Napsins: new human aspartic proteinases

Distinction between two closely related genes

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Abstract cDNA sequences were elucidated for two closely related human genes which encode the precursors of two hitherto unknown aspartic proteinases. The *(pro)napsin A* gene is expressed predominantly in lung and kidney and its translation product is predicted to be a fully functional, glycosylated aspartic proteinase (precursor) containing an RGD motif and an additional 18 residues at its C-terminus. The *(pro)napsin B* gene is transcribed exclusively in cells related to the immune system but lacks an in-frame stop codon and contains a number of polymorphisms, one of which replaces a catalytically crucial Gly residue with an Arg. Consideration is given to whether *(pro)napsin B* may be a transcribed pseudogene or whether its putative protein product undergoes rapid intracellular degradation.

Key words: New human aspartic proteinase; Cloning; Tissue distribution; Northern blot; Reverse transcriptase polymerase chain reaction; Distinction between closely related genes

1. Introduction

Aspartic proteinases contain two internally homologous domains, each of which contributes a catalytic Asp residue to the active site which is located between the two domains [1]. Each aspartic acid residue is found in the hallmark sequence ~Hydrophobic-Hydrophobic-Asp-Thr/Ser-Gly~ which, together with a further ~Hydrophobic-Hydrophobic-Gly~ motif, forms a structural feature known as a psi loop [2]. In some enzymes, the domains are contained within a single polypeptide chain so that each of the motifs occurs twice within the molecule and in the encoding gene [3].

Five aspartic proteinases are well documented to be produced in the human body. Three (pepsin, gastricsin and renin) are secretory enzymes which not only have well-defined physiological roles but have also been associated with pathological states, e.g. cancer [4]. Cathepsin D and cathepsin E are present in intracellular compartments and are readily distinguished by their molecular architecture and cytomorphological compartmentation [5]. With the exception of cathepsin E, crystal structures have been solved for these enzymes and the gene locus for all five enzymes has been identified and mapped [6]. With the advent of human genome projects, ready access to a vast array of human expressed sequence tags (ESTs) is now possible. Interrogation of these databases for the hallmark sequences of aspartic proteinases, i.e. the ~Hydrophobic-Hydrophobic-Asp-Thr/Ser-Gly~ plus ~Hydrophobic-Hydrophobic-Gly~ motifs, gave a preliminary indication that yet further aspartic proteinases were encoded within the human genome. We have called these napsins (for novel aspartic proteinases of the pepsin family).

2. Materials and methods

2.1. Detection and preparation of (pro)napsin A and B clones

The protein sequence of human procathepsin E was used as a probe to search the human EST databases. Inspection of the predicted sequences of the resulting hits indicated that several contained the hallmark ~Hydrophobic-Hydrophobic-Asp-Thr-Gly~ and ~Hydrophobic-Hydrophobic-Gly~ motifs but the intervening sequence was distinct from those of the five known human aspartic proteinases. The initial EST clones that encompassed parts of *(pro)napsin A* were prepared by scientists at Human Genome Services or the Institute for Genomic Research using established EST methods [7,8].

RNA prepared from human lung, kidney and spleen was purchased from Clontech Laboratories Inc., Palo Alto, CA, USA. Clones of *(pro)napsin B* were generated from RNA isolated from human Raji cells using the Tri-reagent kit (Sigma, Poole, UK). First strand cDNA was synthesised by reverse transcriptase using either random hexamers or a modified oligo$(dT)_{17}$ primer as described previously [9]. An initial PCR product spanning most of the *(pro)napsin B* sequence was amplified from random-primed cDNA using specific forward (5′-TGG GTT CAC ACC CGG CT-3′) and reverse (5′-GCC AGT CCC ACT CGT GC-3′) primers. A 3′ product (product 2) extending to the poly(A) tail was generated, using as template cDNA that had been synthesised using the modified oligo$(dT)_{17}$ primer in a PCR amplification which involved an internal primer (5′-CT CAG GGT GAC GTC CGC-3′) together with the modified oligo$(dT)_{17}$ as the reverse primer. Amplified products were all cloned into pGEM-T (Promega, Southampton, UK). PCR amplifications and dideoxy sequencing of plasmids in both strands were performed by standard methods as described previously [9].

