Human meprinβ: O-linked glycans in the intervening region of the type I membrane protein protect the C-terminal region from proteolytic cleavage and diminish its secretion

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Human meprin (hmeprin; *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; EC 3.4.24.18) is a member of the astacin family of zinc metalloendopeptidases. The major site of expression is the brush border membrane of small intestinal and kidney epithelial cells. The enzyme is a type I integral membrane protein composed of two distinct subunits, α and β, which are linked by disulphide bridges. The enzyme complex is attached to the plasma membrane only via the β-subunit. The α-subunit is cleaved in the endoplasmic reticulum in a constitutive manner to remove the C-terminal membrane anchor which leads to secretion of the protein. While the β-subunit of hmeprin remains largely attached to the brush-border membrane some proteolytic processing occurs intracellularly as well as at the cell surface and results in the release of this subunit from the cell. In the present paper, we report that the β-subunit bears multiple O-linked sugar residues in the intervening domain. In contrast, the α-subunit does not contain O-linked oligosaccharides. Our results show that the O-linked carbohydrate side chains in hmeprinβ are clustered around a 13 amino acid sequence that contains the main cleavage site for proteolytic processing of the subunit. Prevention of O-glycosylation by specific inhibitors leads to enhanced proteolytic processing and the consequence is an increased release of hmeprinβ into the culture medium.

Key words: membrane shedding, metzincin, O-glycosylation, proteolytic processing.

INTRODUCTION

Human meprin (hmeprin; *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; PPH; EC 3.4.24.18) is expressed in the brush border membrane of small intestinal and kidney epithelial cells [1]. It was first discovered by its ability to hydrolyse *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid ('PABA-peptide'), a synthetic peptide used for testing exocrine pancreatic function [2]. Hmeprin belongs to the family of astacin zinc metalloendopeptidases [family M12A, clan MB in the MEROPS database for proteolytic enzymes (http://merops.iapc.bbsrc.ac.uk/)] [3–5]. The enzyme comprises two subunits, α and β, both of which are multidomain polypeptides with molecular masses of approx. 95 and 115 kDa, respectively. Meprin α and β are both substantially glycosylated and show the same basic structure (44% identity and 61% similarity of the primary structure), comprising an N-terminal propeptide, the astacin-protease domain with an extended HEXXHXXGFXHE motif [6], the meprin A5 protein tyrosine phosphatase μ-domain (MAM) [7] and the meprin-and-TRAF-homology (MATH) domain [8]. It is speculated that the two latter domains mediate protein–protein interactions. An intervening domain separates all these domains from the C-terminal epidermal-growth-factor-like domain [9], the transmembrane and the cytosolic regions. Both subunits are processed differently in stably transfected MDCK (Madin–Darby canine kidney) and transiently transfected COS-1 cells. The C-terminus of the α-subunit including the membrane anchor region is removed in the rough endoplasmic reticulum (ER) before rapid transport through the cellular compartments and secretion into the culture medium as a complex glycosylated form occurs [10]. The unprocessed form of hmeprinα is retained in the ER and degraded. Although hmeprinβ is largely bound to the brush border membrane, it may also be proteolytically processed, resulting in the release from transfected cells and from intestinal explants in organ culture [11]. Proteolytic processing of hmeprinβ occurs after passage through the Golgi complex and requires a 13-amino-acid sequence (amino acids 595–607) directly adjacent to the epidermal-growth-factor-like region [11]. Due to the fact that the α- and β-subunits form disulphide-linked heterodimers they are predominantly located at the cell surface via membrane-bound hmeprinβ [12]. Cleavage of hmeprinβ does however provide a mechanism for the secretion of the heterodimers. We investigated the glycosylation status of hmeprin α- and β-subunits in view of the possibility that this secretory process may be influenced by particular glycan side chains. Carbohydrate groups may confer a wide variety of important physical and biological recognition properties to glycoproteins such as conformational stability, mediation of appropriate folding, protease resistance, charge and water-binding capacity, protein targeting and host–pathogen, protein–protein and cell–cell interactions (for review see [13–15]). The role of O-linked oligosaccharides is unclear at present since these have not been studied as extensively as N-linked glycans. O-linked sugars are most abundant in mucins and cell-surface glycoproteins but are also found in serum and certain nuclear glycoproteins [16,17]. In mucin-type glycoproteins, O-linked sugar chains are mainly attached via an GalNAc residue to the hydroxy group of threonine or serine. It is not well understood what determines which particular serine or threonine residues are glycosylated but flanking sequences seem to be strongly involved [18–20]. The O-linked carbohydrates are usually clustered within certain regions of the peptide chain. Their occurrence is sometimes found to be coherent with mediating protease resistance as has been shown for human transferrin receptor [21] and even short O-glycosylated sequences have profound effects on the conformation and size of glycoproteins [22]. Involvement in apical targeting of proteins in

Abbreviations used: benzyl GalNAc, benzyl-*N*-acetyl-α-D-galactosamide; endo F, endo-N-glycosidase F; endo H, endo-β-N-acetylglucosaminidase; ER, endoplasmic reticulum; hmeprin, human meprin; MDCK, Madin–Darby canine kidney cells; MEM, minimal essential medium.

