Additions and Corrections

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Page 13374, Table II: This table was printed incorrectly. The correct table is shown below:

TABLE II
Digestion of synthetic peptide substrates by nephrosin

Substrates	Sequences[a]	Hydrolysis
Tosyl-GPK-NA	GPK	ND[b]
Suc-AAPF-NA	AAPF	ND[b]
Suc-AAPL-NA	AAPL	ND[b]
Cbz-GGL-NA	GGL	ND[b]
Substance P	RPKPQQ ↓ F ◆ FGLM-NH_2	Complete
Lys-bradykinin	KRPPG ↓ F ◆ SPFR	Complete
Bradykinin 1–5	RPPGF	ND[b]
Bradykinin 1–6	RPPGFS	ND[b]
Bradykinin 1–7	RPPG ↓ FSP	Partial
des-Arg-bradykinin	PPGF ↓ SPFR	Complete

[a] ↓ indicates bonds cleaved by carp nephrosin, and ◆ indicates major cleavage site.
[b] ND, nondetectable in 60 min.

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Purification and Cloning of Carp Nephrosin, a Secreted Zinc Endopeptidase of the Astacin Family*

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We have purified a secreted proteinase of 23 kDa from carp head kidney by sequential column chromatography on a Reactive Blue 72-agarose dye affinity column and an FPLC Mono-P column. The secretion of this proteinase from carp head kidney can be stimulated by high concentrations of potassium. Since the carp proteinase is present mainly in the head kidney, kidney, and spleen (all of which are lymphohematopoietic organs), it is named nephrosin. The carp nephrosin is most sensitive to metal chelators, but not to inhibitors specific for other classes of proteinases. A cDNA clone has been isolated from a carp head kidney cDNA library by immunoscreening with a polyclonal antiserum raised against purified nephrosin. The cloned cDNA is 1086 base pairs in length and has an open reading frame encoding a protein of 273 amino acids, including a 19-amino acid signal peptide and 56-amino acid propeptide. The deduced amino acid sequence shows moderate levels of identity to medaka HCE1 (52.5%), medaka LCE (50.7%), crayfish astacin (33.2%), murine meprin-α (34%), and murine meprin-β (33.5%), all members of the astacin family of zinc endopeptidases. Nephrosin is the first member of the astacin family found in lymphohematopoietic tissues.

Teleost head kidney consists of interrenal tissue (1–3) homologous to the adrenal cortex, chromaffin cells (4, 5) responsible for epinephrine and norepinephrine secretion, immune cells (6–9) responsible for immunoglobulin M secretion, and hematopoietic cells (6, 10, 11). Bone marrow is not present in fish, whereas hematopoietic tissues and lymphatic tissues coexist in the lymphohematopoietic organs. In addition to the head kidney, spleen and kidney are also lymphohematopoietic organs in teleost fish (6).

The presence of four different types of cells (neurons, endocrine cells, immune cells, and hematopoietic cells) in the head kidney prompted us to study the secreted proteins from head kidney since these cells are all active in secretion. Interacting molecules may be secreted by one cell type in a paracrine mode to exert its effects on neighboring cells or on the extracellular matrix (ECM).[1] Therefore, we attempted to purify the secreted proteins from carp head kidney and eventually to understand the functions of the secreted molecules. Here, we report the purification and cloning of one secreted protein, nephrosin. Nephrosin is a zinc metalloproteinase and is present mainly in the gill, kidney, head kidney, and spleen. We have also demonstrated that secretion of nephrosin can be stimulated by high concentrations of extracellular potassium. Amino acid sequence analysis reveals that nephrosin is most closely related to medaka hatching enzymes, members of the astacin family (12). However, our evidence suggests that nephrosin is probably not a hatching enzyme.

EXPERIMENTAL PROCEDURES

Perfusion of Carp Head Kidney—Common carp (*Cyprinus carpio*) purchased from a local fish market were held in plastic tanks (40 × 45 × 80 cm) under natural temperature and photoperiod for 1 day before experiments. Fish were decapitated between 10 and 11 a.m. The head kidney was carefully removed and placed in the normal perfusion buffer consisting of 125 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$, 10 mM glucose, and 10 mM $NaPO_4$, pH 7.4. The setup of perfusion system was essentially the same as described (13). Following a 90-min stabilization period, perfusate samples were collected for eight 10-min intervals. For the control group, tissue was perfused with the normal perfusion medium throughout the perfusion period, whereas for the experimental group, a high potassium perfusion medium (50 mM potassium, 80 mM NaCl, 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$, 10 mM glucose, and 10 mM $NaPO_4$, pH 7.4) was administered for 20 min starting from collection interval two. Perfusate samples were then frozen at −20 °C until use for immunoblot, zymographic (see below), and lactate dehydrogenase assay (14).

