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Polarised expression of human intestinal *N*-benzoyl-L-tyroxyl-*p*-aminobenzoic acid hydrolase (human meprin) α and β subunits in Madin-Darby canine kidney cells

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N-Benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase (PPH, human meprin), is a peptidase found in the microvillus membrane of human small intestinal epithelial cells. PPH belongs to the astacin family of zinc-metalloendopeptidases and is a protein complex composed of two glycosylated subunits, α and β. The present report describes the cloning of the complete β subunit and the remaining N_2-terminal end of the α subunit for analysis of their primary structures in addition to the examination of their biogenesis in transfected cell cultures. The complete open reading frame of the PPHβ cDNA translates into 700 amino acid residues compared with 746 residues for the PPHα cDNA. The primary structure of β and α subunits are 44% identical and 61% similar. As predicted from their primary structure, the two subunits of PPH have identical modular structures; starting at the N_2-terminus both contain a signal peptide, a propeptide, a protease domain containing the astacin signature, a meprin A5 protein tyrosine phospatase μ (MAM) and a meprin and TRAF homology domain (MATH) domain, an epidermal growth factor(EGF)-like domain, a putative transmembrane anchor domain and a short cytosolic tail. Pulse/chase labelling and immuno-Gold electronmicroscopy of recombinant PPH β and α subunits expressed in transfected Madin-Darby canine kidney (MDCK) cells show that post-translational processing and transport of the two subunits are very different. When expressed alone, the β subunit acquired complex glycan residues, readily formed homodimers and was transported to the plasma membrane. Small amounts of PPHβ were found in the culture medium. In contrast, the cell-bound α subunit, when expressed alone, remained primarily in the high-mannose form, was aggregated and not expressed at the cell surface. However, the bulk of mostly endo-β-*N*-acetylglucosaminidase H-resistant α subunit was found in the filtered culture medium. The proteolytic event that leads to the formation of this soluble transport-competent form occurs in the endoplasmic reticulum (ER). Coexpression of the α subunit with the β subunit allowed the localisation of the α subunit to the plasma membrane. These studies indicate that assembly of the two subunits of PPH is required for the localisation of the α subunit to the plasma membrane. In contrast to rodent meprin, both PPH subunits are apically secreted from MDCK cells.

Keywords: human meprin; *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; astacin family; processing; intracellular transport.

N-Benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase (PPH) is a metalloendopeptidase isolated from the microvillus membrane of human enterocytes [1]. The enzyme was first identified using *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (Bz-Tyr-NBzOH), a substrate used to assess exocrine pancreatic function [2], but it is also capable of hydrolysing a great variety of biologically active peptides including bradykinin, angiotensins and substance P [1] Intestinal PPH comprises two subunits, α and β, with molecular masses of approximately 95 kDa and 105 kDa, respectively. Biogenesis of PPH involves dimerisation through the formation of disulphide bridges, as demonstrated by labelling studies in organ cultured human small intestinal explants [3].

Molecular cloning of the α subunit of PPH has lead to the definition of a new family of metalloendopeptidases, named after astacin, a digestive protease found in the freshwater crayfish *Astacus fluviatilis* [4]. In addition to PPH, members of the astacin family include human bone morphogenetic protein BMP1 [5], UVS.2 from *Xenopus laevis* embryos [6], tolloid from *Drosophila melanogaster* embryos [7], blastula protease 10 (BP10) and SPAN from sea urchin embryos [8, 9], LCE/HCE (low and high choriolytic enzyme) from *Oryzias latipes* (fish embryo) [10], a 1,25-dihydroxyvitamin D3-stimulated peptidase from *Coturnix japonica* (Japanese quail) [11], flavastacin [12] and meprin from rodents. Meprin, also termed endopeptidase 24.18

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Abbreviations. Bz-Tyr-NBzOH, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid; PPH, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; EGF, epidermal growth factor; ER, endoplasmic reticulum; MAM, meprin A5 protein tyrosine phosphatase μ; MATH, meprin and TRAF homology domain; TRAF, tumor-necrosis-factor(TNF)-receptor-associated factors; MEM, minimum essential medium; MHC, major histocompatibility complex; $PhMeSO_2F$, phenylmethyl-sulfonyl fluoride; endo H, endo-β-*N*-acetylglucosaminidase H.

Enzyme. *N*-Benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase (EC 3.4.24.18).

is expressed in microvillus membranes of epithelial cells in kidney proximal tubules and small intestine of mouse and rat [13–15], and has also been found to be excreted in a soluble form in mouse urine [16]. In contrast, PPH has so far only been identified in the human intestine [17], although a similar enzyme activity has recently been found in human kidney also [18].

Expression studies in COS-1 (Simian virus 40 transformed African green monkey kidney) and MDCK (Madin-Darby canine kidney) cells have shown that rat meprin α and a mouse meprin-PPHα chimera form disulphide linked homodimers [17, 19, 20] that are secreted in a complex glycosylated form. However, the cell-associated α subunit was an immature and transport-incompetent protein which did not reach the cell surface. Activation of both the soluble and cell-bound forms of the enzyme could be achieved by limited treatment with trypsin through the proteolytic removal of the propeptide [20].

Here we report the full-length cDNA sequence and predicted primary structure of the β subunit of PPH, together with the 5′ cDNA sequence and corresponding peptide sequence of the α subunit of PPH not previously reported. We further show that when expressed in MDCK cells alone, PPHβ matures and reaches the cell surface. Moreover, only when the two subunits are coexpressed in MDCK cells is PPHα localised to the surface of these cells. C-terminal proteolytic processing of PPHα necessary for its secretion is further shown to occur in the endoplasmic reticulum (ER).

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesised by Microsynth (Switzerland). The UniZAP XR Custom cDNA library, pSG5 mammalian expression vector, XL1-Blue *Escherichia coli* host and *Taq* DNA polymerase were purchased from Stratagene. Hybond C extra nitrocellulose membrane, [γ-^{32}P]dATP (3000 Ci/mmol), [^{35}S]dATP[αS] (1500 Ci/mmol) and L-[^{35}S]methionine/cysteine (1000 Ci/mmol) were from DuPont NEN. Sequenase version 2.0 DNA sequencing kit, M-MLV reverse transcriptase and T4 DNA ligase were from U.S.B. The rRNAsin RNAse inhibitor, dGTP, dATP, dTTP and dCTP were supplied by Promega. Zetaprobe nylon membrane and all SDS/PAGE chemicals were purchased from Bio-Rad Laboratories. Herring sperm DNA, MP agarose, endoglycosidase H were purchased from Boehringer Mannheim. The Nusieve GTG agarose was supplied by FMC Bioproducts. Ficoll 400 and protein-A-Sepharose CL-4B were purchased from Pharmacia. BSA, phenylmethylsulfonyl fluoride (PhMeSO$_2$F), pepstatin, aprotinin, leupeptin, benzamidine and high-molecular-mass protein standards were from Sigma. Polynucleotide kinase and restriction enzymes were from New England Biolabs. Guanidine isothiocyanate, cell culture media, penicillin and streptomycin were obtained from Gibco BRL. Geniticin was from Sigma. Foetal calf serum was from Biological Industries. All other chemicals were analytical grade from Merck.

Cloning of PPHβ cDNA. A 44-amino-acid sequence of detergent-solubilised mature PPHβ, from human intestinal microvillus membrane, was obtained by N_2-terminal sequencing [17] and from overlapping CNBr peptide sequences: NSIIGEKYRWPHTIPYVLEDSLEMNAKGVILNAFERYRLXTXID. The residues in positions 10 and 13 have been corrected from that previously published due to more consistent sequencing data of the N_2-terminus. Several degenerate oligonucleotide PCR primers (19–23 nucleotides) were designed from the codons of the peptide sequence above with the purpose of cloning the corresponding cDNA fragment for subsequent use in screening a cDNA library. First, antisense degenerate primers were used to perform reverse transcription of total RNA prepared from human small intestinal mucosa using a guanidinium thiocyanate method [21]. The resulting single-stranded cDNA was then amplified by PCR using the antisense and sense degenerate primer pair. An expected single PCR product (≈90 bp) was resolved by Nusieve agarose-gel electrophoresis and subsequently cloned into the pGEM-T vector (Promega), amplified in XL1-Blue *E. coli* hosts (Stratagene), isolated over a Qiagen column and sequenced by the Sanger dideoxynucleotide termination method (Sequenase version 2.0) on both strands with M13 universal and reverse primers. The 90-bp PCR product was found to encode a 22 amino acid stretch of the mature N_2-terminal sequence of PPHβ above. Two PPHβ-specific oligonucleotide probes (27 nucleotides) were designed from different regions within this stretch and used as two independent probes for screening the cDNA library and also for cloning the 5′ cDNA end of PPHβ. Approximately 900000 plaques from an human intestinal cDNA library constructed from an eight month jejunum using the lambda ZAP II vector (Stratagene) were amplified in XL1-Blue *E. coli* and screened using standard protocols (Stratagene) [22]. Plaques were lifted onto duplicate nylon membranes and hybridised, respectively, to the two PPHβ-specific oligonucleotide probes that were 5′-end ^{32}P-labelled with polynucleotide kinase. Hybridisations were carried out at 50°C for approximately 16 h in 6× NaCl/Cit (20×Stock solution: 3.0 M NaCl, 0.3 M sodium citrate), 2×Denhardts [100×stock solution: 2% (mass/vol.) bovine serum albumin, 2% (mass/vol.) Ficoll, 2% (mass/vol.) poly(vinyl pyrollidone)], SSPE (20×stock solution: 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 0.02 M Na_2EDTA), SDS 0.05% (mass/vol.), and denatured, fragmented herring sperm DNA 100 μg/ml. Washings were in 6×NaCl/Cit, 0.1% SDS (mass/vol.) at 50°C for 30 min, twice, then in 2×NaCl/Cit, 0.5% SDS (mass/vol.) at 50°C for 10 min. The membranes were then exposed to X-ray film (Fuji) for approximately 16 h at −80°C. Plaques were picked and after two rescreenings, only one isolated clone was found to consistently hybridise both probes. The 2139-bp insert was recovered within the pBluescript SK(−) phagemid by *in vivo* excision (Stratagene), named pPPHβ-3b, and further amplified for analysis. To sequence both strands of pPPHβ-3b, nested deletion mutants were prepared from both ends of the cDNA using the Erase-a-Base System (Promega), the mutants were then amplified in Novablue (Novagen) hosts, isolated and sequenced as previously described with M13 universal and reverse primers. Overlapping sequences of both the sense strand (11 clones) and the antisense strand (9 clones) were obtained for the complete pPPHβ-3b cDNA (Fig. 1) using this method. The PPHβ-3b clone was found to be lacking an in-frame start codon and hence lacked the extreme 5′ end of the complete PPHβ cDNA, as was also the case with the α subunit of PPH (PPHα-22.4) previously characterised [17]. Therefore, anchored PCR was performed to obtain the remaining 5′ cDNA ends of both PPHβ and PPHα as described below.

