TRANSVERSE LOCALIZATION OF GLYCOSYL TRANSFERASES IN ROUGH AND SMOOTH MICROSOMES

Ivan EGGENS and Gustav DALLNER+

Department of Pathology at Huddinge Hospital, Karolinska Institutet and +*Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden*

Received 4 November 1980

1. Introduction

The presence of a number of glycosyl transferases in the membranes of rough and smooth microsomes is well established [1]. The core portion of the oligosaccharide chain is attached to dolichol pyrophosphate during synthesis and transferred as a whole to the endogenous protein acceptor. Completion of the protein-bound oligosaccharide is known to take place at various locations. Transfer of individual sugar residues from the nucleotide-activated form may or may not involve dolichol monophosphate as a lipid intermediate.

In liver cells sugar nucleotides are synthesized in the cytoplasm, but the majority of the proteins synthesized on the endoplasmic reticulum are of the secretory type and are glycosylated at the luminal surface [2]. On the other hand, protein-bound oligosaccharides are also present at the cytoplasmic surface of this organelle and some of the enzymes at this surface have now been identified as glycoproteins [3–7]. One could expect that certain glycosyl transferases or at least a part of the glycosyl transferase system is present at the cytoplasmic surface of the endoplasmic reticulum and, in fact, earlier observations indicated that both mannosyl and glucosaminyl transferases are sensitive to proteolytic treatment of intact microsomal vesicles [8,9].

Here, intact, non-permeable rough and smooth microsomal vesicles were subjected to proteolysis and treated with the non-permeable reagent diazobenzene sulfonate (DABS). Inhibitors of glucosyl transferase reactions were also employed. The results demonstrate that there are several different systems present in the endoplasmic reticulum and that a part of them is localized at the cytoplasmic surface, especially in rough microsomes.

2. Materials and methods

Rough and smooth microsomes were prepared as in [10] from livers of adult male rats (180 g). The fractions were washed in all experiments by recentrifugation in 0.15 M Tris–HCl (pH 8.0). *p*-Diazobenzene sulfonate (DABS) was prepared as in [11]. The incubation mixture contained 50 mM KCl, 50 mM Tris–HCl (pH 7.5); 0.25 M sucrose; 0.75 mM DABS and 18 mg microsomal protein in 3 ml final vol. The incubation time was 2 min in an ice-water bath and the process was terminated by adding 5 ml cold 0.25 M sucrose containing 13 mM $CaCl_2$ and 8 mM $MgCl_2$ and centrifuging at 25 000 × *g* for 15 min.

For proteolysis the incubation mixture contained 50 mM KCl; 50 mM Tris–HCl (pH 7.5); 0.25 M sucrose; 0.45 mg trypsin (Boehringer, Mannheim); 0.45 mg unspecific protease (type VII from *B. amyloliquefaciens*, Sigma, St Louis) and 18 mg microsomal protein in 3 ml final vol. The incubation time was 10 min at 37°C and was terminated by the addition of 5 ml cold 0.25 M sucrose and centrifugation for 60 min at 105 000 × *g*. The incubation mixture for in vitro incorporation of mannose contained 30 mM Tris–HCl (pH 7.8); 1 mM $MnCl_2$; 2 mM AMP; 0.5 μCi GDP-[^{14}C]mannose (80 mCi/mmol. Radiochemical Centre, Amersham) and 2.5 mg microsomal protein in 1 ml total vol. Amphomycin (400 μg) was added where indicated. These incubations were performed at 30°C for 30 min.