Purification of Nephrosin—Carp head kidneys (20 g) were homogenized in 200 ml of 20 mM Tris-HCl containing 5 mM EDTA, pH 8.0, by a glass homogenizer. The supernatant was collected by centrifugation at 12,000 × *g* for 30 min, and 0.3 M NaCl was added to the supernatant. The sample was then applied onto a Reactive Blue 72-agarose column (2.5 × 10 cm; Sigma) equilibrated with 0.3 M NaCl in the homogenization buffer and eluted stepwise by 0.3 M, 0.6 M, and 1.0 M NaCl in the homogenization buffer. All three elution steps would desorb nephrosin from the column but the 0.6 M NaCl fractions contained nephrosin activity of the highest purity. The 0.6 M NaCl fractions were pooled, lyophilized, and dissolved in 25 mM imidazole/HCl buffer, pH 7.1, and applied to an FPLC Mono P HR5/5 column (Pharmacia Biotech Inc.). Purification was achieved by a 10-min elution with 25 mM imidazole, pH 7.1, and a 40-min elution with 10% Polybuffer, pH 4.0 (Pharmacia Biotech Inc.) at a constant flow rate of 0.8 ml/min. The active fractions were pooled, dialyzed against 100 mM ammonium bicarbonate, and lyophilized. Proteolytic activities of nephrosin during each purification step were monitored with the proteolytic zymographic assay (see below). Protein concentrations were determined by a Coomassie Blue binding assay with bovine serum albumin (BSA) as the standard (15).

Gel Electrophoresis—Gel electrophoresis was performed using a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U62621.

§ Both authors contributed equally to this paper.

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[1] The abbreviations used are: ECM, extracellular matrix; PAGE, polyacrylamide gel electrophoresis; RCM, reduced and carboxymethylated; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DIG, digoxigenin; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s); NA, nitroanilide; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Tricine SDS-PAGE system as described previously (16). The gel concentration was 7.5%, and the bisacrylamide to acrylamide ratio was 6%. For proteolytic zymographic assay, Tricine SDS-polyacrylamide gel was prepared as described (17, 18), except that the gel contained 0.1% reduced and carboxymethylated BSA (RCM-BSA). After electrophoresis, the gel was incubated for 1 h at 27 °C with 2% Triton X-100 in 20 mM Tris-HCl, pH 8.0 once and Tris buffer containing 0.1 mM $ZnCl_2$ twice, each for 90 min. The gel was then stained with 0.2% Coomassie Blue R250 in 40% methanol, 7% acetic acid and destained with 30% methanol, 7% acetic acid.

Effects of Proteinase Inhibitors—The effects of various inhibitors against nephrosin were examined using RCM-BSA as a substrate. The enzyme samples (0.1 μg in 10 μl) were preincubated with a particular inhibitor or buffer alone (mock) on ice for 10 min, and the reaction was initiated by the addition of 20 μl of substrate solution (30 μg of RCM-BSA in 20 mM Tris-HCl containing 15 μM $ZnCl_2$, pH 8.0). The reaction was maintained at 27 °C for 1 h and stopped by the addition of 30 μl of 2 × concentrated SDS sample buffer without 2-mercaptoethanol. The reaction mixture was then boiled for 3 min. Four μl of the sample were analyzed by SDS-PAGE, and the amounts of residual RCM-BSA was calculated using an Imaging Densitometer (Bio-Rad GS-670). For each treatment, the amounts of RCM-BSA digested (total RCM-BSA minus residual RCM-BSA) were calculated and compared with those of mock treatment.

Hydrolysis of Small Peptides—The peptides (10 μg in 40 μl) were dissolved in 20 mM ammonium bicarbonate containing 15 μM $ZnCl_2$. The reaction was started by adding 10 μl of enzyme solution (0.1 mg/ml) in 20 mM ammonium bicarbonate, and the mixture was incubated at 27 °C for 1 h. The reaction mixture was then lyophilized, dissolved in 0.1% trifluoroacetic acid, and separated on a reverse-phase C_{18} column eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Each peak was collected, lyophilized, and subjected to amino acid sequencing (model 477A; Applied Biosystems, Forest City, CA).

Chromogenic Assay with Peptide-p-nitroanilide Substrates—The proteolytic activities of nephrosin was determined by using the synthetic peptides, *N*-tosyl-Gly-Pro-Arg-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, *N*-carbobenzoxy-Gly-Gly-Leu-*p*-nitroanilide, and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, respectively (19), as substrates.

Immunization and Immunoblot Analysis—The Mono-P fraction was dissolved in phosphate-buffered saline (PBS) and thoroughly mixed with an equal volume of Freund's complete adjuvant for the first injection or Freund's incomplete adjuvant for the second and third injections. Approximately 100 μg of nephrosin was injected subcutaneously into the back of a guinea pig during each immunization, biweekly. Ten days after the third injection, the blood was collected by heart puncture and serum stored at 4 °C. Western blotting was carried out using guinea pig anti-nephrosin antiserum (1:2000 dilution) and horseradish peroxidase-conjugated second antibody (1:1000 dilution) after electrotransfer to nitrocellulose paper (0.22 μm, Sartorius). Immunoreactive bands were detected by the $NiCl_2$ enhancement method (20).

Artificial Insemination—Female carp were injected twice at the base of pectoral fin with PBS homogenate of one pituitary, each 6 h apart. Ovulation occurred 12 h after the first injection (21). Ovulated eggs were collected and mixed with spontaneously spermiated milt for 5 min and then dispersed with tap water. Fertilized eggs were then allowed to develop at 25 °C with constant compressed air bubbling, and water was replaced twice a day. Embryos were harvested between 70 and 72 h after fertilization and frozen in liquid nitrogen. Hatching liquid was collected at 82 h when more than 80% of the embryos completed hatching, and then frozen at −70 °C.

General Methods in Molecular Biology—Standard procedures in molecular biology were used for preparation of plasmid DNA, restriction enzyme digestion, DNA agarose gel electrophoresis, DNA ligation, and the transformation of bacteria (22).

