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INCORPORATION OF *N*-ACETYLGLUCOSAMINE FROM UDP-*N*-ACETYLGLUCOSAMINE INTO PROTEINS AND LIPID INTERMEDIATES IN MICROSOMAL AND GOLGI MEMBRANES FROM RAT LIVER

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Summary

Rough and smooth microsomes and Golgi membranes incorporate *N*-acetylglucosamine from UDP-*N*-acetylglucosamine into endogenous protein acceptors. A lipid intermediate of the dolichol phosphate type participates in this transfer reaction in the case of both microsomal subfractions, but the nature of lipid glycosylation is different in these two fractions. Glucosamine transfer in Golgi membranes does not appear to involve a lipid intermediate. In contrast to the results obtained under in vivo conditions, no glucosamine label is recovered in nascent ribosomal proteins or on luminal secretory proteins after incubation in vitro. Proteolysis of intact vesicles of the subfractions removes glycosylated dolichol phosphate and protein acceptors to various extents and interferes with transferase activities. This finding suggests the possibility that glycosylation at the cytoplasmic side of the membrane of the endoplasmic reticulum may involve a system separate from that acting at the luminal side of the same membrane.

Introduction

Two types of glycoprotein are found in liver microsomal and Golgi fractions: the secretory type destined to be secreted into the blood and the membranous type which remains within these membranes or is transferred to other intracellular membranes [1]. Since *N*-glycosidic binding between asparagine and *N*-acetylglucosamine (GlcNAc) is the only type of bond connecting protein

Abbreviation: SDS, sodium dodecyl sulphate.

and sugar moieties in the membrane of the endoplasmic reticulum [2], GlcNAc is an obligatory precursor for the synthesis of all microsomal glycoproteins. In addition to the first sugar residue of the oligosaccharide chain various glycoproteins also contain a number of GlcNAc residues in the core and in terminal positions. Thus it is not surprising that the system transferring GlcNAc from UDPGlcNAc to various endogenous and exogenous protein acceptors is found in both rough and smooth microsomes, as well as in Golgi membranes [3—5].

Intensive investigations during recent years on rat liver, myeloma cells, pancreas and hen oviduct have demonstrated that phosphorylated dolichol derivatives are involved in the transfer of GlcNAc to protein acceptors [6]. Most investigations indicate that the dominating reaction is the transfer of GlcNAc and terminal phosphate from UDPGlcNAc to dolichol phosphate, followed by the transfer of a second GlcNAc to form dolichol pyrophosphate *N,N'*-diacetylchitobiose. To date, no evidence for the participation of other types of lipid intermediates in GlcNAc transfer has been reported. It is possible that microsomes possess two pools of lipid intermediates [7], but no experimental evidence demonstrating that different transferase systems are involved in the biosynthesis of different glycoproteins is yet available. On the other hand, it has been demonstrated that the Golgi membranes contain two different CMPsialyl transferases [8].

In the present investigation we studied the nature of GlcNAc transfer to endogenous protein acceptors and the intramembranous localization of these acceptor proteins in rough microsomes, smooth microsomes and the Golgi fraction. The results obtained suggest that this system is asymmetrically distributed in the tranverse plane of the membrane.

Materials and Methods

Adult male albino rats weighing 180—200 g and starved overnight were used. Rough and smooth microsomes were prepared as described earlier [9]. The Golgi fraction was isolated from rats acutely intoxicated with alcohol and the total fraction flotating on the 1.10 M (instead of 1.15 M) sucrose layer was used [10]. The fractions were washed in all experiments by recentrifugation in 0.15 M Tris · HCl/5 mM $MgCl_2$, pH 8.0, which greatly decreased contamination by pyrophosphatase activity by removing adsorbed proteins. For separation of bound ribosomes rough microsomes (4 mg protein/ml) were suspended in deoxycholate (0.5%) and the particulate fraction obtained by centrifugation at 105 000 × *g* for 90 min used. For studying microsomal contents, the subfractions were treated with a modification of the procedure of Kreibich et al. [11,12] to release vesicular secretory proteins. The incubation mixture for in vitro incorporation of GlcNAc contained, in a total volume of 400 µl, 30 mM Tris · HCl buffer, pH 7.8; 2.5 mM EDTA; 10 mM $MnCl_2$; 1.5 mM (rough microsomes), 2.0 mM (smooth microsomes), or 2.5 mM (Golgi membranes) ATP; 0.05 µCi of UDP[^{14}C]GlcNAc (300 mCi/mmol, Radiochemical Centre, Amersham), and 50 µl sample containing 1.5 mg (rough microsomes), 1.0 mg (smooth microsomes) or 0.6 mg (Golgi membranes) protein. The incubations were performed at 30°C for 30 min in thick-walled centrifuge tubes. After incubation the mixture was cooled and centrifuged without dilution at

