RELATIONSHIP OF PROTEIN-BOUND SIALIC ACID TO MICROSOMAL MEMBRANES

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Summary: Intramembranous microsomal protein-bound sialic acid cannot be removed to any great extent by various washing procedures. After in vivo glucosamine-^{3}H prelabeling, rabbit antiserum to rat serum proteins does not precipitate sialic acid associated label. In vivo injected glucosamine-^{3}H shows a peak incorporation after 30 minutes into protein-bound sialic acid of Golgi membranes and of soluble cytoplasmic fraction. The incorporation rate into rough and smooth microsomes is much slower, suggesting a possible transfer of some cytoplasmic glycoproteins to the microsomal membranes.

The microsomal fraction of the rat liver contains glycoproteins (1,2). The main part of these proteins participates in the secretory process of the endoplasmic membrane system and appears in the blood (3). The serum glycoproteins are characterized by the process of sequential synthesis of their oligosaccharide chain, since the core portion is added in the channels of the rough and smooth endoplasmic reticulum, while the terminal sugars, galactose and sialic acid, are attached within the Golgi membranes. It has been proposed that microsomal membranes themselves also have sialic acid containing glycoproteins as constitutive components, which would be of high interest from a structural and functional point of view (4,5). It is believed that structural glycoproteins in erythrocytes span the external and internal side of the membrane, thereby creating bridges in the vertical plane (6). If these proteins are real components of the structural make-up, they should not be removed by various washing procedures, or, in the case of the integral protein, they would even resist high ionic strength (7). It is also expected that membrane components differ immunologically from those of the serum and, furthermore, that their turnover would

reflect the individuality which is characteristic for chemical constituents at various cytological locations. The purpose of this investigation was to find out the relation of sialic acid containing glycoproteins to the various types of cytoplasmic membranes and to provide evidence for their intramembranous localization by analyzing the incorporation pattern of their sugar moiety.

Materials and Methods

Rats starved for 20 hr were used in all experiments. In the case of *in vivo* labeling, glucosamine-^{3}H, specific activity 10 Ci/mmol, from the Radiochemical Centre, Amersham, in sterile physiological solution was injected in nembutal anaesthesia into the vena portae. When indicated, 20 mg non-labeled glucosamine in 0.25 ml was also injected into the vena portae 10 min after the administration of label as chase. Total microsomal fraction, microsomal subfractions, and Golgi membranes were prepared as earlier (8,9). Supernatant was prepared after perfusion of the liver with 0.25 M cold sucrose. The postmicrosomal supernatant was recentrifuged for 3 hr at 105,000 g to remove all particulate components.

To remove adsorbed and luminal proteins, the fractions were first washed with alkaline buffer, followed by warming and cooling in hypotonic solution, and finally subjected again to washing with alkaline buffer (10).

The antiserum treatment of the microsomes was performed essentially as described by Redman and Cherian (11). Microsomes or microsomal subfractions were dissolved in 0.5 % deoxycholate and centrifuged for 60 min at 105,000 g. The supernatant was treated with rabbit antiserum to rat serum protein by incubation for 60 min at 37^{o}C, followed by storage at 4^{o}C overnight. The antiserum used was that found to be necessary for maximal effect. The precipi-

Table 1. Effect of the various treatments on washed microsomes

Treatments	PL, mg per g liver	NANA, µg per g liver	NANA, µg / PL, mg	cpm in NANA / g liver
None	7.5	40.5	5.4	24,077
Tris-buffer (Tris-HCl 0.01 M) pH 7.5	7.5	37.7	5.0	23,250
Tris-buffer + 1.5 M KCl	7.5	36.8	4.9	23,355
Tris-buffer + 1.5 M KCl + DOC 0.2 %	3.9	19.0	4.9	13,724
Tris-buffer + sonication	6.4	27.9	4.4	17,335
Tris-buffer + 1.5 M KCl + sonication	5.9	31.2	5.3	18,780
Tris-buffer + 1.5 M KCl + DOC 0.2 % + sonication	3.7	19.4	5.2	13,965
Citrate-phosphate buffer 0.05 M, pH 5.0	5.8	28.9	5.1	14,542

