Biosynthesis and shedding of epiglycanin: a mucin-type glycoprotein of the mouse TA3Ha mammary carcinoma cell

Torunn THINGSTAD, Hans L. VOS and John HILKENS[1]

Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Epiglycanin is a mucin-type glycoprotein present at the surface of TA3Ha mouse mammary tumour cells. It is a long rod-like glycoprotein with a molecular mass of 500 kDa. Its function has not yet been established but its overexpression can affect cell–cell and cell–matrix adhesion. To understand better the biological function of epiglycanin, we have studied the biochemical structure and biosynthesis of epiglycanin in TA3Ha cells. Pulse–chase labelling experiments with [^{3}H]threonine revealed an early precursor with a molecular mass of approx. 300 kDa containing approx. 5–10 kDa of N-linked glycans. The precursor was gradually converted into a high-molecular-mass mature form, owing mainly, if not entirely, to O-glycosylation. The mature molecule consists of two major glycoforms that differ in sialylation. Unlike secreted mucins, epiglycanin did not form cysteine-bound multimers, providing further evidence that epiglycanin belongs to the class of membrane-associated mucins. The mature form, but not the precursor form, is shed from the cell surface. The half-life of epiglycanin on the cell surface was found to be approx. 60 h. These results provide the first detailed analysis of the biochemical structure and biosynthesis of epiglycanin.

Key words: epiglycanin precursor, human carcinoma-associated antigen, membrane-associated mucins, O-glycosylation, tumour markers.

INTRODUCTION

Epiglycanin is a sialylated, membrane-associated glycoprotein with a large mucin-like extracellular domain protruding 500 nm above the cell surface [1]. Epiglycanin consists of a polypeptide backbone that is rich in threonine, serine and proline residues [1]. The large number of proline residues in the extracellular domain is responsible for the extended structure, which becomes rigid as a result of the attachment of numerous O-linked glycans to serine and threonine residues. A similar model has been proposed for another murine cell-surface-bound mucin, episialin or muc-1 [2,3]. Epiglycanin is present in large quantities at the surface of the TA3Ha mouse mammary tumour cells. Because of its length and high levels of expression, epiglycanin has been suggested to mask surface molecules, such as class I histocompatibility antigens, and it is therefore considered to be responsible for the ability of TA3Ha cells to grow in allogeneic mouse strains despite their expression of class I MHC antigens [4,5]. Moreover, shielding of MHC class I molecules more easily allows a syngeneic tumour to escape immune attack. Epiglycanin has also been shown to be extremely potent in preventing cell–cell and cell–matrix adhesion [6]. These anti-adhesion effects suggest a possible role for epiglycanin in facilitating the invasion and metastasis of tumour cells.

Several monoclonal antibodies (mAbs) and polyclonal antibodies (poAbs) have been raised against epiglycanin [7,8]. They all share the characteristic that carbohydrates constitute part of their epitopes. Some of these antibodies also detect a human mucin-like molecule, human carcinoma-associated antigen (HCA), which might be the human counterpart of epiglycanin [7,9].

The carbohydrate composition and the structure of the many O-linked carbohydrates bound to the protein backbone have been extensively studied [1,10–12]. However, very little is known about the protein structure of the molecule. In the present study we examined the biosynthesis of epiglycanin by pulse–chase analysis in the TA3Ha cell line to reveal the structural features that have until now been difficult to identify. The results show that epiglycanin is generated through an approx. 300 kDa precursor protein containing only N-linked glycans. The fully mature form is shed from the cell surface with a half-life of approx. 60 h.

EXPERIMENTAL

Cells

Mouse TA3Ha mammary carcinoma cells were maintained in ascites by intraperitoneal inoculation of 4×10^4 cells in syngeneic strain A mice. The cells were harvested on day 7, treated with ery-lysis mix containing 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4, to remove erythrocytes, then washed several times with PBS. TA3Ha cells *in vitro* and TA3St cells were cultured in RPMI 1640 medium supplemented with 10 % (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

Monoclonal antibodies and polyclonal antisera

The Armenian hamster mAbs A23 and C21 against glycosylated epiglycanin were described by Kemperman et al. [6]. The mouse mAb AE3 against glycosylated epiglycanin was described by Haavik et al. [8].

Rabbit antiserum reacting with the polypeptide backbone of epiglycanin (poAb) was raised as follows. The gel fragment containing the precursor of epiglycanin was excised from an

Abbreviations used: ER, endoplasmic reticulum; HCA, human carcinoma-associated antigen; mAb, monoclonal antibody; poAb, polyclonal antibody.

[1] To whom correspondence should be addressed (e-mail jhi@nki.nl).

SDS/5% polyacrylamide gel, rehydrated for 5 min in 50 mM NH_4HCO_3 (400 mg/ml) containing a cocktail of protease inhibitors and crushed. The gel suspension was boiled for 5 min, shaken for 1 h at 37 °C, centrifuged for 10 min at 10000 ***g***, freeze-dried and stored at −20 °C. New Zealand white rabbits were immunized by three subsequent subcutaneous injections, once every 4 weeks, with the eluted precursor dissolved in 0.9% NaCl and emulsified in Freund's adjuvant. Serum was obtained from blood collected on day 7 after the third injection.

