Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 1828–1831, April 1980
Biochemistry

Different half-lives of the carbohydrate and protein moieties of a 110,000-dalton glycoprotein isolated from plasma membranes of rat liver

(methionine/galactose/fucose/*N*-acetylneuraminic acid/turnover)

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Communicated by Hans Popper, December 26, 1979

ABSTRACT **By using a four-step procedure (*i*, solubilization with Triton X-100; *ii*, affinity chromatography on concanavalin A-Sepharose; *iii*, affinity chromatography on wheat germ lectin-Sepharose; *iv*, preparative sodium dodecyl sulfate gel electrophoresis) a glycoprotein was isolated from rat liver plasma membrane. The molecular weight is 110,000 and the isoelectric point is 5.8. It contains L-fucose, *N*-acetylneuraminic acid, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and considerable quantities of aspartate, threonine, serine, and leucine. In pulse–chase experiments the half-lives of methionine and arginine, representing the half-life of the protein, were determined as 70 hr and 78 hr, respectively. The half-lives of the terminal carbohydrates L-fucose and *N*-acetylneuraminic acid were 12.5 and 33 hr, respectively. The galactose half-life was 20 hr. From this it is concluded that terminal sugars turn over several times in the life-span of this protein molecule. This process may be operative during membrane recycling mechanisms.**

Glycoproteins are important constituents of cell surface membranes. Their multiple roles include receptor function, enzymatic activity, transport, cell–cell interaction, and antigenicity (1–3). Because the carbohydrate moieties, especially the terminal carbohydrates (3–6), determine the biological activity of glycoproteins, the turnover of terminal carbohydrates in membrane glycoproteins may participate in the regulation of intracellular and intercellular processes. Malignant transformation, for example, is accompanied by alterations of the dynamic state of terminal carbohydrates in cell membrane glycoproteins (2, 7–9). Half-lives of different constituents (amino acids and terminal carbohydrates) have been measured in the total liver plasma membrane. Protein-bound *N*-acetylneuraminic acid turns over homogeneously (10), whereas protein-bound L-fucose shows a heterogeneous turnover (11). The proteins show a highly heterogeneous turnover (12, 13). Though several glycoproteins have been isolated from rat liver plasma membrane (14–19), the half-lives of both the carbohydrate and protein moieties of an isolated membrane glycoprotein have not yet been determined. This paper describes the isolation of a glycoprotein from rat liver plasma membrane and the determination of the half-lives of its carbohydrate and protein moieties.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing 180–200 g, were fed a commercial diet, containing 19% protein, and given water ad lib. Plasma membranes were prepared according to Neville (20) with some modifications (21) or by a zonal centrifugation method (22) and checked for purity as described (21).

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Purification of the 110,000-Dalton Glycoprotein (TC2W2E3) of the Plasma Membrane (Fig. 1). Plasma membranes were homogenized in a loose-fitting Dounce-type homogenizer with 10 strokes in buffer A [0.01 M Tris·HCl/0.15 M NaCl/0.001 M $MgCl_2$/0.001 M $CaCl_2$/0.02% NaN_3 (wt/vol)/1.5% Triton X-100 (vol/vol), pH 7.2] and solubilized for 12 hr at 10°C. After centrifugation (30,000 × *g* for 20 min) the supernatant (fraction T) was applied to a concanavalin A-Sepharose 6B column (5 ml of sedimented gel for the solubilized proteins from 20 mg of plasma membranes). With buffer B [composition as buffer A, but only 0.5% (vol/vol) Triton X-100] the unbound proteins were washed out (fraction TC1). The bulk of the retained glycoproteins was eluted with 0.2 M methyl α-D-mannopyranoside solubilized in buffer B (fraction TC2). By using higher concentrations of methyl α-D-mannopyranoside, low additional amounts of these glycoproteins could be obtained (fraction TC3 and TC4). The glycoproteins of TC2 were immediately fractionated on a wheat germ lectin-Sepharose 6 MB column. The glycoproteins not bound to this lectin were washed out with buffer B (fraction TC2W1). The retained glycoproteins were eluted with 0.2 M *N*-acetyl-D-glucosamine in buffer B (fraction TC2W2). The respective protein fractions were dialyzed against distilled water overnight and lyophilized. The detergent was removed by extraction with absolute ethanol and the precipitated proteins were pelleted. In order to isolate the pure glycoprotein the TC2W2 polypeptides were separated on 7.5% $NaDodSO_4$/polyacrylamide gels. By scanning the gels in a recording spectrophotometer at 280 nm the polypeptides could be localized. The pure glycoprotein (TC2W2E3) was eluted from the gel with 2.5% (vol/vol) Triton X-100 (for 12 hr at 10°C). After filtration of the glycoprotein solution and lyophilization, the detergent was extracted again with absolute ethanol. By boiling in 80% (vol/vol) ethanol for 5 min the salts were dissolved and the glycoprotein was precipitated.

