DIFFERENTIAL LOCALIZATION IN THYROID MICROSOMAL SUBFRACTIONS OF A MANNOSYLTRANSFERASE, TWO N-ACETYLGLUCOSAMINYLTRANSFERASES AND A GALACTOSYLTRANSFERASE

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SUMMARY : Two groups of glycosyltransferases have been differentially localized in microsomal subfractions from sheep thyroid. In rough microsomes an N-acetylglucosaminyltransferase and a mannosyltransferase incorporate internal carbohydrates to an endogeneous light precursor of thyroglobulin. In a smooth microsomal subfraction now obtained rich in identifiable Golgi structures, an other N-acetylglucosaminyltransferase and a galactosyltransferase catalyze the transfer of less internal carbohydrates to light precursors of thyroglobulin and to polymeric 18 S molecules.

Glycoprotein biosynthesis in mammalian tissues proceeds by a serial addition of monosaccharides to newly made polypeptides (cf. 1). It is interesting to determine the precise subcellular site(s) and the properties of the membrane-located glycosyltransferases involved. If the most internal asparagine-linked N-acetylglucosamine of glycoproteins such as thyroglobulin, serum glycoproteins and immunoglobins is attached very soon after peptide synthesis, either to completed chains or to nascent chains emerging from within the ribosomal structure (cf. 2-4), one may expect to find the corresponding transferase activity in rough microsomes. This has been found to be the case with liver by Molnar et al. (5) whereas other reports are controversial (6,7). In a preceding study (8) we have reported on the preferential localization in smooth microsomes from sheep thyroid of two glycosyltransferase activities extractable with Triton X-100 : a galactosyltransferase and an N-acetylhexosaminyl transferase. Further investigations were needed to find out if the latter was involved in the incorporation of the peptide-linked

N-acetylglucosamine residues of thyroglobulin (the major glycoprotein synthesized in the gland) or only of the more peripheral ones.

METHODS

UDP [UL-^{14}C] galactose (245 mC/mmole) and UDP-N-acetyl [I-^{14}C] glucosamine (42 mC/mmole) were from NEN, GDP [UL-^{14}C] mannose (143 mC/mmole) from the Radiochem. Ctr.

Mild homogenization of sheep thyroid slices was done with an Ultra-Turrax mixer (half max. speed twice for 8 sec) in 4 vol. of 0.4 M sucrose containing 1 % Dextran 500 (Pharmacia) as a stabilizing agent for Golgi membranes (9,10) and either 0.1 M Na phosphate buffer pH 7.2 or 0.04 M TRIS-HCl buffer pH 7.2 with 0.005 M $MgCl_2$. All operations were carried out at 0-4°. A 10000 x g supernatant was centrifuged for 1 h at 226000 x g (Spinco rotor 50). Microsomes were gently resuspended in 1.5 M buffered sucrose without Dextran and subfractionated according to a modification of a procedure used by others (11,10) : 4 ml corresponding to ∿ 6 g thyroid were overlaid sequentially with 2.7 ml of 1.3 M, 1.15 M and 1.05 M buffered sucrose and with 0.8 ml of 0.4 M buffered sucrose. Gradients were centrifuged for 4 h at 38000 rpm (Spinco rotor SW 40). The material at each interphase wa pipetted off, diluted to a 0.25-0.5 M sucrose concentration and respun at 226000 x g for 1 h, giving microsomal subfractions M1 to M4 whereas the dense pellet (eventually resuspended and sedimented again) was called M5.

For electron microscopy microsomal pellets were fixed for 1 h in 2 % OsO_4 in 0.177 M Na phosphate buffer pH 7.2. After dehydration in alcohols and embedding in Epon, ultra-thin sections are doubly stained with uranium and lead salts. E.M. are taken with a Siemens Elmiskop 101.

Microsomal pellets, stored frozen for up to 1-2 weeks without appreciable loss of activities, were suspended in cold H_2O for assays ; frozen solutions are now somewhat less stable. The transfer of [^{14}C] monosaccharides to protein (endogeneous acceptors unless otherwise specified) in enzymic assays (legend Table I) was measured as radioactivity precipitated by 5 % trichloracetic acid. For the characterization experiments essentially similar assays but on a larger scale were processed according to already described methods (2,8,12). Chemical determinations were done as previously described.

