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

Joyce A. ELDERING1, Jürgen GRÜNBERG1, Dagmar HAHN1, Huib J. E. CROES2, Jack A. M. FRANSEN2 and Erwin E. STERCHI3

1 Institute of Biochemistry and Molecular Biology, and Department of Pediatrics, University of Berne, Switzerland
2 Department of Cell Biology and Histology, University of Nijmegen, The Netherlands

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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-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 N2-terminus both contain a signal peptide, a propeptide, a protease domain containing the astacin signature, 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.
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 excrated 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-PPH 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 PPHβ localised to the surface of these cells. C-terminal proteolytic processing of PPHβ necessary for its secretion is further shown to occur in the endomembrane reticulum (IER).

EXPIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesised by Microsynth (Switzerland). The UniZAP XR Custom cDNA library, SGS mammalian expression vector, XL-1-Blue Excherichia coli host and T7a DNA polymerase were purchased from Stratagene. Hybond C extra nitrocellulose membrane, [γ-32P]dATP (3000 Ci/mmol), [35S]dATP (1500 Ci/mmol) and [-35S]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 rRNA in RNAase inhibitor, dGTP, dATP, dTTP and dCTP were supplied by Promega. The Nusieve GTG agarose was supplied by Boehringer Mannheim. The Nusieve GTG agarose-gel electrophoresis and subsequently cloned into the pGEM~T vector (Stratagene) were amplified in XL-1-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 32P-labelled with polynucleotide kinase. Hybridisations were carried out at 50°C for approximately 16 h in 6X NaCl/Cit (20Xstock solution: 3.0 M NaCl, 0.3 M sodium citrate), 2XDenhardts [100Xstock solution: 2% (mass/vol.) bovine serum albumin, 2% (mass/vol.) Ficoll, 2% (mass/vol.) poly(vinyl pyrrolidone)], SSPE (20Xstock solution: 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 0.02 M Na2EDTA), SDS 0.05% (mass/vol.), and denatured, fragmented herring sperm DNA 100 μg/ml. Washings were in 5XNaCl/Cit, 0.1% SDS (mass/vol.) at 50°C for 20 min, twice, then in 2XNaCl/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 pHPlβ-3b, and further amplified for analysis. To sequence both strands of pHPlβ-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 Novablaue (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 pHPlβ cDNA (Fig. 1) using this method. The pHPlβ-3b clone was found to be lacking an in-frame start codon and hence lacked the extreme 5' end of the complete pHPlβ 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 PHPlβ 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),
The PCR products for the PPHβ (≈ 325 bp) and PPHα (≈ 175 bp) 5′ cDNA ends were then ligated directly into the pGEM-T vector and amplified in Novabluet. Two clones from each ligation reaction were isolated and sequenced on both strands using M13 universal and reverse primers. The sequence of the PPHβ 5′ cDNA end overlapped with the PPHα-3b cDNA sequence by 50 nucleotides. The sequencing strategy of the complete PPHβ cDNA, is shown in Fig. 1.

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

Construction of expression plasmids. The assembled PPHβ expression plasmid is shown in Fig. 2A. First, PCR primers were designed from the predicted start codon region of PPHβ 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 EcoRI 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 XbaI site. Using the pGEM-T plasmid containing the 5′ cDNA end of PPHβ 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 XbaI–BamHI insert [containing the major portion of the PPHβ cDNA from the XbaI site downstream to and including the poly(A) tail] was isolated from pPPHβ-3b by restriction digest and agarose-gel isolation. Finally, these two PPHβ cDNA fragments, together comprising the complete reading frame of the PPHβ cDNA, were then ligated into the EcoRI and BgIII (BamHI compatible) sites of the mammalian expression vector, pSG5 (Strategene) thus creating the complete open reading frame of the PPHβ subunit cDNA.

Using the newly cloned PPHβ 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 PstI–HindIII PPHα cDNA fragment (1200 bp) was obtained from pPPHα-22.4 [17]. Finally, a BamHI–HindIII fragment (1600 bp) containing the moirop portion of the PPHα cDNA, was removed and replaced by the two PPHβ cDNA fragments isolated above. The resulting expression plasmid, pPPHα, contained the complete open reading frame of the PPHβ subunit cDNA.

