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Immunohistochemical Localization of Napsin and Its Potential Role in Protein Catabolism in Renal Proximal Tubules

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Summary. **In a previous *in situ* hybridization study, we demonstrated the mRNA expression of napsin, an aspartic protease of the pepsin family, in the kidney, lung, and lymphoid organs of mice. However, findings on the cellular localization of napsin at the protein level are controversial, and no information on the subcellular localization is available. The present immunohistochemical study revealed the cellular and subcellular localization of napsin in mice and rats, and also analyzed the influences of chemical-induced proteinuria on the renal expression of this enzyme in rats. Immunohistochemistry using a polyclonal antibody against mouse napsin showed that napsin immunoreactivity was noticeable in lysosomes of renal proximal tubule cells and in lamellar bodies of pulmonary type II alveolar cells. In the lung, immunoreactivity was also found in lysosomes of alveolar macrophages and on the surface of type I alveolar cells; the immunoreactivities in these cells may be due to the uptake and adhesion of napsin secreted from type II alveolar cells, since they did not express napsin mRNA. Conversely, immunoreactivity for napsin was undetectable in B lymphocytes with intense mRNA expression. In puromycin- or doxorubicin-induced proteinuria, napsin mRNA expression was markedly elevated in renal proximal tubules, showing characteristic distribution patterns. Immunostaining of kidneys with protcinuria showed intense immunoreactivity for napsin in congested and enlarged lysosomes, called protein absorption droplets. These results indicate that napsin functions as a lysosomal protease and is involved in protein catabolism in renal proximal tubules.**

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Napsin, an aspartic protease of the pepsin family, was originally isolated from the mouse kidney by MORI et al. (1997), and its homologues have been found in humans (TATNELL et al., 1998) and rats (SCHAUER-VUKASINOVIC et al., 2000b). The cellular expression of mouse napsin mRNA was revealed in our previous study using *in situ* hybridization: the renal proximal tubules, pulmonary type II alveolar cells, and B lymphocytes intensely express napsin mRNA (MORI et al., 2001). Genes for human napsin possess two isoforms: *napsin A* is expressed in the kidney and lung, and *napsin B* is expressed in the spleen. The lack of a stop codon in napsin B cDNA suggests that *napsin B* is a pseudogene. Although two immunohistochemical studies have examined the localization of human napsin A, their findings on the precise distribution are contradictory. SCHAUER-VUKASINOVIC et al. (1999) reported a positive immunoreactivity in proximal and collecting tubules of the kidney, type II alveolar cells, and some splenic lymphocytes, while HIRANO et al. (2000) detected immunoreactivity in alveolar macrophages and pancreatic exocrine glands and ducts as well as renal tubules and type II alveolar cells. Biochemically, napsin has been considered to be present as a membrane-associated protein or a lysosomal enzyme (SCHAUER-VUKASINOVIC et al., 1999, 2000a), but there is no morphological evidence regarding the subcellular localization of napsin.

The renal proximal tubule, where napsin mRNA is expressed most abundantly, is an essential site for protein absorption and catabolism (WALL and MAACK, 1985; MAACK et al. 1992). In this catabolic pathway, certain proteases appear to participate in the disintegration of proteins filtered through glomerular capillaries (CORTNEY et al., 1970; MAACK et al.,

1979; POSNER et al., 1982; OLBRIGHT et al., 1986). Cathepsin D, one of the predominant lysosomal proteases, is assumed to share such a catalysis (BARICOS and SHAH, 1984). However, cathepsin D appears ubiquitously in many cells and organs (FAUST et al., 1985; TAN and WONG, 1987); in the kidney it is found predominantly in cortical collecting tubules (YOKOTA et al., 1985). Several investigators have elucidated the clinical importance of napsin in some pathologic conditions, such as renal dysfunction (SCHAUER-VUKASINOVIC et al., 2000c) and lung adenocarcinomas (CHUMAN et al., 1999). The concentrations of napsin in urine were reported to decrease in diseased kidneys compared with those of healthy individuals, while the tissue expression of napsin was elevated in lung malignancies. *In vitro* characterization of napsin revealed its substrate-specificity and pH-optimum (ROSSÉ et al., 2000; SCHAUER-VUKASINOVIC et al., 2000a; GRÜNINGER-LEITCH, 2002), but no information is available regarding its *in vivo* function.