2.2. Northern blotting and distinction between (pro)napsin A and B transcripts

Human multiple tissue Northern blots (Clontech) were hybridised with a *Bam*HI/*Sal*I fragment of 267 bp derived from the 3′ end of product 2, described above. This probe, which hybridises to both (pro)napsin A and B messages because of their sequence identity (>90%), was radiolabelled by incorporation of [^{32}P]dCTP (RadPrime DNA Labelling System, Life Technologies Inc., Grand Island, NY, USA) and purified using a CHROMA SPIN+TE-30 column (Clontech). After a 1 h prehybridisation, hybridisation was carried out for 2 h using ExpressHyb buffer (Clontech) at 68°C. The membrane was washed twice in 2×SSC, 0.05% SDS for 20 min, twice in 0.1×SSC, 0.1% SDS at 50°C for 20 min and exposed to X-ray film at −70°C with two intensifying screens. Distinction between specific mRNAs for (pro)napsins A and B was accomplished by RT-PCR using forward (5′-T GGT GGA ATC AAG GGT GC-3′) and reverse (5′-AA GTA

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Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction

PII: S0014-5793(98)01522-1

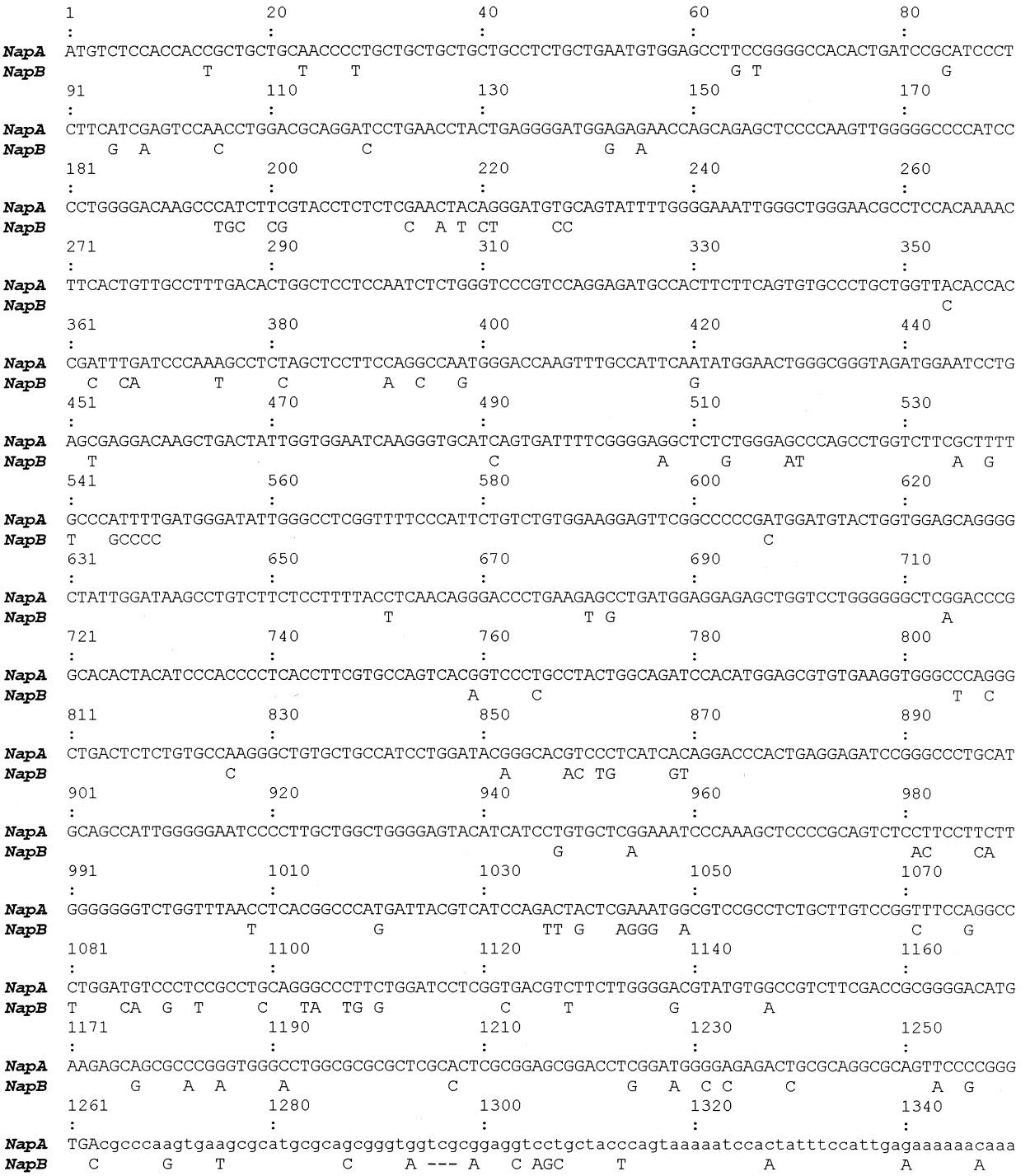