Determination of Half-Lives. In pulse–chase experiments the half-life was measured from the disappearance of protein-bound radioactivity in TC2W2E3, which follows the equation $A(t) = A_0\, e^{-kt}$, in which $A(t)$ is the specific radioactivity at time t, A_0 is the original specific radioactivity, and k is a constant. In this equation the half-life ($\ln 2/k$) is not changed when the specific radioactivity is replaced by a value that is proportional to $A(t)$.

The peak area of TC2W2E3 was measured by scanning $NaDodSO_4$ gels of fraction TC2W2 at 280 nm. The gel slice corresponding to this glycoprotein was cut out and after incubation with 2.5% (vol/vol) Triton X-100 (for 12 hr at room temperature) the radioactivity was measured.

With this method the amount of protein, the peak area, and the radioactivity were proportional to each other. Therefore, the measured value cpm/cm^2 peak area is proportional to the specific radioactivity dpm/mg of protein.

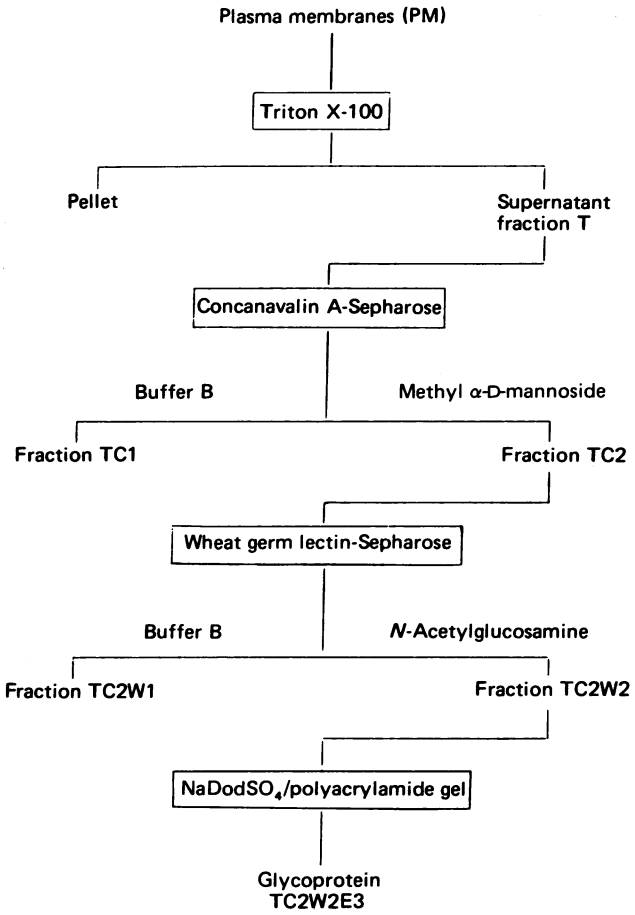

FIG. 1. Isolation of the 110,000-dalton glycoprotein (TC2W2E3) of rat liver plasma membrane.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The gels were prepared as described (23–25). The protein samples were solubilized and the gels were stained and destained as described (13). For analytical purposes 10% gels were used; for preparative purposes, 7.5% gels.