Anchored PCR. Total RNA was prepared from human small intestinal mucosa with a guanidinium thiocyanate method [21] followed by the isolation of polyadenylated mRNA, from 35 μg total RNA, using a Dynabeads Oligo $(dT)_{25}$ kit (Dynal). The preparation of mRNA was then used in the 5′ RACE System (Gibco BRL) to clone the 5′ cDNA ends of both PPHβ and PPHα. Briefly, the mRNA was split into two aliquots, denatured at 70°C for 10 min to reduce secondary structures and the unknown 5′ mRNA ends were reverse transcribed with gene-specific antisense primers for PPHβ and PPHα cDNAs, respectively. The resulting single-stranded cDNAs were then isolated apart from the primers and tailed with dCTP. The tailed cDNAs were then amplified by PCR using nested gene-specific antisense primers and the sense 5′ RACE Anchor Primers (Gibco BRL).

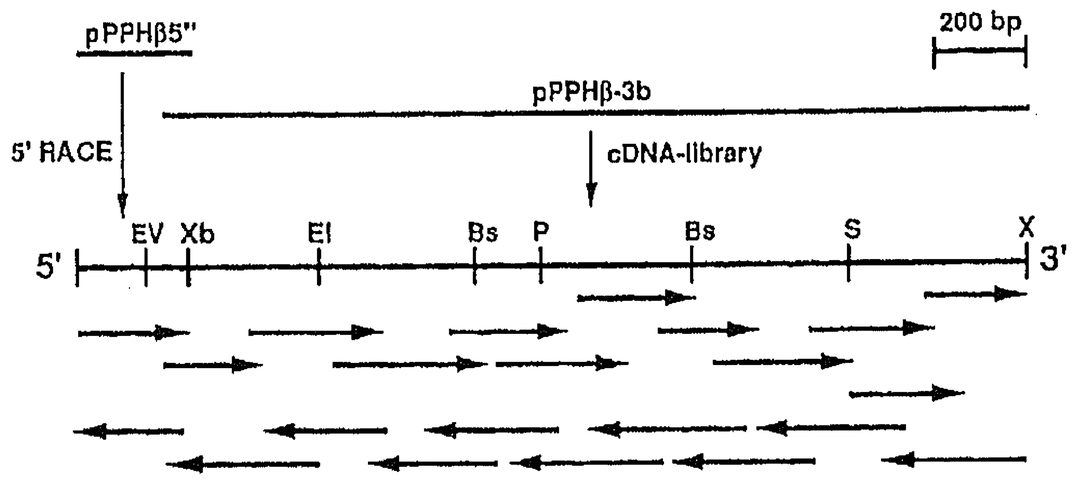

Fig. 1. Cloning and sequencing strategy of the complete cDNA for the *β* subunit of human PPH. Two overlapping clones were obtained encoding the complete cDNA of 2326 bp. The 5′ cDNA end, PPH*β*5′, obtained by reverse transcription and anchored PCR of human small intestinal mRNA, was cloned into the pGEM-T vector and sequenced on both strands with M13 universal and reverse primers. The major portion of the PPH*β* cDNA including the poly(A) tail, PPH*β*-3b, was obtained from a human intestinal cDNA library and subcloned into pBluescript vector. Nested deletion mutants were prepared from each end of PPH*β*-3b for sequencing. Arrows indicate the approximate position and direction of each sequence obtained from the deletion mutants. Restriction enzyme sites shown were both predicted from the cDNA sequence and experimentally verified: *Eco*RV (EV), *Xba*I (Xb), *Eco*RI (EI), *Bst*XI (Bs), *Pst*I (P), *Sac*I (S), *Xho*I (X).

The PCR products for the PPH*β* (≈ 325 bp) and PPH*α* (≈ 175 bp) 5′ cDNA ends were then ligated directly into the pGEM-T vector and amplified in Novablue. Two clones from each ligation reaction were isolated and sequenced on both strands using M13 universal and reverse primers. The sequence of the PPH*β* 5′ cDNA end overlapped with the PPH*β*-3b cDNA sequence by 50 nucleotides. The sequencing strategy of the complete PPH*β* cDNA, is shown in Fig. 1.

The previously unknown sequence of the PPH*α* 5′ cDNA end was obtained using the same strategy as above.

Construction of expression plasmids. The assembled PPH*β* expression plasmid is shown in Fig. 2A. First, PCR primers were designed from the predicted start codon region of PPH*β* in order to obtain the 5′ cDNA end apart from the dGTP-rich anchor region. The sequence of the sense primers (26 nucleotides) was specific for a site 16 nucleotides upstream from the predicted start codon and contained an *Eco*RI site. The sequence of the antisense primers (29 nucleotides) was specific for a region located 260 nucleotides downstream from the predicted start codon and contained a gene-specific *Xba*I site. Using the pGEM-T plasmid containing the 5′ cDNA end of PPH*β* as a template, PCR was performed and the expected product (≈ 275 bp) was isolated from an agarose gel, digested with the respective restriction enzymes, followed by an additional gel isolation step. Secondly, an approximately 2050-bp *Xba*I−*Bam*HI insert [containing the major portion of the PPH*β* cDNA from the *Xba*I site downstream to and including the poly(A) tail] was isolated from pPPH*β*-3b by restriction digest and agarose-gel isolation. Finally, these two PPH*β* cDNA fragments, together comprising the complete reading frame of the PPH*β* cDNA, were then ligated into the *Eco*RI and *Bg*III (*Bam*HI compatible) sites of the mammalian expression vector, pSG5 (Stratagene) thus creating the expression plasmid, pPPH*β*.

The expression plasmid, pPPH*α*, was created by a similar strategy and is shown in Fig. 2B. First, PCR primers were designed from the 5′ cDNA region of PPH*α*. The sequence of the sense primer (21 nucleotides) was specific for the start-codon region of PPH*α* and contained a *Bam*HI site just upstream of the start codon, whereas, the sequence of the antisense primers (20 nucleotides) was specific for a region 160 nucleotides downstream from the predicted start-codon that contained a *Pst*I site.

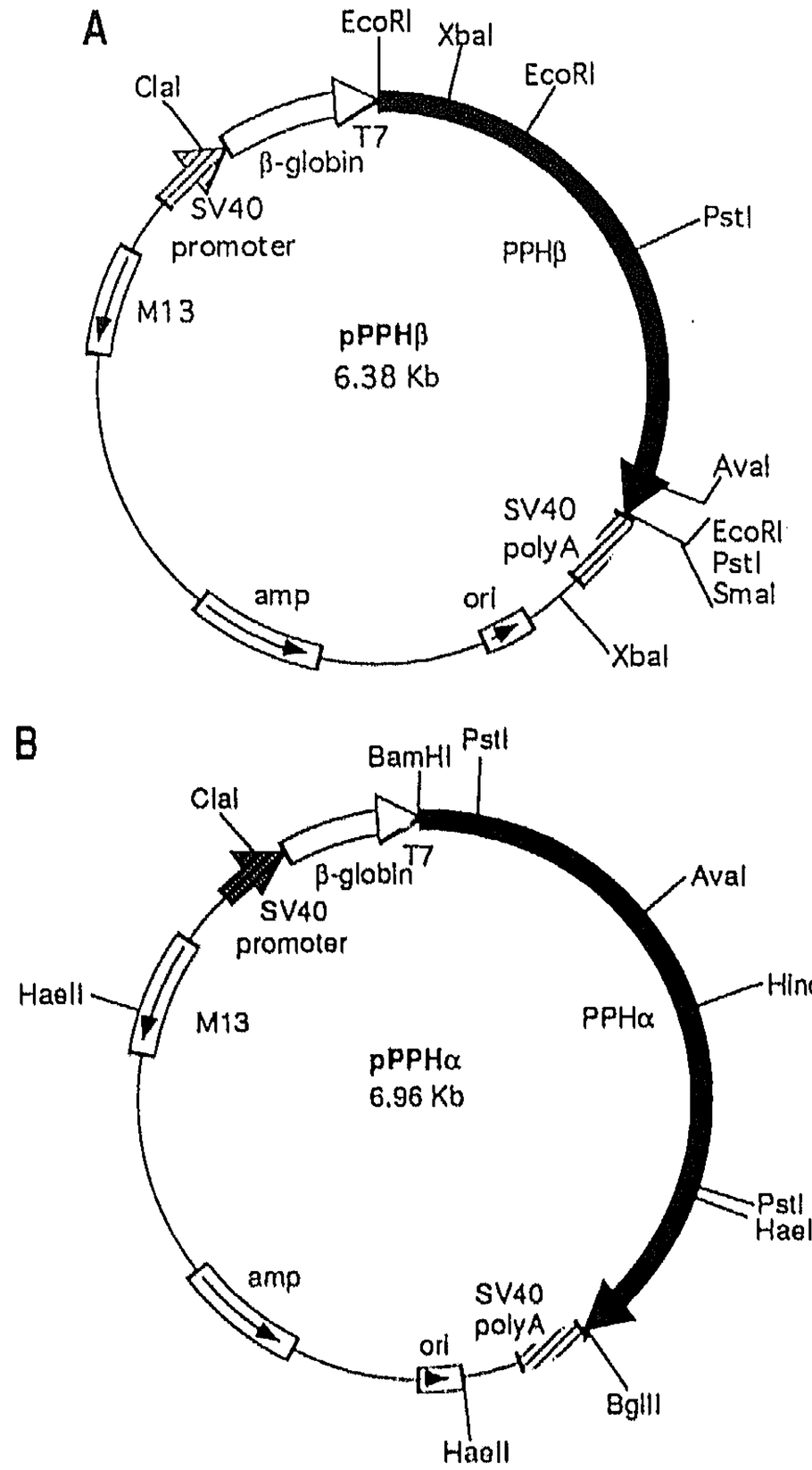

Fig. 2. Construction of expression plasmids, pPPH*β* and pPPH*α*. (A) The expression plasmid, pPPH*β*, was constructed by ligating an *Eco*RI−*Xba*I PCR fragment (275 bp), from pPPH*β*5′, and a *Xba*I−*Bam*HI fragment (2050 bp), from pPPH*β*-3b, into the *Eco*RI−*Bg*III sites of the expression vector, pSG5. The resulting plasmid, pPPH*β*, contained the complete open reading frame of the PPH*β* subunit cDNA. (B) The expression plasmid, pPPH*α*, was constructed by ligating a *Bam*HI−*Pst*I fragment (175 bp) from pPPH*α*5′ and a *Pst*I−*Hin*dIII fragment (1205 bp) from pPPH*α*-22.4 into the *Bam*HI/*Hin*dIII sites of pSGMP*α* which contained the remaining 3′ cDNA portion of the PPH*α* cDNA (1440 bp) and the expression vector, pSG5. The resulting expression plasmid, pPPH*α*, contained the complete open reading frame of the PPH*α* subunit cDNA.

Using the newly cloned PPH*α* 5′ cDNA end as a template, PCR was performed and the resulting PCR product (≈175 bp) was isolated and digested with the respective restriction enzymes. Secondly, a *Pst*I−*Hin*dIII PPH*α* cDNA fragment (1200 bp) was obtained from pPPH*α*-22.4 [17]. Finally, a *Bam*HI−*Hin*dIII fragment (1600 bp) containing the meprin portion of the PPH-meprin*α* expression plasmid, pSGMP*α* [20], was removed and replaced by the two PPH*α* cDNA fragments isolated above. The resulting expression plasmid, pPPH*α*, contained the complete open reading frame of the PPH*α* cDNA in the expression vector, pSG5. Both expression plasmids, pPPH*β* and pPPH*α*, were sequenced to confirm the correct sequence of an in-frame 5′ cDNA end.

