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EVIDENCE FOR COUPLING BETWEEN TRANSPORT OF UDP-GLUCOSE AND ITS SYNTHESIS BY MEMBRANE-BOUND PYROPHOSPHORYLASE IN GOLGI APPARATUS OF CAT LIVER

FLORENCE PERSAT, GÉRARD AZZAR, MARIE-BÉNÉDICTE MARTEL and RENÉ GOT *

Laboratoire de Biochimie des Membranes, LBTM-CNRS No. 5421, Université Claude Bernard, Lyon I, 69622 Villeurbanne Cedex (France)

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Incubation of sealed vesicles of cat-liver Golgi apparatus with UDP[^{14}C]glucose showed that the vesicles accumulated radioactivity. After Triton X-100 treatment or sonication of washed vesicles, soluble radiolabeled species were released and identified by paper chromatography as UDP[^{14}C]glucose, [^{14}C]glucose 1-phosphate and free glucose. In the incubation medium, UDPglucose was effectively protected by addition of dimercaptopropanol and UTP. Presence of glucose 1-phosphate and glucose within the vesicles most probably arose from luminal pyrophosphatase and phosphatase. A portion of the [^{14}C]glucose moiety became covalently linked to endogenous acceptors. Uptake of UDPglucose was saturable and dependent on time and on the concentration of sugar nucleotide. Together, these results were consistent with a transport system for UDPglucose in Golgi vesicles. Furthermore, penetration rate was considerably higher with UDPglucose synthetized in situ from glucose 1-phosphate by membrane-bound pyrophosphorylase than from added UDPglucose: V_{max} values were respectively 10 and 2 pmol/15 min per mg protein. This result allows the conclusion that a coupling between translocase and synthetase is involved in UDPglucose transport through Golgi apparatus membranes. The mechanism of this 'kinetic advantage' is discussed.

Introduction

One of the major functions of glycosyltransferases of the Golgi apparatus is the terminal glycosylation of proteins destined for secretion. There is evidence [1–3] to suggest that the orientation of these enzymes is probably toward the lumen. This spatial arrangement poses a problem in logistics because glycosyltransferases utilize as substrates sugar nucleotides which are apparently unable to cross the membrane, taking into account their charged and hydrophilic characters. To solve this problem, we will consider at least two possibilities.

The first possibility involves isoprenoid carrier lipids which might mediate transfer of activated glycose moieties from one side of the membrane to the other by 'flip-flop'. But, from recent biochemical data [4], it is apparent that the liver Golgi complex does not contain detectable activities of enzymes acting on dolichyl phosphate.

The possibility that sugar nucleotides permeate Golgi apparatus vesicles has also been investigated; a specific uptake mechanism appears to function in the production of an intraluminal pool of CMP-NeuNAc [5,6] and GDPfucose [7].

We have approached this problem of the transmembrane movement of activated sugars by determining if sugar nucleotides relevant to glycolipid and glycoprotein synthesis are generated with

* To whom correspondence should be addressed.

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the lumen of Golgi apparatus cisternae. We have previously provided evidence that UDPglucose pyrophosphorylase is associated with liver Golgi apparatus [8–10]. Studies presented in this report suggest that if sugar nucleotide uptake by a putative transporter can be detected, there is a coupling between membranous synthesis of UDPglucose and its transport across the Golgi membrane.

Materials and Methods

Radioactive substrates. D-[U-^{14}C]glucose 1-phosphate (227 mCi/mmol) and UDP-D-[U-^{14}C]glucose (233 mCi/mmol) were purchased from Amersham International, U.K.

Preparation of Golgi fraction. A Golgi-rich fraction, isolated as previously described [8–10] from cat liver, was enriched, over homogenate, respectively 2-fold in glucose-6-phosphatase activity (2% yield of total homogenate activity), 27-fold in sialyltransferase activity (14% yield) and 4-fold in 5′-nucleotidase activity (3% yield). It was essentially free of cytosol contamination as measured by activities of glucose-6-phosphate dehydrogenase and lactate dehydrogenase. Recovery of the UDPglucose pyrophosphorylase activity in the Golgi-derived vesicles was 75%, with a 1.6-fold enrichment relative to the total smooth microsomes. Vesicle integrity as determined by stimulation of galactosyltransferase after treatment with Triton X-100 [2] was found to be 70%. Golgi vesicles prepared using a $^{2}H_2O$-sucrose gradient, according to procedure of Fleischer [11] had a higher integrity, but they were largely contaminated by cytosol, since they were not washed.

Paper chromatography. Radiolabeled UDPglucose, glucose 1-phosphate and glucose were isolated and identified using descending developments for 17 h on sheets of Whatman No. 3 paper previously washed with 10 mM EDTA (pH 7) and then air-dried. The solvent was ethanol/1 M sodium acetate (pH 3.8) (5 : 2, v/v) [12]. The chromatograms were cut into bands (2 cm wide) and the radioactive sugar nucleotide, degradation products and endogenous glucose acceptor (the protein-bound radioactive glucose remained near the origin and the UDPglucose migrated to 6 cm) were located and measured by counting each band in a scintillation mixture containing 4 g 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene.

