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Expression of the alpha subunit of PABA peptide hydrolase (EC 3.4.24.18) in MDCK cells

Synthesis and secretion of an enzymatically inactive homodimer

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In this paper, we report the expression of PPHα in the polarized cell line MDCK (Madin Darby canine kidney). In these cells, the enzyme was synthesized in an inactive proform, which upon treatment with trypsin was activated. The enzyme isolated from cell extracts was core-glycosylated and appeared to be retained in the ER as a homodimer. No PPHα was detectable on the surface of intact cells by immunofluorescence. However, a complex glycosylated soluble but inactive form was present in the culture medium, suggesting that proteolytic removal of the C-terminal membrane anchoring peptide leads to the secretion of PPHα.

PABA peptide hydrolase; Astacin; Meprin; Intestine; MDCK; Processing

1. INTRODUCTION

PABA peptide hydrolase (PPH) is a metalloendopeptidase of the human intestinal epithelium which belongs to the family of astacin proteases [1–3]. It is composed of two subunits, alpha and beta, which form multimeric structures. In the preceding paper we reported on the primary structure of the α subunit of PPH [4]. In common with other members of the astacin family of proteases [5–12], PPHα displays a modular organization of different structural domains, including the complete astacin protease domain with its characteristic extended zinc-binding motif, an adhesive domain and an EGF-like domain. PPHα is highly homologous to the alpha subunits of mouse and rat meprin [10,12]. The NH_2-terminal regions of these proteases contain putative signal peptide and propeptide sequences and potential membrane anchoring domains which have been identified near the COOH-termini. The three enzymes thus represent isoenzymes having species-specific differences in tissue expression and possibly also subunit assembly. Accordingly, PPHα has only been found in the gastrointestinal tract in human [4], while in the mouse the α subunit of meprin appears kidney-specific [13,14], and in the rat it is expressed both in intestine and kidney [12]. The assembly of subunits seems also different in the three enzyme. While α_4, $\alpha_2\beta_2$, and β_4 configurations have been reported for the mouse enzyme [15], the rat enzyme has been described as a $\alpha\beta$ heterodimer that forms $(\alpha\beta)_2$ tetramers [11]. By transiently expressing PPHα cDNA in COS-1 cells we have demonstrated the formation of α_2 homodimers [4].

Bond et al. have described latent forms of mouse meprin which could be activated in vitro by limited treatment with trypsin [13]. It is likely that the activation of these proteases involves the proteolytic removal of the pro sequence. X-ray crystal structure analysis of the prototype of these proteases, astacin from the fresh water crayfish *Astacus fluviatilis*, indicated that the NH_2-terminal alanine forms a salt-bridge with a glutamic acid residue close to the catalytic center [16,17]. The presence of a propeptide would clearly hinder such an arrangement.

Here, we report on the expression of PPHα in polarized Madin Darby canine kidney (MDCK) cells. We show by amino acid sequencing, that the enzyme is synthesized in an inactive pro-form in these cells and that activation can be brought about by subsequent treatment with trypsin. The membrane-bound form of PPHα appears unable to exit the endoplasmic reticulum, but proteolytic processing leads to the secretion of a soluble form of the enzyme into the culture medium.

2. EXPERIMENTAL

2.1. *Materials*

[^{35}S]Methionine (1,000 Ci/mmol) was from Du Pont NEN. Cell culture media, penicillin and streptomycin were obtained from GIBCO BRL. Fetal calf serum (FCS) was from Biological Industries. Phenylmethanesulphonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, benzamidine, geneticin (G418), sodium butyrate and molecu-

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Abbreviations: PPH, PABA peptide hydrolase; PPHα, alpha subunit of PABA peptide hydrolase; meprinα, alpha subunit of meprin; FITC, fluorescein isothiocyanate.

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lar weight standards were from Sigma Chemical Co. All restriction enzymes, T4 DNA ligase, DNA polymerase I, Klenow fragment, endoglycosidase F, and endoglycosidase H were purchased from Boehringer Mannheim. Calf intestinal alkaline phosphatase was from Promega. The pXT1 and pSG5 vectors were from Stratagene Cloning System. The protein A Sepharose beads were from Pharmacia Biosystem. All other chemicals were p.a. grade from Merck.

2.2. *Cells and cell culture*

MDCK cells (strain II) were kindly provided by Dr. Kai Simons (European Molecular Biology Laboratory). MDCK cells were grown in Eagle's minimal essential medium with Earle's salts (EMEM) supplemented with 5% (v/v) FCS, 100 units/ml of penicillin and 100 μg/ml of streptomycin. The medium was changed every 2 days. Transfected cells were grown in EMEM supplemented with 5% (v/v) FCS and 400 μg/ml G418.

