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Influence of transferrin glycans on receptor binding and iron-donation

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Human bi-bi-antennary transferrin (Tf) was partially deglycosylated by subsequently incubating with one or more of the following exoglycosidases: neuraminidase, β-galactosidase or *N*-Acetyl-β-D-glucosaminidase. Aglyco-Tf obtained from serum of a patient suffering from the Carbohydrate Deficient Glycoprotein syndrome was isolated. Receptor binding and the Tf and iron uptake capacities of the fully glycosylated-, partially deglycosylated- and aglyco-Tf were compared using the human hepatoma cell line PLC/PRF/5. No difference in binding capacity between the iso-Tf fractions could be demonstrated, however, the Tf and iron uptake capacity of aglyco-Tf was clearly reduced compared with the other Tf fractions.

***Keywords*: deglycosylation, iron, transferrin**

Introduction

Transferrin (Tf), the fourth most abundant plasma protein in man, is a monomeric protein consisting of 679 amino acids and a calculated molecular weight of 79 570 Da. The major function of Tf is transporting iron from the site of adsorption to the various cells in the human body. Being the main source of iron, Tf plays a crucial role in cellular iron uptake.

Receptor mediated endocytosis is considered to be the most important iron uptake mechanism. Endocytosis is initiated by binding of diferric Tf to its specific receptor. The Tf receptor is a class II receptor consisting of two identical subunits of 95 kDa each [1–3]. The Tf receptors are concentrated in clathrin-coated pits. At physiological pH the affinity of the Tf receptor is much higher for diferric Tf than for monoferric- or apo-Tf, ensuring an effective use of the Tf receptors. The clathrin-coated pit folds into a vesicle and becomes internalized into the cytoplasm. Once internalized, the coated vesicles lose their clathrin coat and become rapid acidified by means of a proton-pump. This acidification facilitates the release of iron from Tf and also increases the affinity of the Tf receptor for apo-Tf ensuring the binding and subsequent transfer of this carrier protein to the extracellular cavity [4].

Hepatocytes are also able to obtain iron through the asialoglycoprotein receptors [5, 6]. This C-type lectin [7] has a high affinity for galactose terminating oligosaccharides of desialylated glycoproteins including partially desialylated Tf.

Transferrin consists of two domains, the N-terminal and the C-terminal domain, the latter bears two N-linked glycans of the complex type attached to the asparagine residues 413 and 611. These glycans can differ in the degree of branching; bi-antenary, tri-antennary and even tetra-antennary structures have been shown to exist. This variability contributes to the so called microheterogeneity of Tf.

Specific variations in the oligosaccharide moieties of Tf are known to occur in several (patho)physiological conditions such as pregnancy, rheumatoid arthritis, malignancies and alcohol abuse [4, 8–12]. In the Carbohydrate Deficient Syndrome (CDG), first reported by Jaeken *et al.* [13], marked changes in the carbohydrate content of Tf were found; even a subfraction of Tf completely lacking carbohydrates has been described [14, 15]. The consequence of these variations remains obscure, as does the function of their oligosaccharides.

During pregnancy, a shift towards the higher branched glycans can be seen in the guinea pig as well as in man [8, 16, 17]. For humans, an affinity increase for the Tf receptors (TfR) towards the higher branched glycans was reported by Rudolph [18], but could not be confirmed by other authors [8, 19].

It was reported by Hu [20] that deglycosylation of rat Tf decreases the iron-donating ability of the protein to rat hepatocytes. This was not ascribed to a decreased affinity of the TfR for the deglycosylated protein, but it was postulated that the rat Tf glycan promotes the interaction of Tf with