When dolichol phosphate (DP)-[^{14}C]mannose was used as substrate, the incubation mixture contained

30 mM Tris–HCl (pH 7.8); 1 mM $MnCl_2$; 2 mM AMP; 0.4% Triton X-100; DP-[^{14}C]mannose (80 000 cpm) and 2.5 mg microsomal protein in 1 ml total vol. DP-[^{14}C]mannose was prepared enzymically and purified by chromatography. The incubation time was 30 min at 30°C. Extractions with chloroform–methanol (lipid I) and with chloroform–methanol–H_2O (lipid II) were performed as in [8]. The radioactivity in the lipid extracts and in the protein pellet was determined as in [8]. The incubation mixture for in vitro incorporation of *N*-acetylglucosamine (GlcNAc) contained 30 mM Tris–HCl (pH 7.8); 2.5 mM EDTA; 10 mM $MnCl_2$; 1.5 mM ATP; 1.2 μCi UDP-[^{14}C]GlcNAc (300 mCi/mmol, Radiochemical Centre, Amersham) and 2.5 mg microsomal protein in 1 ml total vol. Tunicamycin (10 μg) was added where indicated. The incubation was performed at 30°C for 30 min. When dolichol pyrophosphate (DPP)-[^{14}C]GlcNAc was used as substrate, the incubation mixture contained 30 mM Tris–HCl (pH 7.8); 2.5 mM EDTA; 10 mM $MnCl_2$; 1.5 mM ATP; DPP-[^{14}C]GlcNAc (80 000 cpm) and 2.5 mg microsomal protein in 1 ml. DPP-[^{14}C]GlcNAc was prepared and purified as in [12]. Extractions of lipid I and lipid II and determination of radioactivity of these fractions and of protein were also performed as in [8]. Protein was estimated by the Biuret procedure [13].

3. Results and discussion

Investigation of the transverse asymmetry of enzymes or enzymic systems in microsomal vesicles requires that the permeability barrier of the membrane is not changed. Microsomal membranes are impermeable to both macromolecules and charged substances [14]. It is of basic importance to retain this impermeability even after proteolysis and DABS treatment. When the latency of two enzymes whose active sites are localized at the inner surface of the microsomal vesicles, namely mannose 6-phosphatase and nucleotide diphosphatase, was determined, the results were identical for the treated and untreated membranes. In addition, the intramicrosomal water determined with dextran of 10 000 M_r was 1.3 μl/mg dry wt in both cases. Therefore, the vesicles used in our experiments retained an intact permeability barrier at the end of the treatment with surface probes.

Incubation of rough microsomes with GDP-mannose results in incorporation of mannose into the lipid I fraction (dolichol monophosphate), the lipid II fraction (dolichol pyrophosphate-oligosaccharide) and into protein (table 1). Amphomycin, which is an inhibitor of the interaction of GDP-mannose with dolichol phosphate, completely eliminates mannose incorporation into lipid I; but incorpora-

Table 1
Glycosylation of lipids and proteins of rough and smooth microsomes with GDP-[^{14}C]mannose and DP-[^{14}C]mannose as substrates

Microsomes, treatment	Substrate	Inhibitor	Lipid I	Lipid II % of control	Protein
RM, none	GDP-[^{14}C]mannose	–	100 (2196)	100 (152)	100 (411)
RM, none	GDP-[^{14}C]mannose	Amphomycin	3	18	54
RM, DABS	GDP-[^{14}C]mannose	–	59	40	58
RM, proteolysis	GDP-[^{14}C]mannose	–	63	47	54
RM, proteolysis	GDP-[^{14}C]mannose	Amphomycin	3	11	39
RM, none	DP-[^{14}C]mannose	–		100 (119)	100 (469)
RM, DABS	DP-[^{14}C]mannose	–		59	63
SM, none	GDP-[^{14}C]mannose	–	100 (909)	100 (83)	100 (471)
SM, none	GDP-[^{14}C]mannose	Amphomycin	4	28	97
SM, DABS	GDP-[^{14}C]mannose	–	79	86	94
SM, none	DP-[^{14}C]mannose	–		100 (174)	100 (410)
SM, DABS	DP-[^{14}C]mannose	–		30	45

Abbreviations: RM, rough microsomes; SM, smooth microsomes; DABS, diazobenzene sulfonate; DP, dolichol monophosphate