2.3. *Construction of meprin–PPH-alpha expression vector (pSGMPα)*

The vector for MPα expression was constructed as previously described [4]. Briefly, a ~50 bp *Eco*RI–*Pst*I fragment of PPHα lacking a start codon was substituted with a ~200 bp *Eco*RI–*Pst*I meprinα DNA fragment containing an AUG start codon in pBluescript (Stratagene). The resulting ~2.9 kbp *Bam*HI–*Bgl*II fragment was inserted into the *Bam*HI–*Bgl*II sites of pSG5 yielding the pSGMPα expression vector.

2.4. *Antibodies*

Antibodies against a PPHα–glutathione-*S*-transferase fusion protein were raised in rabbits as previously described [4].

2.5. *Transfection of MDCK cells*

A two vector system was used to establish MDCK cell lines which permanently expressed the meprin–PPHα chimera. The ratio between the expression vector (pSGMPα) and the plasmid containing the neo resistance gene (pXT1) for selection was 10:1. The transfection procedure has been described previously [18].

2.6. *Determination of PPH activity*

MDCK9 cells were grown in 100 mm dishes and stimulated by 8 mM sodium butyrate 16 h prior to enzyme activity determination. PPH activity was assayed according to Sterchi et al. [19]. 50 μl of lysed cells were incubated with 50 μl of *N*-bz-L-Tyr-*p*-amino benzoic acid (20 mmol/l final concentration in 50 mmol/l Tris-HCl,1 mmol/l $MgCl_2$, pH 7.5) for at 37°C for 1 h.

For trypsin activation, lysed MDCK9 cells were incubated with 10 μg trypsin for 1 to 6 h, followed by inactivation of trypsin by the addition of 25 μl soja bean trypsin inhibitor (2 mg/ml). As a negative control, trypsin inhibitor was added together with the trypsin.

2.7. *Metabolic labelling*

Subconfluent MDCK9 cells (60 mm dishes) were stimulated with 8 mM sodium butyrate 16 h before labelling [18]. The cells were incubated in methionine-free EMEM supplemented with 5% (v/v) dialyzed FCS for 1 h and were labelled with 50 μCi [^{35}S]methionine (1 Ci = 37 GBq) for the times indicated in the figure legends. In some experiments the cells were chased after labelling in medium containing 10 mM non-radioactive methionine.

2.8. *Immunoprecipitation*

The cells were washed three times with ice-cold PBS, scraped off in 1 ml ice-cold PBS and collected by low speed centrifugation. After lysis in 330 μl HB (25 mM Tris-HCl, 50 mM NaCl, pH 8.1), containing 1% (w/v) NP40, 1% (w/v) DOC, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 3.5 μg/ml benzamidine and 2 μg/ml aprotinin and removal of cell debris by high speed centrifugation, 7 μl of 20% (w/v) SDS was added and the proteins were denatured by boiling for 5 min. 0.7 ml HB, containing 1% Triton X-100 was added and the probes were precleared with Sepharose-A, followed by immunoprecipitation with rabbit anti PPHα antibody as described previously [4,20].

2.9. *Endoglycosidase H and endoglycosidase F treatment*

After immunoprecipitation the samples were treated with the endoglycosidases as previously described [20,21].

2.10. *SDS-Polyacrylamide gel electrophoresis*

Proteins were analyzed on 7.5% polyacrylamide gels. Electrophoretic separation was performed under reducing or nonreducing conditions. Fixation and fluorography were as described [21].

2.11. *NH_2-terminal amino acid sequence determination*

MPα was immunoprecipitated from non-treated and trypsin-treated MDCK cell lysates as described above, subjected to SDS-PAGE and transblotted onto Problot membrane (Applied Biosystems). Coomassie-stained bands were excised and loaded onto an automatic model 477 sequencer from Applied Biosystems.

2.12. *Immunofluorescence staining*

Postconfluent MDCK9 cells grown on glass coverslips were fixed in 3% paraformaldehyde in PBS$^+$ (PBS with 0.1 mM Ca^{2+} and 0.5 mM Mg^{2+}) for 20 min. The cells were washed three times with PBS$^+$ and incubated with 50 mM NH_4Cl in PBS$^+$ for 20 min. The cells were washed again and incubated with the first antibody (rabbit antiPPHα, diluted 1:20 in PBS supplemented with 2% FCS) for 30 min. The cells were washed twice with PBS–2% FCS and once with PBS, the second antibody (FITC-conjugated swine antirabbit IgG from Dako, Denmark, diluted 1:20 with PBS–2% FCS) incubated for 30 min and washed as described above. The coverslips were covered in mounting medium (Sigma). For intracellular staining, the cells were permeabilized with 0.1% Triton X-100 in PBS after fixation in paraformaldehyde. All procedures were performed at room temperature.