105 000 × *g* for 60 min. The pellet was resuspended in 0.5 ml water and extracted with 3 ml chloroform/methanol (2 : 1, v/v) at 40°C for 20 min with occasional mixing. After centrifugation the upper water-methanol phase was removed and the surface of the lower phase rinsed with "upper phase" [13]. The chloroform phase was then decanted, mixed with 1.5 ml upper phase, centrifuged and the surface again rinsed with upper phase. This washing procedure was repeated twice. The remaining protein was washed three times with 1.5 ml upper phase and extracted twice with 3 ml chloroform/methanol (2 : 1, v/v) at 40°C for 20 min. The three chloroform/methanol extracts were pooled (Lipid 1). The remaining fraction was extracted with 3 × 1 ml chloroform/methanol/H_2O (1 : 1 : 0.3) (Lipid 2). The protein pellet was washed with 1 ml H_2O and solubilized in 1 ml 2% sodium dodecylsulphate (SDS). The two lipid extracts were evaporated and radioactivity determined after addition of 10 ml Bray's solution [14].

For the estimation of β-glucosaminidase activity the incubation medium contained 0.2 ml 0.05 M citrate/phosphate buffer, pH 4.0 or 6.0, or 0.08 M Tris-buffer, pH 7.8; 20 μl 1% Triton X-100; 100 μl 10 mM *p*-nitrophenyl-*N*-acetyl-β-glucosamine pyranoside; and fractions in a final volume of 0.5 ml. After incubation at 37°C for 30 min the reaction was stopped with 0.5 ml 12% trichloroacetic acid, centrifuged and 0.5 ml 2 M Trisma base added to the supernatant. Absorption was measured at 400 nm.

Gel filtration was performed at room temperature on a column (1.8 × 70 cm) of Sephadex G-150 preswollen in 1% SDS [7]. The protein fraction was solubilized in 5 ml 2.5% SDS. After centrifugation at 105 000 × *g* for 1 h the supernatant was applied to the column and eluted with 1% SDS at a flow rate of 5 ml/h. 5 ml fractions were collected. Aliquots of each fracton were mixed with Bray's solution and radioactivity determined.

Protein was estimated using the Biuret reaction [15]. The values presented in the tables and figures are the means of 5—8 experiments, with the exception of the data shown in Fig. 2, which is the mean of 2 experiments.

Results

Incorporation in vitro

Rough and smooth microsomes and Golgi membranes all incorporate *N*-acetylglucosamine (GlcNAc) into the lipid-depleted insoluble fraction upon incubation with UDPGlcNAc in vitro (Table I). Compared to the radioactivity appearing in the protein fraction, a large amount of radioactivity is also present in the chloroform/methanol soluble fraction from rough microsomes. The majority of this radioactivity behaves chromatographically like dolichol bound sugar. The radioactivity in the Lipid 1 fraction of smooth microsomes is lower and GlcNAc appears to be transferred to protein acceptors of the Golgi membranes without the involvement of lipid intermediates. The radioactivity recovered in the chloroform/methanol/water (1 : 1 : 0.3, v/v) soluble fraction, which contains chiefly dolichol pyrophosphate oligosaccharide [16], is low for all three subfractions. ATP is obviously required for incorporation, both into lipid intermediates and protein. The presence of Triton in the incubation medium increases GlcNAc incorporation into the Lipid 1 fraction of rough

TABLE I

IN VITRO INCORPORATION OF [^{14}C]GlcNAc INTO CYTOPLASMIC MEMBRANES

The composition of the complete incubation system is given in Materials and Methods. In all cases, the reaction was stopped by the addition of chloroform/methanol (2 : 1,v/v) without previous centrifugation of the samples. The concentration of Triton X-100 was 0.4% and the amount of dolichol phosphate added was 5 nmol. Results expressed in cpm/mg protein.