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epithelial cells has also been discussed in the case of neurotrophin receptor [23,24], interleukin-2 receptor [25], sucrase–isomaltase [26] and dipeptidyl peptidase IV [27]. A typical structural motif found in membrane proteins is a relatively short O-glycosylated sequence of 20–70 amino acids [22], the so-called stalk which separates the membrane domain from the functional domain as in sucrase–isomaltase [28], maltase–glucoamylase [29], low-density lipoprotein receptor [30] or decay-accelerating factor [31]. These structural elements provide a spacer between the cell membrane and the functional domains and the O-linked glycans are thought to protect these spacer sequences against proteolytic attack [32].

In this paper we show that the hmeprin β-subunit, in contrast with the α-subunit, bears O-linked oligosaccharides in the intervening region. When O-glycosylation of the β-subunit is inhibited a significant increase in secretion can be observed, demonstrating that these glycans are involved in protecting this subunit against post-translational proteolytic processing.

MATERIALS AND METHODS

Reagents and materials

Minimal essential medium (MEM)/Earle's Hepes medium without L-glutamine supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin and 29.2 mg/ml L-glutamine were obtained from Gibco-BRL. L-[^{35}S]Methionine (1000 Ci/mmol) was from DuPont NEN. Endo-β-N-acetylglucosaminidase (endo H), endo-N-glycosidase F (endo F; 'PNGase F'), neuraminidase and O-glycosidase were obtained from Roche. Protein A insoluble cell suspension was from Sigma and Protein A–Sepharose beads were from Amersham Biosciences. All chemicals for gel electrophoresis and the DC (detergent-compatible) Protein Assay Kit were purchased from Bio-Rad. DMSO was from Sigma. Polyclonal antibodies were raised in rabbit against glutathione S-transferase fusion protein from a 330-amino-acid N-terminal fragment of hmeprin α-subunit. O-glycosylation inhibitor benzyl-*N*-acetyl-α-D-galactosamide (benzyl GalNAc) was obtained from Oxford GlycoSystems. Medical X-ray films were from Fuji.

Mutagenesis

Recombinant PCR [33] was used to delete the 13-amino-acid sequence of hmeprinβ that was identified by Pischitzis et al. [11] to be necessary for hmeprinβ shedding. Using pPPHβ [12] as a template (10 ng/μl in a 50 μl final volume) two separate PCR rounds were performed with the following primer pairs: (i) PPHβ832, 5′-AAATGTGTGTGGCATGATCC-3′, and PPH β1805-13s, 5′-ACCTCAACTCTACATGCTCAAAAACCAC-CTGTAAAAATGACG-3′; and (ii) PPHβ2135, 5′-TTCATTA-GCTGGCCATATTGTCG-3′, and PPHβ1805-13a, 5′-GTGG-TTTTTGAGCATGTAGAGTTGAGGTGAGATATGTC-3′. The PCR conditions were as follows. Initial denaturation (4 min at 94 °C) prior to 10 amplification cycles (1 min at 94 °C, 1 min at 63 °C and 1.5 min at 72 °C), followed by 25 amplification cycles (1 min at 94 °C, 1 min at 57 °C and 1.5 min at 72 °C). After combining these two PCR products, a third round of PCR was performed using only the outside primers (PPHβ832 and PHβ2135) under the following conditions. Initial denaturation (4 min at 94 °C) followed by 30 cycles (1 min at 94 °C, 1 min at 57 °C and 1.5 min at 72 °C). The final product was gel-purified, cut with *Eco*NI and *Sac*I and ligated into pPPHβ cut with the same enzymes, yielding plasmid hmeprin$\beta\Delta Q_{595}$-L_{607}. Sequencing was performed to confirm the mutation.

Cell culture

MDCK cells were transfected with pPPHα or pPPHβ [12] for permanent protein expression as described elsewhere [34]. Cells were grown in MEM/Earle's Hepes medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 29.2 mg/ml glutamine. The medium was changed every second day. COS-1 cells, transiently transfected with pPPHβ or the hmeprinβQ_{595}-L_{607} mutant, were grown in MEM/Earle's Hepes medium including 10% fetal calf serum and the supplements mentioned above.

Metabolic labelling

Stable transfected MDCK cells (stimulated with a final concentration of 8 mM sodium butyrate for 12 h) and COS-1 cells grown in 60 mm culture dishes were starved for 1 h in 2 ml of cell-culture medium lacking methionine before pulse-labelling with 75 μCi of [^{35}S]methionine for 60 min. Chase was performed in 2.5 ml of complete medium containing 10 mM L-methionine for the indicated times. The media were collected and filtered through 0.2 μm filters (Millipore). The cells were washed three times in cold PBS (0.15 M NaCl/0.05 M sodium phosphate, pH 7.4), scraped off the dishes and pelleted by centrifugation (1 min at 15000 ***g*** and 4 °C) before immunoprecipitation.