Metabolic labelling

For metabolic labelling studies we used TA3Ha cells freshly harvested from murine ascites fluids and kept in culture for the indicated periods. TA3St cells used for metabolic labelling were maintained *in vitro*. Cells (5×10^6) were cultured for 30 min in RPMI medium supplemented with 1% (v/v) fetal calf serum and containing 10% of the usual concentration of glucose (0.45 mg/ml). Subsequently, 50 μCi of [^{3}H]glucosamine was added to label mainly glycoproteins. For [^{3}H]threonine labelling, 5×10^6 cells were starved in 2 ml of threonine-free RPMI medium supplemented with 1% (v/v) fetal calf serum for 30 min, and subsequently labelled for the appropriate duration by the addition of 50 μCi of [^{3}H]threonine. In pulse–chase experiments, the labelled cells were washed in PBS and further cultured in RPMI medium supplemented with 10% (v/v) fetal calf serum for the indicated duration, washed again in PBS and lysed on ice for 30 min in 1 ml of lysis buffer (per 5×10^6 cells) containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% (v/v) Nonidet P40 and protease inhibitors (1 μl/ml aprotinin and 0.2 mM PMSF). After centrifugation, the supernatant was collected and used for further experiments.

Treatment of tissue culture cells with tunicamycin

To inhibit N-linked glycan incorporation, TA3Ha cells (5×10^6) were starved and labelled metabolically as described above in the presence of 10 μg/ml tunicamycin.

Treatment of tissue culture cells with *O*-sialoglycoprotein endopeptidase and benzyl-α-GalNAc

O-Sialoglycoprotein endopeptidase was purchased from Cedarlane Laboratories (Ontario, Canada). Benzyl-α-GalNAc was purchased from Sigma Chemicals. TA3Ha cells (5×10^6) harvested from ascites fluids were incubated with the glycoprotease (12 μg/ml) in RPMI medium at 37 °C for 30 min, to remove all existing epiglycanin. After being washed, the cells were cultured for 2 days in RPMI medium containing 2 mM benzyl-α-GalNAc and subsequently starved of threonine and metabolically labelled in the presence of 2 mM benzyl-α-GalNAc for 30 min, as described in the section on metabolic labelling.

Immunoprecipitation procedures

Rabbit polyclonal antiserum and hamster mAbs A23 and C21 were bound to Protein A–Sepharose CL-4B beads (Amersham Pharmacia Biotech AB). Mouse mAb AE3 was bound to anti-mouse/rat coated cellulose (Sac-cel beads; Wellcome Diagnostics). Coating was performed by incubating 100 μl of 10% bead suspension with 20 μl of antiserum and 5 μl of affinity-purified mAbs for 1 h at 4 °C. The coated beads were collected by centrifugation at 10000 ***g*** for 30 s and washed three times in PBS containing 0.5% (v/v) Nonidet P40. Lysates of labelled cells were added to the beads and incubated at 4 °C for 1–2 h on a rocker platform. The beads were collected by centrifugation at 10000 ***g*** for 30 s and subsequently washed four times in PBS containing 0.5% (v/v) Nonidet P40. Bound proteins were removed by resuspending the immunoprecipitates in Laemmli sample buffer, followed by boiling for 5 min. Unless indicated otherwise, the samples were reduced by adding 7% (v/v) 2-mercaptoethanol to the sample buffer before boiling.

Enzyme treatments

Treatment with neuraminidase

Immunoprecipitates were resuspended in a solution of 20 m-units of neuraminidase from *Vibrio cholerae* (Boehringer Mannheim) in 50 mM sodium acetate buffer, pH 5.5, containing 154 mM NaCl and 9 mM $CaCl_2$, then incubated for 1 h at 37 °C.

Treatment with O-glycosidase

Neuraminidase-treated immunoprecipitates were washed and resuspended in a solution of 2 μl of 0.5 unit/ml O-glycosidase (Boehringer Mannheim) in PBS with the addition of 0.2 mM PMSF, 1 μl/ml aprotinin, 0.5 μl of pepstatin (0.4 μg/μl) and 1.5 μl of trypsin inhibitor (1 μg/μl) and incubated at 37 °C for the durations indicated.

Treatment with endoglycosidase H

Immunoprecipitates were resuspended in a solution of 10 m-units of endoglycosidase H (Boehringer Mannheim) in 50 mM sodium citrate buffer, pH 5.5, with the addition of 10 mM EDTA, 0.02% SDS and 1% (v/v) 2-mercaptoethanol, then incubated for 1 h at 37 °C.

Treatment with N-glycosidase F

Immunoprecipitates were resuspended in a solution of 1 unit of N-glycosidase F (Boehringer Mannheim) in 100 mM sodium phosphate buffer with the addition of 10 mM EDTA, 0.2% SDS, 1% (v/v) Nonidet P40 and 1% (v/v) 2-mercaptoethanol, then incubated for 1 h at 37 °C. The enzyme-treated immunoprecipitates were washed twice in PBS containing 0.5% (v/v) Nonidet P40 and centrifuged at 10000 ***g*** for 30 s.

SDS/PAGE

Lysates of labelled cells or immunoprecipitated material were dissolved in Laemmli sample buffer, boiled for 5 min and separated on a 3% (w/v) stacking/5% (w/v) separating polyacrylamide gel. Prestained SDS/PAGE molecular mass markers (broad range; Bio-Rad) were used as a reference. After electrophoresis, the stacking gels were usually removed, and gels were fixed and stained in methanol/acetic acid/water (4:1:5, by vol.) containing Coomassie Brilliant Blue, then destained in methanol/acetic acid/water (3:2:35, by vol.). The gels were then incubated for 30 min in Amplify (Amersham Life Science), and then dried and exposed to Kodak X-OMAT AR films at −70 °C. In some cases the stacking gel was not removed; in those cases the boundary between the stacking and separating gels is indicated with an arrow on the photographs of the autoradiograms.