Computer analysis of DNA and protein sequences. Nucleotide sequences were read with the USEDIT program and Reader (C.B.S. Scientific Company) and initial comparisons made using the DNAid program (F. Dardel and P. Bensoussan,

Ecole Polytechnique, Palaiseau cedex, France). For computer-assisted analysis of DNA and protein sequences, programs in the GCG (Genetics Computer Group, Madison, Wisconsin) software package were used.

Cell culture. MDCK cells were a generous gift from Dr Kai Simons (EMBL, Heidelberg). Wild-type MDCK cells were grown as monolayers in minimum essential medium (MEM) Earles Hepes medium supplemented with 5% (mass/vol.) foetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air. MDCK cell clones were grown in the same medium except with geneticin (400 μg/ml, Sigma) instead of the penicillin/streptomycin mixture. Medium was changed every 2–3 days and cells passaged when confluent unless otherwise stated.

Transfection of MDCK cells. Subconfluent MDCK cells of a low passage number (under 25) were used for $CaPO_4$-DNA transfection with PPHα and PPHβ expression vectors, separately or together. Methods are as described by Grünberg et al. [20]. Briefly, 1×10^6 cells were plated onto a 10-cm dish and incubated overnight followed by trypsinisation and resuspension in 2 ml medium supplemented with 10% (by vol.) foetal calf serum in preparation of each DNA transfection reaction. A $CaPO_4$-DNA precipitate was prepared containing 2 μg of the neomycin-selectable marker plasmid pXT1 (Stratagene), together with 20 μg of the experimental plasmids, pPPHα and pPPHβ, separate or in combination. The cell suspension and $CaPO_4$-DNA precipitate were gently mixed and allowed to stand at room temperature for 20 min. 10 ml medium [10% (by vol.) foetal calf serum] was then added and the cells incubated for 16 h at 37°C. The transfection medium was removed and the cells shocked with 15% (mass/vol.) glycerol in NaCl/Hepes (50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.1) for 1 min. The cells were rinsed several times and incubated in medium supplemented with 10% (by vol.) foetal calf serum and penicillin/streptomycin mixture overnight. The cells were split 1:10 and selection started with medium [with 5% (by vol.) foetal calf serum] containing Geneticin (400 μg/ml). After 2 weeks approximately 20 single colonies/transfection reaction were isolated using cloning rings for propagation. Each cloned cell line was screened for PPH subunit expression by metabolic labelling with [^{35}S]methionine and immunoprecipitation with PPHα-specific antisera [17] and PPHβ-specific mAb [23]. The immunoprecipitates were analysed by SDS/PAGE and fluorography (data not shown). Three expressed PPHα alone, two clones expressed PPHβ alone, and one clone was found to express PPHα and PPHβ. The cell clones with the highest expression rates, PPHα-4, PPHβ-11 and PPH$\alpha\beta$-12 were chosen for subsequent experimentation and not used beyond 30 passages.

Metabolic labelling of transfected MDCK cells. The transfected cells were plated 1:4 onto plastic culture dishes and treated the next day with sodium butyrate (8 mM) for 16 h. The normal culture medium was replaced with methionine-free medium containing 5% (by vol.) dialyzed foetal calf serum for 1 h. The cells were then labelled with 50 μCi L-[^{35}S]methionine/cysteine for 15 min or overnight. The cells labelled only 15 min were either harvested immediately after labelling (0 min chase) or incubated in chase medium (methionine-free medium supplemented with 10 mM methionine) for different time periods before harvesting. The cells were harvested for immunoprecipitation, endo-β-*N*-acetylglucosaminidase H (endo H) treatment, and analysis by SDS/PAGE and fluorography. The media were collected and filtered (0.2 μm) to remove any dead cells.

Antibodies. All cell and medium samples were subjected to immunoprecipitation with one of two antibodies; a mouse monoclonal antibody (mAb), HBB 3/716/36 [23], which recognises only the native PPHβ subunit, or with a rabbit antisera (antiPPHα) raised against denatured PPHα subunit [17], and which recognises denatured PPHβ subunit to a lesser extent. Antibody specificity was previously established by immunoprecipitation of labelled native and denatured proteins from organ culture of human small intestinal mucosa and also from cells transfected with pPPHβ or pPPHα (unpublished results). In immuno-gold electron microscopy two polyclonal antibodies, A/1 (specific for the α subunit) and LDi/1 (recognising the α and β subunits), were used.

Immunoprecipitation. Samples were harvested for immunoprecipitation as previously described [24] with minor modifications. At the times indicated above, labelled cells were washed three times with ice-cold NaCl/P_i (138 mM, pH 7.3) and scraped with a rubber policeman into 1 ml ice-cold NaCl/P_i. The pelleted cells were solubilised in 300 μl buffer A (Tris/HCl 25 mM, NaCl 50 mM, pH 8.0) containing 1% (by vol.) Nonidet P-40, 1% (mass/vol.) sodium deoxycholate and protease inhibitors (1 mM $PhMeSO_2F$, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 3.5 μg/ml benzamidine, 2 μg/ml aprotinin) and incubated 30 min on ice followed by microcentrifugation (15000 *g*, 5 min at 4°C) to remove nuclei and dense cellular debris. Only protein samples to be immunoprecipitated with the anti-PPHα rabbit sera were first denatured by boiling 5 min with SDS 0.5% (mass/vol.) before continuing. The cell lysates and collected media were then diluted in 3 volumes of homogenization buffer with 1% (mass/vol.) Triton X-100 and precleared of non-specific binding proteins with 50 μl protein A insoluble Sepharose suspension (Staph A cell suspension) (Gibco) for a minimum of 30 min at 4°C followed by microcentrifugation (15000 *g*) for 1 min to remove the Staph A cell suspension. The rabbit antisera (20 μl) were coupled to Sepharose protein-A beads (50 μl) in 1 ml of 0.1 M sodium phosphate, pH 8.2, for a minimum of 2 h at 4°C and washed twice in the same buffer before being added to the appropriate samples for immunoprecipitation for 16 h at 4°C. Immunoprecipitates were then washed in NaCl/P_i with non-ionic detergents as previously described [24] before endo-β-*N*-acetylglucosaminidase H (endo H) treatment [25] and SDS/PAGE.

Cell surface immunoprecipitation. Transfected MDCK cells were metabolically labelled 16 h then washed in NaCl/P_i 37°C and incubated for 30 min at 37°C in serum-free medium. After washing the cells four times in ice-cold NaCl/P_i, 20 μl anti-PPHβ mAb in 1 ml NaCl/P_i was added and incubated 30 min at 4°C with gentle shaking. The cells were washed and 400 μg of unlabelled transfected cell protein in 1 ml NaCl/P_i was added to saturate the remaining free binding sites of the antibody. After 15 min at 4°C the cells were washed again, harvested and lysed as above. After centrifugation to remove cell debris, the antigen-antibody complex was adsorbed to protein-A beads and analyzed by SDS/PAGE.

Carbonate extraction of microsomal proteins. Carbonate extractions of microsomal proteins was essentially as described by Fujiki et al.[26]. Briefly, membranes were prepared by homogenisation in 0.1 M Na_2CO_3, pH 11, low-speed centrifugation to remove large cellular components, followed by ultracentrifugation at 15000 *g* for 1 h. The pellet was resuspended in carbonate buffer, left on ice for 30 min and ultracentrifuged again. Pellet and supernatant were analysed for labelled PPH protein by immunoprecipitation, SDS/PAGE and fluorography.

SDS/PAGE. Immunoprecipitates were dissolved in 40 μl 2× sample buffer [0.16 M Tris/HCl, pH 6.8, 4% (mass/vol.) SDS, 20% (by vol.) glycerol, 0.002% (mass/vol.) bromophenol blue] and boiled 5 min in the absence or presence of dithiothreitol (1 mM) which reduces disulphide bonds. Proteins were resolved by SDS/PAGE [7.5% (mass/vol.) polyacrylamide] according to Laemmli. The M_r standards used were myosin, 205000; β-galac-

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                                                                1 CAACCCTGAATGTCATAGTTAGCTACTTTCAACTGGAAGCTACAAC      46

47  ATGGATTTATGGAATCTGTCTTGGTTTCTGTTCTTGGATGCTCTTCTCGTGATTTCTGGCTTGGCAACTCCAGAAAACTTTGATGTAGAT     136
 1  M  D  L  W  N  L  S  W  F  L  F  L  D  A  L  L  V  I  S  G  L  A  T  P  E  N  F  D  V  D      30
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137  GGCGGAATGGACCAGGACATATTTGATATCAATGAAGGTTTGGGACTGGATCTTTTTGAGGGTGACATCAGACTTGATAGGGCACAAATT     226
 31  G  G  M  D  Q  D  I  F  D  I  N  E  G  L  G  L  D  L  F  E  G  D  I  R  L  D  R  A  Q  I      60

227  AGAAATTCCATCATTGGAGAAAAGTATAGATGGCCTCATACCATTCCATATGTTCTAGAAGATAGCTTGGAAATGAATGCTAAGGGAGTT     316
 61  R [N...S...I...I...G...E...K...X...R...W...P...H...T...I...P...Y...V...L...E...D...S...I...E...M...N...A...K...G...V      90
     °

317  ATCCTCAATGCATTTGAACGTTATCGCCTTAAAACATGTATTGACTTTAAGCCTTGGGCTGGAGAAACAAACTATATATCAGTGTTCAAG     406
 91  I...L...N...A...F...E...R...Y...R...L...K...T...C...I...D  F  K  P  W  A  G  E  T  N  Y  I  S  V  F  K     120

407  GGCAGTGGCTGCTGGTCTTCAGTAGGAAATAGGCGGGTTGGGAAGCAAGAACTTTCCATCGGGGCAAACTGTGACCGAATAGCAACAGTT     496
121  G  S  G  C  W  S  S  V  G  N  R  R  V  G  K  Q  E  L  S  I  G  A  N  C  D  R  I  A  T  V     150

497  CAACACGAGTTCCTCCACGCTCTGGGATTCTGGCATGAGCAGTCGCGTTCTGACCGGGATGACTATGTCAGGATAATGTGGGACAGAATT     586
151  Q  H  E  F  L  H  A  L  G  F  W  H  E  Q  S  R  S  D  R  D  D  Y  V  R  I  M  W  D  R  I     180
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587  CTGTCAGGCAGAGAGCACAATTTTAACACCTATAGTGACGATATATCAGATTCCCTGAATGTTCCCTATGATTACACTTCAGTAATGCAC     676
181  L  S  G  R  E  H  N  F  N  T  Y  S  D  D  I  S  D  S  L  N  V  P  Y  D  Y  T  S  V  M  H     210

677  TACAGTAAAACTGCATTCCAAAATGGAACAGAGCCGACAATTGTCACAAGAATCTCAGACTTTGAGGATGTGATCGGCCAACGAATGGAT     766
211  Y  S  K  T  A  F  Q  N  G  T  E  P  T  I  V  T  R  I  S  D  F  E  D  V  I  G  Q  R  M  D     240
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767  TTCAGTGACTCTGATCTCCTAAAGTTGAATCAACTGTATAACTGCTCCTCTTCCTTGAGTTTTATGGACTCATGCAGTTTTGAACTGGAA     856
241  F  S  D  S  D  L  L  K  L  N  Q  L  Y  N  C  S  S  S  L] S  F  M  D  S  C  S  F  E  L  E     270
                                  ********