Benzyl GalNAc treatment

Benzyl GalNAc (3 μl/ml of medium; 800 mM in DMSO stock solution [35]) was added to the cells to reach the maximum inhibition concentration of 2.4 mM during 1 h of methionine starving, 1 h of pulse-labelling and during the indicated chase times. To cells not treated with inhibitor DMSO (3 μl/ml of medium) was added alone.

Immunoprecipitation

Hmeprin subunits were immunoprecipitated from cells and media for analysis by SDS/PAGE [36]. Cells were lysed in homogenization buffer (25 mM Tris/HCl, pH 8.0/50 mM NaCl) containing 1% deoxycholic acid and 1% Nonidet P-40, in the presence of protease inhibitors (1 μg/ml pepstatin, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 17.4 μg/ml benzamidine and 1.7 mM PMSF) for 30 min on ice. Proteins were denatured by boiling for 5 min in the presence of SDS at a final concentration of 0.5%. Homogenization buffer containing 1% Triton X-100 was added to a final SDS concentration of 0.1%. Preclearing was performed in the presence of 50 μl of *Staphylococcus aureus* A cell suspension for 1 h at 4 °C followed by immunoprecipitation specific polyclonal antibody (20 μl) coupled to Protein A–Sepharose beads for 1.5 h at 4 °C. Immune complexes were collected by centrifugation (2 min, 15000 ***g***, 4 °C) and washed three times with 0.5% Nonidet P-40/0.05% deoxycholic acid/0.05% SDS in sodium phosphate, pH 7.2, and twice with 125 mM Tris/HCl, pH 8.2/500 mM NaCl/0.5% Nonidet P-40/1 mM EDTA. The media were treated as the cell lysates.

Endo H treatment

Immunoprecipitated proteins were eluted by boiling the Protein A–Sepharose beads in 30 μl of 150 mM sodium citrate/1% SDS before addition of 60 μl of 150 mM sodium citrate/1 mM PMSF. The samples were divided into two equal aliquots. High-mannose core glycosylations were digested by incubation for 20 h at 37 °C with 1 unit of endo H.

Endo F and combined O-glycosidase/neuraminidase/endo F treatment

Immunoprecipitated proteins were collected by boiling the Protein A–Sepharose beads in 25 μl of 20 mM sodium phosphate, pH 7.2/0.5 % SDS. The samples were diluted with 100 μl of 20 mM sodium phosphate, pH 7.2, 1 % Nonidet P-40 and 50 mM EDTA and then divided into two equal aliquots. N-Glycans were digested by incubation for 20 h at 37 °C with 1 unit of endo F. Combined digestion of O-linked oligosaccharides and N-glycans was by incubation for 20 h at 37 °C with 2 units of neuraminidase, 2.5 units of O-glycosidase and 1 units of endo F, respectively.

SDS/PAGE

Immunoprecipitates treated with endo H, endo F or neuraminidase/O-glycosidase/endo F or left untreated were analysed by SDS/PAGE (7.5 % gels) under reducing conditions according to Laemmli [36]. The gels were fixed, soaked in 1 M sodium salicylate for 25 min, dried and subjected to fluorographic analysis.

Determination of enzyme activity and protein concentration

MDCK cells permanently expressing hmeprinβ were incubated for 12 h in the presence of 2.4 mM benzyl GalNAc or DMSO alone. Cells were scraped off the 60 mm dishes in 0.5 ml of homogenization buffer and sonicated three times for 10 s. Media were collected and filtered through 0.2 μm filters (Millipore). Trypsin activation of meprinβ was by incubation overnight with 20 μg/ml trypsin. After adding soya bean trypsin inhibitor to a final concentration of 80 μg/ml, enzyme activities of meprinβ were measured by monitoring the hydrolysis of azocasein into dye-containing peptides according to the procedure described by Wolz and Bond [37]. The protein concentrations were determined using the DC Protein Assay Kit.

Densitometric measurements and molecular-mass determination

Densitometric measurements and determination of molecular masses were carried out with Molecular Analyst v1.4 system from Bio-Rad. Fluorographic signals were scanned densitometrically with 42 μm resolution at 600 d.p.i. (dots per inch) with the 700 Imaging Densitometer (Bio-Rad) and calculated to adjusted volume per absorbance unit $\times$ mm^2 using the Molecular Analyst v1.4 software. To calculate total protein amount the adjusted volume per absorbance unit $\times$ mm^2 values of each hmeprinβ signal from cell extract and corresponding medium were added up and set to 100 %. Molecular masses were determined with Profile Analyser software (Bio-Rad).