In vivo labeled microsomes were prepared after 60 min of injection of 0.2 mCi glucosamine-^{3}H into the vena portae of 150 g rats. The tris-water-tris washed microsomes were recentrifuged after different treatments (3 hr, 105,000 g) and the pellets were analyzed. In the case of deoxycholate, the concentration of protein was 5 mg/ml and sonication was performed with a Branson sonicator at 3.5 A during 2 min. PL, NANA, and DOC represent phospholipid, sialic acid and deoxycholate, respectively.

tate was removed by centrifugation, and total radioactivity and radioactivity in protein-bound sialic acid were measured both in supernatant and pellet. The amount of sialic acid can not be quantified in the supernatant, because of the high content of this sugar in antiserum.

Protein, phospholipid, sialic acid determinations, trypsin and neuraminidase treatments, as well as radioactivity measurements were made as before (5,8,10).

Results and Discussion

The radioactivity incorporated into protein bound sialic

Table 2. Treatment of microsomes with rabbit antiserum to rat serum protein

	Radioactivity in membranes cpm/mg liver			Radioactivity in sialic acid cpm/g liver		
Microsomes	Total	Non-precipitable	Precipitable	Total	Non-precipitable	Precipitable
Total	232	161	44	9,322	10,241	0
Rough	108	73	22	2,747	2,890	0
Smooth	103	78	12	6,707	8,351	0

Rats were injected with glucosamine-^{3}H as described in Table 1 and were decapitated after 60 min. Microsomes and microsomal subfractions were prepared and washed as in Table 1. Treatment with antisera according to Redman and Cherian (11). The radioactivity of precipitated and non-precipitated fractions was determined. Also, sialic acid was extracted from these two fractions for analysis of radioactivity.

acid after intraportal injection of glucosamine-^{3}H is tightly attached to microsomal membranes and cannot be removed to any large extent with various washing procedures (Table 1). High ionic strength has no significant influence, and sonication, which removes both sialic acid and radioactivity, also decreases membrane phospholipid content. The most pronounced effect is exhibited by deoxycholate, which, however, removes all membrane components to about the same extent, consequently leaving the sialic acid per phospholipid ratio practically unchanged.

The tris-water-tris procedure utilized in these experiments is very effective in removing adsorbed and luminal secretory proteins, but some serum glycoproteins, i.e. those which are not completed and participate in the enzyme-substrate complex during the biosynthetic process, may be present in bound form. In order to test this possibility, washed microsomes were treated with an-