FACScan analysis

TA3Ha cells (2×10^5) were washed and resuspended in PBS or PBS containing a 1:20 dilution of neuraminidase from *Vibrio cholerae*. After incubation for 30 min at 4 °C, the cells were centrifuged and resuspended in 200 μl of RPMI medium without

fetal calf serum but containing the hamster mAbs A23 and C21 at saturating concentrations. The cells were incubated for 1 h at 4 °C, washed and resuspended in 150 μl of a 1:40 dilution of FITC-labelled rabbit anti-hamster immunoglobulin (Nordic, Tilburg, The Netherlands). After incubation for 30 min at 4 °C, the cells were washed and resuspended in 200 μl of PBS. Flow cytometric analysis was performed with a FACScan (Becton–Dickinson). A scatter window was set to eliminate dead cells.

RESULTS

Identification of epiglycanin by SDS/PAGE

The amino acid composition analysis by Codington and Haavik [1] showed that threonine is one of the most abundant essential amino acids present in epiglycanin (15%). We therefore used ^{3}H-labelled threonine to label epiglycanin preferentially. When a lysate of TA3Ha cells labelled for 3 h with [^{3}H]threonine was analysed by SDS/PAGE, autoradiography revealed that almost all of the incorporated label was concentrated in a diffuse high-molecular-mass band barely penetrating the gel (Figure 1A, first lane) and in some minor bands of low molecular mass (less than 100 kDa). mAbs A23, C21 and AE3, which are directed against epiglycanin [6,13], immunoprecipitated [^{3}H]threonine-labelled molecules with slightly dissimilar mobilities, but all were clearly within the region of the diffuse high-molecular-mass band from the lysate (Figure 1A). Moreover, the high-molecular-mass band was not present in the [^{3}H]threonine-labelled lysate of the closely related TA3St cells, which are known to have lost epiglycanin expression [14] (Figure 1B). Preclearing of the radiolabelled lysate with the same mAbs as the actual immunoprecipitation revealed that this protein was almost completely removed by each antibody (Figure 1A, lanes labelled cl). These results indicate that the diffuse high-molecular-mass band represents primarily if not exclusively epiglycanin, and that almost all [^{3}H]threonine label is incorporated into epiglycanin and some unknown proteins with a molecular mass of less than 100 kDa.

Although mAbs A23 and C21 are both directed against epiglycanin, as has been described previously [6], the slightly different mobilities of the immunoprecipitated molecules on SDS gels suggest that they detect different glycoforms of epiglycanin (see below). Both mAbs are hamster IgGs and the specificity of mAb A23, which is mainly used in this study, is shown in Figure 1(B). mAb A23 immunoprecipitates high-molecular-mass molecules from TA3Ha cells but no similar molecules from the closely related TA3St cell line. Neither does normal hamster serum precipitate the high-molecular-mass molecules from the TA3Ha cells (Figure 1B). mAbs C21 and AE3 showed very similar specificity patterns on TA3Ha and TA3St cells (results not shown).

The AE3 epitope has been described in detail by Haavik et al. [13] and consists of clustered T (Galβ1-3GalNAc-O-Ser/Thr) disaccharides with a specific spacing dependent on the peptide backbone. The precise epitopes recognized by the hamster mAbs are not known but treatment of TA3Ha cells with neuraminidase completely abolished the reaction with the C21 antibody but not that with the A23 and AE3 mAbs, as measured in a FACScan analysis (Figure 1C). This demonstrates that sialic acid is part of the epitope of the C21 antibody but not of the A23 and AE3 antibodies. This is in accordance with the results shown in Figure 1(A), in which mAbs A23 and AE3 immunoprecipitated mainly the molecules represented by the most slowly migrating upper part of the diffuse band, representing less sialylated forms of the protein, whereas C21 immunoprecipitated faster-migrating molecules, which are highly sialylated (see below).

