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Initial glycosylation of proteins with acetylgalactosaminylserine linkages

(epithelial glycoprotein/nascent peptides/acetylgalactosaminitol)

GER J. A. M. STROUS

State University, Department of Histology and Cell Biology, Nic. Beetsstraat 22, 3511 HG Utrecht, The Netherlands

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ABSTRACT **Epithelial glycoprotein like that produced by the gastric surface consists of a polypeptide chain rich in serine and threonine; to these amino acid residues oligosaccharide chains of variable length are linked. The linking sugar is acetylgalactosamine. To find out whether the initial glycosylation takes place at the ribosomal level, I treated purified peptidyl-tRNA, derived from rat gastric membrane-bound polysomes, with alkali in the presence of boro[^{3}H]hydride. Alkali specifically splits glycosidic bonds between serine or threonine and oligosaccharide side chains (β-elimination reaction). The linking sugar is converted to an alditol and simultaneously labeled. GalNAc was identified as the linking sugar by paper chromatography. Furthermore, nascent peptides with lengths between 40 and 60 amino acid residues already contained this linking sugar. Gel filtration on Bio-Gel P-2 of ^{3}H-labeled saccharides revealed that nascent chains contained mainly monosaccharides, but some di- or trisaccharides were found with GalNAc as the linking sugar. These findings demonstrate that initial glycosylation of epithelial glycoprotein occurs at the ribosomal level rather shortly after the nascent peptide chain has reached the cisternal lumen of the endoplasmic reticulum.**

The majority of glycoproteins occurring in mammalian cells either contains glycosylamine linkages between GlcNAc and asparagine or glycosidic linkages between GalNAc and serine (threonine). The first type is generally present in serum glycoproteins, the second one in epithelial glycoproteins, also called mucins (1). Glycoproteins like human chorionic gonadotropin (2), fetuin (3), and the erythrocyte membrane glycoprotein glycophorin (4) contain both glycosylamine and glycosidic linkages, the carbohydrates of the latter being bound to serine or threonine residues of the peptide chain by GalNAc as the linking sugar.

Most studies on the initial glycosylation of proteins have been carried out on glycoproteins with a glycosylamine binding between GlcNAc and asparagine (5–16). In that case the initial glycosylation results from the transfer of an oligosaccharide, consisting of 2 *N*-acetylglucosamine, 8–12 mannose, and possibly 1 or 2 glucose residues, from a lipid intermediate to the appropriate asparagine residues of the growing peptide chain (5, 7, 9, 10). Little is known about the way GalNAc residues of the epithelial glycoproteins are attached to serine and threonine. Because the oligosaccharide chains of these glycoproteins show a great variety in length and in composition, from disaccharides up to components with 20 sugar residues, it is unlikely that their biosynthesis takes place in a way analogous to the GlcNAc-Asn type glycopeptides.

Biosynthetic studies in a number of laboratories have suggested that initial glycosylation of epithelial glycoproteins takes place in the microsomal fractions (17–22), but up till now the precise subcellular site has not been determined. The aim of this study was to establish the moment of the initial glycosylation in nascent epithelial glycoprotein in living cells and the nature of the linking sugar.

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Rat gastric mucosa predominantly synthesizes an epithelial glycoprotein that contains glycosidic sugar-peptide bonds. The glycoprotein consists of a polypeptide backbone rich in serine and threonine, all of which are provided with oligosaccharide chains of about 20 sugar residues (R. Spee-Brand, G. Strous, and M. Kramer, unpublished data). As is known from structural studies on porcine gastric and some other epithelial glycoproteins, the oligosaccharide chains are bound to the polypeptide via a GalNAc residue (23, 24). The glycosidic bond of GalNAc to serine or threonine can be split in the presence of 0.05 M sodium hydroxide at room temperature, sugar bonds in the oligosaccharide chains as well as the GlcNAc-Asn bonds being alkali resistant. If this cleavage (β-elimination) is carried out in the presence of boro[^{3}H]hydride, the linking GalNAc residue is converted to radioactive acetylgalactosaminitol by reduction; the method releases the oligosaccharides and simultaneously labels their reducing linking sugars. By use of purified gastric peptidyl-tRNA as a source of nascent glycopeptides, this method has been applied to determine whether or not the first sugar is added while the peptide chain is nascent to identify the linking sugar, to determine the minimal peptide chain length required for initial glycosylation, and to examine the size of the initial oligosaccharide.

