Molecular cloning and biochemical characterization of a new mouse testis soluble-zinc-metallopeptidase of the neprilysin family

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Because of their roles in controlling the activity of several bioactive peptides, members of the neprilysin family of zinc metallopeptidases have been identified as putative targets for the design of therapeutic agents. Presently, six members have been reported, these are: neprilysin, endothelin-converting enzyme (ECE)-1 and ECE-2, the Kell blood group protein, PHEX (product of the phosphate-regulating gene with homologies to endopeptidase on the X chromosome) and X-converting enzyme (XCE). In order to identify new members of this important family of peptidases, we designed a reverse transcriptase–PCR strategy based on conserved amino acid sequences of neprilysin, ECE-1 and PHEX. We now report the cloning from mouse testis of a novel neprilysin-like peptidase that we called NL1. NL1 is a glycoprotein that, among the members of the family, shows the strongest sequence identity with neprilysin. However, in contrast with neprilysin and other members of the family which are type II integral membrane proteins, NL1 was secreted when expressed in cultured mammalian cells, likely due to cleavage by a subtilisin-like convertase at a furin-like site located 22 amino acid residues in the C-terminus of the transmembrane domain. The recombinant enzyme exhibited neprilysin-like peptidase activity and was efficiently inhibited by phosphoramidon and thiorphan, two inhibitors of neprilysin. Northern blot analysis and *in situ* hybridization showed that NL1 mRNA was found predominantly in testis, specifically in round and elongated spermatids. This distribution of NL1 mRNA suggests that it could be involved in sperm formation or other processes related to fertility.

Key words: enkephalin degradation, furin-like processing, germ cells, metallopeptidases, NL1.

INTRODUCTION

Peptides are used by cells from yeast to mammals to elicit physiological responses. The use of peptides as messengers usually involves the following steps: 1) production and release of the peptide by a specific cell, 2) interaction of the peptide with a receptor on the surface of the target cell, and 3) degradation of the peptide to terminate its action. The first and last steps of this scheme require the participation of proteases/peptidases. There is increasing evidence that membrane-associated zinc-metallopeptidases play important roles in both of these steps. Although activation of inactive prohormone precursors into bioactive peptides is generally performed by proteases of the subtilisin family located either in the *trans*-Golgi network or in secretory granules of the cell (for a review see [1]), a few peptides require a final processing step after secretion. This step involves the action of membrane-associated zinc metallopeptidases. Two enzymes are particularly well characterized: angiotensin-converting enzyme (ACE), which cleaves inactive angiotensin I into angiotensin II (for a review see [2]), and endothelin-converting enzyme (ECE), which cleaves isoforms of big endothelin into endothelins (for a review see [3]). In addition to their role in peptide activation, cell-surface zinc-metallopeptidases have also been implicated in the termination of the peptidergic signal by degrading the active peptides into inactive fragments. One of the best known of these peptidases is probably neprilysin (also known as neutral endopeptidase or endopeptidase 24.11) which has been implicated in the physiological degradation of several bioactive peptides (for reviews see [4,5]). In the brain, neprilysin controls the half-life of the enkephalins and substance P, two neuropeptides involved in pain control [6,7]. In the kidney, the enzyme is believed to participate in the regulation of blood pressure by locally controlling the activity of the atrial natriuretic peptide, bradykinin and endothelin-1 [7–10]. Neprilysin has been shown to be present in many other tissues although its role is not always well understood.

Interestingly, neprilysin and the ECEs show significant structural similarities and appear to belong to a family of peptidases that includes PHEX (product of the phosphate-regulating gene with homologies to endopeptidase on the X chromosome; formerly known as PEX), a peptidase involved in the regulation of phosphate homoeostasis (reviewed in [11]), the Kell blood group protein [12] and X-converting enzyme (XCE), a newly discovered enzyme present in the nervous system [13]. ACE, despite being a cell-surface zinc-metallopeptidase with some similarities to neprilysin, is not strictly speaking a member of the

Abbreviations used: ACE, angiotensin-converting enzyme; ECE, endothelin-converting enzyme; PHEX, product of the phosphate-regulating gene with homologies to endopeptidases on the X chromosome; XCE, X-converting enzyme; RT, reverse transcriptase; NL, neprilysin-like; endo H, endoglycosidase H; PNGase F, peptide: N-glycosidase F; RER, rough endoplasmic reticulum; α1-PDX, α1-anti-trypsin Portland variant; 5′-RACE, rapid amplification of 5′ cDNA ends; SEP, soluble secreted endopeptidase; poly(A)$^+$, polyadenylated.

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same family [14]. Because of their important role as regulators of bioactive peptide activity, these enzymes (more specifically neprilysin and the ECEs) have been identified as putative targets for therapeutic intervention, similar to the way inhibition of ACE is used to control blood pressure. However, the recent discovery of PHEX and XCE, and the possibility that yet other unknown members still exist, raise the problem of the specificity of the therapeutic agents to be developed.

To search for other members of the neprilysin family, we designed a reverse transcriptase (RT)–PCR strategy based on conserved regions of neprilysin, ECE-1 and PHEX. In the present paper, we report the molecular cloning, tissue distribution, expression and partial biochemical characterization of a new neprilysin-like peptidase that we called NL1.

MATERIALS AND METHODS

DNA manipulations

All DNA manipulations, phage library screening and plasmid preparations were performed according to standard protocols [15,16]. Site-directed mutagenesis was performed using a PCR-based strategy as described previously [17].

mRNA purification and RT–PCR protocol for identification of new members of the neprilysin family

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). First-strand cDNA was synthesized from 1 μg of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech).

Two degenerate sense primers, oligonucleotide 3817 (5′-TGGATGGA$^{T}/_{C}$G$^{A}/_{C}$IG$^{G}/_{A}$IACI$^{A}/_{C}$A-3′) and oligonucleotide 3719 (5′-$^{A}/_{G}$TIGTITT$^{T}/_{C}$CCIGCIGGI$^{A}/_{G}{}^{T}/_{A}$I$^{C}/_{T}$T$^{G}/_{C}$CA-3′), corresponding to amino acid residues 459–465 and 552–560 of neprilysin sequence respectively, and one antisense primer, oligonucleotide 3720 (5′-AIICCICCI$^{A}/_{T}{}^{C}/_{T}{}^{A}/_{G}$TCIGCI$^{G}/_{A}{}^{C}/_{T}{}^{A}/_{G}$TT-$^{T}/_{C}$TC-3′), corresponding to amino acid residues 646–654 (see Figure 1), were synthesized. PCR was performed with 5 μl of cDNA template and 1 μl of *Taq* DNA polymerase (5 units) in a final volume of 100 μl, containing 1 mM $MgCl_2$, 2 μM each of oligonucleotide 3817 and 3720, 200 μM of each dNTP and 5% (v/v) DMSO. Cycling profiles included an initial denaturation step of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 40 °C and 1.5 min at 72 °C, and a final extension step at 72 °C for 10 min. One-half of the amplified DNA was separated on a 2% (w/v) agarose gel and fragments ranging in size from 500–700 bp were purified and resuspended in a final volume of 50 μl. A second round of PCR was done with primers 3719 and 3720, using either 10 μl of the first PCR reaction or 5 μl of the purified fragments as template, and the new PCR products were ligated into the pCR2.1 vector (Invitrogen). Several identical clones corresponded to a potential new member of the neprilysin family. We called this member NL1 for neprilysin-like 1.