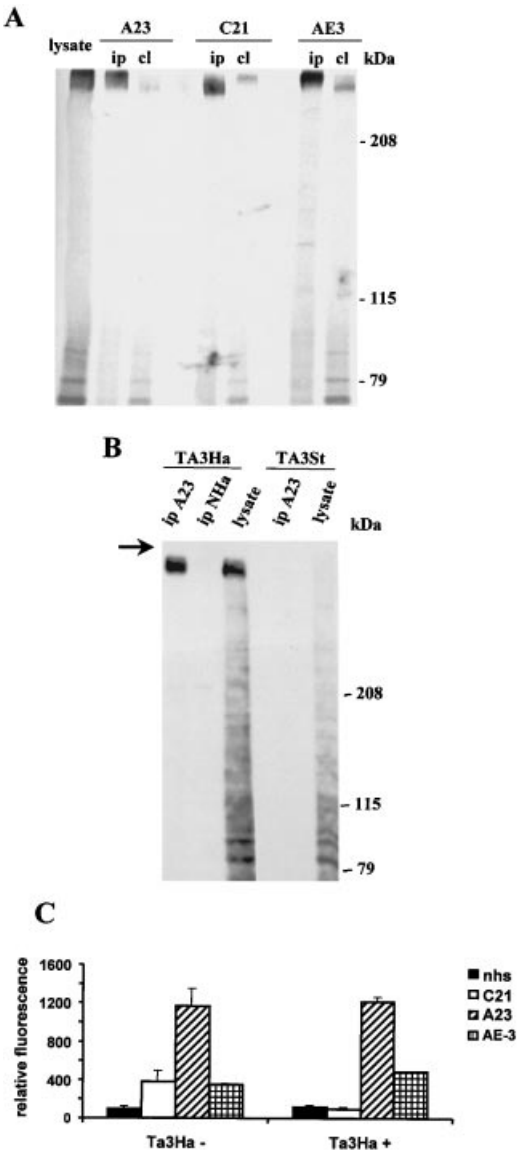

Figure 1 Identification of epiglycanin

(**A**) TA3Ha cells were labelled for 3 h with [^{3}H]threonine and lysed in lysis buffer; epiglycanin was immunoprecipitated from the cell lysate with the mAbs A23, C21 and AE3. The cell lysate, immunoprecipitated material (ip) and the cleared cell lysate after two subsequent immunoprecipitations with the same mAbs (cl) were analysed on an SDS/5% polyacrylamide gel and detected by autoradiography. (**B**) Epiglycanin-positive TA3Ha cells and epiglycanin-negative TA3St cells were labelled for 3 h with [^{3}H]threonine and lysed in lysis buffer; epiglycanin was immunoprecipitated from the cell lysate with mAb A23 or normal hamster serum (NHa). The non-treated lysate is shown as control. In (**A**) and (**B**) the positions of molecular mass markers are indicated at the right. (**C**) Sensitivity of the A23, C21 and AE3 epitopes to treatment with neuraminidase. Ta3Ha cells were first treated with neuraminidase at 4 °C for 30 min (TA3Ha+) or PBS only (TA3Ha−); the cell surface expression of epiglycanin was then measured by FACScan analysis with mAbs A23, C21 and AE3. C21 did not detect epiglycanin after treatment with neuraminidase. Normal hamster serum (nhs) was used as control. Each bar represents the mean of three separate cell samples. The S.D. is indicated.

Identification of a precursor of epiglycanin by pulse–chase analysis

To study the structure of epiglycanin in more detail, we performed pulse–chase experiments to identify possible precursor proteins. TA3Ha cells were labelled with [^{3}H]threonine for 10 and 30 min,

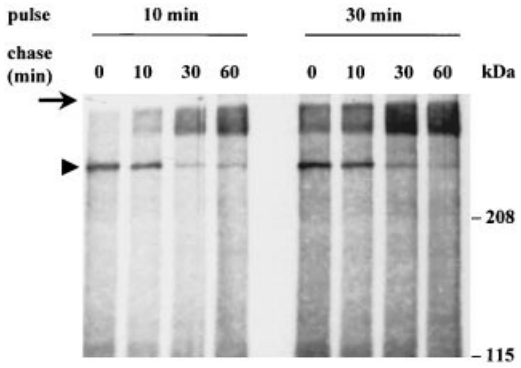

Figure 2 Pulse–chase analysis

TA3Ha cells were pulse-labelled with [^{3}H]threonine for 10 and 30 min and chased for the durations indicated. The cells were lysed in lysis buffer and the cell lysates were analysed on an SDS/5% polyacrylamide gel. An arrowhead indicates the position of the 300 kDa precursor; an arrow indicates the boundary between the stacking and separating gels. The positions of molecular mass markers are indicated at the right.

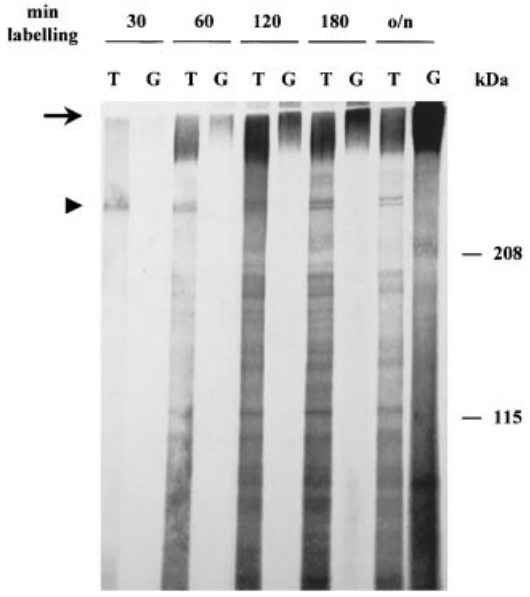

Figure 3 Glycosylation of epiglycanin

TA3Ha cells were labelled with [^{3}H]threonine (T) and with [^{3}H]glucosamine (G) for the durations indicated and cell lysates were analysed on an SDS/5% polyacrylamide gel. An arrowhead indicates the position of the precursor band. Note that the band appearing after 60 min of labelling with [^{3}H]threonine just above the precursor band is non-specific and is not related to the precursor. An arrow indicates the boundary between the stacking and separating gels. Abbreviation: o/n, incubation overnight. The positions of molecular mass markers are indicated at the right.

followed by different chase periods in medium containing an excess of unlabelled threonine. After 10 min of labelling, we observed a single band of approx. 300 kDa; after 30 min of labelling the diffuse high-molecular-mass bands representing mature epiglycanin were present in addition to the 300 kDa band (Figure 2). During the chase periods, the band representing the 300 kDa protein was gradually replaced by the bands representing the high-molecular-mass form of epiglycanin. The 300 kDa protein had all the characteristics expected of a precursor of a mucin: it had a high molecular mass, it was the molecule most strongly labelled by [^{3}H]threonine (reflecting a high threonine content), as is the mature molecule, and it was being chased into a form with a much higher molecular mass, indicating extensive post-translational modifications. We tentatively concluded that this band represented a precursor of epiglycanin. The band representing the precursor molecule seemed to be less intense than the bands representing the mature molecules after the chase, even though the cells had been labelled for the same duration in all instances. This apparent discrepancy was most probably due to the higher sensitivity of the underglycosylated precursor molecule to proteolytic degradation during and after cell lysis than the highly glycosylated mature form, as has also been shown for MUC1/episialin [15].