MATERIALS AND METHODS

Isolation of Gastric Polysomes. Male Wistar albino rats, weighing 150–200 g, were kept from food for 24 hr and killed by decapitation. All subsequent steps were performed at 0–4°C. Gastric scrapings were obtained as described (25) and homogenized in 0.25 M sucrose/50 mM Tris·HCl, pH 7.4/25 mM KCl/5 mM 2-mercaptoethanol/5 mM magnesium acetate (TKM buffer), supplemented with 0.2 mg of cycloheximide per ml. The homogenate was centrifuged at 2000 × *g* for 10 min at 2°C. The supernatant was layered on a discontinuous gradient consisting of 3 ml of 2 M sucrose and 3 ml of 1.5 M sucrose made in an SW-41 centrifuge tube and centrifuged at 100,000 × *g* for 16 hr. After centrifugation the interface between 2 M and 1.5 M sucrose, containing the rough microsomes, was diluted with TKM buffer and supplemented with 1% Triton X-100 and deoxycholate to a final concentration of 0.3%. The detergent-treated microsomes were layered onto 2 M sucrose in TKM medium and centrifuged for 5 hr at 200,000 × *g*. The pellet contained the gastric membrane-bound polysomes.

Isolation of Peptidyl-tRNA. For the isolation of peptidyl-tRNA from the gastric polysomes, the previously described (26) procedure was used (see legend to Fig. 1). The fractions containing the peptidyl-tRNA were purified by extensive dialysis against 0.15 M NaCl in 50 mM Tris·HCl (pH 7.4) and application onto a column (20 × 1 cm) of DEAE-Sephadex in the same buffer. After the column was washed with the starting buffer, the peptidyl-tRNA was eluted with a linear NaCl gradient. The peptidyl-tRNA eluted between 0.4 and 0.5 M NaCl. These fractions were dialyzed and lyophilized.

Preparation of Labeled Oligosaccharides and Analysis. Hydrolysis (β-elimination) and labeling of saccharides derived from peptidyl-tRNA were performed as described by Aminoff *et al.* (27). Purified peptidyl-tRNA (0.5 A_{260} units) was dissolved in 25 μl of 50 mM NaOH containing 0.1 M NaB^3H_4 (Radiochemical Centre, Amersham, England; specific activity 7 Ci/mmol, 1 Ci = 3.7×10^{10} becquerels). The mixture was incubated at 20°C for 16 hr. After the incubation, the reaction mixture was neutralized with 50 mM HCl and dried under reduced pressure. The excess boric acid was removed as methyl borate. In some experiments the products of the β-elimination and labeling procedure were hydrolyzed with 2 M HCl for 6 hr at 100°C. Deacetylated amino sugars were isolated by passage on a Dowex-50 column by the procedure of Boas (28). *N*-Acetylation was carried out with acetic anhydride as described by Distler *et al.* (29). Before fractionation of the nascent peptides on a Bio-Gel P-6 column, the peptides were split off from the peptidyl-tRNA by treatment with 1.8 M Tris·HCl (pH 8.0) for 60 min at 37°C (30).