In the present study, the immunohistochemical localization of napsin protein was investigated at light and electron microscopic levels and compared with mRNA expression. We also examined the change in napsin expression in rat glomerulopathy models, based on the hypothesis that napsin is involved in the protein catabolic pathway in the proximal tubules.

MATERIALS AND METHODS

Animals

Male ddY mice (SLC, Shizuoka) weighing 20–30 g and male Wistar rats (SLC) weighing 180–190 g were used. All animals were fed standard laboratory chow and given free access to water. Glomerulopathy was induced in rats by a single intravenous injection of puromycin aminonucleoside (50 mg/kg, Sigma-Aldrich, St. Louis, MO) or doxorubicin (7.5 mg/kg, Adriacin®, Kyowa Hakko, Tokyo). Control rats received solvent alone. All control and treated rats were housed in metabolic cages and 24 h-urine was collected 14 or 28 d after injection. It has been reported that the urinary protein excretion reaches a maximum 14 d after puromycin injection (DIAMOND and KARNOVSKY, 1986) and 28 d after doxorubicin injection (BERTANI et al., 1982), and thereafter decreases gradually. Therefore, puromycin-injected rats were sacrificed at 14 d, and doxorubicin-injected and control rats at 28 d. The care of animals and experimental procedures were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

Antibody

To raise a polyclonal antiserum against mouse napsin, a 42 amino acid peptide corresponding to 378–419 of mouse napsin was synthesized. It was expressed as a glutathione S-transferase (GST) fusion protein, using the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and BL21 cells (Stratagene, La Jolla, CA). The fusion protein was purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech), cleaved with thrombin, and then purified by removal of GST by absorption with glutathione-Sepharose 4B. Antigen was emulsified with Freund's complete adjuvant (Difco, Dctroit, MI) and injected subcutaneously into a female New Zealand White rabbit (450 μg antigen per injection) at intervals of two weeks. Two weeks after the sixth injection, the immunoglobulin fraction was purified using protein-G Sepharose (Amersham Pharmacia Biotech). Immunoglobulins specific to napsin were affinity-purified using the synthetic fusion protein coupled with cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech).

Immunoblot

The kidneys of normal mice were snap-frozen in liquid nitrogen and homogenized in a cold buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM ethylenediaminetetraacetic acid (EDTA) and a proteinase inhibitor cocktail. The samples were centrifuged (10,000 g, 4℃, 20 min), and the supernatant was collected. Equal amounts of protein (10 μg per lane) were loaded and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was then incubated with 5% skimmed milk in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Tween 20 and an affinity-purified anti-napsin antibody (1.34 μg/ml) for 1 h, and a horseradish peroxidase-linked goat anti-rabbit IgG (1 : 10,000 in dilution, Jackson Labs, Bar Harbor, ME) for 1 h. The antibody-bound protein was visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Immunohistochemistry

Adult mice were perfusion-fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4), and the kidney, lung, and lymphoid organs were removed and immersed in the same fixative for an additional 6 h. Fresh kidneys were obtained from rats and immersion-fixed with 4% paraformaldehyde fixative overnight. The fixed tissues were immersed in a 30%

sucrose solution overnight at 4℃ and frozen in liquid nitrogen, or embedded in paraffin according to the conventional method. For light microscopy, frozen sections were prepared and dipped in PBS containing 0.3% Triton X-100 for 1 h. The frozen sections and dewaxed paraffin sections were then incubated with 0.3% H_2O_2 in absolute methanol for 15 min to block any endogenous peroxidase activity. After blocking with 10% normal goat serum for 30 min, the sections were incubated with an anti-napsin antibody (2.6 μg/ml) overnight, followed by incubation with biotinylated goat anti-rabbit IgG (Histofine kit, Nichirei, Tokyo) and streptavidin-peroxidase complex (Vectastain ABC Kit, Vector Labs, Burlingame, CA), each for 1 h. Immunoreaction was visualized with 0.01% 3,3'-diaminobenzidine and 0.002% H_2O_2.

For immunoelectron microscopy, tissues were processed for a pre-embedding silver-enhanced immunogold method. Frozen sections were incubated with anti-napsin antibody (9 μg/ml), and subsequently reacted with goat anti-rabbit IgG covalently linked to 1 nm gold particles (BBInternational, Cardiff, UK). After silver enhancement (HQ silver, Nanoprobes, Yaphank, NY), sections were osmificated, dehydrated, and embedded in Epon 812 (Nisshin EM, Tokyo). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined under a JEM-100SX transmission electron microscope (JEOL, Tokyo).