The precursor band of epiglycanin could be observed after only 5 min of labelling with [^{3}H]threonine. In addition, upon continued labelling the intensity of the precursor band gradually decreased and was fully replaced by the high-molecular-mass form after 2 h (results not shown). Only this high-molecular-mass form of epiglycanin could be immunoprecipitated by mAbs A23, C21 and AE3. Thus a positive identification of the 300 kDa precursor band by immunoprecipitation was not possible because the mAbs require post-translational modifications for their reactivity (as discussed above for C21 and AE3).

Post-translational modifications of epiglycanin

When TA3Ha cells were labelled for 60 min with [^{3}H]threonine or [^{3}H]glucosamine, the major radioactive product appeared in both cases as a diffuse band at a similar position in the gel, just below the boundary between the stacking and separating gels (Figure 3). The band increased in intensity after longer labelling periods with both labels. mAb A23 immunoprecipitated precisely the same band, barely penetrating the separating gel, from both [^{3}H]glucosamine-labelled and [^{3}H]threonine-labelled cell lysates. The [^{3}H]threonine-labelled and [^{3}H]glucosamine-labelled molecules could be almost entirely cleared from the lysate by mAb A23 (results not shown), indicating that this [^{3}H]glucosamine-labelled band was also epiglycanin. The relatively strong labelling with [^{3}H]glucosamine and the heterogeneity of the band was indicative of extensive glycosylation of the mature form of epiglycanin. Glucosamine is rapidly converted into *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid; therefore the radioactivity of [^{3}H]glucosamine can be incorporated into both N-linked and O-linked glycans. However, on labelling with [^{3}H]glucosamine, no label was incorporated in the precursor protein represented by the 300 kDa band at any labelling time. Even after labelling overnight, when glycosylated proteins could be seen at almost all positions in the gel, no band at the position of the precursor could be observed. This result suggests that the 300 kDa precursor was not glycosylated. However, epiglycanin has been reported to contain, in addition to O-linked glycans, at least two N-linked glycans [11], and the latter must already be present in the precursor. The number of N-linked glycans is probably too small to show a radioactive signal after labelling with [^{3}H]glucosamine.

N-glycosylation

Tunicamycin inhibits the attachment of N-linked sugars to the protein backbone, which results in a lower molecular mass of the protein. To confirm the presence of N-linked glycans in epiglycanin, cells were labelled for 30 min with [^{3}H]threonine in the presence or absence of tunicamycin and the labelled lysates were

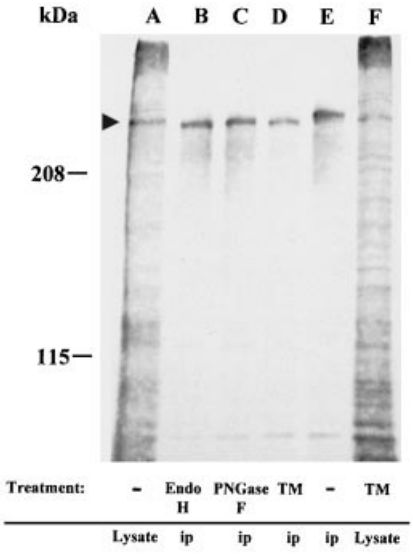

Figure 4 N-glycosylation of epiglycanin

TA3Ha cells were labelled for 30 min with [^{3}H]threonine and lysed in lysis buffer. Lane A, cell lysate; lane B, cell lysate immunoprecipitated with the poAb raised against the precursor and subsequently digested with endoglycosidase H (Endo H); lane C, lysate immunoprecipitated with the anti-precursor poAb and digested with N-glycosidase F (PNGase); lane D, lysate of TA3Ha cells labelled in the presence of tunicamycin and immunoprecipitated with the anti-precursor poAb; lane E, lysate immunoprecipitated with the anti-precursor poAb; lane F, lysate of TA3Ha cells labelled in the presence of tunicamycin. The samples were analysed by SDS/PAGE [5% (w/v) gel]. Abbreviations: ip, immunoprecipitated material; TM, lysates from tunicamycin-treated cells. The positions of molecular mass markers are indicated at the left.

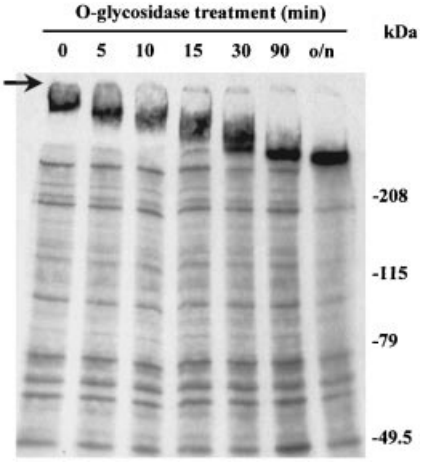

Figure 5 O-glycosylation of epiglycanin

TA3Ha cells were treated with O-glycoprotein endopeptidase to cleave all existing epiglycanin from the cell surface and then cultured for 2 days in medium containing benzyl-α-GalNAc. The treated cells were labelled for 3 h with [^{3}H]threonine in the presence of benzyl-α-GalNAc and lysed in lysis buffer. Epiglycanin was immunoprecipitated from the cell lysate with mAb AE3 and the immunoprecipitates were treated with neuraminidase followed by O-glycosidase for the durations indicated. o/n: overnight incubation. The samples were analysed on an SDS/5% polyacrylamide gel. An arrow indicates the boundary between the stacking and separating gels. The positions of molecular mass markers are indicated at the right.

analysed by SDS/PAGE (Figure 4). As expected, treatment with tunicamycin resulted in a small shift in molecular mass of epiglycanin's precursor, indicating that it did indeed contain N-linked glycans.

