Mechanism of UDP-sugar transport into intracellular vesicles

Occurrence of UDP-GlcNAc/UDP and UDP-Gal/UDP antiports

Roméo Cecchelli, René Cacan and André Verbert*

Laboratoire de Chimie Biologique, Unité Associée au CNRS no. 217, Université de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cedex, France

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The mechanism of translocation of UDP-GlcNAc, UDP-Gal and UDP-Glc into intracellular vesicles has been studied using thymocytes whose plasma membranes have been permeabilized with isotonic ammonium chloride. It has been previously shown that the intracellular vesicles have specific carriers for UDP-GlcNAc and UDP-Gal. We now report that the translocation of these two sugar nucleotides occurs via UDP-GlcNAc/UDP and UDP-Gal/UDP antiports. The entry of UDP-GlcNAc or UDP-Gal into vesicles was specifically dependent on the exit of UDP from these vesicles. In contrast, no antiport mechanism has been recovered with UDP-Glc for which no transport and accumulation into intracellular vesicles were observed.

UDP-sugar transport *(Permeabilized cell)* *Antiport*

1. INTRODUCTION

Most glycosyltransferase activities occur within the lumen of intracellular vesicles [1–3] while the sugar nucleotide precursors are located in the cytoplasm [4], thus it is apparent that translocation of sugar nucleotides is required for the glycosylation process to occur. Such transmembrane movements could take place while the sugars are attached to either nucleotide or dolichol phosphate and thus could be mediated respectively by a specific transport system or by the glycosylation process itself. Recent studies from our laboratory have shown that treatment of mouse thymocytes with an isotonic concentration of ammonium chloride renders the plasma membrane permeable to sugar nucleotides without affecting the permeability barrier of intracellular membranes as reported in [5]. In fact, in ammonium chloride-treated thymocytes, the latency of glycosyltransferase activities is maintained [5], the endogenous pool of CMP-NeuAc and GDP-Fuc remains intralumenal [6] and exogenous labelled sugar nucleotides can be translocated and accumulated [6,7]. The Golgi location of these accumulations has been strongly suggested by the effect of monensin [8]. These accumulations are saturable, temperature dependent and inhibited by nucleoside mono- or diphosphate, demonstrating the occurrence of a specific carrier. Subsequent to translocation into the intracellular vesicles, the sugar moieties are transferred onto endogenous acceptors facing the lumen. These findings raise an interesting topological problem in understanding how inhibitory products of the glycosylation process, such as UDP, are removed from the lumen so that even at high rates of glycosylation no inhibition is observed. Capasso and Hirschberg [9] have reported that translocation of GDP-Fuc across Golgi membranes occurs via a coupled equimolar exchange with the corresponding monophosphate, GMP

* To whom correspondence should be addressed

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coming from the degradation of GDP generated by the fucosylation process.

The purpose of this work is to study the mechanism of translocation of UDP-sugars such as UDP-GlcNAc, UDP-Gal and UDP-Glc.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All reagents were of analytical grade. The labelled products were purchased from Amersham International (Amersham, England) and used at the following specific activity: UDP-*N*-acetyl-D-[^{14}C]glucosamine (6.4 TBq/mol), uridine diphospho-D-[^{14}C]glucose (8.25 TBq/mol), uridine diphospho-D-[6-^{3}H]galactose (1.38 TBq/mol), [5-^{3}H]uridine 5′-diphosphate (2.7 TBq/mol) and [5-^{3}H]uridine 5′-monophosphate (2.7 TBq/mol). AMP, UMP, UDP, UDP-D-galactose, UDP-*N*-acetyl-D-glucosamine, UDP-D-glucose and CMP-*N*-acetylneuraminic acid were obtained from Sigma (St. Louis, USA). Tunicamycin was a generous gift from Dr W.F.J. Cuthbertson (Glaxo Research Ltd, Stoke Poges, England).

