated a distribution pattern for cauxin that was very similar to that observed by *in situ* hybridization (Figure 6B). Strong staining was seen in the Golgi of epithelial cells of the distal tubules (Figure 6E).

Esterase activity staining of renal tissues from mature and immature cats

To investigate the localization of esterase proteins in the kidneys of mature and immature cats, we stained tissue sections for 1-NA esterase activity. Esterase activity was clearly detected in the epithelial cells of the proximal tubules of both mature and immature cats (Figures 7A and 7C). In contrast the epithelial cells of the distal tubules of the mature cats showed weak staining, despite high-level expression of cauxin in these cells (Figure 7B). Immunohistochemical analysis of immature cat kidney revealed that cauxin was not expressed in the epithelial cells of the distal tubules (Figure 7D).

DISCUSSION

The data presented here demonstrate that a high concentration (approx. 0.5 mg/ml) of cauxin is physiologically excreted as the major urinary protein in the urine of healthy male and female cats. Based on an estimated daily excretion of 100 ml/day of urine for a healthy cat, approximately 50 mg of cauxin is excreted per day. Although various urinary proteins have been identified, the cauxin-like urinary proteins, which are excreted physiologically at high concentrations in the urine, have not been reported in other normal mammals. Proteinuria is most commonly recognized as one of the indications of renal abnormality; however, our results show that the excretion of cauxin in urine is a physiological phenomenon that is unique to normal cats.

The amino acid sequence of cauxin shares 47% identity with the rat liver carboxylesterases, and the serine hydrolase-specific active site (GX SXG motif) and carboxylesterase-specific sequence (EDCLY motif) are conserved. However, it should be noted that cauxin is a novel carboxylesterase-like protein that differs from the mammalian carboxylesterases in certain features. Carboxylesterases, which are members of the superfamily of serine hydrolases, hydrolyse xenobiotics containing ester, thioester, or amide groups [21,22]. The physiological functions of mammalian carboxylesterases have not been fully elucidated because of their very broad substrate specificities. One of

Figure 8 Phylogenetic tree of cauxin, BRAWH2002191, NT2RI2001010 protein and mammalian carboxylesterases

The tree was constructed by the neighbour-joining method using a total of 17 amino acid sequences. The branch lengths are proportional to the relative phylogenetic distances between the proteins. Bootstrap percentages that support a given partitioning are shown on the branches. The sequence identity percentages (shown in parentheses) are expressed relative to the amino acid sequence of cauxin. The secretory-type proteins are marked by an asterisk. The accession numbers of the analysed sequences are as follows: rat hydrolase A (X51974), hydrolase B (U10697), hydrolase C (U10698) and hydrolase S (M20629); mouse pEs-N (P23953), Es-22 (Q64176), and Es-male (S64130); human CE-1 (P23141) and CE-2 (U60553); pig (Q29550); rabbit form 1 (P12337) and form 2 (P14943); hamster AT519 (D28566).

the important physiological functions of carboxylesterases is the detoxification of drugs [22–24]. Furthermore, some of the carboxylesterase isozymes, which specifically hydrolyse palmitoyl-CoA, acyl-carnitine, and mono- and diacylglycerols, are thought to be involved in the regulation of cellular lipids [25,26]. Typical mammalian carboxylesterases, which include the 57–66 kDa glycoproteins [27], are present in various organs, such as the kidney, liver, lung, small intestine, testis, brain, and blood [28–33]. Based on the analysis of the renal tissue sections using 1-NA esterase activity staining (Figures 7A and 7C), the cat kidney carboxylesterase is probably localized in the epithelial cells of the proximal tubules in an age-independent manner, as reported for other mammalian kidney carboxylesterases [28]. In contrast to the mammalian carboxylesterases, cauxin expression is specific for the epithelial cells of the distal tubules, and cauxin is secreted into the urine. The expression of cauxin appears to have developed uniquely in cats during the process of evolution.

