Napsin A, a member of the aspartic protease family, is abundantly expressed in normal lung and kidney tissue and is expressed in lung adenocarcinomas

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Abstract A pair of 35 kDa polypeptides (TAO1/TAO2) are expressed in more than 90% of all primary lung adenocarcinomas but not in other major malignancies. Mass spectrometry of tryptic peptides showed that TAO1/TAO2 is identical to napsin A, a recently described member of the aspartic proteinase family. The site of processing of pronapsin A to the mature form was located. Napsin expression was detected in human lung adenocarcinoma tumors, compatible with the marker nature of TAO1/TAO2 in the diagnosis of primary lung adenocarcinoma. This is important since identification of markers which can distinguish primary lung adenocarcinomas from distant metastases is desirable. Northern blot analysis showed expression of napsin also in normal lung and kidney tissue, and in situ hybridization showed expression in type II alveolar cells of the lung. This protease is concluded to have a specific functional role in the normal alveolar epithelium and is a candidate protease for the proteolytic processing of surfactant precursors.

Key words: Lung adenocarcinoma; Aspartic protease; Tumor diagnosis; Mass spectrometry

1. Introduction

Lung cancer is the most frequently diagnosed major cancer in the world and the most common cause of cancer death [1]. The trend is now a decline for most histological types of lung cancer among males, whereas the incidence does not decrease among women [1]. Non-small cell lung cancer (NSCLC) accounts for 70–80% of all lung cancers and includes squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma and large cell carcinoma [2]. Adenocarcinoma develops from type II pneumocytes and from bronchiolar non-ciliated secretory cells (Clara cells) [3,4]. The lung is also a common site for metastasis from tumors growing at other sites in the body. There is a need for markers which can distinguish primary lung adenocarcinomas from distant metastases to the lung, since treatment protocols differ between primary lung cancer and metastatic disease. Some such markers have been described, including surfactant proteins [3], TTF-1 (thyroid transcription factor-1) [5] and members of the mucin family of glycoproteins [6]. However, better markers are needed to distinguish between primary and metastatic lung adenocarcinoma.

A pair of polypeptide markers for lung adenocarcinoma has been described using two-dimensional gel electrophoresis (2-DE) [7,8]. These 35 kDa markers, TAO1/TAO2, have slightly different isoelectric points. Examination of 57 different primary lung carcinomas using 2-DE showed that 52 (91%) expressed TAO2 [8]. We have determined the amino acid sequence of tryptic peptides derived from TAO2 and report that these polypeptides are identical to a recently described [9] aspartic protease, napsin A, uniquely expressed in lung and kidney cells. In situ hybridization detected napsin A in type II pneumocytes in normal lung tissues, and napsin A expression was found in lung adenocarcinomas. The expression of napsin A in defined cells in the normal lung epithelium suggests an important functional role for this protease which may involve the proteolytic processing of lung surfactant protein precursors.

2. Materials and methods

2.1. Two-dimensional electrophoresis (2-DE) and mass spectrometric analysis of TAO1 and TAO2

Lung adenocarcinomas were obtained shortly after resection, and the cells were isolated [10], solubilized and subjected to 2-DE [11]. The TAO1 and TAO2 spots were excised from Coomassie brilliant blue stained gels, destained, and digested with trypsin (1 pmol/μl) (Promega). The resulting fragments were analyzed by MALDI-TOF, and sequenced by capillary HPLC-tandem mass spectrometry using an ion trap instrument (Finnigan MAT LCQ) equipped with a micro-electrospray ionization source [12].

2.2. EST clones and human fetal lung cDNA library screening

Four EST clones (W19120, T54068, R11458, AA359075) that contained the sequences corresponding to the TAO1/2 peptide sequences were obtained from the American Type Culture Collection. In order to identify a full length clone, a human fetal lung cDNA library (Clontech) was screened with a 565 bp probe from the W19120 clone or a 517 bp probe from the T54068 clone purified after polymerase chain reaction (PCR) and labeled with [α-^{32}P]-dCTP (NEN) using a random priming kit (Stratagene) [13]. Three positive clones were selected from the secondary screening using W19120, and DNA sequencing indicated that only one was a full length clone (W3). The coding region of this clone was sequenced for both strands using

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overlapping primers. The probe from T54068 detected several positive candidates which were selected from the secondary screening. Three of the candidate clones were found to have sequences identical to those of W19120. None matched the sequence of T54068, R11458 or AA359075.