A poAb raised against the 300 kDa precursor of epiglycanin (see the Experimental section) specifically immunoprecipited the precursor of epiglycanin and did not react with the mature protein (Figure 4, lane E). Immunoprecipitation of the lysates prepared from tunicamycin-treated cells with the newly developed rabbit antiserum showed that this poAb still reacted with the precursor of epiglycanin, thus proving that the antiserum was directed against a peptide epitope in epiglycanin (Figure 4, lane D). When the precursor was immunoprecipitated with this poAb and was subsequently treated with endoglycosidase H and N-glycosidase F (enzymes that remove high-mannose and complex forms of N-linked glycans respectively), the molecular mass of the early precursor decreased to the same extent as after treatment with tunicamycin (Figure 4). The sensitivity to treatment with endoglycosidase H indicates that the N-linked carbohydrates in the precursor were still present in the high-mannose configuration and had not undergone modification to more complex forms. This confirms the notion that the 300 kDa precursor form of epiglycanin was present in the endoplasmic reticulum (ER).

O-glycosylation

To show directly the presence of O-linked glycans on mature epiglycanin, TA3Ha cells were labelled with [^{3}H]threonine in the presence of the sugar analogue benzyl-α-GalNAc as described in the Experimental section. Next, epiglycanin was immunoprecipitated with mAb A23 and treated for different periods with neuraminidase and O-glycosidase; combined with the treatment with benzyl-α-GalNAc, this removed O-linked glycans. Benzyl-α-GalNAc inhibits O-glycosylation by competing with endogenous Tn (GalNAc-O-Ser/Thr) disaccharides and, after conversion into benzyl-α-GalNAc-Gal, with endogenous T (Galβ1-3GalNAc-O-Ser/Thr) disaccharides as acceptors for the glycosyltransferases that add sialic acid and galactose to the GalNAc of O-linked glycoproteins [16–20]. It has been shown that treatment with benzyl-α-GalNAc causes the accumulation of mucins with Tn and particularly T carbohydrate structures [16,21]. The T antigen is the substrate for O-glycosidase. SDS/PAGE analysis shows that the band corresponding to epiglycanin shifted to a lower molecular mass (Figure 5), demonstrating that the mature form of epiglycanin contains a large number of O-linked glycans. The molecular mass of epiglycanin after this treatment was decreased almost to that of the precursor, indicating that the major shift in molecular mass during biosynthesis must have been the result of O-glycosylation. The remaining difference was most probably due to residual GalNAc residues on the protein backbone, although additional post-translational modifications cannot be excluded.

Epiglycanin consists of at least two major sialylated forms

To investigate the presence of sialic acid on the mature molecule, lysates of TA3Ha cells labelled for 30 min and 3 h with [^{3}H]threonine were immunoprecipitated with mAb A23 and subsequently treated with neuraminidase. The neuraminidase treatment resulted in a single narrow high-molecular-mass band with a very low mobility on the SDS/polyacrylamide gel (Figure 6A). Because SDS binds poorly to highly glycosylated proteins, the mobility of these molecules in SDS-containing gels is determined mainly by the negatively charged sialylated glycans [15]. Therefore the removal of sialic acid residues from highly glycosylated proteins such as mucins results in a lower mobility in SDS/polyacrylamide gels. In this way, the shift in mobility of mature epiglycanin demonstrates that mature epiglycanin does

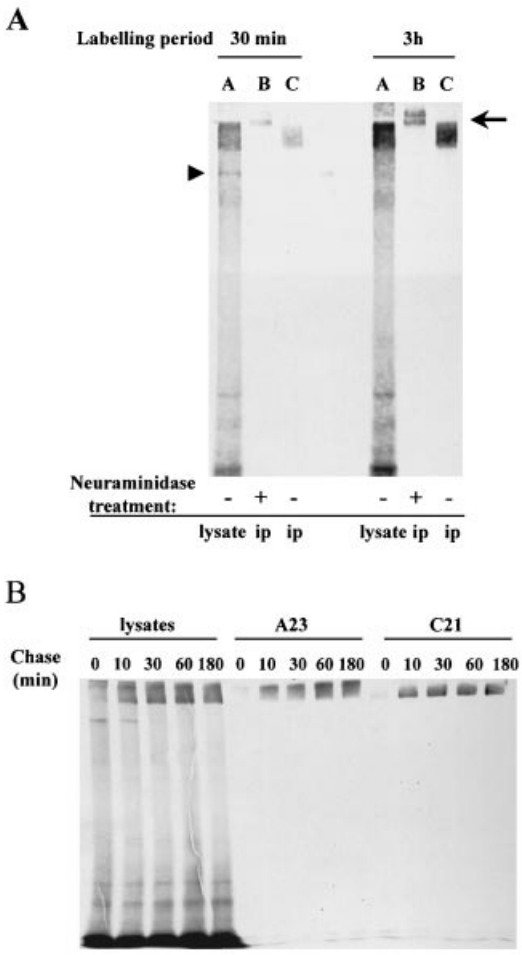

Figure 6 Sialylation of epiglycanin

(**A**) TA3Ha cells were labelled with [^{3}H]threonine for 30 min or 3 h. Lanes A, cell lysate; lanes B, cell lysate immunoprecipitated with mAb A23 and digested with neuraminidase; lanes C, cell lysate immunoprecipitated with mAb A23. The samples were analysed on an SDS/5% polyacrylamide gel. An arrowhead indicates the precursor of epiglycanin and an arrow the boundary between the stacking and separating gels. Note that the treatment with neuraminidase of cells labelled for 3 h with [^{3}H]threonine seems to give rise to two bands on the SDS/polyacrylamide gel. However, this is a single band separated into two by the boundary of the stacking and separating gels. (**B**) Two major sialylated forms of epiglycanin. TA3Ha cells were labelled with [^{3}H]threonine for 3 h and subsequently cultured in unlabelled medium for the durations indicated. Cell lysates and epiglycanin immunoprecipitated with mAbs A23 and C21 were analysed by 5% (w/v) PAGE.

indeed contain numerous glycans with terminal sialic acids and that the differential mobilities of the two forms of the mature molecule are the result of variation in sialylation.

