Human napsin A: expression, immunochemical detection, and tissue localization

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Abstract A novel aspartic proteinase, called napsin, has recently been found in human and mouse. Due to high similarity with cathepsin D a structural model of human napsin A could be built. Based on this model a potential epitope SFYLNRDPEEPDGGE has been identified, which was used to immunize rabbits. The resulting antibody was employed in monitoring the expression of recombinant human napsin A in HEK293 cell line. Western blot analysis confirmed the specificity of the antibody and showed that human napsin A is expressed as a single chain protein with the molecular weight of approximately 38 kDa. Immunohistochemical studies revealed high expression levels of napsin A in human kidney and lung but low expression in spleen.

Key words: Human napsin A; Expression; Napsin A antibody; Immunohistochemistry; Human kidney; Human lung

1. Introduction

Aspartic proteinases belong to the class of endopeptidases. They contain two Asp residues in the active site, each in a distinct domain of the enzyme [1,2]. Other members of this family of enzymes are cathepsin D and E, renin, pepsin, gastricsin, and bovine chymosin. Many of these enzymes have been extensively studied because of their well-defined physiological roles and their involvement in pathological states related to cancer [3] or other diseases [4].

Recently a new aspartic proteinase has been described in humans and mouse, respectively [5,6]. This new human enzyme, called napsin, is found in two isoforms, napsin A and B, which share high sequence homology (85%). While napsin A seems to be a functional aspartic proteinase predominantly expressed in lung and kidney, napsin B is transcribed exclusively in cells associated with the immune system. Tatnell et al. suggest that napsin B might be a transcribed pseudogene lacking a stop codon and as a result it is possible that the protein product is rapidly degraded and, therefore, difficult to detect [5]. Human aspartic proteinases pepsin and gastricsin are secreted by the stomach, cathepsin E is found in the endoplasmatic reticulum of erythrocytes and stomach mucosa cells [7], whereas cathepsin D is located in lysosomes of various cell types. The fact that mRNA of napsin A is specifically localized in kidney and lung tissue suggests a possible difference in its biological function from other aspartic proteinases. In situ hybridization of kidney-derived aspartic proteinase (KAP), a mouse homolog of napsin, resulted in strong signals in the outer stripe of the outer medulla predominantly localized in the proximal straight tubule [6]. It is of interest whether human napsin A has a similar tissue localization as KAP.

The goal of this work was to express human napsin A and obtain a specific polyclonal antibody which was then employed in immunochemical studies using Western blots and in tissue localization studies.

2. Materials and methods

2.1. Modeling

A sequence alignment of napsin A and sequences of aspartic proteinases with known 3D structure revealed highest sequence identity (49%) with human cathepsin D. The structure of pepstatin-inhibited human cathepsin D [8] was chosen as template to build a 3D model of human napsin A using an SGI O2 workstation and our in-house modeling software Moloc [9]. The correctness of the model was checked with Moloc and a program by Luthy et al. [10].

2.2. Design and synthesis of a polyclonal antibody

A napsin A-specific polyclonal antibody was produced by immunizing rabbits with a 15 amino acid long peptide SFYLNRDPEEPDGGE. Immunization of rabbits and serum preparation was performed by Genosys Biotechnology, Cambridgeshire, UK. Serum was collected after several bleedings and tested for detection of napsin A (1:1000 dilution) transblotted on nitrocellulose membrane from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex, San Diego, CA, USA). For evaluation of putative cross-reactivity the corresponding napsin B-specific antigenic peptide SFYFNRDPEVADGGE was synthesized.

2.3. DNA construction, cell culture and lysate preparation

The mouse sequence of kidney-derived aspartic proteinase ([5]; Accession number: em_ro:mmd8991) was used to search the Incyte TM database for homologous human EST clones. Two subgroups were identified which represented two human isoforms of the mouse protein. One EST clone harboring the putative translational start codon was obtained from Incyte and sequencing revealed that the insert contained the entire coding region for napsin A. This insert was further subcloned into pcDNA3.1, an expression vector (Invitrogen Corp., San Diego, CA, USA) containing a CMV promotor and a marker for G418 selection.