Cloning of full-length NL1 cDNA

The cloned NL1 PCR fragment was used as a probe to screen a mouse testis λ Uni-ZAP^{TM}XR cDNA library (Stratagene). Twelve out of a hundred positive phages were plaque purified and subcloned into the pBS SK vector (Stratagene). As the longest clone analysed presented an incomplete open reading frame (pBS-NL1A), rapid amplification of cDNA 5′ ends (5′-RACE) with primers located in the vector (5′-TAGTGGATCC-CCCGGGCTGCAG-3′, sense primer) and NL1 (5′-ACCAAA-CCTTTCCTGTAGCTCC-3′, antisense primer, nt 1303–1324 of NL1; see Figure 2) was subsequently performed on the DNA of the remaining semi-purified positive clones. Amplification was performed with 1 μl of Vent polymerase (New England BioLabs, Beverley, MA, U.S.A.) in a final volume of 100 μl containing 50 ng of DNA, 4 mM $MgSO_4$, 1 μM of each oligonucleotide, 200 μM of each dNTP and 10% DMSO. Cycling parameters included an initial denaturation step of 1 min at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. A PCR fragment of the expected length was subcloned into pCR2.1 vector (clone pCR-NL1A), but sequencing revealed no initiator ATG codon. A nested 5′-RACE was then performed on mouse testis cDNA using the Marathon Ready cDNA kit (Clontech) with sense oligonucleotides AP1 and AP2 (from the kit) and NL1 antisense oligonucleotides 5′-CCTGAGGGCTCGTTTTACAACCGTC-CT-3′ (nt 503–529 of NL1; see Figure 2) and 5′-CTCATCC-CAGGAGAAGTGTAGCAGGCT-3′ (nt 475–502 of NL1; see Figure 2) as recommended by the supplier. The resulting fragment was cloned into pCR2.1 vector (pCR-NL1B). Since only 10 bp were missing for the initiator ATG codon, we reconstructed the 5′ end of the cDNA by PCR-amplifying clone pCR-NL1A with sense primer 5′-CCACCATGGTGGAGAGAGCAGGCTGG-TGTCGGAAGAAG-3′ (nt 332–364 of NL1; see Figure 2; the 10 missing nucleotides are underlined) and antisense primer 5′-ACCAAACCTTTCCTGTAGCTCC-3′ (nt 1303 to 1324 of NL1; see Figure 2) using Vent polymerase as described above. The DNA fragment was then inserted into pCR2.1 (clone pCR-NL1C). The entire open reading frame was reconstituted following digestion of pBS-NL1A and pCR-NL1C with *Eco*RI and *Pfl*MI. The 5′ end of NL1 cDNA was excised from pCR-NL1C and ligated into pBS-NL1A at the corresponding sites, resulting in plasmid pBS-NL1B.

For expression studies, a *Bam*HI/*Apa*I fragment generated from pBS-NL1B, corresponding to the full length cDNA of NL1, was inserted into the pCDNA3/RSV [18] vector.

Production of polyclonal antibodies

A plasmid for the production in *Escherichia coli* of a glutathione S-transferase (GST)-fusion protein with NL1 was constructed using the pGEX-4T-3 expression vector (Pharmacia Biotechnologies). A 255 bp fragment from NL1 was amplified by PCR with Vent polymerase using sense primer 5′-GCTACG-GGATCCGTGGCCACTATGCTTAGGAA-3′ (nt 1139–1158; see Figure 2) and antisense primer 5′-CGATTGCTCGAG TGGGAACAGCTCGACTTCCA-3′ (nt 1377–1396; see Figure 2). Both pGEX-4T-3 and the PCR product were digested with *Bam*HI and *Xho*I and ligated. The recombinant protein was produced and purified according to the supplier's instructions. Five-week-old female balb/c mice were immunized at monthly intervals for 3 months with 20 μg of the recombinant NL1 fragment in Freund's adjuvant and antisera were subsequently collected.

Cell culture and transfection

Human embryonic kidney (HEK-293) cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum and supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Transfections of cells with appropriate plasmids were performed by the calcium/phosphate-DNA co-precipitation method [19]. To establish permanent cell lines, G418 (geneticin) selection was initiated 48 h after the transfections at 400 μg/ml for 12 days and gradually decreased to 100 μg/ml.

LLC-PK$_1$ cells transfected with pRcCMV-sNEP (where sNEP stands for soluble neprilysin) were maintained as described previously [20].

Immunoblot analysis

For immunoblot analysis, cells were incubated for 16 h in synthetic Dulbecco's modified Eagle's medium containing 2 mM sodium butyrate. Cellular proteins were solubilized as described previously [21]. Secreted proteins recovered in culture media were concentrated approx. 10-fold by ultrafiltration. Immunoblot analysis were performed using the NEN Renaissance kit with the polyclonal antibody specific to NL1 or the α1-anti-trypsin inhibitor antibody (Calbiochem, LaJolla, CA, U.S.A.) followed by the appropriate horseradish peroxidase-conjugated IgG (Vector Laboratories).

For the glycosylation studies, proteins were incubated with endoglycosidase H (endo H) or peptide:N-glycosidase F (PNGase F) as suggested by the distributor (New England BioLabs).

Enzymic activity assays

NL1 activity was monitored and compared with soluble-neprilysin activity using [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin (50 Ci/mmol; Research Products International Inc.), as described previously [21,22]. K_m values were determined by the isotope-dilution method. The inhibitory effects of phosphoramidon and thiorphan were also assessed as described previously [21].

HPLC analysis of the hydrolysis of Leu-enkephalin

Leu^5-enkephalin (5 μg) was incubated at 37 °C for 1 h in 50 mM Mes, pH 6.5, with a concentrated culture medium of HEK-293 cells expressing NL1 (approx. 300 μg of total proteins) or LLC-PK$_1$ cells expressing soluble neprilysin (approx. 30 μg of total proteins), in the absence or presence of 0.1 mM phosphoramidon. Hydrolysis products were separated by reverse-phase HPLC as described previously [23]. Tyr-Gly-Gly and Phe-Leu were both identified by elution profiles of synthetic marker peptides.

Northern blot analysis

A mouse multiple-tissue poly(A)$^+$ mRNA (polyadenylated mRNA) blot (Clontech) was hybridized with a [^{32}P]dCTP random-primer labelled probe in ExpressHyb solution (Clontech). The blot was washed according to the manufacturer's recommendations and exposed to Fuji RX film for 7 days at −80 °C with intensifying screens.

RT–PCR screening of mouse tissues

First strand cDNA synthesis was performed with 1 μg of total RNA from mouse tissues and oligo(dT) as the primer, using Gene Amp RNA PCR Core Kit (Perkin–Elmer). For the PCR reactions, primers 5′-TGGCGAGAGTGTGTCAGCTATGTC-3′ and 5′-CTTCCAAAATGTAGTCAGGGTAGCCAATC-3′ were used with *Taq* polymerase. One-tenth of the PCR products were visualized on a 4% agarose gel.

In situ hybridization

To construct a plasmid for the synthesis of cRNA probes for *in situ* hybridization, pCR-NL1A was used as a template to amplify a 452 bp fragment by PCR with sense primer 5′-GGAGCCATAGTGACTCTGGGTGTC-3′ (nt 416–439; see Figure 2) and antisense primer 5′-GACGCTCAGCAGGGGCTCAGAGTC-3′ (nt 842–865; see Figure 2). The amplification product was inserted into pCRII vector (Invitrogen). Synthesis of riboprobes and protocols for *in situ* hybridization were as described previously [24].

RESULTS

Cloning and sequence analysis of mouse NL1 cDNA

In order to isolate cDNAs for new members of the neprilysin family, we developed an RT–PCR strategy based on the fact that neprilysin, ECE-1 and PHEX share regions of significant sequence identity (Figure 1). Following RT–PCR on testis mRNAs with nested primers, a DNA fragment of approx. 300 bp was amplified. This DNA fragment was cloned and the plasmids from 24 independent colonies were sequenced: three clones had no insert, four clones had DNA fragments not related to the neprilysin family, seven clones had sequences corresponding to mouse neprilysin and three clones had sequences corresponding to mouse PHEX, showing that our approach efficiently allowed the identification of members of the family. Moreover, seven identical clones had a new cDNA presenting sequence similarities to members of the neprilysin family. The full-length cDNA was subsequently obtained by screening a mouse testis λ cDNA library followed by 5′-RACE, as described in the Materials and methods section. The nucleotide and deduced amino acid sequences shown in Figure 2 confirm that we cloned a novel neprilysin-like protein, referred to thereafter as NL1.

