LOCALIZATION OF NEWLY SYNTHESIZED GLYCOPROTEINS IN THE MEMBRANE OF THE ROUGH ENDOPLASMIC RETICULUM OF RAT LIVER

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Received 29 August 1977

1. Introduction

Recent investigations have established that the ER of rat liver synthesizes not only secretory glycoproteins (GP) but GP destined to become integral components of the ER membrane as well [1]. These latter proteins are localized either at the outer or at the inner surface of the membrane [2] and biosynthesis is carried out by a complex process involving several lipid intermediates and glycosyl transferases [3,4]. Experimental data indicate that the biosyntheses of different oligosaccharide chains do not follow the same pattern. Sugar transfer may be regulated partially by the specificity of individual polyprenyl phosphates and partially by the specificity of different transferases for different acceptors [5]. Thus, the oligosaccharide chains of various membrane proteins may follow specialized mechanisms of synthesis: they may be completed at a single location [6]; they may be completed sequentially at different locations during a transport process [7]; and finally, these chains may be synthesized by the latter procedure and subsequently be relocated to the site where the biosynthetic process was initiated [8]. In this investigation we have studied the location of newly synthesized GP in the transverse plane of the ER membrane in order to gain insight into the mechanism of biosynthesis of these proteins.

Abbreviations: ER, endoplasmic reticulum; GP, glycoproteins; Gal, galactose; GlcN, glucosamine; NANA, *n*-acetylneuraminic acid

2. Materials and methods

Rats starved for 20 h were used. D-[1-^{3}H]Glucosamine (2000 mCi/mmol) and D-[1-^{3}H]galactose (5000 mCi/mmol), purchased from the Radiochemical Centre, Amersham, and dissolved in 0.9% NaCl, were used in the in vivo experiments. 0.2 μCi (for GlcN labeling) or 0.5 μCi [^{3}H]GlcN (for NANA labeling) or 0.2 μCi [^{3}H]Gal was injected into the portal vein of each rat under pentobarbital anesthesia. In the time course experiments non-labeled GlcN (5 mg/rat for GlcN labeling, 10 mg/rat for NANA labeling) or non-labeled Gal (5 mg/rat) was injected into the portal vein 5 min after administration of the radioactive pulse.

Rough microsomes were prepared and washed with 0.15 M Tris–HCl, pH 8.0, as described earlier [9]. For trypsin treatment 50 μg trypsin (Boehringer, Mannheim) per mg protein was added to a suspension of rough microsomes in 0.25 M sucrose and 50 mM Tris–HCl buffer, pH 7.5, and this suspension was then incubated for 15 min at 30°C. The incubation was stopped by addition of trypsin inhibitor (1.5-fold excess) and by cooling in an ice-water bath. Control microsomes were treated in an identical manner but without trypsin. Microsomes were pelleted by centrifugation at 105 000 × *g* for 60 min and this pellet was used for analysis. The microsomes were suspended in water and extracted with chloroform/methanol (2:1) and chloroform/methanol/H_2O (1:1:0.3) as described earlier [6]. After hydrolysis, NANA, Gal and GlcN were purified by ion-exchange chromatography as previously [2]. Radioactivity in

the purified sugars was measured after the addition of scintillator. GlcN and Gal were quantitated by gas liquid chromatography of the alditol acetate derivatives [10,11]. Determination of NANA was carried out with the Warren procedure [12]. Protein determination was performed with the Biuret reaction [13] and phospholipids were analyzed as described earlier [14].

3. Results and discussion

The chemical composition of rough microsomes is shown in table 1. These determinations were made using Tris–water–Tris washed rough microsomes; the washing procedure removes all non-membraneous proteins, i.e., both secretory and adsorbed proteins. Trypsin treatment removes about half of the membrane protein without solubilizing phospholipids. A sizeable portion of the protein-bound sugar residues is also liberated. Trypsin treatment removes 40% of rough microsomal GlcN and Gal and about 30% of the NANA. In exp. 2 and 3, table 1, the pattern of GlcN labeling in washed rough microsomes both before and after removal of the luminal soluble protein from the inside of rough microsomal vesicles is given. Both after 20 min and 60 min of labeling in vivo a significant amount of radioactivity (40–50%) is associated with the soluble proteins in the vesicle lumen, i.e., with secretory proteins. In subsequent time course studies with various precursors the luminal proteins were not removed by water treatment, since this washing procedure slightly alters the membrane structure [17], which is disadvantageous in such membrane studies as these.

Table 1
Chemical composition of rough microsomes and incorporation of [^{3}H]glucosamine into rough ER in vivo

Exp.		Rough microsomes	
		Control	Trypsin-treated
1	Protein[a]	6.1	3.15
	Phospholipid[a]	2.65	2.46
	GlcN[b]	7.65	4.42
	Gal[b]	3.08	1.81
	NANA[b]	4.20	2.96
2	GlcN (20 min label)[c]		
	Total protein	45 800	
	Luminal soluble protein	20 100	
3	GlcN (60 min label)[c]		
	Total protein	86 900	
	Luminal soluble protein	31 000	

[a] mg/g wet wt liver
[b] μg/mg phospholipid
[c] cpm in protein-bound GlcN/g wet wt liver