Isoelectric Focusing. Gel solution for 3 gels: 0.2 ml of 40% Ampholines (pH 2–11), 0.8 ml of solution A (28.38 g of acrylamide, 1.62 g of *N,N'*-methylenebisacrylamide, H_2O to 100 ml), 3.0 ml of protein solution in Triton X-100, 0.01 ml of 10% (wt/vol) ammonium peroxydisulfate in H_2O, 0.005 ml of *N,N,N',N'*-tetramethylethylenediamine. Anode solution: 0.01 M H_3PO_4; cathode solution: 0.02 M NaOH. The focusing was done at constant voltage, initially 50 V, later increasing the voltage stepwise up to 200 V. The proteins were fixed in 12.5% (wt/vol) trichloroacetic acid and stained and destained as described for the NaDodSO$_4$ gels. The final concentration of Triton X-100 in the gels could be varied between 0.1 and 1% (vol/vol), the acrylamide concentration (normally 6%) between 4% and 8% without impairing the result of the focusing.

Carbohydrate Analysis. The hydrolysis of the glycoprotein and the determination by gas/liquid chromatography of the neutral sugars was done as described (26). D-Ribose or L-arabinose were used as internal standards. The respective alditol acetates were identified by mass spectrometry and mass fragmentography. The amino sugars were eluted from Dowex H^+ with 1 M HCl and measured by an amino acid analyzer using a LiCl buffer system, which separates amino sugars from amino acids. Correction for loss was made by measuring the radioactivity of the internal standard (D-[^{14}C]Galactosamine).

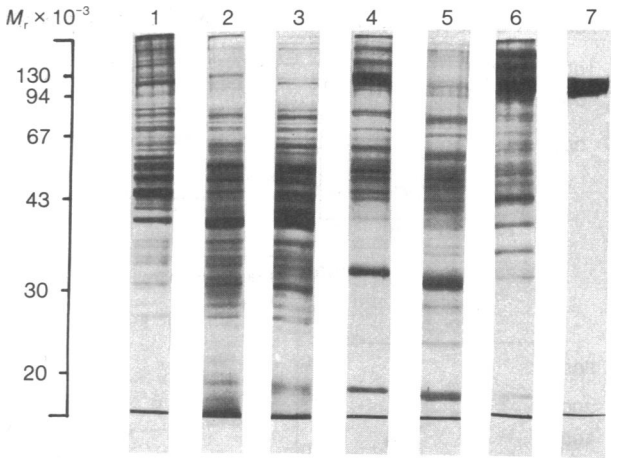

FIG. 2. Polypeptide pattern of the fractions obtained during the isolation of TC2W2E3: 10% NaDodSO$_4$/polyacrylamide gels, stained with Coomassie brilliant blue. Lane 1, plasma membrane; lane 2, fraction T; lane 3, fraction TC1; lane 4, fraction TC2; lane 5, fraction TC2W1; lane 6, fraction TC2W2; lane 7, glycoprotein TC2W2E3.

Amino Acid Analysis. After hydrolysis in 6 M HCl for 24 hr at 110°C, followed by removal of the HCl, the amino acids were measured with an amino acid analyzer.

Protein Determination. The method of Lowry *et al.* (27) was used with bovine serum albumin as standard. The purified glycoprotein was quantified by amino acid analysis.

Determination of the Radioactivity. Radioactivity was measured in a liquid scintillation spectrometer in Bray's solution (28).

Chemicals and Isotopes. Chemicals were purchased from Roth (Karlsruhe, West Germany), Merck (Darmstadt, West Germany), and Calbiochem. Ampholytes and calibration proteins for isoelectric focusing were from Serva (Heidelberg, West Germany), calibration proteins for molecular weight determination on NaDodSO$_4$ gels from Boehringer Mannheim. Isotopes were obtained from the Radiochemical Centre (Amersham, England).