Immunoscreening of a Carp Head Kidney cDNA Library—A carp head kidney cDNA library prepared from poly(A)-enriched RNA by unidirectional insertion of cDNA into λ-ZAP II (23) was purchased from Stratagene (La Jolla, CA). The λ-ZAP phages were plated at a density of 5×10^4 plaques/agar plate (150 mm, inner diameter). A total of 60 plates was screened. After incubation for 3 h at 42 °C, the plates were overlaid with nitrocellulose filters (pore size, 0.45 μm; Micron Separations, Westboro, MA) that had been impregnated with 10 mM isopropyl-1-thio-β-D-galactopyranoside. Incubation was continued overnight at 37 °C. The filters were then removed, washed with PBS at room tem-

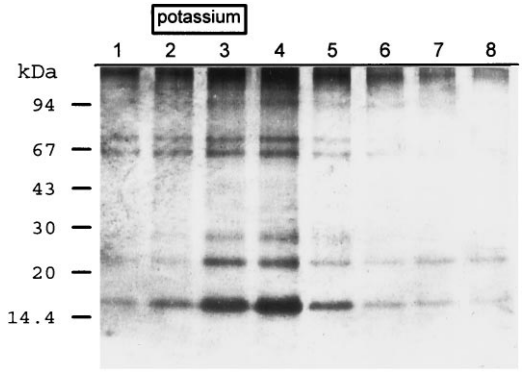

FIG. 1. **Secretion of proteins from carp head kidney perfused *in vitro*.** After tissue was equilibrated with fish saline for 90 min, samples of perfusate were collected for eight 10-min intervals. Perfusion medium was changed from normal fish saline to that containing 50 mM potassium for 20 min at the beginning of interval 2. The perfusate samples were analyzed by SDS-PAGE and silver staining.

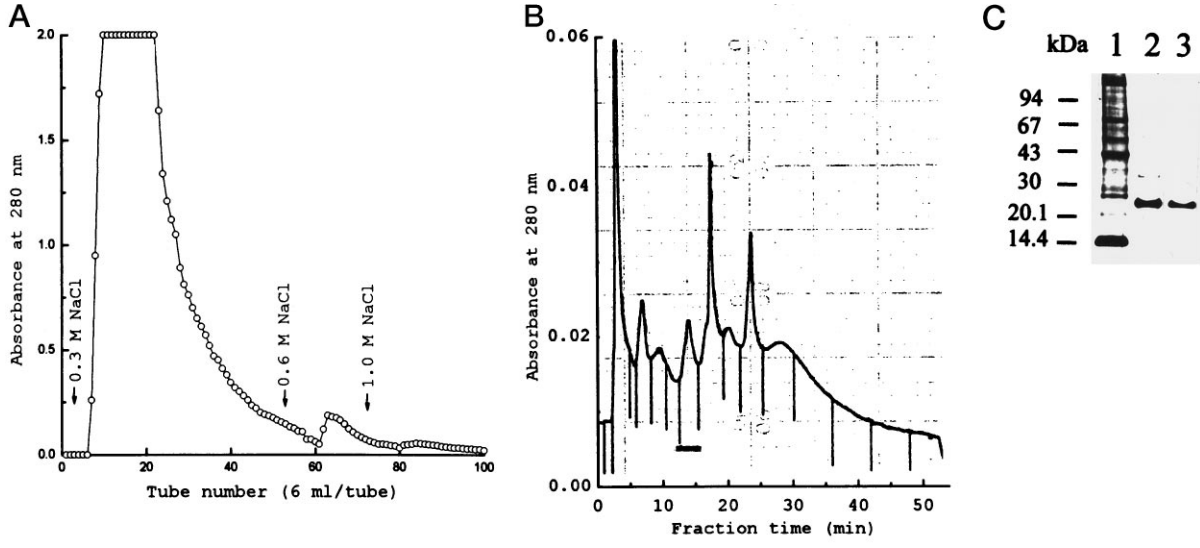

FIG. 2. *A* and *B*, elution profiles of nephrosin from Reactive Blue 72-agarose (*A*) and Mono-P columns (*B*). Head kidney extract (200 ml) was chromatographed on a Reactive Blue 72-agarose column. Fractions of 6 ml were collected at a flow rate of 25 ml/h. Proteins obtained from the first column (80 μg) were chromatographed on a Mono-P column equilibrated with 20 mM imidazole, pH 7.1, and eluted with 10% Polybuffer, pH 4.0. Fractions containing nephrosin were collected as indicated by *bar* on the figure. Crude extract (*lane 1*) and samples from the Reactive Blue 72-agarose column (*lane 2*) and from Mono-P column (*lane 3*) were analyzed by SDS-PAGE and silver staining (*C*).

TABLE I
Summary of purification of nephrosin from carp head kidney

Step	Volume	Protein	Activity[a]	Specific activity	Yield	Purification
	ml	*mg*	*units*	*units/mg*	*%*	*-fold*
Extract[b]	200	2207	119.2	0.054	100	1
Dye column	48	0.376	6.28	16.70	5.27	172
Mono-P	4	0.098	1.78	18.16	1.49	187

[a] One unit of activity is defined as the amount of nephrosin causing the same proteolytic activity as 1 unit of chymotrypsin in the RCM-BSA zymographic assay.
[b] A total of 20 g of carp head kidney was used in this preparation.