2.2. *Cell preparation and permeabilization technique*

Thymocytes were prepared from thymus of 4-week-old Swiss mice. To render the plasma membrane permeable to sugar nucleotides, thymocytes were resuspended in the following medium: 0.154 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$ according to Cecchelli et al. [5]. The total duration of this treatment was 25 min at 20°C followed by a 5 min centrifugation at 1000 × *g*. Under these conditions, 90% of the cells were permeabilized as judged by the trypan blue exclusion test.

2.3. *Incubation medium for the determination of transport and transfer activities*

For transport and glycosyltransferase assays, the thymocytes were resuspended in the medium described by Cox and Peters [10] at 5×10^8 cells/ml. All assays were performed in 100 μl of this medium, containing 20 μM of either labelled sugar nucleotides or nucleotides, 5 mM $MgCl_2$ and 2 mM $MnCl_2$ and in the case of UDP-GlcNAc, 1 μg/ml of tunicamycin to inhibit use of this sugar nucleotide in the dolichol cycle.

The methodology for the assays of transport capacity of thymocytes has been detailed in [6,7]. Briefly, after incubation, the cells were passed through a layer of immersion oil to remove the non-entrapped radioactive precursor. The total cell-associated radioactivity entrapped within intracellular vesicles (the transport capacity) and the trichloroacetic acid-precipitable radioactivity (the transfer activity onto endogenous acceptors) were determined. The acid-soluble radioactive material was the difference between the total cell-associated radioactivity and the acid-precipitable radioactivity. The nature of the soluble material was determined as follows: after the final washing, the cell pellet was extracted with ethanol/water (2:1, v/v) and the extract was analysed by chromatography on Whatman 3 MM paper using the solvent pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) for sugar nucleotides and degradation products, or by electrophoresis on Whatman 3 MM paper (0.02 M citrate buffer, pH 3.5) for UPD, UMP and uridine.

3. RESULTS AND DISCUSSION

3.1. *Study of the exchange between UDP-GlcNAc and UDP*

In previous studies, we have shown that UDP-GlcNAc accumulated into intracellular vesicles via a specific carrier [6] and Perez and Hirschberg [11] have shown that UDP-GlcNAc was translocated across the membranes of Golgi vesicles. We set out to determine whether translocation of UDP-GlcNAc was coupled with an exchange of nucleotides from the lumen as previously described for GDP-Fuc/GMP [9].

3.1.1. Exit of entrapped UDP-[^{14}C]GlcNAc

Ammonium chloride-treated thymocytes were preloaded with UDP-[^{14}C]GlcNAc. As can be seen in fig.1, there was a time-dependent accumulation of radiolabelled solutes within the intracellular vesicles that became constant after 10 min. As identified previously [6], the acid-soluble radioactivity was uncleaved UDP-[^{14}C]GlcNAc. The addition of 1 mM UMP led to the exit of 50% of the soluble radioactivity within 1 min in agreement with preliminary studies by Capasso and Hirschberg [9]. Furthermore, addition of 1 mM UDP led to the complete exit of entrapped radioac-

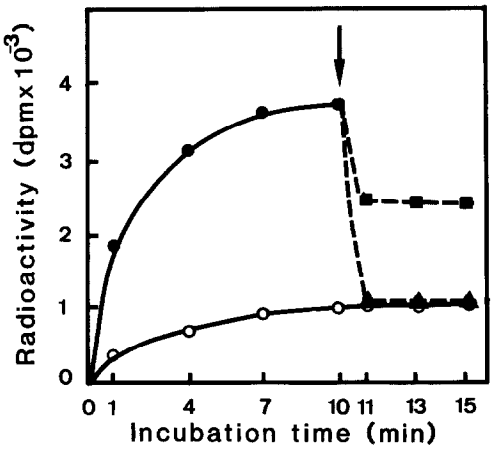

Fig.1. Effect of UMP and UDP on the accumulation of UDP-[^{14}C]GlcNAc into intracellular vesicles. Total transport of UDP-[^{14}C]GlcNAc (●) into intracellular vesicles and covalently bound [^{14}C]GlcNAc (○) were measured during the first 10 min, then 1 mM UMP (■) or 1 mM UDP (▲) were added (arrow), and transport and transfer were measured.