Fraction	Incubation mixture	Lipid 1	Lipid 2	Protein
Rough microsomes	Complete system	2 251	149	1 949
	— ATP	360	68	173
	+ Triton X-100	5 379	123	812
	+ Dolichol phosphate and Triton X-100	15 023	127	1 048
Smooth microsomes	Complete system	816	152	6 459
	— ATP	181	98	507
	+ Triton X-100	675	133	1 215
	+ Dolichol phosphate and Triton X-100	3 545	139	1 198
Golgi membranes	Complete system	240	15	12 612
	— ATP	125	10	560
	+ Triton X-100	232	10	1 374
	+ Dolichol phosphate and Triton X-100	1 034	15	1 403

microsomes. Incorporation into protein of rough and smooth microsomes, as well as of Golgi membranes, is greatly decreased by this detergent. Addition of dolichol phosphate demonstrates that the two microsomal subfractions can glycosylate this lipid intermediate but the exogenous dolichol phosphate does not significantly enhance the transfer of sugar to protein acceptors.

Using the conditions employed here incorporation of radioactivity into protein is linear up to 2 mg microsomal protein per sample. The first lipid intermediate in the subcellular fractions is saturated with GlcNAc within the first 5 min of incubation, while incorporation into protein is still increasing somewhat after 30 min. To avoid excessive damage of the membrane, e.g. by endogenous phospholipases, the incubations were terminated after 30 min.

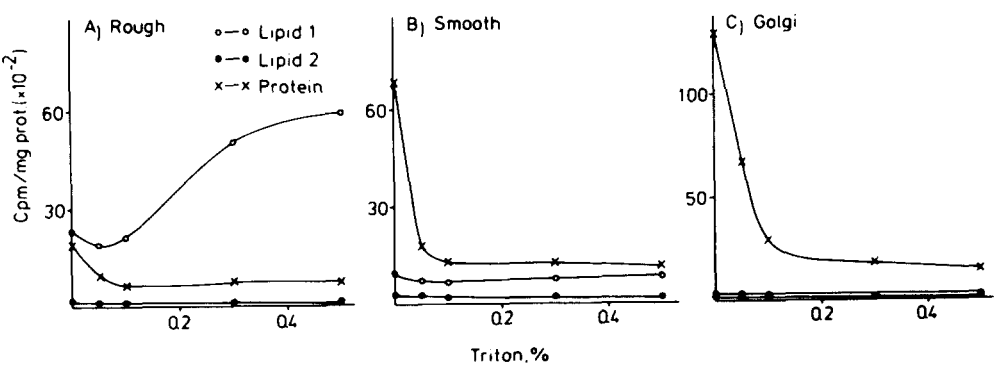

Fig. 1. Effect of Triton X-100 on the incorporation of [^{14}C]GlcNAc in vitro. (A) Rough microsomes; (b) smooth microsomes, and (C) Golgi membranes. In all cases, the samples were extracted without previous centrifugation.

TABLE II

EFFECT OF CENTRIFUGATION ON THE RECOVERY OF RADIOACTIVE GlcNAc IN MEMBRANES

The incubation mixture was either extracted directly after incubation (non-sedimented) or first cooled and centrifuged at 105 000 X *g* for 60 min and the resulting pellet extracted after resuspension (sedimented). L1 and L2 = Lipid 1 and 2. Results expressed in cpm/mg protein.

	Non-sedimented			Sedimented		
	L1	L2	Protein	L1	L2	Protein
Rough microsomes	2196	152	2 036	1497	25	1003
Smooth microsomes	796	165	6 525	747	27	4853
Golgi membranes	221	15	12 127	90	10	5437

Effect of Triton

Increasing concentration of Triton X-100 reveals characteristic differences among the subfractions. At a concentration of 0.1%, incorporation into the Lipid 1 fraction is unchanged and into protein decreases in the case of rough microsomes (Fig. 1A). Higher detergent concentrations stimulate incorporation into the Lipid 1 fraction of rough microsomes. With smooth microsomes 0.1% Triton decreases incorporation into protein by 85% but the radioactivity in the two lipid intermediates is unaffected by the detergent (Fig. 1B). The behavior of the Golgi membranes is similar to that of the smooth microsomes (Fig. 1C).