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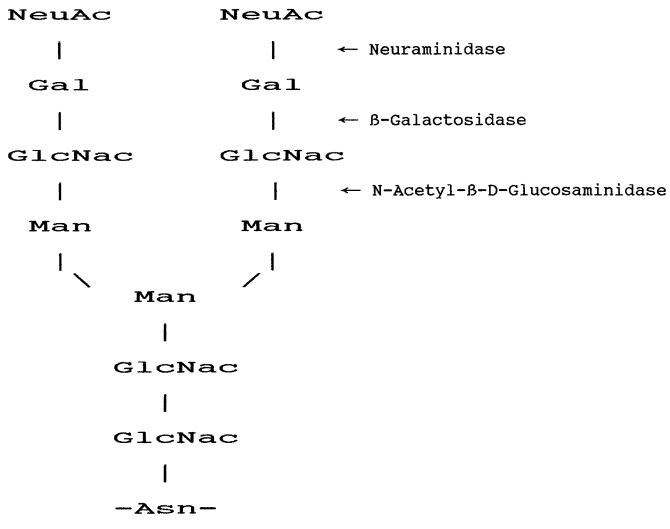

Figure 1. Biantennary glycan chain of the complex type showing the cleavage sites of the exoglycosidases used for partial deglycosylation.

low-affinity binding sites on the rat hepatocytes. No significant differences were found between rat aglyco-Tf and rat Tf with respect to the affinity for iron and the kinetics of the release of this metal from the N- and C-lobe of rat Tf.

Mason *et al.* [21] reported that there were no differences in the receptor binding and iron donating capacities of aglyco-Tf, obtained by site-directed mutagenesis, and commercially available transferrin in HeLa S_3 cells.

In this study, we investigated the influence of Tf oligosaccharides on receptor binding and iron uptake by the human hepatoma cell line PLC/PRF/5, otherwise known as the Alexander cell line. This cell line was chosen because of the absence of the asialoglycoprotein receptor [22], facilitating the comparison of Fe-transport properties of Tf in partially deglycosylated Tf and aglyco-Tf.

Binding and iron uptake studies were performed using bi-bi-antennary Tf, partially deglycosylated subfractions of this protein, and aglyco-Tf. Deglycosylation was performed by step-wise removal of the sugars up to the mannose bifurcation (Figure 1) using exoglycosidases; resulting in three partially deglycosylated Tf subfractions. These subfractions will be referred to as asialo-Tf, agalacto-Tf, and aglucosamine-Tf. Obviously, 1 mol of aglucosamine-Tf still contained 4 mol of glucosamine in the chitobiose core.

Aglyco-Tf was isolated from serum of a patient suffering from the CDG syndrome, the amino acid composition proved to be identical with normal Tf (unpublished observations). The lack of oligosaccharides does not influence the iron binding sites of Tf as seen on an Electron Paramagnetic Resonance (EPR) spectrum [23].

Materials and methods

Partial deglycosylation was performed step-wise, each step was followed by a purification of the incubated Tf either by using a Sepharose-bound anti-Tf IgG column [24] or preparative iso-electric focusing [25, 26]. Isolated fractions intended for cell incubations were focused twice ensuring a pure batch.

Bi-bi-antennary Tf

Commercially obtained Tf (Behringwerke) was separated into tri-tri-, bi-tri- and bi-bi-antennary subfractions by means of a Sepharose ConA column [27].

Neuraminic acid removal

Bi-bi-antennary Tf was incubated at 37 °C with *Clostridium perfringens* neuraminidase type X (Sigma) in sodium-citrate buffer (pH 5.0) for at least 24 h in a ratio of 1 mU enzyme per mg Tf.

Asialo Tf was isolated from partially desialylated Tf by preparative iso-electric focusing [25] on an Ultrodex gel (Pharmacia, LKB).

Galactose removal

Asialo Tf (20 mg ml^{-1}) was incubated with *Diplococcus pneumoniae* β-galactosidase (Boehringer Mannheim) for at least 24 h.

Incubations were performed in sodium-citrate buffer (pH 6.5) at 37 °C, using 2.5 mU enzyme for each mg Tf. Following incubation the Tf was purified by preparative iso-electric focusing and the galactose content of the sample was determined. A decrease in the galactose content of 95% or more was considered successful, otherwise the enzyme incubation was repeated.

Removal of the distal glucosamine

Agalacto Tf was incubated with *Diplococcus pneumoniae N*-acetyl-β-D-glucosaminidase (Boehringer Mannheim) at 37 °C in sodium-citrate buffer (pH 4.8) for at least 24 h. The incubation was performed with a Tf concentration of approximately 20 mg ml^{-1} and 30 mU enzyme for each mg Tf. After the incubation the Tf was purified by Sepharose-bound anti-Tf IgG affinity chromatography. The results of these incubations were examined by determining the glucosamine content.