NL1 cDNA spans 2925 nt, including a 5′-untranslated region of 331 nt, an open reading frame of 2295 nt from nt 332–2626, and a 3′-untranslated region of 299 nt. The sequence surrounding the proposed initiator ATG conforms to the Kozak consensus [25]. The deduced amino acid sequence of NL1 reveals a putative-type II transmembrane protein of 765 amino acid residues encompassing a short N-terminal cytoplasmic tail, a unique putative-transmembrane domain, and a large C-terminal extracellular domain. The ectodomain contains nine potential N-glycosylation sites (Asn-X-Ser/Thr) and ten cysteine residues corresponding to those conserved among all the members of the

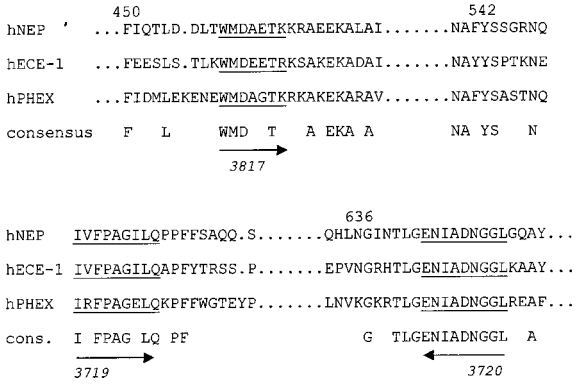

Figure 1 Partial amino acid sequence alignment of human neprilysin, ECE-1 and PHEX

Numbers above the sequences identify residues in the neprilysin sequence. Regions corresponding to degenerate oligonucleotides used for the nested PCR are underlined. Arrows below the sequences represent the degenerate oligonucleotides and the numbers correspond to the nomenclature used in the text.

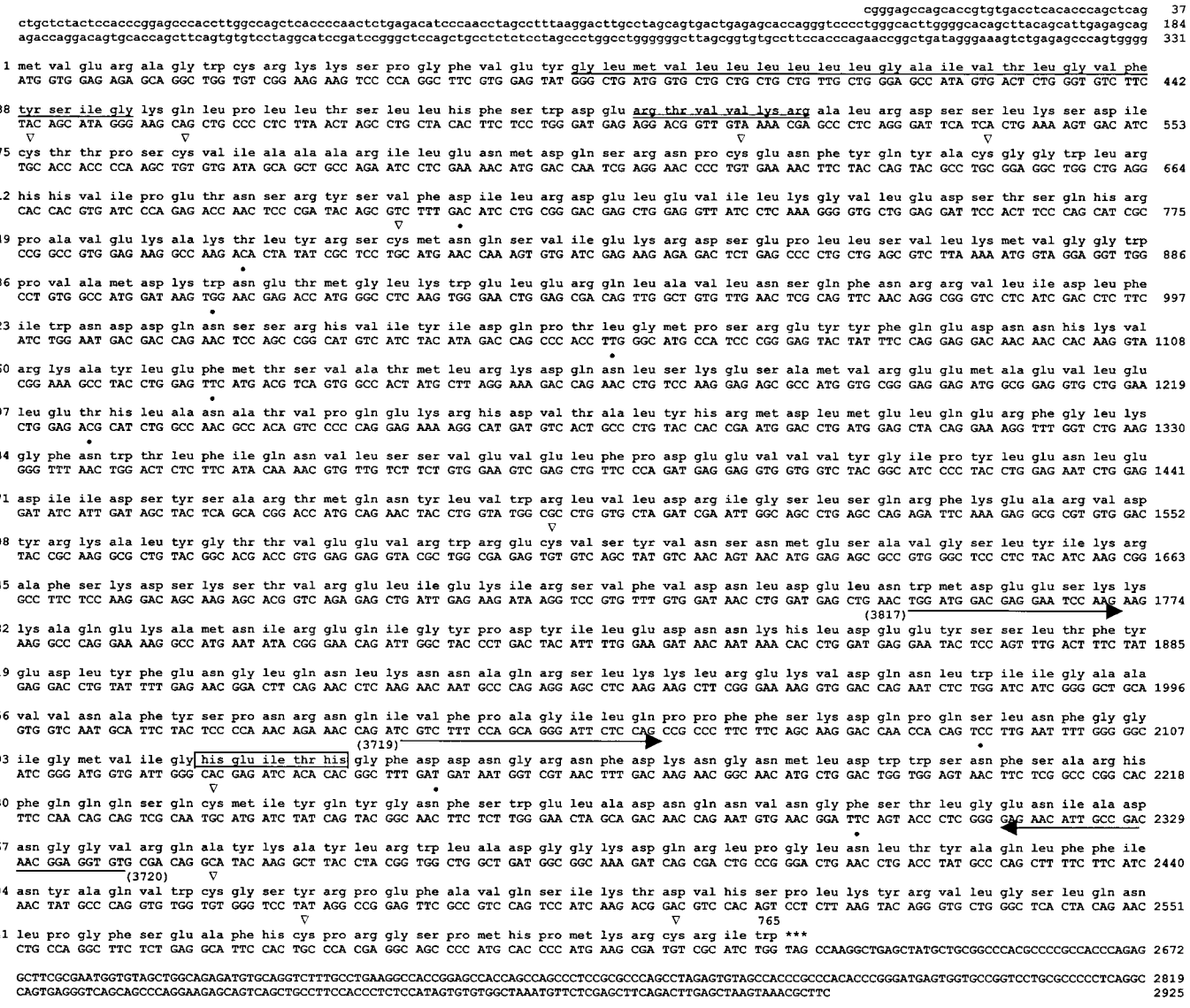

Figure 2 Nucleotide and deduced amino acid sequences of NL1 cDNA cloned from mouse testis

The predicted transmembrane domain of NL1 is underlined and the putative furin/prohormone-cleavage site is double underlined. The conserved cysteine residues are indicated by an arrowhead, the nine potential N-glycosylation sites are indicated with a dot (●) and the HEXXH zincin consensus is boxed. Arrows and numbers in brackets show the position of the oligonucleotide primers used in PCR.

family, which are presumably involved in proper folding and in maintenance of the protein in an active conformation. All amino acid residues known to be part of the active site of neprilysin are present in NL1 (see Figure 3). The predicted protein sequence presents greater similarities to neprilysin than to any other member of the family (Table 1).

Although NL1 shares numerous features with proteins of the neprilysin family, a notable aspect distinguishes it from the others: the first conserved cysteine residue of the ectodomain is more distant (34 amino acid residues) from the predicted transmembrane domain in NL1 than it is in neprilysin (nine amino acid residues) or any other members of the family. Moreover, we noticed a putative furin-cleavage site (-Arg_{58}-Thr-Val-Val-Lys-Arg_{63}-) between the end of the transmembrane domain and the first cysteine (Figure 2). This observation suggests that NL1 could exist as a secreted rather than a membrane-bound protein.

NL1 expression in HEK-293 cells

HEK-293 cells were transfected with a pCDNA3/RSV expression vector containing NL1 cDNA, and a permanent cell line was established as described in the Materials and methods section (HEK/NL1 cells). Immunoblotting with a polyclonal antibody showed that after 16 h of culture, most NL1 was present in the culture medium with small amounts of the enzyme in the cell extract (Figure 4). Secreted and cell-associated NL1 had apparent