Effect of centrifugation

The experiments described above were performed in the accepted manner, that is, at the end of the incubation lipid extraction and protein separation were carried out by partition. On the other hand, when the particles were

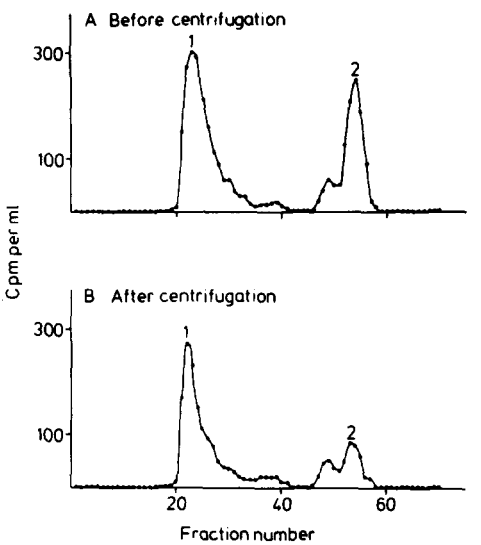

Fig. 2. Sodium dodecyl sulphate-Sephadex G-150 chromatography of the protein fraction from rough microsomes after incubation with UDP[^{14}C]GlcNAc in vitro. A, rough microsomes were extracted with chloroform/methanol (2 : 1, v/v) and chloroform/methanol/H_2O (1 : 1 : 0.3, v/v), solubilized in 2.5% sodium dodecyl sulphate (at a concentration of 5 mg protein/ml) and subjected to chromatography. B, rough microsomes were centrifuged (105 000 X *g*, 60 min) after incubation and, after resuspension, treated as in A. For explanation of peaks 1 and 2, see the text.

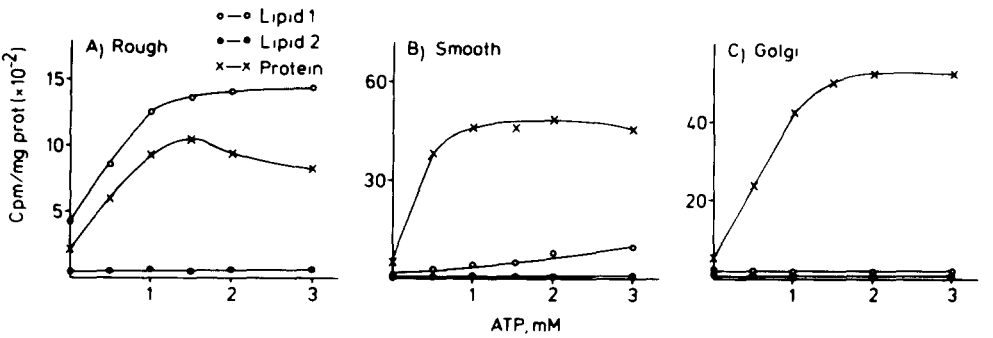

Fig. 3. Effect of ATP on the incorporation of [^{14}C]GlcNAc in vitro. Rough microsomes (A), smooth microsomes (B) and Golgi membranes (C) were incubated with UDP[^{14}C]GlcNAc in vitro in the presence of different concentrations of ATP. At the end of the incubation the particles were sedimented (105 000 × *g*, 60 min), and the pellets resuspended and extracted.

sedimented by ultracentrifugation at the end of the incubation prior to lipid extraction, the incorporation pattern was different from that seen above (Table II). The radioactivity both in lipid intermediates and protein is significantly decreased after sedimentation. In Golgi membranes more than 50% of the radioactivity in the protein fraction can be removed by centrifugation prior to extraction. The experiment described in Table II demonstrates that a sizeable portion of the radioactivity in the lipid extracts and in protein is not associated with the membrane, but is water soluble and can be removed by sedimentation. This situation may be similar to that occurring after incubation with GDPmannose, where free oligosaccharides are coprecipitated with the protein [7]. To test this possibility the insoluble residue before and after centrifugation was subjected to Sephadex G-150 chromatography in the presence of sodium dodecylsulphate (Fig. 2). The size of peak 1, representing the protein fraction, is not significantly influenced by centrifugation; but peak 2, consisting of free oligosaccharides, is decreased to a great extent by sedimentation. Thus, in order to remove coprecipitating oligosaccharides present in the insoluble residue centrifugation or gel filtration must be performed.