HEK293 cells were maintained in growth medium MEM++, supplemented with 10% FCS, L-glutamine and penicillin/streptomycin (Life Technologies Inc., Gaithersburg, MD, USA) in a humidified incubator with 5% CO_2 at 37°C. To create stable lines these cells were transfected with the expression vector containing human napsin A and carrying the G418 resistance gene using lipofectamine (Life Technologies) according to the manufacturer's protocol. After 48 h the cells were split and grown in growth medium supplemented with 0.5 mg/l of G418 (Sigma, St. Louis, MO, USA). Medium was changed twice a week and resistant colonies were isolated 16 days after transfection. Lysate from several colonies was prepared by sonication of the cellular pellet in PBS containing 20 mM EDTA and 1 mM PMSF. From such lysates a crude pellet fraction was isolated by centrifugation at 14000 rpm in an Eppendorf centrifuge cooled to 4°C. The

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Abbreviations: HEK, human embryonic kidney; MEM, minimal essential medium; FCS, fetal calf serum

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pellet fraction was further eluted in the same buffer containing 1% Elugent (Calbiochem-Novabiochem, La Jolla, CA, USA). After a second centrifugation step the lysates were split into aliquots and stored at 4°C for immediate use or at −80°C for long term storage. The total protein concentration was determined and the samples were analyzed for the appearance of recombinant human napsin A using Western blot analysis.

2.4. Electrophoresis and Western blot analysis

SDS-PAGE was carried out by following the method of Laemmli (1970) [11]. In brief, cell homogenate was heated at 95°C for 5 min in reducing sample buffer and subjected to SDS-PAGE using 4–20% Tris-glycine gels (Novex). Electrophoresis was performed for 1 h at a constant 200 V. Proteins were subjected to electroblotting onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) for 2 h at 25 V using 25 mM Tris/192 mM glycine/20% (v/v) methanol as a buffer system. See-Blue prestained standards (Novex) were used as molecular mass standards. The membrane was blocked using 20% newborn fetal serum in PBS supplemented with 0.1% (v/v) Tween-20 (PBSt) for 1 h at room temperature, washed with PBSt, and incubated for 1 h at room temperature with anti-napsin A antibody (1:1000, v/v). After washing the membrane with PBSt (1×15 min and 2×5 min), the membrane was incubated for 1 h at room temperature with HRP-conjugated anti-rabbit Ig from donkey (1:5000, v/v) (Amersham), and the IgG was detected with the enhanced chemiluminescence system (Amersham) in accordance with the manufacturer's instructions.

2.5. Immunohistochemical analysis

The cellular localization and the expression pattern of napsin A were studied by different immunohistochemical techniques in normal human kidney, lung and spleen. The human kidney tissue ($n=7$) was either obtained from tumor-remote parts of tumor resections or from donors without history of medical problems or medications who died in a motor vehicle accident. Normal lung tissue ($n=3$) was also obtained from brain-dead donors, whereas the spleen tissue ($n=2$) was obtained from patients dying from bronchopneumonia. The tissue was either fixed in 4% neutral-buffered formalin or in methyl carnoy's fixative prior to embedding in paraffin. For the detection of napsin A on 4 μm thick paraffin sections of kidney and lung, we used the DAKO Envision system (K4010) with peroxidase/diaminobenzidine as the enzyme/chromogen solution as well as the immunogold silver staining method (Zymed Histogold Kit 95-6149) which eliminates the problem of endogenous enzyme activity. When the Envision system was applied, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Unspecific background staining was blocked by incubating the sections with goat serum and by additionally preabsorbing the primary and the labeled polymer conjugated with the secondary antibody with human serum (1:2 dilution) overnight at 4°C. For the immunogold silver staining method, a non-immune serum blocking solution was used to eliminate non-specific protein binding followed by the anti-napsin A antibody, which was preabsorbed overnight with human serum at 4°C. Then an anti-rabbit secondary antibody, conjugated to 5 nm colloidal gold was added. The gold signal was intensified by applying the silver enhancing solution offered in the kit. The stain was either viewed by transmission light microscopy in which a positive signal appeared as a very intense gray to black signal or by epipolarization using a fluorescent microscope with an A-Pol filter set (polarizer, neutral beam splitter, analyzer) which can increase sensitivity by a factor greater than 100. The immunohistochemical detection of napsin A on the spleen tissue was done by LifeSpan BioSciences (Seattle) using a Vector ABC-alkaline phosphatase kit (AK5002) with the Vector Red substrate kit (SK-5100) to produce a fuchsia-colored red deposit. Counterstaining was performed using Mayer's hematoxylin. Negative controls included staining with the preimmune serum or with non-immune rabbit IgG in the absence of the napsin antibody.