The radioactive labeled components were identified by paper chromatography in ethyl acetate/acetic acid/formic acid/water, 18:3:1:4 (vol/vol) on Whatman no. 1 paper or in ethyl acetate/pyridine/0.5 mM boric acid, 3:2:1 (vol/vol) on DEAE-cellulose strips, that had been sprayed to uniform wetness with a solution of 0.5 mM sodium tetraborate and then dried, following the method of Conrad (31). The chromatograms were cut into 1-cm segments, which were put into counting vials; 1 ml of water was added to the paper strips and then 10 ml of Lumagel. The ^3H radioactivity was measured in a liquid scintillation counter with an efficiency of 58%. Acetylgalactosaminitol and galactosaminitol, prepared as described by Crimmin (32), were used as standards. The samples were deionized with 2 ml of a mixed-bed ion-exchange resin, type MB-1 (Rohm & Haas Co.) before chromatography. The reference sugars were stained with periodate/benzidine reagent (33).

Gel filtration of nascent peptides derived from peptidyl-tRNA was performed on 90 × 1 cm column packed with Bio-Gel P-6 (200–400 mesh); Bio-Gel P-2 of the same particle diameter was used to fractionate ^3H-labeled oligosaccharides prepared from the nascent peptides by the β-elimination reaction. The eluting buffer was 0.1 M ammonium acetate.

RESULTS

Isolation of Nascent Peptides. Polysomes derived from rough microsomes, isolated from rat gastric scrapings, were loaded onto a discontinuous gradient the upper phase of which contained Mg^{2+} ions whereas the lower phase was supplemented with EDTA. Polysomes carrying peptidyl-tRNA dissociate into their subunits when they enter the EDTA-containing phase. The centrifugation time was such that the ribosomal subunits were centrifuged to the bottom of the tube. Fig. 1 illustrates the results of the centrifugation of dissociated gastric membrane-bound polysomes. The ratio of the absorbance at 260 and at 280 nm of the material in fractions 7 and 8 was 2.04, indicating a highly pure tRNA preparation. These fractions were further purified on a DEAE-Sephadex column from which the peptidyl-tRNA could be eluted with 0.4 M NaCl. This is characteristic of peptidyl-tRNA.

To measure the amount of completed epithelial glycoprotein present in the purified peptidyl-tRNA preparation, I mixed gastric [^3H]glycoprotein (10 mg, 10^5 dpm), labeled with [^3H]galactose in *ex vivo* perfusion of a rat stomach, with 1 mg of gastric polysomes. After purification of the peptidyl-tRNA as described in this section, less than 10 dpm of the radioactive glycoprotein was present in the preparation. This result demonstrates that the peptidyl-tRNA prepared from purified membrane-bound polysomes can be considered free of epithelial glycoprotein.

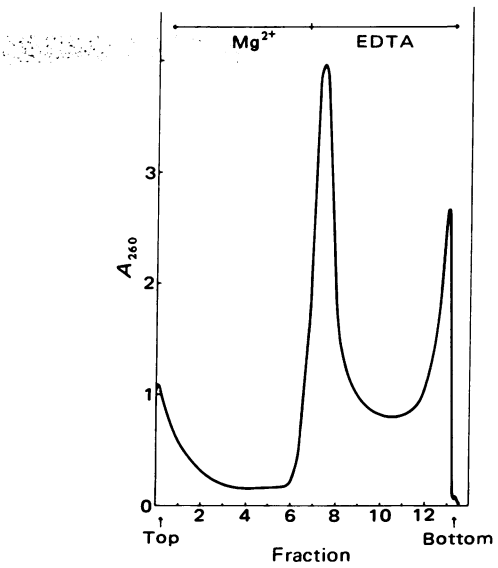

FIG. 1. Sedimentation profile of peptidyl-tRNA from rat gastric polysomes. The gradient, consisting of a lower layer (6 ml) that contained 50 mM Tris·HCl (pH 7.4)/80 mM KCl/10 mM EDTA in a 20–30% (wt/vol) linear sucrose gradient and an upper layer (6 ml) that contained 50 mM Tris·HCl (pH 7.4)/80 mM KCl/5 mM magnesium acetate/5 mM 2-mercaptoethanol in 10% sucrose, was prepared in an SW-41 gradient tube. About 5 mg of polysomes, derived from rough microsomes, was applied to the gradient. Centrifugation was performed for 16 hr at 25,000 rpm, and the gradient was monitored with a Gilford spectrophotometer.