tivity. We further examined whether the exit of UDP-[^{14}C]GlcNAc from intracellular vesicles was specifically dependent on the entry of UDP into the vesicles. To ammonium chloride-treated thymocytes preloaded by a 10 min incubation with 20 μM UDP-[^{14}C]GlcNAc, various concentrations of either AMP, UMP or UDP were added and incubation carried out for 1 min. Fig.2 shows that only UDP stimulated the total exit of UDP-[^{14}C]GlcNAc (100% at 25 μM). UMP was much less effective (50% at 50 μM) and AMP had no effect.

The exit of UDP-GlcNAc from the intracellular vesicles appears concomitant with the entry of UDP, but the possibility that the selectivity of the translocator may not be absolute cannot be ruled out. Further experiments were developed to check the reversibility of the exchange.

3.1.2. Exit of entrapped [^{3}H]UDP

As shown in fig.3, permeabilized thymocytes were able to accumulate [^{3}H]UDP from an external to a lumenal compartment. The accumulation of the radioactivity became constant after 10 min. However, when the same experiment was performed with [^{3}H]UMP, no accumulation was observed as reported by Barthelson and Roth [12].

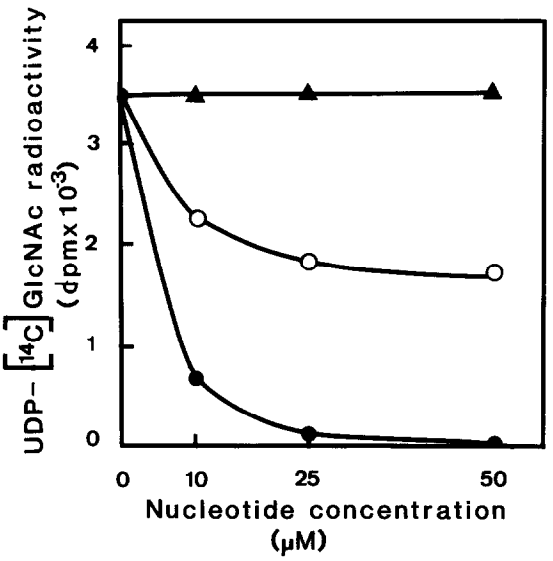

Fig.2. Effect of nucleotide concentrations on the exit of entrapped UDP-[^{14}C]GlcNAc. The permeabilized cells were allowed to accumulate UDP-[^{14}C]GlcNAc for 10 min. At this time various concentrations of AMP (▲), UMP (○) or UDP (●) were added and the UDP-[^{14}C]GlcNAc remaining entrapped was measured after 1 min.

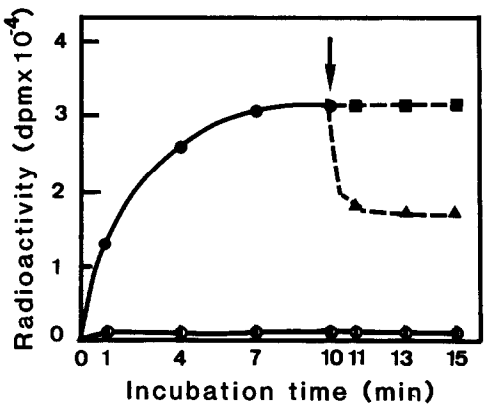

Fig.3. Effect of CMP-NeuAc and UDP-GlcNAc on the exit of entrapped [^{3}H]UDP. Accumulation of [^{3}H]UDP (●) and [^{3}H]UMP (○) into intracellular vesicles was measured during 10 min. The arrow indicates the addition of 1 mM CMP-NeuAc (■) or UDP-GlcNAc (▲); the remaining entrapped labelled material was measured.

