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Purification and Cloning of an Endogenous Protein Inhibitor of Carp Nephrosin, an Astacin Metalloproteinase*

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Nephrosin is a newly discovered member of the astacin family. It is a secreted proteinase and is present in carp head kidney, kidney, and spleen, all of which are responsible for immune and hematopoietic functions in fish. A complex formed by nephrosin and its inhibitor was purified from carp kidney extract by heparin affinity column chromatography. The presence of the nephrosin-inhibitor complex in different tissues was examined by immunoblotting with polyclonal antisera against the purified nephrosin inhibitor and nephrosin. Both nephrosin and the nephrosin inhibitor were present mainly in gill, head kidney, kidney, and spleen. In addition, we have cloned the cDNA encoding the nephrosin inhibitor. There are two different cDNA clones possibly resulting from two different genes, and the long form contains unique tandem repeat sequences in the 3′-end. The deduced primary structure of nephrosin inhibitor is similar to that of fetuin-A, a mammalian protein present in blood, liver, cerebrospinal fluid, and cerebral cortex during fetal development. Treatment with both *N*-glycosidase F and *O*-glycosidase removed the carbohydrate moiety of the nephrosin inhibitor and decreased the apparent molecular mass from 40 to 30 kDa. The nephrosin inhibitor seems to be synthesized in liver and then secreted to the blood as a precursor. When it was distributed into hematopoietic tissues, it was processed from 67 to 40 kDa and acquired inhibitory activity. This processing phenomenon of fetuin has not been reported elsewhere. Importantly, the presence of an endogenous inhibitor of nephrosin is the first report of this kind for astacin enzymes. It is very likely that endogenous tissue inhibitors may also be present for the regulation of other astacin enzymes.

Nephrosin is a zinc metalloendopeptidase that is synthesized mainly in the lymphohematopoietic tissues of teleosts; head kidney, kidney, and spleen (1). The protein is secreted from the head kidney, and its secretion can be increased by potassium-induced membrane depolarization. The deduced amino acid sequence of nephrosin shows >50% identity to medaka hatching enzymes (2) and 30–40% identity to the proteinase domain of members of the astacin enzyme family (3). The astacin enzymes include a group of metalloendopeptidases involved in diverse biological functions, including protein digestion in crayfish (astacin) (4), dorsal/ventral determination in vertebrates and invertebrates (tolloid) (5–7), morphogenesis in hydra (*Hydra* metalloproteinase-1) (8), degradation of the hard chorion before hatching in fish (hatching enzymes) (9, 10), and processing and degradation of peptides at the microvillar membrane of mammalian kidney and intestine (meprins) (11–13). However, most members of the astacin family are involved in developmental processes, and only a few of them (meprins, astacin, and nephrosin) are expressed in mature organisms in a tissue-specific manner.

Astacin enzymes are synthesized as preproprotein precursors, the signal sequence of which directs them to either membrane-bound or secreted forms (3). Analyses of the N-terminal sequences of purified astacin, hatching enzymes, meprin A, bone morphogenetic protein-1, and nephrosin indicate that the preproproteins of these enzymes have been processed to remove the prosequence, which would give rise to precisely processed and fully active enzymes (14). Together with matrix metalloproteinases (also called matrixins), adamalysins/reprolysins, and serralysins, they have been grouped into a superfamily of metzincins on the basis of almost superimposable topology and a conserved methionine residue beneath the active-site metal (15). The catalytic function of metzincin enzymes depends on a zinc ion in the active site. This catalytic zinc is coordinated by three histidine residues and a water molecule anchored by a glutamic acid residue in matrixins and adamalysins (16–20). These four ligands surround the catalytic zinc in a trigonal pyramidal coordination sphere. However, in the astacins and serralysins, a tyrosine residue immediately following the conserved methionine (-MHY-) with its hydroxyl oxygen provides the fifth ligand to the catalytic zinc (21–24). These five ligands ligate the catalytic zinc in a trigonal bipyramidal geometry.

As for regulation of enzyme activities, most studies have focused on matrixins among the four classes of metalloproteinases. First, most matrixins are synthesized and secreted in a form of zymogen whose activation requires precise removal of the propeptide domain (25). Another important mechanism for the regulation of the activities of matrixins is mediated by endogenous inhibitors like α-macroglobulin in body fluid and

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the tissue inhibitors of metalloproteinases (TIMPs)[1] in tissues (26). Up to now, four different TIMPs (TIMP1–TIMP4) have been identified in vertebrates (26). All TIMPs are able to form a 1:1 tight complex with multiple matrixins, albeit with differences in binding affinity (27, 28). It has been predicted that snake venom zinc endopeptidase adamalysin II, but not astacins and serralysins, can also be inhibited by TIMP in a similar manner (29). In fact, TIMP3 inhibits ADAM-17 (30), ADAM-TS4 and ADAM-TS5 (31), and ADAM-10 (32), which is also inhibited by TIMP1. In this study, an endogenous protein inhibitor of carp nephrosin has been purified from the enzyme-inhibitor complex. N-terminal sequencing and cDNA cloning data indicate that the inhibitor is derived from fetuin by proteolytic processing. In addition, the processing activities are associated only with the tissues in which the nephrosin inhibitor can be found. This is the first demonstration of an endogenous tissue inhibitor of an astacin enzyme. Surprisingly, the nephrosin inhibitor is derived from an unexpected protein, fetuin.

EXPERIMENTAL PROCEDURES

Purification of Nephrosin and the Nephrosin Inhibitor—Carp kidneys (20 g) were homogenized in 200 ml of TE buffer (20 mM Tris-HCl containing 5 mM EDTA, pH 8.0) in a glass homogenizer. The supernatant was collected by centrifugation at 20,000 × *g* for 30 min. The sample was then applied to a heparin-HyperD column (70-μm particle size, 2.5 × 10 cm; BioSepra Inc.) equilibrated with TE buffer and eluted stepwise by 0.3, 0.45, 0.6, and 1.0 M NaCl in TE buffer. The 0.6 M NaCl fractions were saved for further purification of nephrosin. The 0.45 M NaCl fractions containing the nephrosin-inhibitor complex were pooled, dialyzed against deionized water, lyophilized, dissolved in TE buffer, and applied to a heparin-HyperD FPLC column (20-μm particle size, 0.5 × 5 cm; BioSepra Inc.). Purification was achieved by elution with 0.3, 0.45, 0.6, and 1.0 M NaCl in TE buffer (10 min for each elution) at a constant flow rate of 0.8 ml/min. The 0.45 M NaCl fractions containing the nephrosin-inhibitor complex were pooled, dialyzed against 0.1 M acetic acid, lyophilized, dissolved in TE buffer, and immediately applied to the heparin-HyperD FPLC column for a second time. The elution program was the same as described above, and the unretarded fractions (p40) containing the nephrosin inhibitor were pooled, dialyzed against deionized water, and lyophilized. The nephrosin inhibitor precursor was purified from carp serum. 10 ml of carp serum was mixed with an equal volume of TE buffer and then applied to a reactive brown-Sepharose column (1.5 × 8 cm) equilibrated with TE buffer. The bound proteins were eluted stepwise by 0.3, 0.6, and 1.0 M NaCl in TE buffer. The 0.3 and 0.6 M NaCl fractions contained the nephrosin inhibitor precursor (p67).

Gel Electrophoresis—Gel electrophoresis was performed using a Tricine/SDS-PAGE system as described previously (33). The gel concentration was 7.5%, and the bisacrylamide/acrylamide ratio was 6%. The protein solution was mixed with an equal volume of 2-fold concentrated nonreducing sample buffer, incubated at room temperature for 10 min, and subjected directly to electrophoresis when the nephrosin-inhibitor complex was to be preserved. Otherwise, the sample in nonreducing sample buffer was boiled for 5 min to completely dissociate the complex. For reverse proteolytic zymographic assay, Tricine/SDS-polyacrylamide gel was prepared as described, except that the gel contained 0.2% gelatin. After electrophoresis, the gel was incubated twice for 30 min at 27 °C with 2% Triton X-100 in 20 mM Tris-HCl, pH 8.0, and for 3–4 h with Tris buffer containing 0.01 mM $ZnCl_2$ and 0.2 μg/ml nephrosin. For diagonal two-dimensional gel electrophoresis, the first dimension gel electrophoresis was conventional SDS-PAGE, except that the protein mixture in the SDS sample buffer was incubated at room temperature for 5 min instead of being boiled. Before the second dimension gel electrophoresis, the gel slice was either incubated with stacking gel buffer at room temperature for 10 min or boiled in stacking gel buffer for 3 min. The second dimension gel electrophoresis was also conventional SDS-PAGE. The gel was then stained with GelCode blue solution (Pierce).