Isolation of carbohydrate deficient Tf

Aglyco-Tf was isolated from a patient suffering from Carbohydrate Deficient Glycoprotein syndrome by means of Sepharose-bound anti-Tf IgG affinity chromatography and preparative iso-electric focusing. Complete lack of glycans in this isolated subfraction was demonstrated by carbohydrate analysis.

Carbohydrate analysis

To monitor the result of the enzymatic incubations using β-galactosidase and *N*-acetyl-β-D-glucosaminidase, carbohydrate analyses were performed after each step on purified Tf samples.

For determination of the galactose content following incubation with β-galactosidase, 100 to 400 µl aliquots of Tf solution with a known protein concentration were hydrolysed at 100 °C in 2 M trifluoroacetic acid for 24 h at pressures < 1 mm Hg. The hydrolysate was evaporated at room temperature, the residue was dissolved in 100 µl distilled water and mixed with 900 µl 100% ethanol. The hydrolysate was cleaned up with 6 µl analytical resin suspension (Ultra pac 11, Pharmacia/LKB), followed by partition chromatography with a cation-exchange resin using 90% ethanol as eluent and tetrazolium chloride as reagent [28].

N-Acetyl glucosamine was determined as glucosamine after special hydrolysis in 3 M p-toluenesulfonic acid over 24 h at 110 °C and $P < 1$ mm Hg, using a slightly adapted amino acid analysis program (Biochrom 20, Pharmacia/LKB, Cambridge, UK) [29, 30].

Electrophoresis and isoelectric focusing

SDS-Page and isoelectric focusing were performed on a Phast-system (Pharmacia, LKB) according to the manufacturer's instructions.

Labelling of the Tf subfractions

The Tf subfractions used for the binding studies were labelled with ^{125}I [31], 1 mg of diferric Tf was reacted for 10 min at room temperature with 0.83 mCi ^{125}I in a glass test tube coated with 100 µg Iodo-Gen according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Free iodine was separated from the radiolabelled Tf by passing the solution down a disposable Sephadex G-25 PD-10 column (Pharmacia), followed by extensive dialysis against 0.1 M Tris (pH 8.2). For the uptake studies the Tf subfractions were also labelled with ^{59}Fe, using $^{59}FeCl_3$/NTA, Na^{125}I and ^{59}Fe were purchased from Amersham.

Hepatoma cells

The human hepatoma cell line PLC/PRF/5 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were cultured in 35 mm cell culture cluster dishes (Costar) in a Salvis Biocenter 2001 and kept at 37 °C under an atmosphere of 5% CO_2 in DMEM containing 20 mM Hepes to which was added 10% FKS, 4 mM L-Glutamine and Gentamycin (0.05 mg ml^{-1}).

Transferrin binding

Approximately 4 days before a binding study, the cells were disseminated in the culture dishes. Experiments were performed as soon as the cells formed a monolayer.

The culture medium was removed and the cells were washed twice with PBS of 4 °C. To minimize the expression of Tf receptors saturated with endogenous Tf, the cells were incubated with PBS at 37 °C for 15 min and subsequently washed three times with ice cold PBS.

The PBS was replaced by incubation medium (DMEM containing 20 mM Hepes and 4 mM L-glutamine). A concentration range from 0.25 µg ml^{-1} to 3 µg ml^{-1} of the ^{125}I labelled differric Tf subfractions was added to the dishes and the cells were incubated for 90 min at 0 °C.

All enzymatically treated Tf subfractions were compared simultaneously in duplicate. Carbohydrate deficient Tf was compared with one of the partially deglycosylated subfractions. Nonspecific binding was determined by the binding of ^{125}I labelled Tfs in the presence of a 50-times excess of unlabelled Tf of the same subfraction. Unfortunately, we were unable to determine nonspecific binding for the carbohydrate deficient Tf because of the very small amounts available.