857  AATGTGTGTGGCATGATCCAAAGTTCAGGAGATAATGCTGACTGGCAACGGGTTTCACAGGTTCCCAGGGGGCCAGAGAGTGATCACTCC     946
271  N  V  C  G  M  I  Q  S  S  G  D  N  A  D  W  Q  R  V  S  Q  V  P  R  G  P  E  S  D  H  S     300

947  AACATGGGCCAGTGCCAAGGTTCTGGTTTCTTCATGCATTTCGATAGCAGCTCTGTAAATGTGGGGGCCACAGCAGTGCTGGAAAGTAGA    1036
301  N  M  G  Q  C  Q  G  S  G  F  F  M  H  F  D  S  S  S  V  N  V  G  A  T  A  V  L  E  S  R     330

1037  ACGCTGTACCCTAAAAGAGGATTTCAGTGCCTGCAATTTTACTTATATAACAGTGGCAGTGAAAGTGATCAACTGAACATCTATATCAGG    1126
 331  T  L  Y  P  K  R  G  F  Q  C  L  Q  F  Y  L  Y  N  S  G  S  E  S  D  Q  L  N  I  Y  I  R     360

1127  GAGTATTCTGCAGACAATGTGGATGGCAATTTAACCCTTGTGGAAGAAATAAAAGAAATACCCACTGGGAGCTGGCAACTTTATCATGTA    1216
 361  E  Y  S  A  D  N  V  D  G  N  L  T  L  V  E  E  I  K  E  I  P  T  G  S  W  Q  L  Y  H  V     390
                                  ********

1217  ACATTGAAAGTGACCAAGAAGTTTAGAGTGGTGTTTGAAGGACGCAAAGGCTCTGGTGCATCACTGGGTGGTCTGTCTATTGATGACATC    1306
 391  T  L  K  V  T  K  K  F  R  V  V  F  E  G  R  K  G  S  G  A  S  L  G  G  L  S  I  D  D  I     420

1307  AATCTTTCGGAAACACGGTGCCCTCATCATATCTGGCATATAAGGAATTTCACACAGTTCATTGGCAGCCCAAATGGAACTCTGTATAGC    1396
 421  N  L  S  E  T  R  C  P  H  H  I  W  H  I  R  N  F  T  Q  F  I  G  S  P  N  G  T  L  Y  S     450
      ********                                 ********                    ********

1397  CCTCCATTTTACTCTTCTAAAGGTTATGCCTTTCAGATTTACTTAAATCTAGCCCATGTGACTAATGCAGGGATATATTTCCACTTGATC    1486
 451  P  P  F  Y  S  S  K  G  Y  A  F  Q  I  Y  L  N  L  A  H  V  T  N  A  G  I  Y  F  H  L  I     480

1487  TCTGGAGCCAATGATGATCAATTACAGTGGCCATGTCCTTGGCAACAAGCCACAATGACACTTTTGGATCAAAATCCTGACATTCGACAG    1576
 481  S  G  A  N  D  D  Q  L  Q  W  P  C  P  W  Q  Q  A  T  M  T  L  L  D  Q  N  P  D  I  R  Q     510

1577  CGTATGTCCAATCAGCGGAGTATAACTACAGACCCATTTATGACCACCGATAATGGAAACTATTTCTGGGACAGGCCTTCTAAAGTGGGA    1666
 511  R  M  S  N  Q  R  S  I  T  T  D  P  F  M  T  T  D  N  G  N  Y  F  W  D  R  P  S  K  V  G     540

1667  ACAGTGGCTTTGTTCTCTAATGGAACTCAGTTTAGAAGAGGTGGGGGCTATGGAACCAGTGCCTTTATAACCCACGAAAGGCTGAAAAGC    1756
 541  T  V  A  L  F  S  N  G  T  Q  F  R  R  G  G  G  Y  G  T  S  A  F  I  T  H  E  R  L  K  S     570
                ********

1757  AGAGATTTTATAAAAGGAGATGATGTTTATATCCTACTGACAGTGGAAGACATATCTCACCTCAACTCTACACAAATCCAGCTAACACCA    1846
 571  R  D  F  I  K  G  D  D  V  Y  I  L  L  T  V  E  D  I  S  H  L  N  S  T  Q  I  Q  L  T  P     600
                                                                ********

1847  GCCCCTAGTGTTCAAGACCTCTGCTCAAAAACCACCTGTAAAAATGACGGTGTCTGCACTGTTCGAGATGGCAAAGCTGAGTGCAGGTGC    1936
 601  A  P  S  V  Q  D  L  C  S  K  T  T  C  K  N  D  G  V  C  T  V  R  D  G  K  A  E  C  R  C     630
                          ═══════════════════════════════════════════════

1937  CAGTCAGGGGAAGACTGGTGGTACATGGGAGAAAGGTGTGAAAAGAGAGGCTCCACCCGAGACACCATAGTCATTGCTGTTTCATCTACT    2026
 631  Q  S  G  E  D  W  W  Y  M  G  E  R  C  E  K  R  G  S  T  R  D  T  I  V  I  A  V  S  S  T     660
      ═══════════════════════════════════════

2027  GTTGCTGTGTTTGCCTTGATGCTGATCATCACCCTTGTCAGTGTCTATTGCACCAGGAAGAAATATCGTGAAAGGATGAGCTCAAATCGA    2116
 661  V  A  V  F  A  L  M  L  I  I  T  L  V  S  V  Y  C  T  R  K  K  Y  R  E  R  M  S  S  N  R     690

2117  CCAAATTTGACTCTGCAAAATCATGCTTTTtgaagattaactcgacaatatggccagctaatgaaattaaaaaggattcttcatcatggа    2206
 691  P  N  L  T  L  Q  N  H  A  F                                                                  700
      ********

2207  tttcgcctaagtgatattacagccacctcattcttctaaaagtggatatttttctgtaaatagctggaaatattataaatcactattttg    2296
2297  gtcaaagtcaaaaaaaaaaaaaaaaaaaaa    2326
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Fig. 3. cDNA and deduced amino acid sequence of the *β* subunit of human intestinal PPH. The 2326 bp PPH/*β* cDNA contains an open reading frame of 700 amino acids beginning from an AUG start-codon and ending at a TGA stop codon. The dotted underline indicates the amino acid sequence obtained by sequencing of the N_2-terminus and of an overlapping CNBr-peptide fragment of immunoprecipitated detergent-solubilised PPH. The start of the N_2-terminal sequence (Asn62) of mature PPH/*β* is indicated by an open circle. In brackets is the astacin domain containing the zinc-binding site (HEXXH) and astacin signature which is double underlined. The single underline indicates the EGF-like domain. Conserved cysteines in the astacin and EGF-like domains are in bold type. Potential N-glycosylation sites are underlined with stars. The putative membrane anchor domain is italicised.

tosidase, 116000; phosphorylase *b*, 97400; albumin, 66000; ovalbumin, 45000; carbonic anhydrase, 29000. Gels were stained with Coomassie blue R-250 (Bio-Rad) in 10% (by vol.) acetic acid and 40% (by vol.) isopropanol then destained. For fluorography, gels were rinsed 5 min in distilled H_2O, incubated in 1 M sodium salicylate 20 min, rinsed again and vacuum dried at 80°C before exposure to X-ray film (Fuji) at −80°C and fluorography. Exposure times of the fluorographs were varied with each experiment to maximise visualisation of the protein bands.

Immunocytochemistry. For the immunolocalisation of PPH in stable transfected MDCK cells, cells were fixed 5 days after confluence in 1% (by vol.) formaldehyde (freshly prepared from para-formaldehyde) in 0.1 M sodium phosphate, pH 7.4, for 1 h at room temperature. After fixation cells were washed and gently scraped with a spatula with a rubber tip, pelleted in 10% (mass/vol.) gelatin, post-fixed and stored for at least 24 h at 4°C in 1% (by vol.) formaldehyde in 0.1 M sodium phosphate buffer. Ultrathin cryosections were cut on a Leica cryoultramicrotome and incubated with specific polyclonal antibodies A/1 and LDi/

1 against PPHα and PPHβ subunits, respectively, and with protein A complexed to colloidal gold particles [27]. Sections were observed in a JEOL JEM1010 electron microscope operating at 80 kV.

RESULTS

PPHα 5′ cDNA cloning. The 5′ end of PPHα cDNA contained an open reading frame starting with the consensus AUG start-codon and overlapped by 60 nucleotides with the near-full-length PPHα cDNA sequence previously obtained [17]. Thus, the complete open reading frame of the PPHα cDNA of 2920 bp encodes 746 amino acid residues (EMBL sequence database, accession no. M82962). The complete cDNA-deduced amino acid sequence of PPHα is shown in Fig. 4 in alignment with the PPHβ subunit. It contains a putative signal peptide and propeptide as predicted by sequence alignment with sequences of chimeric mouse-PPHα [20], mouse meprinα [28] and rat meprinα [29].

PPHβ cDNA cloning. The complete cDNA sequence and deduced amino acid sequence for the β subunit of PPH is shown (Fig. 3). The open reading frame (nucleotides 47–2149) starts with the consensus sequence RXXAUGG for the initiation of translation in vertebrates [30] and ends with a TGA stop codon. The primary structure of PPHβ contains 700 residues. The 44-amino-acid sequence of the N_2-terminus and overlapping CNBr fragment of the mature β subunit isolated from human intestine is present in the deduced sequence (Fig. 3) thus, confirming the identity of this cDNA. Hydropathy analysis [31] of PPHβ revealed a hydrophobic region (Leu6–Leu21) containing several potential signal-peptidase-cleavage sites [32]. The N_2-terminal sequence (LATPENE...) of one such putative propeptide of PPHβ aligns with that empirically determined for mouse meprinβ [28]. Another hydrophobic stretch consisting of 25 residues (Thr652–Tyr676) exists near the C-terminus and constitutes a potential membrane anchor domain similar to that found in the predicted sequence of the α subunit [17]. These findings are also consistent with observations in the intestinal mucosa that PPH is an integral microvillar membrane protein with an extracellular orientation of its catalytic site [1].

The proteolytic astacin domain of the β subunit spans 198 residues starting from the N_2-terminus of the mature intestinal subunit (Asn62) and ending with Leu259. This astacin domain contains the extended zinc-binding motif, HEXXHXXGFXHE, which is found within the extended astacin signature sequence, HEXXHXXGFXHE(Q,H)XRXDRDX(Y,H)(V,I)X(I,V) specific for zinc-metallo-endopeptidases of the astacin family [4, 33]. Also within the astacin region are the four conserved cysteine residues (Cys103, Cys124, Cys144, Cys255) which in astacin have been shown to form two disulphide bonds important in protein folding [34, 35].

