Cellular Distribution of Napsin (Kidney-Derived Aspartic Protease-like Protein, KAP) mRNA in the Kidney, Lung and Lymphatic Organs of Adult and Developing Mice

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Received June 2, 2001

Summary. Kidney-derived aspartic protease-like protein (KAP), initially identified in the mouse kidney, is a novel aspartic protease exclusively expressed in the lung and spleen as well as the kidney. Its orthologues have been identified in the human and rat, and termed napsin. We performed *in situ* hybridization analysis to determine the cellular expression of napsin mRNA in the kidney, lung, and lymphatic organs of adult mice and to demonstrate, for the first time, its expression patterns in ontogeny. In the adult mouse kidney, extremely intense signals for napsin mRNA were observed in the proximal straight and convoluted tubules, in agreement with a previous study. The first signals for napsin mRNA during nephrogenesis occurred selectively in mesonephric tubules at embryonic day 13, and in metanephric tubules from embryonic day 14. In the lung, a distribution restricted to type II alveolar cells or their precursors was found from embryonic day 15, at the onset of type II cell differentiation, to the adult stage. In the spleen, the mRNA was expressed in lymph nodes of the white pulp and the marginal zone – namely, B-lymphocyte-rich regions – from postnatal day 0 to adult. The lymph node and Peyer's patch displayed similar expression patterns, but T cell-dependent areas in these organs and the thymus lacked such signals. These findings suggest that mouse napsin possesses crucial functional roles not only in the kidney but also in the lung and lymphatic tissues, even during fetal stages.

Aspartic proteases constitute a subclass of endopeptidases and commonly contain two essential aspartic acid residues at the active site (TANG and WONG, 1987; KHAN and JAMES, 1998). They include well-known enzymes such as cathepsins D and E, pepsin, gastricsin, chymosin, renin, and β -site APP-cleaving enzyme (BACE). Aspartic proteases have been extensively studied because of their important physiologi-

cal roles and involvement in various pathological events (ROCHEFORT, 1992; KAY et al., 1998; VASSAR et al., 1999).

The cDNA of kidney-derived aspartic protease-like protein (KAP), a novel mouse aspartic protease, has been isolated as a gene potentially involved in the physiological functions of the kidney (MORI et al., 1997). Subsequently, orthologues of mouse KAP, called napsin, have been identified in the human (TATNELL et al., 1998) and rat (SCHAUER-VUKASINOVIC et al., 2000b). The high sequence homology among these mammals (72–88% of nucleotide residues) suggests that KAP and napsin belong to the same family; therefore, we use the name napsin in the present paper. There are two isoforms, napsins A and B, only in humans (TATNELL et al., 1998). The human *napsin A* gene encodes a functional aspartic protease predominantly expressed in the kidney and lung, while the human *napsin B* gene, transcribed exclusively in the spleen, lacks a stop codon; therefore, it is not clear whether napsin B is expressed as a functional enzyme. Northern blot analysis for napsin has revealed a restricted distribution in the kidney, lung, and spleen of these three mammals (MORI et al., 1997; TATNELL et al., 1998; SCHAUER-VUKASINOVIC et al., 2000b), suggesting functions distinct from other aspartic proteases. Histochemically, human napsin A has been demonstrated predominantly in the proximal urinary tubules of the kidney and the type II alveolar cells of the lung (CHUMAN et al., 1999; SCHAUER-VUKASINOVIC et al., 1999; HIRANO et al., 2000), although previous studies reported some discrepant results concerning its cellular localization. In contrast, nothing is known about the cellular distribution of napsin in rodents except for its expression in the proximal urinary tubules (MORI et al., 1997); its

localization and functional significance in the spleen and other lymphatic tissues remain undetermined.

We here report the precise cellular distribution of napsin mRNA in the kidney, lung, and lymphatic tissues of mice during developing and adult stages as demonstrated by *in situ* hybridization, and also provide evidence for its predominant expression in B lymphocytes.