When TA3Ha cells were labelled briefly with [^{3}H]threonine and subsequently chased with unlabelled medium for different periods, the broad smear representing mature epiglycanin was manifested by two pronounced bands, which slowly merged into one diffuse band with lower mobility (Figure 2). To examine whether the upper epiglycanin band represented an undersialylated intermediate precursor, TA3Ha cells were labelled with [^{3}H]threonine and chased with unlabelled threonine for different periods from 0 to 180 min. Next epiglycanin was immunoprecipitated with mAbs A23 and C21, which detect different sialylated forms of epiglycanin. In the chosen time range, the time point of appearance of the upper band, immunoprecipitated by mAb A23, did not differ from that of the lower band immunoprecipitated by mAb C21 (Figure 6B). Thus both the undersialylated and sialylated molecules were probably formed at the same time point and their intensities accumulated at the same rate with longer chase times.

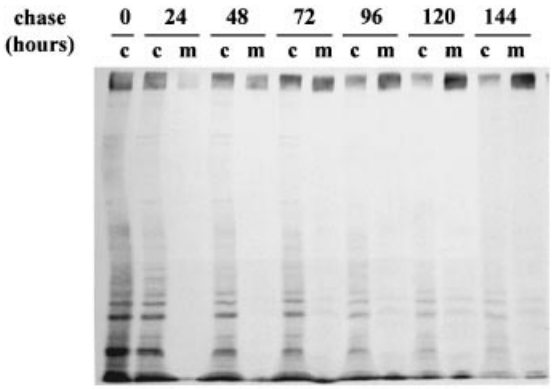

Figure 7 Shedding of epiglycanin from the cell surface

TA3Ha cells were labelled with [^{3}H]threonine for 3 h and subsequently cultured in unlabelled medium for the durations indicated (chase). Culture medium (m) and cell lysates (c) were analysed by SDS/PAGE [5% (w/v) gel].

Epiglycanin does not form cysteine-linked multimers

No difference could be seen in the mature epiglycanin bands on the SDS/polyacrylamide gel when lysates of [^{3}H]threonine-labelled TA3Ha cells were analysed under reducing and under non-reducing conditions (results not shown). This proves that epiglycanin, unlike secreted mucins, does not consist of S–S-bound multimers, which is in accordance with the lack of cysteine residues in the amino acid composition [1].

Shedding from the cell surface

Epiglycanin is shed from the cell surface into the medium [1,22]. To study the half-life of epiglycanin on the cell surface, TA3Ha cells were labelled for 3 h with [^{3}H]threonine and subsequently grown in medium containing unlabelled threonine for various periods ranging from 0 to 6 days, followed by SDS/PAGE analysis (Figure 7). Radiolabelled epiglycanin was detectable in the culture medium after 24 h of incubation. The amount of radiolabelled epiglycanin in the medium increased with the duration of the chase, while the amount of labelled mucin in the cells decreased. The same result was obtained by the immunoprecipitation of epiglycanin from cell lysates and media containing mAb A23 (results not shown), indicating that the background of labelled, non-epiglycanin molecules was negligible. Radiolabelled epiglycanin in the medium of the TA3Ha cells could have been the result of non-specific cell death or could have been present on shed membrane vesicles rather than by active shedding. However, the percentage of dead cells, as determined by Trypan Blue exclusion, was only 2% or less during the first 4 days of the chase and then gradually increased to 11% on day 6 of the chase. This low percentage of dead cells could not have been responsible for the observed rate of shedding and the large amount of epiglycanin in the medium. Moreover, small membrane vesicles or fragments were removed by centrifugation. Densitometric analysis of three independent experi-

ments revealed that 50 % of the shedding occurred within approx. 60 h. The 300 kDa precursor was not detected in the culture medium. This is consistent with the concept that the 300 kDa polypeptide is the precursor form of epiglycanin, which is mainly restricted to the ER and possibly to the early Golgi.

DISCUSSION

Here we report the detailed analysis of the structure and biosynthesis of epiglycanin, which has been reported to be a member of the class of cell surface associated mucins [1]. This identification was based on electron microscopy, but concrete biochemical evidence was lacking. Our experiments show that neither the mature form nor the precursor of epiglycanin can form S–S-bound multimers, which is one of the characteristics of secreted gel-forming mucins. This finding therefore provides conclusive evidence that epiglycanin belongs to the class of membrane-bound mucins. Epiglycanin is not identical with the two best-known membrane-associated mucins, the mouse counterpart of human episialin/MUC1, or rat and human sialomucin complex/asialoglycoprotein/MUC4 [23,24], because no RNA for these molecules is expressed in the TA3Ha cells (H. L. Vos, S. Van der Valk and J. Hilkens, unpublished work). Epiglycanin is also unlikely to be identical with MUC3 [25] because it is not present in normal murine intestines, as detected with the mAbs, and the amino acid compositions of murine Muc-3 and epiglycanin are different (compare [1] and [26]). However, we cannot exclude the possibility that epiglycanin is the murine counterpart of the recently identified MUC11 and MUC12 [27], which are both also membrane-bound mucins.