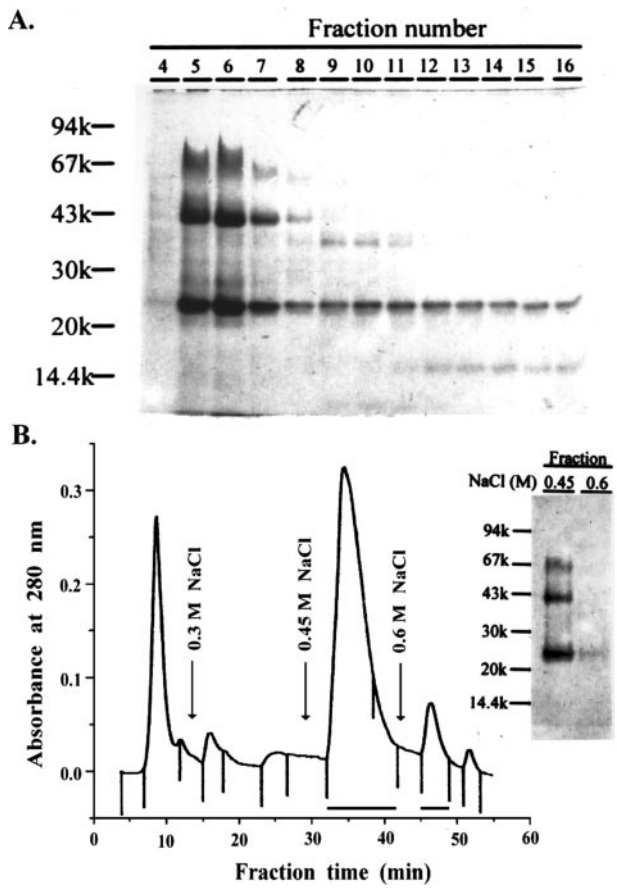

FIG. 1. **Purification of the nephrosin-inhibitor complex from carp kidney.** *A*, carp kidney extract was applied to a heparin affinity column (2.5 × 10 cm) equilibrated with TE buffer and eluted with 0, 0.3, 0.45, 0.6, and 1.0 M NaCl in TE buffer. The 0.45 M NaCl fractions were analyzed by SDS-PAGE and Coomassie Blue staining. *B*, fractions 4–8 in *A* were combined and applied to a heparin FPLC column (0.5 × 5 cm) equilibrated with TE buffer and eluted with 0, 0.3, 0.45, 0.6, and 1.0 M NaCl in TE buffer. Protein peaks were pooled as indicated by the *bars*. *Inset*, the 0.45 and 0.6 M NaCl fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

Immunization and Immunoblot Analysis—Purified p67 and p40 were dissolved in phosphate-buffered saline 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 50 μg of nephrosin inhibitor was injected subcutaneously into the back of a guinea pig during each biweekly immunization. 10 days after the third injection, the blood was withdrawn by heart puncture, and serum was stored at 4 °C. Carp tissues were homogenized in 10 volumes of TE buffer, pH 8.0, by a glass homogenizer. The supernatant was collected by centrifugation at 20,000 × *g* for 30 min. The tissue extracts were then stored at −70 °C after glycerol was added to a final concentration of 10% for preservation of the nephrosin-inhibitor complex. Otherwise, the tissue extracts were mixed with an equal volume of 2-fold concentrated SDS sample buffer, boiled for 5 min, and stored at −70 °C. Immunoblotting was carried out using anti-nephrosin inhibitor or anti-nephrosin antiserum (1:2000 dilution) and horseradish peroxidase-conjugated second antibody (1:10,000 dilution) after diffusion blotting onto polyvinylidene difluoride (PVDF) membrane (34). Immunoreactive bands were detected by the $NiCl_2$ enhancement method (35).

Cross-linking and Glycoprotein Deglycosylation—Cross-linking of nephrosin and the nephrosin inhibitor was achieved by mixing both proteins (0.5 mg/ml) and 0.125–2 mM bis(sulfosuccinimidyl) suberate (BS^3; Pierce) in 20 mM sodium phosphate buffer, pH 7.1, at room temperature for 30 min. The reaction was terminated by adding Tris buffer to a final concentration of 50 mM. Protein deglycosylation was carried out using *N*-glycosidase F (recombinant enzyme from *Flavobacterium meningosepticum*, Roche Applied Science) and *O*-glycosidase (*Diplococcus pneu-*

[1] The abbreviations used are: TIMPs, tissue inhibitors of metalloproteinases; FPLC, fast protein liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PVDF, polyvinylidene difluoride; LC, liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

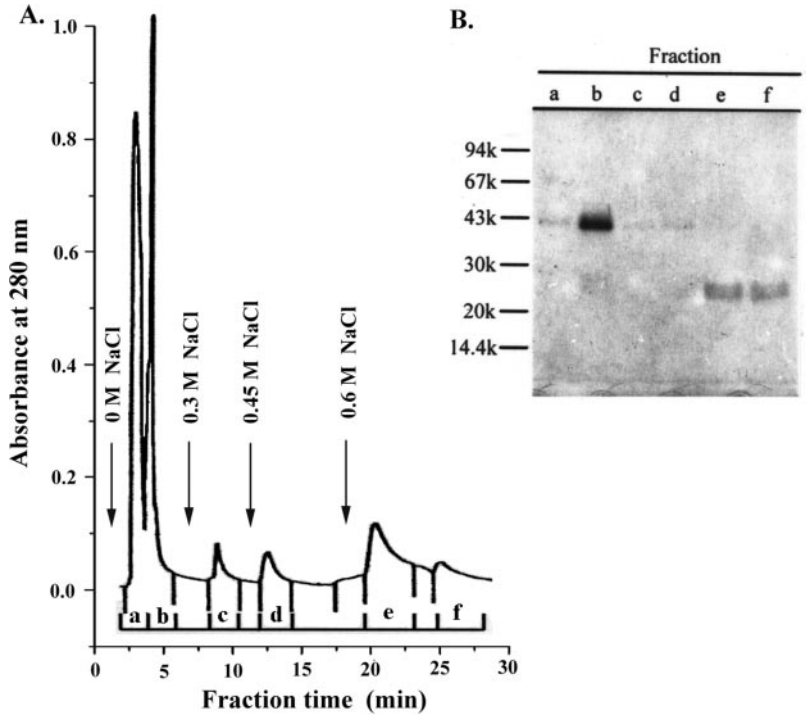

FIG. 2. **Purification of the nephrosin inhibitor from the nephrosin-inhibitor complex.** *A*, the purified nephrosin-inhibitor complex obtained from the 0.45 M NaCl fractions in Fig. 1*B* was treated with 1% acetic acid; lyophilized; applied to a heparin FPLC column (0.5 × 5 cm) equilibrated with TE buffer; and eluted with 0, 0.3, 0.45, 0.6, and 1.0 M NaCl in TE buffer. Fractions were collected as indicated by the *letters* under each peak. *B*, the indicated fraction from each peak in *A* was analyzed by SDS-PAGE and Coomassie Blue G-250 staining.

moniae, from Roche Applied Science) according to the procedures provided by the manufacturer.