Since cauxin is excreted in the urine, but not detected in the blood by Western blotting, it appears that this protein is secreted from the apical compartment of the epithelial cells of the distal tubules into the renal tubular space (Figure 5B). The N-terminal amino acid sequence contains the proposed signal peptide of 28 highly hydrophobic residues, which provides a targeting signal for the endoplasmic reticulum (ER). Cauxin is transported to the plasma membrane via the ER and Golgi apparatus, and modified by the addition of *N*-linked oligosaccharide chains. Modification by *N*-glycosylation is supported by a decrease in molecular mass after deglycosylation with endoglycosidase F (Figure 1C). After this modification, it is thought that cauxin is transported to the apical membrane for secretion. Comparisons of the C-terminal amino acid sequence of cauxin with typical mammalian carboxylesterases indicate that the C-terminal sequence of cauxin lacks the unique C-terminal ER retention sequence His-Asp-Glu-Leu (HDEL), which functions in protein retrieval from the Golgi apparatus and in protein retention in the ER lumen. The lack of

the C-terminal retention sequence might be explained as an efficient molecular mechanism to allow cauxin secretion from the epithelial cells of the distal tubules into the urine. In addition, we suggest that cauxin contains a targeting signal for apical transport. It has been reported that the *N*-glycans and glycosyl phosphatidylinositol anchor comprise the apical sorting signal of proteins [34–36]. Further studies on the secretion mechanism of cauxin are warranted.

In mammals, urinary proteins that are secreted from the renal tubules into the urine play certain physiological roles in the urine. For example, the Tamm–Horsfall urinary glycoprotein, which is secreted from the distal tubules, is thought to be essential in protecting the kidneys from bacterial infection [37]. Nephrocalcin, which is secreted from the proximal tubules, acts as an inhibitor of calcium oxalate monohydrate crystallization [4]. Since cauxin is excreted in large amounts in cat urine, we suggest that it performs physiological functions in cat urine, as do other urinary proteins that are secreted from the renal tubules.

In this investigation, we uncovered the enzymological properties of cauxin, i.e., the hydrolysis of the ester bonds of *p*-NPA and 1-NA. Furthermore, the substrate specificity of cauxin seems to be different from those of typical mammalian carboxylesterases. Since cauxin is not expressed in the immature cat kidney, it seems to be involved in physiological functions that are specific for mature cats. If cauxin functions as an esterase in cat urine, the physiological substrates for cauxin should be present in mature cat kidneys and/or urine. To elucidate this question, we are currently examining cat-specific substances in the kidneys and urine. Recently, we identified kidney-specific acylglycerols (medium-chain-length fatty acids), which appear in mature cat kidneys but not in other cat organs or in immature cat kidneys. Free medium-chain-length fatty acids are present in cat urine, but not detected in cat kidney (M. Miyazaki, K. Osada, M. Suzuki, A. Suzuki, H. Taira and T. Yamashita, unpublished

work). Some carboxylesterase isozymes reportedly hydrolyse certain lipids, such as palmitoyl-CoA, acyl-carnitine, and mono- and diacylglycerols [25,26]. We postulate that one of the physiological functions of cauxin is to hydrolyse kidney-specific acylglycerols in the urine of mature cats. The urine of mature cats has a uniquely strong odour. The free medium-chain-length fatty acids released by the hydrolysis of kidney-specific acylglycerols in mature cats may be the source of the urine odour, which is used by mature cats for territory marking. Further analysis of cauxin substrates that are present in cat kidneys and urine will aid our understanding of the physiological roles of cauxin.

Based on the results of the NEDO Human cDNA Sequencing Project, cauxin-like cDNAs were found from human brain and teratocarcinoma cell. Although the biochemical and physiological functions of these cDNAs encoded proteins are unknown, it has been speculated that cauxin-like proteins are expressed in the kidney and/or other organs of humans. To clarify the evolutionary lineage of cauxin, we constructed a phylogenetic tree using the neighbour-joining method and a total of 17 amino acid sequences (Figure 8). The structure of the tree suggested the existence of four distinct groups: Group 1, the major group of nine carboxylesterases, which includes rat hydrolase A [38], rat hydrolase B [28], rat hydrolase C [28], rat hydrolase S [39], mouse carboxylesterase Es-22 [40], mouse carboxylesterase pEs-N [41], pig carboxylesterase [42], human carboxylesterase 1 [43], rabbit carboxylesterase form 1 [44], and duck thioesterase B [17]; Group 2, three carboxylesterases that include human carboxylesterase 2 [45], rabbit carboxylesterase form 2 [46], and hamster carboxylesterase AT51p [47]; Group 3, cauxin, the BRAWH-2002191 protein, and the NT2RI2001010 protein; and Group 4, mouse carboxylesterase Es-male [48]. The phylogenetic tree analysis also indicates that cauxin is more closely related to the BRAWH2002191 protein and the NT2RI2001010 protein than to thioesterase B or the secretory-type carboxylesterases, such as rat hydrolase S and mouse carboxylesterase pEs-N. These results suggest that cauxin, the BRAWH2002191 protein, and the NT2RI2001010 protein are categorized as a novel group (we designated as cauxin family) of carboxylesterase multigene family. Future studies may well uncover cauxin-like proteins in other animal species.

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