Polarised expression of human intestinal
N-benzoyl-L-tyrosyl-p-aminobenzoic acid hydrolase (human meprin)
a and b 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, a and b. The present report describes the cloning of the complete b subunit and the remaining N-terminal end of the a 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 PPH/a cDNA translates into 700 amino acid residues compared with 746 residues for the PPH/a cDNA. The primary structure of b and a 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 A5 protein tyrosine phosphatase (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 immunogold electron microscopy of recombinant PPH/1 and a 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 b subunit acquired complex glycan residues, readily formed homodimers and was transported to the plasma membrane. Small amounts of PPH/a were found in the culture medium. In contrast, the cell-bound a 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-b-N-acetylgalactosaminidase-resistant a 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 a subunit with the b subunit allowed the localisation of the a subunit to the plasma membrane. These studies indicate that assembly of the two subunits of PPH is required for the localisation of the a 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, a and b, 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 a 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 BMP5 [5], UV5.2 from Xenopus laevis embryos [6], tolloid from Dro sophila 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 embryos) [10], a 1,25-dihydroxyvitamin D3-stimulated peptide from Coturnix japonica (Japanese quail) [11], flavastacin [12] and meprin from rodents. Meprin, also termed endopeptidase 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 excrated in a soluble form in mouse urine [16]. In contrast, PPH has so far only been identified in the human intestine [17]. Similarly, 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 metrin α and a mouse metrin-PHPα chimera form disulfide 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 (IER).

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesised by Microsynth (Switzerland). The UniZAP XR Custom cDNA library, SGS mammalian expression vector, XL1-Blue Escherichia coli host and Tag DNA polymerase were purchased from Stratagene. Hybrid C extra nuclease cellulose membrane, [γ-32P]dATP (3000 Ci/mmol), [α-32P]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, R 5'-primer (1000 Ci/mmol), |; W S|dATP|«S1 (1500 Ci/mmol) and ¡methionine/ poly(vinyl pyrollidone), SSPE (20Xstock solution: 3.6 M sodium chloride, 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 6XNaCl/Cit, 0.1% SDS (mass/vol.) at 50°C for 30 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 approximatey 16 h at –80°C. Plaques were picked and after two screenings, 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 pPPH3β-3b, and further amplified for analysis. To sequence both strands of pPPH3β-3b, nested deletion mutants were prepared from both ends of the cDNA using the Erase-a-Base System (Promega), named pPPH3β-3b, and further amplified for analysis. To sequence both strands of pPPH3β-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 Novabla (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 pPPH3β-3b cDNA (Fig. 1) using this method. The PPH3β-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),

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 (∼100 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-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 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), 2XDenhardt's (100Xstock solution: 2% (mass/vol.) bovine serum albumin, 2% (mass/vol.) Ficoll, 2% (mass/vol.) poly(vinyl pyrollidone)), 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 6XNaCl/Cit, 0.1% SDS (mass/vol.) at 50°C for 30 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 approximatey 16 h at –80°C. Plaques were picked and after two screenings, 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 pPPH3β-3b, and further amplified for analysis. To sequence both strands of pPPH3β-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 Novabla (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 pPPH3β-3b cDNA (Fig. 1) using this method. The PPH3β-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.
The previously unknown sequence of the PPHβ 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 generated from the predicted 5' cDNA 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 XbaI site. The sequence of the antisense primers (29 nucleotides) was specific for a region located 40 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 BglII (BamHI compatible) sites of the mammalian expression vector, pSG5 (Stratagene) thus creating the expression plasmid, pPPHβ.

The expression plasmid, pPPHβ, was created by a similar strategy and is shown in Fig. 2B. First, PCR primers were designed from the 5' cDNA region of PPHα. The sequence of the sense primer (21 nucleotides) was specific for the start-codon region of PPHα and contained a BamHI 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 PstI site.

Using the newly cloned PPHα 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 (17). Finally, a BamHI—HindIII fragment (1600 bp) containing the 5' cDNA portion of the PPHα cDNA, was then ligated into the EcoRI and BglII (BamHI compatible) sites of the mammalian expression vector, pSG5 (Stratagene) thus creating the expression plasmid, pPPHα.