2.3. Northern blotting

Human multiple tissue Northern blots and a human RNA master blot were obtained from Clontech. The cDNA probe was amplified by PCR using a cloned cDNA prepared from the fetal lung cDNA library with a sense primer, 5′-TCAAGCGCTATGTCTCC-ACCACCGCTGCT-3′ (nucleotide 1–20, napsin A), and an antisense primer, 5′-CAGACTGAATTCTCACCCGGGGAACTGCGCCTG-3′ (nucleotide 1243–1263), and labeled with [α-^{32}P]-dCTP (NEN) by random primed labeling. After a 1 h prehybridization, hybridization was performed overnight at 42°C, and blots were washed in 2×SSC, and 2×SSC, 1% SDS, at room temperature, and 0.2×SSC, 0.1% SDS, at 42°C for 5 min, then autoradiographed at −80°C.

2.4. 2-DE, SDS/PAGE and immunoblotting

Rabbit polyclonal antibody specific for napsin A was raised against the napsin A sequence 64–80Cys (i.e. KPIFVPLSNYRDVQYFGC) conjugated to keyhole limpet hemocyanin through an added carboxy-terminal cysteine residue. Tissue samples were obtained from CHTN (Cooperative Human Tissue Network, Philadelphia, PA, USA). Frozen tissues were thawed and homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 0.5 mM PMSF, 25 mM β-glycerophosphate. Protein concentration of the supernatant was measured using the BioRad protein assay. For SDS/PAGE, 20 μg protein for each sample was loaded per lane on a 10% Tris-glycine polyacrylamide gel (Novex) and electrophoresed at 125 V for 90 min at room temperature, while 2-DE was performed with 50 μg protein [11]. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting. After blocking of membranes in 5% BSA in PBS/Tween-20 at room temperature for 1 h, napsin A was detected by a 1 h incubation with an immunopurified rabbit polyclonal antibody against the N-terminal napsin A peptide at 1:2000 dilution. This was followed by incubation with an antirabbit secondary antibody conjugated to horseradish peroxidase at room temperature for 30 min. Bands were visualized by Super Signal Chemiluminescent Substrate (PIERCE) and exposed to Kodak BioMax MR film for 10–15 min.

2.5. In situ hybridization

In situ hybridization was performed on samples of formalin fixed, paraffin embedded tumor tissue. In brief, 6 μm tumor sections were