In situ hybridization

Two non-overlapping antisense oligonucleotides corresponding to rat napsin cDNA (GenBank accession no. AJ251299) +387 to +431 bp and +914 to +958 bp were used as probes for *in situ* hybridization analysis. These oligonucleotides were labeled with [^{35}S]-dATP to a specific activity of 0.5×10^9 dpm/μg DNA, using terminal deoxyribonucleotidyl transferase (Promega, Madison, WI). Tissues were freshly removed from rats and frozen in liquid nitrogen. Frozen 10 μm thick sections were prepared and mounted on glass slides pre-coated with 3-aminopropyltriethoxysilane. They were fixed with 4% paraformaldahyde for 10 min and acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4×SSC (1×SSC; 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (BSA), 0.6 M NaCl, 0.25% SDS, 200 μg/ml yeast transfer RNA, 1 mM EDTA, and 10% dextran sulfate, and then dehydrated. Hybridization was performed at 42℃ for 10 h in the prehybridization buffer supplemented with 10,000 cpm/ml of the ^{35}S-labeled oligonucleotide probes and 100 mM dithiothreitol. The slides were washed at room temperature for 20 min in 2×SSC containing 0.1% sarkosyl and twice at 55℃ for 40 min in 0.1× SSC containing 0.1% sarkosyl. Finally, the sections were exposed to Kodak BioMax MR film (Kodak, Rochester, NY) for 1 week.

Measurement of urinary protein

Protein was determined by the Lowry method with a Bio-Rad *DC* Protein Assay Kit (Bio-Rad, Hercules, CA). The results were analyzed by the unpaired Student's t test.