Following the incubations, the cells were washed three times with PBS (4 °C), lysed by addition of distilled water and collected with a rubber 'Policeman'. The dishes were rinsed with 0.1% Triton X-100. The samples were homogenised by sonication for 20 s in melting ice and samples were taken for protein determination according to the method described by Bradford [32]. Surface bound radioactivity was measured with a Packard 500C autogamma spectrometer.

Transferrin and iron uptake

Uptake studies were performed in similar culture dishes to those used for the binding studies. The cells forming a monolayer were washed twice with PBS (37 °C) and incubated with incubation medium as used in the binding studies. At $t = 0$ doubly labelled Tf (^{59}Fe and ^{125}I) was added to a final concentration of 3 µg ml^{-1}. At noted times the incubation medium was removed and the cells were washed three times with ice cold PBS. To remove all surface bound Tf, the cells were incubated in acetate buffer (25 mM sodium acetate, 0.15 M NaCl, 20 mM $CaCl_2$, pH 4.5, 4 °C) for 8 min followed by a 2 min incubation in PBS (4 °C). This procedure was repeated once.

Subsequently the cells were lysed and collected as described in the binding studies. Intracellular concentrations of ^{59}Fe and ^{125}ITf were determined with a Packard 500C autogamma spectrometer. As in the binding studies, the partially deglycosylated Tf subfractions were compared simultaneously, and the carbohydrate deficient Tf was compared with one of the subfractions.

Mathematical analysis of the Tf binding experiments

A nonlinear curvefit program (Statgraphics, Statistical Graphics Corporation) was used to evaluate the data obtained in the Tf binding assays according to the Langmuir equation for equilibrium binding; $T = (B_{max}{*}x)/(x + K_d) +$ non-specific binding. T (total binding, see Figure 4) represents the fraction of Tf that is bound, B_{max} is the saturation level for the specific binding, x is the concentration of the free Tf and K_d is the concentration of Tf at which the specific binding has reached half its maximum. The specific binding (S, see Figure 4) is characterized by $(B_{max}{*}x)/(x + K_d)$. The nonspecific binding is a linear function of x.

Results

Partial deglycosylation results in a slight decrease in molecular mass, as can be seen on the SDS polyacrylamide gel shown in Figure 2. Figure 3 shows that the iso-electric point of the deglycosylated Tfs changes only when the sialic acid is removed, and not with further treatment.

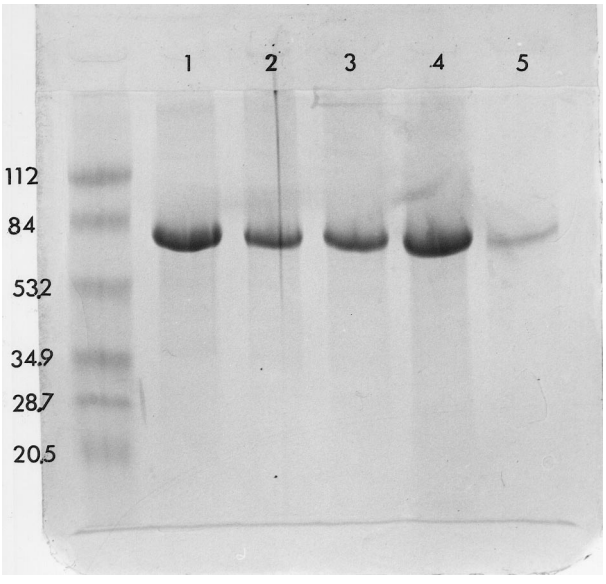

Figure 2. SDS PAGE (PhastGel Homogeneous 12.5) showing the different iso-Tf fractions. Lane 1, fully glycosylated-Tf; lane 2, asialo-Tf; lane 3, agalacto-Tf; lane 4, aglucosamine-Tf; lane 5, aglyco-Tf. Markers are indicated in kDa. Staining was done with Coomassie Brilliant Blue R250.