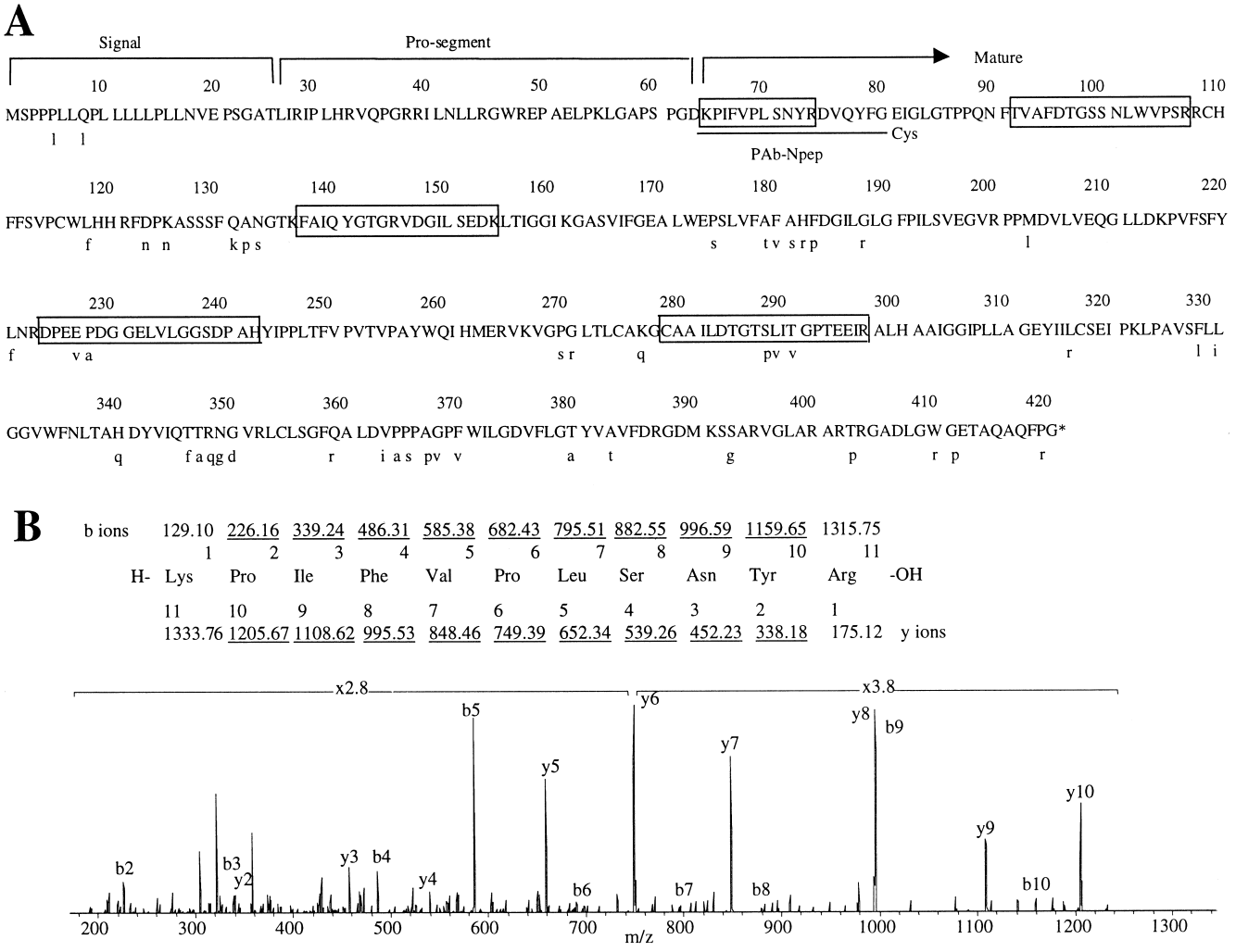

Fig. 1. Amino acid sequence (A) and analysis (B) of human pronapsin A. The continuous sequence in A is deduced from the nucleotide sequence of a cDNA clone (W3). For pronapsin B the corresponding amino acid exchanges are shown below the pronapsin A sequence. The positions of the five peptides identified by mass spectrometry are boxed and the peptide used for raising antibodies (Pab-Npep) is underlined. In B, the identification, by ion trap mass spectrometry, of the sequence of the amino-terminal peptide of the mature form is shown (top part) with the observed m/z values of the fragmentation products underlined as deduced from the fragmentation mass spectrum of the same peptide (bottom part). Segments of the profile which were expanded in the direction of the *y*-axis by 2.8 or 3.8× are indicated and both the b and y ions are clearly identifiable.