Processing of the Nephrosin Inhibitor—To 50 μg of nephrosin inhibitor precursor (p67) in 0.22 ml of 50 mM $NaHCO_3$ was added 0.1 mg of sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce) in 25 μl of *N*,*N*-dimethylformamide. The reaction was carried out for 2 h on ice. Unreacted biotin was removed by dilution with 20 mM HEPES, pH 7.1, and concentration using a Centricon centrifugal filter (Millipore Corp., Bedford, MA). The processing reaction contained 10 μl of biotinylated precursor (0.1 μg), 10 μl of HEPES, 8 μl of tissue extract (10 μg), and 2 μl of 25 mM $CaCl_2$/$MgCl_2$. The reaction was carried out for 50 min at 28 °C and stopped by the addition of 30 μl of 2-fold concentrated SDS sample buffer. 10 μl of each reaction product was resolved by SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was blocked with 3% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 and probed with horseradish peroxidase-conjugated streptavidin (0.01 mg/10 ml) in phosphate-buffered saline containing 0.05% Tween 20. Streptavidin-reactive bands were detected by the $NiCl_2$ enhancement method (35).

Mass Spectrometry—The purified nephrosin-inhibitor complex, the nephrosin inhibitor, and nephrosin were resolved by SDS-PAGE. After electrophoresis, the gel was stained with GelCode blue solution. The gel bands of the nephrosin-inhibitor complex, nephrosin, and the nephrosin inhibitor were excised, destained, reduced, carboxymethylated, and subjected to in-gel digestion with trypsin. Tryptic peptides were extracted with 0.1% trifluoroacetic acid in 50% acetonitrile, 0.1% trifluoroacetic acid in 100% acetonitrile, 0.1% trifluoroacetic acid in 50% acetonitrile, and 0.1% trifluoroacetic acid in 100% acetonitrile. The eluted peptides were subjected to liquid chromatography (LC)/electrospray ionization/tandem mass spectrometry analysis on an integrated nano-LC/tandem mass spectrometry system (Micromass) comprising a 3-pumping Micromass/Waters CapLC® system with an autosampler, a stream select module configured for a precolumn plus an analytical capillary column, and a Micromass Q-Tof Ultima® API mass spectrometer fitted with nano-LC sprayer operated under MassLynx® 4.0 control.

Isolation of Nephrosin Inhibitor cDNA from Carp Liver—N-terminal amino acid sequences (Applied Biosystems Model 477A) were obtained from the nephrosin inhibitor precursor (ASLPTGTDVVYKCQK) and from a peptide fragment copurified with the nephrosin inhibitor (HEILVTKCPDCPGLL) after transfer to a PVDF membrane. Degenerate primers corresponding to the two heptapeptide sequences (underlined) were designed as follows: 5′-GCNAGYCTNCCNACNGGNAC-3′ as the forward primer and 5′-GGRCARTCNGGRCAYTTNGT-3′ as the reverse primer (where R is A and G; Y is T and C; and N is A, T, C, and G). mRNA from liver was purified with the QuickPrep Micro mRNA purification kit (Amersham Biosciences AB, Uppsala, Sweden). Complementary DNA was synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen). All of the above experiments were carried out according to the procedures included with the purchased items. PCR with 0.5 μg of both primers, 0.1 μg of cDNA, 10 μl of 10-fold concentrated *Taq* polymerase buffer, 2 μl of 10 mM dNTP, and 2.5 units of *Taq* polymerase in a final volume of 100 μl was performed at 94 °C for 3 min; at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s for 35 cycles; and for a final extension at 72 °C for 15 min. A DNA fragment of ~400 bp was synthesized in this reaction. The PCR product was purified following agarose gel electrophoresis using an Ultrafree-DA centrifugal filter (Millipore Corp.). The 5′- and 3′-ends of nephrosin inhibitor mRNA were obtained with the Marathon cDNA amplification kit (Clontech) using 5′-primers (5′-CTCATCACTGGAGATGGTCACATTACA-3′ and 5′-CATGGCGGTGATGGTCATTAATGAAGC-3′) and 3′ primers (5′-TCAGGACAAGATTGCTACATTAGAAGC-3′ and 5′-CTAGAGGAAACTGAGTGCCACATTGTG-3′).

Northern Blot Analysis—Total RNA was isolated from carp brain, gill, heart, head kidney, intestine, kidney, liver, muscle, ovary, and spleen with the RNAzol B kit (Biotecx Laboratories, Houston, TX). 20 μg of total RNA from each tissue was fractionated on 1% formaldehyde-agarose gel in MOPS and transferred onto a Hybond-N^+ membrane (Amersham Biosciences). After prehybridization at 68 °C in hybridization buffer (Clontech) for 1 h, blots were hybridized with a ^{32}P-labeled cDNA probe for 2 h under identical conditions. Blots were washed twice with 2× SSC containing 0.1% SDS at room temperature for 5 min. After autoradiography, the blots were stripped by boiling in 0.5% SDS solution and reused for another hybridization experiment. The cDNA probes used for Northern blotting were derived from carp fetuin-L sequence corresponding to nucleotides 276–781 and 788–1305. They were synthesized with *Taq* polymerase and labeled with Klenow enzyme and [α-^{32}P]dATP. A cDNA probe of the carp β-actin gene (nucleotides 2419–2769) was used as an internal control. Standard procedures in molecular biology were used for preparation of plasmid DNA, restriction enzyme digestion, DNA-agarose gel electrophoresis, DNA ligation, and transformation of bacteria (36).

Expression of Recombinant Fetuin Proteins—We constructed expression vectors encoding the insert segment of the long form of fetuin (amino acids 261–422) and the full-length short form of fetuin (amino acids 17–300, lacking the signal peptide). The DNA fragment was prepared by PCR amplification using a 5′-primer containing a synthetic BamHI site (5′-CACGGATCCTGCTCAGTCAAGAAGA-3′, nucleotides 821–836 for the former; and 5′-ACTGGATCCGCCAGCCTCCCGACAG-3′, nucleotides 87–102 for the latter) and a 3′-primer containing a synthetic HindIII site (5′-GCTAAGCTTCAGGCACTGATTCTGG-3′, complementary to nucleotides 1290–1306 for the former; and 5′-GTGAAGCTTGTTACCAGTTGGACGT-3′, complementary to nucleotides 924–938 for the latter) with the cloned cDNA as a template. The PCR

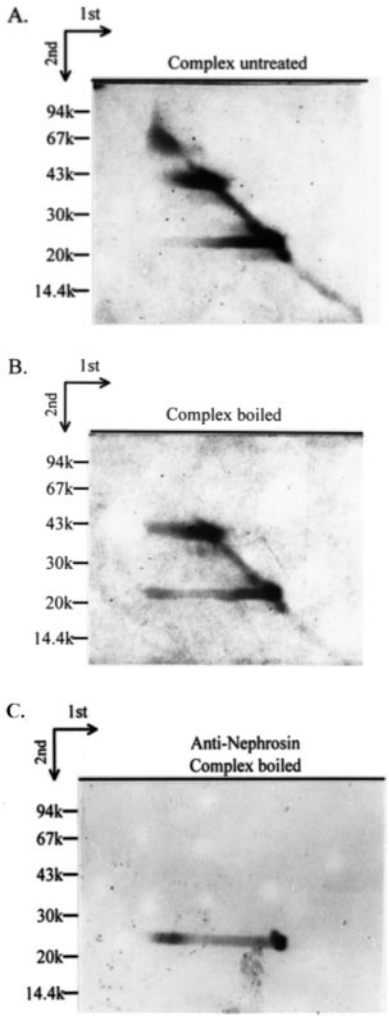

FIG. 3. **Diagonal gel electrophoresis of the nephrosin-inhibitor complex.** 2 μg of nephrosin-inhibitor complex mixed with an equal volume of 2× concentrated SDS sample buffer was subjected to conventional SDS-PAGE for the first dimension electrophoresis. The gel slice was removed with a razorblade and subjected to another conventional SDS-PAGE for the second dimension electrophoresis. Between the two gel electrophoresis steps, the gel slice was incubated at room temperature (*A*) or boiled for 3 min in stacking gel buffer (*B* and *C*). *A* and *B*, Coomassie Blue G-250 staining; *C*, immunoblotting with anti-nephrosin antiserum.

products were digested with BamHI and HindIII and subcloned into the corresponding sites of expression vectors pQE31 and pQE30, respectively (QIAGEN Inc.). The constructed vector was transformed into *Escherichia coli* strain M15. An overnight culture carrying the expression construct was diluted 1:100 into 100 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The culture was grown at 37 °C until A_{600} = 0.5–0.7, and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. The cells were incubated for an additional 4–5 h, harvested by centrifugation at 4000 × *g* for 20 min, and resuspended in 10 ml of cold lysis buffer (20 mM Tris-HCl and 100 mM NaCl, pH 8.0). The cells were sonicated on ice until the solution become clear, and the supernatant was removed after centrifugation. The pellet fraction was dissolved in column equilibration buffer (20 mM Tris-HCl, 8 M urea, and 0.3 M NaCl) and passed through TALON™ metal affinity resin (Clontech) at room temperature to purify the His-tagged proteins. The column was washed with 5 volumes of column equilibration buffer and eluted with 25, 50, 100, and 200 mM imidazole and 200 mM EDTA in column equilibration buffer. Both His-tagged proteins were eluted in the 200 mM imidazole fraction.