TABLE II
Digestion of synthetic peptide substrates by nephrosin

Substrates	Sequences[a]	Hydrolysis
Tosyl-GPK-NA	GPK	ND[b]
Suc-AAPF-NA	AAPF	ND[b]
Suc-AAPL-NA	AAPL	ND[b]
Cbz-GGL-NA	GGL	ND[b]
Substance P	RPKPQQ ↓ F⇓FGLM-NH_2	Complete
Lys-Bradykinin	KRPPG ↓ F⇓SPFR	Complete
Bradykinin 1–5	RPPGF	ND[b]
Bradykinin 1–6	RPPGFS	ND[b]
Bradykinin 1–7	RP ↓ PGFSP	Partial
Des-Arg-bradykinin	PP ↓ GFSPFR	Complete

[a] ↓ indicates bonds cleaved by carp nephrosin, and ⇓ indicates major cleavage site.
[b] ND, nondetectable in 60 min.

TABLE III
Effect of proteinase inhibitors on the enzymatic activities of nephrosin
The purified enzyme was preincubated with each reagent at the indicated concentration for 10 min at 0 °C and then with RCM-BSA for 1 h at 27 °C. Residual enzyme activities were calculated as the ratio of the amount of RCM-BSA digested in the presence of inhibitor to that in the absence of inhibitor.

Inhibitor	Concentration	Residual activity
	mM	*%*
None		100
1,10-Phenanthroline	5	8.2
	1.25	19
EDTA	5	26
	2.5	35
Captopril	5	15
	2.5	61
Diethyldithiocarbamate	5	6.2
	2.5	23
Pepstatin	20 μg/ml	90
PMSF	5	100
Iodoacetate	10	70
	5.5	67

perature, and blocked with 3% skim milk in PBS for 1 h at room temperature. Following blocking, filters were probed with a polyclonal antiserum specific for carp nephrosin at a 1:500 dilution in PBS containing 3 mg/ml bovine serum albumin, 1 mM EDTA, and 0.4% Triton X-100 at 4 °C for 16 h. The filters were then washed three times at room temperature in PBS and incubated with horseradish peroxidase-conjugated anti-guinea pig IgG (Sigma) for 1 h at room temperature. After three washes with PBS, the immune complexes were incubated in PBS containing 0.2 mg/ml diaminobenzamidine and revealed by adding H_2O_2 (10 μl in 10 ml of PBS). Phages displaying stronger signals were isolated for secondary screening. One positive clone was isolated after screening 3×10^6 plaques, and pBluescript plasmids containing cDNA inserts were obtained by *in vivo* excision according to the protocols provided by Stratagene.

DNA Sequencing and Sequence Analysis—Plasmids carrying cDNA inserts were sequenced in both directions using the T7 Sequenase version 2.0 (U.S. Biochemical Corp.) and the dideoxy chain termination method (24). A search for related sequences in GenBank™, EMBL, SWISS-PROT, and Protein Identification Resource was carried out with an IFIND program using the FASTA algorithm of Pearson and Lipman (25). Alignment of the deduced amino acid sequences with known members of the astacin family was accomplished with the Clustal W multiple alignment program (26).

Northern Blot Analysis—Total RNA was isolated from carp blood cells, brain, gill, heart, head kidney, intestine, kidney, liver, ovary, spleen, and late embryos with the RNAzol B kit (Biotecx, Houston, TX). Twenty μg of total RNA from each tissue was fractionated on 1% formaldehyde-agarose gel in MOPS buffer and transferred onto a Hybond-N membrane (Amersham Corp.). Following prehybridization for 1 h in 50% formamide, 5 × SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-laurylsarcosine, 0.02% SDS at 50 °C for 1 h, blots were hybridized with a polymerase chain reaction-generated digoxigenin (DIG)-labeled cDNA probe for 20 h under identical conditions. Blots were washed twice for 5 min with 2 × SSC containing 0.1% SDS at room temperature and twice for 15 min at 68 °C with 0.1 × SSC containing 0.1% SDS. Detection of DIG signals was accomplished using the DIG luminescent detection kit (Boehringer Mannheim). The cDNA probe used for Northern blotting was synthesized by the polymerase chain reaction DIG probe synthesis kit (Boehringer Mannheim) with two primers derived from nephrosin sequence; F1, CGGAGCCGTCCTGTTGAGGA (nucleotides 75–94) and R1, CCCCGTAAAGGAGTTTAGGCC (nucleotides 378–397).

RESULTS

Purification of Nephrosin—During the perfusion of carp head kidney, the spontaneous release of proteins or leakage of proteins due to handling damage of the tissue tended to decrease with time (data not shown). However, in response to a 50 mM potassium stimulation, higher amounts of protein were released by the carp head kidney (Fig. 1). The amounts of protein of 15, 23 (p23), 28, 64, and 70 kDa in the perfusate samples following the potassium treatment were elevated for 20–30 min and then returned to a lower level. Using a zymographic assay, we found that a proteinase activity was associated with the electrophoretic mobility of p23. Therefore, we attempted to purify this secreted proteinase from carp head kidney. By sequential chromatography on a Reactive Blue 72-agarose (Fig. 2*A*) and a FPLC-Mono P column (Fig. 2*B*), we purified the 23-kDa proteinase to near homogeneity (Fig. 2*C* and Table I). N-terminal protein sequencing of this protein revealed a single sequence: NADP*X*TARR*X*KWRKSRNGLV-. The low recovery of nephrosin by the purification procedures was because we collected the fraction with the highest purity (0.6 M NaCl) only. However, by this purification scheme, the whole procedure can be accomplished within 10 days.