RESULTS

In earlier experiments we have shown that the expression of hmeprinα in MDCK and COS-1 cells leads to the secretion of a proteolytically processed and complex glycosylated form into the culture medium. Proteolytic processing occurs in the ER and is necessary for further transport and secretion of the α-subunit [10]. A minor fraction of the hmeprin β-subunit is also proteolytically processed post-Golgi and is subsequently released into the medium [12].

To study glycosylation and processing of human meprins α and β in more detail two approaches were used: (i) biochemical analysis of the glycosylation status using endo H and endo F to remove high-mannose and complex N-glycans respectively and neuraminidase/O-glycosidase digestion to remove O-linked oligosaccharides, and (ii) *in vitro* inhibition of O-glycosylation in cell cultures using benzyl GalNAc.

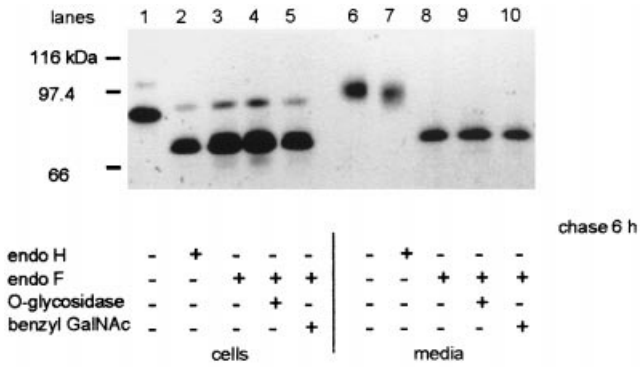

Figure 1 Pulse–chase labelling of hmeprin α-subunit in transfected MDCK cells

MDCK cells permanently expressing hmeprinα were metabolically labelled with [^{35}S]methionine for a 60 min pulse followed by a chase of 6 h. Cells and corresponding media were harvested and proteins immunoprecipitated under denaturing conditions with polyclonal antibody PPH/Nt-1-330. Immunoprecipitates were divided in two equal aliquots and treated with the indicated enzyme(s). For *in vitro* inhibition of O-glycosylation 2.4 mM benzyl GalNAc or DMSO alone per dish was added for 1 h in methionine-free medium, 1 h of pulse-labelling and during chase time. Immunoprecipitates were analysed by SDS/PAGE (7.5 % gels) and submitted to fluorographic analysis. Positions of the molecular-mass markers are indicated on the left.

Hmeprin α-subunit expressed in MDCK cells does not bear O-linked oligosaccharides

Cells were metabolically labelled with [^{35}S]methionine for 1 h and the chase time was 6 h. Intracellularly, a minor 104 kDa form and a predominant 88 kDa form of hmeprinα were found (Figure 1, lane 1), both of which showed endo H sensitivity by shifting to 81 and 74 kDa respectively (Figure 1, lane 2). Digestion of these two forms by endo F did not result in a further molecular-mass shift (Figure 1, lane 3), indicating that these are the two core-glycosylated ER forms of the 104 kDa precursor and the 88 kDa proteolytically processed meprin α-chain respectively. Digestion of these two forms with neuraminidase/O-glycosidase/endo F (Figure 1, lane 4) did not lead to further molecular-mass shifts compared with the endo H-digested sample. In cells treated with benzyl GalNAc and subsequent endo F digestion of the isolated protein no shift in molecular mass was observed compared with endo H-sensitive forms (Figure 1, lane 5). In the culture medium a 95 kDa form was found (Figure 1, lane 6), which showed a slight downwards shift after endo H digestion (Figure 1, lane 7). This indicates that in contrast to the cell-associated hmeprinα, secreted hmeprinα is complex glycosylated. Digestion of this form by endo F resulted in a shift from 95 to 74 kDa (Figure 1, lane 8), the same molecular mass as that found after endo H digestion of the proteolytically processed form in the cell extract. Treatment with neuraminidase/O-glycosidase/endo F did not show any further shift in molecular mass (Figure 1, lane 9). Also, meprinα isolated from the medium of cells treated with benzyl GalNAc and subsequent digestion of the isolated protein by endo F did not show any further downward shift (Figure 1, lane 10). Taken together these results show that the mature form of hmeprinα does not bear any O-linked glycans and is only N-glycosylated.