**Computer analysis of DNA and protein sequences.** Nucleotide sequences were read with the USEEDIT program and Reader (C.B.S. Scientific Company) and initial comparisons were made using the DNASIS program of T. Dardel and P. Bensoussan.
The cells were then labelled with 50 jaCi analysis by SDS/PAGE and fluorography. The media were collected, endo-β-N-acetylglucosaminidase H (endo H) treatment, and before harvesting. The cells were harvested for immunoprecipitation with 10 mM methionine for different time periods before combination. The cell suspension and CaPO₄DNA precipitate was prepared containing 2 pg of the neomycin-selectable marker plasmid pXT1 (Stratagene), together with 20 single colonies/transfection reaction were isolated using cloning and PPH//-specific mAb [23]. The immunoprecipitates were analysed by SDS/PAGE and fluorography (data not shown). Three cell clones were found to express PPH// and PPH//-1. 1 and PPH//-specific mAb [23]. The immunoprecipitates were washed in NaCl/Pj with non-ionic buffer A (Tris/HCl 25 mM, NaCl 50 mM, pH 7.1) for 1 min. The cells were rinsed several times and incubated in medium supplemented with 5% (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 Genetin (400 µg/ml, Sigma) instead of the penicillin/streptomycin mixture. Medium was changed every 2-3 days and cells passed 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 PPHHa and PPH//-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 pX7T1 (Strategene), together with 20 µg of the experimental plasmids, pPPHHa and pPPH//-. The cells 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 NaH₂PO₄, 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 Genetin (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 [³⁵S]methionine and immunoprecipitation with PPHHa-specific antisera [17] and PPH//-specific mAb [23]. The immunoprecipitates were analysed by SDS/PAGE and fluorography (data not shown). Three expressed PPHHa alone, two clones expressed PPH//- alone, and one clone was found to express PPHHa and PPH//-. The cell clones with the highest expression rates, PPHHa-4, PPH//-1 and PPHHa//-2 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 [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 3/7/16/36 [23], which recognizes only the native PPH//- subunit, or with a rabbit antisera (antiPPHHa) raised against denatured PPHHa subunit [17], and which recognises denatured PPH//- 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 pPPHHa or pPPH//- (unpublished results). In immun-gold electron microscopy two polyclonal antibodies, A/1 (specific for the a subunit) and LDV/1 (recognising the a and b 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-PPHHa rabbit sera 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 pre- cleared 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-PPH Ha/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 proteins was essentially as described by Fujiki et al.[26]. Briefly, membranes were prepared by homogenisation in 0.1 M Na₂CO₃, 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 PPHHa 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 dihtiothreitol (1 mM) which reduces disulphide bonds. Proteins were resolved by SDS/PAGE [7.5% (mass/vol.) polyacrylamide] according to Laemmli. The M, standards were myosin, 205000; β-galac-
Eldering et al. (Eur J. Biochem. 247)

**Fig. 3. cONA and deduced amino acid sequence of the β subunit of human intestinal PPII.** The 2326 bp PPII cDNA contains an open reading frame of 700 amino acids beginning from an AUG start-eodon and ending at a TGA stop codon. The dotted underline indicates the amino acid sequence of mature PPMI/.

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tosidase, 45000; carbonic anhydrase, 29000. Gels were

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**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.) formaldehyde (by vol.) in 0.1 M sodium phosphate buffer.

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Potential N-Glycosylation sites are underlined with stars, The putative membrane

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Potentially dystrophic collagen is the astacin and EGF-like domains are in bold type. Conserved

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Potential N-glycosylation sites are underlined with stars, The putative membrane

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RESULTS

PPHβ 5′ cDNA cloning. The 5′ end of PPHc cDNA contained an open reading frame starting with the consensus AUG start codon and overlapped by 60 nucleotides with the near-full-length PPHc cDNA sequence previously obtained [17]. Thus, the complete open reading frame of the PPHc cDNA of 2920 bp encodes 746 amino acid residues (EMBL sequence database, accession no. M82962). The complete cDNA-deduced amino acid sequence of PPHc 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-PPHc [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 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-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. Hydrophathy analysis [31] of PPHβ revealed a hydrophobic region (Leu6–Leu21) containing several potential signal-peptide-cleavage sites [32]. The N-terminal sequence (LATPE) of one such putative propeptide of PPHβ aligns with that empirically determined for mouse meprin [28]. Another hydrophobic stretch consisting of 25 residues (Thr652–Thr676) 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 membrane protein of the astacin family [4, 33].