Substrate Specificity of Nephrosin—The proteolytic activity of nephrosin could be demonstrated using SDS-PAGE zymography with gelatin, RCM-BSA, or RCM-fibrin as a substrate-(data not shown). To determine the preference of cleavage sites by nephrosin, we examined various peptide-nitroanilides and peptides. None of the peptide-nitroanilides, including tosyl-GPK-NA, succinyl-AAPF-NA, succinyl-AAPL-NA and *N*-carbobenzoxy-GGL-NA, were digested by nephrosin (Table II). Obviously, nephrosin did not hydrolyze the amide bond of these nitroanilides. However, nephrosin cleaved certain peptide bonds of synthetic substance P and Lys-bradykinin. When bradykinin derivatives of different length were tested for their digestion by nephrosin, it was found that there is a length

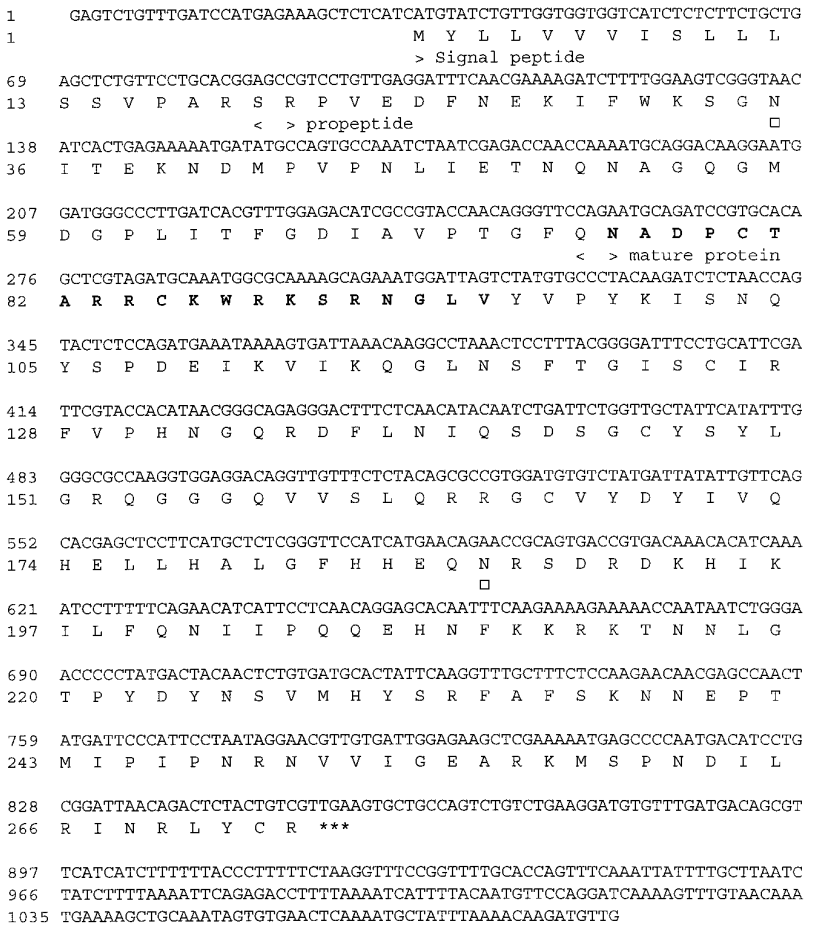

FIG. 3. **Nucleotide sequence and predicted amino acid sequence of nephrosin.** The open reading frame encodes a protein of 273 amino acids, including a 19-amino acid signal peptide and a 56-amino acid propeptide. Amino acid residues in *boldface* match with those determined from purified protein. Potential *N*-glycosylation sites are indicated by *squares*.

requirement for being a substrate. Nephrosin did not cleave the terminal peptide bonds of bradykinin 1–5 and bradykinin 1–6. Among the digestible substrates, nephrosin cleaved at the Gln-Phe and Phe-Phe bonds of substance P and at the Gly-Phe and Phe-Ser bonds of bradykinin derivatives with a preference for phenylalanine at the P_1 position and proline at the $P_2/P_3/P_4$ position. Therefore, nephrosin mainly functions as an endopeptidase with little exopeptidase or amidase activity.

Effects of Proteinase Inhibitors—Various inhibitors were tested for their effects on nephrosin with RCM-BSA as a substrate (Table III). The chelating agents including 1,10-phenanthroline, EDTA, captopril, and diethyldithiocarbamic acid inhibited the proteolytic activity of nephrosin to a great extent, especially 1,10-phenanthroline and diethyldithiocarbamic acid. Inhibitors of other classes of proteinases were ineffective. The results indicate that nephrosin is a metalloproteinase.

cDNA Cloning of Nephrosin—After screening 3×10^6 plaques with a polyclonal antiserum raised against purified nephrosin, one positive clone was isolated. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. The clone has an insert of 1086 bp including 32 bp of the 5′-untranslated region, an open reading frame of 821 bp, and 232 bp of the 3′-untranslated region. The putative initiating ATG codon, which agrees with Kozak's rule (27), is at nucleotide 33. The open reading frame is predicted to encode a protein of 273 amino acids, including a 19-amino acid signal peptide and a 56-amino acid propeptide. The N-terminal 20 amino acid residues determined from purified nephrosin matched with the deduced amino acid sequence. The deduced amino acid sequence also confirms the presence of a propeptide sequence (see also sequence comparison data). The theoretical molecular mass of the mature protein is 23089.35 Da, which is close to the estimated molecular mass from SDS-PAGE analysis.