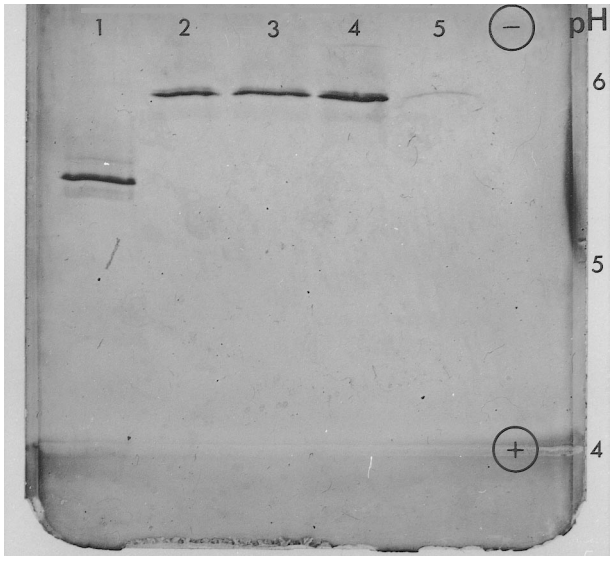

Figure 3. IEF (PhastGel IEF 4–6.5) showing the different iso-Tf fractions. Lane 1, fully glycosylated-Tf, lane 2, asialo-Tf; lane 3, agalacto-Tf lane 4, aglucosamine-Tf; lane 5, aglyco-Tf (faint, but present). Staining was done with Coomassie Brilliant Blue R250.

Carbohydrate analysis data of the deglycosylated subfractions and the aglyco-Tf are shown in Tables 1 and 2. Very low galactose content was detected in the agalacto-Tf and the *N*-acetylglucosomine content of aglucosamine Tf was halved compared to its concentration in bi-bi-Tf. Very little galactose or *N*-acetylglucosamine was detected in the aglyco-Tf. Table 2 also shows that the amino acid

Table 1. Galactose and mannose determinations on agalacto-Tf and aglyco-Tf. The galactose content of agalacto-Tf was determined by comparing with normal bi–bi-antennary-Tf using mannose as a standard. For the glycan analysis of aglyco-Tf, rhamnose was added to the Tf solution as an internal standard in a molar ratio of 7.34 to 1. Values are given in mol sugar per mol protein.

	Reference values serum bi–bi-antennary-Tf	*agalacto-Tf*	*aglyco-Tf*
Rhamnose	0.00	0.00	7.34
Mannose	5.49	5.51	0.52
Galactose	4.31	0.14	0.42

Table 2. *N*-Acetyl glucosamine determination of aglucosamine-Tf and aglyco-Tf. Values are given in mol amino acid per mol protein. Normal serum Tf was used as a reference.

	Reference values serum bi-bi-antennary-Tf	*Aglucosamine-Tf*	*Aglyco-Tf*
N-Acetyl glucosamine	8.0	4.1	0.8
Threonine	30.0	29.8	30.7
Serine	41.0	40.1	40.6
Glutamate + NH_2	59.0	57.9	60.0
Glycine	50.0	50.3	48.8
Alanine	57.0	49.4	57.3
Valine	45.0	44.9	46.8
Methionine	9.0	7.2	8.6
Isoleucine	15.0	14.7	15.4
Leucine	58.0	56.1	59.3
Tyrosine	26.0	24.9	26.2
Phenylalanine	28.0	27.3	28.4
Aspartate + NH_2	79.0	75.3	81.5
Histidine	19.0	18.6	18.8
Lysine	58.0	57.3	62.3
Arginine	26.0	24.4	26.4

