Localization of GTP-stimulated Core Glycosylation to Fused Microsomes

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ABSTRACT Purified rough microsomes from liver maximally incorporated N-acetyl-[3H]glucosamine into endogenous acceptors from UDP-N-acetyl-[3H]glucosamine substrate, providing the associated ribosomes were removed and 0.5 mM GTP was added. These conditions also led to the coalescence of microsomes into large fused membranes. By measurement of membrane profiles on electron micrographs, a correlation was observed between GTP-stimulated glycosylation and microsomal membrane length ($r^2 = 0.92$). Membrane fusion was not observed in the absence of GTP, with sugar transfer inhibited by >90% for acid-resistant acceptors (protein), and ~50% for acid-labile acceptors (lipid-linked intermediates). When radiolabeled acceptors were localized by electron microscope radioautography, high concentrations of silver grains (83 grains/100 μm membrane length) were observed over fused membranes with lower grain densities observed over unfused membranes in the same preparation (20 grains/100 μm). These studies directly link microsomal membrane fusion to GTP-stimulated core glycosylation. The observations extend the suggestion of Godelaine et al. (1979, Eur. J. Biochem. 96:17-26) that physiological levels of GTP promote the translocation of substrate across endoplasmic reticulum membranes which, we propose, occurs via a membrane fusion phenomenon.

A central problem in asparagine-linked core glycosylation is the mechanism by which nucleotide sugars located in the cell gain access to glycoprotein acceptors located within the lumina of the endoplasmic reticulum. As the lipid-linked oligosaccharide precursors of the core sugar glycopeptides are also intraluminally oriented (1) then sugar translocation must occur before or during the synthesis of the lipid-linked oligosaccharides. In 1977, Godelaine and colleagues (2) discovered that physiological concentrations of GTP enabled the translocation of sugar substrate across intact membrane vesicles derived from the rough endoplasmic reticulum (ER), provided these membranes had been stripped of their ribosomes (2–4). Paiement, with these same investigators (5) showed that physiological concentrations of GTP coincidentally promoted fusion of the same stripped rough microsomes.

To ascertain whether the GTP-specific membrane fusion phenomenon was merely coincidental or more causally linked to core glycosylation, we have compared membrane fusion and glycosylation quantitatively and attempted to localize the sites of sugar translocation to acceptor glycolipids and glycopeptides. Electron microscope radioautography of ER vesicles after incubation with GTP and uridine diphosphate-N-acetyl-[3H]glucosamine (UDP-[3H]GlcNAc) has revealed silver grains, indicative of glycosylated lipid-linked oligosaccharides and glycopeptides, predominantly over fused ER membranes, thereby providing a direct link between membrane fusion and core glycosylation. Portions of this work have been reported in abstract form (6).

MATERIALS AND METHODS

Preparation of Microsomal Subfractions: Microsomes from rat liver were prepared by differential centrifugation by using a slight modification of the procedure of Bergeron et al. (7). After an initial centrifugation at 8,700 g for 13 min, the supernatant was centrifuged at 25,000 rpm for 6 min 40 s in the Beckman type 50 rotor (8). Total microsomes were separated from the supernatant by centrifugation at 100,000 g for 60 min and then resuspended in 0.25 M sucrose with 4 mM imidazole buffer, pH 7.4. These rough microsomes were stripped of their ribosomes with 5 mM sodium pyrophosphate as previously outlined (2) and the resulting membrane preparation was designated stripped rough microsomes. Protein concentr-
The total acid-precipitable products were washed twice with cold 5% trichloroacetic acid. The term "microsomes" refers to membrane fractions that are resistant to precipitation with 8% glycogen, and "prepared rough microsomes" are microsomes stripped of ribosomes with 0.5 M sucrose. The vesicle profiles are homogeneous in appearance and size (measurement of 1,002 μm of membrane yielded a mean perimeter of 550 nm) and are surrounded by numerous electron-dense ribosomal particles. A peroxisomal core is indicated by the arrow, × 22,000.

