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SYNTHESIS, MEMBRANE INSERTION AND GLYCOSYLATION OF THYROGLOBULIN IN A COMPLETELY HETEROLOGOUS SYSTEM

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SUMMARY: Thyroglobulin has been synthesized, inserted into membranes and glycosylated in a completely heterologous, reconstituted system consisting of a protein synthesizing extract, stripped dog pancreas microsomal membranes and calf thyroid RNA.

There is now considerable evidence to support the hypothesis that secreted glycoproteins are synthesized on polysomes which become membrane-bound following synthesis of an N-terminal hydrophobic signal sequence. (1-5) This signal "pre" sequence has been found on more than 20 nascent proteins destined for secretion, ovalbumin being the only known exception. (6) The signal sequence penetrates the membrane vesicle and is thought to be cleaved by a specific peptidase located in the membrane. (7,8) The remainder of the peptide encoded by the RNA follows into the vesicle where post translational modification continues, e.g., glycosylation. (9-12)

Thyroglobulin, a glycoprotein which serves as a prohormone for thyroxine and triiodothyronine, is well suited to studies of this hypothesis. Thyroglobulin comprises 60% of the protein in the thyroid, is synthesized on membrane-bound polysomes (13) and is glycosylated within the rough and smooth endoplasmic reticulum. (14) According to the signal hypothesis, the nascent thyroglobulin peptide should be synthesized in a "prepro" form. However, discrimination between 'prepro' and 'pro' thyroid hormone is almost impossible because the basic subunit has a molecular weight of at least 300,000. (15) Small differences in molecular weight in this size range cannot be determined accurately.

However, an alternative approach is to demonstrate nascent thyroglobulin transfer and subsequent glycosylation across microsomal membranes in a completely

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heterologous system. Glycosylation is essential for release of thyroglobulin into the follicular lumen so that adequate production of thyroid hormone depends on this process.

Materials and Methods

All glassware was sterilized by autoclaving and all buffers were treated with 0.1% diethylpyrocarbonate and boiled before use. Sigma supplied chemicals for cell-free systems. Schwarz/Mann supplied RNAase-free sucrose. Wellcome supplied donkey anti-rabbit precipitating serum. Radioisotopes were used in the following quantities for each 50 µl assay: D-(1-^{3}H) mannose, specific activity 2.7 Ci/mMol (Amersham) 1.0 µCi per assay. L-leucine (3,4,5-^{3}HN), specific activity 80 Ci/mMol (New England Nuclear) 0.5 µCi per wheat germ assay, 1.0 µCi per reticulocyte lysate assay. Guanosine diphosphate mannose, (mannose-1-^{3}H(N)) - specific activity 12.6 Ci/mMol (New England Nuclear) 0.625 µCi/assay. Guanosine diphosphate mannose, (mannose-^{14}C(U)) - specific activity 192 µCi/mMol (New England Nuclear) 0.25 µCi/assay.

RNA Preparation

Calf thyroid RNA was prepared from Triton-treated membrane-bound polysomes. (18) The supernatant after 27,000 x g centrifugation for 5 minutes was made 6 M with respect to guanidine hydrochloride and the pH was adjusted to 5.5 with acetic acid. A half volume of ethanol was added and the mixture was allowed to stand for 2 hours at -20°C. Following centrifugation at 4,000 x g for 20 minutes, the RNA pellet was dissolved in 6 M guanidine hydrochloride, 20 mM sodium acetate pH 5.5. Ethanol precipitation was repeated with centrifugation after 30 minutes. This extraction was repeated 3 times. The RNA was washed with ethanol:water, 2:1 and with 95% ethanol, dried and dissolved in distilled water. RNA was heated to 60°C for 3 minutes and rapidly cooled immediately prior to use in translation systems.

Preparation of Dog Pancreas Microsomal Membranes

Stripped dog pancreatic membranes were prepared by the method of Katz et al (19) (preparation 1) and the method of Shields and Blobel (4) (preparation 2). The A_{260nm}:A_{280nm} ratios were 1·75. The membranes were treated with micrococcal nuclease as recommended by Shields and Blobel (4) and adjusted to 50 A_{260} per ml in 0.25 M sucrose.

Cell-Free Systems

Rabbit reticulocyte lysate was prepared by the method of Crystal et al.(20) Each 50 µl incubation contained 40 µl of lysate containing the incubation mixture described by Pelham and Jackson (21) 5 µl of stripped dog pancreatic membranes and 5 µl of thyroid RNA (3 mg/ml). The lysate was not treated with micrococcal nuclease. The wheat germ lysate was prepared as described (22) with the following modification. The gel filtration buffer contained 3 mM magnesium acetate because the higher magnesium concentration used was found to exceed that optimal for synthesis. A 50 µl incubation mixture contained 25 µl of wheat germ extract, 15 µl of incubation cocktail(22) and 800 µM spermidine 5 µl stripped dog pancreatic membranes and 5 µl of thyroid RNA (3 mg/ml). Incubation was for 90 minutes at 30°C.