RESULTS

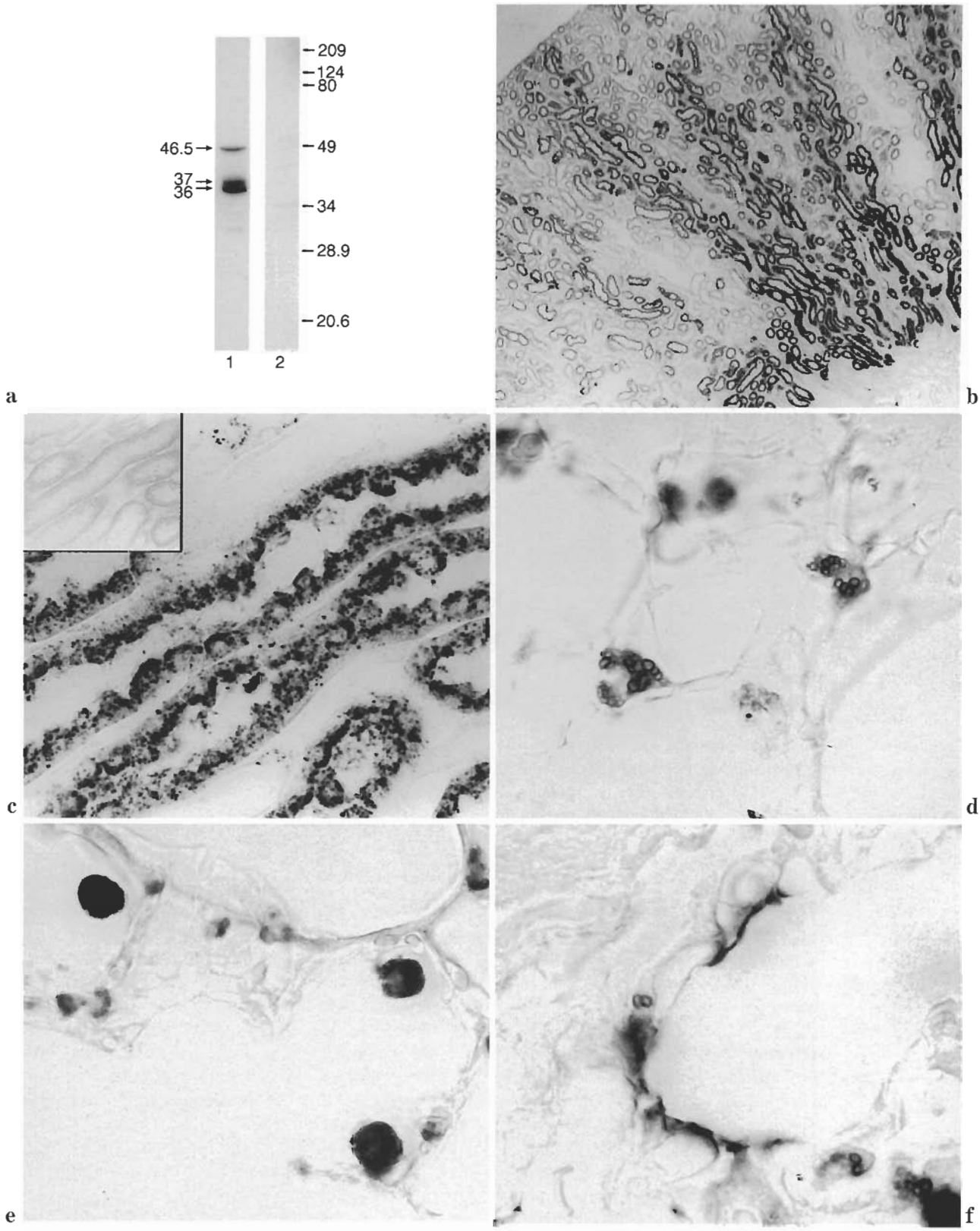
Specificity of napsin antibody

A polyclonal antibody against napsin was raised in a rabbit and affinity-purified. Immunoblot analysis using this antibody revealed two major bands (approximately 36 and 37 kDa) and a minor band (46.5 kDa) in an extract from the mouse kidney (Fig. 1a). The major bands are comparable with a predominant immunoreactive band (about 38 kDa) detected in the human kidney (SCHAUER-VUKASINOVIC et al., 1999). The distribution pattern of immunoreactivity shown by immunostaining of the kidney (Fig. 1b) was consistent with that of napsin mRNA (MORI et al., 2001). Furthermore, the immunoreactivities in immunoblotting and immunostaining were completely abolished using the primary antibody preabsorbed with antigen (Fig. 1a, c, inset). All of these results indicate the specificity of this antibody.

Immunohistochemical localization of napsin

Since we obtained identical immunohistochemical findings in mice and rats, the data described here are those from mice. In the mouse kidney, the napsin immunoreactivity was intense in the proximal straight tubules but less so in the proximal convoluted tubules (Fig. 1b). The immunoreactive structures in both regions were granular in appearance and tended to gather at apical regions of cells (Fig. 1c). Immunoelectron microscopy demonstrated aggregations of immunogold particles for napsin in lysosomes in epithelial cells of the proximal tubules (Fig. 2a, b). No immunoreactivities were detectable in other components of the nephron.

In the lung, type II alveolar epithelial cells and alveolar macrophages stained positively for napsin (Fig. 1d, e), although the mRNA has previously been