**TABLE I**

| Fraction          | Protein (mg/g) | RNA (mg/g) | Glucose-6-phosphatase (U/g) | Acid phosphatase (U/g) |
|-------------------|----------------|------------|-----------------------------|------------------------|
| Homogenate        | 188.35 ± 16.31 | 8.00 ± 1.22| 14.96 ± 3.64                | 6.41 ± 0.26            |
| Microsomes        | 27.71 ± 3.59   | 3.50 ± 0.55| 7.60 ± 1.65                 | 0.97 ± 0.12            |
| Rough microsomes  | 6.97 ± 1.28    | 1.48 ± 0.15| 2.02 ± 0.47                 | 0.15 ± 0.02            |
| Stripped rough microsomes | 3.54 ± 0.69 | 0.52 ± 0.06| 1.66 ± 0.34                 | 0.10 ± 0.01            |

Fractions were isolated as described in Materials and Methods and assayed for various constituents. Values are given as means ± SD. Protein and RNA determinations from seven experiments are given in milligrams. Glucose-6-phosphatase and acid phosphatase from four experiments are expressed as micromoles of phosphate liberated per minute per milligram of protein. Values are expressed per gram of liver wet weight.

**Morphological Procedures:** Stripped rough microsomes were incubated in the endogenous glycosylation medium and then fixed and processed for electron microscopy as previously described (15). Stereology was carried out as previously described (15) using a Zeiss MOP-3 digitizer (Carl Zeiss, Inc., Don Mills, Ontario, Canada). For electron microscope radioautography, subcellular fractions were incubated with UDP-[3H]-GlcNAc for 120 min in the presence or absence of 0.5 mM GTP, then fixed and embedded in Epon. Losses of radioactivity in the various buffers and dehydrating solvents were monitored and found to be 1% of the total incorporated radioactivity for samples incubated with 0.5 mM GTP and 2.9% of the incorporated radioactivity for samples incubated in the absence of GTP (data not shown). Thin sectioning, application of nuclear emulsion, and radioautographic development for "fine" silver grains were as described previously (15). Morphometry was carried out on radioautographs having a final magnification of ×30,000 as calibrated with a grating replica of 54,800 lines/inch (J. B. EM Services Inc., Montreal, Canada). Using preparations developed for radioautography as indicated above, many of the silver grains consisted of a single, compact, round silver deposit, others consisted of two or four small silver deposits packed closely to one another. For grain distribution analysis, we have defined silver grains in two ways (6) and have carried out separate determinations based on these two definitions. In the first case, silver grains consisted of a single, compact, round silver deposit, others consisted of two to four small silver deposits packed closely to one another. For grain distribution analysis, we have defined silver grains in two ways (6) and have carried out separate determinations based on these two definitions. In the first case, silver grains consisted of a single, compact, round silver deposit, others consisted of two to four small silver deposits packed closely to one another. For grain distribution analysis, we have defined silver grains in two ways (6) and have carried out separate determinations based on these two definitions. In the first case, silver grains consisted of a single, compact, round silver deposit, others consisted of two to four small silver deposits packed closely to one another. For grain distribution analysis, we have defined silver grains in two ways (6) and have carried out separate determinations based on these two definitions. In the first case, silver grains consisted of a single, compact, round silver deposit, others consisted of two to four small silver deposits packed closely to one another.
RESULTS

Purified rough microsomes were analyzed morphologically (Fig. 1) revealing an homogenous population of ribosome-studded vesicles with a mean sectioned perimeter of 550 nm. Biochemical studies (Table I) indicated a recovery of 13.5% of homogenate glucose-6-phosphatase activity in this fraction, 3.7% of protein, and 16.8% of homogenate RNA, with an RNA/protein ratio of 0.21. Approximately 2% of the homogenate acid phosphatase activity was recovered in the fraction (Table I). Ribosomes were removed by washing with sodium pyrophosphate (2). This resulted in stripped rough microsomes (Fig. 2) with a mean perimeter of 508 nm of the sectioned vesicles. Biochemical studies (Table I) revealed a loss of 50.8% of protein and 64.8% of the RNA by the sodium pyrophosphate washing procedure, but with less of a loss of glucose-6-phosphatase activity (17.8%) and acid phosphatase (33.3%).