RESULTS

Purification of the Nephrosin Inhibitor—Several potential heparin-binding domains are present in the sequence of carp nephrosin: ^{8}RRCKWRKSR^{16}, ^{118}KHIK^{121}, and ^{136}KKRK^{139}. Therefore, we tried to use heparin affinity column chromatography in our purification procedures for nephrosin. Nephrosin indeed bound to heparin-HyperD gel in TE buffer and was eluted by the addition of 0.6 M NaCl in the same buffer. However, 5–10% of the nephrosin was eluted in the earlier 0.45 M NaCl fractions along with two major proteins of 40 kDa (p40) and 65 kDa (p65) and other minor components (Fig. 1*A*). Rechromatography of the 0.45 M NaCl fractions (fractions 4–8) on a heparin FPLC column removed the contaminated minor proteins, but not p40 or p65 (Fig. 1*B*). Attempts to remove p40 and p65 from nephrosin by additional gel filtration or ion exchange column chromatography were not successful. The purified complex possessed no proteolytic activity for reduced and carboxymethylated bovine serum albumin, which can be readily digested by nephrosin (1). The FPLC 0.45 M NaCl fractions were then dialyzed against 1% HOAc for 48 h, lyophilized, dissolved in TE buffer, and immediately applied to the same heparin FPLC column. The 40-kDa protein and nephrosin were found in the unretarded fractions and in the 0.6 M NaCl fractions, respectively (Fig. 2). However, in the 0.45 M NaCl fractions, we saw only trace amounts of any proteins. The unretarded fractions (p40) were collected, lyophilized, and used for nephrosin inhibitor assay.

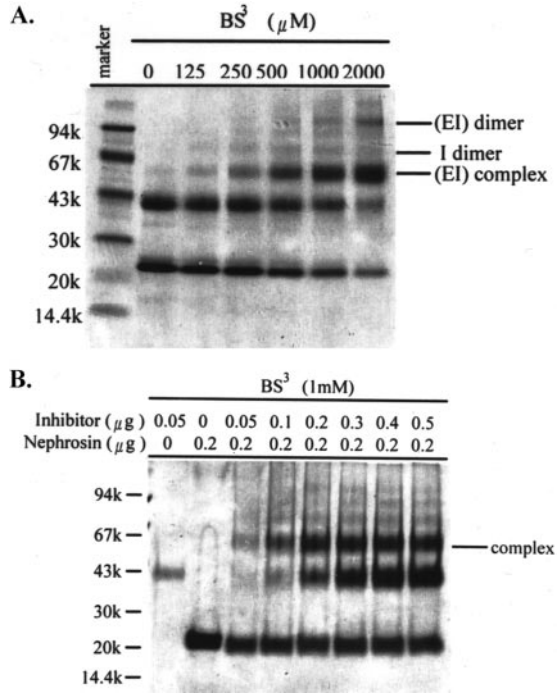

FIG. 4. **Chemical cross-linking of nephrosin and the nephrosin inhibitor.** *A*, 400 ng of purified nephrosin-inhibitor complex was dissolved in 20 mM sodium phosphate buffer, pH 7.1, containing various concentrations of BS^3 (0–2000 μM) and incubated at room temperature for 30 min. The reaction was stopped and analyzed by SDS-PAGE and Coomassie Blue staining. *(EI) dimer*, enzyme-inhibitor dimer; *I dimer*, inhibitor dimer. *B*, the purified nephrosin inhibitor (0–0.5 μg) was mixed with 0.2 μg of nephrosin and cross-linked with 1 mM BS^3 under the conditions described for *A*. The cross-linked sample was boiled and analyzed by SDS-PAGE and Coomassie Blue G-250 staining.

p40 as a Protein Inhibitor of Nephrosin—We noticed that p65 was completely lost in our purification procedures of p40. Interestingly, the relative molecular mass of p65 is close to the calculated sum of those of p40 and nephrosin (p23). To test the speculation that p65 is an SDS-resistant complex of p40 and nephrosin, we analyzed the purified complex by modified diagonal two-dimensional gel electrophoresis. The first dimension gel electrophoresis was conventional SDS-PAGE, except that

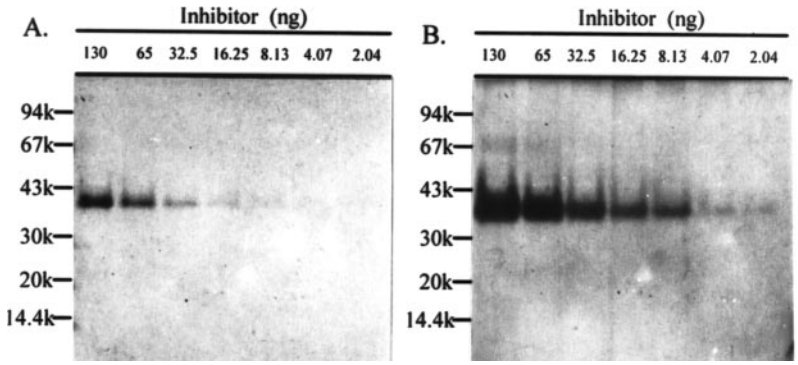

FIG. 5. **SDS-PAGE and reverse zymographic assay for the purified nephrosin inhibitor.** The purified nephrosin inhibitor (130 ng and serial dilutions) was subjected to gel electrophoresis on a plain polyacrylamide gel and then stained with Coomassie Blue G-250 (*A*) or on a polyacrylamide gel containing 0.2% gelatin, renatured in 2% Triton X-100, incubated in buffer containing nephrosin at room temperature for 3 h, and stained with Coomassie Blue G-250 (*B*).

the protein mixture in the SDS sample buffer was incubated at room temperature for 3 min instead of being boiled. Before the second dimension gel electrophoresis, the gel slice was either incubated with stacking gel buffer at room temperature for 10 min (Fig. 3*A*) or boiled for 3 min in stacking gel buffer (Fig. 3*B*). The second dimension gel electrophoresis was also conventional SDS-PAGE. The original diagonal two-dimensional gel electrophoresis was designed to align disulfide bond linkage by isolating peptides linked with the disulfide bonds (37). The modified method was designed to align protein partners associated by SDS-resistant noncovalent bonds, which can be dissociated before the second dimension electrophoresis due to the boiling treatment. Therefore, p65 was no longer detected after boiling, and spots of nephrosin (as indicated by anti-nephrosin immunoblotting in Fig. 3*C*) and p40 appeared, but away from the diagonal line. It is evident that the nephrosin-p40 complex partially dissociated during the first dimension electrophoresis as judged by the continuation of protein staining along the electrophoresis track. Based on these results and that the purified complex did not display proteolytic activity, we speculate that p40 serves as an inhibitor of nephrosin and that p65 is a complex of nephrosin and its inhibitor.