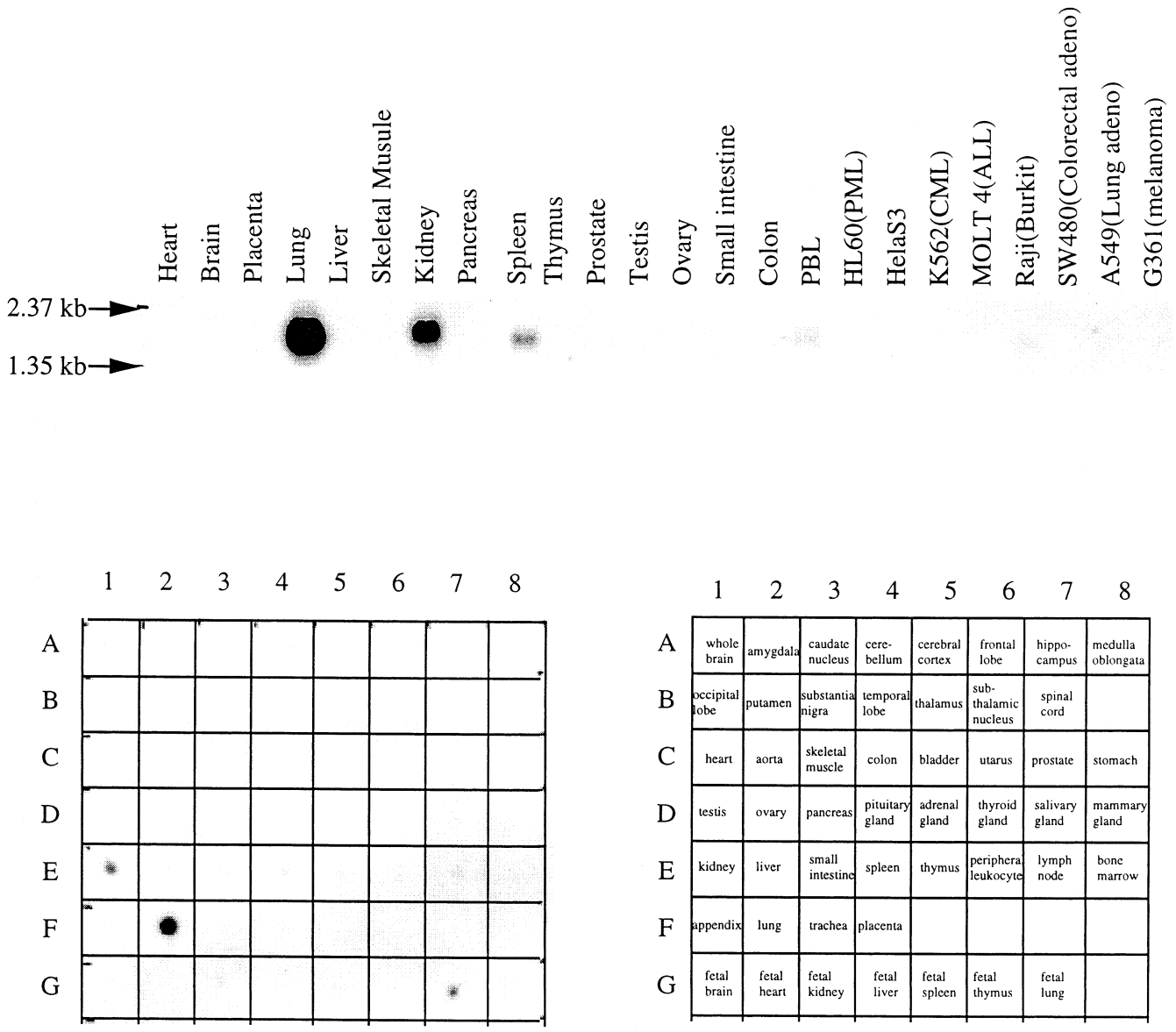

Fig. 2. Expression of napsin mRNA in human tissues and cancer cell lines. A: Northern blot analysis showing strong signals in lung and kidney tissues and weak signals in spleen, PBL, HL60 and Raji. Marker fragments of 2.37 and 1.35 kb migrated as indicated. B: Dot blot analysis of differentiated and fetal human tissues. Napsin mRNA is expressed predominantly in adult lung and kidney and in fetal lung.

deparaffinized with xylene, treated with proteinase K (1 μg/ml, 30 min, 37°C), transferred to 0.1 M triethanolamine buffer (5 min), and treated with triethanolamine containing 0.25% acetic anhydride for 10 min. Sections were washed in 2×SSC, dehydrated, and allowed to air-dry. After overnight hybridization (2×SSC, 50% formamide, 10% dextran sulphate, 55°C) with a ^{35}S-labeled RNA probe (1.16×10^5 cpm/μl), sections were washed (the most stringent step being 0.1×SSC, 15 min at 60°C) and RNAse A treated (20 μg/ml, 30 min, 37°C). Finally, the slides were dehydrated, air-dried, dipped in Kodak NTB emulsion, exposed for 6 or 19 days, and were developed and hematoxylin-eosin stained. The probes were made using T3 (antisense) and T7 (sense) RNA polymerase (Promega) from a pCMS-EGFP vector (Clontech) containing an *Nhe*1/*Eco*R1 napsin A insert.

