LOCALIZATION IN SMOOTH MICROSOMES FROM SHEEP THYROID OF BOTH A GALACTOSYLTRANSFERASE AND AN N-ACETYLHEXOSAMINYLTRANSFERASE

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Summary : A galactosyltransferase and, to a lesser extent, an N-acetylhexosaminyltransferase, are predominantly localized in a smooth subfraction from sheep thyroid microsomes (possibly including Golgi elements). Triton X-100 "solubilizes" these membrane-bound enzymes, and endogeneous acceptors presumed to be intermediates in the biosynthesis of secreted thyroid glycoproteins.

Recent studies of thyroglobulin biosynthesis have suggested a particle located stepwise addition of individual monosaccharides, occuring after completion of the peptide moiety, largely if not entirely at a non-ribosomal site and perhaps at different stages in the elaboration of the protein molecule from its subunits precursors (1-5). After incubation of sheep thyroid slices with [^{14}C] glucosamine, both rough and smooth microsomes have been found to be labeled (6).

The precise subcellular sites of incorporation of individual monosaccharides, to global or specific glycoproteins, have been difficult to define in studies using prelabeled tissues, largely because of artefactual relocations during homogenization. Thus it remains questionable whether N-acetylglucosamine, a sugar present at two distinct positions in the carbohydrate units of several glycoproteins, including thyroglobulin*, begins to be incorporated at the polysomal stage or later on, when the newly made polypeptides successively traverses the channels of the rough and smooth

*The large type of carbohydrate units in thyroglobulin consists of linear sequences sialic acid (or fucose) → galactose → N-acetyl-glucosamine, attached to an inner core containing several mannoses and peptide-linked N-acetylglucosamine. The smaller type of carbohydrate units contains only mannose and N-acetylglucosamine.

endoplasmic reticulum (refs. in 4,7). On the other hand, electron microscope radioautographic studies after [^{3}H]glucose or [^{3}H]galactose injection have indicated the (smooth-surfaced) Golgi apparatus of several cells as an exclusive site of glycosylation (8,9), a view partly modified more recently when it was observed that, in the thyroid gland, [^{3}H]mannose would be taken up at a site closer to ribosomes (10). Some of the ambiguities inherent to the above-mentioned approaches have been avoided in a few recent studies of the subcellular localization of specific glycosyltransferases (11,12).

This communication describes the preferential localization in smooth thyroid microsomes of the following glycosyltransferases : 1/ an enzyme catalyzing the transfer of galactose from UDP [^{14}C] galactose to endogeneous incompletely glycosylated acceptor(s), 2/ one or more enzyme(s) catalyzing the transfer to endogeneous acceptors of glucosamine (probably as N-acetylglucosamine) from a biosynthetic UDP-N-acetyl [^{14}C]hexosamine.

METHODS

UDP-galactose is from Sigma. UDP [UL-^{14}C]galactose (245 mC/mmole) is from NEN ; UDP-N-acetyl [1-^{14}C]hexosamine (1.8 mC/mmole ; 70 % UDP-N-acetylglucosamine and 30 % UDP-N-acetylgalactosamine) has been isolated from sheep thyroid slices incubated with [1-^{14}C]glucosamine (13).

Sheep thyroid microsomes are subfractionated either according to a modification of the Dallner's method (6) or using a multilayered sucrose gradient without added ions. All operations are carried out at 0-4°. In the first case, 7 ml of a microsomal suspension in 0.25 M sucrose, corresponding to 4.7 g thyroid, are made 15 mM in CsCl and layered over 5 ml of 1.3 M sucrose also 15 mM in CsCl. In the second case, 6 ml of the microsomal suspension are layered over a gradient composed of 3 layers, 2 ml each, of 1.5 M, 1.3 and 1.1 M sucrose. Both types of gradients are centrifuged at 144000 g for 3.5 h (Spinco rotor 40). In the Dallner's method two main fractions are obtained : a pellet of rough microsomes, R, and a fluffy layer around the 1.3 M sucrose interphase ; the latter is pipetted off, diluted to a 0.25-0.5 M sucrose concentration and respun at 144000 g for 2 h, giving a pellet of smooth microsomes, S. In the other procedure, the material present at each interphase is pipetted off and respun as above, giving the microsomal subfractions I, II and III, whereas the dense material at the bottom of the tube is called subfraction IV.