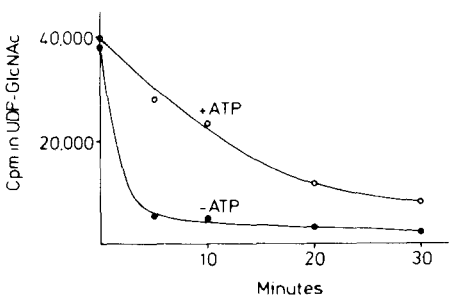

Fig. 4. Hydrolysis of UDPGlcNAc by rough microsomes. After incubation, extraction and partition were performed and aliquots of the water phase spotted on Whatman No. 1 paper. The chromatograms were developed in ethanol/ammonium acetate (0.5 M, pH 3.9) (5 : 2, v/v) [18]. The spot corresponding to UDPGlcNAc was cut out and placed in a scintillation vial with 10 ml of Bray's solution.

Pyrophosphatases and glycosidases

In order to inhibit pyrophosphatases [17] present in the various fractions ATP was included in the incubation medium (Fig. 3). The effect is dramatic; incorporation into protein in the three membrane fractions is stimulated 10—15 times. A significant stimulation of the incorporation into the Lipid 1 fraction occurs only with rough microsomes; this effect is small with smooth microsomes and not seen at all with Golgi membranes.

Since cytoplasmic membranes contain high levels of pyrophosphatase activity, it was important to determine the amount of substrate present in the incubation medium under our conditions. Rough microsomes were incubated in the medium containing UDP[^{14}C]GlcNAc for various periods of time and, after partition, the substrate remaining in the water-phase was determined (Fig. 4). In the absence of ATP only 15% of the substrate was recovered after 5 min incubation, but loss of substrate in the presence of ATP was much slower.

Another antagonistic enzyme is β-*N*-acetylglucosaminidase, which may attack the products formed. It is known that, in addition to the lysosomal enzymes, there are glycosidases in other cellular membranes and in the soluble cytoplasm [19]. This hydrolytic activity was measured at pH 4.0, 6.0, and 7.8 (the pH of the incubation medium) (not shown in table). There is detectable activity at pH 4.0 in all fractions, and hydrolysis is greatly decreased at pH 6.0. At pH 7.8 the activity is decreased to such an extent that is may be regarded as negligible.

Labeling of ribosomes

Previous experiments demonstrated that under in vivo conditions the *N*-glycosidic bond between asparagine and GlcNAc may be formed at the ribosomal level [20]. Ribosomes labeled with [^{3}H]glucosamine in vivo contain labeled nascent polypeptides which are released upon incubation with puromycin (Table III). After incubation with UDP[^{14}C]GlcNAc in vitro the label

TABLE III

RELEASE OF NASCENT PEPTIDES LABELED WITH GLUCOSAMINE FROM RIBOSOMES BY PUROMYCIN

In the in vitro experiments rough microsomes were incubated with UDP[^{14}C]GlcNAc and ribosomes were prepared. To release the nascent peptides, the isolated ribosomes were treated with puromycin [20]. The ribosomal pellet was suspended (0.5 g/ml) in a medium containing 0.1 M KCl, 0.04 M Tris · HCl, pH 7.6, 5 mM $MgSO_4$ and 1 mM EDTA. Puromycin was added to a final concentration of 0.5 mM and the mixture was incubated at 0°C for 30 min. At the end of the incubation additional medium (but containing 0.8 M KCl) was added, the preparation centrifuged at 105 000 × *g* for 90 min and the radioactivity in the supernatant determined. The control was treated as above but without addition of puromycin. In the in vivo experiments rats (180 g) were injected with 0.2 mCi [^{3}H]glucosamine into the portal vein 5 min before decapitation. Rough microsomes were isolated and the bound ribosomes were prepared and treated as above.