MATERIALS AND METHODS

Animals

Adult male and female ddY mice (10-weeks old) and fetal and neonatal mice (from embryonic day 11 to postnatal day 7; day 0 of the gestational period was determined by the appearance of a vaginal plug) (Japan SLC, Shizuoka, Japan) were used in the present study. In the experimental protocols for animal care and handling, the investigators adhered to the guidelines of Hokkaido University. For *in situ* hybridization using ^{35}S -labeled probes, mice were sacrificed by bloodletting from the cervical artery under deep anesthesia with pentobarbital, and tissues were freshly removed and frozen in liquid nitrogen. Frozen sections, 10 μm in thickness, were prepared and mounted on silane-coated glass slides. For *in situ* hybridization using digoxigenin-labeled probes, anesthetized adult mice were perfusion-fixed via the aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were removed and immersed in the same fixatives for an additional 6 h at 4°C. After fixation, tissues were embedded in paraffin and 3 μm -thick sections were prepared and mounted on silane-coated glass slides.

In situ hybridization

Two non-overlapping 45-mer antisense oligonucleotide probes were synthesized for the mouse KAP cDNA (nucleotide residues 555–599 and 1086–1130, Accession no. D88899, MORI et al., 1997). The oligonucleotides were labeled with ^{35}S -dATP to a specific activity of 0.5×10^9 dpm/ μg DNA, using terminal deoxynucleotidyl transferase (Promega, Madison, WI). *In situ* hybridization analysis was performed as previously described (MOWA and IWANAGA, 2000). In brief, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h by adding ^{35}S -labeled oligonucleotide probes at a final concentration of 0.5×10^7 cpm/ml in 50% formamide containing 30 mM Tris-HCl (pH 7.6), 0.6 M NaCl, 1 mM EDTA, 100

mM dithiothreitol, 1×Denhardt's solution, 0.25% sodium dodecyl sulfate (SDS), 10% dextran sulfate and 200 $\mu\text{g}/\text{ml}$ yeast tRNA. Control hybridization was performed in the presence of a 20-fold excess amount of unlabeled antisense probes. Slides were rinsed at room temperature for 30 min in 2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate) containing 0.1% sarkosyl, twice at 55°C for 40 min in 0.1×SSC containing 0.1% sarkosyl, dehydrated through a graded series of ethanol, and air-dried. Slides were exposed to Hyperfilm β -max film (Amersham, Buckinghamshire, UK) for 2 weeks, or dipped into autoradiographic emulsion (NTB-2; Kodak, Rochester, NY), exposed at 4°C for 4 weeks and counterstained with hematoxylin.

To determine the restricted localization of signals, *in situ* hybridization using non-radioisotope probes was performed. The 756bp fragment of mouse KAP cDNA (nucleotide residues 253–1008) was obtained from the kidney by the reverse transcriptase-polymerase chain reaction method using the primer pair (5'-CTTCAACGAATCCACCTTGG-3', 5'-TGTGTC TAGGATGGCACTGC-3'). Reverse transcription was performed using Superscript II Reverse Transcriptase (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's protocol. The fragment was ligated with and cloned in pGEM-T easy vector (Promega, Madison, WI). The cRNAs were synthesized with T7 and SP6 RNA polymerases as sense and antisense riboprobes in the presence of digoxigenin-labeled UTP using a DIG RNA Labeling Kit (Roche Diagnostics, Tokyo, Japan), respectively (FURUTA et al., 1990; KON et al., 1993). Within these probes, antisense RNA was designed to hybridize with KAP mRNA on the paraffin-embedded sections. Deparaffinized and subsequently air-dried sections were processed for *in situ* hybridization by a modification of Kon's method (KON et al., 1993). Briefly, they were digested with 20 $\mu\text{g}/\text{ml}$ proteinase K in 0.1 M Tris-HCl buffer (pH 7.5) for 15 min at room temperature, fixed with 10% formalin for 5 min, rinsed with DEPC H_2O , and then dried. Hybridization was performed overnight at 58°C by adding 1% sense or antisense RNA probes in the buffer of 50% formamide, 10 mM Tris-HCl (pH 7.6), 600 mM NaCl, 1 mM EDTA (pH 8.0), 1×Denhardt's solution, 0.25% SDS, 10% dextran sulfate and 200 $\mu\text{g}/\text{ml}$ tRNA. Slides were rinsed at 50°C with 2×SSC, 1×SSC and 0.5×SSC for 10 min, respectively. After preincubation with 1% blocking reagent (Nucleic Acid Detection Kit; Roche Diagnostics, Tokyo, Japan) for 30 min at room temperature, they were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments diluted 1:400 with a