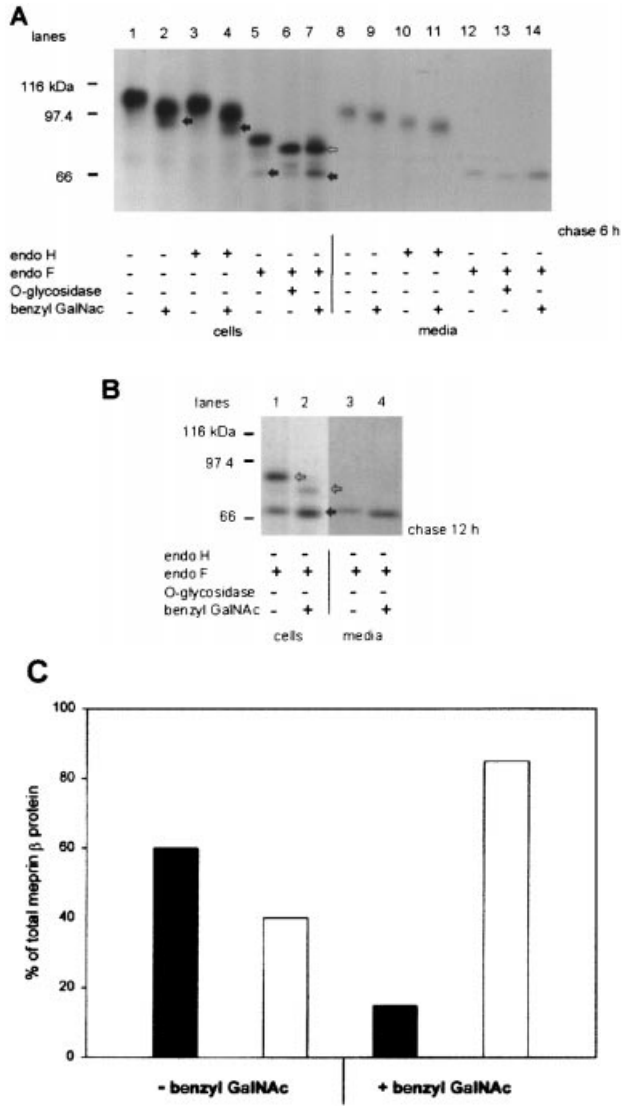

Figure 2 Pulse–chase labelling of hmeprin β-subunit in transfected MDCK cells

MDCK cells permanently expressing hmeprinβ were metabolically labelled with [^{35}S]methionine for a 60 min pulse followed by a chase of 6 h (**A**) and 12 h (**B**). Cells and corresponding media were harvested and proteins immunoprecipitated under denaturing conditions with polyclonal antibody PPH/Nt-1-330. Immunoprecipitates were divided into two equal aliquots and treated with the indicated enzyme(s). For *in vitro* inhibition of O-glycosylation 2.4 mM benzyl GalNAc or DMSO alone per dish was added for 1 h in methionine-free medium, 1 h of pulse-labelling and during chase time. Immunoprecipitates were analysed by SDS/PAGE (7.5% gels) and submitted to fluorographic analysis. Positions of the molecular-mass markers are indicated on the left. In (**A**) the uncleaved and cleaved intracellular forms of hmeprinβ are indicated by a white and black arrows respectively. The fluorograph shown in (**B**) was densitometrically scanned (**C**). The values of cell extract and medium for each condition were summed up and set as 100% of total protein. The relative amounts of uncleaved (■) and cleaved (□) hmeprinβ isolated from treated and non-treated cells after 12 h of chase are shown as a percentage of the total hmeprinβ protein.

Hmeprinβ expressed in MDCK cells bears O-linked oligosaccharides

Cells were metabolically labelled with [^{35}S]methionine for 1 h and chase times were as indicated. In cells not treated with benzyl GalNAc, the hmeprinβ found after 6 h of chase with an apparent molecular mass of 115 kDa (Figure 2A, lane 1) was partially sensitive to endo H (Figure 2A, lane 3). After endoglycosidase F digestion we observed a major downward shift to 83 kDa (Figure 2A, lane 5), which shows that the hmeprinβ isolated from cell extract was complex glycosylated. This form contributes to the mature uncleaved form. A second 70 kDa hmeprinβ form was visible after endo F digestion, which represents the cleaved hmeprinβ (Figure 2A, lane 5). Quantitatively, the uncleaved species was the predominant form. Combined treatment with endoglycosidase F/neuraminidase/O-glycosidase resulted in a further downward shift of both forms to 78 and 68 kDa respectively (Figure 2A, lane 6). The same molecular-mass pattern was found if hmeprinβ isolated from cells treated with benzyl GalNAc was digested with endo F (Figure 2A, lane 7). This shows that, in contrast with the α-subunit, hmeprinβ contains O-linked sugar chains.

Inhibition of O-glycosylation leads to increased intracellular cleavage and subsequent secretion of hmeprinβ

Significant amounts of cleaved hmeprinβ were only found in cells treated with O-glycosylation inhibitor (Figure 2A, lanes 2, 4 and 7). After 12 h of chase under inhibitory conditions the uncleaved form disappeared almost completely in favour of the cleaved form (Figure 2B, lanes 1 and 2).

Under control conditions only a minor fraction of hmeprinβ was secreted into the culture medium (Figure 2A, lanes 8, 10 and 12). Treatment of cells with benzyl GalNAc to inhibit O-glycosylation resulted in increased secretion of cleaved hmeprinβ into the medium (Figure 2A, lanes 9, 11 and 14). The effect of *in vitro* inhibition of O-glycosylation was more prominent after a chase time of 12 h (Figure 2B, lanes 3 and 4). The level of secreted hmeprinβ was thus clearly increased after inhibition of O-glycosylation during cell culture.