	Control		Puromycin treated	
	cpm/g liver	%	cpm/g liver	%
Ribosomes labeled in vitro	1825	100	1750	96
Ribosomes labeled in vivo	2910	100	1950	67

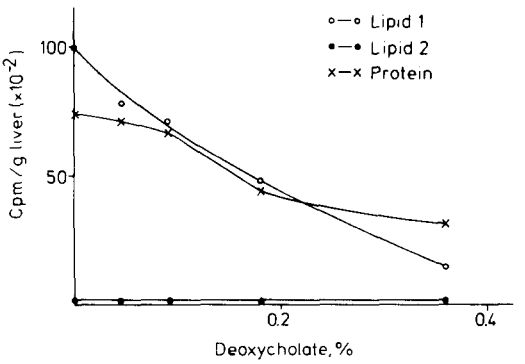

Fig. 5. Deoxycholate treatment of rough microsomes labeled in vitro. Rough microsomes were incubated with UDP[^{14}C]GlcNAc, pelleted by centrifugation and resuspended in 0.25 M sucrose. Deoxycholate was added to aliquots of resuspended rough microsomes (2.2 mg protein/ml) and the suspensions were centrifuged at 105 000 × *g* for 4 h. The pellets were resuspended and extracted as described in Materials and Methods.

appearing in the ribosomal fraction is not released by puromycin, indicating that under these conditions no glycosylation of nascent peptides occurs. This conclusion is further supported by the fact that puromycin treatment of rough microsomes during incubation with UDP[^{14}C]GlcNAc (not shown in table) does not affect GlcNAc incorporation into protein, i.e., newly synthesized polypeptides at the ribosomal level are not part of the protein acceptor pool.

Deoxycholate treatment

The relation of the proteins labeled in vitro to the membranes was studied

TABLE IV

COMPARTMENTALIZATION OF THE *N*-ACETYLGLUCOSAMINE TRANSFERASE SYSTEM OF ROUGH MICROSOMES

In Expt. 1 rough microsomes were treated with trypsin (50 μg trypsin/mg protein, 30°C, 15 min), deoxycholate at 0°C or at 30°C in the absence or presence of trypsin, recentrifuged and the resuspended pellet was used for incubation. After incubation the mixtures were centrifuged prior to extraction. In Expt. 2 the rough microsomes were incubated with UDP[^{14}C]GlcNAc, centrifuged, resuspended in 0.15 M Tris · HCl, pH 8.0—5 mM $MgCl_2$, centrifuged and the resuspended pellet was treated as in Expt. 1.

Expt.	Treatment	Lipid 1 (cpm)		Lipid 2 (cpm)		Protein (cpm)	
		g liver	%	g liver	%	g liver	%
1	Treatment before incubation						
	None	13 952	100	233	100	9348	100
	Trypsin	12 836	92	242	104	5141	55
	Deoxycholate, 0°C	17 998	129	289	124	9815	105
	Deoxycholate, 30°C	8 790	63	142	61	5048	54
	Deoxycholate+ trypsin, 30°C	5 441	39	86	37	2430	26
2	Treatment after incubation						
	None	12 562	100	198	100	8476	100
	Trypsin	10 426	83	164	83	7035	83
	Deoxycholate, 0°C	12 311	98	188	95	8646	102
	Deoxycholate, 30°C	10 522	84	152	77	7459	88
	Deoxycholate + trypsin, 30°C	8 668	69	127	64	5933	70

by exposure to increasing concentrations of deoxycholate. Deoxycholate at low concentrations specifically removes enzyme proteins loosely bound to the microsomal membrane, while other more tightly bound enzymes are not solubilized [21]. It is apparent from Fig. 5 that both the glycosylated protein acceptors and lipid intermediate are present partially in the membrane compartment which is solubilized at low detergent concentrations. It is remarkable that even at the highest deoxycholate concentration (0.36%, 2.2 mg protein/ml), where a large portion of the phospholipids are solubilized, about 40% of the glycosylated protein is still membrane-bound.