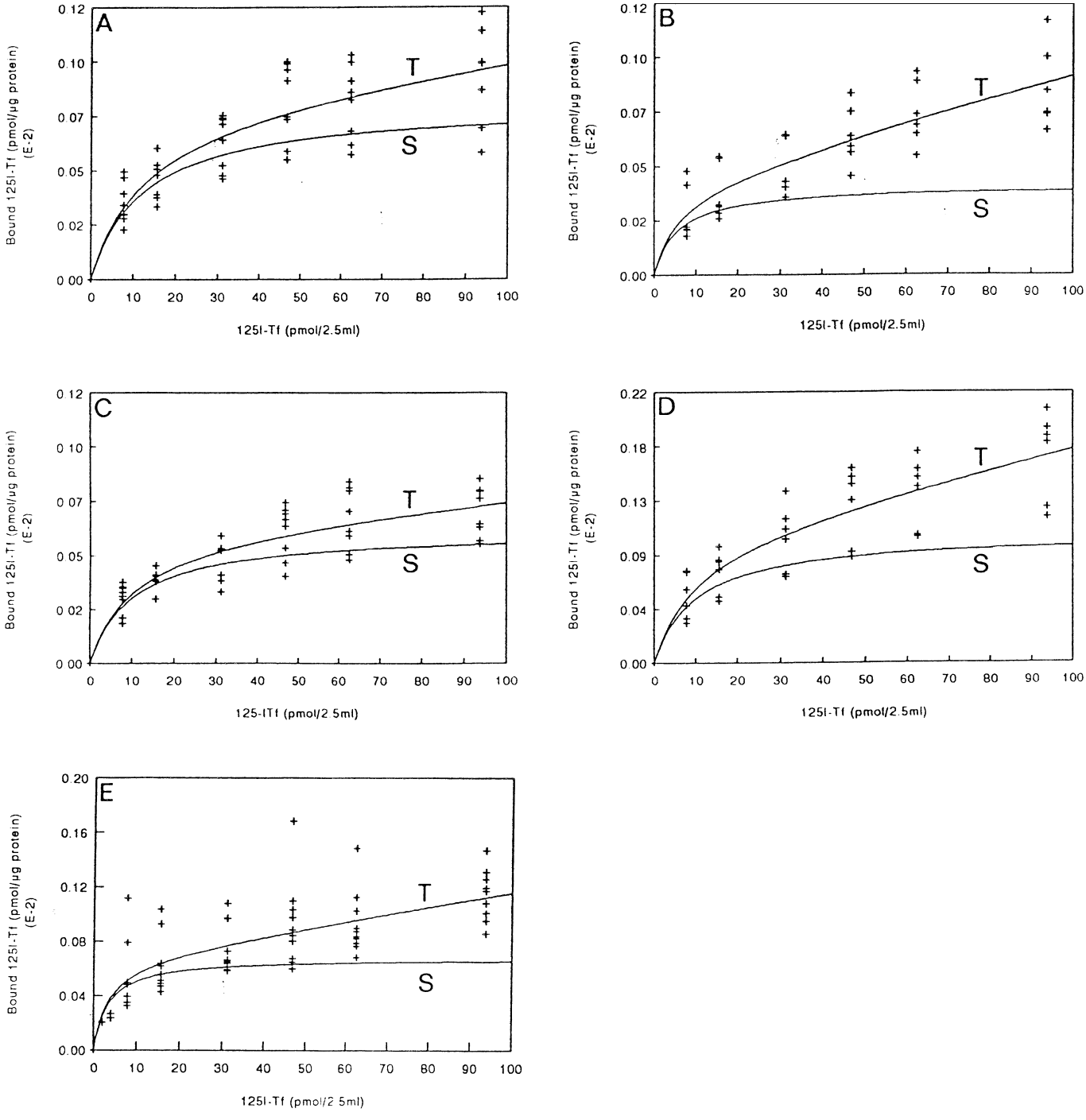

Figure 4. Total binding (T) and specific binding (S) of ^{125}I–Tf by PLC/PRF/5 cells. A, fully glycosylated Tf; B, asialo-Tf; C, agalacto-Tf; D, aglucosamine-Tf; E, aglyco-Tf. The total binding curve is fitted to the given data points using the function $y = (B_{max}*x)/(x + K_d)$ + nonspecific binding. The nonspecific binding for each Tf subfraction was determined by incubating the PLC/PRF/5 cells with the ^{125}I labelled Tf subfraction in the presence of a 50 times excess of the same (unlabelled) subfraction (data points not given). The nonspecific binding is a linear function of x ($y = ax$). The specific binding is calculated by subtracting the nonspecific binding from the total binding. In other words, the specific binding is represented by $y = (B_{max}*x)/(x + K_d)$. Four experiments were performed with bi-bi-Tf, agalacto-Tf and aglyco-Tf. Three experiments were performed with asialo-Tf and aglucosamine Tf.

composition of the different proportions was similar. The Tf binding curves are shown in Figure 4 and the K_d values in Table 3. The data was very variable. There was no significant difference between the dissociation constants (K_d) of the different Tfs preparations using a one way analysis of variance (Statgraphics, Statistical Graphics Corporation).