Glycosylation of Nascent Peptides. Linkages between GalNAc and serine or threonine can be split by alkali treatment. To ascertain the usefulness for labeling of mono- or oligosaccharides bound to peptidyl-tRNA, I tested the β-elimination reaction on ovine submaxillary mucin from which 40% of the sialic acid had been removed by neuraminidase. Ovine submaxillary mucin contains a large amount of the disaccharide GalNac-sialic acid. The paper chromatographic analysis of the reaction products is shown in Fig. 2. The radioactive material indicated by the arrow is the disaccharide acetylgalactosaminitol-sialic acid because treatment of this material with neuraminidase causes a shift of the radioactivity to the acetylgalactosaminitol position. The ratio of GalNAc to GalNAc-sialic acid is about 2:3, indicating that under the conditions used both saccharides are split off to the same extent in spite of the decrease of electron-attacking capacity of the leaving group caused by the presence of sialic acid. The total amount of radioactive acetylgalactosaminitol was about 2 mCi; this means that nearly all GalNAc was hydrolyzed from the mucin during the reaction.

If *O*-glycosylation of polypeptides starts while they are nascent and still bound to tRNA, the peptidyl-tRNA chains isolated from epithelial cells should contain an oligosaccharide glycosidically bound to serine or threonine. To examine whether indeed an amino sugar is already attached to the nascent chains and to identify this amino sugar, I treated the purified peptidyl-tRNA with NaOH in the presence of boro[^3H]hydride. After hydrolysis with HCl to dissociate possible oligosaccharides into monosaccharides, the deacetylated amino sugars were isolated with a Dowex-50 column. The purification procedure was necessary to remove radioactive components other than hexosamines that interfere with the chromatographic analysis (e.g., amino acids). Reacetylation was performed with acetic anhydride and the radioactive material was analyzed by paper

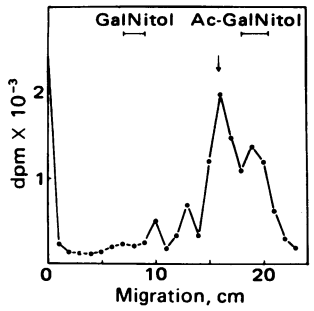

FIG. 2. Paper chromatographic analysis of ^{3}H-labeled disaccharides derived from ovine submaxillary mucin. One milligram of the mucin was incubated with 0.1 unit of neuraminidase at pH 5 for 5 hr and exhaustively dialyzed. Sialic acid was determined before and after neuraminidase treatment by the method of Warren (34). Forty percent of the sialic acid appeared to be removed from the mucin. The β-elimination reaction was carried out in the presence of NaB^3H_4. Amino acids and peptides were removed by Dowex-50 (H^+) treatment. An aliquot was analyzed by paper chromatography on Whatman 1 paper for 8 hr with ethyl acetate/acetic acid/formic acid/water, 18:3:1:4 (vol/vol) as solvent. The arrow indicates radioactive material susceptible to treatment by neuraminidase. After this treatment, the radioactivity reached the acetylgalactosaminitol (Ac-GalNitol) position during chromatography.

chromatography (Fig. 3). The radioactivity peak corresponded to the site of chemically synthesized acetylgalactosaminitol (Fig. 3*A*). After elution of the radioactive material from the paper and subsequent hydrolysis with HCl, the radioactive material behaved like galactosaminitol (Fig. 3*B*).