3. Results

3.1. Identification of TAO1 and TAO2 and cloning of the corresponding cDNA

Viable tumor cells were prepared from freshly excised primary tumors as described [10]. Cell lysates were subjected to two-dimensional gel electrophoresis using immobilized pH gradient (IPG) electrophoresis in the first dimension. After staining the gels with Coomassie brilliant blue, the protein spots corresponding to TAO1 and TAO2 [7,8] were excised and pooled for in-gel digestion. Mass spectrometry of fragments revealed several peptides common to TAO1 and TAO2 tryptic digests, and five were subjected to collision-induced dissociation (CID). Interpretation of these spectra (Fig. 1B) yielded peptide sequences which were used to search the human EST (expressed sequence tags) GenBank database. Four EST clones were identified that contained TAO1/2 sequences. The first clone, of 495 bp (W19120), had the correct sequence of two out of the five peptides; however, the last three clones (T54068, R11458, AA359075) were overlapping to each other and contained all the peptide sequences but with several amino acid exchanges (Fig. 1A) and lacked an in-frame stop

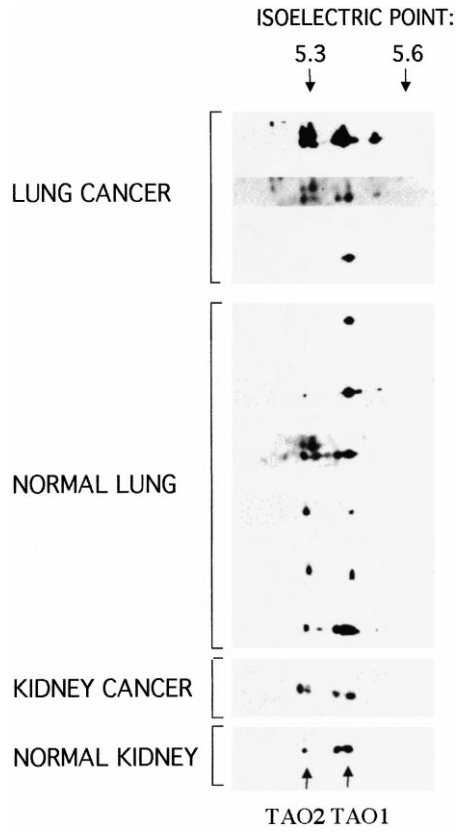

Fig. 3. 2-DE profiles of a panel of normal and tumor cell extracts from lung and kidney. Polypeptides corresponding to TA01 and TA02 were revealed by immunoblotting with an immunopurified antibody to a synthetic amino-terminal peptide. No other spots were observed in the 2-DE gels.

codon. Screening a human fetal lung cDNA library yielded a full length cDNA clone, which had a sequence identical to the recently described aspartic protease napsin A [9]. Interestingly, we were unable to identify peptides in our tryptic digest, or a clone in the lung cDNA library, corresponding to a second gene (napsin B), which apparently is transcribed exclusively in cells of the immune system [9].

3.2. Expression of napsin mRNA

The distribution of napsin mRNA was examined by Northern blot (Fig. 2A) and dot blot (Fig. 2B) analyses using a full length cDNA probe. The probe annealed to a 1.6 kb transcript which was highly abundant in lung, and also expressed in the kidney, spleen and peripheral blood leukocytes (Fig. 2A). In fetal tissue, again the most abundant expression was detected in lung, with low levels also in fetal kidney (Fig. 2B). No detectable amount of the mRNA was found in other tissues. Napsin mRNA expression was detected in HL60 promyelocytic leukemia and in Raji Burkitt lymphoma cells, but not in the A549 lung adenocarcinoma cell line. None of these cell lines express the TAO1/2 (napsin A) protein. It appears likely that cells derived from the immune system express only the napsin B message which differs from napsin A in the coding sequence, and probably in the sequence of the 5′-flanking region in order to achieve such specific cell type expression.