Thus, it was possible to examine whether addition of unlabelled UDP-GlcNAc would stimulate exit of entrapped UDP. Fig.3 shows that addition of 1 mM UDP-GlcNAc led to a 50% decrease in radioactivity remaining in the vesicles. In contrast, when 1 mM CMP-NeuAc was added to the mixture, no exit of radioactivity was detected, even though CMP-NeuAc is known to enter vesicles rather efficiently [7,13]. Due to the activity of Golgi surface phosphatases [14], the nature of the radioactive products in the incubation medium and in the intracellular vesicles before and after the addition of UDP-GlcNAc had to be checked. In control experiments, after 10 min incubation most of the extravesicular UDP was degraded (2% remaining) into UMP (92%) and uridine (6%) although the radioactivity entrapped in the vesicles still contained mainly UDP (63%). When 1 mM UDP-GlcNAc was added after the 10 min [^{3}H]UDP-preloading period, the radioactivity remaining entrapped represented mainly uridine (96%). The data reported in table 1 demonstrate that addition of UDP-GlcNAc leads to the exit of UDP only. Fig.4 shows that the exit of [^{3}H]UDP is dependent on the entry of UDP-GlcNAc (100% at 250 μM) and that CMP-NeuAc has no effect on the accumulated [^{3}H]UDP. Since the extent of the endogenous pool of UDP-GlcNAc is not known in our system, the stoichiometry of the antiport mechanism cannot be calculated.

We therefore hypothesize that UDP-GlcNAc enters efficiently into intracellular vesicles via a coupled exchange with the corresponding nucleoside diphosphate: UDP.

3.2. *Study of the exchange between UDP-Gal and UDP*

Previous studies from our laboratory and another [7,15] have shown that UDP-Gal is translocated into intracellular vesicles. To obtain evidence of a similar exchange mechanism in the translocation of UDP-Gal, ammonium chloride-treated thymocytes were first incubated with 20 μM UDP-[^{3}H]Gal for 10 min, then different unlabelled nucleotide derivatives were added to the cell suspension for 1 min. Fig.5 shows that AMP has no effect on the entrapped UDP-Gal, UMP has little effect (around 10%), but UDP stimulates the exit of the entrapped UDP-Gal (85% at 150 μM). Thus the exit of UDP-Gal from intracellular vesicles appears to be concomitant with the entry of UDP specificity.

To confirm the exchange between UDP-Gal and UDP, thymocytes were preloaded with [^{3}H]UDP. After 10 min, different concentrations (50–250 μM UDP-Gal) were added to the incubation mixtures. Fig.4 shows that UDP-Gal stimulates the exit of UDP entrapped into the vesicles (100% at 150 μM).

Our results demonstrate that the mechanism of exchange described for UDP-GlcNAc/UDP also occurs for UDP-Gal/UDP. The antiport

Table 1

Distribution of the soluble radioactivity in the incubation medium and in the intracellular vesicles after addition of sugar nucleotides

	Incubation medium	Intracellular vesicles	
		Control	+UDP-GlcNAc
Uridine	9919 ± 410 (6)	7824 ± 263 (36)	7743 ± 99 (96)
UMP	1579899 ± 102832 (92)	217 ± 20 (1)	241 ± 31 (4)
UDP	31633 ± 1040 (2)	13692 ± 620 (63)	119 ± 33 (1.5)

The data represent means ± SD from three different experiments. The radioactivity is expressed in dpm and the number in parentheses indicates the percent of the total radioactivity

