SUBCELLULAR SITE OF GLYCOPROTEIN SYNTHESIS IN LIVER

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Received September 19, 1969

SUMMARY

The smooth and rough microsomal fractions of rat and rabbit liver were shown to carry out transfer of N-acetylhexosamine, galactose and mannose to endogenous protein acceptors from corresponding sugar-nucleotides. The rough microsomal fraction was more active than the smooth microsomal fraction for the incorporation of hexosamine and mannose, but only one-third as active for the incorporation of galactose. Data also are presented which suggest that among the subcellular fractions only the microsomal fractions are active in the hexosamine and mannose transfer reactions.

Regarding the mechanism of glycoprotein formation in mammalian tissues, researchers agree that the polypeptide backbone of these complex proteins is assembled according to the well established pathway of protein synthesis. As to the glycosylation of protein and completion of the oligosaccharide side chains two hypotheses have been suggested. According to one the carbohydrate chain is built up in the Golgi cisternae of mammalian cells by a multienzyme complex of carbohydrate transferases, which is localized in the membranes of this region (single-site hypothesis). This idea is based on the original observations of Neutra and Leblond (1) who injected ^{3}H-labeled glucose or galactose in rats, and found that macromolecules, other than glycogen, were labeled

[1]This investigation was supported by Public Health Service Grant No. PHS RO1-HD-02591 and a Career Development Award 5-K3-Ca-11, 158.

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only in the Golgi zone of liver and other tissues. Supporting this interpretation, Wagner and Gynkin (2), and Caccam, Jackson, and Eylar (3) have reported that incorporation of N-acetylglucosamine and mannose in endogenous proteins is carried out by the smooth microsomal fractions (rich in Golgi fragments) but not by the rough microsomal fractions of rat and rabbit liver. Similar observations were reported by using HeLa cells (4).

According to the other hypothesis glycosylation of proteins is initiated in liver (5-7) and plasma cell tumor (8) during the growth of the polypeptide chain on the ribosomes. However, the remainder of the oligosaccharide chain is assembled within the membranes (9) of the rough and smooth endoplasmic reticulum (which may include the Golgi region, multi-site hypothesis).

The purpose of the present experiments is to show that transfer of sugars to endogenous acceptors are carried out by both the rough and smooth microsomal fractions under in vitro conditions, thus supporting the multi-site hypothesis derived from experiments in vivo.

MATERIALS AND METHODS

Smooth and rough microsomal fractions were prepared by the Dallner procedure (10) from the livers of rats and rabbits which had been starved for 20 hr. Isolation of subcellular fractions, enzyme assays, and measurements of protein, RNA and radioactivity were described previously (5). UDP-N-acetylhexosamine-^{14}C (4-5 μC/μmole, glucosamine-^{14}C:galactosamine-^{14}C = 3:1) was prepared by incubating Ehrlich Ascites Tumor with glucosamine-^{14}C (11); UDP-galactose-^{3}H and GDP-mannose-^{14}C (both 10 μC/μmole) were purchased from New England Nuclear Corporation.

RESULTS AND DISCUSSION

In order to determine which of the subcelluar components

TABLE I

DISTRIBUTION OF SUGAR TRANSFERASE, AND MARKER ENZYME-ACTIVITIES AMONG SUBCELLULAR FRACTIONS OF RAT LIVER

Fraction	Sugar Transferase[2]		Marker Enzyme		
	hNAc[3]	Man	g-6-Pase	Scc.Dh	βGal.ase
Debris[1]	18	13	15	24	11
Heavy mitochondria	20	27	20	55	32
Light mitochondria	12	20	16	16	41
Microsomes	48	40	45	5	8
106,000g supernatant	2	0	4	0	8

Results are expressed in percent of recovered activity.

[1]When the debris fraction was further separated according to Blobel and Potter (16) to nuclei and mixed membranous fraction (cell membrane; residual mitochondria and microsomes) no sugar transferase, or glucose-6-Pase activity was found in the nuclear fraction.

[2]The mixtures in 0.5 ml volume contained: 5mM $MgCl_2$, 5 mM $MnCl_2$, 1mM EDTA, 0.1M Na-maleate, pH 6.9, 0.01M KCl, 1% Triton X-100 (Triton caused 2-3 fold increase in the incorporation of sugars for each fraction), 3 mg membrane protein and 35,000 dpm of UDP-N-acetylhexosamine-^{14}C (4 μC/μmole) or 100,000 dpm of GDP-mannose-U-^{14}C (10 μC/μmole). Reactions were carried out at 30° for 15 min and stopped by the addition of 2 ml of 10% CCl_3COOH. The precipitates were washed 5 times with 5 ml portions of 10% CCl_3COOH, once with chloroform-methanol (2:1) and then with 95% ethanol; they were finally dissolved in 0.5 ml of 0.2N NaOH and counted in a liquid scintillation counting system.

[3]hNAc, acetylhexosamine; Man, mannose; G-6-Pase, glucose-6-Pase; Scc.Dh, succinic dehydrogenase; βGal.ase, β-galactosidase.

could carry out measurable sugar transfer reactions into endogenous acceptors, livers of rats and rabbits were fractionated to yield debris, heavy mitochondrial, light mitochondrial, microsomal and cell sap fractions. Incorporation of hNAc-^{14}C, mannose-^{14}C as well as marker enzymes were determined (succinic dehydrogenase for mitochondria, 12; β-galactosidase for lysosomes, 13; and glucose-6-Pase for microsomes, 5). The results of a representative experiment (Table I) show that the incorporation activity for both sugars parallels the distribution of glucose-6-