Microsomal subfractions are suspended in the following cold medium : 0.25 M sucrose, 0.005 M $MgCl_2$, 0.025 M KCl, 0.02 M Tris buffer pH 7.5, and treated for 20-30 min with 0.25 % (v/v) Triton X-100 (Rohm and Haas Co.). Particulate material is removed by a 2 h centrifugation at 144000 g. Supernatants which include vesicle contents and "solubilized" membranes (more than 50 % of the initial protein content and $\sim$ 80 % of the phospholipid content) are dialyzed against cold H_2O, lyophilized and stored at - 20° before use. A variety of other extraction procedures proved to be less suitable (14).

Methods for chemical determinations have been described previously (4,6).

Transfer of $[^{14}C]$monosaccharides to protein in enzymic assays (legend Table I) is measured as radioactivity precipitated by trichloracetic acid.

RESULTS AND DISCUSSION

General characteristics of the enzymic systems : they have been studied with extracts obtained from unfractionated sheep thyroid microsomes. The galactosyltransferase requires Mn^{2+} for maximal activity (optimum 2.5 mM), a property also reported by Spiro and Spiro (15) and shared by other galactosyltransferases ; Mg^{2+} is roughly $\frac{1}{10}$ as effective. The pH optimum in cacodylate buffer is around 6.4. The N-acetylhexosaminyltransferase activity also requires Mn^{2+} (optimum 2.5-5 mM), Mg^{2+} being $\frac{1}{3}$ as effective, at a 10 mM concentration. The pH optimum in cacodylate buffer is very broad and is around 7.4. These properties are reminiscent of those exhibited by a goat colostrum N-acetylglucosaminyltransferase (16). In any case, several distinct hexosamine transfer enzymes may be present.

Incorporation of radioactivity varies as a function of time and is proportional to the amount of microsomal extract in the assay. In such microsomal extracts the glycosyltransferases are not saturated in the endogeneous acceptors presumed to be very largely intermediates in the biosynthesis of the major thyroid protein, thyroglobulin. Table I shows that the addition of increasing amounts of either a pre-heated microsomal extract or ribosomes stimulates the incorporation. It is worth noticing the absence in ribosomes of galactosyltransferase as well as of N-acetylhexosaminyltransferase. The most likely interpretation of the stimulation of the galactosyltransferase activity by ri-

Table I

Incorporation of radioactivity from UDP [^{14}C]galactose or from UDP-N-acetyl [^{14}C]hexosamine to microsomal protein acceptors.

	prep. nb.	microsomal extract mg protein	added acceptors: pre-heated microsomal extract mg protein	added acceptors: ribosomes mg	^{14}C dpm incorporated
UDP [^{14}C]gal.	1	0.0	0.5		51
		0.4	0.0		950
		0.4	0.5		1740
		0.4	1.0		2590
		0.4	2.0		3530
	2	0.0		0.6	40
		0.4		0.0	1500
		0.4		0.6	2250
		0.4		1.2	2716
UDP-N-ac. [^{14}C]hexosamine	1	0.0	0.5		34
		0.4	0.0		348
		0.4	0.4		420
		0.4	1.0		870
	2	0.0			28
		0.4			355
		0.4			438

Enzymic assays for galactosyltransferase contain the following components (mμmoles in 0.325 ml) : UDP [^{14}C]galactose, 24 (9166 dpm/mμmole) ; cacodylate-HCl pH 6.4, 16120 ; $MnCl_2$, 3250 ; β-mercaptoethanol, 2000 and a lyophilized extract from unfractionated microsomes, 0.4 mg protein. Assays for N-acetylhexosaminyltransferase contain : UDP-N-acetyl [^{14}C]hexosamine, 44 (3970 dpm/mμmole) ; cacodylate-HCl pH 7.4, 16120 ; $MnCl_2$, β-mercaptoethanol and enzymic extract as above. Added acceptors : either a microsomal extract pre-heated for 15 min at 100°, or ribosomes (prepared by adding 1%, w/v, Na deoxycholate to a suspension of thyroid microsomes in saline sucrose and sedimenting for 18h at 144000 g through sucrose layers ; 2 M sucrose inferior layer ; protein/RNA ≃1.6). At the end of the incubation (1h at 37°), assays are diluted to 3 ml (with an addition of serum albumin if necessary) chilled in ice, and made 5% (v/v) in trichloracetic acid. Precipitates are washed 3 times with 3 ml of 5% trichloracetic acid. Dried precipitates are dissolved at 60° in 0.5 ml of hyamine hydroxide before adding 10 ml of a scintillator solution. Countings are done in a Packard Tri-Carb liquid scintillation spectrometer.