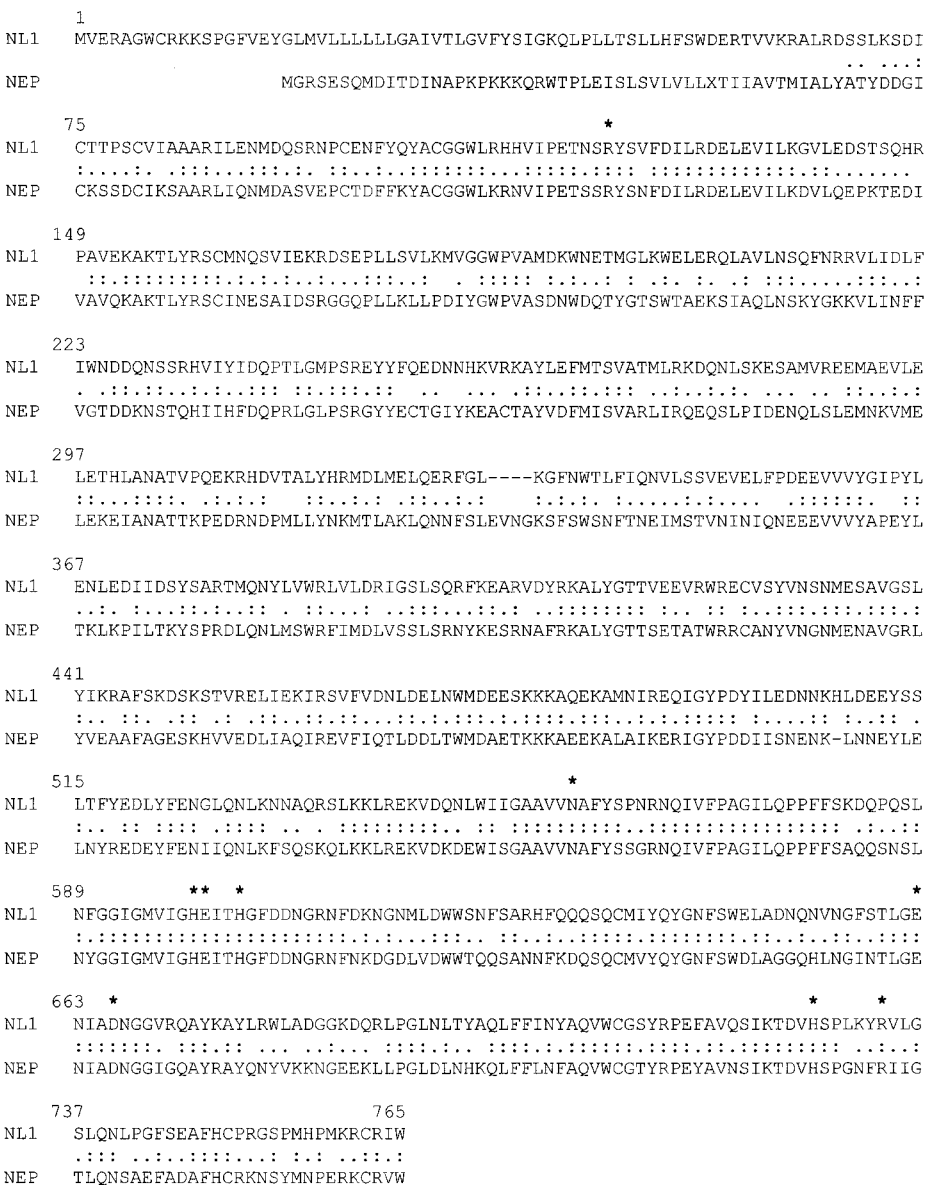

Figure 3 Amino acid sequence comparison of NL1 and neprilysin

Single dots stand for similarity and double dots for identity. Residues known to be important for enzyme activity, including the three zinc ligands (His^{583}, His^{587} and Glu^{646} in neprilysin), the catalytic Glu residue (Glu^{584} in neprilysin), the transition-state-stabilizing His residue (His^{711} in neprilysin), the residues involved in substrate binding (Arg^{102}, Arg^{717} and Asn^{542} in neprilysin) and the Asp residue implicated in structure maintenance of the active site (Asp^{650} in neprilysin) are indicated with asterisks (*).

molecular masses of approx.125 kDa and 110 kDa respectively. To characterize the glycosylation state of NL1, we next submitted the recombinant protein to deglycosylation by PNGase F and endo H. PNGase F removes high mannose as well as most complex N-linked oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side chains of the high-mannose type found on proteins in the rough endoplasmic reticulum (RER) but which have not yet transited through the Golgi complex; thus, resistance to endo H can be used as an indication that the protein has travelled through the Golgi complex. PNGase F treatment showed that the cell-associated and secreted NL1 were N-glycosylated, since their electrophoretic mobility increased following digestion (Figure 4). However, the secreted NL1 migrated as a doublet after PNGase F treatment, with one band co-migrating with the cell-associated form and the second having a slower rate of migration. Since untreated and endo H-digested secreted NL1 are seen as single bands by SDS/PAGE, our observation suggests that a proportion of secreted NL1 undergoes further post-RER post-translational modification that renders some of the N-linked oligosaccharides resistant to PNGase F digestion.

In contrast with secreted NL1, NL1 from cell extracts was sensitive to endo H treatment. This result shows differences in the glycosylation state of the two species and suggests that the cell-associated form observed in transfected cells is an intracellular species that has not travelled through the Golgi complex.

Table 1 Comparison of NL1 with other members of the neprilysin family

The region covering amino acids 70 to 765 of NL1 was compared with the corresponding sequences of other members of the neprilysin family.

Protein	Identity (%)	Similarity (%)
Mouse neprilysin	55	74
Rat neprilysin	54	73
Human neprilysin	55	74
Rat ECE-1	39	60
Human ECE-1	39	60
Bovine ECE-2	38	57
Mouse PHEX	39	58
Human PHEX	39	58
Human Kell blood group protein	26	46
Human XCE	36	56

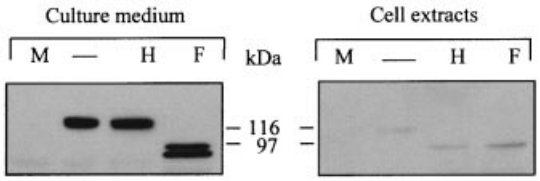

Figure 4 Immunoblot analysis of NL1-transfected HEK-293 cells

Proteins from culture medium and from cell extracts (corresponding to one-fiftieth of a 150-mm-diameter dish) were separated by SDS/PAGE [8% (w/v) gels], transferred to nitrocellulose membranes and revealed with an NL1-specific polyclonal antibody. M, mock-transfected cells; —, NL1-transfected cells; H, endo H-treated NL1-transfected cells; F, PNGase F-treated NL1-transfected cells. Molecular mass marker positions are shown in the centre.

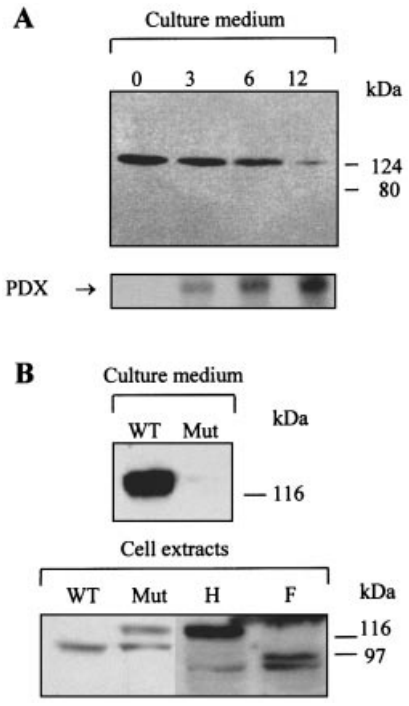

Figure 5 Analysis of NL1 processing in HEK-293 cells

(**A**) Proteins from the culture medium of HEK-293 cells co-expressing NL1 and α1-PDX were separated as in the legend to Figure 4 and revealed with either NL1 antibodies (upper panel) or α1-anti-trypsin inhibitor antibodies (lower panel). The amount in μg of plasmid pCDNA3.1/CMV/PDX used in the transfections is indicated above each lane. (**B**) Proteins from the culture medium or extracts of cells expressing either wild-type NL1 (WT) or NL1 mutated in the furin-cleavage site (Mut) (corresponding to one-twentieth of a 150-mm-diameter dish) were analysed as in Figure 4. H, Mut proteins treated with endo H; F, Mut proteins treated with PNGase F. Lanes H and F were exposed three times longer. Molecular mass marker positions are shown on the right (B and C).

Processing of NL1 by a subtilisin-like convertase

To determine whether a member of the mammalian subtilisin-like convertase family is responsible for NL1 presence in the culture medium of transfected cells, we transiently co-transfected HEK-293 cells with a constant amount of plasmid pCDNA3/RSV/NL1 and increasing amounts of plasmid pCDNA3/CMV/PDX [26]. This latter vector promotes the expression of the α1-anti-trypsin Portland variant (α1-PDX), a known inhibitor of subtilisin-like convertases [27]. Immunoblot analysis of the culture media of cells expressing both NL1 and α1-PDX indicated that NL1 secretion was strongly inhibited by the presence of α1-PDX (Figure 5A); a relationship was observed between the amount of α1-PDX and the level of inhibition of NL1 secretion.