Effect of trypsin

Trypsin treatment of intact microsomal vesicles solubilizes proteins located at the cytoplasmic surface. When microsomal vesicles are made permeable to macromolecules with low concentrations of deoxycholate, the vesicle content is released; and at the same time, it becomes possible to introduce trypsin into the vesicle [11,12]. This approach makes it possible to investigate the distribution of chemical and enzymatic components both at the cytoplasmic and the inner surface. In order to study the transverse distribution of the UDPGlcNAc transferase system in rough microsomes, proteolytic treatment of this fraction was performed both before and after incubation with the substrate (Table IV). Trypsin pretreatment of rough microsomes decreases the transfer of sugar to the Lipid 1 fraction by 8% and the incorporation into protein acceptors by 45%, which is probably an effect on the transferase activity. To a smaller extent the solubilization of protein acceptors also contributes to the effect which is demonstrated by the removal of labeled protein by trypsin treatment after incubation with the sugar nucleotide. Deoxycholate treatment at 0°C does not remove any label, indicating thereby that the newly glycosylated protein is not located in the lumen. Incubation with deoxycholate at 30°C was necessary to obtain hydrolysis at the inner surface of the vesicles by trypsin, but the detergent treatment itself damages the transferase system at this temperature. In spite of this undesirable effect it appears that trypsin causes some inactivation of the transfer of GlcNAc to protein by acting on the inside surface.

TABLE V

COMPARTMENTALIZATION OF THE *N*-ACETYLGLUCOSAMINE TRANSFERASE SYSTEM OF SMOOTH MICROSOMES

Smooth microsomes were treated and incubated as described for rough microsomes in Table IV.

Expt.	Treatment	Lipid 1 (cpm)		Lipid 2 (cpm)		Protein (cpm)	
		g liver	%	g liver	%	g liver	%
1	Treatment before incubation						
	None	2265	100	102	100	14 035	100
	Trypsin	2242	99	107	105	13 333	95
	Deoxycholate, 0°C	3488	154	151	148	7 018	50
2	Treatment after incubation						
	None	2077	100	110	100	13 237	100
	Trypsin	1039	50	47	43	9 928	75
	Deoxycholate, 0°C	1745	84	95	86	11 913	90

TABLE VI

COMPARTMENTALIZATION OF THE *N*-ACETYLGLUCOSAMINE TRANSFERASE SYSTEM OF GOLGI MEMBRANES

Golgi membranes were treated and incubated as described for rough microsomes in Table IV.

Expt.	Treatment	Lipid 1		Lipid 2		Protein	
		cpm / g liver	%	cpm / g liver	%	cpm / g liver	%
1	Treatment before incubation						
	None	78	100	5		1903	100
	Trypsin	39	50	4		1123	59
2	Treatment after incubation						
	None	69	100	6		1866	100
	Trypsin	69	100	5		1325	71

In the case of smooth microsomes incorporation into protein acceptors is high, while in the lipid fractions it is lower than with rough microsomes (Table V). Trypsin treatment before incubation does not interfere with the transferase system to any substantial extent. 25% of the protein-bound GlcNAc label is removed, however, from smooth microsomes when they are subjected to proteolysis after incubation. Only a small amount of radioactivity is removed by deoxycholate treatment, i.e., the soluble proteins in the vesicle lumen are not glycosylated under in vitro conditions.

In the case of Golgi membranes trypsin has a very well defined effect (Table VI). If incubation is performed on Golgi membranes after trypsin treatment, the number of counts incorporated into the protein fraction is decreased by 40%. In addition, about 30% of the protein-bound GlcNAc label is liberated when Golgi membranes are treated with trypsin after incubation. Clearly, a sizeable portion of the acceptor proteins for GlcNAc are distributed on the cytoplasmic surface of the Golgi membranes.