RESULTS AND DISCUSSION

Some properties of the microsomal subfractions. The pale yellow fraction M1 is essentially free from RNA as well as from identifiable ribosomes when studied by E.M. It represents as an aver. from 7.6 % of the recovered microsomal proteins (Na phosphate medium, 3 exp.) to only 3.4 % ($MgCl_2$ medium, 2 exp.). With the phosphate medium E.M. reveals (Fig. 1) the presence of nume-

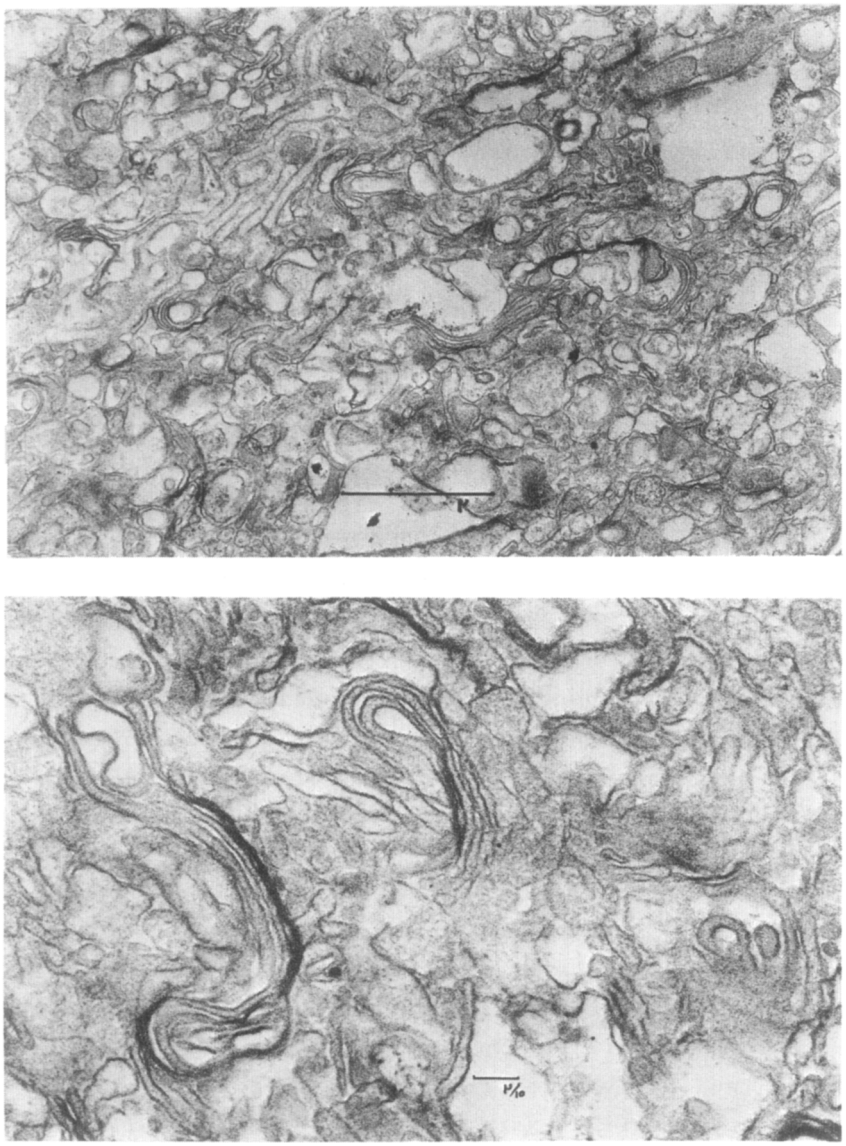

Fig. 1 - Representative sections from a M1 pellet prepared in the Na phosphate medium : Golgi stacks and saccules (full of a dense material) various smooth-surfaced vesicles.

rous Golgi elements i.e. components of classical dictyosomes ; more confused pictures are obtained with the other medium. During their attempts to isolate the Golgi apparatus from plant cells, Morre and Mollenhauer (9) have already noticed the preserving effect of Na^+ (particularly of Na phosphate). The RNA/protein ratio increases from M3 to M5 (up to 0.15) suggesting the presence of ribosomes and probably of rough microsomes. E.M. confirms this interpretation. A better preservation of rough microsomes is found in the M5 samples prepared with $MgCl_2$.