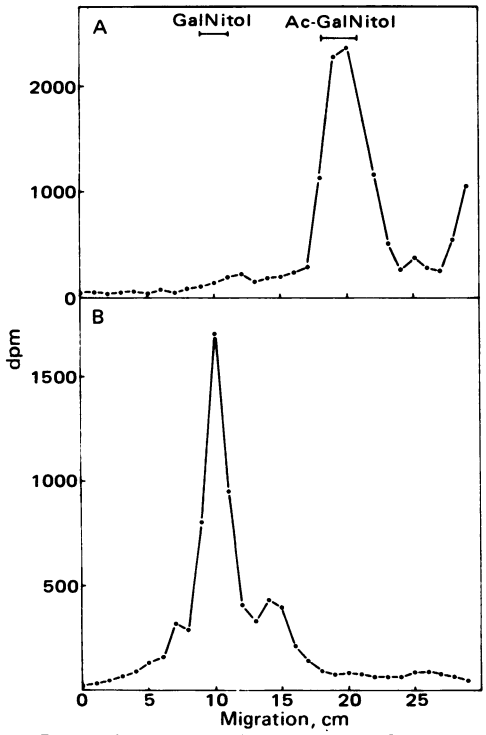

FIG. 3. Paper chromatographic analysis of ^{3}H-labeled linking sugars derived from peptidyl-tRNA. Peptidyl-tRNA was treated with 50 mM NaOH in the presence of NaB^3H_4. After hydrolysis with HCl, isolation of amino sugars on Dowex-50, *N*-acetylation with acetic anhydride, and deionization with mixed-bed ion-exchange resin, the radioactive material was applied to Whatman 1 paper (*A*). Chromatography was carried out for 10 hr. The radioactive material present in the acetylgalactosaminitol position was eluted with 2 M HCl, hydrolyzed for 5 hr at 100°C, and again chromatographed (*B*). Reference sugars were stained with periodate/benzidine.

Because chromatography as described above does not distinguish between acetylgalactosaminitol and acetylglucosaminitol, separation in another chromatographic system was performed with boric acid and DEAE-cellulose strips. In that case the radioactivity comigrated with acetylgalactosaminitol; no radioactivity was detected in the acetylglucosaminitol position (not shown). These findings indicate that GalNAc is a linking sugar in nascent peptides from gastric polysomes and that the synthesis of at least part of the oligosaccharides starts on the peptidyl-tRNA level.

The Moment of Glycosylation. In order to determine the minimal length of the growing polypeptide chain required for glycosylation, I cleaved the nascent peptides from the tRNA and separated them on a Bio-Gel P-6 column (Fig. 4). Peptides present in the void volume with M_r of 6000 and larger, peptides with M_r between 5000 and 4000, and peptides with M_r between 4000 and 1000 were pooled. The three fractions, designated A, B, and C, respectively, were treated with alkali in the presence of boro[^{3}H]hydride. The radioactive material was not hydrolyzed with HCl, but directly analyzed by paper chromatography. The results are shown in Fig. 5. Acetylgalactosaminitol was found in fractions A and B, but practically none was found in fraction C. This observation demonstrates that the glycosylation becomes operative when the polypeptide chains have a molecular weight of 4000–6000.

The total amount of [^{3}H]galactosaminitol in fraction A was about 3 nmol and in fraction B about 1 nmol. Assuming that 1 A_{260} unit contains 50% aminoacyl-tRNA and 25% peptidyl-tRNA bearing nascent peptides for epithelial glycoprotein, that the average peptide length is 200 amino acid residues, and that about 40 GalNAc residues are present on a nascent peptide, rough calculation indicates that 16 nmol of GalNAc would be linked to 1 A_{260} unit of peptidyl-tRNA.

The reaction with radioactive sodium borohydride produced several other labeled components (Fig. 5). A rather large amount of radioactivity was identified as glucitol by paper electrophoresis in 50 mM borate (pH 9.2) (not shown). The material migrating just behind galactosaminitol and at the start is apparently oligomers that have glucitol in the reducing position because hydrolysis of them with HCl resulted in a shift to the glucitol R_F value. This is indicative for glycogen-like degradation products.