To confirm that proteolysis by the subtilisin-like convertase occurred at the putative furin-cleavage site identified in the NL1 ectodomain (-Arg^{58}-Thr-Val-Val-Lys-Arg^{63}-), the amino acid residues Asn^{62}-Gly^{63} were substituted for Lys^{62}-Arg^{63} by site-directed mutagenesis in vector pCDNA3/RSV/NL1 and the mutated vector was used to establish HEK-293 cells expressing the mutant protein (HEK/NL1mut cells). Immunoblot analysis of the culture media of HEK/NL1mut cells showed that the mutation totally abolished secretion of NL1 (Figure 5B). Furthermore, an additional form of NL1 with a molecular mass of 127 kDa was detected in the extract of these cells (Figure 5B). This new species was resistant to endo H digestion (Figure 5B) and was found associated with membranes when HEK/NL1mut cells were fractionated according to Chidiac et al. [28] (results not shown).

NL1 enzymic activity

Culture media from HEK-293 and HEK/NL1 cells were tested for enzymic activity using [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin (a known neprilysin substrate) as substrate. Activity was detected in the culture medium of HEK/NL1 cells but not in that of HEK-293 cells. The activity increased linearly with the amount of NL1 (Figure 6A) and with the incubation period (Figure 6B), indicating that degradation of the substrate was due to NL1 enzymic activity.

We then characterized the enzymic parameters of NL1 using the same substrate and compared them with those of an engineered soluble form of neprilysin [29]. NL1 affinity for [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin was slightly higher than that of soluble neprilysin, as shown by their K_m values of 18 μM and 73 μM respectively (Table 2). Inhibition assays showed that phosphoramidon had similar effects on NL1 and soluble-neprilysin activity, with IC_{50} values of 0.9 nM and 0.5 nM respectively, and that thiorphan, a specific inhibitor of neprilysin, inhibited NL1 with an IC_{50} of 47 nM, compared with an IC_{50} of 8 nM for neprilysin (Table 2).

Very low levels of phosphoramidon-sensitive activity were detected in extracts of HEK/NL1 cells (results not shown), consistent with the small amounts of NL1 observed by immunoblotting.

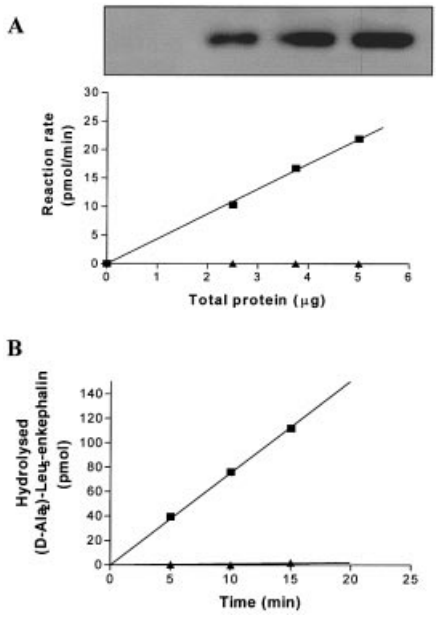

Figure 6 NL1 enzymic activity as a function of (A) the amount of protein and (B) time

(**A**) Increasing volumes of culture medium from HEK-293 or HEK/NL1 cells were tested for peptidase activity using [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin as substrate. The amount of NL1 detected by immunoblotting in the aliquots used to measure activity is shown in the panel above the graph. (**B**) Aliquots of culture medium from HEK-293 or HEK/NL1 cells containing 2.5 μg of total protein were incubated for the indicated time periods with the same substrate as in (**A**). ■, NL1; ▲, mock.

To determine whether NL1 had cleavage-site specificity similar to neprilysin, we incubated Leu^5-enkephalin in the presence of NL1 recovered from the medium of HEK/NL1 cells (Figure 7B) or in the presence of soluble neprilysin (Figure 7C), and analysed the degradation products by reverse-phase HPLC. Peaks co-migrating with standard Tyr-Gly-Gly and Phe-Leu peptides were observed in both reverse-phase HPLC profiles, indicating that both enzymes cleaved the substrate at the Gly^3-Phe^4 peptide bond. This enkephalin-degrading activity was completely inhibited by phosphoramidon (1 μM; Figure 7A).

Tissue and cellular distribution of NL1 mRNA

Tissue distribution of NL1 mRNA was determined by Northern blot analysis with a specific probe corresponding to the 5′end of the coding region of NL1 cDNA. A single transcript of 3.4 kb was detected exclusively in testis among all the mouse tissues tested (Figure 8A). Mouse tissues were also screened by RT–PCR. Using this more sensitive technique, expression of NL1 was observed in several other tissues including heart, brain, spleen, lungs, liver and kidney (Figure 8B). Consistent with the Northern blot results, RT–PCR analysis, although not strictly quantitative, detected more NL1 mRNA in testis than in other tissues.

To gain more insight into NL1 mRNA distribution, we examined by *in situ* hybridization cryostat sagittal sections from a 4-day-old newborn mouse, as well as sections from a 16-day-old animal (p16) and adult tissues (heart, brain, spleen, lungs, liver, kidney and testis). The presence of NL1 mRNA was detected only in adult testis (Figure 9). Only the germinal cells in the luminal face of the seminiferous tubules were labelled (Figures

Table 2 Comparison of the enzymic parameters of NL1 and soluble neprilysin with D-Ala^2-Leu^5-enkephalin

Each value is the mean ± S.E.M. for three (*) or four (**) independent experiments. See the Materials and methods section for details.

Enzyme	K_m* (μM)	IC_{50}** Phosphoramidon (nM)	IC_{50}* Thiorphan (nM)
NL1	18 ± 10	0.9 ± 0.3	47 ± 12
Soluble neprilysin	73 ± 18	0.5 ± 0.1	8 ± 2

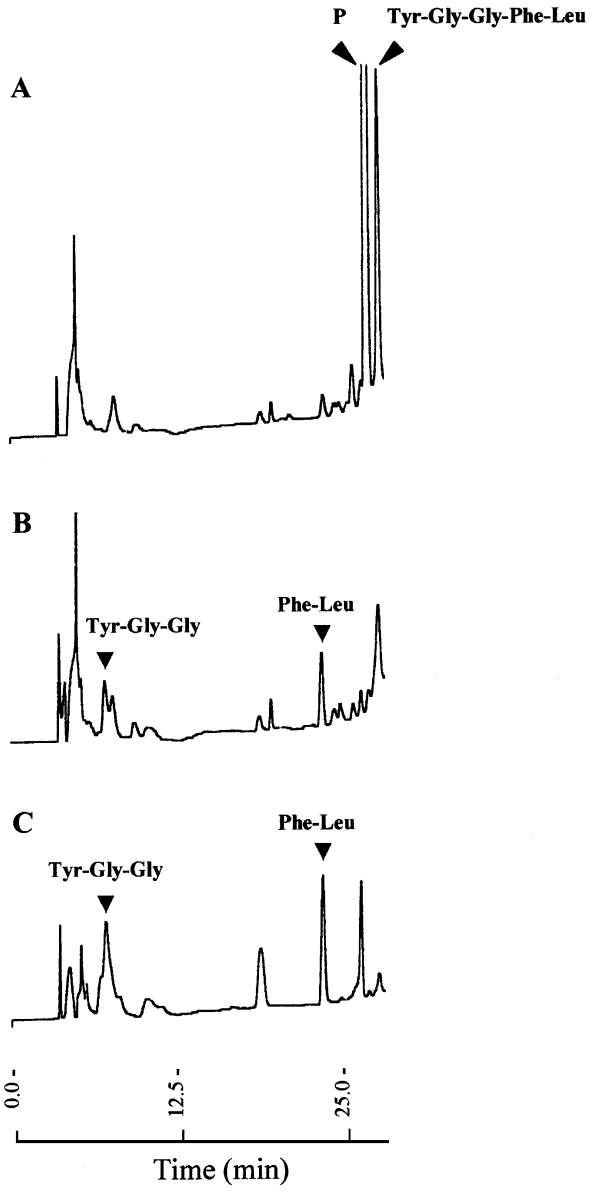

Figure 7 HPLC analysis of degradation products of Leu$_5$-enkephalin

Leu$_5$-enkephalin was incubated with concentrated culture medium from HEK/NL1 cells in the presence (**A**) or absence (**B**) of phosphoramidon (P), or with concentrated medium from soluble-neprilysin-transfected LLC-PK$_1$ cells (**C**), and the hydrolysis products were resolved by HPLC. Tyr-Gly-Gly and Phe-Leu were both identified with HPLC profiles of synthetic marker peptides.

