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N-Linked Oligosaccharides of Human Transferrin Are Not Required for Binding to Bacterial Transferrin Receptors

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Derivatives of human transferrin (hTf) with removed or modified N-linked oligosaccharides were compared with native hTf with respect to their binding to bacterial hTf receptors from *Neisseria meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenzae*. Partially and fully deglycosylated hTf were prepared by enzymatic deglycosylation with glycopeptidase F and isolated by concanavalin A-Sepharose affinity chromatography. Oligosaccharide-modified hTf was prepared via mild periodate oxidation. Competition and direct binding experiments with the hTf derivatives demonstrated that the hTf oligosaccharides are not essential for binding to the bacterial hTf receptors.

Essentially all forms of life require iron for growth (4). Although iron is abundant in animal hosts, free iron is not readily available for microbial use during the host-microbe interaction because most is found intracellularly or bound to the iron-binding proteins transferrin and lactoferrin (4).

To compete successfully for free iron within the host, many bacterial pathogens have developed systems of iron acquisition involving siderophores. Siderophores are iron chelators with extremely high affinity for ferric ions (14). They compete with and capture iron from host iron-binding factors. Once having bound iron, the siderophores are then internalized via specific outer membrane protein receptors (8). In contrast, *Neisseria meningitidis* and *N. gonorrhoeae* do not produce siderophores (27). However, both *N. meningitidis* (1, 15) and *N. gonorrhoeae* (13) are capable of utilizing iron from human transferrin (hTf) and human lactoferrin. Similarly, *Haemophilus influenzae* does not appear to produce functional siderophores, yet it is able to utilize hTf iron, but not human lactoferrin iron (7). Each of the above bacteria requires direct contact between the bacteria and the iron-binding proteins.

It is now known that *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* possess specific protein receptors for binding to hTf and human lactoferrin which have been isolated and characterized recently (10, 19–23). Competition dot binding assays, using membranes and whole cells from iron-starved bacteria, have demonstrated that the hTf and human lactoferrin receptors are specific for the human form of transferrin and lactoferrin in these bacteria. Competition dot binding assays also demonstrate that both iron-loaded and iron-free hTf (apo-hTf) are capable of binding to the receptor but that iron-saturated hTf is capable of effectively blocking the receptor at concentrations 10-fold lower than those required by apo-hTf (22).

The structure of hTf is well known. hTf is a monomeric glycoprotein that can reversibly bind two ferric ions per molecule. The primary amino acid sequence of hTf contains 678 amino acids with a calculated molecular weight of 79,570: 6% of the total weight of hTf is contributed by the carbohydrate chains (12, 25). It has a bi-lobed structure, with both of the hTf N-linked oligosaccharides being found on the C lobe, at amino acids 413 and 611 (2).

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The role of these N-linked oligosaccharides in the receptor-mediated iron removal and subsequent uptake of iron from transferrin by bacteria is not known. However, results of a competition dot binding assay for determining the specificity of the transferrin receptor in meningococci (22) indicated that mild periodate oxidation used under conditions reported to chemically modify the oligosaccharides of glycoproteins selectively (26) caused a drastic loss in the binding of hTf to its receptor. This finding prompted us to investigate the role of the hTf oligosaccharides in the binding of hTf to the bacterial hTf receptors further.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. meningitidis* B16B6, *N. gonorrhoeae* W003, and *H. influenzae* (serotype b) OA104 were grown on iron-sufficient and iron-deficient broth essentially as described previously (19, 21, 22).

Preparation of membranes. Crude membranes were prepared from iron-deficient cells as described by Schryvers and Morris (22).

Chemicals. Peroxidase-conjugated hTf was obtained from Jackson Immunoresearch Labs, Avondale, Pa. Iron-loaded and iron-free hTf, concanavalin A (ConA), horseradish peroxidase (HRP), methyl-α-D-mannopyranoside, and prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standard (27,000 to 180,000) mixture were obtained from Sigma Chemical Co., St. Louis, Mo. HRP color developing reagent was obtained from Bio-Rad Laboratories, Richmond, Calif. Periodic acid was obtained from Fisher Scientific Co., Fair Lawn, N.J. Tris hydrochloride, Tris base, and glycine were all purchased from Terochem Laboratories Ltd., Edmonton, Alberta, Canada. ConA-Sepharose was obtained from Pharmacia (Canada) Inc., Dorval, Quebec. Biotin reagent (biotin-NHS) was obtained from Pierce Chemical Co., Rockford, Ill. Glycopeptidase F and endoglycosidase F were obtained from Boehringer Mannheim Canada, Dorval, Quebec. Streptavidin-agarose was purchased from Bethesda Research Laboratories, Gaithersburg, Md.