Elongation of Oligosaccharide Chains. Whether oligosaccharide elongation also takes place at the ribosomal level is not clear from the results in the preceding section because the nature of the sugars possibly attached to GalNAc may vary, influencing the R_F value of di- or trisaccharides. Moreover,

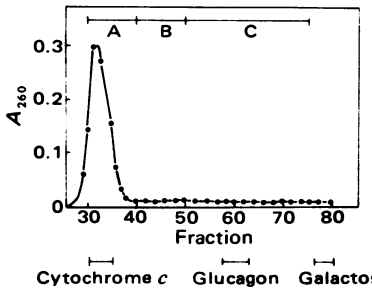

FIG. 4. Gel filtration of nascent peptides derived from peptidyl-tRNA on a Bio-Gel P-6 column. Peptidyl-tRNA (1.0 A_{260} unit) was treated with 1.8 M Tris-HCl (pH 8.0) at 37°C for 1 hr to split the peptidyl-tRNA bond. The material was applied to a column packed with Bio-Gel P-6 that had been calibrated with cytochrome *c*, glucagon, and galactose. The void volume contained the tRNA. Three fractions were collected: A, $M_r > 6000$; B, M_r between 4000 and 6000; and C, M_r between 1000 and 4000.

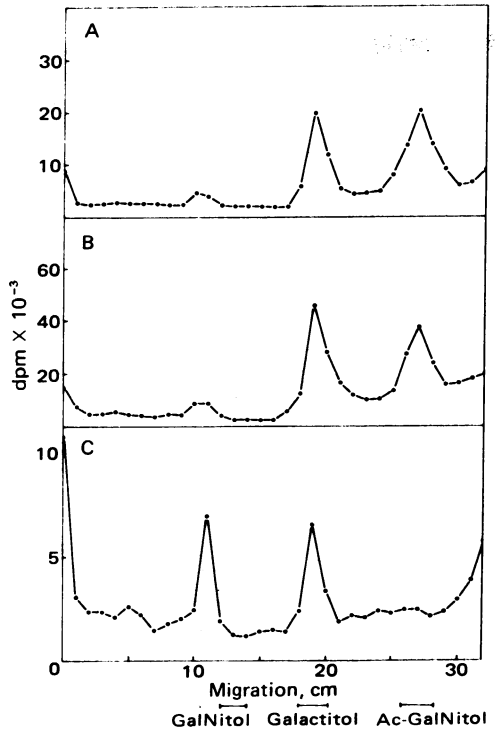

FIG. 5. Paper chromatographic analysis of ^3H-labeled saccharides derived from nascent peptides. The three fractions designated in Fig. 4 were subjected to β-elimination by NaOH and reduction with NaB^3H_4. Borate was removed as methyl borate, and the fractions were deionized with mixed-bed resin in order to remove radioactive peptides, lyophilized, and dissolved in water. An aliquot was applied on the paper. Chromatography was carried out for 16 hr as described in the legend to Fig. 2.

larger oligomers will probably not migrate in the chromatographic systems used. Therefore, the presence of oligosaccharides on the peptidyl-tRNA was studied by examining the molecular weight of the labeled material containing GalNAc at the reducing position.

Peptidyl-tRNA was again treated with alkali in the presence of boro[^3H]hydride. All volatile radioactivity was removed by evaporation under reduced pressure, and amino acids and peptides were removed by Dowex-50 (H^+) treatment. The resulting radioactive material was chromatographed on a Bio-Gel P-2 column. The radioactivity pattern is shown in Fig. 6. Not all radioactive material derived from peptidyl-tRNA in fractions 55–70 could be identified as oligosaccharides with acetylgalactosaminitol in the reducing position. Part of it consisted of oligomers with glucitol in the reducing position as shown in Fig. 5. Therefore, the ratio of acetylgalactosaminitol-containing species in the different column fractions was determined after hydrolysis and paper chromatography. The fractions were pooled as indicated in the figure and analyzed by paper chromatography (Fig. 7). The experiment was done quantitatively. It can be concluded that more than 80% of the GalNAc residues are present as monosaccharides on the peptidyl-tRNA (fraction 1). Less than 20% occurs in di- or trisaccharides bound to peptidyl-tRNA (fraction 2). Higher oligomers are practically absent (<1%) (fractions 3 and 4).