Transport assays of UDPglucose. Penetration assays were performed with added UDPglucose or with membrane-synthesized UDPglucose. Freshly prepared Golgi vesicles (1–2.5 mg of protein) were incubated at 38°C with 5.4 mM $MgCl_2$, 0.1 M Bicine buffer (pH 7.8), 0.25 M sucrose, 5 mM 2-mercaptoethanol, 2.9 mM UTP, 5 mM dimercaptopropanol and radioactive substrates, in a total volume of 1 ml. Reactions were stopped by placing samples on ice. After addition of 8 ml ice-cold buffer, these were centrifuged for 1 h at $120\,000 \times g_{av.}$ at 4°C. Pellets were washed by suspension in 9 ml buffer containing 5 mM dimercaptopropanol. After centrifugation, the pellets were homogenized in 1 ml buffer and treated by Triton X-100 (1.8%, w/v) or sonicated for 5 min in a Branson Sonifier (20 W continued) at room temperature. The suspensions were centrifuged at $120\,000 \times g_{av.}$ for 1 h and 200-μl portions of the resulting supernatants were counted for radioactivity in TM 299 (Packard). Other portions were analysed by paper chromatography. The last pellets were dissolved in 200 μl formic acid for 24 h and radioactivity was determined in the same scintillator.

Results

Only freshly prepared Golgi vesicles of liver were utilized in the experiments; quick-freezing followed by storage in liquid nitrogen was avoided.

The study of sugar nucleotides transport was hampered by the presence, in animal tissues, of highly active nucleotide pyrophosphatase and alkaline phosphatase which act sequentially to degrade sugar nucleotides substrates. UDPglucose was thoroughly degraded after 10 min of incubation. The combination of dimercaptopropanol with the concentration of UTP utilized for UDPglucose pyrophosphorylase reaction provided a high degree of UDPglucose and glucose 1-phosphate protection. This combination allowed more than 80% of UDPglucose to remain intact for 1 h. Furthermore, washing of the pellets with resuspension eliminated radioactivity adsorbed or soluble in the pellet volume that was outside the vesicles. Incubation of Golgi vesicles with added

UDP[^{14}C]glucose or synthesized UDP[^{14}C]glucose from [^{14}C]glucose 1-phosphate by membrane-bound pyrophosphorylase resulted in the accumulation of radioactivity within such vesicles, even after two washings. Most of the radioactivity entraped (95%) was released by treatment with Triton X-100 – which caused vesicles to become permeable to charged molecules [13] or solubilized endogenous acceptor – or, to a lesser extent, by sonication. Analyses of the detergent solubilized radioactive species by paper chromatography showed the presence of UDP[^{14}C]glucose and [^{14}C]glucose covalently bound to Golgi proteins (this sugar was released from the radioactivity remaining at the origin of paper chromatography by acid hydrolysis, 2 M HCl, 1 h at 100°C). [^{14}C]Glucose 1-phosphate and free [^{14}C]glucose also were identified. The time-course of UDPglucose uptake is presented in Fig. 1.

Importantly, mechanical means of vesicle disruption by sonication released in a supernatant the same amount of UDPglucose, glucose 1-phosphate and glucose as with detergent, while glucose covalently bound to glycoproteins remained in the membranous pellet. This result supports the membrane-bound character of endogenous acceptor.

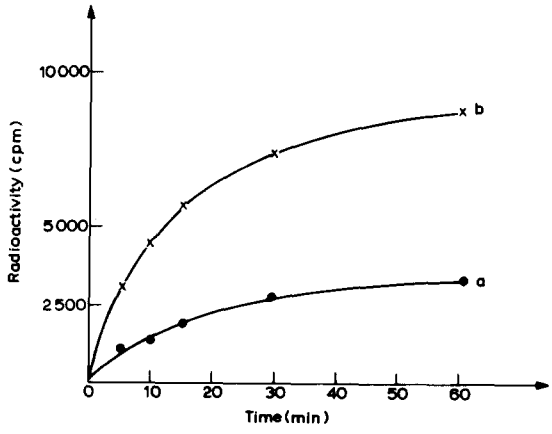

Fig. 1. Time-course of UDPglucose uptake. UDP[^{14}C]glucose (11 μM) or [^{14}C]glucose 1-phosphate (44 μM) were incubated with Golgi vesicles (2.4 mg/ml) as described in Materials and Methods. After washings, pellets were treated by Triton X-100 (1.8%, w/v) for 10 min at room temperature and supernatants obtained by centrifugation at 120000 × g_{av} for 1 h were analysed for their content in UDP[^{14}C]glucose. (a) Added UDPglucose. (b) UDPglucose synthesized by membranous pyrophosphorylase from glucose 1-phosphate and UTP (2.9 mM).