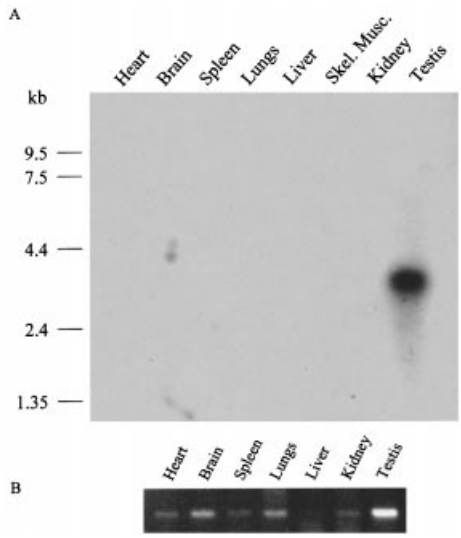

Figure 8 Tissue distribution of mouse NL1 mRNA by Northern blot and RT–PCR analysis

(**A**) The membrane purchased from Clonetech containing 2 μg of poly(A)$^+$ mRNA of various tissues was hybridized with a 1 kb specific cDNA probe corresponding to the 5′-coding region of NL1. The blot was exposed for autoradiography for 7 days. (**B**) Total RNA (1 μg) was used for the RT–PCR reactions. Amplified DNA fragments were resolved on a 4% agarose gel.

9A and 9B). These cells were identified as round and elongated spermatids in all spermiogenesis maturational stages (Figures 9B1, 9B2, 9B3 and 9B4). Neither spermatozoa (Figures 9B and 9B5) nor spermatocytes, spermatogonies or Sertoli cells were labelled (Figure 9B). Interstitial cells were also negative. Controls were performed with sense riboprobes, which produced only non-specific background (results not shown). The 4-day-old mouse sagittal sections and all other tissues tested were negative.

DISCUSSION

The great interest in members of the neprilysin family as putative therapeutic targets and the recent discovery of new members of this important family of peptidases led us to investigate whether additional members of the family remained to be identified. Using a PCR-based strategy, we cloned, from mouse testis, a partial cDNA encoding a new neprilysin-like enzyme that we called NL1. Analysis of the amino acid sequence encoded by the full-length NL1 cDNA revealed that this member of the family most resembled neprilysin, with 55% identity and 74% similarity. Recently, the primary structure of a new zinc metallopeptidase from total mouse embryo was reported [30]. This enzyme, called soluble secreted endopeptidase (SEP), is found as either a soluble or a cell-associated form, due to alternative splicing. NL1 shows only three amino acid differences from the soluble form of SEP indicating that secreted SEP and NL1 are the same enzyme. Our cloning strategy did not allow characterization of the cell-associated form of NL1, which is a minor species in mouse testis [30].

The amino acid sequence of NL1 predicts a topology of a type II integral-membrane glycoprotein that is similar to the other members of the family. Treatment of the recombinant protein with PNGase F showed that NL1 possesses N-linked carbohydrate side chains. However, it is not possible to determine precisely whether all nine putative N-glycosylation sites are used, although the 30 kDa decrease in molecular mass upon PNGase F treatment suggests that most are glycosylated. It has already been shown that all asparagine residues in an Asn-X-Ser/Thr consensus are glycosylated in rabbit neprilysin expressed in COS-1 cells and that sugar moieties increase the stability and enzymic activity of the protein and facilitate its intracellular transport [31]. Three of neprilysin glycosylated Asn residues (Asn^{145}, Asn^{285} and Asn^{628}) are conserved in NL1 (Asn^{163}, Asn^{303} and Asn^{643}). Amongst these residues, Asn^{145} and Asn^{628} have been reported to influence neprilysin enzymic activity [31]. In the same work, it has also been shown that the effect of sugar addition on folding and intracellular transport of neprilysin is an accumulative effect of all glycosylation sites rather than a contribution of any particular one. Glycosylation of NL1 may share similarities with that of neprilysin, since we found their primary structures and enzymic activities to be very similar.

Surprisingly, expression of the cDNA by transfection of HEK-293 cells showed that most of the enzyme was secreted in the culture medium. The small amount of NL1 associated with the cells was endo H-sensitive, suggesting that the cell-associated enzyme is a species that has not yet left the RER. The presence of a furin-cleavage site in the NL1 sequence between the predicted transmembrane domain and the first conserved cysteine residue of the ectodomain led us to believe that a member of the mammalian subtilisin-like family of convertases was responsible for the presence of NL1 in the culture medium. These enzymes are involved in processing a variety of precursor proteins such as growth factors and hormones, receptors, plasma proteins, matrix metalloproteinases, metalloproteases-disintegrins and viral envelope glycoproteins (for a review see [32]). Site-directed mutagenesis of the furin-cleavage site (-Arg^{58}-Thr-Val-Val-Lys-Arg^{63}-) and expression of α1-PDX, a potent inhibitor of mammalian subtilisin-like convertases [27], confirmed that a member of this family of endoproteases was involved in NL1 secretion, presumably by cleaving at the C-terminus of Arg^{63}. There are only a few examples of proteins that are processed from a membrane-bound precursor to a secreted form following cleavage by subtilisin-like convertases; these include meprin and collagen XVII [33,34]. Three members of the subtilisin-like family of convertases, namely furin, PC4 and PC7, are known to be expressed in germ cells [35–38]. Whether one of these convertases generates secreted NL1 from its membrane form is under current investigation. In any case, NL1 is the only known member of the neprilysin family that is secreted. This unique feature suggests that NL1 plays its physiological role in a context different from that of the membrane-bound peptidases, thereby diversifying the role of the peptidases of the neprilysin family. It is of interest that circulating forms of neprilysin in blood and urine have been described, but they have generally been related to pathological or stressful conditions [39–43].

We have observed in cells expressing NL1 mutated at the furin-cleavage site, the appearance of a species resistant to digestion by endo H. This mutated protein was associated with cellular membranes. Taken together, these results indicate that NL1 is first synthesized and inserted into the RER membrane as a type II-transmembrane protein. During intracellular transport, NL1 is converted into a soluble form by the action of a member of the mammalian subtilisin-like convertases. The identity of the cellular compartment where this process occurs is not known. However, mammalian subtilisin-like convertases are usually active in post-Golgi compartments of the secretory pathway, suggesting that processing of NL1 from the membrane-bound form to the soluble form is a post-Golgi event.

Despite almost total abrogation of NL1 secretion, we observed only a slight accumulation of endo H-resistant NL1 in cells either co-expressing α1-PDX and NL1 (results not shown) or expressing

