Quantitative aspects of mucus glycoprotein biosynthesis in rat gastric mucosa

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The synthesis of the polypeptide backbone of mucus glycoproteins in rat stomach was studied. CsCl centrifugation of the homogenate of [^3H]serine pulse-chase labelled stomach or mucosal scrapings showed that [^3H]serine was mainly incorporated into molecules having a density identical to that of proteins and that only 8–12% was incorporated into macromolecules with the density of mucus glycoproteins. [^3H]Galactose, however, was almost exclusively incorporated into macromolecules with a density identical to that of mucus glycoproteins. Electrophoretic analysis of the CsCl fraction containing the mucus glycoprotein revealed that 78% of the [^3H]serine-labelled macromolecules had an electrophoretic behaviour identical to that of mucus glycoproteins. Thus, only a small portion (about 6–10%) of incorporated [^3H]serine was present in the backbone of the mucus glycoprotein. Translation in a wheat germ cell-free system of total RNA derived from both whole stomach and superficial mucosal scrapings, using either [^{35}S]methionine or [^{35}S]cysteine as radioactive amino acid, yielded a wide range of proteins. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, one major translation product of whole stomach RNA had an apparent M_r (43 000) identical to that of rat pepsinogen. As this polypeptide could not be found amongst the translation products of RNA from scrapings it probably was pepsinogen. The present data provide strong evidence that the backbone polypeptide of mucus glycoproteins only accounts for a small part of the proteins synthesized by mucus-producing cells.

Mucus is synthesized and secreted by a specialized cell type, the mucous cell. These epithelial cells are easily recognized by their typical shape and special staining characteristics as a consequence of mucus storage. Gastric mucus glycoproteins from different species have been isolated and characterized (Schrager & Oates, 1971; Starkey *et al.*, 1974; Spee-Brand *et al.*, 1980; Pearson *et al.*, 1980). They consist of a peptide containing numerous oligosaccharide side chains, 14–18 sugar residues long and *O*-glycosidically linked to serine or threonine residues (Allen, 1978).

In order to investigate the effect of prostaglandins on the synthesis of mucus glycoprotein, we recently reported that PGE_2 increased [^3H]galactose incorporation up to 4-fold in the fundic surface mucous cells of the vascular perfused rat

stomach (Jentjens *et al.*, 1984). Light-microscopic autoradiography was used to quantify [^3H]galactose incorporation. The effect of PGE_2 on total protein synthesis in the surface mucous cells was monitored by measuring the incorporation of [^3H]serine. Serine is a quantitatively important amino acid of the mucus glycoprotein backbone, accounting for 13% of the amino acid residues present in this polypeptide (Spee-Brand *et al.*, 1980). If PGE_2 indeed stimulates the synthesis of mucus glycoproteins, we would have expected to find increased [^3H]serine incorporation. However, we were not able to show this after PGE_2 treatment. In addition, analysis of purified mucus glycoproteins did not reveal an effect of PGE_2 on carbohydrate composition, oligosaccharide chain size, or buoyant density of mucus glycoproteins (Jentjens *et al.*, 1984). The observation that PGE_2 stimulates [^3H]galactose, but not [^3H]serine, incorporation into mucous cells led us to assume that PGE_2 has no stimulatory effect on the overall protein synthesis of the mucous cell, but only

Abbreviations used: SDS, sodium dodecyl sulphate; PGE_2 , 16,16-dimethylprostaglandin E_2 .

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stimulates the synthesis of the polypeptide backbone of the glycoprotein. We suggested that the synthesis of backbone polypeptides in mucus-producing cells contributes only little to the total protein synthesis in these cells.

In this study we present biochemical evidence for this hypothesis. Pulse-chase experiments using [^3H]galactose and [^3H]serine show that incorporation of [^3H]serine into mucus glycoproteins is only a small part of the total [^3H]serine incorporation. Moreover, translation *in vitro* of RNA isolated from either whole stomach or from gastric mucosal scrapings does not reveal a polypeptide that might be considered as the backbone of the glycoprotein molecule. This indicates that the backbone polypeptide is not a quantitatively important product of the gastric mucous cell.

Materials and methods

Chemicals

Fluorocarbon FC-75 was obtained from the 3M Company (St. Paul, MN, U.S.A.). Bovine serum albumin and amino acids were from Sigma. Radiochemicals were purchased from Amersham International. All other chemicals were of analytical grade from Merck.

Experimental animals

To synchronize and precondition them, male Wistar rats weighing 200–250 g were subjected to a strict fasting-feeding protocol, which consisted of a 2-h fasting period followed by free access to food for 2 h and another fasting period for 16 h. They had water *ad libitum*.