Preparation of fully and partially deglycosylated hTf. Partially and fully deglycosylated forms of hTf were obtained by enzymatic treatment to remove the N-linked oligosaccharides followed by separation of the two forms by ConA-Sepharose chromatography. A 200-μl (10-U) portion of endoglycosidase F stock solution was added to 2 mg of hTf

or apo-hTf in 200 μl of 100 mM sodium phosphate buffer, pH 8.5, and the mixture was incubated at 37°C for approximately 4 to 5 days. After incubation, the buffer was exchanged by passing the sample through a gel filtration column (10DG, 10 ml; Bio-Rad) equilibrated with ConA buffer (50 mM sodium acetate, pH 6.9, containing 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$). This preparation of partially and fully deglycosylated hTf was applied to a column (1 by 5 cm) containing ConA-Sepharose equilibrated in ConA buffer and washed with 20 ml of the equilibrating buffer, followed by a 20-ml gradient of 0 to 0.2 M methyl-α-D-mannopyranoside in ConA buffer to elute off any bound hTf. All chromatographic steps were performed at room temperature.

Mild periodate oxidation of hTf. hTf was subjected to mild periodate oxidation, to modify the oligosaccharides, essentially as described previously (26), except that the reaction was stopped by the addition of glycine to 1% (wt/vol) and the reagents were removed by gel filtration on an acrylamide column followed by ultrafiltration with an Amicon Centriflo membrane cone (Amicon Corp., Danvers, Mass.).

Biotinylation of hTf. hTf was biotinylated as described previously by Schryvers and Morris (23) with few modifications. Commercially purchased iron-loaded or iron-free hTf or their derivatives were dissolved in 50 mM Tris hydrochloride, pH 7.5, buffer to a final concentration of 1 mg/ml. For every milliliter of hTf solution, 20 μl of a 12.5-mg/ml concentration of biotin-NHS in dimethylformamide was added. The reaction was stopped by the addition of 100 μl of a 10-mg/ml concentration of glycine to each 1-ml portion, and the mixture was incubated for an additional 2 h with agitation at 4°C. Upon completion of the reaction, the samples were applied to a gel filtration column to remove excess reagent and then concentrated by ultrafiltration with an Amicon Centriflo membrane cone and stored at 4°C.

Small-batch affinity isolation of receptor. Affinity isolation of receptor proteins was performed essentially as described previously (22). A 10-μg portion of biotinylated apo-hTf or biotinylated hTf derivatives was added to 1 mg of iron-deficient *N. meningitidis* B16B6 membranes in 1 ml of 50 mM Tris hydrochloride buffer, pH 8.0, containing 100 mM NaCl (low-salt buffer). After binding biotinylated ligand, the membranes were washed, solubilized, and mixed with streptavidin-agarose. The streptavidin-agarose was washed twice with high-salt buffer (50 mM Tris hydrochloride, pH 8.0, containing 1 M NaCl) and once with high-salt buffer containing 250 mM guanidine hydrochloride, followed by two washes with high- and low-salt buffers, respectively. The bound receptor proteins were then eluted in SDS-PAGE sample buffer and analyzed by SDS-PAGE.

Analytical methods. SDS-PAGE was performed routinely, using a discontinuous gel system with a Tris hydrochloride-glycine buffer system based on the method described by Laemmli (9). Silver staining of gels was performed as described previously (16).

Electroblotting onto Immobilon transfer membrane (Millipore Corp., Bedford, Mass.) was performed with a Bio-Rad Transblot apparatus at 90 V for 1 h or 15 V overnight in 25 mM Tris hydrochloride–0.2 M glycine (pH 8.3)–20% methanol. The Immobilon membrane was removed from the apparatus and developed with either amido black stain (17) to detect protein or ConA to detect oligosaccharide. The ConA binding assay was performed essentially as described previously by Hawkes (6) with the exception that our blocking solution consisted of 1.5% fish gelatin, pretreated by periodate oxidation (5), in 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl buffer.

Protein concentrations were determined by the method of Lowry et al. (11), with bovine serum albumin as the standard. Protein concentrations of whole-cell suspensions and outer membrane preparations used in the dot assay were performed by using the rapid method described previously by Rylatt and Parish (18), with bovine serum albumin as the standard.