Following the astacin domain in PPHβ are several potentially important functional domains in protein adhesion, MAM, MATH, and an epidermal-growth-factor (EGF)-like domain, that also exist in PPHα. The MAM domain spans a 170 amino-acid-segment (Ser260–His429) and is named after the functionally diverse proteins meprin, A5, protein tyrosine phophatase μ which contain the extensive consensus sequence [36]. A sequence of 75 amino acid residues following the MAM domain has approximately 30% identity to tumor-necrosis-factor(TNF)-receptor-associated factors (TRAF) and has been named meprin- and TRAF-homology (MATH) domain [37]. After an intervening segment of approximately 100 amino acids and abutting the putative membrane anchor is an EGF-like domain with six conserved cysteines (Cys608–Cys643). Extending beyond the hydrophobic region is then the C-terminus composed of 24 mostly polar residues (Cys677–Phe700) constituting the putative cytosolic domain.

```
  1 .....MDLWNLSWFLFLDALLVISGLATPENFDVDGGMDQDIFDINEGL 44
          :: :.:::   | :.|. |:..:  |.| | :.|| :|| :
  1 MAWIRSTCILFFTLLFAHIAAVPIKHLPEENVHDADFGEQKDISEINLAA 50

 45 GLDLFEGDIRLDRAQIRNSIIGEKYRWPHTIPYVLEDSLEMNAKGVILNA 94
    |||||:||| |::.  ||:: :.. ||. .|||:|.|.|::||||.|| |
 51 GLDLFQGDILLQKS..RNGLRDPNTRWTFPIPYILADNLGLNAKGAILYA 98

 95 FERYRLKTCIDFKPWAGETNYISVFKGSGCWSSVGNRRVGKQELSIGANC 144
    ||.:|||.|:||||:.||..|| . . .||||.||:.:|| |::|||..|
 99 FEMFRLKSCVDFKPYEGESSYIIFQQFDGCWSEVGDQHVG.QNISIGQGC 147

145 DRIATVQHEFLHALGFWHEQSRSDRDDYVRIMWDRILSGREHNFNTYSDD 194
    .  |.::||:||||||:|||||.||||||.| ||.|||| :|||:||.|.
148 AYKAIIEHEILHALGFYHEQSRTDRDDYVNIWWDQILSGYQHNFDTYDDS 197

195 ISDSLNVPYDYTSVMHYSKTAF.QNGTEPTIVTRISDFEDVIGQRMDFSD 243
    : ..||.||||.|:||| . .| .|:. |||..:|.:|:.:||||:|||.
198 LITDLNTPYDYESLMHYQPFSFNKNASVPTITAKIPEFNSIIGQRLDFSA 247

244 SDLLKLNQLYNCSSSLSFMDSCSFELENVCGMIQSSGDNADWQRVSQVPR 293
    || :||.:|||... .::| |.|| .|:|||||:. |:.||.:  : :.
248 IDLERLNRMYNCTTTHTLLDHCTFEKANICGMIQGTRDDTDWAH.QDSAQ 296

294 GPESDHSNMGQCQGSGFFMHFDSSSVNVGATAVLESRTLYPKRGFQCLQF 343
    :.| ||. :||| |.|:||:|..||...:..|:|||||.|||||  |||||
297 AGEVDHTLLGQCTGAGYFMQFSTSSGSAEEAALLESRILYPKRKQQCLQF 346

344 YLYNSGSESDQLNIYIREYSADNVDGNLTLVEEIKEIPTGSWQLYHVTLK 393
    :.  .||.||.| :::|  ....   .|. |:.:.: .. .|.: ||.||
347 FYKMTGSPSDRLVVWVRRDDSTGNVRKLVKVQTFQGDDDHNWKIAHVVLK 396

394 VTKKFRVVFEGRKG.SGASLGGLSIDDINLSETRCPHHIWHIRNFTQFIG 442
    ..||| :|:| || ...| ||: :|||.|.||.||  :| :|||.|.::
397 EEQKFRYLFQGTKGDPQNSTGGIYLDDITLTETPCPTGVWTVRNFSQVLE 446

443 ..SPNGTLYSPPFYSSKGYAFQIYLNLAHVTNAG...IYFHLISGANDDQ 487
      |..:.| ||.||.|.||:|.: |  .  ...|   : ||:.||.||.
447 NTSKGDKLQSPRFYNSEGYGFGVTLYPNSRESSGYLRLAFHVCSGENDAI 496

488 LQWPCPWQQATMTLLDQNPDIRQRMSNQRSITTDPFMTTD..NGNYFWDR 535
    |:||.. .|..:|:|||:||:|.|||. . :||..  |..  |:. :|||
497 LEWPVENRQVIITILDQEPDVRNRMSSSMVFTTSKSHTSPAINDTVIWDR 546

536 PSKVGTVALFSNGTQFRRGGGYGTSAFITHERLKSRDFIKGDDVYILLTV 585
    ||:|||   : .:..  |: :.| |:||.|:.||.|.|:|.||:.|:..
547 PSRVGT...YHTDCNCFRSIDLGWSGFISHQMLKRRSFLKNDDLIIFVDF 593

586 EDISHLNSTQI.....................QLTPAPSVQDL..... 607
    |||.||. |::                     |:....|...:
594 EDITHLSQTEVPSKGKRLSPQGLILQGQEQQVSEEGSGKAMLEEALPVSL 643

608 ............................CSKTTCKNDGVCTVRDGKAE 627
                                |....|.|||:|.  .|.|.
644 SQGQPSRQKRSVENTGPLEDHNWPQYFRDPCDPNPCQNDGICVNVKGMAS 693

628 CRCQSGEDWWYMGERCEKRGSTRDTIVIAVSSTVAVFALMLIITLVSVYC 677
    ||| ||..::| |||||:. :   ..:.:.:::|.:|: | : |. :
694 CRCISGHAFFYTGERCQSAEVHGSVLGMVIGGTAGVIFLTFSIIAILSQR 743

678 TRKKYRERMSSNRPNLTLQNHAF 700
    .||
744 PRK..................... 746
```

Fig. 4. Optimal alignment of the amino acid sequences of the β subunit (upper) and α subunit (lower) of human PPH. The complete primary structure of the β subunit and the α subunit were aligned using the algorithm of Needleman and Wunsch [38]. Gaps (···) were inserted for optimal alignment. A line is present between identical residues, two dots are present between chemically very similar residues, one dot between similar residues.

Consistent with previous experiments showing PPH from intestinal biopsies to be glycosylated [3], the primary structure of the β subunit contains 10 potential N-glycosylation sites. Optimal alignment (GAP program) [38] of the deduced amino acid sequences of PPH and meprin showed the β subunits to be homologous, with the mouse equivalent being 78% identical and 86% similar, and the rat equivalent being 78% identical and 87% similar.

Fig. 4 shows optimal alignment of the complete translated reading frames of PPH β and α subunits with an overall 44% identity and 61% similarity. Although the multidomain structure

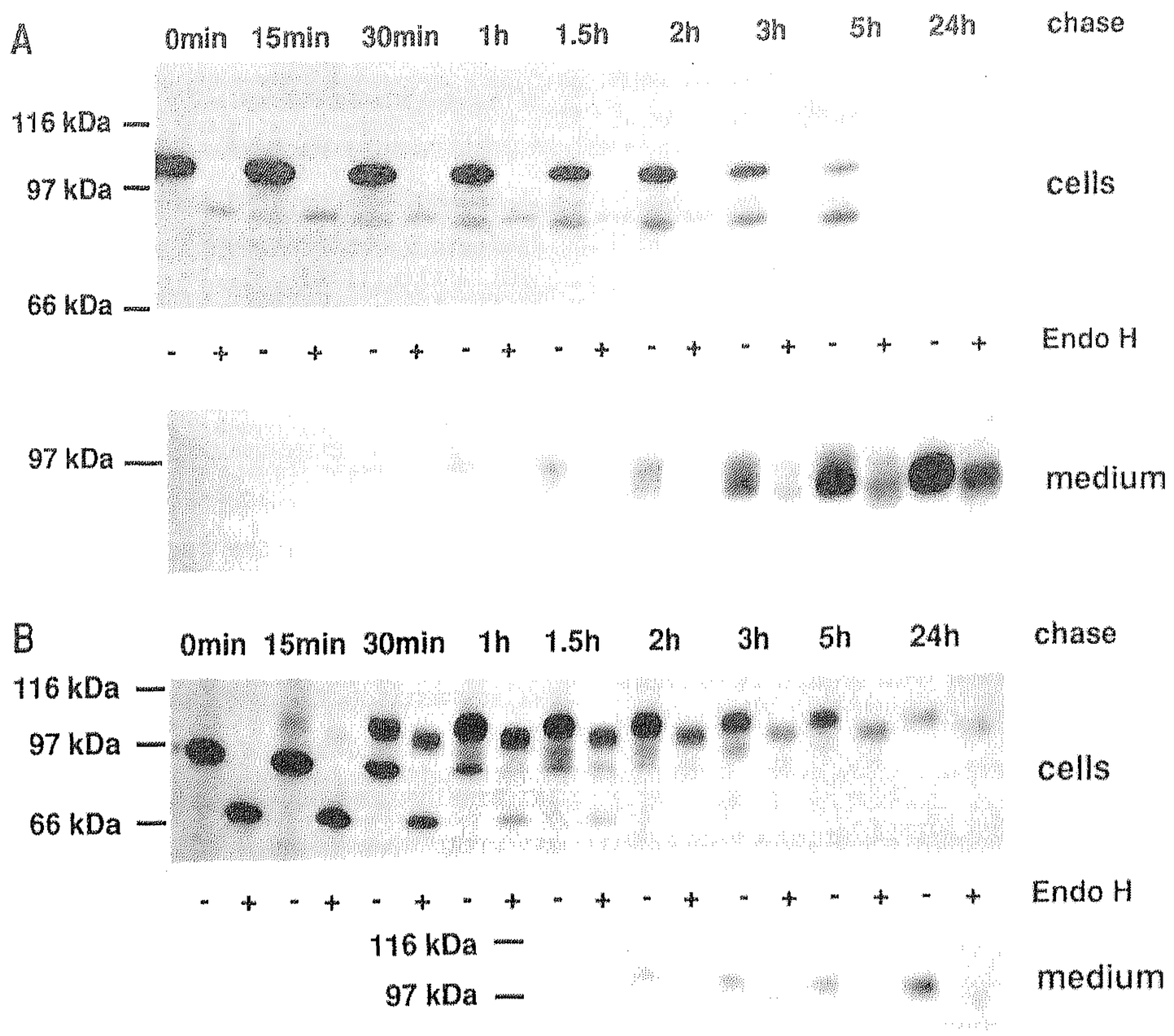

Fig. 5. Pulse-chase labelling of the PPHα subunit (A) and the PPHβ subunit (B) in transfected MDCK cells. MDCK cells, permanently-expressing either PPHα (A) or PPHβ (B), were metabolically labelled with [^{35}S]Met in a 15-min pulse followed by a chase of 0 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 5 h, and 24 h in Met-free medium. Cells and media from each time point were harvested and proteins immunoprecipitated with subunit-specific antibodies and divided into two aliquots. One aliquot was reduced with dithiothreitol 0.1 M, the other was treated with endo-β-*N*-acetylglucosaminidase H before being reduced with dithiothreitol. The immunoprecipitates were resolved by SDS/PAGE and visualised by fluorography. Molecular mass (kDa) markers are indicated on the left.

is identical between the two subunits, two distinguishing features are readily detectable in their primary structures and may contribute to differential functions. One being the putative cytosolic tail in which that of the β subunit is longer by 18 residues and contains at least two potential phosphorylation sites (Tyr682 and Ser688) [39] compared with that of the α subunit (Ser741–Lys746). The second difference is the presence of two additional segments (19 and 39 amino acids in length) in the α subunit, just upstream from the EGF-like domain, that are missing in the β subunit.