3. Results and discussion

3.1. Design of anti-napsin A-specific antibody

The amino acid identity of the mature region of napsin A [5] with other human aspartic proteinases is quite high (40–50%). Crystal structures of many aspartic proteinases have been solved and showed rather high similarity in the overall folding [8,12–14]. Of all proteins with known 3D structure human cathepsin D has the highest amino acid identity with napsin A. Therefore, we used the structure of cathepsin D [8] as template to build a 3D model of napsin A. With the help of this model we have identified a peptide, SFYLNRDPEEPDGGD (first aspartate in the DPEEPD sequence corresponds to position 158 in pepsin numbering), suitable for the immunization of rabbits yielding polyclonal antibodies. The underlined DPEEPD sequence is likely to be located within a loop. We assume the two glutamates to be involved in favorable antigen-antibody interactions while the two flanking prolines are likely to rigidify the loop and, therefore, decrease the number of loop conformations. A comparison with other aspartic proteinases revealed that the DPEEPD sequence is only found in napsin A (Fig. 1). This should provide high antibody specificity for this enzyme compared to other human aspartic proteinases, including the napsin B isoform.

NapA	S	F	Y	L	N	R	D	P	E	E	P	D	.	.	G G E
NapB	S	F	Y	**F**	N	R	D	P	E	**V**	**A**	D	.	.	G G E
CatD	S	F	Y	L	**S**	R	D	P	**D**	**A**	**Q**	**P**	.	.	G G E
Renin	S	F	Y	**Y**	N	R	D	**S**	E	**N**	**S**	**Q**	S	L	G G **Q**
CatE	S	**V**	Y	**M**	**S**	**S**	**N**	P	E	**G**	**G**	**A**	.	.	G **S** E
Pepsin	S	**V**	Y	L	**S**	**A**	D	.	**D**	**Q**	.	**S**	.	.	G **S** **V**

Fig. 1. Alignment of the peptide sequence used as the antigen to raise anti-napsin A polyclonal antibody to the equivalent sequences of napsin B, cathepsin D, renin, cathepsin E, and pepsin. Gaps are introduced for maximal alignment. Sequences are listed with decreasing similarity from top to bottom. Napsin A unique sequence is underlined. Residues different to those in human napsin A are shown in bold.

3.2. Expression of the napsin A gene

In order to generate immunoreactive protein for in vivo studies, we expressed human napsin A in human embryonic kidney cells (HEK293) as described in Section 2. The expression vector contained the full cDNA sequence of napsin A

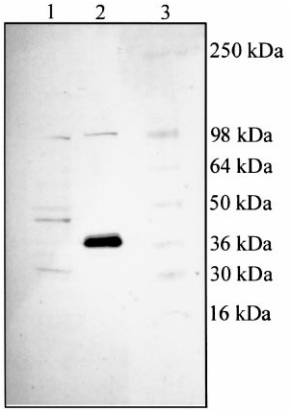

Fig. 2. Western blot of recombinant human napsin A expressed in HEK293 cells using anti-napsin A polyclonal antibody raised against the peptide SFYLNRDPEEPDGGE. See text for details. Lanes 1 and 2 contain detergent treated pellet fraction of non-transfected HEK293 cells and HEK293 cells expressing recombinant human napsin A, respectively. The molecular mass standards corresponding to myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa) are shown in lane 3.