RESULTS

Isolation and Analysis of the 110,000-Dalton Glycoprotein. The gel electrophoretic analysis of the protein fractions obtained during the isolation of the glycoprotein is shown in Fig. 2. Two major glycoproteins were determined in fraction TC2, with molecular weights of 110,000 and 27,000. In fraction TC2W2 the 110,000-dalton glycoprotein was highly enriched. The next

Table 1. Carbohydrate and amino acid analysis of TC2W2E3

Residue	nmol/mg protein	Residue	nmol/mg protein
Fuc	47	Pro	460
NeuAc	73	Gly	835
Man	306	Ala	665
Gal	250	Cys	145
Glc	251	Val	555
GlcN	267	Met	130
GalN	43	Ile	380
		Leu	730
Asx	1030	Tyr	375
Thr	605	Phe	380
Ser	840	Lys	515
Glu	1035	His	160
		Arg	340

Tryptophan was not determined.

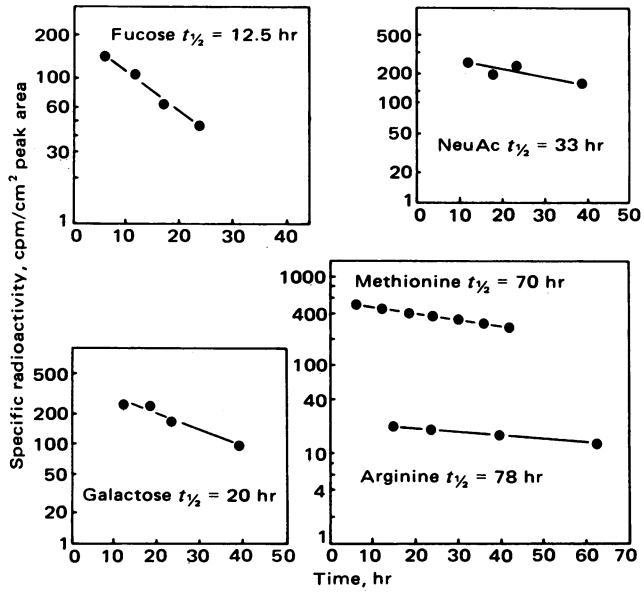

FIG. 3. Determination of half-lives in 110,000-dalton glycoprotein. For fucose, 0.55 mCi (1 Ci = 3.7×10^{10} becquerels) of L-[6-^{3}H]-fucose (15,000 mCi/mmol) per 100 g of body weight was injected intraperitoneally. Two hours after the label and then every 12 hr, 50 mg of fucose was given as a chase. Each point represents the protein-bound radioactivity in TC2W2E3 isolated from the pooled plasma membranes of three livers. In one experiment the half-life was calculated as 10.8 hr, in the other one, 14.2 hr. The mean is 12.5 hr. For half-life determination of protein-bound *N*-acetylneuraminic acid and galactose, 1.3 mCi of *N*-[^{3}H]acetylmannosamine (500 mCi/mmol) per 100 g of body weight or 0.167 mCi of D-[1-^{14}C]galactose (60 mCi/mmol) per 100 g of body weight was injected, intraperitoneally. Two hours after the label and then every 12 hr, 50 mg of the respective sugar was given as a chase. One point represents the measurement from one rat liver. For half-life determination of protein-bound methionine, 0.5 mCi of L-[^{35}S]methionine (1040 Ci/mmol) per 100 g of body weight was injected. Two hours after the label and then every 12 hr, 10 mg of methionine was given as a chase. Each point represents the protein-bound radioactivity in TC2W2E3 isolated from the pooled plasma membranes of two livers. For arginine, 0.250 mCi of L-[*guanido*-^{14}C]arginine (55.3 mCi/mmol) per 100 g of body weight was given. Each point represents results from one liver.

lane shows the pure glycoprotein (TC2W2E3), which forms a single band on NaDodSO$_4$ gels of different acrylamide concentrations. The apparent molecular weight on NaDodSO$_4$ gels was 110,000, estimated by use of marker proteins. Isoelectric focusing of the glycoprotein showed a sharp band at the pH of 5.8, estimated by use of marker proteins. Table 1 presents the carbohydrate and amino acid composition of the isolated glycoprotein.