Sequence Homology—The deduced amino acid of nephrosin shows moderate sequence identity (29–52.5%) with members of the astacin family (Fig. 4) such as medaka HCE1 (52.5%), LCE (50.7%; Ref. 28), crayfish astacin (33.2%; Ref. 29), murine meprin-α (34%; Ref. 30), and murine meprin-β (33.5%; Ref. 31). The N-terminal regions, including the signal peptide and propeptide sequences, are most variable. All members are likely to be secreted and further processed as evidenced by the presence of the signal and propeptide sequences. The junction for the propeptide and mature protein in nephrosin is at a similar position to that in other members. Stretches of highly conserved sequences were found, especially in the unique zinc binding motif, HE*XX*HALGF*X*HE*XX*R*X*DRD. From x-ray structural analyses of crayfish astacin (32), zinc ion is ligated by three histidine residues, one tyrosine residue, and one water molecule H-bonded to a glutamic acid residue. Five residues involved in the penta-coordination of zinc are conserved in all members. Most members of the family have extra domains C-terminal to the proteinase domain except crayfish astacin, medaka hatching enzymes, and carp nephrosin, which consist of only ~200 amino acid residues in the mature forms.

Tissue Distribution—The presence of nephrosin in different tissues was examined by immunoblotting (Fig. 5) with a polyclonal antiserum against purified nephrosin and by Northern blotting (Fig. 6) with nephrosin partial cDNA as a probe. Only samples of gill, kidney, head kidney, and spleen contained an immunoreactive protein of a similar molecular mass. Because of the limited distribution of nephrosin in the kidney, head kidney, and spleen, it was named nephrosin herein until we discover the true physiological functions of this metalloproteinase. The DIG-labeled cDNA probe for nephrosin hybridized

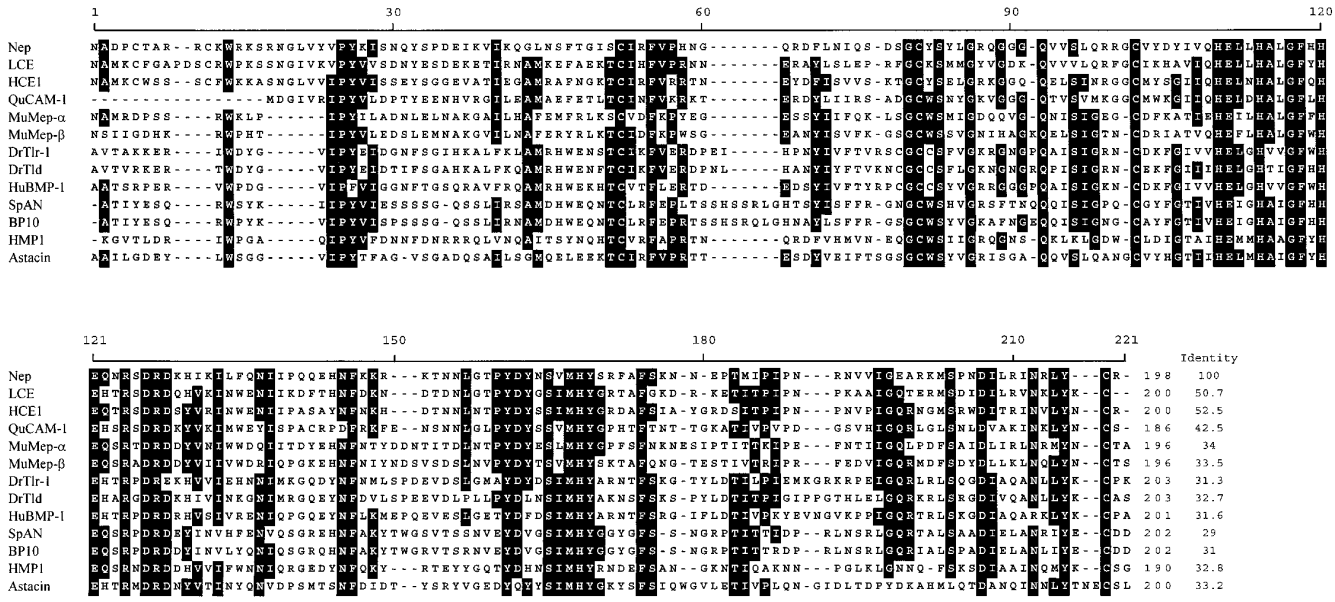

FIG. 4. **Sequence comparison of members of the astacin family.** Sequences of the proteinase domain of the astacin family members were aligned by Clustal W, version 1.6 (26). Alignments highlight residues that are in the majority for all sequences. *Nep*, nephrosin; *LCE*, medaka low choriolytic enzyme; *HCE-1*, medaka high choriolytic enzyme-1; *QuCAM-1*, quail chorioallantoic metalloproteinase-1; *MuMep-α*, mouse meprin-*α*; *MuMep-β*, mouse meprin-*β*; *DrTlr-1*, *Drosophila* Tolloid-related-1; *DrTld*, *Drosophila* Tolloid; *HuBMP-1*, human bone morphogenetic protein-1; *SpAN*, sea urchin SpAN; *BP10*, sea urchin blastula protease-10; *HMP1*, hydra metalloproteinase 1; *astacin*, crayfish astacin. The degree of overall identity to carp nephrosin is shown at the end.

with a single 1.2-kilobase band in all samples except intestine and ovary, and with highest levels in head kidney and kidney. This size of mRNA is in good agreement with that of nephrosin clone (1086 bp). The lack of a protein signal in heart extract is unusual, since its mRNA level was similar to that of spleen.