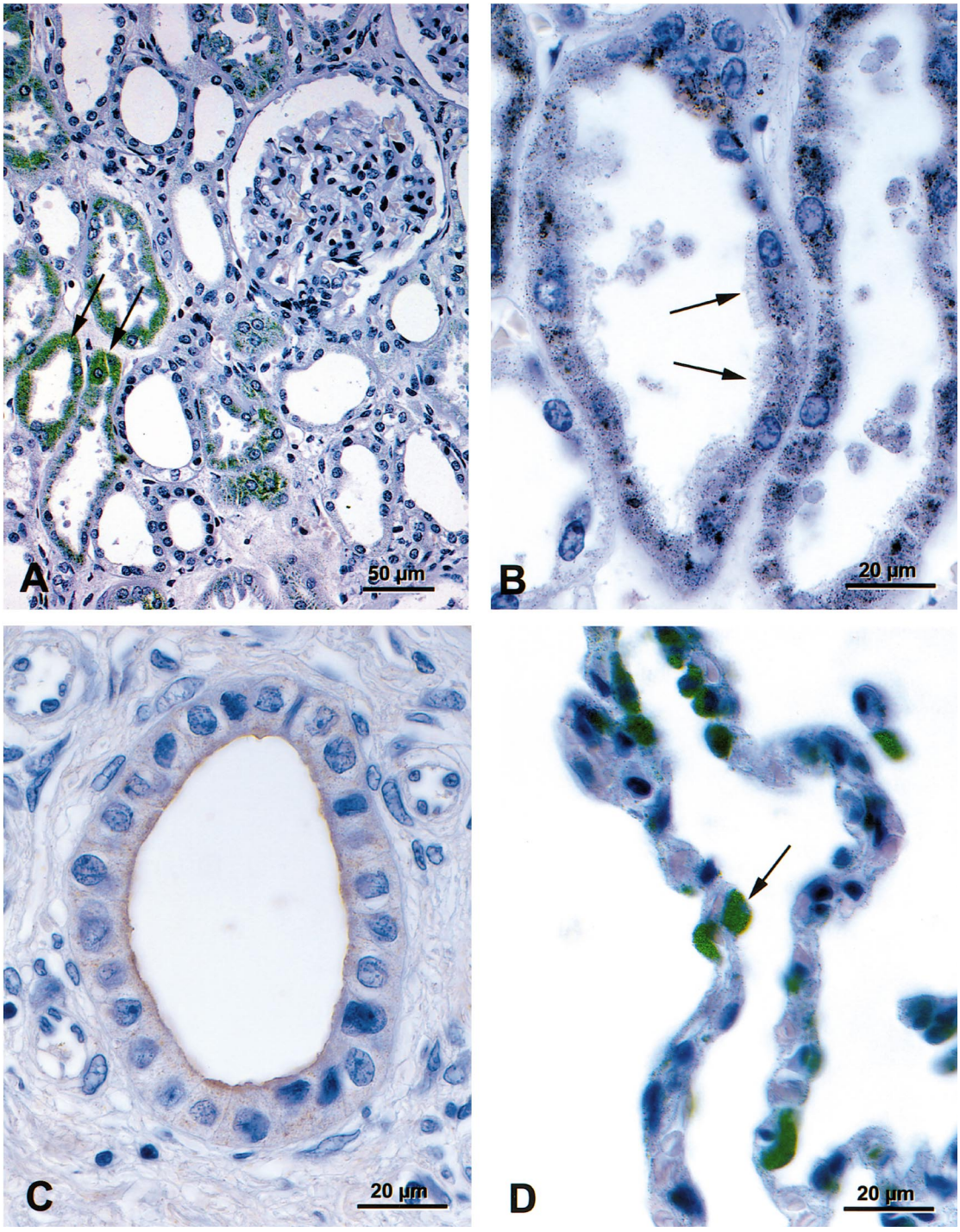

Fig. 3. Immunohistochemical localization of napsin A in human kidney and lung. A: Part of the renal cortex demonstrating intense staining for napsin A in proximal convoluted tubules (arrows), whereas no significant staining is found in the glomerulus (detection by immunogold silver staining, epipolarization). B: Proximal convoluted tubules at higher magnification with clearly visible brush borders (arrows) are strongly stained with the napsin A antibody (immunogold silver staining, no epipolarization). C: Collecting duct in the medulla shows positive staining for napsin A along the luminal border (DAKO Envision detection system with peroxidase/DAB). D: Type II pneumocytes (arrow) in the alveolar epithelium show strong staining for napsin A (immunogold silver staining, epipolarization). All sections were counterstained with Mayer's hematoxylin.

encoding for 420 amino acids. The sequence comprises the signal peptide, propeptide and the mature enzyme [5].