Computer analysis of DNA and protein sequences. Nucleotide sequences were read with the USEEDT program and Reader (C.B.S. Scientific Company) and initial comparisons made using the DNAid program [18]. Dardel and P. Bensoussan,
The cells were then labelled with 50 μCi collected and filtered (0.2 μm) to remove any dead cells. Analysis by SDS/PAGE and fluorography. The media were supplemented with 10 mM methionine for different time periods.

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 Heps medium supplemented with 5% (mass/vol.) foetal calf serum, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO₂ 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 CaPO₄-DNA transfection with PPHα and PPHβ expression vectors, separately or together. Methods are as described by Grünberg et al. [20]. Briefly, 1×10⁶ 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 CaPO₄-DNA precipitate was prepared containing 2 μg of the neomycin-selectable marker plasmid pX71 (Stratagene), together with 20 μg of the experimental plasmids, pPPHα and pPPHβ, separate or in combination. The cell suspension and CaPO₄-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 NaHPO₄, 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 PPHα subtype expression by metabolic labelling with [¹⁴C]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 PPHα alone, and one clone was found to express PPHα and PPHβ. The cell clones with the highest expression rates, PPH4-4, PPHβ-11 and PPHβ-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 30 μCi [³⁵S]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 (antiPPHβ) raised against denatured PPHβ 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 immunogold electron microscopy two polyclonal antibodies, A/1 (specific for the α subunit) and LD/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, (138 mM, pH 7.3) and scraped with a rubber policeman into 1 ml ice-cold NaCl/P. 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 PMSF, 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-PPHβ 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 precloned 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, 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, 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, 20 μl anti-PPHβ mAb in 1 ml NaCl/P, 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, 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 protein was essentially as described by Fujiki et al. [26]. Briefly, membranes were prepared by homogenisation in 0.1 M NaCl, 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 PPHβ 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 diethylthritol (1 mM) which reduces disulphide bonds. Proteins were resolved by SDS/PAGE [7.5% (mass/vol.) polyacrylamide] according to Laemmli. The M, standards used were myosin, 205000; β-galac-
The cysteines in the astacin and EGF-like domains are in bold type. Potential N-glycosylation sites are underlined with stars.

Fig. 3. cONA and deduced amino acid sequences of the \( \alpha \) subunit of human intestinal PPII. The 2326 bp PPH/\( \alpha \) eDNA 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-terminal sequence (Asn62) of mature PPM/\( \alpha \). In brackets is the astacin domain containing the zinc-binding site (HEXXH) and astacin signature which is double underlined. The simile underline indicates the FGF-like domain. Conserved sequences were cut on a Leica ultramicrotometer and incubated with specific polyclonal antibodies A1 and LDL.

**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 40% (by vol.) formaldehyde in 0.1 M sodium phosphate buffer). Immunolocalisation of PPH was performed with a polyclonal antibody raised against a synthetic peptide (NH\(_2\) -GTLQGGLARSS-(Eiu: J. Bloch cm. 247)-COOH) and visualised with the ABC method (Vector Laboratories). Immunolocalisation studies were cut on a Leica ultramicrotometer and incubated with specific polyclonal antibodies A1 and LDL.

**N-Glycosylation sites.** Potential N-glycosylation sites are underlined with stars. The putative membrane anchor domain is italicised.

todisase, 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 \( \text{H}_2\text{O} \), 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 fluorograms were varied with each experiment to maximise visualisation of the protein bands.
RESULTS

PPHβ 5' cDNA cloning. The 5' end of PPHβ cDNA contained an open reading frame starting with the consensus AUG start-codon and overlapped by 60 nucleotides with the near-full-length PPHβ cDNA sequence previously obtained [17]. Thus, the complete open reading frame of the PPHβ cDNA of 2920 bp encodes 746 amino acid residues (EMBL sequence database, accession no. M82962). The complete cDNA-deduced amino acid sequence of PPHβ 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 meprin [28] and rat meprin [29].

PPHβ cDNA cloning. The complete cDNA sequence and deduced amino acid sequence for the β subunit of PPHβ is shown (Fig. 3). The open reading frame (nucleotides 47–2149) starts with the consensus sequence RXXAUGG for the initiation of protein translation [34, 35]. The open reading frame for the // subunit of PPHβ is shown in Fig. 4 in alignment with the // subunit and the β subunit were aligned using the algorithm of Needleman and Wunsch [38]. Gaps (•••) were inserted against PPHβ and PPHα subunits, respectively, and with proteolytically cleaved // subunit and the β subunit were aligned using the algorithm of Needleman and Wunsch [38]. Gaps (•••) were inserted

Fig. 4. Optimal alignment of the amino acid sequences of the β subunit (upper) and α subunit (lower) of human PPHβ. 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.
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 cytotoxic tail in which that of the β subunit is longer by 18 residues and contains at least two potential phosphorylation sites (Tyr682 and Ser788) [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, pPPHa and pPPHβ, and cell lines that permanently expressed the subunits of PPH in a polarised mammalian cell line were identified. Two subunits 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-acetyl-

...glucosaminidase H that cleaves only the high mannose species found primarily in the ER.