Binding assays. Aliquots (2 μl) of a membrane suspension of iron-starved *N. meningitidis* B16B6, *N. gonorrhoeae* W003, or *H. influenzae* OA104 (0.5 mg/ml) were applied to nitrocellulose-cellulose acetate paper (0.45 μm, HA paper; Millipore Ltd.) and dried, and the paper was blocked by incubation at 37°C for 1 h in blocking solution. Competition binding assays were performed essentially as described previously (22), using mixtures of unconjugated hTf or its deglycoglylated derivatives and HRP-conjugated hTf.

Direct binding assays were performed as described previously (22) except that the initial binding mixture contained biotinylated forms of hTf rather than an HRP conjugate and that a second binding step with HRP-streptavidin was included. Serial twofold dilutions of biotinylated hTf derivatives were prepared in blocking solution starting with a concentration of 1.3 μg/ml (lanes A to G), with the control (lane H) containing none. After 30 min of incubation at 37°C, the paper was washed three times with Tris-buffered saline. This was followed by a second incubation with 200 ng of HRP-conjugated streptavidin per ml in blocking solution for another 30 min at 37°C. The paper was washed with Tris-buffered saline and then developed with HRP color developing reagent as described previously (22).

RESULTS

Preparation of hTf derivatives. To prepare hTf with the N-linked oligosaccharides removed, glycopeptidase F was selected since it removes the entire oligosaccharide chain (24). Preliminary experiments were performed with a commercial preparation of glycopeptidase F. Subsequent experiments were performed with a commercial preparation of endoglycosidase F, which differs from glycopeptidase F in that it cleaves oligosaccharides between the di-*N*-acetylchitobiose moiety of some asparagine-linked carbohydrate chains, mainly those that contain high content of mannose-type residues (24), resulting in a single monosaccharide unit remaining attached to the asparagine residue. The glycopeptidase F activity was strongly favored by the selection of a high pH. The commercial preparation of endoglycosidase F contained 100 to 150 U of glycopeptidase F for every 6 U of endoglycosidase F.

Initially, experiments were performed to determine the conditions that would yield the maximum amount of deglycosylated hTf, using the minimum amount of enzyme. Although preliminary experiments demonstrated that the addition of a reducing agent (2-mercaptoethanol) drastically enhanced enzymatic deglycosylation of hTf, particularly the production of fully deglycosylated hTf (f-hTf), we chose to use deglycosylation conditions without reducing agents to preserve the native structure of the hTf. Time course experiments (data not shown) demonstrated a stepwise removal of one oligosaccharide to give partially deglycosylated hTf (p-hTf), which occurred relatively rapidly, and a more gradual and incomplete removal of the second oligosaccharide to give f-hTf. After a few days of incubation, using moderate amounts of the enzyme, the sample mixture contained some p-hTf and some f-hTf, with no detectable native hTf remaining. Prolonged incubation periods did not decrease the approximate maximum 4:6 ratio of p-hTf/f-hTf

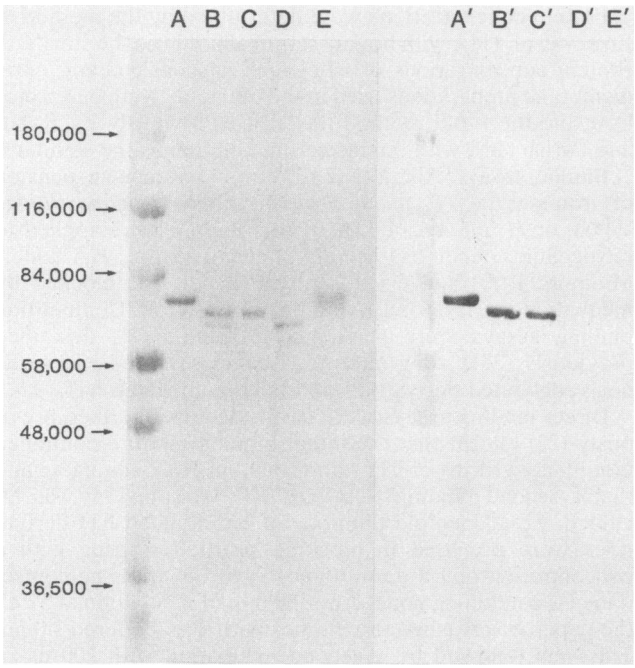

FIG. 1. Analysis of deglycosylated derivatives of hTf. SDS-PAGE analyses of enzymatically and chemically treated, iron-loaded hTf were done in duplicate. The polyacrylamide gel was electroblotted; one set was stained for protein with amido black (lanes A to E) and the other set was developed with ConA to detect oligosaccharides (lane A' to E'), as described in Materials and Methods. Lanes A and A', Control hTf sample without enzyme or chemical treatment. Lanes B and B', Glycopeptidase F-treated hTf prior to ConA-Sepharose affinity chromatography. Lanes C and C', Purified partially deglycosylated hTf. Lanes D and D', Purified fully deglycosylated hTf. Lanes E and E', Mild periodate-oxidized hTf.