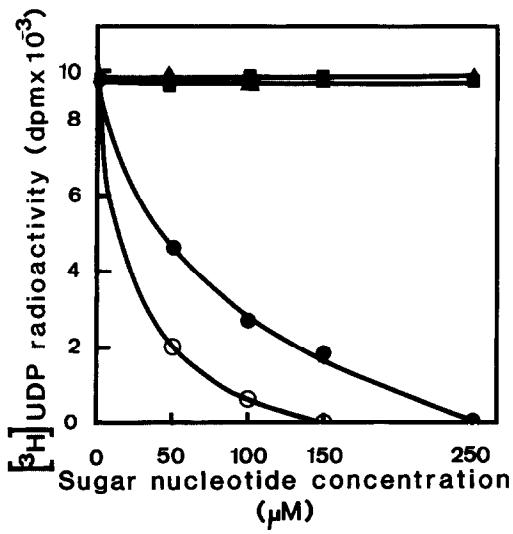

Fig.4. Effect of sugar nucleotides on the exit of entrapped [^{3}H]UDP. Thymocytes were allowed to accumulate [^{3}H]UDP during 10 min. At this time various concentrations of CMP-NeuAc (■), UDP-Glc (▲), UDP-GlcNAc (●) or UDP-Gal (○) were added and the remaining entrapped [^{3}H]UDP was measured after 1 min.

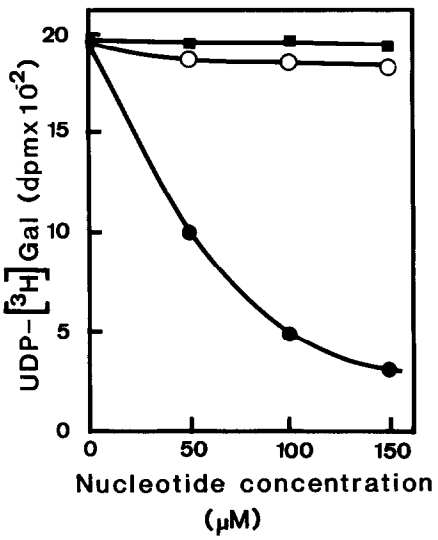

Fig.5. Effect of nucleotide concentrations on the exit of entrapped UDP-[^{3}H]Gal. Thymocytes were allowed to accumulate UDP-[^{3}H]Gal for 10 min, then various concentrations of AMP (■), UMP (○) or UDP (●) were added and the remaining entrapped UDP-[^{3}H]Gal was measured after 1 min.

mechanism allows the precursors to enter intracellular vesicles but also decreases the lumenal accumulation of UDP which is a potent inhibitor of glycosyltransferases. Another way to eliminate UDP was described by Kuhn and White [14] and Fleischer and Brandan [15] on rat mammary gland and rat liver respectively, in which intracisternal UDPases hydrolyzed UDP into UMP which probably re-enters the cytosol. In our model, as shown in fig.3 no accumulation of UMP was demonstrated in contrast to what was observed by Brandan and Fleischer [16].

Since UDP-GlcNAc and UDP-Gal did not inhibit translocation of each other (not shown), we hypothesize that the translocators in the membrane of the Golgi apparatus are different for UDP-GlcNAc and UDP-Gal, but that both exchange their sugar nucleotides with UDP.

3.3. *Is UDP-Glc translocated by a similar mechanism?*

Persat et al. [17] have shown that Golgi membranes derived from cat liver can transport UDP-Glc from the incubation medium to the lumen. Perez and Hirschberg [18] demonstrated that UDP-Glc was translocated into rough endoplasmic reticulum vesicles via a coupled exchange with lumenal UMP, but in Golgi vesicles translocation of UDP-Glc could not be detected. Thus we examined whether UDP-Glc could be translocated into intracellular vesicles in our model using ammonium chloride-treated thymocytes incubated with UDP-[^{14}C]glucose. Irrespective of the incubation medium, incubation time or concentration of sugar nucleotide, no difference was observed between the total cell-associated radioactivity and the trichloroacetic acid-precipitable radioactivity (not shown) demonstrating that no intracellular pool of UDP-Glc could be observed; however, it may be considered that UDP-Glc entered the vesicles and was immediately used for glucosylation. Fig.4 shows that UDP-Glc has no effect on the accumulation of [^{3}H]UDP in intracellular vesicles, indicating that the translocation of glucosyl units does not occur via an exchange mechanism between UDP-Glc and UDP. The question is still open as to whether UDP-Glc is translocated as a whole via a UDP-independent carrier mechanism, in contrast to what we observed for UDP-GlcNAc and UDP-Gal, or whether Glc-P-Dol only is involved.

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