Perfusion system

The vascular perfusion system as described by Van Huis & Kramer (1979) was used with some minor modifications. Rats were anaesthetized by intraperitoneal injection of pentobarbitone sodium (50 mg/kg body wt.). After a 1 min perfusion with Tyrode's solution, perfusion was continued with 'perfusion medium', and maintained for 1 h. The perfusion medium consisted of a Tyrode's solution containing 5% (w/v) bovine serum albumin and 10% (w/v) fluorocarbon FC-75, emulsified beforehand. Amino acids were added at plasma concentrations. During the perfusion, the medium was continuously oxygenated and thermostabilized at 37°C. The pH of the medium was maintained at 7.4.

Incorporation of precursors

After 30 min of preperfusion, D-[1- ^3H]galactose (22 Ci/mmol) or L-[3- ^3H]serine (29 Ci/mmol) was added to the perfusion medium at a final concentration of 10 $\mu\text{Ci/ml}$. The perfusion was stopped

after a labelling period of 30 min, but for chase labelling was continued for a further 2 h with fresh perfusion medium lacking the labelled precursor.

The stomach was removed from the perfusion system, immediately cut open along the greater curvature and washed in 0.1 M-phosphate buffer (pH 7.4). The fundus was quickly frozen in liquid N_2 and stored at -20°C for later biochemical determinations. Alternatively, the mucosal surface was scraped off with a razor blade and the scrapings were stored at -20°C . Microscopic examination of the scraped stomach revealed that only surface and pit mucous cells and adhering mucus had been removed.

Isolation of mucus glycoproteins

The fundus or fundus scrapings were thawed and homogenized in 1 mM-EDTA/0.5 M-2-mercaptoethanol in a Polytron PCU-2 tissue homogenizer. The homogenate was centrifuged at 1700 g for 15 min to remove nuclei, cell debris and undissolved mucus. The supernatant was dialysed against 1 mM EDTA/0.1 M-2-mercaptoethanol, freeze-dried and used for further analysis.

Fundic mucus glycoproteins were isolated by equilibrium centrifugation (60 h; 150 000 g) on CsCl as described by Spee-Brand *et al.* (1980). The starting density of the CsCl was 1.42 g/ml (40% w/w). After centrifugation, each gradient was collected as 0.25 ml fractions and CsCl was removed by dialysis against water.

Total hexose was assayed by colorimetry, using the orcinol/ H_2SO_4 reagent (François *et al.*, 1962). A mixture of galactose and fucose reflecting the ratio (2:1) as determined for gastric glycoproteins was used as a standard (Spee-Brand *et al.*, 1980). Total protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Preparation of RNA from rat stomach

Total RNA was isolated according to the procedure described by Chirgwin *et al.* (1979). Whole stomach or scrapings were homogenized (Polytron PCU-2) in 10 ml of 4 M-guanidinium thiocyanate/25 mM-sodium citrate (pH 7.0)/0.5% sodium *N*-laurylsarcosine/0.1 M-2-mercaptoethanol containing 1–2 drops of antifoam (Sigma). The homogenate was centrifuged at 1700 g and 15°C for 10 min to remove cell debris. The supernatant (5 ml) was layered on to 1 ml of sterilized 5.7 M-CsCl/0.1 M-EDTA (pH 7.0) and centrifuged at 142 000 g and 15°C in a SW 50 rotor for 16 h. The top layer was carefully removed and the CsCl decanted. The pellet was rinsed twice with 70% (v/v) ethanol (-20°C) and resuspended in 200 μl of Tris/HCl (5 mM, pH 7.4). After addition of 20 μl of potassium acetate (2 M, pH 5.0) and 400 μl of 70%

ethanol and storage for 5 h at -20°C the tube was centrifuged, the supernatant decanted, the pellet rinsed once with 100% ethanol and resuspended in 100 μl of water.

Translation in vitro

A wheat germ cell-free translation was prepared according to Roberts & Paterson (1973). Translation incubations (25 μl) containing 7.5 μl of wheat germ cell-free translation preparation, 20 mM-Hepes (pH 7.0), 2.5 mM-ATP, 0.32 mM-GTP, 20 mM-phosphocreatine, creatine kinase (0.06 $\mu\text{g}/\text{ml}$), 5 mM-dithiothreitol, 60 mM-potassium acetate, 0.76 mM-spermidine, 0.12 μM of each amino acid (with the exception of the radioactive amino acid) 15 μCi of [^{35}S]methionine or 25 μCi of [^{35}S]cysteine, and 1–2 μg of total RNA, were carried out at 24°C for 90 min.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis of the CsCl fractions and the translation products was performed on 10% polyacrylamide slab gels in the presence of 0.1% SDS, according to Laemmli (1970). Samples to be analysed by SDS/polyacrylamide-gel electrophoresis were prepared in a small volume of SDS sample buffer containing 0.05 M-2-mercaptoethanol and boiled for 3 min. Gels were routinely stained with Coomassie Blue for protein or with the HIO_4 /Schiff reagent for glycoprotein. Gels were prepared for fluorography as described by Bonner & Laskey (1974).