DABS and proteolytic treatments were performed as in section 2. Where indicated amphomycin (400 μg/ml) was added to the incubation medium. Lipid I represents the chloroform:methanol (2:1) extract and Lipid II the chloroform:methanol:H_2O (1:1:0.3) extract. The values in parenthesis show the incorporation in cpm/mg protein taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of the control

tion into protein is still 50% of the original amount. When the surface of intact microsomes is attacked by DABS or proteases, incorporation into dolichol phosphate and into the endogenous protein acceptor are both decreased by ~40%. Amphomycin further decreases the transfer of mannose into microsomal protein after protease treatment, indicating that the dolichol-mediated pathway is present both in the protease-insensitive and -sensitive compartments of the vesicles. Incubation of rough microsomes with DP-mannose also resulted in incorporation both into the lipid II and protein fractions and again both of these were partially inhibited by DABS treatment. The pattern in the case of smooth microsomes is very different. Amphomycin eliminates completely the transfer of mannose to dolichol phosphate in this subfraction; but in contrast to rough microsomes, incorporation into protein is not decreased. DABS treatment has only a small effect on mannose transfer to the lipid I fraction and, again, no inhibition of incorporation into protein is observed. On the other hand, mannose transfer from DP-mannose to DPP-oligosaccharide and to protein is inhibited to a large extent by DABS-treatment of smooth microsomes.

Transfer of glucosamine from the nucleotide-activated form to dolichol monophosphate is inhibited completely by tunicamycin, a well known inhibitor of this reaction (table 2). However, about half of the protein glycosylation was retained. Both DABS and proteolytic treatment significantly reduced the incorporation into both lipid fractions and protein. In addition, tunicamycin-sensitive glycosylation of protein was also present in rough microsomes after protease treatment. DABS treatment also reduced the transfer of glucosamine from the DPP-bound form to the lipid-bound oligosaccharide and to the endogenous protein acceptor. Tunicamycin eliminates sugar transfer to dolichol monophosphate in smooth microsomes as well and the same transfer reaction is strongly inhibited by DABS treatment. However, these compounds had no effect on protein glycosylation. On the other hand, when DPP-glucosamine is used as substrate, incorporation into both lipid II fraction and protein was strongly reduced.

This investigation demonstrates that both rough and smooth microsomes possess several glycosylating pathways, but that the two subfractions differ significantly. The dominating pathway for mannosyl and glucosaminyl transfer in rough microsomes involves dolichol phosphates. The initial steps are present on

Table 2
Glycosylation of lipids and proteins of rough and smooth microsomes with UDP-[^{14}C]GlcNAc and DPP-[^{14}C]GlcNAc as substrates

Microsomes, treatment	Substrate	Inhibitor	Lipid I	Lipid II % of control	Protein
RM, none	UDP-[^{14}C]GlcNAc	–	100 (1234)	100 (79)	100 (772)
RM, none	UDP-[^{14}C]GlcNAc	Tunicamycin	4	16	56
RM, DABS	UDP-[^{14}C]GlcNAc	–	36	43	49
RM, proteolysis	UDP-[^{14}C]GlcNAc	–	36	40	48
RM, proteolysis	UDP-[^{14}C]GlcNAc	Tunicamycin	5	21	36
RM, none	DPP-[^{14}C]GlcNAc	–		100 (41)	100 (243)
RM, DABS	DPP-[^{14}C]GlcNAc	–		49	39
SM, none	UDP-[^{14}C]GlcNAc	–	100 (962)	100 (65)	100 (781)
SM, none	UDP-[^{14}C]GlcNAc	Tunicamycin	15	27	89
SM, DABS	UDP-[^{14}C]GlcNAc	–	49	48	93
SM, none	DPP-[^{14}C]GlcNAc	–		100 (29)	100 (89)
SM, DABS	DPP-[^{14}C]GlcNAc	–		46	46