Indirect Immunoprecipitation of Thyroglobulin

2.5 µl of rabbit antiserum to thyroglobulin was added to the sample which was made 1% in Triton X-100, in sodium deoxycholate (DOC), and 0.1% in sodium dodecyl sulphate. The sample volume did not exceed 0.5 ml. The mixture was

incubated at 20°C for 2 hours. 25 µl of donkey anti-rabbit serum was added and incubation repeated. The mixture was layered over 0.5 ml of 1 M sucrose in 1% Triton X-100, 1% DOC in phosphate buffered saline (PBS, 10 mM phosphate buffer, 100 mM NaCl) in a 1 ml Fisher microfuge tube and centrifuged for 5 minutes at 7,000 x g. The pellet was washed 3 times with 1 ml of 1% Triton, 1% DOC in PBS and extracted with $CHCl_3$:CH_3OH:H_2O, 1:1:0.3 to remove glycolipids.

Liquid Scintillation Counting

Radioactive samples were solubilized in NCS (Amersham) and counted in an omnifluor-toluene scintillation coctail in a Searle Mark III liquid scintillation counter with a dpm accessory. Double-labelled samples were counted for 100 minutes and all background radioactivities have been subtracted.

RESULTS: Glycosylated peptides were measured by the incorporation of radiolabelled mannose into TCA precipitable material by protein synthesizing systems programmed with thyroid RNA and supplemented with stripped dog pancreatic membranes. Protein synthesis was concomitantly monitored by radiolabelled leucine incorporation.

Table I shows the amount of [^{14}C] leucine and [^{3}H] mannose incorporated into TCA precipitable material by the wheat germ system programmed with total thyroid RNA in the presence and absence of dog membranes. The ratio of [^{3}H]:[^{14}C] activity more than doubled when membranes were included in the incubation suggesting that thyroid RNA specifically stimulated sugar uptake in the presence of membranes.

Immunoprecipitation using thyroglobulin antibody of the products of wheat germ translation labelled with [^{3}H] GDP-mannose in the presence and absence of dog membranes is shown in Table II. There was considerably greater activity in the immunoprecipitate derived from incubation in the presence of membranes, wheat germ lysate and RNA than in either of the control incubations.

The percentage of immunoprecipitable mannose-labelled material was very low in the wheat germ system. The known stimulation of endogenous wheat germ mRNA translation by the addition of exogenous RNA may explain the low values. Considerable incorporation of [^{3}H] mannose also occurred when wheat germ was incubated alone which may indicate contamination with membranes. Furthermore, glycosylation of dog pancreatic proteins may be occurring within the vesicles even after dissociation of ribosomes as has been shown to occur for thyroid microsomes.(23) Since a thyroglobulin specific antibody was used, these possibilities do not invalidate the results in the wheat germ system. Nevertheless, it was decided to use reticulo-

TABLE I

INCORPORATION OF [^{3}H] MANNOSE AND [^{14}C] LEUCINE IN THE WHEAT GERM SYSTEM WITH AND WITHOUT DOG MEMBRANES

WHEAT GERM	THYROID RNA	MEMBRANE	[^{14}C] dpm	[^{3}H] dpm	[^{3}H]/[^{14}C]
+	+	+	850	18,479	21.7
+	+	-	157	1,587	10.1

Control values of incorporation of wheat germ with membranes and wheat germ alone have been subtracted.

TABLE II

^{3}H GDP MANNOSE INCORPORATION INTO THYROGLOBULIN IMMUNOPRECIPITATES SYNTHESIZED BY THE WHEAT GERM SYSTEM

Wheat Germ	Thyroid RNA	Dog Membranes	dpm
+	+	+	1,346
+	-	+	939
+	-	-	777

cyte lysate for further experiments. This system has the advantage that globin, the major endogenous product of lysate protein synthesis, is not glycosylated.

To show entry of thyroglobulin into vesicles, at the end of incubation the reaction mixture was further incubated at 37°C with ribonuclease (10 µg/ml for 5 min), trypsin (25 µg/ml for 15 min) and trypsin inhibitor (50 µg/ml for 15 min), respectively. The vesicles were lysed by detergents and their contents indirectly immunoprecipitated with thyroglobulin antibody. The results are shown in Table III. There was a consistently higher proportion resistant to trypsin in the presence of dog membranes.

