Comparison of desialylation of rat transferrin by cellular and non-cellular methods

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We have previously shown that the liver endothelium can desialylate the glycoprotein transferrin (Tf). In the present work we provide evidence that asialotransferrin obtained by this means behaves differently on *Ricinus communis* agglutinin (RCA₁₂₀) lectin affinity chromatography from asialotransferrin obtained by either neuraminidase treatment or acid hydrolysis. Purified rat transferrin was radiolabelled either with ¹²⁵I (protein moiety) or with ³H (sialyl residues), and subsequently saturated with iron. It was then passed through an RCA₁₂₀-agarose column to isolate the fully sialylated component. Sialylated Tf was then desialylated either by incubation with purified rat liver endothelium or, *in vitro*, by neuraminidase treatment or by acid hydrolysis. The protein was again subjected to RCA₁₂₀ column chromatography. Although both neuraminidase treatment and acid hydrolysis almost completely desialylated the glycoprotein (as evidenced by near absence of ³H label), the glycoprotein was not retained by the RCA₁₂₀-agarose column. By contrast, liver endothelium partially desialylated the glycoprotein, but this desialylated fraction was retained by the RCA₁₂₀-agarose column. These results suggest that desialylation with neuraminidase or acid hydrolysis may be inadequate for functional studies of asialotransferrin.

INTRODUCTION

Transferrin (Tf) is a glycoprotein that is responsible for the delivery of iron to iron-requiring cells. The delivery occurs by binding of Fe-Tf to specific membrane receptors that bind the protein moiety of Tf molecule. This is followed by internalization of the ligand–receptor complex. Within the cell, iron is dissociated from Tf, and Tf is then recycled. This scheme has been shown for several cell systems, including hepatocytes (Baker & Morgan, 1969; Karin & Mintz, 1981; Bleil & Bretscher, 1982; Light & Morgan, 1982; Sibille *et al.*, 1982; Van Renswoude *et al.*, 1982; Klausner *et al.*, 1983; Lamb *et al.*, 1983; Dickson *et al.*, 1983; Enns *et al.*, 1983; Harding *et al.*, 1983; Hopkins, 1983; Iacopetta & Morgan, 1983; Iacopetta *et al.*, 1983; Ciechanover *et al.*, 1983; Octave *et al.*, 1983; Stein *et al.*, 1984; Willingham *et al.*, 1984; Yamashiro *et al.*, 1984; Larrick *et al.*, 1985; Young *et al.*, 1985; Bomford *et al.*, 1986; Hunt & Marshall-Carlson, 1986; Irie & Tavassoli, 1987). There is some evidence that internalization is actually not necessary for the release of iron to the cell (Thorstensen & Romslo, 1988).

Several reports have indicated that plasma Tf may be partially desialylated (Stibler & Borg, 1981; Regoeczi *et al.*, 1984; Storey *et al.*, 1987) and that the presence of desialylated Tf can be correlated with iron-storage disease in the liver, suggesting that this form of Tf may deliver iron to hepatocytes through a mechanism different from that of Tf–receptor interaction. It is possible that desialylated Tf is removed by asialoglycoprotein receptors of hepatocytes that are lectin-like molecules recognizing penultimate galactosyl residues of the glycoprotein (Tavassoli *et al.*, 1986a; Irie *et al.*, 1988).

However, when Tf is desialylated by neuraminidase

treatment, its uptake by hepatocytes has not been different from that of sialylated Tf (Ashwell & Morell, 1974). Both seem to enter hepatocytes via Tf–receptor interaction. The reason for this discrepancy is not clear. Since we have previously demonstrated that liver endothelium also can desialylate Tf (Tavassoli, 1986; Irie *et al.*, 1988), in the present work we prepared desialylated Tf by various means. We then studied the behaviour of these desialylated preparations by RCA₁₂₀ lectin affinity chromatography, which binds the penultimate galactosyl residues exposed after desialylation. We now provide evidence that the asialo-Tf obtained by ‘chasing’ it through the liver endothelium interacts differently with this lectin from the asialo-Tf obtained by either neuraminidase treatment or acid hydrolysis.