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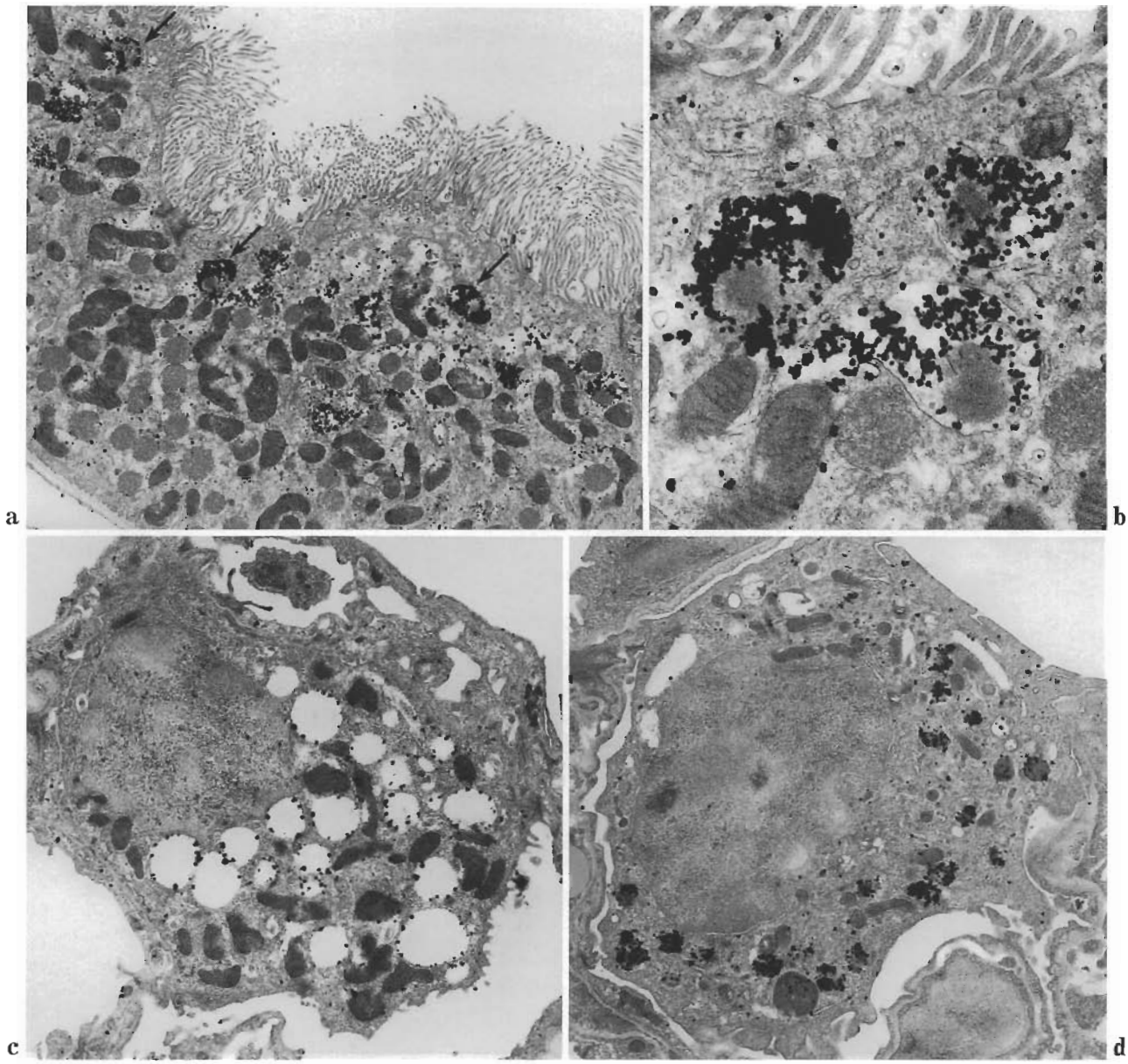

Fig. 2. Immunoelectron microscopy for napsin. **a** and **b.** Immunogold particles densely aggregate on lysosomes (*arrows* in **a**) in the renal proximal tubule. **b** is a higher magnification of **a**. **c.** The rim of lamellar bodies is selectively labeled in a pulmonary type II alveolar cell. **d.** Dense labelings with gold particles are seen in lysosomes of an alveolar macrophage. a: ×5,000, b: ×20,000, c: ×6,800, d: ×7,000

Fig. 1. Immunoblot analysis (**a**) and immunostaining (**b**–**f**) of mouse napsin. **a.** The napsin antibody detects two major bands of approximately 36 and 37 kDa, and one minor band (46.5 kDa) in lysate of the mouse kidney (*lane 1*), whereas the signals completely disappear when using the antibody preabsorbed with the antigen (*lane 2*). **b** and **c.** In the kidney, proximal straight and convoluted tubules are selectively labeled (**b**), and the immunoreactivity displays a granular pattern (**c**). **d f.** In the lung, the rims of large granules in type II alveolar cells (**d**), cytoplasm of alveolar macrophages (**e**), and surface of type I alveolar cells (**f**) are stained positively. b: ×14, c: ×130, inset: ×46, d: ×480, e: ×390, f: ×800