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bosomes, a fact also reported in the study of a liver microsomal enzyme by Sarcione and Carmody (17), is that these ribosomes are strongly contaminated with incompletely glycosylated proteins, probably released when the membranes are disrupted by deoxycholate (4). The stimulation of the N-acetylhexosaminyltransferase might,

at least in part, be similarly explained, although nascent peptide chains might now be implicated too.

The radioactivities incorporated have been characterized after pronase digestion of labeled trichloracetic acid precipitates. Non dialyzable glycopeptides have been subjected to an acid hydrolysis (dialyzable glycopeptides have not yet been studied). In the case of galactosyltransferase assays, paper chromatographic analysis with butanol-ethanol-water shows that essentially all the radioactivity migrates as galactose. With an N-acetylhexosaminyltransferase assay, most of the label has been found to correspond to glucosamine during paper chromatography in pyridine-ethyl acetate-water, but some radioactivity has trailed in the region corresponding to galactosamine.

Distribution of the galactosyltransferase and N-acetylhexosaminyltransferase activities between the extracts derived from microsomal subfractions. As shown in Table II, both types of glycosyltransferase activities are the highest in the extracts prepared from the smooth microsomes S, or from the light subfraction I, also expected to be enriched in smooth microsomes. Table III shows the protein content and the RNA/protein ratio of the microsomal subfractions before their extraction with Triton X-100. As discussed in a preceding article (6), from a morphological point of view, the Dallner's subfractions are somewhat heterogeneous ; the subfraction S may contain, among smooth-surfaced vesicles from several origins, elements derived from the Golgi apparatus. A further subfractionation of the smooth microsomes in the presence of divalent cations (6) have shown once that the transferases are exclusively located in those smooth vesicles aggregated by Mg^{2+}.

It appears unambiguously that the extracts derived from smooth microsomes not only have the highest galactosyltransferase activity (as measured per mg protein) but also account for most of this activity in thyroid microsomes. As for the N-acetyl hexosaminyltransferase activity, its location in smooth membranes is perhaps not exclusive. These preliminary results support the idea that, in the course of intracellular migration and co-ordinated achievement of thyroid glycoprotein, the attachment of galactose and of an important part of the N-acetylglucosamine residues occurs at a site remote from that of peptide synthesis, within smooth membranes possibly related to the Golgi system. Experi-

TABLE II

Distribution of galactosyltransferase and N-acetylhexosaminyl-transferase between extracts derived from microsomal subfractions.

extract from sub-fract. (a)	added acceptors	dpm incorpor./mg protein (b) enzyme extr.		protein of the extract mg/g thyr. (c)
		galactosyl-transferase	N-acetyl-hexosaminyl-transfse	
S	0	4700	1005	0.79
	+	8250	1998	
R	0	660	448	1.41
	+	1300	612	
I	0	5120	892	0.66
	+	7850	1540	
II	0	1380	457	0.64
	+	2350	605	
III	0	498	211	0.33
	+	860	286	
IV	0	85	86	0.41
	+	168	182	

(a) Abbreviations defined in Methods. (b) Enzymic assays as in Table I ; added acceptors : pre-heated microsomal extract, 0,6 mg protein. Incubation time : 30 min for the galactosyl-transferase, 60 min for the N-acetylhexosaminyl transferase. (c) Operational values.

TABLE III

Protein content and RNA/protein ratio of the initial microsomal subfractions

subfraction	protein mg/g thyroid	RNA/protein
S	1.38	0.04
R	2.32	0.13
I	0.95	0.07
II	1.05	0.10
III	0.67	0.12
IV	0.53	0.14

ments are being done (using highly labeled UDP-N-acetyl $[^{14}C]$ glucosamine) to find out from studies of reaction products whether or not the smooth microsomes, in addition to a transferase invol-

ved in attaching the less internal N-acetylglucosamine residues, also contain an enzyme for the transfer of the peptide-linked residues.

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