solution containing 100 mM Tris-HCl and 150 mM NaCl (pH 7.5) for 2 h at room temperature. After three rinses with the same buffer for 5 min, they were incubated with a color-substrate solution containing 4.5 μ l of nitroblue tetrazolium and 3.5 μ l of X-phosphate (0.75 ml 5-bromo-chloro-3-indolyl phosphate and 50 mg/ml toluidinium in dimethylformamide) in 1 ml of a solution composed of 100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂ (pH 9.5) in a dark room for 2 h at room temperature. Sections were rinsed with both a buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) and distilled water, and finally mounted with glycerin-gelatin.

Immunohistochemistry

Fresh frozen sections adjacent to those used for *in situ* hybridization were mounted on poly-L-lysine-coated glass slides, respectively fixed with both ice-cold acetone and 10% neutral-buffered formalin for 10 min, and rinsed with 0.01 M phosphate-buffered saline (pH 7.4). After preincubation with 10% normal goat serum for 30 min at room temperature, the sections were incubated overnight at 4°C with a biotin-conjugated rat anti-mouse B220 monoclonal antibody specific for mouse B lymphocytes (Cedarlane Laboratories, Ontario, Canada) diluted at 1:400, followed by incubation with avidin-peroxidase complex (Histofine kit; Nichirei, Tokyo, Japan) for 60 min at room temperature. The immunoreaction was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.002% H₂O₂, and examined with a light microscope.

RESULTS

Expression in adult mice

In situ hybridization using two non-overlapping antisense oligonucleotide probes exhibited consistent labeling in the kidney, lung, and lymphatic organs. The specificity of hybridization was also confirmed by the disappearance of the signals when an excess dose of cold probes was added to the hybridization fluid. In the adult mouse kidney, napsin mRNA was expressed predominantly in the outer stripe of the outer medulla, and weaker signals radiated toward the cortex, as clearly shown on X-ray films (Fig. 1). Light microscopic observation of hybridized sections (microautoradiography) revealed strong labeling in the proximal straight tubules and less intense labeling in the proximal convoluted tubules (Fig. 2a). No significant signals were recognized in the glomeruli, loops of Henle, distal urinary tubules, or collecting ducts. The same distribution pattern of napsin mRNA was obtained

Fig. 1. *In situ* hybridization of mouse napsin mRNA in an adult mouse. X-ray images of the ³⁵S-labeled antisense probe show the intense expression of the mRNA in the kidney (*Kid*), lung (*Lung*), spleen (*Spl*), lymph node (*Lym*), and Peyer's patch (*PP*), while the thymus (*Thy*) and liver (*Liv*) are negative. $\times 3.5$

by *in situ* hybridization using the digoxigenin-labeled cRNA probe (Fig. 2b).

Autoradiography on X-ray film showed diffuse and weak signals throughout the adult lung (Fig. 1). Light microscopically, signals were distributed in a small number of cells along the alveolar epithelium (data not shown), but identification of the types of the expressing cells with microautoradiography was not possible. Use of the antisense riboprobe revealed significant expression in type II alveolar cells that were round or cuboidal (Fig. 2c). No significant labeling was recognizable in any other elements, including type I alveolar cells, epithelia of the terminal and respiratory bronchioles, or alveolar macrophages.