3.3. Expression of TAO1/2 (napsin) protein

In order to analyze the protein expression, we raised a rabbit polyclonal antibody against the amino-terminal peptide of the mature form (PAb-Npep) (Fig. 1A). Western immunoblotting was performed, initially after one-dimensional electrophoresis (1-DE) SDS/PAGE. Control experiments showed that the antibody recognized a thioredoxin-napsin A fusion protein expressed in *Escherichia coli*. The results of the Western analyses showed that the immunopurified antibody detected TAO1/2 (napsin A) in six/six normal lung samples and two/four normal kidney samples, but not in breast or colon tissues. In cancerous samples, three/three lung and one/six kidney tissues expressed the TAO1/2 (napsin A) protein, whereas three breast, one colon, four lymphomas and seven lung cancer cell lines did not express the protein. Immunoblotting with the immunopurified antibody was also carried out following 2-DE. Both lung and kidney tissues are positive for 35 kDa TAO1/2 spots (Fig. 3).

3.4. Napsin expression in normal lung tissue and in adenocarcinomas

Napsin expression was studied in tissue sections using in situ hybridization. Both sense and antisense probes were synthesized and hybridized to all sections. Hybridization with sense probes resulted in a diffuse background, and did not localize specifically to cells. As shown in Fig. 4A and B, only some cells of the normal alveolar epithelium expressed napsin (indicated by arrows). These cells showed the characteristics of type II pneumocytes. Three intermediately or intermediately/poorly differentiated lung adenocarcinomas, and one highly differentiated lung adenocarcinoma, showed strong napsin mRNA expression (Table 1). In contrast, specific hybridization was not observed to two poorly differentiated tumors/areas. Napsin mRNA expression in a highly differentiated area of tumor 94-9235 and in the intermediately differentiated tumor 94-9979 is shown in Fig. 4C and D.

4. Discussion

We have identified and sequenced five peptides derived from the TA01/2 spots. Using these sequences as probes, we established that the TA01/2 protein is napsin A and that a mature 35 kDa form, lacking a propeptide is prevalently expressed both in normal and cancerous cells. Napsin A was described [9] as a new member of the aspartic protease family during the time that this work was in progress. The cDNA sequences of two closely related genes were reported, pronapsin A predominantly expressed in lung and kidney, and pronapsin B exclusively expressed in cells derived from the immune system, but lacking an in-frame stop codon and containing several amino acid exchanges [9]. We completely agree on those results. We also provide detailed analysis at the protein level. Processing of pronapsin A to the mature form is now concluded to occur between Asp-63 and Lys-64, since cleavage to generate peptide KPIFVPLSNYR could not be the result of trypsin digestion. Napsin A showed three to four isoelectric forms in 2-DE. Two forms with a slightly higher molecular weight were also found in some samples. Three potential N-glycosylation sites are predicted in the mature enzyme and could be responsible for such heterogeneity.

Our results obtained from in situ hybridization establish that napsin mRNA is highly expressed in normal type II