3. RESULTS AND DISCUSSION

We have transfected MDCK cells with a cDNA construct coding for a chimeric enzyme, MPα, composed of the entire mature alpha subunit of human PPH and the NH_2-terminus from the alpha subunit of mouse meprin A which included the putative signal sequence and part of the putative propeptide sequence (Fig. 1). Clone MDCK9 which permanently expressed this chimeric protein was isolated and used for the studies described here.

3.1. *Synthesis of an inactive proenzyme and activation by trypsin*

MDCK9 cells and culture medium exhibited no PPH activity using *N*-Benzoyl-L-tyrosine-*p*-amino benzoic acid as substrate. However, treatment of lysed MDCK9 cells with trypsin lead to activation of the enzyme (Fig. 2). NH_2-terminal amino acid sequencing of the inactive form of MPα revealed the sequence VSIKHL (under-

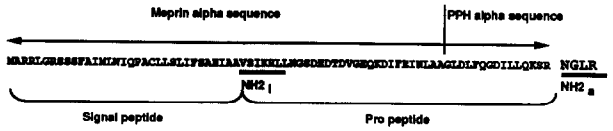

Fig. 1. NH_2-terminal amino acid sequence of MPα with cleavage sites for proteloytic processing. The amino acid sequence from meprina is indicated. For detail of construction of the meprin–PPHα chimera (MPα) see Dumermuth et al. [4]. The NH_2-terminal amino acid sequences (underlined) of inactive MPα (NH_2i) and active PPHα are VSIKHL and NGLR respectively. In addition, the signal peptide and the propeptide sequences are indicated.

lined in Fig. 1), thus confirming the site of the signal peptide cleavage. The NH_2-terminal sequence of the detergent solubilized mature PPHα (NGLR) is also indicated in Fig. 1. An activating process involving proteolytic removal of a propeptide by a trypsin-like enzyme is thus a crucial step in the biogenesis of active PPH. This is in agreement with recent reports on the X-ray crystal structure of astacin [17,22]. Clearly, MDCK cells lack the enzyme for this processing step. Comparison of the NH_2-terminal amino acid sequence of PPHα and PPHβ [4], solubilized from microvillus membrane of human enterocytes by nonionic detergents, indicate that, in the intestine, both subunits are proteolytically processed. This is in contrast to meprin isolated from mouse kidneys, where processing has only been shown for the alpha subunit [23]. The site of proteolytic processing and thus activation is not known. Processing of both subunits in the human intestine suggest that this process might be mediated by pancreatic trypsin.

3.2. *A membrane-bound form of the enzyme is retained in the ER*

Metabolic labelling of MDCK9 cells, followed by immunoprecipitation from solubilized cell extracts led to the isolation of an immature form of the enzyme. A major protein with an apparent $M_r \approx 100$ kDa was synthesized which was sensitive to treatment with endoglycosidase H (Fig. 3A). This sensitivity to Endo H digestion was retained for prolonged chase times, indicating that the protein was retained in the ER. Pulse chase labelling experiments, followed by analysis of immunoprecipitated MPα by SDS-PAGE under non-reducing conditions revealed the occurence of disulfide bridged homodimers (Fig. 3B). The rapid formation of these homodimers confirms our previous findings from organ cultured human small intestinal explants [2] and from transiently transfected COS-1 cells [4]. It is also consistent with the classical function of protein disulfide isomerase, an ER enzyme which mediates protein folding [24].

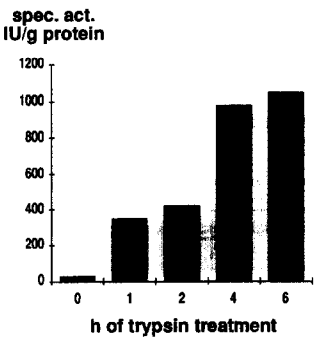

Fig. 2. Activation of PPH activity by trypsin. Transfected MDCK9 cells were lysed with nonionic detergents 16 h after stimulation with sodium butyrate (see methods). Activity of MPα was measured against *N*-benzoyl-L-tyrosine-*p*-aminobenzoic acid (PABA peptide) before and after treatment with trypsin.