Fig. 1. Nucleotide sequences encoding *(pro)napsins A (NapA)* and *B (NapB)*. The *NapA* sequence is given in full. For *NapB*, at any position where no base is shown, sequence identity with *NapA* exists. (-) indicates the absence of a nucleotide. The TGA stop codon (bp 1261–1263) in *NapA* is TGC in *NapB*.

AAA GGA GAA GAC AGG-3′) primers that permit amplification of the exon 5 region (bp 471–662; Fig. 1) of both messages. Following cloning of the resultant amplicons into pGEM-T, restriction digestion with *Nco*I/*Sal*I confirmed the presence of *(pro)napsin A/B* inserts. A *Bst*XI site is present in this region of the *(pro)napsin A* but not the *(pro)napsin B* gene. Digestion with *Bst*XI released fragments of 164 or 129 bp depending on insert orientation from *(pro)napsin A* clones. Conversely, an *Msp*AI site present in the *(pro)napsin B* sequence is absent in the *(pro)napsin A* clone. Digestion with *Msp*AI releases diagnostic fragments of 331+144 bp or 412+63 bp, depending on insert orientation, from *(pro)napsin B* clones compared to an intact fragment of 475 bp from plasmids containing the *(pro)napsin A* amplicon. Representative positive clones of each type were cloned and sequenced.

3. Results and discussion

Interrogation of the EST databases identified a number of entries that contained the hallmark ~Hydrophobic-Hydrophobic-Asp-Thr-Gly~ and ~Hydrophobic-Hydrophobic-