Results of the pPPHa-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 (~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 (~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 sulphur-linked homodimers (data not shown).

Fig. 5B shows 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...
immediately after synthesis as shown by analysis or together with the same time traces of PPH/β were detected in the culture medium. Cosylated and complex glycosylated species appeared and at the ging pels and was secreted in dimerised form into the medium (Fig. 6 A). PPH/β also formed disulphide-bridged homo cli  inters

dium compartment, SDS/PAGE was under reducing conditions (see Ex-

pPPlia were continuously labelled for 24 h and

This was further verified by SDS/PAGE under non-reducing conditions (data not shown). Furthermore, heterodimerization was also observed by immunoprecipitation of intracellular forms and is thus an early event.

PPH/β is secreted from the apical membrane domain. MDCK cells expressing PPH/β or together with PPH/β were cultured on filter supports (see Experimental Procedures), labelled and the basolateral and apical membrane 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 electron microscopy. Figs 7 and 8 summarise immuno-gold labelling data in cryosections of transfected MDCK cells. The results in Fig. 7 were obtained using the LD/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. 7 A – D). Some labelling was seen in vacuolar structures (Fig. 7C). 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 require 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. 9 A, 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

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occurred 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).

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Fig. 7. Immunocytochemical localisation of PPHβ. Immunocytochemical localisation in MDCK cells stably transfected with PPHβ alone (A, C) or together with PPHα (B, D). PPH was localised in ultrathin cryosections using the polyclonal antibody Ldr/1 raised against the β subunit followed by incubation with protein A complexed colloidal gold particles. Arrows indicate the basolateral membrane. Bar = 0.2 µm; V, vacuole; N, nucleus.

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

To investigate this further PPHα-, PPHβ-, and PPHαβ-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. 9C. The 100-kDa precursor of PPHα was found to be membrane bound (Fig. 9C, lane 1), while the 90-kDa intermediate form was soluble (Fig. 9C, lane 2). In contrast, PPHβ was exclusively membrane bound (Fig. 9C, lane 3). Analysis of cells coexpressing the two subunits showed the 90-kDa intermediate form of PPHα also to be membrane bound (Fig. 9C, lane 5), a clear indication that the processed PPHα was associated with the membrane-bound PPHβ.

**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 PPHβ subunit contains the same series of domains that have mostly been discussed for the PPHα subunit [17]. The predicted primary structure confirms that this subunit is a type 1 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-termini of PPHβ and PPHα 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-termini of the mouse subunits with the predicted sequences of PPHα and PPHβ 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 meprinβ, 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 propeptide forms of a meprin/PPHα chimeric enzyme [20] and also of the mouse meprinβ subunit in which tryptic digestion in vitro leads to their activation.
A potential cleavage site for a trypsin-like protease (Arg-Asn) exists at the mature N-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 α 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 stabilization of dimers. Alternatively, it may mediate binding of PPH/meprin to other, 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 morphogenetic protein 1 [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-aminobutyric 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-PPH chimera in MDCK cells [20] and with rat meprin in COS-1 cells [19]. The present coexpression studies denon-
Fig. 9. Low temperature incubations, temperature-shift assay and sodium carbonate extraction of PPH subunits. (A) Metabolic labelling at 15 °C and 18 °C. PPHα-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 PPHα was immunoprecipitated from the cells (lane 1–4) and the media (lane 5–7). Lane 1, PPHα after 30 min pulse at 37 °C; lane 2 and 5, PPHα after 24 h chase at 37 °C; lane 3 and 6, PPHα after 41 °C (lane 4 and 9) and 6 h (lane 5 and 10). SDS/PAGE was under reducing conditions (see Experimental Procedures) followed by fluorography. (B) Temperature-shift assay. MDCK cells stable transfected with PPHα 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 PPHα after overnight incubation at 15 °C. Lanes 2–5 and 7–10 show the amount of PPHα 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 PPHα (lane 1, 2), PPHβ (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.

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