Dried gels were exposed to Kodak SB-5 X-ray films at -70°C . Alternatively, lanes of the dried gel were sliced into 1-cm segments, rehydrated with 0.1 ml of water and incubated at 50°C overnight in 1 ml of Lumasolve (Baker Chemicals). Radioactivity in the gel segments was determined in a liquid-scintillation counter with a toluene-based scintillation mixture.

Results

CsCl equilibrium centrifugation

Mucus glycoproteins were isolated from whole stomach by equilibrium centrifugation in a CsCl density gradient. The distribution of hexose- and protein-containing material in the gradient is shown in Fig. 1(a). After labelling a stomach for 30 min in the presence of [^3H]galactose, the CsCl gradient profile revealed that [^3H]galactose radioactivity banded at the same density (i.e. 1.48 g/ml) as the hexose-containing material (Figs. 1a and 1b).

However, when [^3H]serine was used as radioactive precursor, the radioactive macromolecules showed a completely different distribution on a CsCl gradient. Almost all radioactivity was pre-

Fig. 1. Fractionation of stomach or mucosal scrapings homogenate on CsCl-density gradients

(a) Whole stomach homogenate: ●, hexose; ○, protein. DNA in fractions 1 and 2 accounts for the high hexose content in these fractions. (b) Whole stomach homogenate from a stomach perfused for 30 min with either [^3H]galactose (▲) or [^3H]serine (△) (c) Mucosal scrapings from a stomach perfused for 30 min with [^3H]serine; label time was 30 min (□) or 30 min with a 2 h chase period (■). The starting density of the gradients was 1.42 g/ml. The top of (a) shows the density of the fractions after equilibrium centrifugation (60 h, 150000g).

sent in the protein-containing fractions and only a small amount could be detected in the hexose-containing ones (Figs. 1a and 1c).

The light microscopic autoradiographic studies (Jentjens *et al.*, 1984) showed that [^3H]serine incorporation in chief (pepsinogen producing) cells was about five times higher than in mucous cells. Consequently the incorporation in these cells

might obscure the incorporation in other cells. As chief cells are located deep in the mucosa, we carefully scraped the top layer after labelling the stomach with [^3H]serine, in order to obtain the mucus-producing cells without the chief cells. After a 30 min pulse and 2 h chase, we analysed the (glyco)proteins from the scrapings on a CsCl gradient. The results are shown in Fig. 1(c). Fractions 4–9 (hexose-containing fractions) contained only 8 and 12% of the radioactivity present in the total gradients of pulse-labelled and chased stomachs, respectively.

Polyacrylamide-gel electrophoresis

To identify the radioactive macromolecules present in the CsCl gradient, we analysed two peak fractions (7 and 14) of material derived from scrapings after pulse-labelling with [^3H]serine followed by a chase period of 2 h, on a 10% SDS/polyacrylamide gel. The gel was sliced; the amount of radioactivity in each segment is presented in Fig. 2. As can be seen from this

Figure, 78% of the total radioactivity present in fraction 7 was confined to the top of the gel (stacking and 2 cm below the top), which also stained with HIO_4 /Schiff reagent. This indicates that about 6–10% of the [^3H]serine was incorporated in macromolecules that have a buoyant density and electrophoretic mobility identical to that of mucus glycoproteins. The radioactivity in fraction 14 was largely present in proteins having an $M_r < 30000$.

Cell-free protein synthesis

In order to find out whether the polypeptide backbone of the mucus glycoproteins can be identified amongst the other synthesized polypeptides, RNA derived from either whole stomach or mucosal scrapings was translated in a wheat germ cell-free system in the presence of [^{35}S]methionine or [^{35}S]cysteine. Fig. 3 shows an analysis of the translation products obtained on a 10% SDS/polyacrylamide gel. It is clear that translation resulted in a large number of different

Fig. 2. *Polyacrylamide-gel electrophoresis of gradient fractions*

Fractions 7 (○) and 14 (●) of the CsCl gradient after 30 min of labelling with [^3H]serine and a 2 h chase were analyzed on a 10% SDS/polyacrylamide gel. The radioactivity was determined in 1 cm segments. The top of the Figure shows the electrophoretic profile of fraction 7 after staining for glycoproteins with the HIO_4 /Schiff reagent.