The analysis in exp. 1 was performed on fractions which were subjected to the Tris–water–Tris washing procedure [9] to remove both adsorbed and secretory proteins. The rough microsomes in exp. 2 and 3 were only washed with alkaline Tris buffer. After in vivo labeling with GlcN the luminal soluble protein, localized inside the rough microsomal vesicles, was removed by the modified procedure of Kreibich et al. [15,16]. Each value represents the mean of 5–7 experiments

After injection of the appropriate precursor in vivo the appearance of radioactivity in protein-bound sugar residues isolated from washed intact rough microsomal vesicles was followed (fig.1). Radioactivity in GlcN increases continuously during the time period studied (60 min). The appearance of radioactivity bound to trypsin-sensitive proteins exhibits a very different pattern. During the first 30 min there is no labeling of these proteins, but later there is a slow increase in labeling, which reaches 15% of the total at 60 min. Making correction for the presence of non-membraneous secretory proteins in the lumen of the vesicles, the value for the trypsin-sensitive radioactivity is 25% of the total membrane-bound radioactive glucosamine. In the case of Gal no label is apparent in rough microsomes during the first 30 min. After this period there is a rapid increase in radioactivity both in trypsin-sensitive and -insensitive membrane proteins, and the label liberated by trypsin after a 1 h labeling period is 25% of the total incorporated. After injection of [^{3}H]GlcN labeling in NANA is low initially and increases after 10 min. At all time points, trypsin-sensitive radioactivity is significant and about 25% of the total.

There are several possible explanations for the presence and delayed appearance of newly synthesized GP at the cytoplasmic surface of rough microsomes. One or two enzyme systems in this fraction may synthesize two different GP, one with a low and the other with a high rate. Another possibility is that the primary site of synthesis is at the inner surface and at

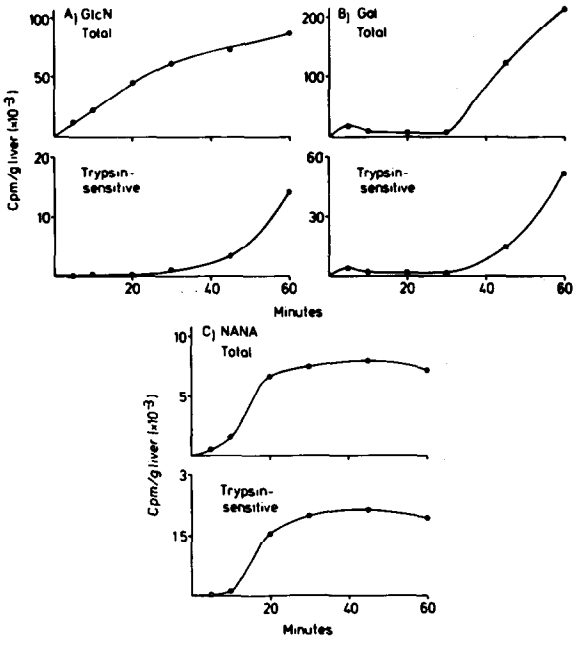

Fig.1. Labeling of rough ER in vivo. The rats were injected into the portal vein with [^{3}H]glucosamine (A and C) or [^{3}H]galactose (B). The control values represent the radioactivity in purified sugar fractions. (A) GlcN; (B) Gal; (C) NANA. The delipidated protein was hydrolyzed and purification was achieved by ion-exchange chromatography [2]. The trypsin-sensitive protein represents the difference between the control pellet and the pellet obtained after trypsin treatment (50 μg trypsin/mg protein, 15 min, 30°C). In all three cases more than 90% of the radioactivity was found to be associated with the original monosaccharide upon chromatography. The values represent the means of 5–6 experiments.

some later stage the newly synthesized protein is translocated to the outer surface by a 'flip-flop' motion. Both of these alternatives are improbable because of the lag phase in the appearance of radioactivity in trypsin-sensitive protein; and furthermore, 'flip-flop' of membrane proteins has not yet been demonstrated. Secondary incorporation of new proteins into the outer surface by 'back flow' from the Golgi system or by incorporation of soluble proteins from the vesicle lumen were excluded in previous studies [18]. The experimental findings in this study are, on the other hand, consistent with a biosynthetic pathway by which some GP are synthesized on bound ribosomes; their sugar moiety is then completed during transport to the Golgi system; the completed protein is discharged into the cytoplasm as a lipoglycoprotein complex and subsequently incorporated into the ER by an exchange process [8,18–20]. The presence of radioactive GlcN in trypsin-insensitive protein during the early phase of labeling is very reasonable, because this sugar is an obligatory component of the core portion of the oligosaccharide chain [21]. The appearance of this label in trypsin-sensitive protein after 30 min may be explained by incorporation from the cytoplasm into the ER of protein completed at the Golgi level. The majority of Gal residues occupy terminal positions in the oligosaccharide chain and are added at the level of smooth ER and Golgi membranes [7]. Consequently, radioactivity is apparent in rough microsomes only after relocation through the cytoplasm of the protein released from the Golgi. One complete cycle requires 30–40 min [18]. The fact that incorporation of radioactive NANA is seen earlier than that of Gal and GlcN is in good agreement with the presence of acceptor proteins at the surface of Golgi vesicles [8] which, after sialidation, appear in the cytoplasm and are shortly thereafter transferred to the rough ER.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council and the Swedish Society of Medical Sciences.

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