EXPERIMENTAL

Protein and labelling

Rat Tf was obtained as an immunoelectrophoretically pure preparation from Cooper Biomedicals Laboratories (Malvern, PA, U.S.A.). Its purity was further demonstrated in our laboratory by isoelectric focusing, which revealed the existence of a mixture of slow-migrating and fast-migrating components, in turn corresponding to the bi- and tri-antennary glycan chains reported to be present in the molecule (Schreiber *et al.*, 1979). The protein was radiolabelled with ¹²⁵I by a solid-phase lactoperoxidase method (David, 1972) or ³H-labelled by a consecutive oxidation and reduction method (Van Lenten & Ashwell, 1971) as modified and described by us in detail previously (Kishimoto & Tavassoli, 1986). The specific radioactivities of ¹²⁵I-labelled and ³H-labelled materials were

Abbreviations used: Tf, transferrin; RCA₁₂₀, *Ricinus communis* agglutinin; PBS, phosphate-buffered saline (0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4); D-PBS, Dulbecco's phosphate buffer (0.14 M-NaCl/6 mM-sodium phosphate/1.5 mM-potassium phosphate buffer, pH 7.4).

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respectively 12 $\mu\text{Ci/nmol}$ and 0.5 $\mu\text{Ci/nmol}$, with a ratio of 24:1.

To remove any desialylated Tf from the labelled preparations, both ^{125}I -labelled and ^3H -labelled preparations were applied (maximum load 40 mg) to *Ricinus communis* agglutinin (RCA_{120})-agarose affinity columns (see below). All the fractions eluted by the equilibration buffer were pooled, dialysed and concentrated by ultrafiltration (Amicon; $M_r > 10000$). These fractions were considered to be fully sialylated, because they were not retained by RCA_{120} , which binds the penultimate galactosyl residues of the glycoprotein when they are not covered by sialyl residues (Pardoe *et al.*, 1968; Nicholson & Blaustein, 1972; Baenziger & Fiete, 1979; Irie & Tavassoli, 1986). The presence of sialyl residues was confirmed by desialylating the glycoprotein [see method *b* below] and measuring the sialyl content of the protein.

Desialylation procedures

Fully sialylated Tf, thus obtained by column chromatography, was subjected to desialylation by method (*a*), (*b*) or (*c*), as follows.

(*a*) Chemical desialylation was performed by heating the glycoprotein (5 mg/ml) in the presence of 0.05 M- H_2SO_4 for 1 h at 80 °C (Hatton *et al.*, 1979).

(*b*) Enzymic desialylation was performed by incubating the glycoprotein (5 mg/ml) in the presence of 0.5 unit of neuraminidase (type VIA, *Clostridium perfringens*; Sigma Chemical Co.) attached to agarose beads. The reaction was allowed to proceed for 48 h at 37 °C in 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM- CaCl_2 , and it was terminated by removal of the enzyme by centrifugation (Kishimoto & Tavassoli, 1986).

Glycoprotein, thus treated by methods (*a*) or (*b*), was recovered by gel filtration on a Sephadex G-25 column (1.2 cm $\times$ 28 cm) equilibrated and eluted with phosphate-buffered saline (PBS). In each case the column eluate was monitored spectrophotometrically for its protein (280 nm) and sialyl content (see below).

(*c*) Desialylation was also performed by incubation with rat liver endothelium (Irie & Tavassoli, 1986; Tavassoli *et al.*, 1986a). Endothelial cells were prepared as described previously (Seglen, 1976; Kishimoto & Tavassoli, 1985). The purity of the cell preparation was higher than 92%, with viability higher than 97%. Endothelial cells (1×10^7 suspended in PBS containing 0.5% bovine serum albumin) were incubated for 30 min at 4 °C in the presence of the ^{125}I -labelled or ^3H -labelled Tf that was saturated with iron (Takahashi & Tavassoli, 1982) to a final protein concentration of 150 $\mu\text{g/ml}$. At the end of the incubation period the supernatant, containing unbound radiolabelled Tf, was removed and the cells were washed thrice in ice-cold PBS. The surface-bound radiolabelled glycoprotein was then 'chased' inside and through the cells by incubating cells in Dulbecco's PBS (D-PBS) containing an excess concentration of unlabelled glycoprotein for 2 h at 37 °C, under gentle agitation. This temperature permits internalization of surface-bound ligand, which has been demonstrated to take place by fractionation of the surface-bound and internalized ligand (Kishimoto & Tavassoli, 1985). At the end of the incubation period, the cell suspension was centrifuged and the supernatant was removed (Irie *et al.*, 1988) for affinity chromatography.

Lectin affinity chromatography

Radiolabelled glycoprotein, after desialylation by each of the three procedures, was subjected to separation by RCA_{120} lectin affinity chromatography. Desialylated Tf obtained by method (*a*) or (*b*) was also saturated by iron before being applied to the column. Labelled glycoproteins were dissolved in 250 μl of PBS and applied to an RCA_{120} -agarose (Sigma Chemical Co.) column (10 mm $\times$ 25 mm). The column was equilibrated and eluted with D-PBS at a flow rate of 2 ml/h at room temperature. Retained components were eluted with D-PBS containing 0.2 M- β -D-galactose (Sigma Chemical Co.). Fractions were analysed for radioactivity, protein concentration (spectrophotometrically at 280 nm) and their sialyl content by using thiobarbituric method (Kishimoto & Tavassoli, 1986). The sialyl/protein ratio was expressed as a molar ratio. In Fig. 1 shown below the elution patterns of ^{125}I -labelled and ^3H -labelled protein are superimposed in order to make the comparison possible.