In comparison with radioactive material derived from peptidyl-tRNA, intact epithelial glycoprotein purified from gastric scrapings was labeled with alkali boro[^3H]hydride and analyzed on the Bio-Gel P-2 column after removal of radioactive polypeptides with Dowex-50 (H^+). A considerable amount of radioactivity was present in the void volume of the column (Fig. 6). This result demonstrates, first, that longer oligosaccharides

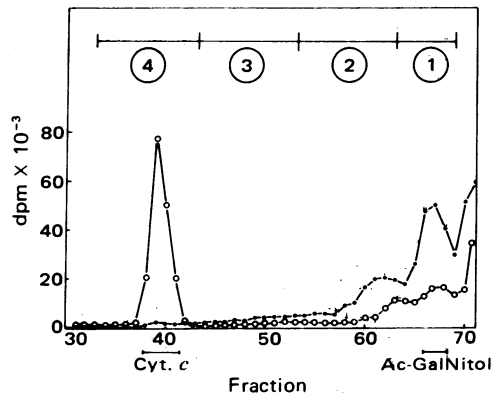

FIG. 6. Gel filtration on a Bio-Gel P-2 column of ^3H-labeled saccharides derived from peptidyl-tRNA (●) or purified gastric glycoprotein (○). Peptidyl-tRNA (0.5 A_{260} unit) was hydrolyzed in 25 μl of 50 mM NaOH in the presence of NaB^3H_4. After incubation the mixture was neutralized, dried under reduced pressure, treated with Dowex-50 (H^+), and lyophilized. The radioactive material was applied to the column and fractions of 2 ml were collected. For measurement of radioactivity, aliquots of 0.01 ml were used. Fractions 65–70, 55–64, 45–54, and 36–44 were pooled and designated 1, 2, 3, and 4, respectively. Ten micrograms of purified gastric glycoprotein (R. Spee-Brand, G. Strous, and M. Kramer, unpublished data) was hydrolyzed and chromatographed in the same way.

are also split off under the conditions used and, second, that the peptidyl-tRNA preparation is free of completed epithelial glycoprotein.

DISCUSSION

The aim of the present investigation was to answer the question of when and where the glycosylation of glycoproteins with glycosidic GalNAc-Ser(Thr) linkages starts. The fact that glycosidic sugar-amino acid bonds can be split by alkali offers the opportunity to label specifically the linking sugar by reduction with boro[^3H]hydride. By using purified peptidyl-tRNA already synthesized *in vivo*, it was unambiguously shown that the