Distribution of the glycosyltransferases between microsomal subfractions. As shown in Table I, the galactosyltransferase specific activity (S.A.) is by far the highest in the Golgi-rich M1. Conversely the mannosyltransferase S.A. is the highest in either M3, M4 and M5 prepared with the Na phosphate medium or in M5 prepared with the $MgCl_2$ medium. As for the N-acetylglucosaminyltransferase S.A., it is high not only in M1 but also in either M3, M4, M5 (Na phosphate medium) or in M5 ($MgCl_2$ medium). If the results are expressed for each subfraction as a percentage of the recovered microsomal activity the dual localization of the N-acetylglucosaminyltransferase activity is again well evidenced with the Na phosphate medium. With the $MgCl_2$ medium the proportion of both the N-acetylglucosaminyltransferase and the galactosyltransferase in M1 is comparatively decreased (as a result of a decreased yield in M1). This fact is reminiscent of a previous observation (8) of an aggregation by 10 mM Mg^{2+} of those Dallner's smooth membranes in which galactosyltransferase is present.

In a single experiment the mitochondrial fraction sedimenting between 1000 and 10000 x g for 10 min in the Na phosphate medium was compared to the corresponding microsomal fraction and found to contain almost one half of the total particulate activities. It is probable that the amount of Golgi apparatus recovered in the mitochondrial fraction varies depending on the degree of homogenisation as has been concluded for the thyroid ergastoplasm (2).

Increased carbohydrate transfers in the presence of various incompletely glycosylated proteins. Addition in various amounts of a pre-heated microsomal extract obtained with Triton X-100 (8) allows substantial increases in the galactose incorporation. The N-acetylglucosamine transfer is similarly increased in the light microsomal subfractions but less so in the dense subfractions.

TABLE I

Distribution of the glycosyltransferases between unextracted microsomal subfractions

	Sub-fraction	Na phosphate medium (a)		$MgCl_2$, TRIS medium (b)	
		dpm incorp./ h/mg protein (S.A.)	% recovered activity	dpm incorp./ h/mg protein (S.A.)	% recovered activity
UDP [^{14}C] Gal.	M1	31060	54.8	12717	21.1
	M2	5060	8.7	2600	10.4
	M3	3033	11.4	2035	16.8
	M4	1816	8.1	1643	25.6
	M5	1495	17.0	1329	26.0
UDP [^{14}C] GNAc	M1	3911	16.5	2282	4.9
	M2	1266	5.2	791	3.9
	M3	1926	17.3	765	7.6
	M4	1958	20.6	1395	26.1
	M5	1488	40.4	2420	57.4
GDP [^{14}C] Man.	M1	4555	1.3	2120	0.6
	M2	13100	4.4	1240	0.8
	M3	28600	21.2	5350	7.5
	M4	28183	24.7	9052	24.2
	M5	21510	48.5	19950	67.0

(a) average 3 exp. ; mg protein (per g thyr.) recovered in M1 : 0.135, M2 : 0.131, M3 : 0.289, M4 : 0.340, M5 : 0.876.
(b) av. 2 exp. ; mg protein in M_1 : 0.037, M_2 : 0.089, M_3 : 0.184, M_4 : 0.348, M_5 : 0.440. Enzymic assays contain 160 µg microsomal protein and the following components (mµmoles in 0.130 ml) : either UDP [^{14}C] galactose : 0.166 ; UDP-N-acetyl [^{14}C] glucosamine : 0.95 or GDP [^{14}C] mannose : 0.28 (0.04 µC each) ; cacodylate-HCl respectively at pH 6.4, 7.7 and 7.0 : 6500 ; β-mercaptoethanol : 800 ; $MnCl_2$: 325 for the galactosyl- and N-acetylglucosaminyl transferases, $MnCl_2$ + $MgCl_2$: 325 each for the mannosyltransferase. At the end of the incubation (45 min at 37°) assays are diluted to 3 ml and receive 2 mg serumalbumin. They are immediately made 5 % (w/v) trichloracetic acid, processed and counted as already described (8).