When stripped rough microsomes were incubated with UDP-[3H]GlcNAc in the endogenous glycosylation medium, incorporation of label into total acid-precipitable products was greatly increased by the addition of 0.5 mM GTP (Fig. 3), in agreement with the work of Godelaine et al (2, 3). Furthermore, GTP enabled transfer of label to hot acid-resistant, precipitable material. GTP-stimulated incorporation of label into hot acid-resistant, precipitable material of stripped rough microsomes has previously been shown to be associated with endogenous

\[ \text{Parameter measured} \begin{array}{ll}
+\text{GTP} & -\text{GTP} \\
[3H]GlcNAc & 15.63 \times 10^6 \pm 3.62 \times 10^6 * \\
\text{Acid-resistant incorporation} & 5.29 \times 10^4 \pm 0.36 \times 10^4 * \\
\text{Fused membranes} & 43.9 \pm 0 \\
\text{Unfused membranes} & 56.1 \% \\
\text{Grain density over fused membranes} & 83.4 \pm 100 \\
\text{Grain density over unfused membranes} & 20.4 \pm 14.3 \\
\end{array} \]

\[ 125 \, \text{Ci} \text{ of UDP-[3H]GlcNAc} \text{ (24 Ci/mmol) was incubated with 60} \mu \text{g of} \text{ protein of stripped rough microsomes in a total volume of 0.5} \text{ ml of the endogenous glycosylation medium in the presence or absence of GTP. At the end of a 2-h incubation, triplicate 20-} \mu \text{l samples were removed and assayed for total incorporation into ice-cold trichloroacetic acid precipitates, as well as incorporation into hot acid-resistant precipitates as described in Materials and Methods. The remaining material was fixed and filtered for random analysis as described in Materials and Methods. A total of 685 grains were counted over fused membranes in the samples incubated in the absence of GTP. Statistical analysis of mean grain densities indicated that the difference between the grain density over fused membranes (+GTP) and that over unfused vesicles was not statistically significant with or without corrections for section thickness (not shown).} \]

\[ \text{* Disintegration per minute (milligrams of protein-}2\text{h)}^{-1} \]

\[ \text{§ Silver grains per 100} \mu \text{m length.} \]
glycopeptides (22). Morphometric analysis of the same stripped rough microsomes incubated in the endogenous glycosylation medium with additional GTP revealed a temporal increase in the mean perimeter of the sectioned vesicles (Fig. 3, right-hand ordinate). Linear regression analysis defined a high correlation between the GTP-stimulated incorporation of UDP-[3H]-GlcNAc into total acid-precipitable products and increases in the mean membrane length of stripped rough microsomes. The coefficient of correlation \( r = 0.956 \) was found to be statistically significant at \( P < 0.01 \), as evaluated by the \( t \) test.

To test if the increase in vesicle size was directly related to the GTP-stimulated glycosylation, electron microscope radioautographic analysis was carried out. Stripped rough microsomes were incubated with UDP-[3H]-GlcNAc in the presence or absence of GTP and assayed for total incorporation into ice-cold trichloroacetic acid precipitates as well as hot acid-resistant precipitates (Table II). Samples from the same incubations were processed for electron microscopy radioautography (Figs. 4a, b). Morphometric analysis revealed a high grain density over fused membranes produced after incubation with GTP (83 grains/100 \( \mu \)m, Table II). Unfused membranes in the same preparation had a lower grain density (20 grains/100 \( \mu \)m, Table II). In samples incubated without additional GTP (Fig. 4b), no membrane profiles were considered fused and the grain density was low (14 grains/100 \( \mu \)m, Table II).