Fig. 3. Polyacrylamide-gel electrophoresis of translation products

Translation products of RNA derived from whole stomach or scrapings were analysed on a 10% SDS/polyacrylamide gel and visualized by fluorography. Lane 1, whole stomach, [^{35}S]methionine; lane 2, whole stomach, [^{35}S]cysteine; lane 3, mucosal scrapings, [^{35}S]methionine; lane 4, mucosal scrapings, [^{35}S]cysteine. Standard proteins with M_r 30000–200000 were co-electrophoresed.

bands. Lanes 1 and 2 (whole stomach) show a prominent band having an apparent M_r of 43000. This protein was not synthesized in RNA derived from scrapings (lanes 3 and 4). Apart from the afore-mentioned band in whole stomach, there is

no other prominent band that might be considered as the backbone of the glycoprotein molecule.

Discussion

Native gastric mucus glycoprotein has an M_r of 2×10^6 , and consists of four subunits, each with an M_r of 5×10^5 . Oligosaccharides comprise over 80% by weight of the mucus glycoprotein (Allen *et al.*, 1982) and thus it can be estimated that the M_r of the polypeptide backbone amounts to no more than 100000. The amino acid composition of the backbone has been determined for rat, pig and human gastric mucus glycoproteins (Spee-Brand *et al.*, 1980; Scawen & Allen, 1977; Schrager & Oates, 1971). More than 25% of the amino acid residues are threonine or serine, which are all provided with oligosaccharide chains *O*-glycosidically linked via *N*-acetylgalactosamine.

The biosynthesis of the protein core is thought to take place in the rough endoplasmic reticulum (Kramer & Geuze, 1977). Sugar addition starts in the rough endoplasmic reticulum and continues in the cisternae of the Golgi complex (Strous, 1979). In the latter compartment the bulk of glycosylation occurs, resulting in subunits of M_r 500000 (Kramer & Geuze, 1977). Quantitative electron-microscopic autoradiography has shown that in rat gastric mucous cells 30–40% of all incorporated [^3H]leucine can be found over the rough endoplasmic reticulum within 5 min, and is transported to the Golgi complex within 40 min and to the secretory granules in about 2 h; almost 50% of the incorporated radioactive leucine is localized over the cytosol. Within 10 min more than 70% of the [^3H]galactose is located over the Golgi compartment; within 1 h more than 40% of the incorporated galactose is present over the secretory granules (Kramer & Geuze, 1977).

The biochemical analyses reported in this paper show that after 30 min of pulse labelling almost all incorporated [^3H]galactose is found in mucus glycoproteins. Of the radioactivity incorporated into macromolecular material during pulse-labelling of the perfused stomach with [^3H]serine, 6–10% had a buoyant density and electrophoretic behaviour identical with that of mucus glycoproteins. Although the time required for radioactive macromolecules to be transported from the rough endoplasmic reticulum to the Golgi complex is about 40 min (Kramer & Geuze, 1977), [^3H]serine incorporation only increased from 8% to 12% after a chase-period of 2 h.

Our conclusion is that gastric mucous cells produce a very limited amount of backbone polypeptide as compared with the total protein synthesis taking place in these cells. This conclusion is not only based on the physical properties of the labelled macromolecules, as determined by

equilibrium centrifugation in CsCl and SDS/polyacrylamide-gel electrophoresis, but is also supported by RNA translation studies *in vitro*.

Gel electrophoresis of the labelled translation products of mRNA from whole stomach yielded a prominent band with an apparent M_r of 43000. This band was not detected in the translation products of mRNA from scrapings. In cells isolated from rat gastric mucosa that had been incubated with [3 H]leucine, Trotman & Greenwell (1979) also detected a highly labelled band with an apparent M_r of 43000, amongst many other radioactively labelled proteins. In gastric mucosa, pepsinogen-producing cells (the chief cells) are confined to the lower half or third of the glandular tubule. Since superficial mucosal scrapings do not contain these cells and since pepsinogen has an M_r of 43000, which is identical with the M_r of the band present in the translation products of whole stomach RNA, the suggestion is warranted that this prominent band is pepsinogen.

As our study failed to identify a possible candidate for the backbone, it cannot be excluded that gastric mucous cells produce more than one (different) backbone polypeptide. Further studies, using antibodies, are needed to identify the backbone polypeptide.

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