If p65 were a complex of nephrosin and its inhibitor (p40), it should be easily demonstrated by chemical cross-linking. The purified complex was subjected to chemical cross-linking by increasing amounts of an amine-reactive cross-linker (BS^3) in phosphate-buffered saline (Fig. 4*A*). A nephrosin-inhibitor complex at a molar ratio of 1:1 formed at the lowest cross-linker concentration tested (0.125 mM). Increasing concentrations of cross-linker gradually increased the amounts of complex formed and also dimers of the inhibitor and dimers of the complex. At 1 mM BS^3, ~70% of the nephrosin inhibitor was cross-linked. The purified inhibitor still retained the ability to form a complex with nephrosin (Fig. 4*B*). Increasing amounts of inhibitor resulted in increased amounts of complex formed. At 0.2 μg of nephrosin and 0.4 μg of inhibitor, ~30% of the inhibitor was cross-linked. In addition, the ability to form inhibitor dimers and complex dimers was lost in the purified nephrosin inhibitor. It appears that the purified inhibitor is only partially active, possibly due to the prolonged acidic incubation used in the procedures.

Proteinase inhibitor activities can also be demonstrated by reverse zymographic assay. In this assay, serially diluted proteinase inhibitors were subjected to SDS gel electrophoresis on a plain polyacrylamide gel (Fig. 5*A*) and also on a polyacrylamide gel impregnated with gelatin (Fig. 5*B*). After electrophoresis, the plain polyacrylamide gel was directly stained for the presence of proteinase inhibitor, and the gelatin-containing gel was renatured and incubated with nephrosin in an appropriate buffer. After incubation, the gel was stained for the presence of gelatin. Due to the proteinase inhibitor activity, the staining sensitivity of the gelatin-containing gel should be much better than that of the plain polyacrylamide gel. This assay can detect the nephrosin inhibitor at a dose as low as 2.04 ng. We also tested whether mammalian TIMP1 and TIMP2 inhibit nephrosin or not. At 30 ng, both proteins showed no inhibitory activity in the assay (data not shown).

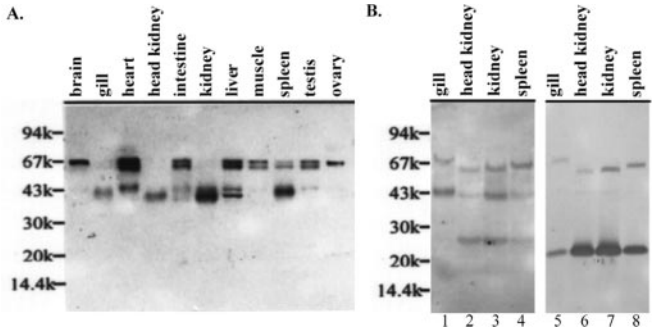

FIG. 6. **Tissue distribution of the nephrosin inhibitor as revealed by immunoblotting.** Proteins from various tissue extracts (10 μg in *A* and 20 μg in *B*) were subjected to SDS-PAGE as follows: *A*, under conditions to completely dissociate the nephrosin-inhibitor complex and probed with anti-nephrosin inhibitor antiserum; *B*, under conditions to preserve the nephrosin-inhibitor complex, transferred to a PVDF membrane, and probed with anti-nephrosin inhibitor antiserum (*lanes 1–4*) or with anti-nephrosin antiserum (*lanes 5–8*).

Tissue Distribution of the Nephrosin Inhibitor—We raised antiserum against purified p40 in guinea pigs for immunoblotting (Fig. 6*A*). Signals of the 67-kDa duplex were detected in brain, heart, intestine, liver, muscle, spleen, testis, and ovary, whereas signals of 40–43 kDa were present in gill, heart, head kidney, intestine, kidney, liver, spleen, and testis. The signals of the 67-kDa duplex originated from blood content in the tissues because the anti-p40 antiserum also detected similar signals in carp serum samples (data not shown). The intensity of the signals of the 67-kDa duplex in the tissue extracts varied in different preparations because of varied blood content within the tissues. Signals of the expected molecular mass (40–43 kDa) were present mainly in tissues where nephrosin could also be detected: gill, head kidney, kidney, and spleen. In these tissues, additional electrophoresis was carried out without sample boiling to preserve the nephrosin-inhibitor complex. Immunoblotting with anti-nephrosin or anti-nephrosin inhibitor antiserum detected the presence of the respective antigen as well as the nephrosin-inhibitor complex (Fig. 6*B*). Notice that a 26-kDa degradation product of the nephrosin inhibitor

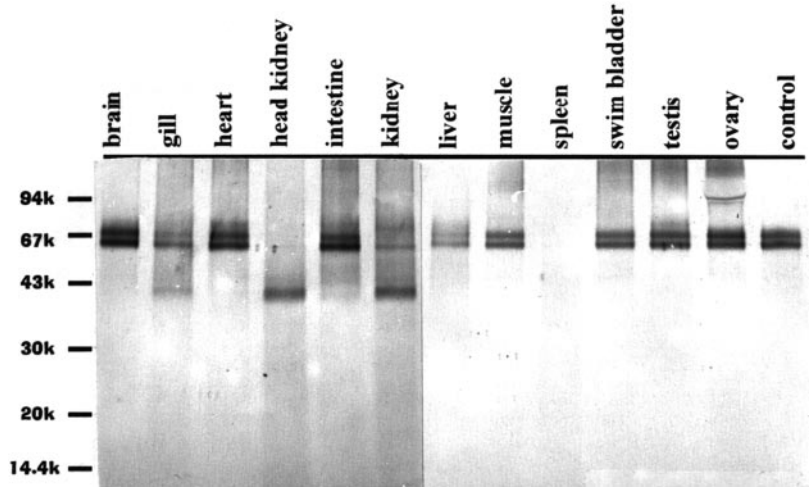

FIG. 7. **Proteolytic processing of the nephrosin inhibitor precursor.** The biotinylated inhibitor precursor (0.1 μg) was incubated with various tissue extracts (10 μg) or with buffer (*control*) at 28 °C for 50 min. The extract was then mixed with SDS sample buffer, resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with peroxidase-conjugated streptavidin.

was detected in Fig. 6*B*, possibly due to prolonged storage of tissue extracts in TE buffer.

We reasoned that the p67 duplex could be the precursor of p40 and therefore purified p67 from carp serum by affinity column chromatography on reactive brown-Sepharose gel. N-terminal protein sequencing of the p67 duplex revealed a single sequence (ASLP**TGTDVVYK**CQK), whereas N-terminal protein sequencing of p40 revealed two sequences ((T/G)(G/T)(T/D)(D/V)(V/V)(V/Y)(Y/K)). Apparently, the N-terminal sequence of p40 begins with the fifth or sixth N-terminal residue of p67, and p40 is a product processed from p67. To confirm this notion, we labeled p67 with biotin and incubated it with various tissue extracts at 28 °C for 50 min. The processed products were examined by blotting with horseradish peroxidase-conjugated streptavidin (Fig. 7). In gill, head kidney, and kidney extracts, p67 were processed into products of 40–43 kDa, whereas in spleen extract, further degradation resulted in no defined products left. However, only p67 signals were detected in other tissue extracts. It appears that appropriate processing of p67 into p40/43 occurs only in gill, head kidney, and kidney. Nevertheless, it remains to be determined whether the processed products truly function as an inhibitor of nephrosin.