obtained after a few days of incubation. Since these conditions resulted in a final reaction mixture containing both p-hTf and f-hTf, we utilized ConA-Sepharose affinity chromatography to separate these two forms of hTf.

To confirm that we had succeeded in removing or modifying the oligosaccharides, the samples were analyzed by SDS-PAGE and electroblotting followed by detection of protein and carbohydrate. Duplicate SDS-PAGE gels were run (Fig. 1) using native hTf (lanes A and A'), enzyme-treated sample before (lanes B and B') and after (lanes C and C' and D and D') ConA-Sepharose affinity chromatography, and periodate-treated hTf (lanes E and E'). The polyacrylamide gels were electroblotted, and one set was developed with amido black stain to detect protein (Fig. 1, lanes A to E) and the other set was developed with ConA to detect oligosaccharides (Fig. 1, lanes A' to E') as described in Materials and Methods. From the amido black stain blot, it is clear that incubation in the presence of the enzyme (lane B) led to the appearance of two new bands, with a loss of the native hTf band. The upper band in lane B is reactive with ConA (lane B'), which identified it as p-hTf. The lower band in lane B is not reactive with ConA (lane B'), which identified it as f-hTf. The sample mixture (lane B) was subjected to ConA-Sepharose affinity chromatography to isolate the p-hTf and the f-hTf derivatives. The pooled fractions from elution with methyl-α-D-mannopyranoside were shown to be p-hTf since the amido black stain showed a band of a lower molecular weight (lane C) than the native, and this band reacted with ConA (lane C'). The pooled fractions from the sample application and buffer wash were shown to be f-hTf since the amido black stain showed a band of even lower molecular weight (lane D) than p-hTf, and this band was not reactive with ConA (lane D'). A diffuse band of the periodate-oxidized hTf (po-hTf) stained with amido black stain (lane E) but only weakly bound ConA (lane E'), which confirmed that oligosaccharide degradation or modification had taken place.

For reasons elaborated below, preparations of deglycosylated apo-hTf were prepared for binding dot assays with apo-hTf. The same conditions were used and essentially the same results as presented in Fig. 1 were obtained. The affinity-isolated forms of partially and fully deglycosylated apo-hTf were used in all subsequent experiments.

Binding assays. To determine the effects of the oligosaccharide removal or modification on hTf binding, competition and direct binding experiments were performed. It should be noted that, in these experiments, derivatives of iron-free hTf (apo-hTf) were used for two reasons. First, periodate oxidation renders hTf incapable of binding iron (13). Second, during preliminary enzymatic deglycosylation reactions, it was observed that the iron-saturated hTf solution lost its typical rusty-brown color to become colorless like the apo-hTf solution, which suggested that hTf had lost its ability to bind iron. Therefore, all subsequent assays comparing the derivatives with the native were performed with apo-hTf so that the effects of oligosaccharide removal or modification could be determined independent of iron status.

Competition binding dot assays were performed in which apo-hTf or its derivatives were premixed with the HRP-hTf conjugate prior to exposure to filter-bound bacterial receptors in the membrane or whole-cell preparations, as described in Materials and Methods. Figure 2 (using iron-starved meningococcal membranes) illustrates that p-hTf and f-hTf forms were as capable as the unmodified apo-hTf of blocking the conjugated hTf from binding to the receptor. On the other hand, the po-hTf showed a drastic reduction in its ability to block binding.

Competition binding dot assays also were performed with membranes or whole cells from *H. influenzae* (serotype b) OA104 and gonococcal strain W003. The findings were identical to those illustrated in Fig. 2 (data not shown).

Direct binding dot assays were performed with membranes or whole cells of *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* (only data for *N. meningitidis* shown; Fig. 3). In these experiments, biotinylated forms of hTf and its derivatives were used. The results clearly demonstrated that the p-hTf and f-hTf derivatives were as capable of binding to the receptor as the native hTf, whereas the po-hTf showed a drastic reduction in binding.