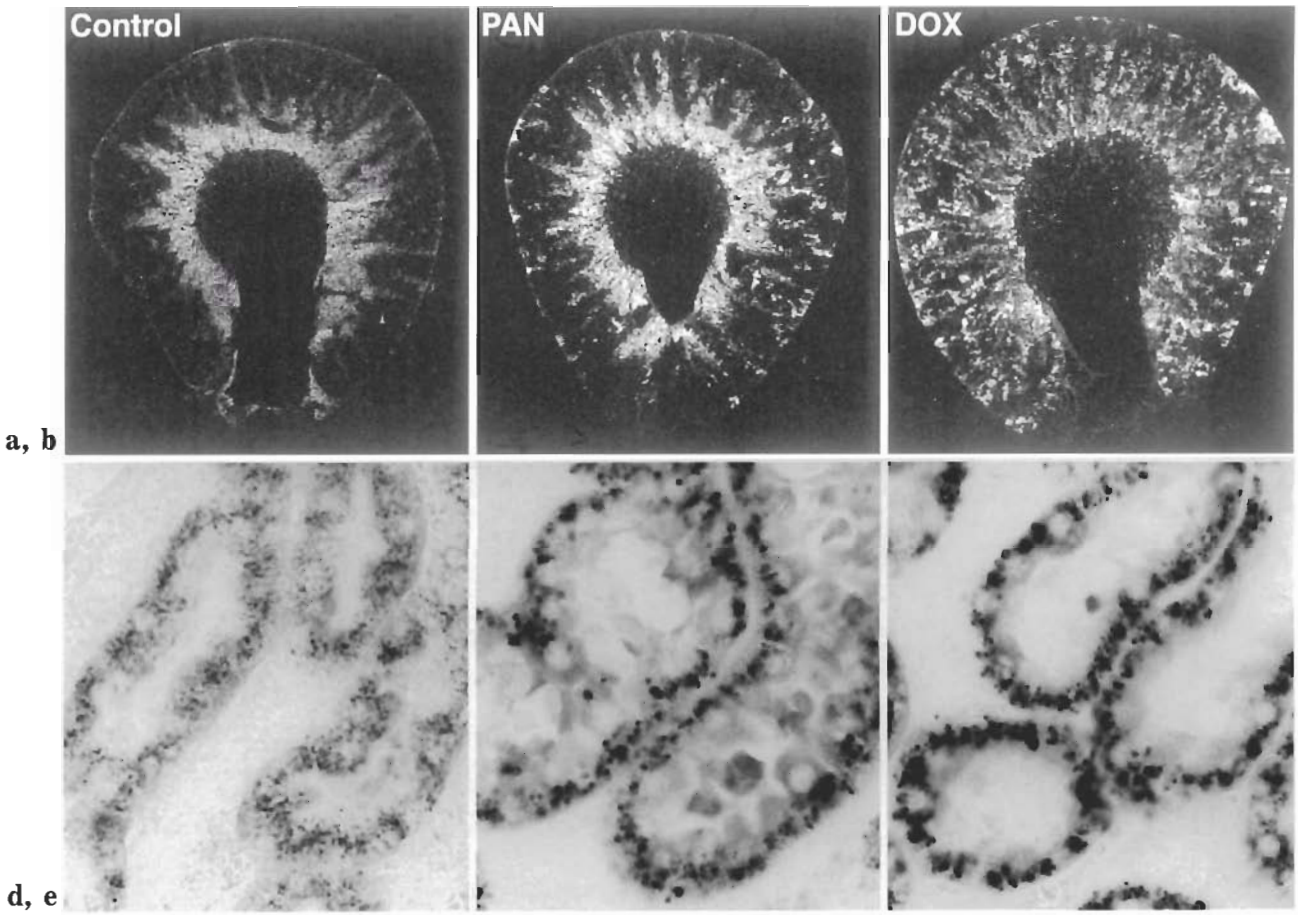

Fig. 3. X-ray film images of napsin mRNA signals (**a**–**c**) and immunohistochemistry for napsin (**d**–**f**) in kidneys of control rats and treated rats with proteinuria. **a.** Napsin mRNA is expressed predominantly in the outer stripe of the outer medulla, and weaker signals radiate toward the cortex in a kidney of a control rat. **b.** Elevated induction of napsin mRNA expression occurs in a puromycin (PAN)-treated rat, compared with a control. **c.** In a kidney of doxorubicin (DOX)-treated rat, napsin mRNA expression is extended throughout the cortex, while the intensity in the outer medulla is somewhat reduced. **d.** Napsin immunoreactivity showing a granular pattern is dispersed in the cytoplasm of proximal tubules in a control. **e** and **f.** Lysosomes are intensely labeled in the proximal tubules of PAN- and DOX-treated rats. In PAN- and DOX-treated rats, immunoreactivity increases in intensity and is coarsely granular. a-c: ×5, d-f: ×200

shown to be undetectable in the latter cells (MORI et al., 2001). Under light microscopy, the immunoreactivity in type II alveolar cells was deposited at the periphery of large granular structures (Fig. 1d), while the cytoplasm of the alveolar macrophages was intensely labeled (Fig. 1e). Significant immunoreactivity was also localized on the surface of several type I alveolar cells, showing sheet-like structures along the alveolar wall (Fig. 1f). By immunoelectron microscopy, immunoreactive gold particles in type II alveolar cells were restricted to the periphery of the lamellar bodies, which were electron-lucent, possibly due to leakage of the contents during preparation and staining (Fig. 2c). The alveolar macrophages contained heavily labeled lysosomes (Fig. 2d).