cDNA Cloning of the Nephrosin Inhibitor—In our preparations of the nephrosin inhibitor (p40), there was one contaminated protein of 26 kDa that could be recognized by the anti-p40 antiserum (data not shown; see Fig. 6*B*). N-terminal peptide sequencing of this protein revealed a single sequence: HEILVTKCPDCPGLL. We therefore used this peptide sequence (TKCPDCP, reverse) along with the N-terminal sequence of p67 (ASLPTGT, forward) to design degenerate primers for PCRs. PCRs carried out with carp liver cDNA generated a product of 400 bp. 5′- and 3′-rapid amplifications of cDNA ends were performed to gain the missing end sequences. Carp nephrosin inhibitor cDNA has two forms of different length. The long form (fetuin-L) has an insert of 1623 bp, including 38 bp of the 5′-untranslated region, an opening reading frame of 1395 bp, and 190 bp of the 3′-untranslated region (Fig. 8). The putative initiation ATG codon, which agrees with the Kozak rule, is at nucleotide 39. The opening reading frame is predicted to encode a protein of 464 amino acids, including a 16-amino acid signal peptide. The N-terminal 15 amino acid residues determined from purified p67 matched the deduced amino acid sequence. The theoretical molecular mass of the mature protein is 50,009.19 Da, which is considerably smaller than the calculated molecular mass of p67. Three potential *N*-glycosylation sites are ^{111}NVT, ^{206}NCT, and ^{253}NIT, and three potential *O*-glycosylation sites are Ser^{411}, Ser^{415}, and Thr^{439}. Indeed, deglycosylation of p67 and p40 by both *N*-glycosidase F and *O*-glycosidase generated products of 55 kDa and 30 kDa, respectively. The short form (fetuin-S) has an insert of 1132 bp, including 38 bp of the 5′-untranslated region, an opening reading frame of 903 bp, and 191 bp of the 3′-untranslated region. The deduced amino acid sequence is largely identical to that of the long form, except for 15 substituted residues and a deletion of a 164-amino acid peptide (residues 264–427 in the long form; referred to as the insert segment). The theoretical molecular mass of the mature protein is 31,809.09 Da, which does not match that of deglycosylated p67. The insert segment in the long form contains three HRPGHGPP repeats and four AGRDPKDKTPDLRGHPEH repeats.

Sequence Homology—The deduced amino acid of the nephrosin inhibitor precursor (fetuin-L) shows moderate sequence identity (15–21%) to members of the mammalian fetuin-A and fetuin-B family (14%) (Fig. 9). Stretches of highly conserved sequences are found especially in the two cystatin-like domains. Cystatin-like domain 1 in carp fetuin starts with residues 27–144, and cystatin-like domain 2 starts with residues 145–260. Two fetuin signatures are found in the carp fetuin sequence, including an LET*X*CH*X*LDPTP motif that is partially conserved (E**ET**E**CH**IVN**PK**P). Mammalian fetuin sequences contain a set of 12 cysteine residues located at fixed distances; 10 of the 12 cysteine residues are conserved in carp fetuin, and the other two cysteine residues can be found in nearby sequences (^{246}CEIY and ^{444}CPLR). A higher extent of sequence identity (64%) is found between fetuin-L and zebrafish fetuin, with much higher conservation within the N-terminal half of the molecule.

Nephrosin Inhibitor Derived from Fetuin Molecules—To further confirm that the nephrosin inhibitor is indeed derived from fetuin, we excised protein bands of the nephrosin-inhibitor complex (p65), the nephrosin inhibitor (p40), and nephrosin by SDS-PAGE and processed them for in-gel trypsin digestion and LC/tandem mass spectrometry analysis. After analysis using the Mascot search engine, 12 peptide fragments from nephrosin and three peptide fragments from fetuin-L were identified in the nephrosin-inhibitor complex band (Table I). The total coverage of sequence for nephrosin and fetuin-L is 42.9% (85/198) and 8.5% (38/447), respectively. Fewer peptide fragments were recovered from fetuin-L (13.8%, 62/447) than from nephrosin (75.2%, 149/198), even when the purified nephrosin inhibitor and purified nephrosin were analyzed by the same procedures. Nevertheless, the data indicate that the nephrosin inhibitor shares identical sequence with fetuin-L, at least within the three peptide fragments identified. Alternatively, bacterially expressed proteins of fetuin-S and the insert

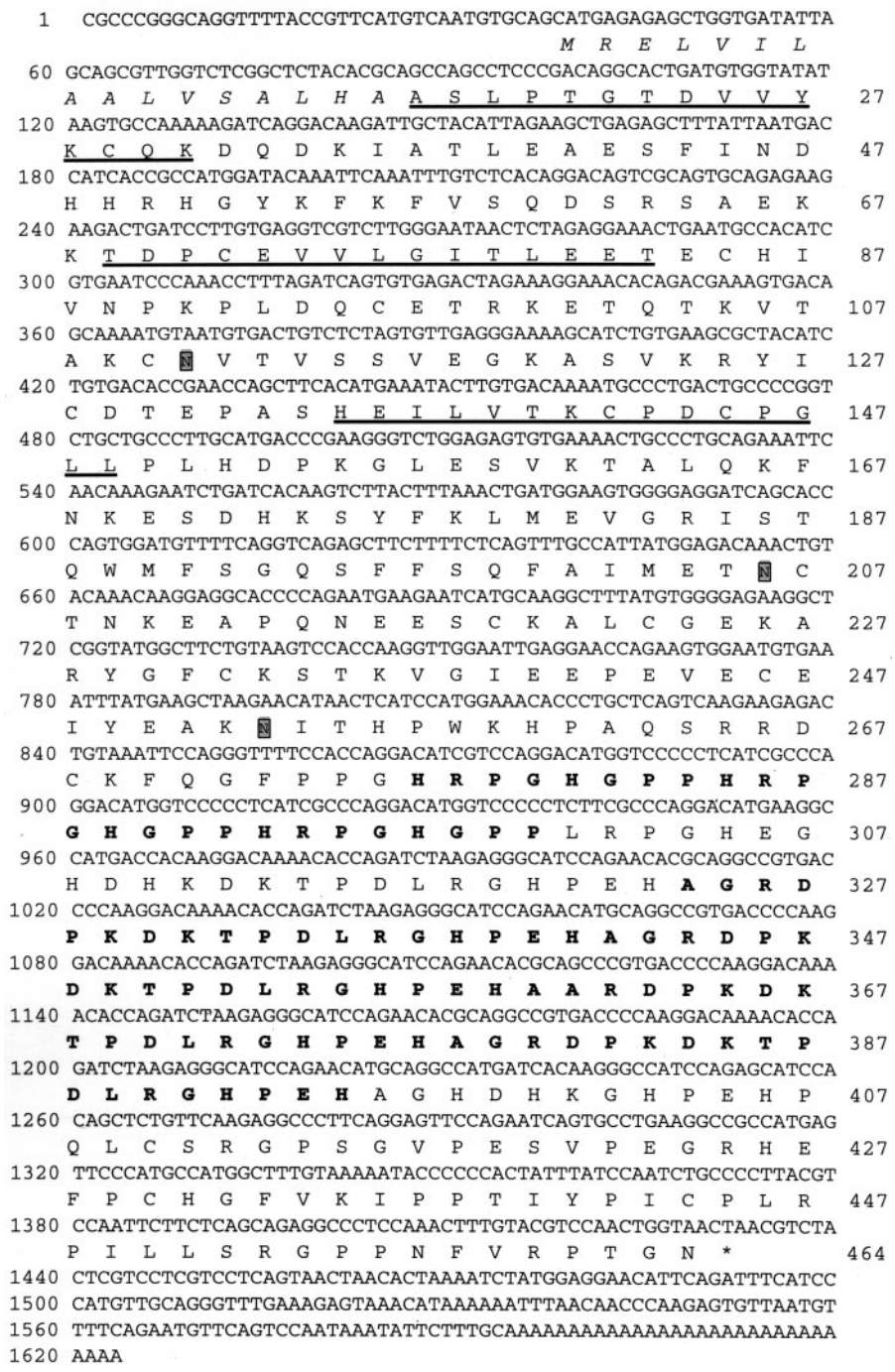

FIG. 8. **Nucleotide and predicted amino acid sequences of carp fetuin.** The open reading frame encodes a protein of 464 amino acids, including a 16-amino acid signal peptide (*italicized*) and three potential *N*-glycosylation sites (*boxed*). The *underlined* residues match those determined from purified proteins. Amino acid residues in *boldface* indicate repeated sequences.

segment of fetuin-L were examined using anti-p40 and anti-p67 antisera in the immunoblot assay (Fig. 10). Both recombinant proteins migrated slower on the polyacrylamide gel than expected. Anti-p67 antiserum recognized both expressed proteins, and anti-p40 antiserum recognized only fetuin-S, but not the insert segment of fetuin-L. The data again confirm that the nephrosin inhibitor is indeed derived from fetuin. In addition, the insert segment of fetuin-L seems to be removed during proteolytic processing.