Table 3. The calculated K_d values for the different Tf binding experiments. The values are given in 10^{-9} M l^{-1}.

Tf-subfraction	K_d
bi-bi-Tf	11.5
	7.2
	2.3
	3.4
asialo-Tf	4.2
	0.7
	4.2
agalacto-Tf	5.5
	3.4
	7.0
	2.0
aglucosamine-Tf	1.8
	9.9
	5.0
aglyco-Tf	2.7
	0.5
	3.7
	0.7

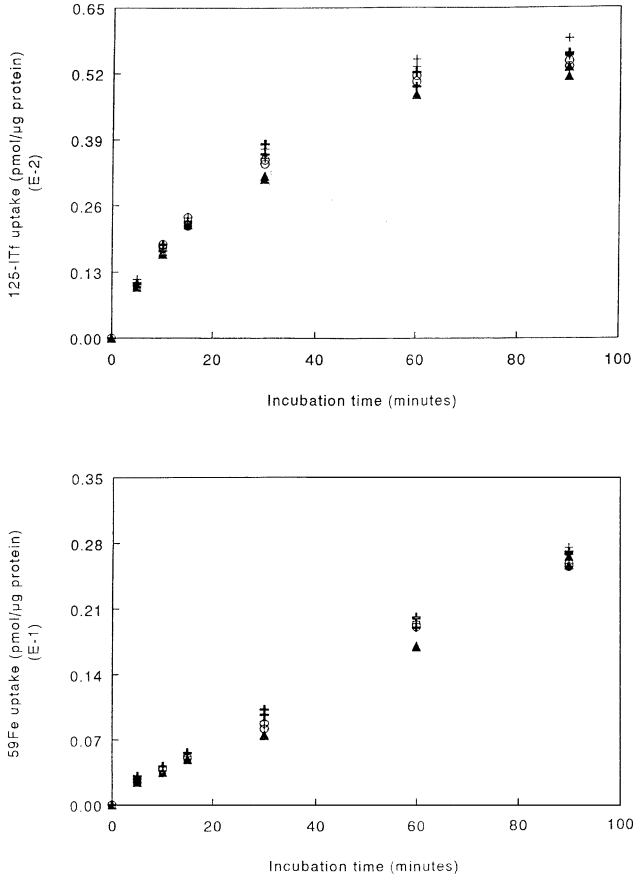

Figure 5. ^{125}I-Tf and ^{59}Fe uptake by PLC/PRF/5 cells. Legend: +, fully glycosylated Tf; ▲, asialo-Tf; ○, agalacto-Tf; +, aglucosamine-Tf. The results of one representative experiment are shown here.