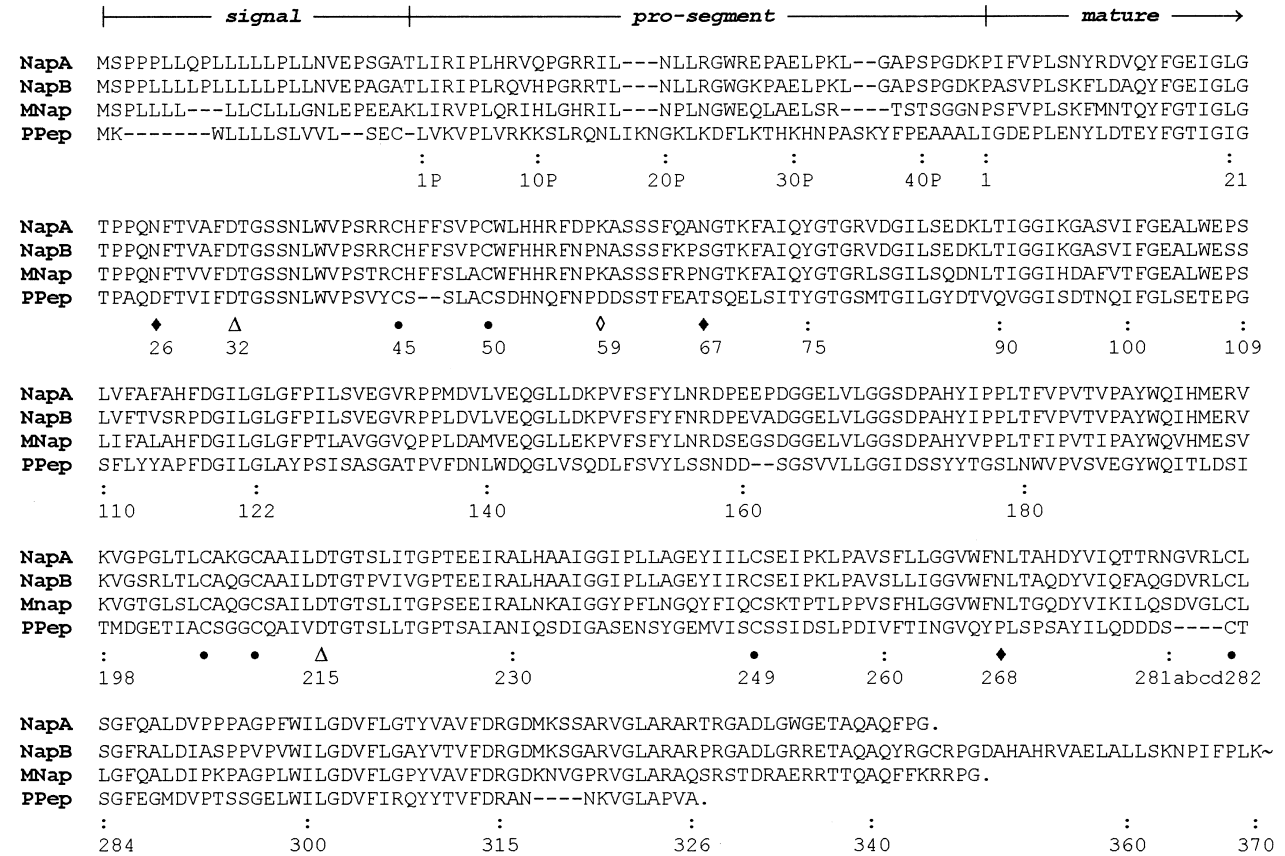

Fig. 2. Alignment of the deduced amino acid sequences of human (pro)napsin A (NapA), (pro)napsin B (NapB), mouse (pro)napsin (Mnap) and pig pepsin(ogen) (PPep). The absence of a residue is indicated by (-) and residues in the (pro)part are designated by a suffix P (pig pepsin numbering). The positions of the active site Asp residues, potential *N*-glycosylation sites and cysteine residues involved in disulfide bond formation are indicated by △, ♦ and ● respectively. The extended C-terminal 'tail' of (pro)napsin B is shown as far as the first of the Lys residues translated from the poly(A) tail of the message.

Gly~ motifs of an aspartic proteinase but the intervening nucleotide sequence predicted a protein that would have a sequence distinct from those of the five known human aspartic proteinases. The full-length nucleotide sequence that was eventually determined for this new aspartic proteinase gene (which we have called *(pro)napsin A*) is given in Fig. 1 with the translated protein product shown in Fig. 2. The 1263-bp open reading frame predicts a 420-amino acid polypeptide consisting of four regions: a signal peptide (~24 residues), a propart region of ~40 residues, the mature enzyme (~336 residues) and a C-terminal extension of 18 residues relative to the known C-termini of the five other human aspartic proteinases (Fig. 2). The functional regions in human (pro)napsin A can be readily identified from their similarity to those in the other human aspartic proteinases. These include (using pig pepsin numbering) the active site ~Ala30-Phe31-Asp32-Thr33-Gly34~ and accompanying ~Ile120-Leu121-Gly122~ motifs; the ~Ile213-Leu214-Asp215-Thr216-Gly217~ and ~Ile300-Leu301-Gly302~ pair and the ~Tyr75-Gly76-X-Gly78 motif that is a conserved feature of the β-hairpin loop that overlies the active site [1]. Retention of these functionally critical residues identifies napsin A as a new human aspartic proteinase. The identity (cDNA and amino acid, respectively) of the mature region of human napsin A (residues 1–344; Fig. 2) was 47.1 and 48.3% with human cathepsin D, 43.6 and 48.4% with cathepsin E, 41.0 and 43.9% with human pepsin, 40.9 and 43.0% with renin, and 37.4 and 41.9% with gastricsin.

The three disulphide bonds characteristic of mammalian aspartic proteinases are predicted to be conserved in the mature enzyme segment of human napsin A (Fig. 2). Three potential N-linked oligosaccharide attachment sites are also predicted in human napsin A. The first of these (at Asn26-Phe27-Thr28) is in the identical location to the glycosylation motif that is present in cathepsin E from all species so far studied [9]. The second (at Asn67-Gly68-Thr69) corresponds in location to glycosylation sites in human renin and cathepsin D. The final site (at Asn268; Fig. 2) does not coincide with glycosylation locations in other human aspartic proteinases.