Our results show that epiglycanin is synthesized from a precursor of approx. 300 kDa comprising the protein backbone with a few attached N-linked glycans. The precursor is evident within 5 min and is converted within approx. 30 min into highly O-glycosylated molecules of high molecular mass, which could be unambiguously identified as epiglycanin by immunoprecipitation with mAbs against glycosylated epiglycanin. Although the mAbs against the high-molecular-mass molecules do not immunoprecipitate the precursor form and the poAb against the precursor does not recognize the mature molecules, all our results indicate that the 300 kDa molecule is an underglycosylated precursor of epiglycanin: (1) the precursor and the mature molecules are the only molecules above 100 kDa that significantly incorporate [^{3}H]threonine; (2) pulse–chase experiments show that the precursor disappears at the same rate and at the same time point as the mature molecule appears; and (3) removing most of the O-linked glycans of the mature molecule decreases its molecular mass to approximately the molecular mass of the precursor.

The molecular mass of the precursor was estimated by the extrapolation of molecular mass markers and should be considered provisional. However, there are additional arguments for such a molecular mass: under shadow-casting electron microscopy, epiglycanin appears as long filaments with an approximate length of 500–550 nm [1]. Jentoft [28] estimated that the length of a 28-residue glycopeptide is 7 nm. This corresponds to a molecular mass of non-glycosylated epiglycanin of approx. 240 kDa, which is in accord with the molecular mass as estimated by SDS/PAGE.

Incubation of the TA3Ha cells with tunicamycin and treatment of the precursor with the enzymes endoglycosidase H or N-glycosidase F proved the presence of N-linked glycans in epiglycanin. The sensitivity for endoglycosidase H shows that the N-linked oligosaccharides of the precursor protein are in the high-mannose configuration, which confirms that this precursor is an early biosynthetic intermediate present in the ER and possibly the early Golgi. The presence of N-linked glycans has been observed in other cell-bound and secreted mucins [15,29] and seems to be a general feature of mucins in addition to the abundant O-glycosylation. N-linked oligosaccharides are added to the polypeptide chain in the ER and have been reported to be important for exit from the ER by inducing proper folding of the protein [30]. However, the N-linked glycans in epiglycanin are essential neither for transport of the precursor from the ER to the Golgi complex nor for further processing of the molecule because in the presence of tunicamycin the precursor matures normally by O-glycosylation and sialylation, which occurs in the Golgi and the *trans*-Golgi network. No clear shift could be observed in the apparent molecular mass of mature epiglycanin by treatment with tunicamycin, endoglycosidase H or N-glycosidase F, probably because the shift in molecular mass was too minute to be detected in the high-molecular-mass region of the gel. Indeed, mature epiglycanin has been shown previously to contain at least one tetra-antennary complex N-linked carbohydrate chain with the composition Man3Gal4GlcNAc5(NeuAc3) in addition to one high-mannose chain [11].

Protein export from the ER is a selective process. Proteins are first concentrated in isolated compartments within the ER before they are transferred to the *cis*-face of the Golgi apparatus, where modification of the N-linked carbohydrates and O-linked glycosylation take place immediately [31–33]. This rate-limiting transport from the ER to the *cis*-Golgi compartment might be the reason that only the early precursor is detected. Real intermediate forms connecting the precursor and the fully glycosylated epiglycanin cannot be detected. Apparently, the transport of epiglycanin through the Golgi is very rapid and does not involve a rate-limiting step.

Mature epiglycanin appears in two major forms. Because only a single epiglycanin precursor was identified, the heterogeneity of the mature glycoprotein must result from other post-translational modifications, most probably O-linked glycosylation. Indeed, the removal of sialic acid residues from the glycans by treatment with neuraminidase results in a discrete band on SDS/polyacrylamide gels, suggesting that the heterogeneity of the mature glycoprotein is due to differences in sialylation. Interestingly, our immunoprecipitation results (Figure 6B) suggest that both the fully sialylated and undersialylated forms are synthesized at the same time and are not interconverted, indicating that the maturation of epiglycanin occurs through two different pathways. However, the possibility cannot be excluded that our mAbs fail to detect earlier glycoforms of epiglycanin that are present in relatively minor quantities.

Our studies show that the 300 kDa precursor is converted into the mature form of epiglycanin within 30 min. A similar timing of the initiation of O-glycosylation has been reported for human episialin/MUC1 [15] and human and rat MUC2 [34–36]. However, the shedding of epiglycanin occurs at a much slower rate than the shedding of episialin/MUC1, which is also a membrane-bound mucin. Only 50 % of epiglycanin is shed during the first 60 h, whereas the half-life of human episialin at the plasma membrane is 16–24 h (M. Boer, S. V. Litvinov and J. Hilkens, unpublished work).

Human episialin/MUC1 and two other well-characterized membrane-bound mucins, rat sialomucin complex (SMC) and its human counterpart, MUC4, are also synthesized through an underglycosylated early precursor. However, these precursors are cleaved proteolytically in the ER within 3–4 min of the initiation of biosynthesis, resulting in non-covalently bound heterodimers [23,37–39]. Although this cleavage does not directly lead to the release of the mucin domain, it might be needed for

shedding and might therefore explain why the mucin-like domains of episialin/MUC1 and MUC4 are relatively more easily released from the carcinoma cells than epiglycanin. Moreover, proteolytic cleavage seems not to be a common step that is critically needed for post-translational processing of membrane-associated mucins, although at least two other mucins of the same class exhibit this feature.

In this first detailed biochemical analysis of epiglycanin, we have demonstrated that this molecule (1) possesses the typical characteristics of a membrane-associated sialomucin, (2) contains a protein backbone with an approximate molecular mass of 300 kDa, (3) is heavily O-glycosylated, and (4) is shed from the cell membrane as a mature molecule at a relatively low rate.

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