When lymphoid organs were immunostained by the same procedures, no napsin immunoreactivity was detectable in the spleen, lymph node, or Peyer's patch (data not shown). This finding is in contrast to a significant expression of napsin mRNA in B lymphocyte-rich regions in these organs (MORI et al., 2001).

Renal napsin expression in glomerulopathy models

The single administration of either puromycin aminonucleocide or doxorubicin caused severe persistent proteinuria (Table 1). The napsin mRNA expression in the kidneys of three experimental groups containing controls was investigated by *in situ* hybridization. Two non-overlapping antisense oligonucleotide probes produced essentially the same labeling patterns in the kidneys of each animal. The specificity of the hybridization was further confirmed by disappearance of the signals when an excess dose of cold probe was added to the hybridization fluid.

In control rats injected with saline, napsin mRNA existed predominantly in the outer stripe of the outer medulla, and moderately intense signals were localized radially in the cortex, as was clearly shown on X-ray films (Fig. 3a). The expression pattern was almost identical to that of the mouse kidney (MORI et al., 2001). In puromycin-treated rats, napsin mRNA expression increased in intensity (Fig. 3b), with the same distribution pattern observed in controls. In contrast, doxorubicin injection affected both the intensity and distribution of signals: napsin mRNA expression in the outer medulla was rather reduced in intensity, while that in the cortex was up-regulated, resulting in a diffuse expression throughout the cortex (Fig. 3c).

Immunoreactivity for napsin among the three groups was compared by immunohistochemistry. The overall distribution of napsin immunoreactivity was consistent with that of mRNA in each of the three groups. In control rats, fine-granular structures with immunoreactivity were dispersed in the cytoplasm of the proximal tubules (Fig. 3d). The proximal tubules in puromycin-treated rats contained intensely labeled coarse granules, namely enlarged lysosomes (Fig. 3e). Also, in doxorubicin-injected rats, massive immunoreactivity occurred in congested and enlarged lysosomes (Fig. 3f).

Table 1. Urinary protein excretion in control, PAN-, and DOX-treated rats[a]

Group	n	Days after injection: 14	Days after injection: 28
		Protein excretion (mg/day)	
Control	5	82±10	71±11
PAN	5	472±124*	
DOX	5	259±37*	632±89*

PAN: puromycin aminonucleoside, DOX: doxorubicin
[a]Results are expressed as mean ± SD.
*$P<0.001$ (vs Control) by the unpaired Student's t test.

DISCUSSION

Localization of napsin

The present immunoblot analysis for napsin revealed three immunoreactive bands at 36, 37 and 46.5 kDa in extracts from the mouse kidney, while immunoreactive human napsin A (SCHAUER-VUKASINOVIC et al., 1999) and rat napsin (SCHAUER-VUKASINOVIC et al., 2000b) were found to be about 38 and 50 kDa as single bands, respectively. Until recently, no study has been available concerning the precise biochemical characteristics of this enzyme in the mouse. However, when considering the molecular mass deduced from the predicted amino acid sequence and possible glycosylation sites in the napsin molecule, a weak band appearing at a molecular weight of 46.5 kDa seems to be a proform of napsin, and two bands at 37 and 36 kDa may be active forms. As for the latter two bands, since the more intense band appeared at 36 kDa, this 36 kDa molecule may be an actual active form, and the upper band at 37 kDa seems to be a molecule on the way to the activation process. Further analyses at the protein level are now seeking to unravel these complexities. The cellular localization of napsin immunoreactivity in the kidney was completely identical to that of its mRNA expression revealed by our *in situ* hybridization analysis (MORI et. al, 2001). Previous studies by SCHAUER-VUKASINOVIC et al. (1999, 2000b) suggested that napsin might be present "in large cellular vacuoles or as a membrane-associated form". The pH-optimum and pH-stability of human napsin A indicated that napsin is a lysosomal enzyme in nature, like cathepsin D (SCHAUER-VUKASINOVIC et al., 2000a; GRÜNINGER-LEITCH et al., 2002). The present study demonstrated the condensed localization of napsin immunoreactivity in lysosomes of the proximal tubules.