Immunoreactivity of expressed napsin A was analyzed by immunochemical detection using anti-napsin A antibody. Electrophoresis of crude HEK293 cell extract containing napsin A in sodium dodecylsulfate (SDS) polyacrylamide gel followed by transblotting of proteins to a nitrocellulose sheet and immunochemical staining with antibody showed a single band with the apparent molecular weight of approximately 38 kDa (Fig. 2). This value is close to the calculated molecular

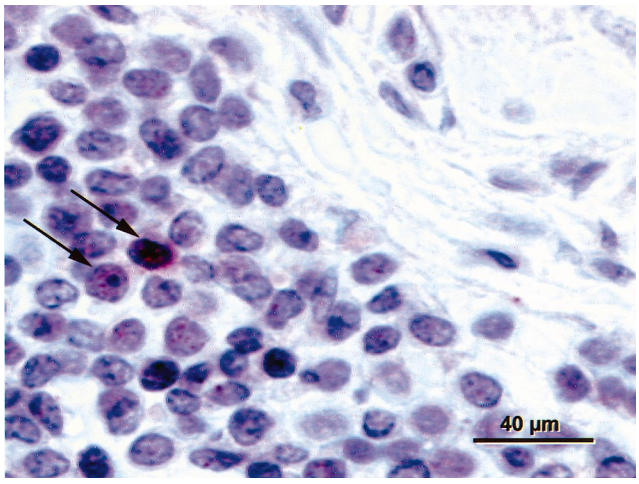

Fig. 4. Occasional lymphocytes (arrows) in the periarterial lymphatic sheat are positively stained with the anti-napsin A antibody (Vector ABC-alkaline phosphatase detection with Vector Red as chromogen).

weight of tentative mature napsin A (~39 kDa). Since no other bands were observed, it can be concluded that the polyclonal antibody specifically recognizes mature napsin A (the antibody did not cross-react with human cathepsin D tested separately, data not shown). However, we can not exclude that the antibody can recognize pronapsin A as well. Absence of the second band corresponding to the zymogen (calculated molecular weight ~43 kDa) suggests that napsin A undergoes maturation. Different ways of maturation of other eukaryotic aspartic proteinases have been described in literature [1,2,15,16]. At present, we can only speculate about a mechanism by which pronapsin A matures.

During the expression procedure in HEK293 cells we have observed by Western blot that not only the cytoplasmic fraction of the cellular lysate but also the remaining cellular pellet contained a band corresponding to napsin A. Moreover, the majority of napsin A seemed to be associated with the pellet. Therefore, in order to release the enzyme into the solution, the cellular pellet was treated with a PBS buffer containing 20 mM EDTA, 1 mM PMSF and 1% Elugent. This detergent treatment resulted in a very efficient extraction of napsin A into the buffer as confirmed by Western blot. Implication of this observation is that napsin A might be present in large cellular vacuoles or membrane associated or that the overexpression in HEK293 cells triggers a mechanism which alters the expression pathway of recombinant napsin A compared to the native one. These assumptions are currently under investigation in our laboratory. It should be pointed out that most of the human aspartic proteinases described so far are not membrane bound. Notable exceptions are the recently described human aspartic proteinases ASP1 and ASP2 (patent number EP848062-A2 and EP855444-A2, respectively), which contain a transmembrane domain at the C-terminus. On the other hand, several aspartic proteinases such as Yapsin 1 and Yapsin 2 from *Saccharomyces cerevisiae* [17,18], and the parasites *Eimeria tenella* [19] and *Plasmodium falciparum* [20] have been described as being membrane anchored. Yeast and *E. tenella* enzymes have long C-terminal tails, which are known to be involved in a glycophosphatidylinositol (GPI) anchoring of these enzymes. Plasmepsins I and II from *P. falciparum* are type II integral membrane proteins, which have a transmembrane domain at the N-terminus of the zymogen [20]. Compared to other human aspartic proteinases, napsin A has a unique C-terminal extension of 18 amino acids [5]. However, this extension does not contain a transmembrane motive nor can it be involved in GPI anchoring [5]. Therefore, we assume membrane association of human napsin A through the C-terminal tail to be highly unlikely. On the other hand, napsin A contains an RGD motif (position 315 in pepsin numbering), which can be involved in integrin binding. Therefore, this type of interaction could potentially be responsible for observed membrane association of napsin A. However, based on our computer model it is not apparent that RGD is in a position to optimally bind to integrin.