To compare the different amounts of cleaved hmeprinβ in cell extract and culture medium under non-inhibitory and inhibitory conditions, the fluorographic signals shown in Figure 2(B) were analysed by densitometric scanning. In untreated cells we found that 60% of the total protein contributed to the uncleaved 83 kDa form and 40% to the cleaved 70 kDa form, respectively, after a 12 h chase (Figure 2C). However, if the cells were treated with benzyl GalNAc to inhibit O-glycosylation *in vitro*, we found a dramatic change in the ratio in favour of the cleaved hmeprinβ form, 85% of which had undergone proteolytic processing, leaving just 15% unprocessed (Figure 2C).

To verify the effect of benzyl GalNAc on the release of hmeprinβ, MDCKβ cells, a stable cell line expressing the hmeprin β-subunit [12], were treated with the inhibitor in three independent experiments. As shown in Figure 3 the amount of hmeprinβ secreted into the medium in cells which were treated with benzyl GalNAc was approx. 4-fold higher compared with medium of untreated cells after 12 h of chase. From the total hmeprinβ protein (cleaved and uncleaved form) 95% contributed to the cell-associated forms and 5% to the secreted form in untreated cells. After inhibition of O-glycosylation, the amount secreted into the medium increased 4-fold to 20% (Figure 3B). O-glycosylation therefore protects hmeprinβ from proteolytic cleavage and reduces secretion of the enzyme.

Inhibiting O-glycosylation does not alter enzymic activity of hmeprinβ

To assess whether O-linked oligosaccharides influence the enzymic activity of the protein, the activity of hmeprinβ against azocasein was determined in cells treated with benzyl GalNAc and compared with non-treated cells. As a negative control wild-type MDCK cells were used (results not shown). Inhibition of O-

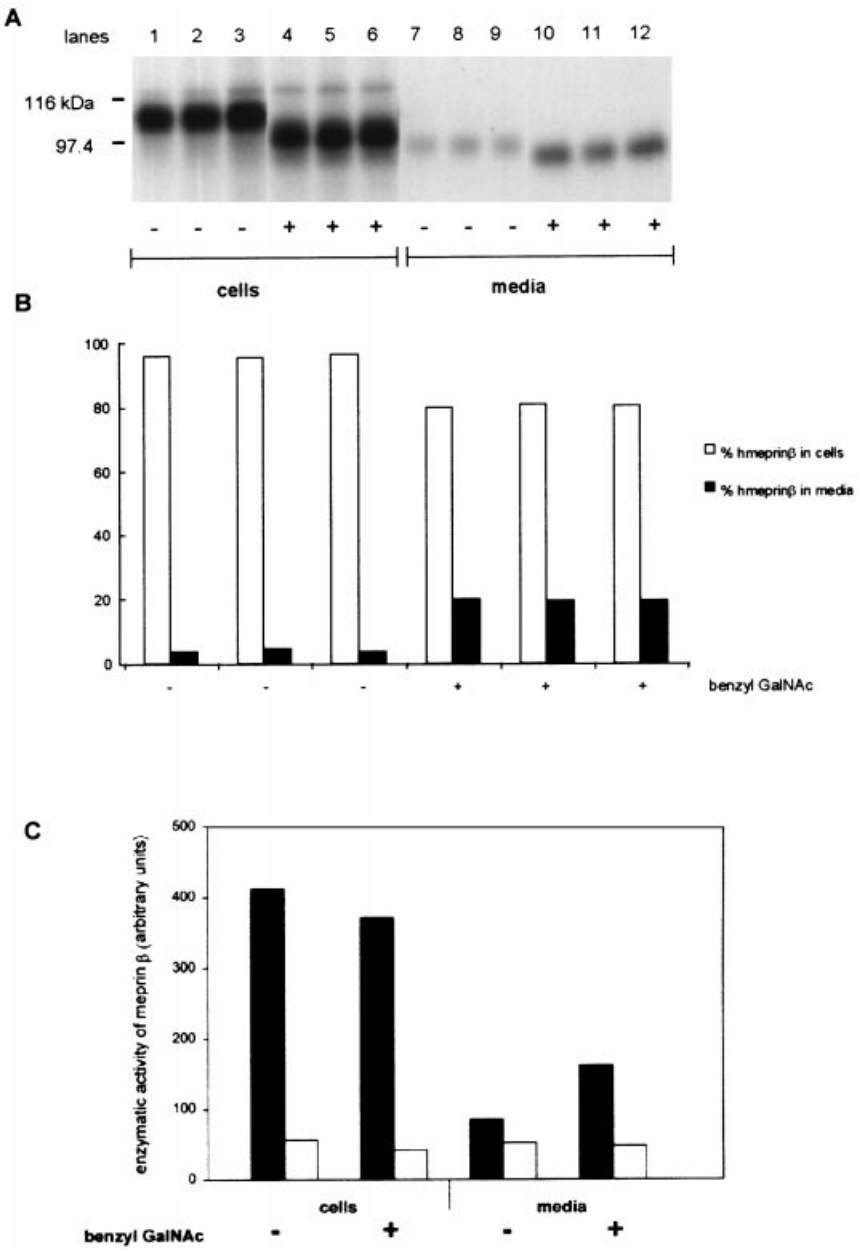

Figure 3 Effect of benzyl GalNAc on the distribution of hmeprinβ protein (A, B) and enzymic activity (C)