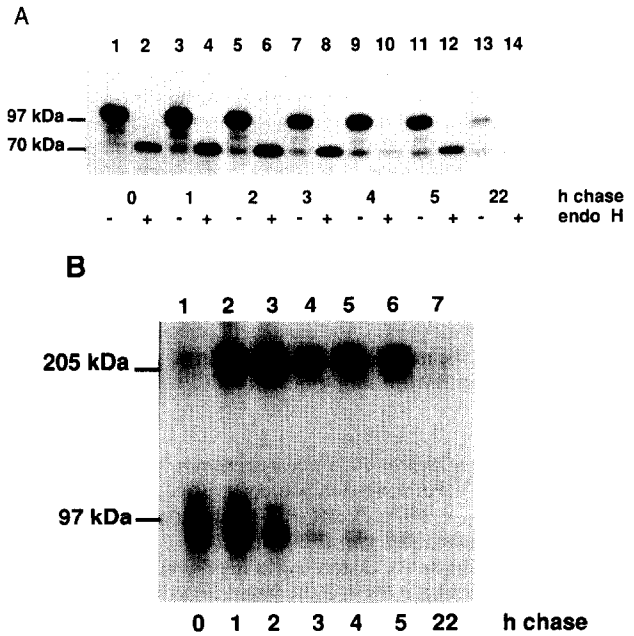

Fig. 3. Metabolic labelling of transfected MDCK9 cells with [^{35}S]methionine. 16 h after stimulation with sodium butyrate, cells were pulse-labelled for 15 min and chased for the times indicated (Fig. 3 A,B), immunoprecipitated and analyzed by SDS-PAGE and fluorography. Endo H treatment of immunoprecipitated proteins was as described [20,21]. (A) Proteins immunoprecipitated from lysed cells and analyzed by SDS-PAGE under reducing conditions (7.5% gel). Position of molecular markers are shown on the left. (B) Proteins immunoprecipitated from lysed cells and analyzed by SDS-PAGE under non-reducing conditions (7.5% gel). Position of molecular markers are shown on the left.

3.3. *No enzyme is found on the cell surface of MDCK9 cells*

Localization of MPα in MDCK9 cells was studied by immunofluorescence labelling, using the PPHα-specific antibodies [4]. When intact MDCK9 cells were labelled, no signal was obtained, indicating that the antibodies had no access to the synthesized protein (Fig. 4B). In contrast, when the cells were permeabilized with Triton X-100 prior to immunofluorescence labelling, a strong signal was obtained (Fig. 4D). The pattern of the labelling obtained with permeabilized cells was characteristic to ER. These findings are consistent with the synthesis of a transport-incompetent membrane-bound form of the enzyme. In human small intestinal mucosa, PPH was localized to the microvillus membrane by immunofluorecence [25] and immuno-electronmicroscopy [1].

3.4. *A soluble form of the enzyme is present in the culture medium*

A decline of the signal was observed after longer chase times and no endo H resistant form was detectable intracellularly. This suggested to us that the newly synthesized protein was either degraded or processed further. To investigate this, the culture medium of

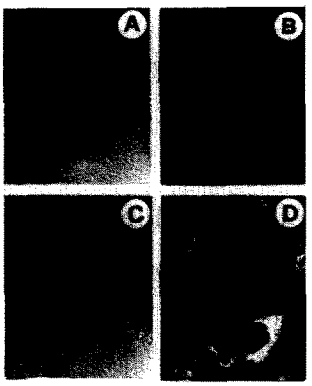

Fig. 4. Immunofluorescence of transfected MDCK9 cells. Cells grown on glass coverslips were fixed with 3% paraformaldehyde as described in Section 2. The first antibody was a rabbit antiPPHα, the second antibody was a FITC-conjugated swine antirabbit IgG. A, cell monolayer of intact MDCK9 cells viewed with Normarsky optics, and B, the same section with immunofluorescence staining. C, cell monolayer of MDCK9 cells permeabilized with Triton X-100 (Normarsky), and D, the same section with immunofluorescence staining.

metabolically labelled MDCK9 cells was immunoprecipitated. Surprisingly, a soluble, endo H resistant form of the enzyme was found (Fig. 5). The enzyme appeared as a double band, both bands displaying a partial resistance to endo H digestion. The nature of these double bands are unknown. This indicates, that complex glycosylation had occurred, but that not all six potential *N*-linked glycosylation sites were affected. For secretion of the enzyme to occur, and assuming that the putative anchor domain actually functions as such, a proteolytic processing step leading to the removal of the anchor sequence must be proposed. The site where this truncation occurs is not known, but the lack of endo H-resistant forms in the cells suggests that this might be an ER event. Transmembrane regions of type I membrane proteins have been implicated as signals for retention in the ER [26]. Maybe the transmembrane domain in PPHα has a similar function, possibly to aid oligomerization and proper folding prior to rapid transport to the cell surface.

Taken together, these data strongly suggest, that a truncated form of PPHα containing the protease domain, the adhesive domain and the EGF-like domain is

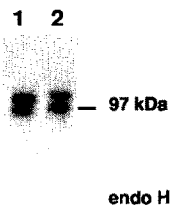

Fig. 5. Immunoisolation of MPα from culture medium of metabolically labelled MDCK9 cells. Metabolically labelled proteins (continuously for 16 h) were immunoprecipitated from the culture medium of transfected MDCK9 cells, and analyzed by SDS-PAGE under reducing conditions (7.5% gel). One sample was treated with endo H prior to analysis (lane 1).

secreted into the lumen of the small intestine. New approaches will be needed to study the role of this multidomain protein in gut physiology.

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