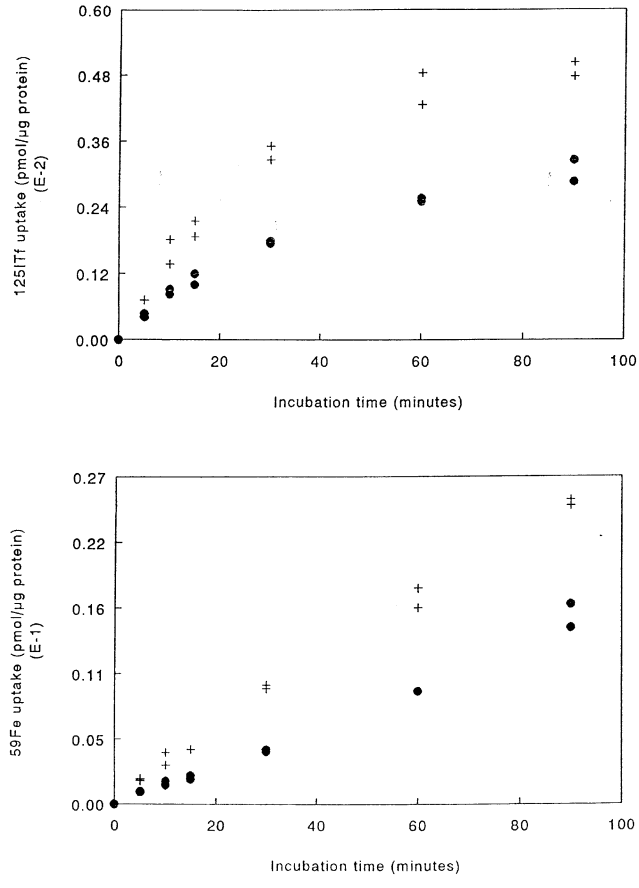

Figure 6. ^{125}I-Tf and ^{59}Fe uptake by PLC/PRF/5 cells. Legend: +, fully glycosylated Tf; ●, aglyco-Tf. The results of one representative experiment are shown here.

In the uptake studies (Figures 5 and 6) a remarkable difference was found between aglyco-Tf and the other iso-Tf fractions. The rate of iron uptake by PLC/PRF/5 cells was much slower from aglyco-Tf than from the other Tf fractions and the latter proteins reached a higher steady-state in the cells than aglyco-Tf. No difference in iron or Tf uptake could be found between the fully and the partially deglycosylated Tfs. The results from one uptake experiment are shown in Figure 5 but are representative of the five experiments that were performed. Some differences in the Tf and iron uptake were seen between experiments, possibly due to differences in cell growth. The results of the comparison between aglyco-Tf and fully-glycosylated Tf shown in Figure 6 are also representative.

Attempts to step-wise deglycosylate Tf further using alpha-mannosidase (Boehringer Mannheim) failed several times under various conditions. Removing $Man_3GlcNac_1$ from aglucosamine Tf following the method described by Maeyama *et al.* [33] using endoglycosidase D (Seikagaku, Tokyo, Japan) also failed several times for unknown reasons.

Discussion

In the present work the effect of the Tf glycan chains on receptor binding and uptake of Tf were studied. Fully glycosylated Tf was compared with partially deglycosylated Tf and aglyco-Tf.

Agalacto-Tf contained less than 5% galactose compared to normal Tf. Aglucosamine Tf contained approximately 50% of the amount of *N*-acetyl glucosamine found in normal Tf, indicating that all distal *N*-acetyl glucosamine had been removed. Only small amounts of sugar were found in aglyco-Tf, approximately 10% of the normal values. This can be easily explained by a slight contamination of the aglyco-Tf subfraction with Tf bearing normal glycans.

The calculated K_d values of the different binding experiments showed a high degree of variability. However, the oligosaccharides of Tf seemed to have no influence on the affinity of the TfR for its ligand. This finding confirmed that previously reported by Mason *et al.* [21].

The carbohydrates on Tf, however, affect the uptake of both the protein and iron by PLC/PRF/5 cells. Iron uptake from aglyco-Tf is diminished compared with partially or fully glycosylated Tf. *In vivo* reduced iron uptake from deglycosylated Tf was previously reported in rats [20]. Our results indicate that the proximal part of the glycan chains i.e. up to and including the mannose bifurcation, is responsible for this difference. Transferrin with oligosaccharides trimmed to the mannose bifurcation has the same iron uptake characteristics as its fully saturated counterpart, whereas the iron uptake ability of aglyco-Tf is diminished. Different uptake rates are also indicated by the different steady-states reached by aglyco-Tf and the other iso-Tfs. The reduced steady-state of aglyco-Tf could be explained by a decreased endocytosis rate or an increased exocytosis rate, whereas the latter is not very likely because of the diminished iron uptake compared with the partially and fully glycosylated Tfs.

The steady-state reached by the aglyco-Tf also shows that this protein takes part in an endocytosis/exocytosis cycle, i.e. is not degraded in the cell.

It can be concluded that the Tf oligosaccharides influence the Tf uptake by PLC/PRF/5 cells, but removing the three distal glycan residues does not alter the function of these oligosaccharides.

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