In the spleen, the expression of napsin mRNA largely coincided with the white pulp, showing a characteristic annular shape (Fig. 1). Under the light microscope, strong signals were confined to the lymphatic nodules — including the germinal centers — where B-lymphocytes densely gathered and to the marginal zone (Fig. 2d, e). No significant signals were found in the periarterial lymphatic sheath or red pulp. When adjacent sections were immunostained using B220, a B-lymphocyte-specific antibody, colocalization of napsin mRNA and the immunoreactivity was clearly demonstrated (Fig. 2d-f). The overall observation of other lymphatic organs by X-ray film revealed intense expression in the lymphatic nodules of both the lymph node and Peyer's patch, but not in the thymus (Fig. 1). In all of these lymphatic organs, intense signals were confined to the lymphatic nodules.

Fig. 2. *In situ* hybridization of napsin mRNA in the adult kidney (**a** and **b**), lung (**c**), and spleen (**d** and **e**) and immunohistochemistry for B220 in the spleen (**f**). **a.** Intense signals detected by the ^{35}S -labeled antisense probe are seen in proximal straight tubules with less intense signals in proximal convoluted tubules. **b.** Proximal straight tubules with a developed brush border are labeled with the digoxigenin-labeled antisense cRNA probe. No significant signals are observed in the loop of Henle (*arrow*). **c.** Type II alveolar cells (*arrowheads*) protruding into the alveolar space express the signals for napsin mRNA. **d** and **e.** Corresponding bright-field (**d**) and dark-field (**e**) images display significant signals in the lymphatic nodule (*L*) of the white pulp and marginal zone (*M*), whereas the periarterial lymphatic sheath (*P*) and red pulp (*R*) are negative. **f.** A section adjacent to **d/e** demonstrates the selective expression of napsin mRNA in B-lymphocytes in the lymphatic nodule and marginal zone of the spleen. a: $\times 12$, b: $\times 86$, c: $\times 150$, d-f: $\times 40$

Fig. 3. Distribution of napsin mRNA during nephrogenesis. **a.** The first signals in the developing kidney are detected in several mesonephric tubules at E13. In contrast, the mesonephric duct (*arrow*) is negative. *M* metanephros. **b.** Expression in the metanephric tubules at E15. No signals are observed in the ureteric duct (*U*). **c** and **d.** Intense signals are seen in many tubules at both E17 (**c**) and P7 (**d**). The glomeruli (*arrowheads* in **c**) are negative. **a:** $\times 70$, **b:** $\times 75$, **c:** $\times 85$, **d:** $\times 33$

Expression in pre- and postnatal mice

The first significant signals for napsin mRNA in the developing kidney were detected in a few mesonephric tubules at embryonic day 13 (E13), whereas the mesonephric and paramesonephric ducts and the metanephros were negative (Fig. 3a). At E14–15, when the metanephros gradually increased in size and moderate numbers of renal vesicles and tubules were present, a significant number of metanephric tubules with cuboidal epithelium expressed mRNA (Fig. 3b). The mesonephros had almost completely regressed by this stage, and few signals could be seen in the mesonephric tubules. By E17, an intense expression was detected in many, but not all, straight or convoluted tubules (Fig. 3c). The neonatal kidney by postnatal day 7 (P7) had almost the same expression pattern of mRNA as in adults; numerous tubules in the outer region of the medulla showed a strong expression, though other components of the medulla

were completely negative (Fig. 3d). Throughout the developing stages, the signals were confined to differentiated tubules, but undetectable in the renal vesicles or S-shaped bodies, glomeruli, or ureteric ducts.