Perhaps the most distinctive features of the human napsin A sequence, however, are located towards the C-terminal end. An RGD motif (residues 315–317) is located immediately in front of a four-amino acid insert that is unique amongst aspartic proteinases. The RGD sequence constitutes the recognition motif for integrin binding and might suggest a novel function for this aspartic proteinase. An RGD sequence has also been detected recently but at different locations in the sequences of cardosin A, a plant aspartic proteinase [10] and in (only) one of seven aspartic proteinases secreted by *Candida albicans* [11]. In addition to this RGD sequence, napsin A also has a C-terminal extension of 18 residues compared to all the other human aspartic proteinases. The func-

tion of this C-terminal 'tail' is not immediately apparent. Crystal structures determined for *Candida* aspartic proteinases with C-terminal extensions of ~9 residues show that these residues contribute an additional strand to the 6-stranded β-sheet that forms the base of the molecular architecture of this family of enzymes [12]. In contrast, even longer tails have been predicted in aspartic proteinases encoded within genes of parasites of mammals, including man [13,14] and poultry (*Eimeria tenella* [15]) and from the yeast, *Saccharomyces cerevisiae* [16,17]. These yeast enzymes (Yap 3 or Yapsin 1; Mkc 7 or Yapsin 2 [16,17]) are known to be GPI-anchored and since the extension in the *E. tenella* enzyme also contains the recognition motif for C-terminal truncation with concomitant attachment of a membrane-targeting GPI anchor [18], these aspartic proteinases may be members of a growing sub-family that are GPI-anchored. However, the highly charged, hydrophilic nature of the napsin A 'tail' suggests that this new human aspartic proteinase cannot be classified into this subfamily.

During our sequencing work on *(pro)napsin A*, it became readily apparent that some human EST clones (e.g. Genbank R11458 and T54068) contained a sequence that was similar to but distinct from that of (pro)napsin A. Thus, mRNA was isolated from human Raji cells (a B-cell line derived from human Burkitt's lymphoma) and reverse-transcribed (see Section 2). The predominant nucleotide sequence elucidated for the *(pro)napsin B* cDNA clones generated is shown in Fig. 1 with the translation product in Fig. 2. However, five polymorphisms were detected in this cDNA sequence. Four of these (AAT/C at nucleotide 666; Fig. 1; CCA/T at 675; GGC/T/G at 711; and GCA/C at bp 1296) were silent. The remaining G/C variation at nucleotide 562 alters the resultant codon from GGC to CGC (Gly to Arg; see below). The overall sequence identities between *(pro)napsin B* and *A* are 91.2% (nucleotide) and 86% (amino acid). The protein sequence predicted by the *(pro)napsin B* gene has two glycosylation motifs (at Asn26 and Asn268; Fig. 2) in common with (pro)napsin A, but the Asn67-Gly68-Thr69 motif in napsin A, renin and cathepsin D is not present. However, an adjacent Asn59-Ala60-Ser61 motif in the predicted napsin B sequence is in a position that does not correspond to any known glycosylation motif in other aspartic proteinases. The predicted napsin B sequence also contains the RGD motif immediately preceding a four-residue insert that was discussed earlier for napsin A.