The relative proportion of each fraction eluted from the column was calculated from the ^{125}I radioactivity, which represented the protein concentration. The relative degree of protein sialylation in each fraction was also calculated by comparing the ^3H and ^{125}I radioactivities. The $^{125}\text{I}/^3\text{H}$ radioactivity ratios were obtained and divided by the ratio of specific radioactivities of the original labelled protein. The reciprocal of the value thus obtained was designated as the sialylation index of each fraction.

RESULTS

As we have reported before (Irie *et al.*, 1988) and confirmed again in the present study, ^{125}I -Tf was resolved by RCA_{120} -agarose affinity chromatography into two major fractions. The fraction eluted by equilibration buffer (before the application of β -D-galactose) consisted of 98% of the applied sample with a sialylation index of 1.0 (Table 1). The sialyl/protein molar ratio in this fraction was calculated to be 2.3:1. This fully sialylated fraction was then used in all the subsequent studies. The fraction eluted with β -D-galactose consisted of only 2% of the applied sample and contained no sialyl residues (Table 1).

To investigate the way in which different desialylation procedures could affect the structure of glycan chains of the protein, the desialylated glycoproteins were again subjected to RCA_{120} lectin affinity chromatography.

As shown in Fig. 1(*a*), neuraminidase-treated Tf gave two components on RCA_{120} affinity chromatography. About 98% of the ^{125}I -labelled Tf applied to the column emerged with the equilibration buffer and appeared to consist of two peaks. These peaks contained very small amounts of sialyl residues, as judged by the elution profile of enzymically treated ^3H -labelled fully sialylated Tf. The sialylation index was calculated to be 0.2 (Table 1). Only 2% of enzymically treated Tf was retained by RCA_{120} -agarose, requiring β -D-galactose for its elution. This component contained no sialyl residues, as suggested by the absence of ^3H label.

A similar chromatographic elution pattern (Fig. 1*b*) was obtained for fully sialylated Tf after desialylation by acid hydrolysis. About 88% of ^{125}I -Tf was eluted with equilibration buffer. As expected, this component contained almost no sialyl residues, as judged by the elution

Table 1. Characteristics of Tf components isolated by RCA₁₂₀-agarose chromatography after various desialylation procedures

For experimental details and method of calculation of the sialylation index see the text.

Type of desialylation	Fractions eluted with:			
	Equilibration buffer		β -D-Galactose	
	% *	Calculated sialylation index	% *	Calculated sialylation index
Non-desialylated	98	1.0	2	0
Liver endothelium	55	1.1	45	0
Neuraminidase	98	0.2	2	0
Acid hydrolysis	88	0.08	12	0

* Sample applied to the column = 100 %.

Fig. 1. RCA₁₂₀-agarose chromatograms of either ¹²⁵I-labelled (●) or ³H-labelled (○) sialylated diferric rat Tf after desialylation by various procedures

Rat Tf was desialylated by neuraminidase treatment (a) or by acid hydrolysis (b). Alternatively, desialylation was done by incubation with purified rat liver endothelium through a pulse-chase experiment (c). Arrows indicate the application of β -D-galactose. For experimental details see the text.

profile of the ^3H label. The sialylation index was calculated to be 0.08 (Table 1). About 12% of the applied material was obtained after elution with β -D-galactose, and this component did not contain any sialyl residues.

The RCA₁₂₀ elution pattern of Tf after desialylation by rat liver endothelial cells is shown in Fig. 1(c). In contrast with the two previous methods, Tf desialylated by this method showed two main peaks of comparable magnitude. The main peak consisted of 55% of the applied sample and was eluted with equilibration buffer. Its sialyl/protein molar ratio was 2.3:1 and the sialylation index was calculated to be 1.1 (Table 1). These are both similar to fully sialylated native Tf, indicating that this component was not desialylated by liver endothelium. The second component (45% of the applied sample) appeared only after elution with β -D-galactose and was fully desialylated, as judged by the absence of ^3H in its elution pattern.