(**A**) Three separate experiments showing MDCK cells stably transfected with hmeprinβ cDNA and metabolically labelled with [^{35}S]methionine for a 60 min pulse followed by a chase of 12 h. Inhibition of O-glycosylation was achieved by addition of 2.4 mM benzyl GalNAc or DMSO alone for 1 h in methionine-free medium, 1 h of pulse-labelling and during chase time. (**B**) The fluorograph shown in (**A**) was densitometrically scanned. Protein bands from each lane were added up and the distribution of total hmeprinβ protein between cells and medium were calculated. (**C**) Enzymic activity of hmeprinβ against azocasein was measured with trypsin (■) and without trypsin (□) activation, as described in the Materials and methods section, after incubation of the cells with 2.4 mM benzyl GalNAC or DMSO alone for 12 h.

glycosylation had no direct influence on the ability of hmeprinβ to hydrolyse azocasein (Figure 3C). The azocasein-hydrolysing activity observed with cell fractions untreated by trypsin represents background and was also observed in wild-type MDCK cells (results not shown). Enzyme activity correlated directly with the distribution of relative protein amounts under inhibitory and non-inhibitory conditions respectively. There was a 2-fold increase of hmeprinβ activity in the medium of benzyl GalNAc-treated cells compared with medium of untreated cells. In cell extract of treated cells we found a slight decrease of activity which is concomitant with the lower meprinβ amount found under inhibitory conditions (Figure 3C).

Identification of O-glycosylation sites in hmeprinβ

The 13-amino-acid sequence (^{595}QIQL**T**PAP**S**VQDL^{607}) identified by Pischitzis et al. [11], which is necessary for proteolytic cleavage of the β-subunit, contains Thr^{599} and Ser^{603} residues (shown in bold) that could represent potential O-glycosylation sites as predicted by computer analysis (Figure 4). By deleting the

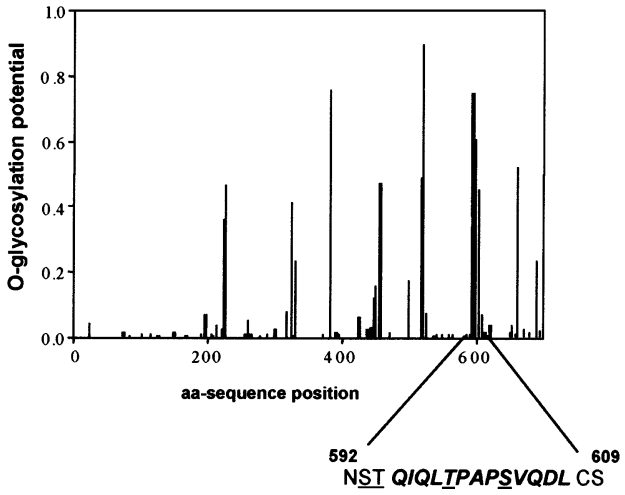

Figure 4 Prediction of O-glycosylated sites in hmeprinβ

The complete amino acid sequence of hmeprinβ was scanned for potential O-glycosylation sites using the NetOglyc prediction engine developed by Hansen et al. [38,39]. The 13-amino-acid sequence involved in C-terminal cleavage of hmeprinβ is shown in bold. Underlines mark the Ser and Thr residues which show a high probability of O-glycosylation.

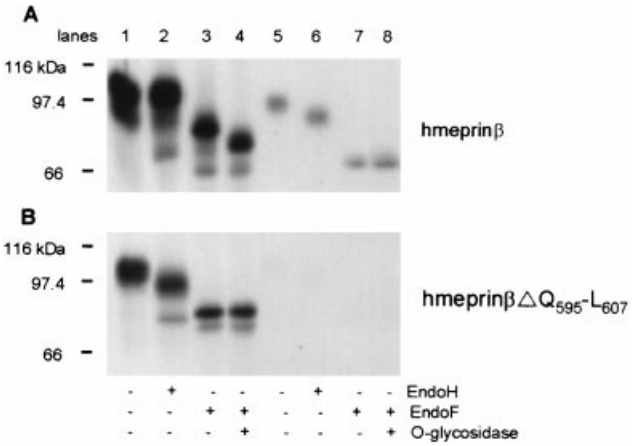

Figure 5 Confirmation of O-glycosylation and cleavage sites in hmeprinβ

COS-1 cells transiently transfected with hmeprinβ (**A**) and hmeprinβQ_{595}-L_{607} (**B**) were metabolically labelled for 1 h, followed by a chase of 3 h. The immunoprecipitated proteins from cells and media were processed as described in the legend to Figure 2.