Most of the other differences between mature napsin A and the predicted sequence of the putative napsin B polypeptide appear to cluster in the region of the active site of the enzyme(s), on the basis of homology modelling (not shown). However, the most unusual features predicted by the *(pro)napsin B* sequence are (i) that it does not contain an in-frame stop codon and (ii) that the residues at positions 120–122 were predicted to be ~Ile-Leu-**Arg**~ in five of 11 independently generated clones but to be ~Ile-Leu-**Gly**~ in the remaining six (Fig. 2). The polymorphism in this CGC/GGC codon was established by analysing *(pro)napsin B* segments of different lengths, generated from different cell types and from genomic as well as cDNA origin. Of the five containing the Arg variant, one clone was isolated from a foetal spleen cDNA library, whilst the other four were independently generated by RT-PCR from RNA extracted from human Raji cells. Of the six Gly containing clones, one was isolated from a foetal spleen cDNA library, and five were generated independently (two from reverse-transcribed adult spleen RNA and three from separate preparations of genomic DNA isolated respectively from human AGS, human Kato III (both gastric adenocarcinoma cell lines) and human white blood cells) using the exon 5 primers, as described in Section 2. The ~Ile-Leu-Gly~ sequence forms the β-strand or thread of the psi loop in the N-terminal domain of all vertebrate/fungal aspartic proteinases [2]. By definition (see Section 1) this sequence *must* be maintained as ~Hydrophobic-Hydrophobic-Gly~ in order for the psi loop to adopt its native conformation and generate the intrinsic activity of an aspartic proteinase. For the Gly-containing variant, this pre-condition is fulfilled. However, in the case of the Arg-containing variant, since this absolute requirement is not met, this would suggest that translation of these *(pro)napsin B* clones would not generate an active enzyme. The possibility exists that these clones may have been derived from a transcribed pseudogene and, if this should prove to be the case, then there would be no requirement to maintain a stop codon under these circumstances. Irrespective of this Gly/Arg polymorphism, an in-frame stop codon was not detected in the sequences determined for any of the *(pro)napsin B* clones.

By comparison with the five other human aspartic proteinases, the cDNA sequences of *(pro)napsin A* and *B* at their respective 3′ untranslated regions are both short, with only 51/48 bp respectively (75% identity) between the TGA stop codon/TGC codon (bp 1261–1263; Fig. 1) of *(pro)napsin A/B* respectively and the AGTAAA putative polyadenylation site motif that is present in both sequences at just over 20 bp upstream from the poly(A) tail. Thus, in the absence of an in-frame stop codon in *(pro)napsin B*, the C-terminal 'tail' that was discussed earlier for mature napsin A would be extended even further by a minimum of 25 residues before the introduction of multiple lysine residues as a consequence of translation of the poly(A) tract in the (pro)napsin B message. A mechanism has been reported in prokaryotic cells [19] for terminating protein translation of defective mRNAs such as those lacking a stop codon, by cotranslational switching of the ribosome to a ubiquitous 10Sa RNA that does have an in-frame stop codon. The consequence of these events is the condemnation of the translated protein product to rapid degradation by energy-dependent bacterial proteinases [20]. It was hypothesised [19] that such a mechanism might also be operative within eukaryotic cells and if this is the case, then any (pro)napsin B protein that is translated may be subject to rapid degradation.

The distribution of (pro)napsin A/B messages in tissues and cells was examined by Northern blotting using a probe derived from the 3′ end of *(pro)napsin B*. The most abundant message was found in lung (Fig. 3, top panel) with a somewhat lower level of transcription apparent in kidney, spleen and leukocytes. The transcript size at ~1.6 kb is consistent with that predicted from the sequence(s) determined. In transformed cell lines, abundant message was detected in promyelocytic leukaemia and Raji cells, both derived from immune-origin lineages (Fig. 3, bottom panel). However, the probe used in these Northerns did not permit distinction between (pro)napsin A and B mRNAs (which have an identity of 86.8% over the length of the probe). Distinction was made possible by a knowledge of the completely conserved exon/intron boundaries in the genomes of the five other human aspartic proteinases (plus genomic DNAs of aspartic protein-

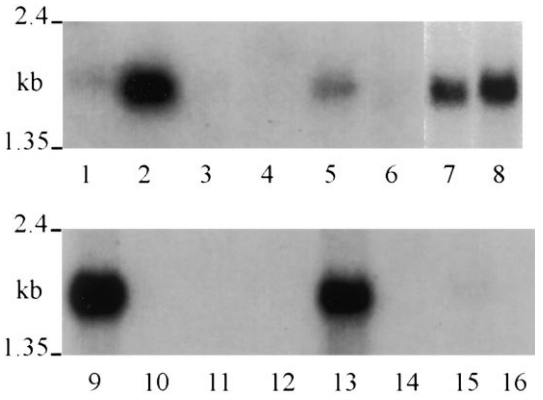

Fig. 3. Expression of the *(pro)napsin A* and *B* genes in human tissues and cancer cell lines. Northern blots probed were from (top): placenta (1); lung (2); liver (3); skeletal muscle (4); kidney (5); pancreas (6); spleen (7); and peripheral blood leukocytes (8); and (bottom): promyelocytic leukaemia HL-40 (9); HeLa S3 (10); chronic myelogenous leukaemia (11); lymphoblastic leukaemia MOLT-4 (12); Raji (13); colorectal adenocarcinoma SW480 (14); lung carcinoma A549 (15) and melanoma G361 (16). Marker fragments of 2.4 and 1.35 kb migrated as indicated. Blots of heart, brain, prostate, testis, ovary, colon mucosa and small intestine were all negative.