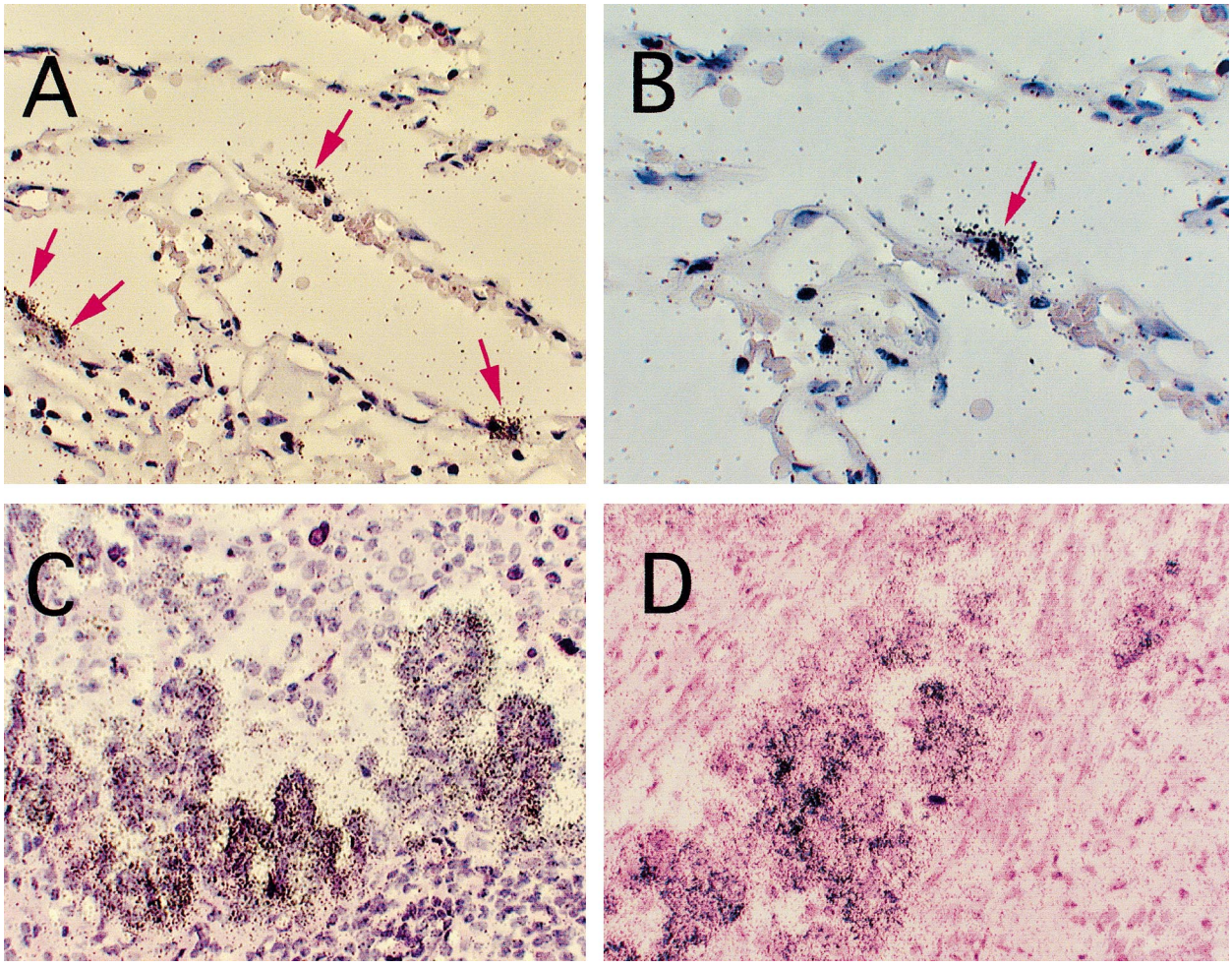

Fig. 4. In situ hybridization using a ^{35}S-labeled antisense napsin RNA probe. A (20× objective) and B (40× objective): normal lung tissue. Type II alveolar cells, with large cytoplasms, show hybridization to the napsin probe (red arrows). C (20× objective): strong napsin mRNA expression in a highly differentiated area of lung adenocarcinoma 94-9235. D (20× objective): strong napsin mRNA expression in lung adenocarcinoma 94-9979.

lung pneumocytes. Type II pneumocytes secrete surfactant in the form of tubular myelin which is converted to a small vesicular form. Both surfactant protein B and C are derived from larger precursors [14], and an aspartic protease has been implicated in the cleavage of pro-surfactant protein B to the mature form [15]. The specific localization of napsin A expression to type II pneumocytes raises the possibility that this aspartic protease is napsin A.