DISCUSSION

The studies presented here have established a link between fusion among isolated liver microsomes and GTP-stimulated core glycosylation. The change in mean membrane length of the microsomal vesicles during glycosylation correlated with GTP-stimulated incorporation of [3H]-GlcNAc into total acid-precipitable products (Fig. 3). The time course of events as shown by quantitation, therefore, supports the previous suggestion (5) that the GTP-induced enhancement of glycosylation could be the result of fusion of the microsomal vesicles. Since fusion correlated with the incorporation of labeled sugar into total acid-precipitable products and not with incorporation into acid-resistant products, these results also agree with the suggestion of Godelaine et al. (2) that the earliest effect of GTP and pyrophosphate treatment on core glycosylation, would be the stimulation of dolichol pyrophosphate \( N,N' \)-diacetylchitobiose (Dol-P-P-GlcNAc-GlcNAc) formation.

To test if the GTP enhancement of glycosylation could be localized to fused membranes, electron microscope radioautography was used to visualize the labeled acceptors. Incubations with 0.5 mM GTP revealed silver grains over unfused as well as fused microsomes but with a four-fold higher grain density over the latter (Table II, Fig. 4a). No significant difference was noted for grain densities over the unfused vesicles in incubations carried out with or without 0.5 mM GTP. Within the limitations of these studies, therefore, GTP-stimulated glycosylation was localized to the fused membrane compartment.

Since fusion and glycosylation of stripped rough microsomal membranes depend on the same factors, exhibit the same kinetic properties, and occur within the same subcompartment we therefore suggest that GTP-stimulated fusion plays a causal role in core glycosylation. Godelaine et al. (3) originally proposed that GTP may stimulate translocation of sugar across the ER membranes. The GTP-stimulated membrane fusion may allow sugar translocation across the membrane, possibly through a change in membrane permeability to activated sugars (5), and/or membrane fusion may reorient the first dolichol derivative of core glycosylation, dolichol pyrophosphate GlcNAc (Dol-P-P-GlcNAc), from a cytosolic to a luminal orientation. This translocation could occur via inverted micelles (25) or inverted cylinders (lamellar-hexagonal phase transition, [26]) known to be formed at points of membrane fusion and suggested to be involved in the translocation of lipid across membranes (27). The location of Dol-P-P-GlcNAc-GlcNAc to the luminal surface of microsomes is well established (28, 29) but the topology of Dol-P-P-GlcNAc during glycosylation is as yet undefined.

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Note Added in Proof: The in vivo counterpart to the membrane fusion

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**Figure 4** Location of endogenous acceptors by electron microscope radioautography of stripped rough microsomes after incubation with UDP-[3H]-GlcNAc in the presence or absence of GTP. Microsomes were incubated for 120 min at 37°C in 0.5 ml of medium (see Table II) then processed for electron microscope radioautography as described in Materials and Methods. Radioautographs were exposed for 42 d. Bars, 1 \( \mu \)m \( \times \) 30,000. (a) Stripped rough microsomes incubated with 0.5 mM GTP for 120 min. The vesicles are highly aggregated. Large irregular membrane-bound fusion products are marked by an asterisk over their lumina. Because of their irregular outlines, such large fusion products can be easily distinguished from the much smaller, more regular unfused vesicles. Unfused vesicles can be seen within some large invaginations (Inv) of the fusion products. Radioautographic "fine" silver grains representing sites of labeled endogenous acceptors are numerous along the length of the fused membranes (notice membrane regions surrounding the asterisks). A particularly high concentration of silver grains is seen along the closely apposed edges of three large fusion products (tangential zone indicated by the curved open arrow). In contrast, few silver grains are observed over regions containing many unfused vesicles (bracketed areas). (b) Stripped rough microsomes incubated without GTP for 120 min. Although the vesicles are aggregated, they resemble unincubated vesicles (see Fig. 2) and had a similar size based on a mean vesicle perimeter of 330 nm. In contrast with a, radioautographic silver grains are few and are randomly distributed over the unfused vesicles.
documented herein may be that core glycosylation is a posttranslational event occurring at points of fusion of ribosome-free regions of rough ER cisternae. A less obvious interpretation is that GTP in vivo may modulate at selected sites beneath ribosomes a local perturbation of membrane lipids allowing for nascent chain translocation and cotranslational core glycosylation.

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