ases from other mammals [9,21]). In this way, primers specific for exon 5 were designed and used in RT-PCR to amplify message(s) prepared from lung, kidney, spleen and Raji cells. These primers do not in themselves permit discrimination but amplify (reverse-transcribed) message(s) for both (pro)napsin A and B. However, the resultant amplicons differ in that the *Bst*XI site present in the (pro)napsin A sequence (at bp 550; Fig. 1) is absent from the (pro)napsin B sequence; and conversely, (pro)napsin A does not contain the *Msp*AI site (at bp 606) that is present in the (pro)napsin B sequence (see Section 2). Analysis of 19 independent clones from human lung revealed that all 19 contained (pro)napsin A inserts. In parallel, 18 of 18 clones from human kidney mRNA were also of (pro)napsin A origin. The message that is present in human lung and kidney as revealed on the Northern blots (Fig. 3) is thus that of (pro)napsin A. In contrast, when 23 independent clones from spleen mRNA were analysed in the same way, all 23 were found to contain (pro)napsin B. Thus, it would appear likely that cells derived from/destined for the immune system do not transcribe the message for (pro)napsin A, but rather produce the (pro)napsin B message. In keeping with this, when human Raji cells were used as one of the sources of mRNA (see earlier), analysis by the exon 5 RT-PCR method revealed that they did not contain (pro)napsin A message. Only (pro)napsin B mRNA was detected (20/20 clones) and this contained the ~Hydrophobic-Hydrophobic-Arg~ variant in the psi loop as discussed earlier. It would thus appear that, although considerable identity in sequence exists along the length of the transcribed messages for (pro)napsins A and B, there must be distinct differences in nucleotide sequence in the 5′ flanking regions of each gene in order to achieve such specific transcription from the *(pro)napsin A* promoter in the lung and kidney yet produce only (pro)napsin B mRNA in immune cells.

During these analyses, it became apparent that yet further EST clones, allegedly of human origin, were in reality derived from mouse (e.g. Genbank AA074707 and AA074174). A full-length cDNA sequence (including the (amended) sequence of I.M.A.G.E. Consortium clones ID 531607 and 644880) was derived in this way for mouse *(pro)napsin*; its translation product is aligned with those for human (pro)napsin A and (pro)napsin B in Fig. 2. While this work was nearing completion, Mori et al. [22] reported the cDNA sequence of a mouse aspartic proteinase-like protein (KAP) which is identical to that of mouse (pro)napsin (Fig. 2). The identities between mouse napsin and human napsin A are 78.8% (nucleotide) and 72.6% (amino acid) respectively and between mouse and human napsin B are 77.2% and 70.1% over the mature enzyme regions. Mouse napsin also contains an RGD motif and does have an in-frame stop codon in a comparable position to that of human napsin A (Fig. 2). We have not, to date, been able to detect any indication of the presence of two closely related genes in mouse so that it would appear that mouse napsin is the equivalent murine proteinase to human napsin A. If human napsins A and B have arisen by gene duplication, then this event appears to have taken place relatively recently in evolutionary terms, after the mouse and human divergence.

The present evidence thus points to the likelihood that napsin A is a functional aspartic proteinase predominantly of lung and kidney, that has not been detected hitherto. In contrast, the possibility exists that human *(pro)napsin B* may be a transcribed pseudogene. Transcription produces a full-length mRNA that maintains the correct reading frame up to, through, and past the equivalent positions (Fig. 1) where the stop codons are located in the open reading frames of mouse and human (pro)napsin A. If this message is translated into (pro)napsin B protein, the absence of an in-frame stop codon suggests that the protein product may be rapidly degraded and thus is unlikely to accumulate to significant levels. Detailed analyses at the protein level are now under way to attempt to unravel these complexities associated with this new type of aspartic proteinase.

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