Tissue-specific Expression of Fetuin mRNA—The presence of fetuin mRNA was investigated in adult female carp by Northern blotting with fetuin partial cDNAs as probes (Fig. 11). One probe derived from the 5′-end cDNA (bp 276–781) hybridized with 2.0- and 1.5-kb bands in liver and with a 2.0-kb band in intestine, spleen, and ovary. The sizes of the mRNA are in good agreement with those of the fetuin clone (1623 and 1132 bp), and both forms were expressed mostly in liver. Another probe derived from the extra sequence found in the long form of the cDNA clone at the 3′-end (bp 788–1305) hybridized only with the 2.0-kb band in liver, intestine, spleen, and ovary. Therefore, the two cDNA clones (long and short forms) are derived from the two mRNAs, and they differ mostly in the extra sequence present in the long form.

DISCUSSION

In this study, we have demonstrated that carp fetuin following proteolytic processing serves as a tissue inhibitor of nephrosin. A complex formed by nephrosin and its inhibitor has been purified from carp kidney extract by heparin affinity chromatography. The complex even at 1 μg did not exhibit detectable proteolytic activity for reduced and carboxymethylated bovine serum albumin, whereas the activity of purified

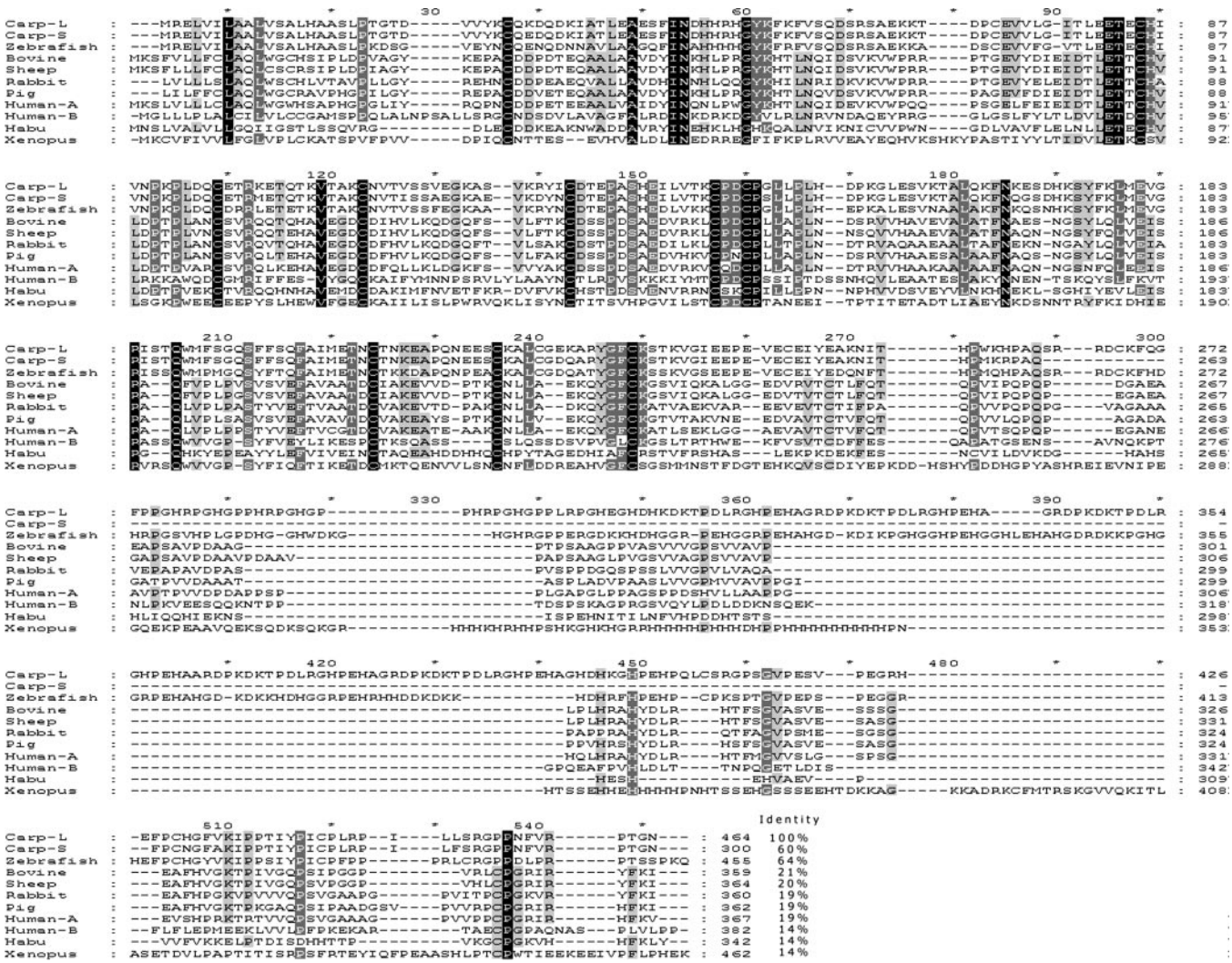

FIG. 9. **Multiple alignment of fetuin from various vertebrates.** Sequences were aligned by ClustalW (Version 1.64). *Carp-L* and *Carp-S* indicate the sequences of the long and short forms, respectively. Identical residues are *boxed*. The degree of overall identity to carp fetuin-L is shown at the end.

nephrosin could be readily detected at 0.05 μg/assay. Unfortunately, reduced and carboxymethylated bovine serum albumin as a substrate does not allow correct measurement of reaction rates because the molecule provides far too many scissile bonds hydrolyzed by nephrosin. To characterize in more detail the inhibition mechanism, we are now searching for a suitable protein substrate that can be attacked only at one site by nephrosin. Notably, some nephrosin-inhibitor complex remained associated upon SDS-PAGE and migrated as if possessing the molecular mass of the sum of the pair. Upon boiling in SDS sample buffer, the complex completely dissociated into nephrosin and the nephrosin inhibitor, suggesting that the binding itself is noncovalent. SDS-resistant proteinase-inhibitor complex has been observed only in pairs of serine proteases and serpins (38). However, the serpin-enzyme complex does not dissociate upon boiling in SDS sample buffer because a covalent bond is involved in the complex formation. Alternatively, the nephrosin-inhibitor complex could be dissociated by prolonged acidic treatments, by which the nephrosin inhibitor was purified. The purified inhibitor still retained nephrosin binding and nephrosin inhibiting activities, indicated by chemical cross-linking and reverse zymographic assay, respectively. Sequence analysis of the cDNA encoding the nephrosin inhibitor revealed that the inhibitor is derived from fetuin molecules synthesized and secreted by liver. Interestingly, both nephrosin and the nephrosin inhibitor were mostly present in kidney and head kidney, where fetuin is proteolytically processed into the nephrosin inhibitor by uncharacterized proteases. Therefore, the nephrosin inhibitor seems to be generated in tissues in which nephrosin is synthesized and secreted. The physiological significance of such a tight regulation of nephrosin is still unknown.

The cDNA of the carp nephrosin inhibitor has two forms of different length. The long form has an insert of 1623 bp, including 38 bp of the 5′-untranslated region, an opening reading frame of 1395 bp, and 190 bp of the 3′-untranslated region (Fig. 8). The short form has an insert of 1132 bp, including 38 bp of the 5′-untranslated region, an opening reading frame of 903 bp, and 191 bp of the 3′-untranslated region. The deduced amino acid sequences are largely identical, except that there are 15 substituted residues and a deletion of a 164-amino acid peptide (corresponding to residues 263–426 in the long form) in the short form. The insert segment found in the long form contains three HRPGHGPP repeats and four AGRDPKDKTPDLRGHPEH repeats. Interestingly, fetuins of lower vertebrates (carp, zebrafish, and *Xenopus*) are longer in sequence than mammalian fetuins by ~100 amino acid residues, including an abundance of histidine and proline residues. Based on structural similarities, fetuins belong to the cystatin superfamily, which comprises cystatins, stefins, kininogens, and histidine-rich gly-

TABLE I
Identified peptide fragments in Coomassie Blue-stained bands
Peptide sequences were identified automatically and verified manually using the Mascot search engine.