Turnover Experiments with the 110,000-Dalton Glycoprotein (TC2W2E3). The half-lives of protein-bound fucose and *N*-acetylneuraminic acid, terminal sugars of carbohydrate chains, of galactose, a subterminal sugar, and of methionine and arginine, representing the half-life of the protein moiety, were measured (Fig. 3). The half-life of L-fucose was 12.5 hr on the average, calculated from two independent experiments (10.8 and 14.2 hr). Half-lives of 33 hr were calculated for *N*-acetylneuraminic acid and 20 hr for galactose. The half-lives of methionine and arginine were 70 and 78 hr, respectively.

DISCUSSION

The glycoproteins previously isolated from liver plasma membrane (14–19) did not seem to be suitable for measurement of half-lives of both the carbohydrate and the protein component for the following reasons. The isolation procedures described were time-consuming or might fragment native glycoproteins due to the action of proteases used in membrane solubilization. Moreover, some of these glycoproteins were not homogeneous on NaDodSO$_4$ gels. Our short isolation procedure produced a membrane glycoprotein that is homogeneous on NaDodSO$_4$ gels. Our main interest was its composition and half-lives rather than its biological function.

The amino acid analysis (Table 1) demonstrated that aspartic acid, threonine, and serine constitute 27 mol % of the amino acids of TC2W2E3. These amino acids can serve as attachment points for carbohydrate chains (1). The high content of leucine, isoleucine, and valine (17 mol %) suggests a hydrophobic region by which this glycoprotein may be embedded into the lipid bilayer as an integral membrane glycoprotein. The hydrophilic site with the constituent carbohydrates emerges from the membrane surface. Besides L-fucose, D-mannose, D-galactose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine, and possibly D-glucose, were found in appreciable amounts. The presence of D-glucose in a membrane glycoprotein had been shown earlier (29).

The function of our 110,000-dalton glycoprotein is not yet known. It does not exhibit any of the following activities of typical plasma membrane enzymes—5′-nucleotidase (EC 3.1.3.5), Na^+,K^+-ATPase, Mg^{2+},Ca^{2+}-ATPase (EC 3.6.1.3), leucine arylamidase (EC 3.4.11.2), nucleotide pyrophosphatase (EC 3.6.1.9), alkaline phosphatase (EC 3.1.3.1), γ-glutamyltransferase (EC 2.3.2.2)—as proven by measurements of enzyme activities in the different glycoprotein fractions and estimation of molecular weights and isoelectric points (data not shown here).

The protein and the carbohydrate moieties of our glycoprotein turn over at different rates. The half-life of L-fucose is 12.5 hr, of *N*-acetylneuraminic acid 33 hr, and of D-galactose 20 hr, whereas the half-life of the protein part (measured after labeling with [^{35}S]methionine and [*guanido*-^{14}C]arginine, respectively) ranges between 70 and 78 hr. Recently, the half-life of the receptor of asialo-orosomucoid has been found to be approximately 88 hr as measured with [^{3}H]leucine (30).

This means that terminal sugars are turning over two to six times in the life span of this glycoprotein. Not only the terminal sugars L-fucose and *N*-acetylneuraminic acid but also the (sub)terminal D-galactose turn over more rapidly than the protein core. Conceivably, a sequential split-off and reattachment of monosaccharides or an *en-bloc* transfer of oligosaccharides is a characteristic feature in the life-span of this glycoprotein.

Terminal sugars are important in the function of glycoproteins (4–6, 31–33). Though the function of our 110,000-dalton glycoprotein is not yet known, its biological activity may be related to the structure of the carbohydrate chain. Terminal sugars may be involved in the conversion of a membrane enzyme or receptor into its active or inactive form, or in the action of glycosyltransferases during cell-to-cell interaction, as suggested by Roseman (34). Possibly terminal sugars are related to the regulation of degradation of membrane glycoproteins, as proven for serum glycoproteins (5, 35, 36), or to the recycling of membrane glycoproteins, as claimed for the receptor of asialo-orosomucoid (37).

We are grateful to Dr. Inge Fromme and R. Wart from the Max Planck-Institut für Immunbiologie and H. Henninger and Cornelia Kuklik for assistance in carbohydrate and amino acid analysis and to Barbara Hassels-Vischer and Roswitha Heussner for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie, Bonn, West Germany. The results have been presented in part at the International Colloquium on Membrane Glycoconjugates in Seillac, France (38).

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