Absence of Nephrosin in Carp Embryos—Due to the highest sequence identity to medaka hatching enzymes (HCE2, HCE1, and LCE), we wish to determine whether nephrosin is a carp hatching enzyme. Soluble proteins and total RNA were prepared from 70-h embryos (a 80-h hatching procedure) and subjected to immunoblot and Northern blot analysis (Fig. 7). In addition, hatching liquid was also harvested after most embryos completed hatching. Using a comparable amount of protein and RNA, both protein and mRNA signals were observed in head kidney, but not in carp embryos or hatching liquid. Therefore, the data suggest that nephrosin is unlikely to be a hatching enzyme analogue, and that carp embryos at this stage expressed very little nephrosin, if any.

Secretion of Nephrosin—Release of immunoassayable nephrosin from carp head kidney fragments was measured using an *in vitro* perfusion system. Fig. 8 shows the effect of potassium-induced secretion of nephrosin. Nephrosin and a degraded product of nephrosin were detected by immunoblotting following a 50 mM potassium treatment (Fig. 8, *upper panel*). The release of nephrosin was almost instantly following induction. The identity of nephrosin in the perfusate samples was confirmed by SDS-PAGE zymography (Fig. 8, *lower panel*). The release of nephrosin was not due to damage of the perfused tissue since lactate dehydrogenase activities in the perfusate samples were not changed during the perfusion experiment (data not shown). The results suggest that nephrosin is secreted by excitable cells in the head kidney in response to membrane depolarization caused by the potassium treatment.

DISCUSSION

Nephrosin is a new member of the astacin family of metalloproteinases based on the following observations: First, the activity of nephrosin is inhibited by metal chelators but not by inhibitors of other classes of proteinases, suggesting that it is a metalloproteinase. Second, cDNA encoding the purified protein was isolated from a carp head kidney cDNA library. One segment of the deduced amino acid sequence matches perfectly with the first 20 amino acids determined from the purified nephrosin. Bacterially expressed protein encoded by the cloned cDNA can be recognized by the same antiserum used for immunoscreening. In addition, the polyclonal antiserum raised against this recombinant protein recognized native nephrosin.[2] Finally, the deduced primary structure of nephrosin resembles members of the astacin family. The most distinguished zinc-binding motif, H*EXXHXX*GF*X*H*EXXRX*DR, is also present in nephrosin. Astacin family members are all secreted proteinases containing a proteinase domain of approximately 200 amino acid residues (23 kDa). Most of the known members contain one or more copies of interaction domains such as EGF (epidermal growth factor)-like or CUB (complement/sea urchin EGF/BMP-1) that are C-terminal to the proteinase domain (12). However, the mature form of nephrosin does not contain any extra sequences C-terminal to the proteinase domain as with astacin and hatching enzymes (HCE1, HCE2, and LCE).

From sequence comparisons, nephrosin is most closely related to medaka hatching enzymes with >50% identity in amino acid sequence. Hatching enzymes are expressed in late stages of embryos in anticipation of hatching and can therefore be harvested from late embryos and hatching liquid (28, 33). However, we were not able to detect nephrosin in late embryos (mRNA and protein) or hatching liquid (protein). Therefore, the high degree of identity between nephrosin and medaka hatching enzymes most likely results from the fact that they are the only sequences from teleost in the astacin family, but not that nephrosin represents a carp hatching enzyme.

Most members of the astacin family are involved in developmental processes. They are hatching enzymes of medaka involved in degrading the egg shell of embryos (28, 33, 34), hydra HMP1 in head regeneration and tentacle differentiation (35),

[2] H.-R. Huang and G.-D. Chang, unpublished results.

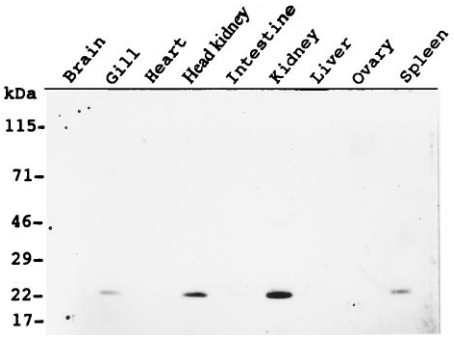

FIG. 5. **Tissue distribution of nephrosin as revealed by immunoblotting.** Proteins from various tissue extracts (10 μg) were electrophoresed on a SDS-polyacrylamide gel and subjected to immunoblotting with an antiserum against purified nephrosin.

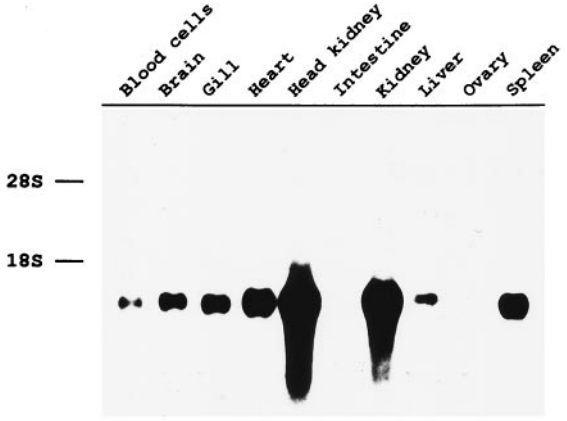

FIG. 6. **Tissue distribution of nephrosin as revealed by Northern blotting.** Total RNA (20 μg) from different tissues were fractionated on a 1% formaldehyde-agarose gel and blotted onto a Hybond-N membrane. The blot was hybridized with a DIG-labeled nephrosin cDNA probe and visualized with luminescent detection.