The proteolytic astacin domain of the β subunit spans 198 residues starting from the N-terminus of the mature intestinal subunit (Asn62) and ending with Lec259. This astacin domain contains the extended zinc-binding motif, HEXXHXXGFXHE, which is found within the extended astacin signature sequence, HEXXXHXXGFXHE (Q,H)XXQRDXDX(Y,P)X(X,H)(V,I) specific for zinc-metallo-endopeptidases of the astacin family [4, 33]. Also within this astacin region are the four conserved cysteine residues (Cys103, Cys124, Cys144, Cys255) which in astacins have been shown to form two disulfide 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 phosphatase 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 (Cys508–Cys643). Extending beyond the hydrophilic region is then the C-terminus composed of 24 mostly basic residues (Cys677–Phe701) constituting the putative cytosolic domain.

Consistent with previous experiments showing PPH from intestinal biopsies to be glycosylated [33], 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...
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 cys- 
osolic 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, pPPHa and pPPHβ, and cell lines that permanently expressed PPHα, PPHβ, and cell lines that permanently expressed PPHα or PPHβ were metabolically labelled with [14C]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.

Results of the pPPHa-transfected MDCK cells are shown in Fig. 5A. Initially, a single PPHα polypeptide (110 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 sulphhydryl-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
PPHα is secreted from the apical membrane domain. MDCK cells expressing PPHα or together with PPHβ 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, PPHα was almost exclusively secreted from the apical plasma membrane domain (Fig. 6B). When expressed together with PPHβ, due to its retention on the cell surface by the β-subunit, only traces of the PPHα 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 LDI/1 antibody (see Experimental Procedures) and show that the β-subunit was present in the microvillus membrane of both cells expressing PPHβ alone or together with PPHα (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 PPHα-specific antibody A/1 (see Experimental Procedures) and show that in MDCK cells expressing only PPHα, this subunit was not present in the microvillus membrane (Fig. 8A). Intranuclearly, the subunit was localised to the nuclear envelope and the ER (Fig. 8C). In MDCK cells expressing both subunits, PPHα was also present on the microvillus membrane (Fig. 8B). In addition, some positive labelling of lysosomes was observed (Fig. 8D). No PPHα was detected on the basolateral membrane (Fig. 8A–C).

Intracellular transport and secretion of PPHα requires proteolytic removal of the transmembrane domain. In pulse/chase experiments with PPHα 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. PPHα-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) was 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. PPHα-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

PPHβ forms heterodimers with PPHα and holds the latter on the cell surface. To examine if the PPH subunits assemble when coexpressed, MDCK cells transfected with pPPHβ alone or together with pPPHα were continuously labelled for 24 h and the subunits immunoprecipitated from the cell surface using PPHα subunit specific antibodies. Fig. 6A shows that in MDCK cells expressing PPHα and PPHβ both forms could be immunoprecipitated from the surface using this PPHβ-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, heterodimerization was also observed by immunoprecipitation of intracellular forms and is thus an early event.

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 PPHβ were detected in the culture medium. The amount of PPHβ in the medium was less than PPHα (Fig. 5A). PPHβ also formed disulphide-bonded 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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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 PPHβ and PPHα show there to be putative prepeptides and propeptides in PPH also. It has been suggested by Gorbach 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 trypsin digestion *in vitro* leads to their activation.

![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 Ldβ/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.](image-url)
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 dimer 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 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 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-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-PPHβ chimera in MDCK cells [20] and with rat meprin in COS-1 cells [19]. The present coexpression studies demon-
strate that PPHα 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, Milliet 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 PPHα 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 to involve the I-domain which is lacking in the β subunit. Two conflicting reports show that in rat meprin cleavage occurs at a furin consensus sequence [50] while in mouse meprin this sequence appears not to be involved [51]. In contrast to rodent kidney meprin/β human PPHβ 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 PPHβ occurs at a later stage than intracellular transport. The significance of this processing of intestinal PPHβ needs further investigation. The difference in processing of PPHα and PPHβ are the subject of work described in the accompanying report by Hahn et al. [52]. The work on processing of off tailswitch mutants strongly suggests that the transmembrane and/or C-terminal domains of PPHα 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 chaperone is involved in ER retention of PPHα. The proteolytic process leading to truncation and subsequent secretion of PPHβ is the subject of further investigation in our laboratory.

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