Mannose transfer is unmodified. Most likely Triton X-100 extracts do not contain the correct acceptors for the transferases located in the rough membranes. As a matter of fact one can obtain a

comparable pattern of stimulations, by adding desialized thyroglobulins partially lacking either galactose or galactose and adjacent N-acetylglucosamine (prepared with thyroid glycosidases, unpublished). Ribonuclease B is a good N-acetylglucosamine acceptor in the presence of M1, but not with M4 or M5.

Characterization of incorporated carbohydrates and of labeled thyroglobulin or precursors after incubation of representative microsomal subfractions with [^{14}C] carbohydrate nucleotides. Carbohydrates have been identified as previously described (8) : 88 to 94 % of the incorporated radioactivity is found associated with the expected monosaccharide.

Characterization of labeled thyroglobulin or related proteins in Na deoxycholate extracts by immunodiffusion and paper electrophoresis will be reported elsewhere. Analysis of digitonin extracts by sucrose density gradient is presented here (Fig. 2). The N-acetylglucosamine and mannose labels obtained with the rough fractions M4 + M5 correspond to a 8-9 S component the nature of which has not yet been precisely studied but likely to represent a precursor of thyroglobulin (13). The N-acetylglucosamine and

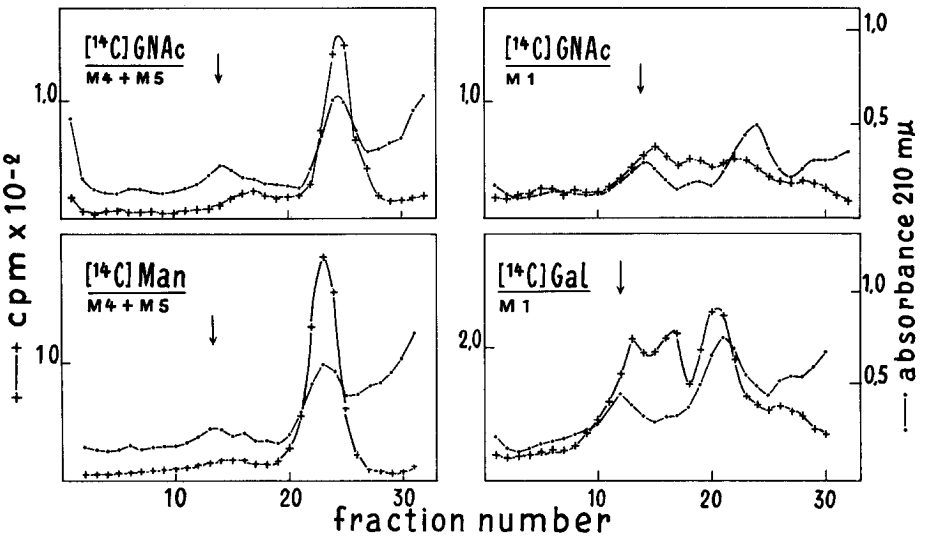

Fig. 2 - Distribution of trichloracetic acid insoluble radioactivities after gradient centrifugation of digitonin microsomal extracts. Microsomal subfractions prepared with Na phosphate ; enzymic incubations for 1 h. Digitonin extraction as in refer. 2. Linear 20-5 % sucrose gradient in buffered saline ; SW40 Spinco rotor, 15 h, 33000 rpm, 4°. Arrows indicate 19 S thyroglobulin.

galactose labeling patterns obtained with a subfraction M1 are stri kingly different : they show not only a 9 S component but also a 13.8 S and a 18 S component, the latter corresponding to fully-sized uniodinated thyroglobulin.

The present work demonstrates unambiguously an already suggested multistep glycosylation of thyroglobulin (1) occuring at different stages in the structural achievement of the molecule (14). Incorporation of the more internal N-acetylglucosamine and of mannose occurs soon after peptide synthesis in the rough E.R. whereas incorporation of the more distal N-acetylglucosamine and of galactose occurs more or less at the time of subunit association, in the Golgi apparatus (recently shown by histochemical methods, in several tissues including the thyroid, to be implicated in the elaboration of glycoproteins (15)). Our biochemical results are also in agreement with recent radioautographic studies after incubating rat thyroids with [^{3}H] galactose and [^{3}H] mannose (16).

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