Small-batch affinity isolation of receptor. Partially deglycosylated apo-hTf and fully deglycosylated apo-hTf were used in the batch affinity isolation of the meningococcal hTf receptor to determine whether deglycosylated biotinylated hTf derivatives could bind and isolate both receptor proteins, as does the native biotinylated hTf (23). Figure 4 illustrates that there was no difference in the ability of the three hTf forms to bind and isolate the hTf binding receptor proteins.

DISCUSSION

Transferrins play a dual role: they transport iron from sites of absorption and storage to sites where it is needed, and in the process they act to withhold iron from microbial agents, thereby inhibiting microbial growth in the host (2). While

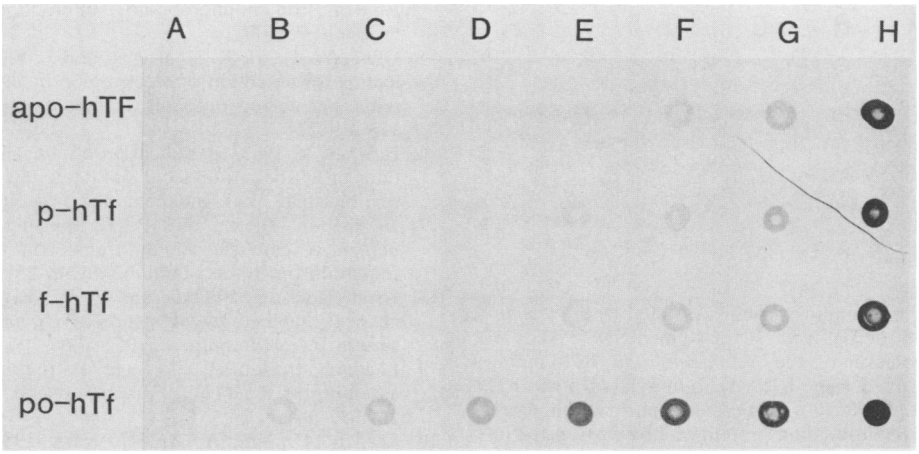

FIG. 2. Competition dot binding assay illustrating the effects of the hTf oligosaccharide removal or modification on hTf binding to bacterial receptors: 1 μg of iron-deficient membranes from *N. meningitidis* B16B6 spotted onto the filter was exposed to mixtures containing labeled HRP-hTf (0.16 μg/ml) and the following concentrations of the indicated unconjugated transferrins; A, 160 μg/ml; B, 80 μg/ml; C, 40 μg/ml; D, 20 μg/ml; E, 10 μg/ml; F, 5 μg/ml; G, 2.5 μg/ml; H, none. After incubation, the binding mixtures were removed, and the filter was washed and developed as described in Materials and Methods.

many bacteria produce siderophores for iron acquisition, *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* have developed specific hTf-binding membrane-bound protein receptors for the removal and uptake of iron from hTf. To date, no details are known regarding the mechanism by which the bacterial receptors are able to bind hTf and subsequently remove and take up iron.

As mentioned in Results, a commercial preparation of endoglycosidase F (containing a high amount of glycopeptidase F) was used in the deglycosylation of hTf. As a result, one potential complication of utilizing a commercial preparation containing the two enzymes with different specificities is that the deglycosylated forms of hTf may have a small proportion of molecules with a monosaccharide unit still attached to the protein. This possibility was limited by use of a high pH favoring glycopeptidase F.

The results in Fig. 1 confirm that we had successfully obtained a preparation of p-hTf (lanes C and C′) and f-hTf (lanes D and D′). The loss of one or both of the glycan chains of hTf results in the shift of the enzyme-treated hTf band(s) (lanes B, C, and D) as compared with the native hTf (lane A) due to a loss of up to about 5,000 daltons. These hTf oligosaccharides have a high content of mannose sugar residues, to which ConA binds with strong affinity and high specificity. Hence, the hTf derivative lacking the carbohydrate moieties, f-hTf (lower band in lane B′ and lane D′), did not bind to ConA.