DISCUSSION

Attention has been focused on desialylated Tf that can be detected in alcoholic patients and patients with certain types of liver disease (Stibler & Borg, 1981; Regoeczi *et al.*, 1984; Storey *et al.*, 1987). It has been postulated that this fraction of plasma Tf may preferentially be taken up by asialoglycoprotein receptors of hepatocytes, rather than by Tf receptors (Irie *et al.*, 1988). Consequently iron is deposited in a compartment of hepatocytes that makes iron less mobilizable. Over a period of time this may lead to iron-storage disease.

We have previously shown that liver endothelium can remove diferric Tf via a receptor-mediated mechanism, internalize it and transport it through a vesicular system and externalize it to the space of Disse, where Tf would be available for uptake by hepatocytes (Tavassoli *et al.*, 1986a; Kishimoto & Tavassoli, 1987). During this transport process iron remains associated with Tf. We have previously shown that this transport process is also associated with partial desialylation of Tf (Irie *et al.*, 1988), and it is possible that this desialylation process is induced by ethanol and can explain the presence of desialylated fraction of Tf in alcoholic patients. Endothelial desialylation is not limited to Tf, since another such glycoprotein, caeruloplasmin, is also modified in the same fashion by the endothelium (Irie & Tavassoli, 1986). It is now known why Tf is only partially desialylated by liver endothelium, since this process is complete in the case of caeruloplasmin, which is similarly removed by liver endothelium via a receptor-mediated mechanism, internalized and transported through a vesicular transport system to the abluminal side, where it is externalized to the space of Disse (Kataoka & Tavassoli, 1985; Tavassoli *et al.*, 1986b). It is possible that the heterogeneity of the glycan chain of Tf may be responsible.

Experimentally, the studies carried out on the behaviour of asialo-Tf are done with materials obtained by treating Tf with commercial preparations of neuraminidase or by acid hydrolysis. Both methods remove sialyl residues of the glycan chain, and our present results are consistent with this conclusion as the desialylated Tf did not contain ^3H label. However, the present study also indicates that the desialylated Tf preparations obtained by these methods behave differently from that obtained after pulse-chase incubation with liver endothelium. The latter form of asialo-Tf is retained by the RCA₁₂₀-agarose

affinity column whereas asialo-Tf obtained by neuraminidase treatment or acid hydrolysis is not.

Desialylation of Tf exposes the penultimate galactosyl residues of the glucan chain. The galactosyl residues have affinity for RCA₁₂₀ and consequently should be retained by the RCA₁₂₀-agarose affinity column. That the material obtained by neuraminidase treatment or acid hydrolysis is not retained suggests that the galactosyl residues exposed by these methods may have been modified by the desialylation so as to make them unavailable for binding to RCA₁₂₀. This is consistent with and supports the work of Hatton *et al.* (1974), who reported that the penultimate galactosyl residues exposed in this fashion are not susceptible to enzymic cleavage by β -galactosidase. Their results were obtained with human Tf, and they reported that this was not the case with Tf of other species such as rabbits, dogs, pig and bovine. In this regard, rat Tf appears to behave similarly to human glycoprotein and can serve as an experimental model for the metabolism of Tf in humans (Regoeczi *et al.*, 1979).

The reason for the difference in the type of material obtained by different desialylation procedures is not immediately clear. It is possible that neuraminidase treatment and acid hydrolysis, in addition to removing sialyl residues, can alter other parts of the molecule including its penultimate galactosyl residues, so as to make them unavailable for binding to RCA₁₂₀ and unsusceptible to enzymic cleavage by β -galactosidase. This concept is supported by our finding that the elution pattern of ^{125}I -protein after treatment with neuraminidase or acid hydrolysis is different from that of fully sialylated protein. Not only does the protein peak appear to have two components, but also the position of the eluted peak is somewhat different from that of non-desialylated peak [compare the first peak in Fig. 1(c) with those of Figs. 1(a) and 1(b)]. The latter two peaks appear to be somewhat delayed compared with the former. This suggests additional alteration in the protein moiety of the molecule. This characteristic appears to be specific for Tf, since other glycoproteins such as caeruloplasmin retain full capacity to bind to the RCA₁₂₀ agarose-column after desialylation (Irie & Tavassoli, 1987, and unpublished work).

An alternative interpretation would be that the penultimate galactosyl residues may be normally modified (e.g. acetylated) so that they would not bind to RCA₁₂₀ after desialylation alone, but liver endothelium, in addition to desialylation, may remove this alteration as well, so that galactosyl residues can now bind to RCA₁₂₀.

At any rate, this study strongly suggests that for functional studies with asialo-Tf desialylation by neuraminidase treatment or by acid hydrolysis may not be optimal. Desialylation by liver endothelium via pulse-chase experiment and purification of the resultant asialo-Tf on an RCA₁₂₀-agarose affinity column may yield materials that are more suitable for functional studies.

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