Expression of PPHα and PPHβ in polarised MDCK cells. With the complete reading frames of both PPHα and PPHβ cDNAs available, individual expression plasmids were constructed for each subunit. To examine the biosynthetic pathways of the subunits of PPH in a polarised mammalian cell line, MDCK cells were transfected with the expression plasmids, pPPHα and pPPHβ, and cell lines that permanently expressed these subunits were established. Newly synthesised proteins were metabolically labelled with L-[^{35}S]methionine/cysteine in pulse/chase experiments designed specifically to differentiate between various subunit species formed early and late during protein processing. PPHα was immunoprecipitated with a rabbit antisera (anti-α) raised against the denatured α subunit, PPHβ was immunoprecipitated with a mAb (HBBM 3/716/36) that is specific for the native β subunit and does not recognise the α subunit. Subunit specificity of the antibodies was verified using immunoprecipitation, western blots and immunofluorescence (data not shown). To follow maturation of the subunits, aliquots of each immunoprecipitate were treated with endo-β-*N*-acetylglucosaminidase H that cleaves only the high-mannose species found primarily in the ER.

Results of the pPPHα-transfected MDCK cells are shown in Fig. 5A. Initially, a single PPHα polypeptide (100 kDa) was observed that was sensitive to endo-β-*N*-acetylglucosaminidase H (90 kDa). During the entire chase period, no endo-β-*N*-acetylglucosaminidase H resistant polypeptide forms were found in cell extracts, indicating that no complex glycosylation occurred. However, the intensity of the 100-kDa polypeptide in cell extracts decreased during the chase period. A second form of PPHα ($\approx$90 kDa) appeared after 15 min of chase which was reduced in size to approximately 70 kDa by endo-β-*N*-acetylglucosaminidase H (Fig. 5A, 5 h chase). The intensity of this form increased over a 5-h chase period. After 24 h of chase no PPHα was detectable in cell lysates. A soluble form of PPHα, ($\approx$95 kDa) that was endo-β-*N*-acetylglucosaminidase H resistant was observed in the filtered medium after 1 h of chase and this increased in intensity until, after 24 h of chase, all the labelled PPHα was in the medium. When these PPHα species were resolved on a non-reducing gel, homodimers were formed almost immediately (data not shown). The species immunoprecipitated from the filtered culture medium were solely in the form of disulphide-linked homodimers (data not shown).

Fig. 5B shows the results obtained with pPPHβ-transfected MDCK cells. The first molecular species found after labelling (0 min chase, Fig. 5B) was an endo-β-*N*-acetylglucosaminidase H sensitive form of 95 kDa which was reduced to 70 kDa upon deglycosylation. After 15 min of chase a new form of 105 kDa appeared which was mostly endo-β-*N*-acetylglucosaminidase H resistant (100 kDa), indicating that complex glycosylation had

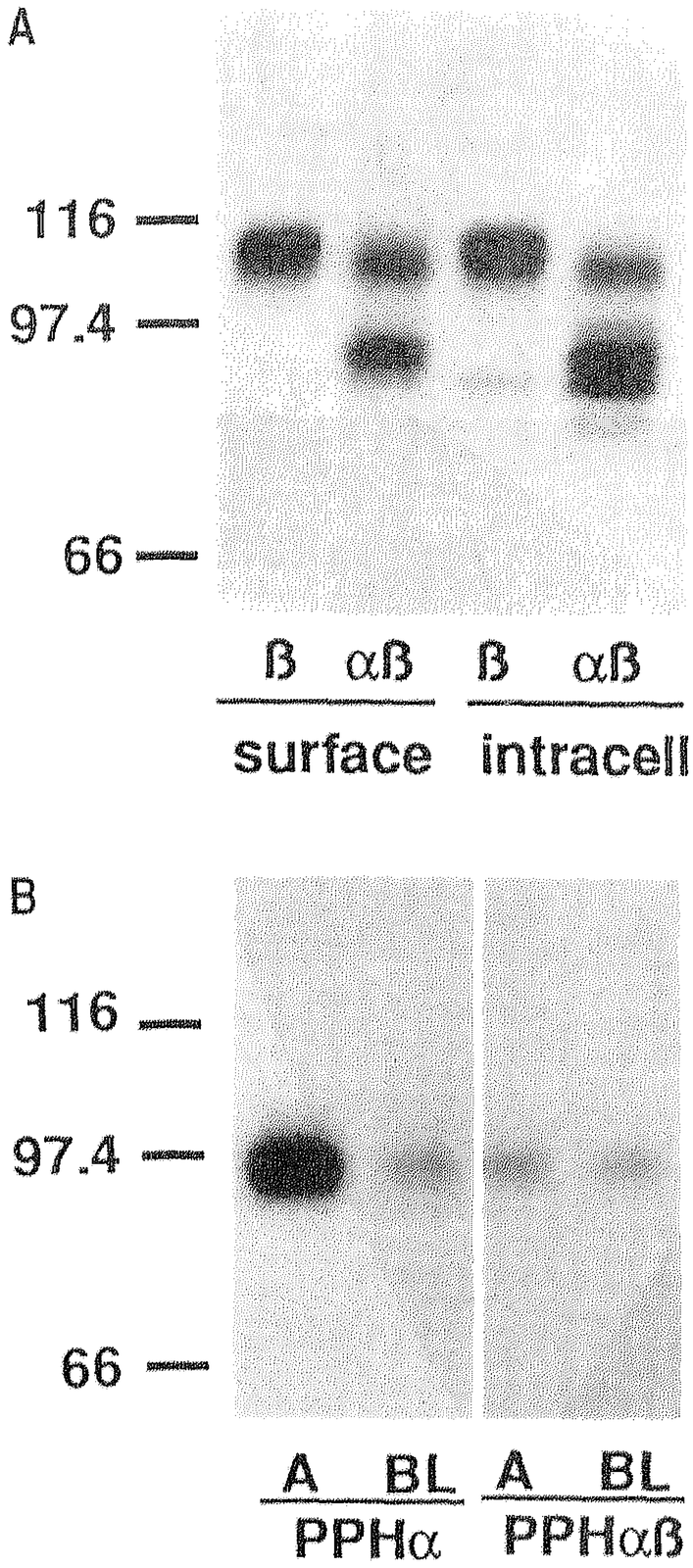

Fig. 6. Surface expression and polarised secretion of PPH in MDCK cells. (A) *Surface expression of PPH subunits in MDCK cells transfected* with PPH*β* or PPH*αβ* cDNA. Cells were labelled for 16 h then washed in NaCl/P_i at 37 °C and serum-free medium, followed by surface immunoprecipitation at 4 °C as described in Experimental Procedures. Intracellular labelled protein was immunoprecipitated in a second step. *β*, PPH*β*-expressing cells; *αβ*, PPH*αβ*-expressing cells. SDS/PAGE was under reducing conditions (see Experimental Procedures) followed by fluorography. (B) Polarised secretion of PPH*α* from filter-grown MDCK cells. Filter-grown cells (see Experimental Procedures) were labelled 16 h and media from the apical and basolateral compartments collected for immunoprecipitation of PPH*α*. PPH*α*, PPH*α*-expressing cells; PPH*αβ*, PPH*αβ*-expressing cells; A, apical medium compartment; BL, basolateral medium compartment. SDS/PAGE was under reducing conditions (see Experimental Procedures) followed by fluorography.

occured but that some coreglycosylated side chains persisted. After 1.5 h of chase an intermediate form between the coreglycosylated and complex glycosylated species appeared and at the same time traces of PPH*β* were detected in the culture medium. The amount of PPH*β* in the medium was less than PPH*α* (Fig. 5A). PPH*β* also formed disulphide-bridged homodimers immediately after synthesis as shown by analysis on non-reducing gels and was secreted in dimerised form into the medium (data not shown).

PPH*β* forms heterodimers with PPH*α* and holds the latter on the cell surface. To examine if the PPH subunits assemble when coexpressed, MDCK cells transfected with pPPH*β* alone or together with pPPH*α* were continuously labelled for 24 h and the subunits immunoprecipitated from the cell surface using PPH*β* subunit specific antibodies. Fig. 6A shows that in MDCK cells expressing PPH*α* and PPH*β* both forms could be immunoprecipitated from the surface using this PPH*β*-specific antibody. As immunoprecipitation was performed under native conditions this clearly shows that the two subunits formed heterodimers. This was further verified by SDS/PAGE under non-reducing conditions (data not shown). Furthermore, heterodimersization was also observed by immunoprecipitation of intracellular forms and is thus an early event.

PPH*α* is secreted from the apical membrane domain. MDCK cells expressing PPH*α* or together with PPH*β* were cultured on filter supports (see Experimental Procedures), labelled and the basolateral and apical media compartments analysed by immunoprecipitation and SDS/PAGE. In cells which only expressed the *α*-subunit, PPH*α* was almost exclusively secreted from the apical plasma membrane domain (Fig. 6B). When expressed together with PPH*β*, due to its retention on the cell surface by the *β*-subunit, only traces of the PPH*α* subunit were detected in the medium.

Intracellular localisation of PPH subunits in MDCK cells by immuno-gold electronmicroscopy. Figs 7 and 8 summarise immuno-gold labelling data in cryosections of transfected MDCK cells. The results in Fig. 7 were obtained using the LDi/1 antibody (see Experimental Procedures) and show that the *β*-subunit was present in the microvillus membrane of both cells expressing PPH*β* alone or together with PPH*α* (Fig. 7A and B). No labelling was observed in the basolateral membrane (Fig. 7A–D). Some labelling was seen in vacuolar structures (Fig. 7C and D). Fig. 8 shows the results obtained with the PPH*α*-specific antibody A/1 (see Experimental Procedures) and show that in MDCK cells expressing only PPH*α*, this subunit was not present in the microvillus membrane (Fig. 8A). Intracellularly, the subunit was localised to the nuclear envelope and the ER (Fig. 8C). In MDCK cells expressing both subunits, PPH*α* was also present on the microvillus membrane (Fig. 8B). In addition, some positive labelling of lysosomes was observed (Fig. 8D). No PPH*α* was detected on the basolateral membrane (Fig. 8A–C).

Intracellular transport and secretion of PPH*α* requires proteolytic removal of the transmembrane domain. In pulse/chase experiments with PPH*α* a smaller form of this subunit (90 kDa) was observed which increased in intensity on fluorographs (Fig. 5A). To investigate the nature of this molecular form and the intracellular localisation where it is formed we performed labelling experiments at reduced incubation temperatures of 15 °C and 18 °C to block intracellular transport. PPH*α*-expressing MDCK cells were pulse-labelled for 30 min at 37 °C and chased at reduced temperatures for zero and 24 h, followed by immunoprecipitation. Fig. 9A shows the 100-kDa form synthesised at 37 °C (Fig. 9A, lane 1). Only a faint band (90-kDa) is seen at this incubation temperature. After 24 h of chase at 37 °C, all protein had left the cells (Fig. 9A, lane 2) and was present in the culture medium (Fig. 9A, lane 5). In contrast, at temperatures of 15 °C (Fig. 9A, lane 3) and 18 °C (Fig. 9A, lane 4), the 90 kDa form had accumulated (Fig. 9A, lanes 3 and 4) and no protein was secreted into the medium (Fig. 9A, lanes 6 and 7). At 18 °C a faint band corresponding to the complex glycosylated form in the medium was detected (Fig. 9A, lane 4).