13 amino acids in hmeprinβ, we were able to demonstrate that these amino acid residues indeed contain the most O-linked glycan chains. In contrast with wild-type hmeprinβ (Figure 5A, lanes 3 and 4), where a shift from the 83 kDa form to 78 kDa was observed upon O-glycosidase treatment, the mutant form lacking the 13 amino acids did not shift downwards (Figure 5B, lanes 3 and 4). This experiment provides further evidence that this 13-amino-acid sequence contains the major cleavage site for proteolytic processing of hmeprinβ, since no secreted form of hmeprin$\beta\Delta Q_{595}$-L_{607} was detectable (Figure 5B, lanes 5–8).

DISCUSSION

The present results show that hmeprinβ bears O-linked oligosaccharides. In contrast, no O-linked glycan chains were found on the α-subunit. Two intracellular protein species of hmeprinβ are found in MDCK cells, an uncleaved and a cleaved form. In the medium only the cleaved form is present. After *in vitro* inhibition of O-glycosylation nearly all of the intracellular hmeprinβ protein was in the cleaved form and an increased secretion into the culture medium was observed. Pischitzis et al. [11] have shown that a 13-amino acid sequence, ^{595}QIQLTPAPSVQDL^{607}, in the C-terminal region is required for cleavage of the β-subunit. Computer analysis of hmeprinβ for the presence of serine and threonine residues bearing potential O-glycosylated carbohydrates using the artificial neural network algorithms developed by Hansen et al. [38,39] supports our experimental findings. A cluster of high O-glycosylation potential was found at Thr^{594} and Ser^{593} just adjacent to the 13-amino-acid sequence and at Thr^{599} and Ser^{603} within that sequence (Figure 4). The molecular-mass shift observed with the cleaved hmeprinβ may be explained by the presence of additional O-linked glycans C-terminal to the cleavage site. The observation that inhibition of O-glycosylation in cells during metabolic labelling results in increased intracellular cleavage of hmeprinβ strongly suggests that one or more O-linked sugar chains efficiently protect this region from proteolytic cleavage. A protective role of O-linked oligosaccharides against proteolysis or proteolytic processing has been shown for several proteins. Human transferrin receptor [21] and decay-accelerating factor [40] are proteolytically cleaved in the absence of O-linked sugars and secreted into the culture medium. Neumann et al. [41] have shown that human insulin-like growth factor-binding protein 6 exhibits greater resistance to proteolysis by chymotrypsin and trypsin if it bears O-linked sugar chains.

O-linked sugar residues are often located within the so-called stalk regions and have been reported for membrane-bound proteins such as sucrase–isomaltase [28], maltase–glucoamylase [29] and low-density lipoprotein receptor [30]. Stalk regions are thought to provide an extension of the functional domains well above the cell surface. O-linked glycans within these regions ensure steric stability and protection against proteolysis when the protein is expressed on the cell surface. Magrane et al. [42] describe such a role of an O-linked sugar domain in the very-low-density lipoprotein receptor. In contrast with the isoform lacking an O-linked sugar domain, which is rapidly cleaved from the cell surface, the O-linked sugar domain of the other isoform seems to be responsible for blocking access to protease-sensitive sites. From previous data we know that both subunits form α/β heterodimers which are associated with the plasma membrane via the β-subunit [12]. *In vivo* hmeprinβ is expressed on the brush border membrane of small intestinal epithelial cells [1] and is thus exposed to a variety of pancreatic proteases in the gut lumen. As only the β-subunit can provide anchoring of the enzyme complex to the plasma membrane, efficient protection of the intervening region against proteases in a hostile environment such as the intestinal lumen is important.

A certain amount of cleaved hmeprinβ is always found in cells not treated with O-glycosylation inhibitor. We have shown that this cleavage process occurs intracellularly after oligosaccharide maturation in the Golgi as well as at the cell surface [11]. It appears to be a regulated process mediated by an as-yet unknown ADAM ('a disintegrin and metalloprotease'; D. Hahn, unpublished work). Aberrant glycosylation resulting in the absence or the incompletion of the O-linked glycan(s) may explain the low steady-state level of cleaved hmeprinβ under control conditions. Several factors such as substrate availability, enzyme (glycosyltransferase) level, enzyme repertoire, residence time of the glycoprotein in a given compartment or the glycoprotein structure determine the glycosylation status of glycoproteins [43,44]. O-linked oligosaccharides protecting hmeprinβ

against cleavage are therefore a determining factor in the release of hmeprinβ homodimers and/or α/β heterodimers from cells.

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