In the developing lung, initial signals were detected in the cuboidal epithelium of acinar tubules at E15, which corresponded to the pseudoglandular period (Fig. 4a). At E16 (the end of the pseudoglandular period) when branching of acinar tubules had further progressed, labeled acinar tubules increased in number (Fig. 4b). When saccules with rather smooth walls appeared from E17 to the postnatal stage, called the terminal-sac period, the intense signals were dispersed within them (Fig. 4c). By P7 (the alveolar period), the epithelium of the alveolar pouches showed an intense and scattered expression (Fig. 4d). The signal intensity in both the saccules and the alveolar pouches was much higher than that in alveoli of adult mice. No significant signals were observed in the

Fig. 4. Distribution of napsin mRNA in the developing lung. **a.** A weak but significant expression of napsin mRNA is detected in the acinar tubules (*arrowheads*) at E15. **b.** Transcripts in acinar tubules extend and increase in intensity at E16, whereas the columnar bronchiolar epithelium (*arrow*) is negative. **c** and **d.** Strong and dispersed signals are detected in the epithelium of the saccules at P0 (**c**), and alveolar septa at P7 (**d**). a: $\times 70$, b: $\times 75$, c, d: $\times 120$

epithelia of bronchial branches throughout the developing stages.

The appearance of napsin mRNA expression in the developing spleen occurred from P0, when the distinction between the red and white pulps became apparent. In any stages, the outer region of the white pulp exhibited a plain expression, showing the same expression patterns as in the adult spleen (data not shown).

DISCUSSION

KAP, consisting of 419 amino acid residues, was identified as a new member of the aspartic protease family in mice (MORI et al., 1997), leading to the subsequent reports of the existence of orthologous human napsin A/B (TATNELL et al., 1998) and rat napsin (SCHAUER-VUKASINOVIC et al., 2000b). In contrast to human napsins, little is known about the

cellular distribution and possible roles of napsin in rodents. Some species differences have been noted, as represented by the expression of *napsin* genes in the spleen: only *napsin B*, recognizable as a pseudogene, is expressed in the human spleen, while the mouse spleen expresses transcribed *napsin*. In the present study, the detailed expression pattern of napsin mRNA in adult mice and ontogenetical development was investigated, and some potential roles suggested.

In the adult kidney, consistent with the data from MORI et al. (1997), we found an abundant expression of napsin mRNA in the proximal straight tubules, and a moderately intense expression in the proximal convoluted tubules. The characteristic distribution of renal napsin mRNA is completely different from other known aspartic proteases produced in the kidney such as renin (KON et al., 1986) and cathepsin D (YOKOTA et al., 1985). As for human napsin A, SCHAUER-VUKASINOVIC et al. (1999) reported its distinct expression in the distal convoluted tubules,

collecting ducts, and Henle loops as well as proximal tubules. Thus, the intrarenal distribution of napsin mRNA differs between the mouse and human. Although no information about the developmental profile of napsin expression is available, the present study revealed an intense expression of napsin mRNA during nephrogenesis: the signals were confined to mesonephric and metanephric tubules (the latter possibly give rise to proximal tubules), while no signals occurred in the primordia of nephrons, i.e., renal vesicles and S-shaped bodies (SAXÉN, 1987; INO et al., 2001). These results suggest that the napsin of urinary tubules may play a crucial role in the reabsorption of urine even in the fetal stage, and possibly in the differentiation of proximal tubules. Data on the function of renal napsin are fragmentary, but gradually increasing. Recent studies by SCHAUER-VUKASINOVIC and co-workers have suggested a non-lysosomal, membrane-associated localization of human napsin A (SCHAUER-VUKASINOVIC et al., 1999; 2000a). It has also been reported that renal napsin A is excreted into the urine, and that some renal diseases are accompanied by a lack or lower concentration of napsin A in the urine (SCHAUER-VUKASINOVIC et al., 2000c). Since some proteases existing in the renal brush border membrane enhance or inhibit H⁺-ATPase activity and Na⁺/H⁺ exchange (SABOLIC et al., 1992; WEINMAN et al., 1993), it is likely that napsin secreted to the lumen promotes the reabsorption of some peptides by digestion of the precursor proteins or directly affecting such transporters.