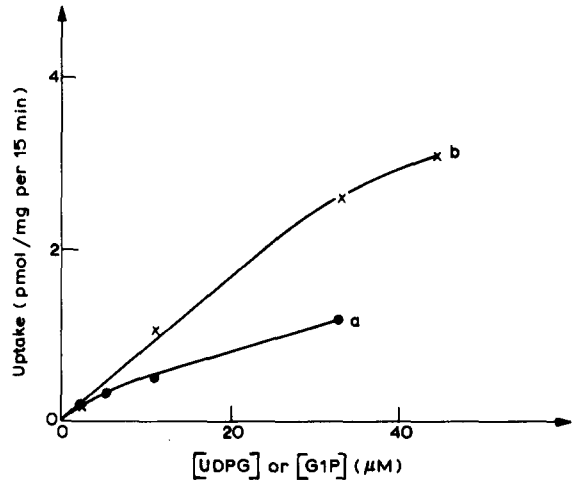

Fig. 2. Concentration dependance of UDPglucose uptake in Golgi vesicles. Golgi vesicles (1.4 mg/ml) were incubated with varying concentrations of UDP[^{14}C]glucose or [^{14}C]glucose 1-phosphate for 15 min. After washings and treatment with Triton X-100 as in Fig. 1, UDP[^{14}C]glucose was determined as described in Materials and Methods. (a) and (b) as in Fig. 1.

Moreover, these vesicles contained a collagen glucosyltransferase functioning with calf-skin collagen as exogenous acceptor (results not shown).

To study the concentration dependence of UDPglucose uptake into Golgi vesicles, such vesicles were incubated with labeled UDPglucose or glucose 1-phosphate at similar concentration. The UDP[^{14}C]glucose and and [^{14}C]glucose 1-phosphate had the same specific radioactivity, respectively 233 and 227 mCi/mmol, and equivalent amounts of radioactivity were used in these experiments. Under these conditions, penetration rates obtained respectively with UDPglucose added or synthesized at membranous level were compared. The latter, however, was in lower amount, since glucose 1-phosphate transformation in UDPglucose was not complete. UDPglucose penetrated Golgi vesicles at considerably higher rates when it was synthesized in situ from glucose 1-phosphate: $V_{max} = 10$ pmol/15 min per mg protein, with an apparent K_m of 75 ± 10 μM as compared with a $V_{max} = 2$ pmol/15 min per mg protein for added UDPglucose (Fig. 2).

The major soluble species of the Golgi pellets were glucose 1-phosphate and, to a lesser extent, free glucose. This probably reflects the breakdown

of internalized UDPglucose by luminal nucleoside diphosphatase and phosphatase [7,14].

Discussion

To evaluate sugar nucleotide transport, nucleotide pyrophosphatases and phosphatases must be controlled, since these enzymes rapidly degrade sugar nucleotides and sugar phosphates. Proper assay conditions must be established for each tissue or enzyme source. With cat-liver Golgi, hydrolytic enzymes were not effectively inhibited by the addition of dimercaptopropanol alone, even at a concentration of 5 mM The presence of 3 mM UTP required for UDPglucose pyrophosphorylase activity provided, in combination with the chelator, a high degree of UDPglucose and glucose 1-phosphate protection; but this protection was restricted to extravesicular space. Glucose 1-phosphate and free glucose released by Triton X-100 or sonication must arise from a compartment inaccessible to pyrophosphatase or phosphatase inhibitors, i.e., intravesicular space. Additionally, the presence of nucleoside diphosphatase and phosphatase has been shown in the lumen of the Golgi vesicles [7,14].

The results of this paper strongly suggest that Golgi membranes can transport UDPglucose from the incubation medium to the lumen. Transport of CMP-*N*-acetylneuraminic acid (CMPNeuAc) and GDPfucose to the lumen of rat-liver Golgi vesicles has been described previously [7]. The V_{max} for UDPglucose, approx. 15-fold less than that for CMPNeuAc, is in good agreement with the value obtained for GDPfucose [7].

These carrier proteins must have recognition sites facing the cytoplasmic side of the Golgi membranes. Previous studies from this laboratory [15–17] had demonstrated the presence in rat-liver Golgi apparatus of a binding protein specific for UDPglucose and its participation in a carrier-mediated transport had been considered [17].

One of the more interesting findings in relation to UDPglucose transport is the role of its synthesis by a membrane-bound pyrophosphorylase. Direct evident has been presented for a higher penetration rate with UDPglucose synthesized in situ. This 'kinetic advantage' has been described by Crane's group [18,19] for the intestinal uptake of glucose from free glucose or disaccharides. The mechanism for the function of sucrose in sugar translocation has been studied by Semenza et al. [20,21] with a reconstituted transport system in lipid membranes.

From the experiments presented in this paper, the most likely mechanism for the function of pyrophosphorylase as UDPglucose translocator is the following. The active site of UDPglucose synthetase may be confined to the cytoplasmic side of Golgi membrane. The products of synthesis are liberated in a microenvironment close to the translocase. High concentrations of UDPglucose could be maintained with a short diffusion time to ensure a higher rate of transport activity.

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