human BMP-1 in bone formation (36, 37), *Drosophila* Tolloid in dorsal-ventral pattern formation (38), and sea urchin BP10 and SpAN in differentiation of ectodermal lineages (39, 40). Few members of the astacin family are expressed in mature organisms in a tissue-specific manner. For examples, crayfish astacin is synthesized in the crayfish hepatopancreas and functions as a digestive enzyme (41). Mammalian meprins are expressed as dimeric proteins (42, 43) in the brush border membranes of kidney and intestine (44, 45). Along with these limited precedents, nephrosin is expressed in the gill, head kidney, kidney, and spleen of mature carp. Although nephrosin and meprins are all present in the kidney, they are not identical as suggested by differences in the primary structure and in the forms of mature protein (dimeric membrane protein *versus* monomeric soluble protein). In addition, meprin exhibits an arylamidase activity toward peptide-*p*-nitroanilide substrates (43) that are shown to be very poor substrates for nephrosin.

Our data show that nephrosin is secreted from carp head kidney and its secretion can be regulated by manipulation of the membrane potentials. The fact that nephrosin is present in the kidney, head kidney, and spleen suggests a role of nephrosin in the lymphohematopoietic system. Whether nephrosin is expressed by the immune system and/or by the hematopoietic system requires critical examinations. We are currently preparing antisera against proteins derived from both systems as cell type markers. Along with the antiserum against nephrosin, we may be in a better position to solve this problem by immunohistochemical staining procedures.

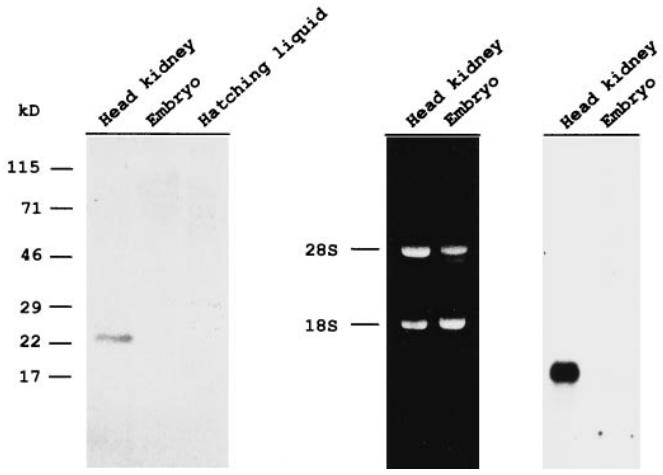

FIG. 7. **Absence of nephrosin in the carp embryos.** Protein (10 μg) from head kidney, 70-h embryos, and hatching liquid were electrophoresed on a 7.5% gel and detected by immunoblotting (*left panel*). Total RNA (20 μg) from head kidney and 70-h embryos were subjected to formaldehyde-agarose electrophoresis in the presence of ethidium bromide (*middle panel*), transferred to a Hybond-N membrane, and probed with a DIG-labeled nephrosin cDNA probe (*right panel*).

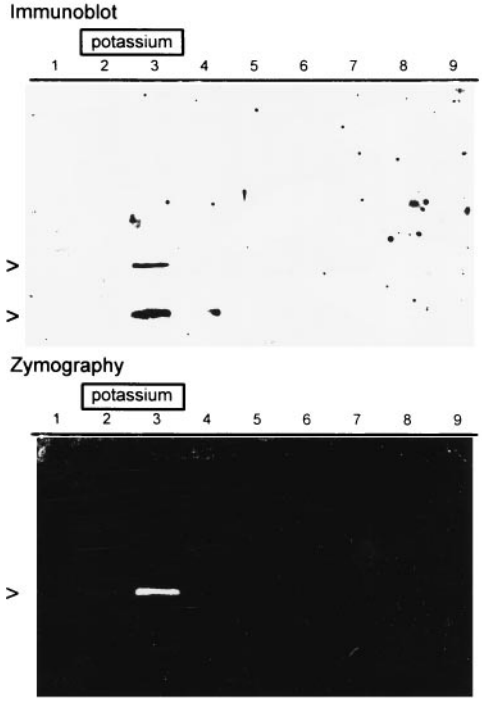

FIG. 8. **Secretion of nephrosin from carp head kidney perfused *in vitro*.** After tissue was equilibrated with fish saline for 90 min, the perfusate samples were collected for nine 10-min intervals. Perfusion medium was changed from normal fish saline to that containing 50 mM potassium for 20 min at the beginning of interval 2. The perfusate samples were analyzed by immunoblotting (*upper panel*) and by proteolytic zymograph (*lower panel*).

We speculate that expression of nephrosin at high levels in the lymphohematopoietic tissues suggests that the proteinase may function in ECM remodeling. One most striking feature pertaining to the tissues is the frequent migration of cells out of and into these sites (46). ECM proteins provide multiple interaction domains mediating adhesion of cells and ECM (47). Although studies on proteolytic remodeling of ECM have been focused largely on matrix metalloproteinases (48), meprin (49), and HMP1 (35) of the astacin family are also effective in de-

grading ECM proteins. In a preliminary experiment,[3] we also found that nephrosin was able to degrade mammalian fibronectin, but not laminin or type IV collagen. In addition, nephrosin can degrade mammalian gelatin in a SDS-PAGE zymographic assay. We are now repeating these experiments using carp ECM as substrates.

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[3] G.-D. Chang, unpublished results.