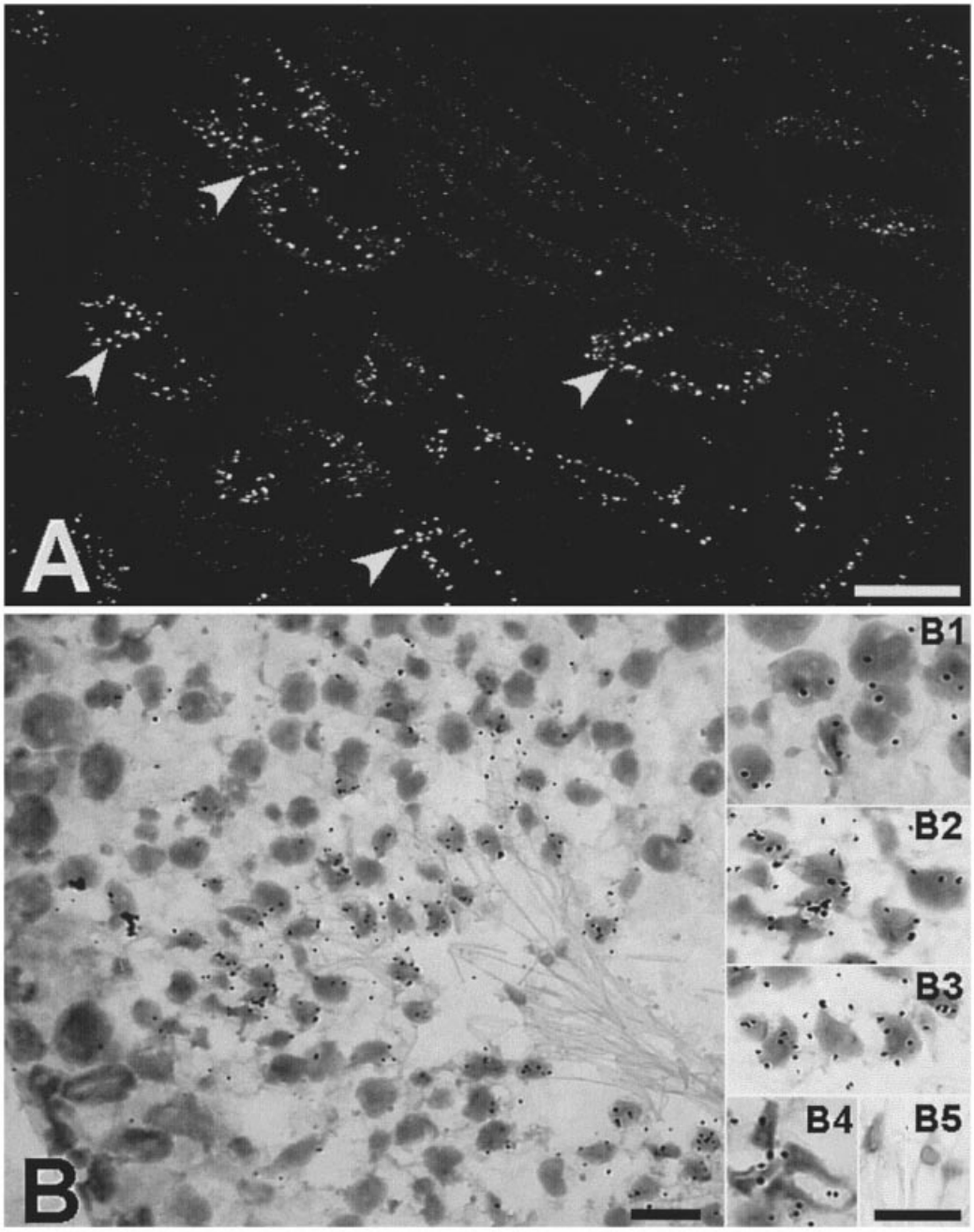

Figure 9 NL1 mRNA distribution in adult mouse testis observed by *in situ* hybridization

Silver labelling is seen as white spots on dark field (**A**) or as dark labelling on bright field (**B**). (**A**) Low-power emulsion autoradiography showing tubular distribution of NL1 mRNA. Some tubules present more evident labelling (arrowheads) indicating a stage-specific tubular distribution. No labelling is seen in the interstitium. (**B**) Sites of NL1 mRNA expression at the cellular level showing labelling in inner germinal cells identified as round and elongated spermatids (higher magnification in **B1**, **B2**, **B3** and **B4**). Note that large cells corresponding to spermatogonies, spermatocytes and Sertoli cells are unlabelled (**B**), as well as spermatozoa (**B** and **B5**). Scale bar = 150 μm (**A**), 15 μm (**B**) and 10 μm [**B1**, **B2**, **B3**, **B4** and **B5** (scale bar in **B5**)].

mutated NL1. This observation suggests that unprocessed NL1 is rapidly degraded. A similar behavior was reported for the Notch1 receptor expressed in the furin-deficient cell line LoVo [44]. The mechanism(s) by which these unprocessed proteins are degraded is still unknown. It is interesting to point out that the spliceoform of SEP that has lost a 23 amino acid peptide, including the furin-cleavage site, generates a cell-associated endo H-sensitive molecule [30].

The most important observation regarding the NL1 primary structure is the conservation of residues which in neprilysin are essential for catalysis and binding of substrates or inhibitors. This finding suggests that NL1 could effectively act as an endopeptidase with a catalytic mechanism similar to that of neprilysin. This hypothesis was supported by the demonstration that [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin, a peptide substrate often used to monitor neprilysin activity, was also an excellent

NL1 substrate. The affinity of NL1 for [3,5-^{3}H]tyrosyl-D-Ala^2-Leu^5-enkephalin was even higher than that of neprilysin, as reflected by a K_m value 4- to 5-fold lower. Furthermore, two well-known neprilysin inhibitors, phosphoramidon and thiorphan, also abolished NL1 activity. Phosphoramidon, which inhibits neprilysin as well as ECE-1 activity, albeit to a lesser extent [45], had very similar effects on NL1 and neprilysin, with an IC_{50} value for NL1 varying not more than 2-fold from the value determined for neprilysin. Thiorphan, considered to be a more specific inhibitor of neprilysin, also inhibited NL1 activity, with an IC_{50} 6-fold greater than that for neprilysin. These results suggest that the active sites of NL1 and neprilysin are similar. This hypothesis is supported by the observation that secreted SEP degraded a set of peptides known to be neprilysin substrates, including substance P, bradykinin and atrial natriuretic peptide [30]. Taken together, these results illustrate the importance of identifying and characterizing other members of the family for the design of highly specific inhibitors.

In agreement with the enzymic parameters demonstrating that NL1 and neprilysin have similar catalytic sites, we have observed that both enzymes cleaved Leu$_5$-enkephalin at the same peptide bond. This result suggests that NL1 hydrolyses peptide bonds on the amino side of hydrophobic amino acid residues as does neprilysin [46]. However, several other peptides will have to be tested to confirm this specificity and to determine whether NL1 has dipeptidyl carboxypeptidase activity as was shown for neprilysin [47–49] and more recently for ECE-1 [50].

RT–PCR experiments with specific primers for the soluble and cell-associated forms of SEP showed a wide tissue distribution of the enzyme with the soluble form of SEP being predominant in testis and the cell-associated form being predominant in other tissues [30]. Our RT–PCR results confirmed the wide tissue distribution of NL1. However, Northern blotting and *in situ* hybridization experiments indicated that expression of NL1 is predominant in germ cells of mature testis. Interestingly, proenkephalin mRNA has been shown to be expressed in germ cells and somatic cells of the testis [36,37,51–54]. Specific functions for testicular enkephalin peptides have not yet been defined, but it is believed that they could act as intratesticular paracrine/autocrine factors. In addition to their putative role as mediators of testicular cell communication, it has also been demonstrated that proenkephalin products synthesized by spermatogenic cells during spermatogenesis are stored in the acrosome of human, hamster, rat and sheep spermatozoa and are released from sperm following the acrosomal reaction [55]. It has thus been proposed that proenkephalin products may act as sperm acrosomal factors during the fertilization process as well as intratesticular regulators secreted by spermatogenic cells. Since Leu$_5$-enkephalin was found to be a good substrate for NL1, opioid peptides originating from proenkephalin could serve as physiological substrates for this new enzyme. In this way, NL1 would serve to regulate the activity of these bioactive peptides.

Testis is the only tissue where the soluble form of SEP is predominant [30], suggesting a testis-specific alternative splicing. Expression of testis-specific molecular species of peptidases or prohormones, arising through diverse mechanisms, has been documented in the past [56,57]. However, the physiological significance of these testis-specific species is not always clear. In the case of NL1 or SEP, it might allow local constitutive secretion by germinal cells of an otherwise cell-associated enzyme, to regulate spermatogenesis much like several other proteolytic enzymes of the seminiferous tubules [58]. Alternatively, it might allow accumulation in the acrosome with proenkephalin peptides and release upon acrosomal reaction. More exhaustive studies concerning NL1 localization and physiological substrate identification will be needed to understand its role in the testis and possibly in the fertilization process.