Abbreviations: RM, rough microsomes; SM, smooth microsomes; DABS, diazobenzene sulfonate; GlcNAc, *N*-acetyl-glucosamine; DPP, dolichol pyrophosphate

DABS and proteolytic treatments were performed as in section 2. Where indicated tunicamycin (10 μg/ml) was added to the incubation medium. Lipid I gives the chloroform:methanol (2:1) and lipid II the chloroform:methanol:H_2O (1:1:0.3) extract. The values in parenthesis show the incorporation in cpm/mg protein taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of the control

the cytoplasmic surface, since they can be inhibited by DABS and proteases. On the other hand, the oligosaccharide product is directed to a large extent to the inner surface. Judging from the effect of inhibitors and proteolytic treatment, it is probable that minor glycosylating pathways with and without the involvement of lipid intermediates are distributed both on the outer and inner surfaces of rough microsomes. Such transferases or transferase systems could be necessary for glycosylation of certain proteins on the cytoplasmic surface of the microsomes [3–7]. The situation with smooth microsomes is very different. The main pathway, studied with two substrates, does not involve dolichol phosphate. In this case the sugar residue is transferred directly to the protein acceptor localized in the inner compartment of the smooth microsomal membrane which is insensitive to trypsin. This particular pathway is probably devoted to the completion of certain oligosaccharide chains and is not involved in the process of core glycosylation. The function of dolichol phosphate in smooth microsomes is not yet clear, but it may be involved in minor routes operative in reactions taking place at the outer and inner surfaces. The distribution and the intramembranous localization of the glycosyl transferase systems in rough and smooth microsomes thus may be one of the main regulating factors in the assembly and transfer of oligosaccharide chains to the acceptor endogenous protein.

Acknowledgements

We wish to thank Dr W. F. J. Cuthbertson, Glaxo-Allenburys Research Co., Sefton Park, England for the tunicamycin and Dr M. Bodansky, Case Western Reserve University, Cleveland, Ohio for the amphomycin. This work was supported by grants from the Magnus Bergwall Foundation and the Swedish Medical Research Council.

References

[1] Schachter, H. and Roseman, S. (1980) in: The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J. ed) pp. 85–160, Plenum Press, New York.
[2] Schachter, H. (1978) in: The Glycoconjugates (Horowitz, M. I. and Pigman, W. eds) vol. 2 pp. 88–181, Academic Press, New York.
[3] Appelkvist, E. L. and Dallner, G. (1978) Biochim. Biophys. Acta 513, 173–178.
[4] Ozols, J. (1972) J. Biol. Chem. 247, 2242–2245.
[5] Haugen, D. A. and Coon, M. J. (1976) J. Biol. Chem. 251, 7929–7939.
[6] Elhammer, Å., Dallner, G. and Omura, T. (1978) Biochem. Biophys. Res. Commun. 84, 572–580.
[7] Imai, Y., Hashimoto-Yutsudo, C., Satake, H., Girardin, A. and Sato, R. (1980) J. Biochem. 88, 489–503.
[8] Nilsson, O. S., De Tomás, M. E., Peterson, E., Bergman, A., Dallner, G. and Hemming, F. W. (1978) Eur. J. Biochem. 89, 619–628.
[9] Bergman, A. and Dallner, G. (1978) Biochim. Biophys. Acta 512, 123–135.
[10] Dallner, G. (1974) Methods Enzymol. 31/A, 191–201.
[11] Nilsson, O. S., DePierre, J. W. and Dallner, G. (1978) Biochim. Biophys. Acta 511, 93–104.
[12] Palamarczyk, G. and Hemming, F. W. (1975) Biochem. J. 148, 245–251.
[13] Gornall, A. G., Bardawill, C. J. and David, H. H. (1949) J. Biol. Chem. 177, 751–766.
[14] Nilsson, R., Peterson, E. and Dallner, G. (1973) J. Cell Biol. 56, 762–776.