TABLE II

EFFECT OF WASHING ON THE ENZYME ACTIVITIES OF THE MITOCHONDRIAL FRACTION

	Sugar Transferase		Marker Enzyme		
	hNAc	Man	G-6-Pase	Scc.Dh.	βGal.ase
Mitochondria	42	51	40	95	88
Microsomes	58	50	60	5	12

Data are given as % of recovered activity

The combined light and heavy mitochondrial fraction of a rabbit liver (about 500 mg protein, containing about 50% of the G-6-Pase and hNAc-transferase activity of the liver fractions) was suspended in 60 ml of 0.25 M sucrose containing 10mM Tris-HCl, pH 7.6, and centrifuged at 15,000 rpm for 10 min (Sorvall RC 2B, SS-34 rotor). The sediment was treated with fresh sucrose two more times. The combined supernatants were centrifuged further at 30,000 rpm for 1 hr (Beckman L2 centrifuge, No. 30 rotor).

phosphatase activity. The relatively high activity of the mitochondrial fractions could be reduced by repeated washings. After three washings 58% of the hNAc and 50% of the mannose transferase activities were recovered in a new microsomal fraction (Table II) devoid of succinic dehydrogenase activity but containing 60% of the original glucose 6-Pase activity. These results suggest that the microsomal structures are the principle cell constituents in the hexosamine and mannose transfer reactions. Thus contamination by microsomes could explain most of the activities of the other subcellular fractions (14).

In subsequent experiments the microsomes were further fractionated to rough and smooth microsomes and incorporation of sugars were determined. In 19 experiments with rats and 5 experiments with rabbits the rough microsomes showed twice as much activity as the smooth microsomes (Table III). Rough microsomes were also more active than smooth microsomes in the incorporation

TABLE III

INCORPORATION OF SUGARS INTO PROTEINS OF SMOOTH AND ROUGH MICROSOMAL FRACTIONS

Additions[1]	Source of Liver	Smooth[2]	Rough	No. of Exp.
UDP-N-Acetyl-hexosamine-^{14}C	Rat	100 (60-140)	200 (113-300)	19
UDP-N-Acetyl-hexosamine-^{14}C	Rabbit	156 (130-200)	270 (255-300)	5
GDP-Mannose-^{14}C	Rabbit	130	190	3
UDP-Galactose-^{3}H	Rabbit	300	90	2
UDP-Galactose-^{3}H + fetuin acceptor[3]	Rabbit	1900	450	1

The results are expressed as dpm/mg protein in average values obtained in the number of experiments indicated.

[1]The reactions were carried out as described in Table I. The activity of UDP-galactose-^{3}H was 100,000 dpm (10 μC/μmole).

[2]The incorporation of sugars by a mixture of smooth and rough microsomal fractions was additive.

[3]Fetuin (CalBiochem) was desialized and its galactose removed after periodate oxidation according to Spiro (17).

of mannose-^{14}C. On the other hand galactose-^{3}H was taken up 3-4 times faster by the smooth microsomes than by rough microsomes. The smooth fraction was also seen to be far more active than the rough fraction for incorporation of galactose when tested with an exogenous acceptor prepared from fetuin.

It is of interest to consider the probable basis for these differences between smooth and rough microsomal fractions. In the liver the most rapidly synthesized carbohydrate containing macromolecules are the plasma glycoproteins (5,6) and their incomplete forms are assumed to be the major acceptors in the present study. In the oligosaccharide chains of most of these pro-

teins, the glucosamine and mannose apparently lie closer to the polypeptide backbone than the galactose, sialic acid, and fucose (15). According to the multi-site hypothesis, based on *in vivo* studies (5,6), it would be expected that glucosamine and mannose would be incorporated more efficiently by the rough microsomal fraction than galactose (or sialic acid, or fucose). The present findings agree with this prediction rather than that of the single-site hypothesis, from which only the smooth microsomes would be expected to display carbohydrate transferase activity.

The results of this paper and those of *in vivo* studies described previously (5,6) disagree with the reports of Wagner and Cynkin (2) and Caccam et al. (3) who claim that the rough microsomal fractions have no glucosamine and mannose transferase activities. Presently we have no explanation for this discrepancy.

References

1. M. Neutra and C.P.Leblond, J. Cell Biol. 30, 137 (1966).

2. R.R. Wagner and M.A. Cynkin, Biochem. Biophys. Res. Commun. 35, 139 (1969).

3. J.F. Caccam, J.J. Jackson and E.H. Eylar, Biochem. Biophys. Res. Commun. 35, 505 (1969).

4. H.B. Bosmann, A. Hagopian and E.H. Eylar, Arch. Biochem. Biophys. 128, 51 (1968).

5. J. Molnar, G.B. Robinson and R.J. Winzler, J. Biol. Chem. 240, 1882 (1965).

6. G.R. Lawford and H. Schachter, J. Biol. Chem. 241, 5408 (1966).

7. J. Molnar and D. Sy, Biochemistry 6, 1941 (1967).

8. F. Melchers and P.M. Knopf, Cold Spring Harbor Symp. Quant. Biol. 32, 255 (1967).

9. E.J. Sarcione, M. Bohne and M. Leahy, Biochemistry 3, 1973 (1964).

10. G. Dallner, Acta Path. Microbiol. Scand. Suppl. 166, (1963).

11. J. Molnar, H. Chao and G. Markovic, Arch. Biochem. Biophys. In press.

12. E.C. Slater and W.D. Bonner, Biochem. J. 52, 185 (1952).

13. T.J. Langley and F.R. Jevons, Arch. Biochem. Biophys. 128, 312 (1968).

14. H.B. Bosmann and S.S. Martin, Science 164, 190 (1969).

15. N. Sharon, Ann. Rev. Biochem. 35, 623 (1966).

16. G. Blobel and V.R. Potter, Science 154, 1662 (1966).

17. R.G. Spiro, J. Biol. Chem. 239, 567 (1964).