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REFERENCES

1 Seidah, N. G. and Chrétien, M. (1995) Methods Enzymol. **244**, 175–188
2 Corvol, P. and Williams, T. A. (1997) in Cell-Surface Peptidases in Health and Disease (Kenny, A. J. and Boustead, C. M., eds.), pp. 99–117, BIOS Scientific Publishers Ltd., Oxford
3 Turner, A. J. (1997) in Cell-Surface Peptidases in Health and Disease (Kenny, A. J. and Boustead, C. M., eds.), pp. 137–153, BIOS Scientific Publishers Ltd., Oxford
4 Kenny, J. (1993) Biochem. Soc. Trans. **21**, 663–668
5 Koehne, P., Schaper, C., Graf, K. and Kunkel, G. (1998) Allergy **53**, 1023–1042
6 Roques, B. P., Fournie-Zaluski, M. C., Soroca, E., Lecomte, J. M., Malfroy, B., Llorens, C. and Schwartz, J. C. (1980) Nature (London) **288**, 286–288
7 Roques, B. P., Noble, F., Dauge, V., Fournie-Zaluski, M. C. and Beaumont, A. (1993) Pharmacol. Rev. **45**, 87–146
8 Ura, N., Carretero, O. A. and Erdos, E. G. (1987) Kidney Int. **32**, 507–513
9 Pham, I., el Amrani, A. I., Fournie-Zaluski, M. C., Corvol, P., Roques, B. and Michel, J. B. (1992) J. Cardiovasc. Pharmacol. **20**, 847–857
10 Yamaguchi, T., Fukase, M., Arao, M., Sugimoto, T. and Chihara, K. (1992) FEBS Lett. **309**, 303–306
11 Tenenhouse, H. S. (1999) Nephrol. Dial. Transplant. **14**, 333–341
12 Marsh, W. L. (1992) Transfusion **32**, 98–101
13 Valdenaire, O., Richards, J. G., Faull, R. M. and Schweizer, A. (1999) Mol. Brain Res. **64**, 211–221
14 Hooper, N. M. (1994) FEBS Lett. **354**, 1–6
15 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D. and Smith, J. A. (1988) Current Protocols in Molecular Biology, Wiley Interscience, New York
16 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
17 Le Moual, H., Dion, N., Roques, B. P., Crine, P. and Boileau, G. (1994) Eur. J. Biochem. **221**, 475–480
18 Jockers, R., Da Silva, A., Strosberg, A. D., Bouvier, M. and Marullo, S. (1996) J. Biol. Chem. **271**, 9355–9362
19 Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. **7**, 2745–2752
20 Lanctôt, C., Fournier, H., Howell, S., Boileau, G. and Crine, P. (1995) Biochem. J. **305**, 165–171
21 Dion, N., Le Moual, H., Fournie-Zaluski, M. C., Roques, B. P., Crine, P. and Boileau, G. (1995) Biochem. J. **311**, 623–627
22 Devault, A., Nault, C., Zollinger, M., Fournie-Zaluski, M. C., Roques, B. P., Crine, P. and Boileau, G. (1988) J. Biol. Chem. **263**, 4033–4040
23 Bawab, W., Aloyz, R. S., Crine, P., Roques, B. P. and DesGroseillers, L. (1993) Biochem. J. **296**, 459–465
24 Ruchon, A. F., Marcinkiewicz, M., Siegfried, G., Tenenhouse, H. S., DesGroseillers, L., Crine, P. and Boileau, G. (1998) J. Histochem. Cytochem. **46**, 459–468
25 Kozak, M. (1986) Cell **44**, 283–292
26 Benjannet, S., Savaria, D., Laslop, A., Munzer, J. S., Chrétien, M., Marcinkiewicz, M. and Seidah, N. G. (1997) J. Biol. Chem. **272**, 26210–26218
27 Anderson, A. D., Thomas, L., Hayflick, J. S. and Thomas, G. (1993) J. Biol. Chem. **268**, 24887–24891
28 Chidiac, P., Nouet, S. and Bouvier, M. (1996) Mol. Pharmacol. **50**, 662–669
29 Lemay, G., Waksman, G., Roques, B. P., Crine, P. and Boileau, G. (1989) J. Biol. Chem. **264**, 15620–15623
30 Ikeda, K., Emoto, N., Raharjo, S. B., Nurhantari, Y., Saiki, K., Yokoyama, M. and Matsuo, M. (1999) J. Biol. Chem. **274**, 32469–32477
31 Lafrance, M. H., Vezina, C., Wang, Q., Boileau, G., Crine, P. and Lemay, G. (1994) Biochem. J. **302**, 451–454
32 Nakayama, K. (1997) Biochem. J. **327**, 625–635
33 Milhiet, P. E., Chevallier, S., Corbeil, D., Seidah, N. G., Crine, P. and Boileau, G. (1995) Biochem. J. **309**, 683–688
34 Schacke, H., Schumann, H., Hammami-Hauasli, N., Raghunath, M. and Bruckner-Tuderman, L. (1998) J. Biol. Chem. **273**, 25937–25943
35 Nakayama, K., Kim, W. S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. and Murakami, K. (1992) J. Biol. Chem. **267**, 5897–5900

36 Torii, S., Yamagishi, T., Murakami, K. and Nakayama, K. (1993) FEBS Lett. **316**, 12–16
37 Seidah, N. G., Day, R., Hamelin, J., Gaspar, A., Collard, M. W. and Chretien, M. (1992) Mol. Endocrinol. **6**, 1559–1570
38 Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros, H., Mbikai, M., Chrétien, M. and Day, R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 3388–3393
39 Almenoff, J., Teirstein, A. S., Thornton, J. C. and Orlowski, M. (1984) J. Lab. Clin. Med. **103**, 420–431
40 Deschodt-Lanckman, M., Michaux, F., de Prez, E., Abramowicz, D., Vanherweghem, J. L. and Goldman, M. (1989) Life Sci. **45**, 133–141
41 Johnson, A. R., Coalson, J. J., Ashton, J., Larumbide, M. and Erdos, E. G. (1985) Am. Rev. Respir. Dis. **132**, 1262–1267
42 Soleilhac, J. M., Lafuma, C., Porcher, J. M., Auburtin, G. and Roques, B. P. (1996) Eur. J. Clin. Invest. **26**, 1011–1017
43 Aviv, R., Gurbanov, K., Hoffman, A., Blumberg, S. and Winaver, J. (1995) Kidney Int. **47**, 855–860
44 Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. and Israël, A. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 8108–8112
45 Turner, A. J. and Murphy, L. (1996) Biochem. Pharmacol. **51**, 91–102
46 Turner, A. J., Matsas, R. and Kenny, A. J. (1985) Biochem. Pharmacol. **34**, 1347–1356
47 Malfroy, B. and Schwartz, J. C. (1982) Biochem. Biophys. Res. Commun. **106**, 276–285
48 Bateman, R. C., Jackson, D., Slaughter, C. A., Unnithan, S., Chai, Y. G., Moomaw, C. and Hersh, L. B. (1989) J. Biol. Chem. **264**, 6151–6157
49 Beaumont, A., Le Moual, H., Boileau, G., Crine, P. and Roques, B. P. (1991) J. Biol. Chem. **266**, 214–220
50 Johnson, G. D., Stevenson, T. and Ahn, K. (1999) J. Biol. Chem. **274**, 4053–4058
51 Kew, D., Jin, D. F., Kim, F., Laddis, T. and Kilpatrick, D. L. (1989) Mol. Endocrinol. **3**, 1191–1196
52 Mehta, N. D., Don, J., Zinn, S. A., Millette, C., Wolgemuth, D. J. and Kilpatrick, D. L. (1994) Endocrinology **135**, 1543–1550
53 Kilpatrick, D. L., Borland, K. and Jin, D. F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5695–5699
54 Kilpatrick, D. L. and Millette, C. F. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5015–5018
55 Kew, D., Muffly, K. E. and Kilpatrick, D. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9143–9147
56 Howard, T. E., Shai, S. Y., Langford, K. G., Martin, B. M. and Bernstein, K. E. (1990) Mol. Cell. Biol. **10**, 4294–4302
57 Jeannotte, L., Burbach, J. P. and Drouin, J. (1987) Mol. Endocrinol. **1**, 749–757
58 Monsees, T. K., Gornig, M., Schill, W. B. and Miska, W. (1998) Andrologia **30**, 185–191

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