Source	Peptide sequences identified	Matched protein
p65	KMSPNDILR	Carp nephrosin
	RNVVIGEAR	
	RNGLVYVPYK	
	KISNQYSPDEIK	
	RQGGGQVVSLQR	
	KNNEPTMIPIPNR	
	KISNQYSPDEIKVIK[a]	
	KILFQNIIPQQEHNFK	
	RFAFSKNNEPTMIPIPNR[a]	
	RDFLNIQSDSGCYSYLGR	
	KNNEPTMIPIPNRNVVIGEAR[a]	
	RNGLVYVPYKISNQYSPDEIK[a]	
p65	KFVSQDSR	Carp fetuin-L
	KIATLEAESFINDHHR	
	RYICDTEPASHEILVTK	
p40	KFVSQDSR	Carp fetuin-L
	KTALQKFNK[a]	
	KSYFKLMEVGR[a]	
	KGLESVKTALQK[a]	
	KIATLEAESFINDHHR	
	RYICDTEPASHEILVTK	
Nephrosin	KMSPNDILR	Carp nephrosin
	RNVVIGEAR	
	RFVPHNGQR	
	RNGLVYVPYK	
	RQGGGQVVSLQR	
	KNNEPTMIPIPNR	
	KQGLNSFTGISCIR	
	KISNQYSPDEIKVIK[a]	
	KILFQNIIPQQEHNFK	
	RFAFSKNNEPTMIPIPNR[a]	
	RDFLNIQSDSGCYSYLGR	
	KTNNLGTPYDYNSVMHYSR	
	KNNEPTMIPIPNRNVVIGEAR[a]	

[a] The search allows one missed cut of trypsin.

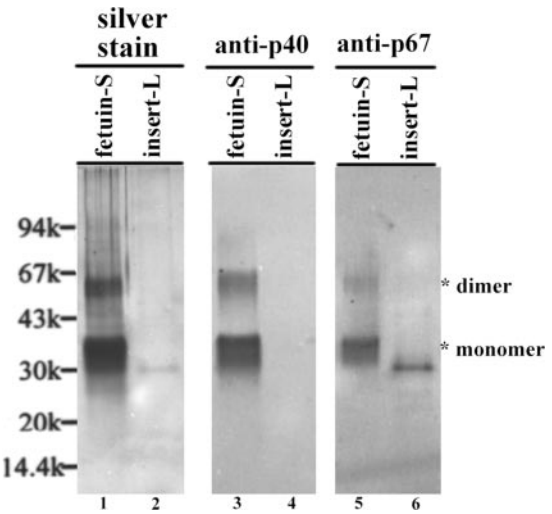

FIG. 10. **Recognition of recombinant fetuin molecules by anti-nephrosin inhibitor antiserum.** Recombinant proteins of fetuin-S (50 ng; *lanes 1*, *3*, and *5*) and the insert segment of fetuin-L (*insert-L*) (10 ng; *lanes 2*, *4*, and *6*) were expressed in *E. coli*, resolved by SDS-PAGE, and subjected to silver staining (*lanes 1* and *2*) or immunoblotting with anti-p40 (*lanes 3* and *4*) or anti-p67 (*lanes 5* and *6*) antiserum.

coprotein (39). Note that histidine-rich glycoprotein is also rich in these amino acid residues. It is believed that the active nephrosin inhibitor is derived from the long form of the precursor because the long-form mRNA is much more abundant. In addition, the size of the circulating nephrosin inhibitor precursor after deglycosylation is ~55 kDa, which is close the calculated molecular size of fetuin-L (50 kDa). It remains to be

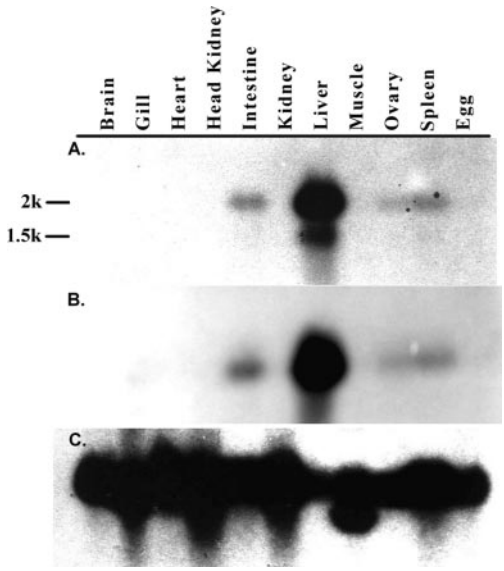

FIG. 11. **Tissue distribution of carp fetuin as revealed by Northern blotting.** Total RNA (20 μg) from different tissues was fractionated on a 1% formaldehyde-agarose gel and blotted onto a Hybond-N$^+$ membrane. *A*, the probe derived from the 5′-end cDNA (bp 276–781) hybridized with 2.0- and 1.5-kb bands in liver and with a 2.0-kb band in intestine, spleen, and ovary. *B*, another probe derived from the extra sequence found in the long form of the cDNA clone at the 3′-end (bp 788–1305) hybridized only with the 2.0-kb band. *C*, the blot was striped and reprobed with a carp β-actin probe.

investigated whether the protein product of fetuin-S is actually synthesized in liver and spleen, where the corresponding mRNA can be detected.

Deglycosylation of the purified nephrosin inhibitor (p40) reduced the molecular size to 30 kDa as estimated by SDS-PAGE. The predicted processing site is near residue 282 because the theoretical molecular size of fetuin-L-(17–282) is 30,017.97 Da. Therefore, much of the insert segment (residues 264–427) is removed during proteolytic processing, which is supported by the data that anti-p40 antiserum recognized recombinant fetuin-S, but not the recombinant insert segment of fetuin-L. Attempts to determine the precise proteolytic site by LC/tandem mass spectrometry have been unsuccessful because of the low coverage of the fetuin sequence by the identified peptide fragments.

Mammalian fetuins are serum proteins, generally with the highest concentrations in serum and body fluids of embryos and fetuses. Human fetuin is a negative acute-phase protein whose concentration in blood drops after inflammation (40, 41). Many functions have been reported to be associated with fetuins: opsonization (42), lipid transport (43), inhibition of the insulin receptor (44), inhibition of apatite formation (45), and regulation of osteogenesis (46–49). However, targeted deletion of the mouse fetuin-A gene results only in unwanted calcification, without causing gross anatomical abnormalities (47). Resistance to envenomation by snakes has been observed in venomous snakes, and protein factors with antihemorrhagic and antineurotoxic effects are present in snake sera (50). Protein inhibitors isolated from sera of the hemorrhagic snakes *Trimeresurus flavoviridis* (named Habu serum factor) and *Bothrops jararaca* (named BJ46a) have been shown to inhibit the corresponding venom metalloproteinases (50, 51). Interestingly, analyses of the primary structures of the two antihemorrhagic factors reveal that they belong to the fetuin family. Therefore, it is not without precedent that members of the fetuin family actually function as inhibitors of metalloproteinases. However, the presence of such a metalloproteinase inhibitor in the serum merely confers resistance of hemorrhagic snakes to snake

venom. Thus, it is a case of adaptive diversity of fetuin function in snakes, but not a general function of fetuin in all animals regarding inhibition of venomous metalloproteinases.

In summary, an endogenous protein inhibitor of carp nephrosin has been purified from the enzyme-inhibitor complex. The data obtained from N-terminal sequencing, cDNA cloning, protein expression, and mass spectrometry indicate that the inhibitor is derived from fetuin by proteolytic processing. In addition, the processing activities are associated with the tissues in which the nephrosin inhibitor can be found. This is the first demonstration of an endogenous tissue inhibitor of an astacin enzyme. It is very likely that endogenous inhibitors may also be present for the regulation of other astacin enzymes or that the nephrosin inhibitor may inhibit other members of the astacin enzyme family, as in the case of TIMPs.

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