Unequivocal evidence of vesicle insertion and, in addition, evidence of glycosylation of thyroglobulin was obtained by incubation in the presence of both

TABLE III

THE EFFECT OF TRYPSIN ON TCA PRECIPITABLE AND IMMUNOPRECIPITABLE ACTIVITY IN A RETICULOCYTE LYSATE IN THE PRESENCE AND ABSENCE OF TOTAL THYROID RNA

RNA	MEMBRANES	TCA PRECIPITABLE BEFORE TRYPSIN dpm	TCA PRECIPITABLE AFTER TRYPSIN dpm	% REMAINING TCA PRECIPITABLE	IMMUNO-PRECIPITABLE dpm
+	+	220,724	175,419	79.5	3,753
-	+	193,580	143,208	74.0	1,975

TABLE IV

DISTRIBUTION OF [^{3}H] GDP-MANNOSE AND [^{14}C] LEUCINE INTO THYROGLOBULIN IMMUNOPRECIPITATES

RNA	MEMBRANES	IMMUNOPRECIPITABLE WITH THYROGLOBULIN ANTIBODY			
		Supernatant		Pellet	
		[^{3}H] dpm	[^{14}C] dpm	[^{3}H] dpm	[^{14}C] dpm
+	+	56	1,834	224	1,573
-	+	145	76	126	204
+	-	0	7,004	47	4,293
-	-	131	853	78	380

[^{3}H] GDP-mannose and [^{14}C] leucine. Vesicles were pelleted after treatment with 0.5 mM puromycin, solubilized with detergents and immunoprecipitated. The results are shown in Table IV. There was incorporation of [^{3}H] GDP-mannose into a [^{14}C] leucine labelled thyroglobulin immunoprecipitate when dog membranes were included in the incubation but not in their absence. The thyroglobulin immunoprecipitate derived from both lysate supernatants after incubation with thyroid RNA with and without membranes showed considerable [^{14}C] activity but no [^{3}H] incorporation.

A second experiment was performed using stripped dog membrane preparation 2 which was less inhibitory to protein synthesis than preparation 1. [^{14}C] GDP-mannose

was used to eliminate any question of spillover from the radiolabelled protein activity and to reduce background controls. After synthesis membranes were added to those samples incubated in their absence as a control for nonspecific contamination of membranes. 5 ml of PBS containing 200 mM sucrose, 10 mM EDTA was added to wash the vesicles which were pelleted by centrifugation for 1 hour at 175,000 x g at 5°C. The results are shown in Table V.

When dog membranes were included in the incubation there was a marked increase in the amount of [^{14}C] GDP-mannose incorporated into the total TCA precipitable activity. The lysate itself either with or without thyroid RNA showed some incorporation of [^{14}C] sugar label which was not pelleted by centrifugation. These glycosylated supernatant proteins however were not precipitable with thyroglobulin antibody.

The proportion of the total [^{14}C] sugar label in the pellets was very much higher when membranes were included indicating that glycosylation was occurring in these vesicles. The proportion of [^{3}H] label in the pellets was low which implies that globin, which constitutes the bulk of the TCA precipitable material, remained in the supernatant. Since we have found that up to 50% of nascent thyroglobulin remains associated with the ribosomal pellet we were not surprised that the TCA and immunoprecipitation data showed only a small increase in [^{3}H] activity in the pellets when membranes were included. However, the incorporation of [^{14}C] label was three times higher in the thyroglobulin immunoprecipitate derived from the pellet after incubation of lysate, membranes and thyroid RNA than in any of the three controls.

CONCLUSIONS: Thyroid polysomal RNA can direct the synthesis of glycosylated thyroglobulin in a reconstituted heterologous system of reticulocyte lysate or wheat germ supplemented with stripped dog pancreas membranes. Glycosylation of thyroglobulin occurs only within these vesicles. These results imply that thyroglobulin mRNA contains the information for synthesis, membrane attachment, membrane insertion and glycosylation. This heterologous cell-free system allows an examination of glycosylation of thyroglobulin free from artifactual problems present

TABLE V

[^{14}C] GDP-MANNOSE AND [^{3}H] LEUCINE UPTAKE BY RETICULOCYTE LYSATES IN THE PRESENCE AND ABSENCE OF THYROID RNA AND STRIPPED DOG PANCREATIC MEMBRANES

RNA	MEMBRANES	TOTAL TCA PRECIPITABLE		TCA PRECIPITABLE IN VESICLES				IMMUNOPRECIPITABLE IN VESICLES			
		[^{3}H] dpm x 10^{-6}	[^{14}C] dpm x 10^{-4}	[^{3}H] dpm x 10^{-5}	% Total	[^{14}C] dpm x 10^{-4}	% Total	[^{3}H] dpm x 10^{-3}	% Pellet	[^{14}C] dpm	% Pellet
+	+	13.12	5.50	3.10	2.36	1.92	34.9	38.22	12.3	230	1.3
-	+	14.53	5.52	2.56	1.76	2.16	38.6	6.98	2.7	79	0.4
+	-	12.90	1.94	2.51	1.95	0.08	4.1	27.89	11.1	63	0.4
-	-	13.52	2.10	3.03	2.2	0.06	3.0	7.68	2.5	20	0.7

when microsomal systems prepared from the thyroid itself are used. (23) Furthermore nascent thyroglobulin is not only glycosylated but also iodinated and exported from the cell thus providing an excellent model system for the requirements of post translational processing.

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