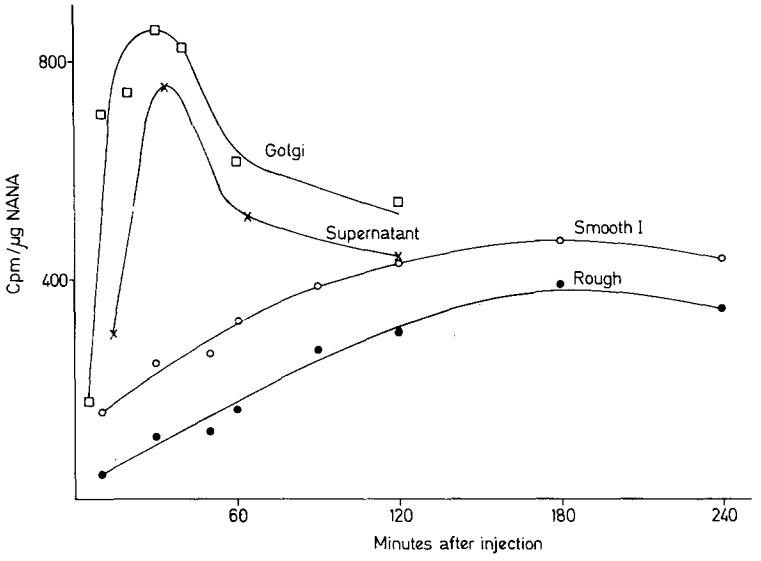

Figure 1. In vivo incorporation of glucosamine-^{3}H into protein-bound sialic acid of different subcellular fractions. Glucosamine-^{3}H, 0.1 mCi/150 g rat was injected as in Table 1. After 10 min, 20 mg of non-labeled glucosamine in 0.25 ml sterile physiological solution was injected into the vena portae as chase. The animals were decapitated after appropriate time points. Golgi fraction and microsomal subfractions were washed as described in Materials and Methods. In the case of supernatant, livers were perfused with 0.25 M sucrose. Each point gives the mean of 5 experiments. NANA represents sialic acid.

tiserum against serum proteins and both total and sialic acid attached radioactivity were determined (Table 2). 10-20 % of total radioactivity is precipitated but no counts associated with sialic acid can be found in the precipitate after antiserum treatment. This finding agrees well with the fact that both rough and smooth microsomes synthesize the core part of export glycoproteins, while sialic acid is added exclusively in the Golgi vesicles. Furthermore, it also means that the presence of protein bound sialic acid in microsomes is not the result of a secondary attachment or contamination.

Neuraminidase treatment at plateau value, both as regards time and concentration, liberates 35 % of bound sialic acid of unwashed microsomes (Table 3). After washing, an additional part

of sialic acid is liberated with this enzyme, but a large part still remains in bound form. Trypsin is also unable to remove the majority of sialic acid. The enzyme treatments again suggest that protein-bound sialic acid participates in the structural make-up of microsomal membranes.

The time course of glucosamine-^{3}H incorporation into protein-bound sialic acid was followed by dilution of the pool with 10,000 times excess of unlabeled glucosamine injected 10 min after the injection of the label. The purpose was to find out if any movement of newly synthesized glycoproteins among subcellular fractions takes place. A rearrangement of newly synthesized phospholipids in vivo has been described recently (12). Fig. 1 shows that the specific radioactivity in sialic acid reaches its maximum in Golgi membranes already after 30 min, after which time a rapid decay occurs. Supernatant of perfused liver also contains protein bound sialic acid (not shown here), corresponding to 4.7 µg per g liver. For this reason, the specific activity was also measured in the TCA precipitated part of the supernatant. The pattern is similar to that of Golgi: high radioactivity at around 30 min and rapid decay thereafter. In contrast to this, both rough and smooth I microsomes display a very different incorporation rate, exhibiting a slow increase during the first three hours. The specific activity at this late time-point is still much lower than that of the Golgi and supernatant at 30 min. The results indicate that glycoproteins with different turnover are present in different subcellular compartments, and, furthermore, a transfer or newly synthesized protein components probably also takes place.

In conclusion, it seems that sialic acid containing glycoproteins are present in microsomes as constitutive membrane components. They are tightly attached and a large part of them can-

Table 3. Effect of trypsin and neuraminidase treatment on microsomal NANA

Microsomes	Treatment	PL, mg / g liver	NANA, µg / g liver
Unwashed	none	5.97	70.00
"	neuraminidase	6.38	45.62
Washed	none	6.51	42.48
"	neuraminidase	5.99	27.77
"	trypsin	6.22	32.27

Tris-water-tris washing, trypsin and neuraminidase treatment of microsomes as previously (5,10). PL and NANA represent phospholipid and sialic acid, respectively.

not be removed by a series of mechanical, chemical, and enzymic treatments. Since attachment of sialic acid to glycoproteins takes place only in Golgi vesicles, the newly synthesized and completed sialoprotein has to be transferred to rough and smooth endoplasmic membranes by one of three ways: a) free luminal transport; b) membrane "back-flow"; or c) through the cytoplasm. The absence of sialic acid in microsomal vesicle content and the similar rate of incorporation into rough and smooth microsomes speaks against the first two possibilities. It appears most plausible, on the basis of incorporation experiments, that some newly synthesized glycoproteins from Golgi vesicles enter into the cytoplasm and are utilized subsequently as structural components of the microsomal membranes.

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