Expression of napsin mRNA in the adult mouse lung was found to be restricted to the type II alveolar cells, in good accord with the results of *in situ* hybridization for napsin A mRNA in the human lung (CHUMAN et al., 1999). On the other hand, immunohistochemistry of human lungs showed positive staining also in alveolar macrophages, a subset of type I alveolar cells, and the bronchiolar epithelium, as well as type II alveolar cells (SCHAUER-VUKASINOVIC et al., 1999; HIRANO et al., 2000). In the developing mouse lung, the first signals for napsin mRNA were detectable linearly along the acinar tubules at the pseudoglandular period. This stage corresponds to the differentiation of type II alveolar cells, i.e., E14–15 (TEN HAVE-OPBROEK et al., 1988). It is generally believed that the acinar tubules, from which the respiratory components differentiate, are composed of only type II alveolar cells, and that this type of cell is the stem cell of type I alveolar cells in fetal (TEN HAVE-OPBROEK, 1979) and adult lungs (ADAMSON and BOWDEN, 1974). At the terminal-sac and following alveolar periods, when type I cells develop (TEN HAVE-OPBROEK, 1981), a more intense but dispersed

expression appeared within the saccules and alveolar pouches. Accordingly, the results presented here definitely show a limited expression of napsin mRNA in type II alveolar cells in adult and developing stages. The type II alveolar cells secrete surfactant proteins, including surfactant proteins B and C, which are derived from larger precursors (JOHANSSON and CURSTEDT, 1997). A cathepsin D-like protease contained in the lung is known to process pro-surfactant protein B into a mature form (WEAVER et al., 1992), and recently CHUMAN et al. (1999) proposed human napsin A as a candidate protease for the cleavage of pro-surfactant proteins. The strong and sustained expression of napsin mRNA from the terminal-sac period when the type II alveolar cells enhance the production of surfactants (TEN HAVE-OPBROEK et al., 1990) suggests an important role for napsin in the proteolytic processing of surfactant proteins.

In both the adult and neonatal spleen, we demonstrated the expression of napsin mRNA in the lymphatic nodules of the white pulp and the marginal zone. The obtained data were not in accord with the reported immunohistochemical findings for human napsin A in the spleen (SCHAUER-VUKASINOVIC et al., 1999), in which positive staining was seen in the sinusoidal and arterial endothelial cells and in subsets of lymphocytes in the periarterial lymphatic sheaths. Furthermore, we first demonstrated distinct expression of napsin mRNA in the lymph node and Peyer's patch; here again, the lymphatic nodules intensely expressed the signals. Observations of serial sections confirmed its abundant expression in mature B lymphocytes. Mouse, rat, and human napsins are known to conserve an RGD motif for integrin binding at the C-terminus (TATNELL et al., 1998; SCHAUER-VUKASINOVIC et al., 2000b). Such an interaction might potentially concern both the signal transmission between B lymphocytes and homing of B lymphocytes in the lymphatic nodules. Alternatively, there is a possibility that napsin in B lymphocytes contributes to the antigen presentation of major histocompatibility complex (MHC) class II molecules. CD4⁺ T lymphocytes recognize antigens that are bound to the peptide-binding groove of MHC class II molecules expressed on antigen-presenting cells such as B lymphocytes, dendritic cells, and macrophages. In these cells, proteases such as cathepsins or unknown proteases participate in the generation of antigenic peptides and degradation of the invariant chain (NAKAGAWA and RUDENSKY, 1999; VILLADANGOS et al., 1999). In the human, napsin B mRNA has been reported to be expressed not only in the spleen but also in peripheral blood leukocytes, promyelocytic

leukaemia HL-40, and Raji cells (a B-cell line derived from human Burkitt's lymphoma), though *napsin B* appears to be a pseudogene (TATNELL et al., 1998). Further comparative analysis in rodents and human is needed to determine the functional significance of napsins and their evolutionary changes in lymphatic organs.

In conclusion, we demonstrated the precise tissue distribution of napsin mRNA in adult, fetal, and neonatal mice. It is now clear that napsin mRNA possesses cell-specific expression patterns unique for each of the kidney, lung, and lymphatic tissues. Furthermore, the consistent expression from developing stages to the adult suggests critical roles of the napsin family in these tissues.

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