The napsin immunoreactivity in type II alveolar cells was restricted to the rim of lamellar bodies in association with the limiting membrane. Although we cannot exclude the possibility that the loss of granular contents affected the immunoreaction, the localization of napsin inside the lamellar bodies suggests an involvement in the processing of granular contents. In type II cells, hydrophobic surfactant proteins B and C are produced from larger precursors through processing by proteases (JOHANSSON and CURSTEDT, 1997). It would appear that napsin is a candidate

protease, such as a cathepsin D-like protease which processes the pro-surfactant proteins into mature forms (WEAVER et al., 1992). Alternatively, on the basis of its membrane-associated localization, napsin may be involved in the cleavage of intramembrane proteins, like the β-site amyloid precursor protein-cleaving enzyme (BACE) (VASSAR et al., 1999; YAN et al., 1999; KITAZUME et al., 2001) and presenilin (STEINER and HAASS, 2000), which are both members of the aspartic protease family. In the lung, napsin protein was also localized on the surface of type I alveolar cells and in lysosomes of alveolar macrophages. However, these cells did not express napsin mRNA in our prevlous study (MORI et al., 2001). Thus, it is reasonable to assume that a considerable amount of napsin is secreted from lamellar bodies together with surfactant, and the adhesion of napsin causes positive staining on the surface of alveolar walls. In the alveoli, the secreted surfactant is finally inactivated by mechanical and biological processes, and phagocytosed by alveolar macrophages for a catabolic pathway (GROSS et al., 1988; JOBE and IKEGAMI, 1993; TRAPNELL and WHISETT, 2002). The immnunoreactivity in lysosomes of the alveolar macrophage may reflect such a catabolic pathway.

Human napsin B gene expressed in the spleen lacks a stop codon, whereas its isoform, *napsin A*, encodes a functional enzyme in the kidney and lung (TATNELL et al., 1998). SCHAUER-VUKASINOVIC et al. (2000b), who identified rat napsin, could not find any isoforms of napsin by searching the Incyte ZooSeq rat EST database. The present study failed to detect napsin protein in lymphatic organs despite the considerable mRNA expression (MORI et al., 2001). Therefore, our findings indicate the possibility that there may be napsin isoforms in the rodents, much like human *napsin B*.

Role of napsin in protein catabolism in the renal proximal tubule

Epithelial cells in the renal proximal tubules can absorb proteins present in the glomerular filtrate. Most of the absorbed proteins are intracellularly transported to lysosomes, where they undergo complete hydrolysis to amino acids that are then returned to the circulation (MAUNSBACH, 1966; WALL and MAACK, 1985; MAACK et al., 1992). This process is essential for the homeostasis of circulating proteins. To evaluate the role(s) of napsin in protein homeostasis, we compared the renal expression of napsin using rat models with severe proteinuria. The injection of puromycin and doxorubicin reportedly resulted in massive proteinuria and overt nephrotic syndrome lasting from 2-4 weeks (BERTANI et al., 1982; DIAMOND and KARNOVSKY, 1986).

In the present *in situ* hybridization, both treated rat models showed a remarkable enhancement of napsin expression in the kidney, although the expression patterns differed between the two models. Puromycin proteinuria simply elevated the napsin expression level in the outer medulla, where napsin is expressed even under normal conditions. Doxorubicin proteinuria extended the expression site to the cortex, and simultaneously reduced the expression in the proximal straight tubules of the outer medulla. Doxorubicin nephrosis is one of the most severe animal models of nephrotic proteinuria, and many studies have indicated that proteins filtered through the glomerular capillary may have intrinsic toxicity (EDDY et al., 1991; MAGIL, 1995; REMUZZI and BERTANI, 1998). Considering that this form of proteinuria invariably progresses to tubulointerstitial nephritis (BERTANI et al., 1986), the reduction of napsin mRNA expression in the proximal straight tubules could be due to cell damage, and might be supplemented by novel expression in the proximal convoluted tubules. A large increase in protein load frequently leads to lysosomal congestion and enlargement in the urinary tubules (MAACK, 1967; MAACK et al., 1992; REMUZZI et al. 1997). The present immunohistochemistry demonstrated the aggregation and enlargement of napsin-containing lysosomes, called protein absorption droplets. Thus, elevated napsin expression followed by the development of napsin-containing lysosomes shows that this proximal tubule-specific aspartic protease significantly participates in protein catabolism.

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