3.3. Immunohistochemical studies

Since immunochemical studies confirmed the specificity of the anti-napsin A polyclonal antibody, further studies have been undertaken in order to determine localization of napsin A in human tissue. It was of interest to elucidate whether the napsin A gene is translated into protein and, if so, in which organs this protein is localized. Tatnell et al. reported expression of the human napsin A gene using Northern blot in normal kidney and lung [5]. We therefore performed immunohistochemical studies on human kidney and lung sections by using anti-napsin A antibody.

The samples of normal human kidney showed prominent staining for napsin A in the cortex region as well as in the outer stripe of the medulla. The staining within the renal cortex was predominantly confined to the epithelial cells of the proximal convoluted and straight tubules as well as the distal convoluted tubules (Fig. 3A, B). The observed staining appeared scattered. The glomeruli, including parietal and visceral podocytes, endothelium of glomerular capillaries, and mesangial cells were negative for staining. Within the renal medulla, occasional collecting ducts and the thin loops of Henle showed faint cytoplasmic staining, but the signal was also found localized along the luminal border of the cell membrane (Fig. 3C). These findings are in good accordance with the results of in situ hybridization of mouse KAP mRNA expression in the mouse kidney [6]. Mori et al. reported abundant expression of mouse KAP in the proximal straight tubule and weak, but significant expression in the proximal convoluted tubule. However, as described above, we have also found expression of napsin A in the distal convoluted tubule, collecting duct, and Henley loops of human kidney. Whether this slight variability in the intra-renal distribution between mouse KAP and human napsin A is due to species differences requires further investigation. In view of the observed staining along the luminal border of the cell membrane in collecting duct and disperse staining within the renal cortex, it would appear that napsin A is membrane anchored. At present the mechanism of membrane association of napsin A is unknown.

The samples of normal adult lung showed positive staining within Type II pneumocytes (Fig. 3D), alveolar macrophages, and a subset of Type I pneumocytes. Positive staining was also observed in respiratory epithelium of the terminal and respiratory bronchioles, plasma cells, and within a subset of lymphocytes. The majority of fibroblasts and smooth muscle cells appeared negative for staining.

We have also performed immunohistochemical study using human spleen tissue. Staining of the spleen was very limited

and less intense compared to kidney and lung. However, positive staining was seen within the sinusoidal endothelial cells of the cords of Billroth, within the endothelial cells of the branches of the central artery, and within subsets of lymphocytes in the periarterial lymphatic sheaths (Fig. 4). Obtained data are not in accordance with the results of Tatnell et al. [5] who reported that only the mRNA encoding napsin B was present in spleen. One possible explanation for this contradiction could be the cross-reactivity of anti-napsin A antibody with napsin B. In order to address this problem napsin B-specific antigenic peptide (see Fig. 1) was immobilized onto the membrane and immunochemical analysis using anti-napsin A antibody was performed. This analysis revealed that the antiserum does not recognize napsin B antigenic peptide (data not shown). Peptide used to raise anti-napsin A antibody was employed as a positive control. These data suggest that the staining observed in spleen is due to the interaction between the antiserum and napsin A but not napsin B. However, only immunochemical study using native or recombinant napsin B as antigen can clearly rule out the possible cross-reactivity. Finally, reported absence of an in-frame stop codon of the napsin B gene [5] does not favor the presence of the napsin B gene product in the spleen, thus making the cross-reactivity unlikely. To explain the appearance of immunoreactive napsin A in the spleen without finding corresponding mRNA further studies are required.

In summary, we have been able to express recombinant human napsin A and to obtain a napsin A-specific polyclonal antibody by taking advantage of the computer modeling approach. This antibody was useful for immunochemical detection of human napsin A and immunohistochemical studies of several human tissues. Results of this study clearly demonstrate localization of human napsin A in kidney and lung. We hope that future studies will elucidate the subcellular localization of napsin A and that these findings, together with the immunohistochemical results presented here, can be useful in investigating the function of this enzyme.

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