Discussion

Glucosamine is a quantitatively sizeable component in rough and smooth microsomes and in Golgi membranes and, as has been demonstrated here, endogenous protein acceptors can be glycosylated by all three subfractions. Also, dolichol phosphate is present in all three of these cytoplasmic membranes as demonstrated in this paper in agreement with previous findings [22]. On the other hand, it is not clear to what extent this substance participates in the transfer of glucosamine to protein. With respect to rough microsomes there is no doubt that the large amount of radioactivity associated with lipid represents incorporation into dolichol phosphate, which has been demonstrated by chromatography. Incorporation in the presence of 5 nmoles of dolichol phosphate is about 10 000 cpm above the endogenous incorporation. Smooth microsomes also contain endogenous glycosylated dolichol phosphate after incubation in vitro and the amount is again greatly increased upon addition of the phosphorylated carrier. In contrast to rough microsomes there is no stimulation by Triton and the stimulation of incorporation by ATP is very

limited and does not follow the pattern exhibited by the incorporation into protein. It is reasonable to suppose that lipid intermediates participate in the transfer of glucosamine from the substrate to the protein acceptors in smooth microsomes, but the type and mechanism of transfer differs from that in rough microsomes. It is also possible, considering recent experimental findings [23], that two different types of polyprenol phosphates are mediating glucosamine transfer in the two different microsomal subfractions. The amount of radioactivity recovered in the lipid extract of the Golgi fraction after centrifugation is extremely small, below 100 cpm per mg protein. This radioactivity chromatographs like dolichol phosphate sugar (with the uncertainty involved because of the low amount of radioactivity) but its presence can be explained by a contamination of the Golgi fraction by other cytoplasmic membranes of less than 10%. In agreement with this finding are recent investigation which, using an indirect approach, showed that the amount of dolichol phosphate in Golgi membranes capable of accepting glucosamine is very small [23]. Characteristically, no stimulation of incorporation into the lipid extract of this fraction is observed in the presence of ATP or Triton. The moderate stimulation obtained upon addition of 5 nmol of dolichol phosphate (800 cpm per mg protein) is probably not significant in light of the high extent of glycosylation of endogenous protein acceptors in the very same fraction. Thus, it is most likely that the transfer of glucosamine to the nearly completed oligosaccharide chain of a glycoprotein at the Golgi level takes place without the involvement of dolichol phosphate.

Glycoproteins which contain glucosamine and are synthesized in microsomes are compartmentalized. Secretory proteins which receive their first sugar residue while still bound to the ribosomes cross the membrane directly and are segregated into the luminal compartment [20]. Under in vitro conditions no labeled sugar appears on the nascent peptides of bound ribosomes and no radioactive glucosamine is incorporated into the luminal secretory proteins. For these reasons we believe that the majority of the proteins glucosaminidated under in vitro conditions are not secretory but membrane proteins. The principle of compartmentalization is also operative for the various types of membrane glycoproteins. There are a number of integral microsomal enzymes which contain sugar residues, including cytochromes b_5 [24] and *P*-450 [25], nucleotide pyrophosphatase [26], adenosine monophosphatase [27] and β-glucuronidase [28]; and these enzymes are localized at different regions of the membrane in the transverse plane [12]. Therefore, the possibility arises that two or more pathways of microsomal glycosylation exist. The experimental data in this study indicate a heterogenous incorporation of sugar residues into protein within the intramembranous space. Newly glycosylated proteins are present both in the deoxycholate soluble and insoluble fractions and significant amounts of newly glycosylated proteins can be removed from the surface of the rough microsomes, more from smooth microsomes, and most from Golgi vesicles by trypsin treatment. Some of the transferases are affected if proteolysis is performed before incubation and, most interestingly, trypsin treatment after in vitro incubation of rough and smooth microsomes solubilizes a portion of the glucosaminidated dolichol phosphate without solubilization of membrane phospholipids. It is possible that complexes (enzyme · dolichol

phosphate · protein acceptor) exist at the outer surface of microsomal membranes and these complexes glycosylate only those enzyme proteins which are located at this same surface.

Only a small amount of newly synthesized protein acceptors, but a large amount of protein-bound glucosamine are removed by trypsin, particularly in rough microsomes [31]. The most attractive explanation for these findings is the proposed mechanism for biosynthesis and transport of some microsomal glycoproteins [29—31]. It appears that some of the newly synthesized proteins, after release from bound ribosomes, move laterally on the cytoplasmic surface of the endoplasmic reticulum to the Golgi system for completion of the oligosaccharide chain. Consequently, at least one of the glucosamine residues may be added by the Golgi apparatus. These proteins are released into the cytoplasm as soluble lipoglycoproteins and then incorporated into the outer surface of microsomal vesicle upon incubation in vitro.

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