Temperature-shift experiments were performed to confirm this precursor/product relationship. PPH*α*-expressing MDCK cells were pulse-labelled for 30 min at 37 °C and chased at 15 °C overnight. Thereafter the incubation temperature was raised to 37 °C and the cells chased for up to 6 h. After the extended

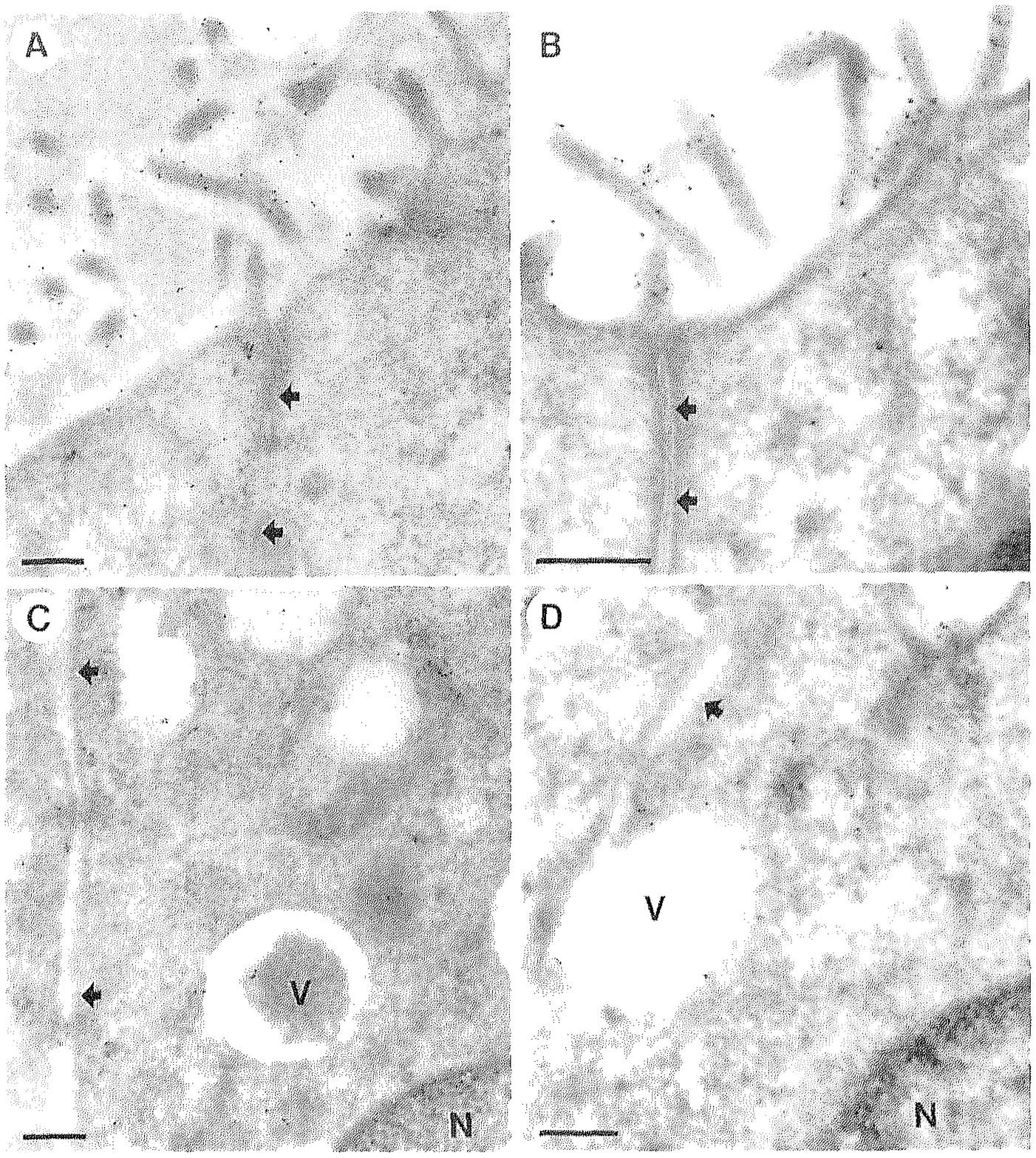

Fig. 7. Immunocytochemical localisation of PPH*β*. Immunocytochemical localisation in MDCK cells stably transfected with PPH*β* alone (A, C) or together with PPH*α* (B, D). PPH was localised in ultrathin cryosections using the polyclonal antibody Ldi/1 raised against the *β* subunit followed by incubation with protein A complexed colloidal gold particles. Arrows indicate the basolateral membrane. Bar = 0.25 μm; V, vacuole; N, nucleus.

15 °C chase an accumulation of the 90-kDa form was observed (Fig. 9 B, lane 1). During the chase period at 37 °C the 100-kDa precursor vanished, while the 90-kDa form was largely retained in a constant amount (Fig. 9 B, lanes 2–5). After the pulse at 15 °C no protein was detectable in the culture medium (Fig. 9 B, lane 6). After raising the temperature to 37 °C increasing amounts of protein were secreted into the medium (Fig. 9 B, lanes 7–10). We conclude from these data that the 90-kDa species is a proteolytically processed form of the 100-kDa PPH*α* precursor.

To investigate this further PPH*α*-, PPH*β*-, and PPH*αβ*-expressing MDCK cells were labelled for 30 min at 37 °C. After harvesting the cells membrane vesicles were prepared which were subsequently extracted with sodium carbonate to separate membrane bound from soluble proteins (see Experimental Procedures). After ultracentrifugation, proteins in the pellet (membrane-bound) and the supernatant (soluble) were denatured and immunoprecipitated prior to analysis by SDS/PAGE. The results are summarised in Fig. 9 C. The 100-kDa precursor of PPH*α* was found to be membrane bound (Fig. 9 C, lane 1), while the 90-kDa intermediate form was soluble (Fig. 9 C, lane 2). In contrast, PPH*β* was exclusively membane bound (Fig. 9 C, lane 3). Analysis of cells coexpressing the two subunits showed the 90-kDa intermediate form of PPH*α* also to be membrane bound (Fig. 9 C, lane 5), a clear indication that the processed PPH*α* was associated with the membrane-bound PPH*β*.

DISCUSSION

With the cloning of the two subunits of PPH complete, questions can now be addressed with respect to processing and assembly of this protein. Also with the knowledge of the deduced protein structures and their putative functions, insight into the physiologic role of this human intestinal peptidase may be obtained.

The present cloning results show that the PPH*β* subunit contains the same series of domains that have mostly been discussed for the PPH*α* subunit [17]. The predicted primary structure confirms that this subunit is a type I transmembrane protein in which the bulk of the domains appear extracellular with a relatively short C-terminus in the cytosol.

Hydropathic analysis of the N_2-termini of PPH*β* and PPH*α* indicate the presence of a signal peptide, expected of membrane-spanning proteins, followed by a hydrophilic propeptide segment. Sequence alignment of the empirically determined N_2-termini of the mouse subunits with the predicted sequences of PPH*α* and PPH*β* show there to be putative prepeptides and propeptides in PPH also. It has been suggested by Gorbea et al. [28] that the propeptide of mouse meprin*β*, a related enzyme subunit from mouse kidney [4], must be proteolytically removed to allow for an active conformation of the protease domain. Recent experiments have established proprotein forms of a meprin-PPH*α* chimeric enzyme [20] and also of the mouse meprin*β* subunit in which tryptic digestion *in vitro* leads to their activation

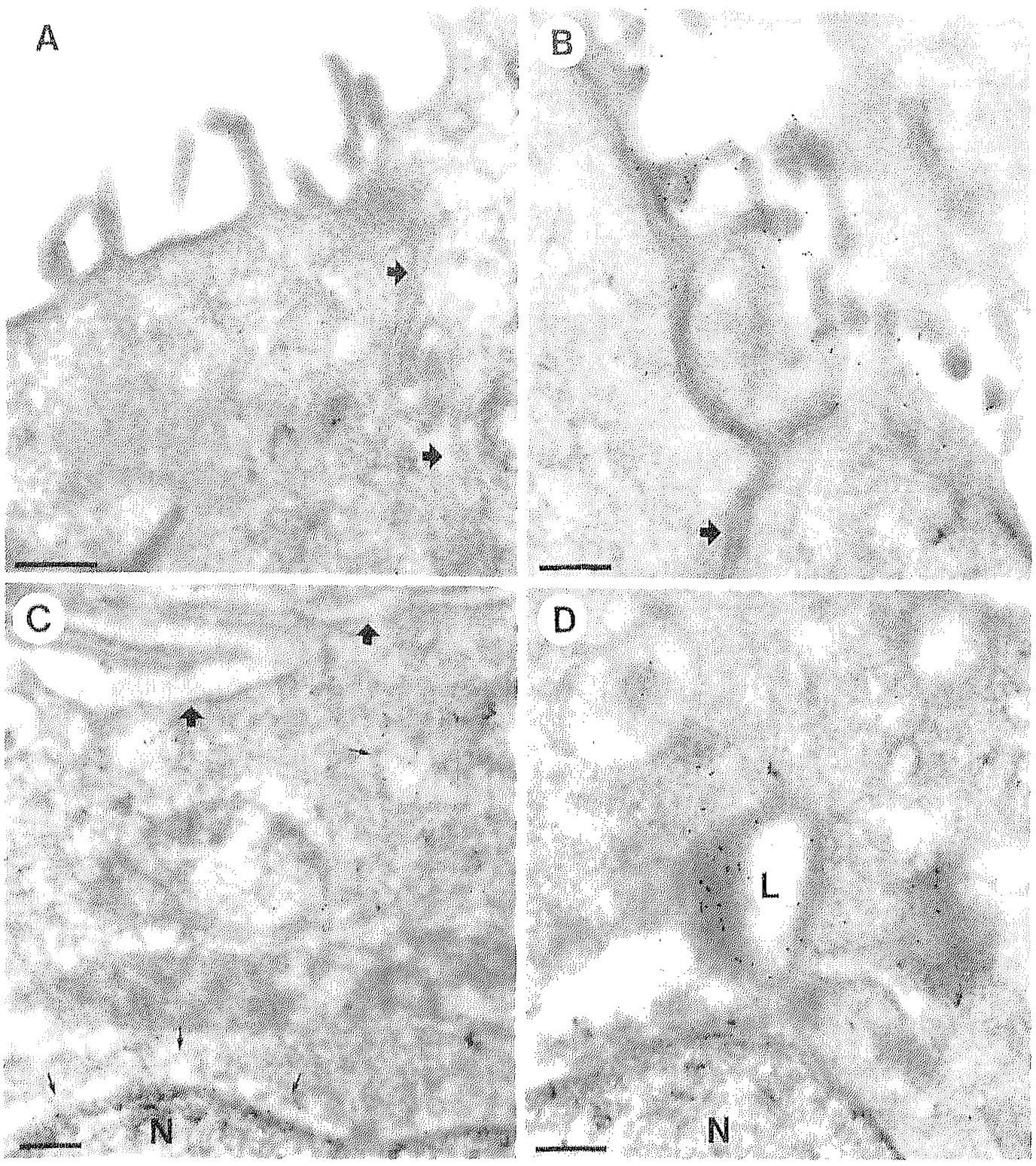

Fig. 8. Immunocytochemical localisation of PPHα. Cryosections of MDCK cells stable-expressing PPHα alone (A, C) or together with PPHβ (B, D). The ultrathin cryosections were incubated with the α-subunit-specific polyclonal antibody A/1 before incubation with protein A complexed gold particles. Bar = 0.25 μm; V, vacuole; N, nucleus; L, lysosome.