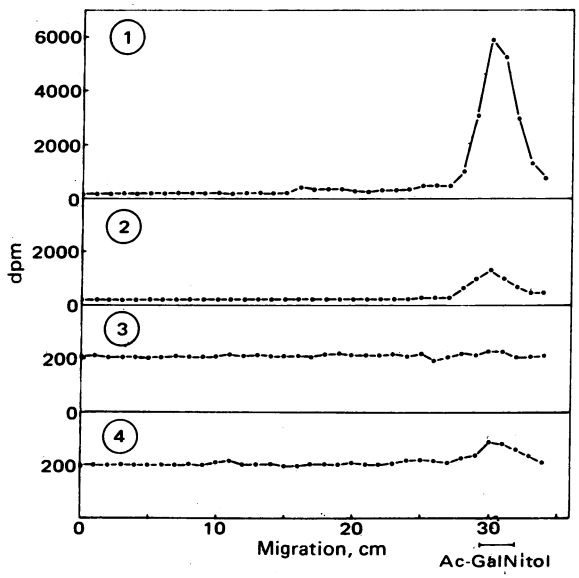

FIG. 7. Paper chromatographic analysis of ^3H-labeled linking sugars derived from peptidyl-tRNA. The radioactive material fractionated as described in Fig. 6 was hydrolyzed with 2 M HCl, reacetylated, deionized, and applied to the paper. Chromatography was performed for 15 hr as described in the legend to Fig. 2. Panels are numbered corresponding to the four fractions indicated in Fig. 6.

initial glycosylation of epithelial glycoproteins in living cells starts at the ribosomal level. However, the experiments do not exclude the possibility that attachment of GalNAc to serine or threonine also occurs to completed polypeptide chains.

Cleavage of the GalNAc-Ser(Thr) bond with alkali by the method of Aminoff *et al.* (27) is quantitative for oligosaccharides with fewer than six sugar residues. We tested the method by using ovine submaxillary glycoprotein containing large amounts of the disaccharide GalNAc-sialic acid. The rate of hydrolysis for the di- and monosaccharide was the same under the conditions used in the experiments. So the method is useful for the aims set in this investigation. As shown in Fig. 7, the amount of di- and trisaccharides is very low in peptidyl-tRNA preparations compared to the monomer.

Studies on glycoprotein synthesis with GlcNAc-Asn linkages indicate that initial glycosylation occurs via a dolichol-oligosaccharide intermediate by which 10–16 sugar residues are added to the protein at the same time (5, 6, 10). This study suggests that the initial glycosylation of GalNAc-Ser type glycoproteins involves only one sugar and not a large group as for the GlcNAc-Asn linked glycoproteins. This can be concluded from the experiments described in Figs. 5 and 7, showing that GalNAc was present as a monosaccharide on most of the nascent peptides with a length of 40 amino acids and more. At this moment, there are no indications for the involvement of lipid carriers. However, the experiments described here do not exclude short-lived intermediates involved in the glycosidic sugar-peptide bond formation.

The minimal length for glycosylation cannot be determined exactly from the experiments with the Bio-Gel P-6 column because contamination of fraction B with material from fraction A cannot be excluded. However, fraction B contains a substantial amount of acetylgalactosaminitol, which would not be expected if fraction B were only a contaminant of fraction A. Assuming that GalNAc is bound to peptides with a molecular weight of 4000–5000 and that about 30–40 amino acid residues of the growing peptide chain (corresponding to 4000 daltons) are shielded in the 60S ribosomal subunit (35), two possibilities are open: the signal peptide contains serine or threonine residues labile to glycosylation, or the signal is cleaved before the first glycosylation takes place. The early glycosylation is in accord with the results obtained for the glycosylation of vesicular stomatitis viral glycoprotein as reported by Rothman and Lodish (7): two oligosaccharide chains are attached to asparagine residues of this glycoprotein during polypeptide synthesis at the moment that the asparagine residues reach the luminal site of endoplasmic reticulum.

The results described here also indicate that oligosaccharide chains are present near the NH_2 terminus of the polypeptide chain. This is in agreement with the structure of bovine cervical mucin, another epithelial glycoprotein, as suggested by Bhushana Rao and Masson (36). Consequently, in the structure of gastric epithelial glycoprotein as proposed by Scawen and Allen (37), the unglycosylated part of the molecule containing the cysteine residues that contribute to the gel structure should be located at the COOH terminus of the molecule.

It is of interest to know whether the mechanism of initial glycosylation as described here for epithelial glycoprotein is also operative for membrane and serum glycoproteins. Preliminary experiments show that liver and pancreas peptidyl-tRNAs derived from membrane-bound polysomes also contain glycosidic GalNAc residues.

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