Strong TAO1/TAO2 expression has been reported [8] in highly differentiated lung adenocarcinomas, whereas dedifferentiated tumors showed weaker expression. Nevertheless, napsin A was detected by 2-DE in a high percentage (>90%) of clinically observed lung adenocarcinomas and may become a useful marker in the differential diagnosis between primary and metastatic lung adenocarcinoma. Our in situ hybridization data demonstrated napsin expression in adenocarcinoma tumor cells, whereas the tumor stroma was negative. Consistent with the report from Hirano et al. [8], we find stronger napsin expression in highly and intermediately differentiated adenocarcinomas than in poorly differentiated adenocarcinomas. The analysis of the titer of napsin A in the serum could be of use in lung cancer diagnosis, in the same manner as analyses of the serine proteases human glandular kallikrein 2 (hK2) and prostate specific antigen (PSA or hK3) are for evaluation of prostate cancer disease progression [16]. Both proteases are highly expressed in normal prostate tissue and in prostate cancer.

In conclusion, we have shown high expression in specific alveolar cells of the protease napsin A. The restricted pattern of expression makes napsin A a marker for primary lung adenocarcinoma and a candidate protease for the cleavage of pro-surfactant proteins.

Table 1
Expression of napsin mRNA in lung adenocarcinomas

Tumor	Histology[a]	Hybridization score
93-13596	intermediate/low	++
93-12807	intermediate/low (some gland formation)	+++
94-9979	intermediate	+++
94-9235	high[b]	+++
	low[b]	–
94-1228	low	–

[a]Evaluated blindly by two independent pathologists. Tumors were classified as showing high/intermediate/low grade of differentiation.
[b]This tumor contained two distinct areas showing different histologies.

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References

[1] Wingo, P.A., Ries, L.A.G., Giovino, G.A., Miller, D.S., Rosenberg, H.M., Shopland, D.R., Thun, M.J. and Edwards, B.K. (1999) J. Natl. Cancer Inst. 91, 675–690.
[2] WHO (1982) Am. J. Clin. Pathol. 77, 123–136.
[3] Mizutani, Y., Nakajima, T., Morinaga, S., Gotoh, M., Shimosato, Y., Akino, T. and Suzuki, A. (1988) Cancer 61, 532–537.
[4] Linnoila, R.I., Mulshine, J.L., Steinberg, S.M. and Gazdar, A.F. (1992) J. Natl. Cancer Inst. 13, 61–66.
[5] Holzinger, A., Dingle, S., Bejarano, P.A., Miller, M.A., Weaver, T.E., DiLauro, R. and Whitsett, J.A. (1996) Hybridoma 15, 49–53.
[6] Patton, S., Gendler, S. and Spicer, A. (1995) Biochim. Biophys. Acta 1241, 407–424.
[7] Hirano, T., Franzén, B., Uryu, K., Okazawa, K., Alaiya, A.A., Vanky, F., Rodriguez, L., Ebihara, Y., Kato, K. and Auer, G. (1995) Br. J. Cancer 72, 840–848.
[8] Hirano, T., Fujioka, K., Franzén, B., Okuzawa, K., Uruy, K., Shibanuma, H., Numata, K., Konaka, C., Ebihara, Y., Takahashi, M., Kato, H. and Auer, G. (1997) Br. J. Cancer 75, 978–985.
[9] Tatnell, P.J., Powell, D.J., Hill, J., Smith, T.S., Tew, D.G. and Kay, J. (1998) FEBS Lett. 441, 43–48.
[10] Franzén, B., Okuzawa, K., Linder, S., Kato, H. and Auer, G. (1993) Electrophoresis 14, 382–390.
[11] Bergman, A.-C., Benjamin, T., Alaiya, A.A., Waltham, M., Sakaguchi, K., Bergman, T., Linder, S., Wirth, P.J., Auer, G., Jörnvall, H. and Appella, E. (1999) Electrophoresis, in press.
[12] Arnott, D., O'Connell, K.L., King, K.L. and Stults, J.T. (1998) Anal. Biochem. 258, 1–18.
[13] Wahl, G.M. and Berger, S.L. (1987) Methods Enzymol. 152, 415–423.
[14] Johansson, J. and Curstedt, T. (1997) Eur. J. Biochem. 244, 675–693.
[15] Weaver, T.E., Lin, S., Bogucki, B. and Dey, C. (1992) Am. J. Physiol. 263, 95–103.
[16] Daher, R. and Beaini, M. (1998) Clin. Chem. Lab. Med. 36, 671–681.