[15, 20, 28]. A potential cleavage site for a trypsin-like protease (Arg-Asn) exists at the mature N_2-terminal sequence of both PPH subunits suggesting that proteolytic processing by such a protease occurs for intestinal PPH. Such a mode of activation for PPH may take place *in vivo* either within the enterocyte or in the lumen of the small intestine known to have abundant trypsin activity. Further studies are necessary to determine the mechanisms and sites of propeptide processing during PPH biosynthesis.

Three potential functional domains, MAM, MATH and EGF-like, exist in both subunits of PPH and may play a role in protein binding. MAM, occurs in several functionally diverse type I transmembrane proteins and has been suggested to be involved in protein-protein interactions [36]. Conserved cysteine residues within the MAM domain have recently been identified which are involved in the covalent dimers formation of the a and β subunits [40, 41]. The MATH domain has only recently been identified by Uren and Vaux as a meprin- and TRAF-homology domain [37]. Due to the different membrane orientation of PPH/meprin and TRAF proteins (tumour-necrosis-factor-receptor-associated proteins) the significance of this sequence conservation is not clear. In TRAF proteins, the TRAFC (MATH) domain is probably involved in protein-protein interactions required for binding of TRAF proteins to their receptors. Thus, the MATH domain in PPH/meprin may serve a similar function, allowing stabilisation of dimers. Alternatively, it may mediate binding of PPH/meprin to other, at present unidentified proteins some of which may resemble proteins that bind TRAF proteins. The other domain, EGF-like, is better characterised [42] and appears to be involved in receptor-ligand binding or calcium binding [43]. Some members of the astacin family that contain the EGF-like domain are also known to function in morphogenetic processes such as human bone morphogenic protein I [5, 44], tolloid of fruit fly [7], and BP10 of sea urchin [45]. Thus, it may be speculated that PPH is involved in the regulation of cell growth and differentiation during rapid proliferation that occurs in the epithelium of the human intestine, by proteolytically modifying peptide growth factors.

It has been reported that assembly of specific subunits of the 4-amino butyric acid receptor determine sorting of the receptor in polarised cells [46]. The present experiments, expressing the PPH subunits individually and together in polarised MDCK cells, show PPH to be a model for studying sorting and assembly mechanisms. Differences in post-translational processing and trafficking of the α and β subunits were confirmed using two approaches; pulse/chase labelling and immunocytochemical staining. The β subunit alone was shown to be synthesised and transported through the Golgi to the plasma membrane, whereas, the α subunit, when expressed alone, was either cell bound in the ER or *cis*-Golgi, or was rapidly secreted via the Golgi. The inability of the α subunit, expressed alone, to be found at the plasma membrane has previously been observed with the mouse meprin-PPHα chimera in MDCK cells [20] and with rat meprinα in COS-1 cells [19]. The present coexpression studies demon-

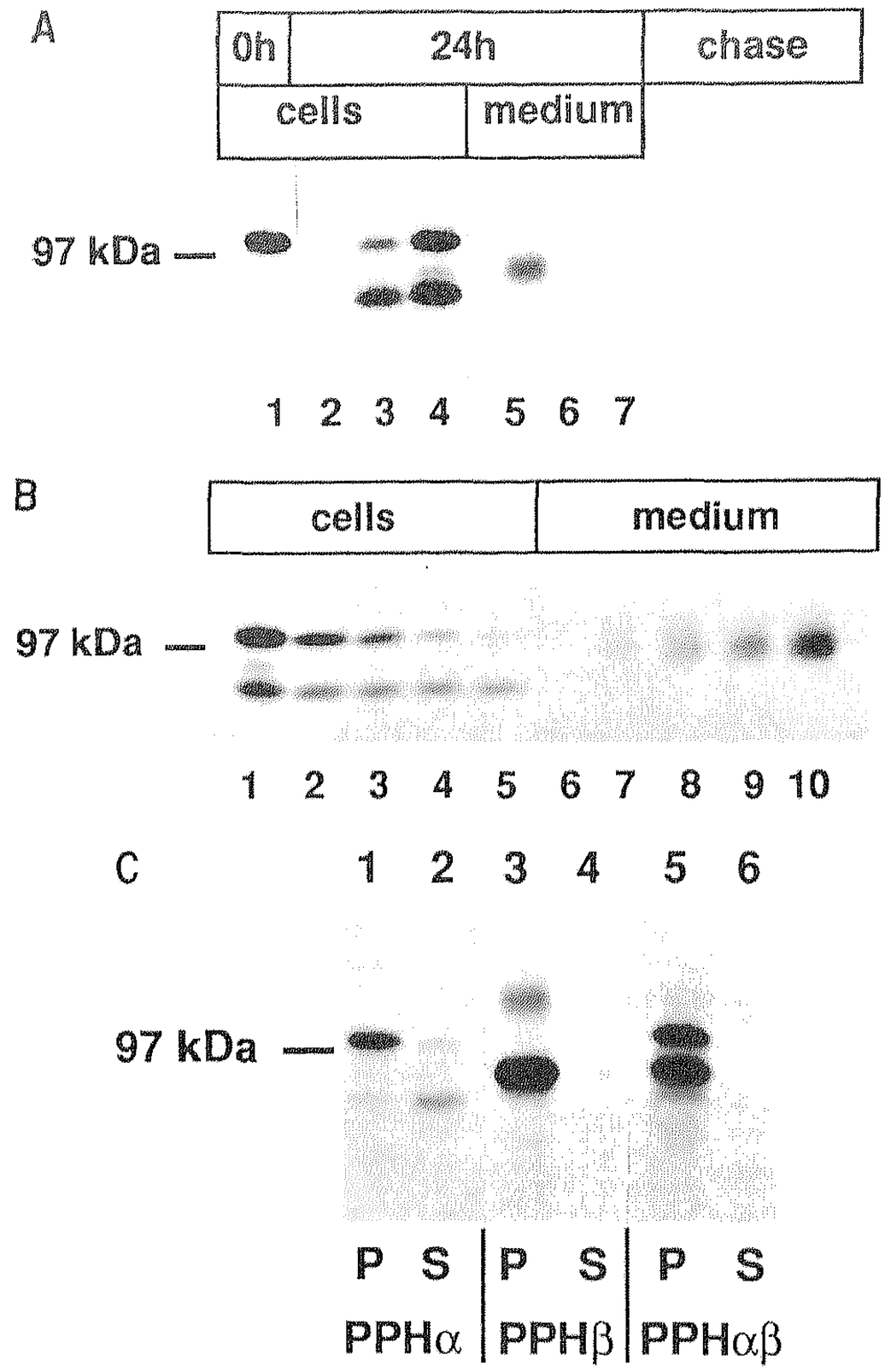

Fig. 9. Low temperature incubations, temperature-shift assay and sodium carbonate extraction of PPH subunits. (A) Metabolic labelling at 15°C and 18°C. PPH*α*-expressing cells were pulse-labelled for 30 min at 37°C and chased for 24 h at low temperatures, after which cells were lysed and PPH*α* was immunoprecipitated from the cells (lane 1–4) and the media (lane 5–7). Lane 1, PPH*α* after 30 min pulse at 37°C; lane 2 and 5, PPH*α* after 24 h chase at 37°C; lane 3 and 6, PPH*α* after 24 h chase at 15°C; lane 4 and 7, PPH*α* after 24 h chase at 18°C. SDS/PAGE was under reducing conditions (see Experimental Procedures) followed by fluorography. (B) Temperature-shift assay. MDCK cells stable transfected with PPH*α* were pulse-labelled for 30 min at 37°C and chased at 15°C overnight. The incubation temperature was raised to 37°C and the cells were incubated for additional 1 h (lane 2 and 7), 2 h (lane 3 and 8), 4 h (lane 4 and 9) and 6 h (lane 5 and 10). Lane 1 and 6 show the immunoprecipitated PPH*α* after overnight incubation at 15°C. Lanes 2–5 and 7–10 show the amount of PPH*α* during the chase at 37°C present in the cells and the media, respectively. SDS/PAGE was under reducing conditions (see Experimental Procedures) followed by fluorography. (C) Sodium carbonate extraction. MDCK cells expressing PPH*α* (lane 1, 2), PPH*β* (lane 3, 4) or both PPH subunits (lane 5, 6) were labelled for 30 min at 37°C and membrane vesicles were prepared followed by extraction with sodium carbonate and ultracentrifugation, thereby separating membrane-bound from soluble proteins. PPH was immunoprecipitated from the membrane-bound fraction (P) and the soluble fraction (S) and analysed by SDS/PAGE under reducing conditions and fluorography.

strate that PPH*α* and *β* subunits are capable of assembling together and that this affects the trafficking of the *α* subunit thus allowing it to be located at the cell surface.

Using a different approach, Milhiet et al. [47] and Johnson and Hersh [48] came to similar conclusions for rat meprin*α* and *β* subunits expressed in COS-1 and 293 cells (an adenovirus-transformed human kidney cell line). They demonstrated that the *α* subunit could be released from intact cotransfected cells by enzymatic and chemical treatment. It was hypothesised that the *α* subunit lacks a membrane-spanning anchor and is thus held in place at the cell surface by disulphide bonds to the *β* subunit. This appears also to be the case for PPH, as the *α* subunit can be removed from the cell surface of intact cotransfected cells by dithiothreitol treatment (Grünberg, J., unpublished results).

The deduced amino acid sequences of the *α* subunits of PPH and meprin show a potential membrane-spanning domain at the C-termini. Thus, a proteolytic event within the *α* subunit probably occurs which removes this membrane anchor. In support of this notion, Marchand et al. [49] have shown that the mature *α* subunit of mouse meprin lacks an epitope near the C-terminus. As evidenced by our pulse/chase experiments, low-temperature incubations, temperature-shift assays, and carbonate extraction we predict that such an event occurs early in protein processing, in the ER, and is required for the *α* subunit to become transport competent. The formation of a proteolytically processed soluble and endo-*β*-*N*-acetylglucosaminidase H-sensitive 90-kDa PPH*α* species was observed in the cells which represents the truncated precursor form of the complex glycosylated 95-kDa species that is secreted from the apical domain of polarised cells. The exact cleavage site is not known, but it has been shown for rodent meprin*α* to involve the I-domain which is lacking in the *β* subunit. Two conflicting reports show that in rat meprin*α* cleavage occurs at a furin consensus sequence [50] while in mouse meprin*α* this sequence appears not to be involved [51]. In contrast to rodent kidney meprin*β* human PPH*β* is also found in the apical culture medium of transfected MDCK cells. A proteolytically processed form was detected in cell extracts. Due to the glycosylation status of the precursor and cleaved forms, both were complex glycosylated, it is concluded that processing of PPH*β* occurs at a later stage during intracellular transport. The significance of this processing of intestinal PPH*β* needs further investigation. The difference in processing of PPH*α* and PPH*β* are the subject of work described in the accompanying report by Hahn et al. [52]. The work on processing of *α*/*β* tailswitch mutants strongly suggests that the transmembrane and/or C-terminal domains of PPH*α* contain an ER-retention function allowing efficient proteolytic processing prior to rapid transport to the cell surface and secretion into the medium. Transient association of PPH via the *α* C-terminal region with calnexin strongly suggests that this chaperonine is involved in ER retention of PPH*α*. The proteolytic process leading to truncation and subsequent secretion of PPH*β* is the subject of further investigation in our laboratory.

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