The competition and direct binding experiments illustrated in Fig. 2 and 3, respectively, demonstrated that the differences in the binding ability of the native, p-hTf, and f-hTf appear to be negligible, whereas the po-hTf shows a great loss in binding the receptor. Although periodate preferentially oxidizes accessible vicinal hydroxyl groups which are present in oligosaccharide side chains, it is not completely specific even under the relatively mild conditions used in our experiments. There is evidence that periodate can target tyrosine residues in transferrin, rendering it incapable of binding to iron (3). This, combined with the fact that complete removal of both of the carbohydrate chains does not alter binding of hTf, strongly suggests that the loss in

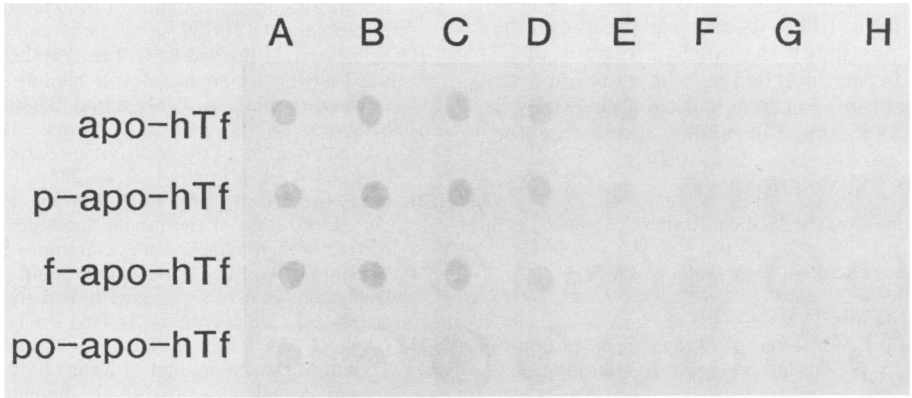

FIG. 3. Direct binding dot assay performed with biotinylated forms of apo-hTf and its derivatives. A 1-μg amount of iron-deficient membranes from *N. meningitidis* B16B6 spotted onto the paper was exposed to solutions containing the following concentrations of the indicated biotinylated transferrins: A, 1.2 μg/ml; B, 600 ng/ml; C, 300 ng/ml; D, 150 ng/ml; E, 75 ng/ml; F, 38 ng/ml; G, 19 ng/ml; H, none. After incubation, the binding solutions were removed, and the filter was washed and then exposed to a binding solution containing HRP-streptavidin. After the second incubation step, the binding solution was removed, and the filter was washed and developed as described in Materials and Methods.

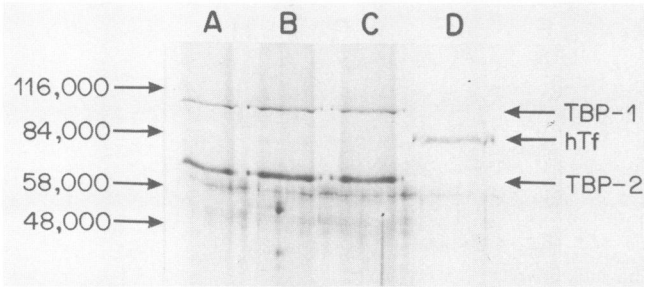

FIG. 4. Analysis of hTf-binding proteins isolated from *N. meningitidis* by hTf and its derivatives: silver-stained SDS-PAGE of small-batch affinity isolation of the *N. meningitidis* hTf-binding proteins, using biotinylated f-apo-hTf (A), biotinylated p-apo-hTf (B), and biotinylated apo-hTf (C). Lane D is control biotinylated apo-hTf. TBP-1, Transferrin-binding protein 1; TBP-2, transferrin-binding protein 2.

binding observed for po-hTf is not a result of oligosaccharide degradation but is a result of alteration in some protein structural component(s). From the results illustrated in Fig. 2 and 3 and from results with membranes or whole cells from *N. gonorrhoeae* and *H. influenzae* (data not shown), we conclude that the hTf oligosaccharides are not essential for the binding of hTf to its bacterial receptors.

Evidence to date shows that the *N. meningitidis* hTf receptor may consist of at least two proteins that were isolated by affinity purification by using biotinylated transferrin (19, 23). In *N. meningitidis* B16B6, these proteins include a high-molecular-weight 95-kilodalton protein (transferrin-binding protein 1) and a lower-molecular-weight 65-kilodalton protein (transferrin-binding protein 2). Since we do not know the relative contribution of these two proteins to the binding reaction observed with membranes or intact cells, we could not be certain that removal or alteration of the oligosaccharides would effect binding to one of the proteins. Our results (Fig. 4) clearly indicate that the partially and fully deglycosylated apo-hTf forms are able to isolate transferrin-binding proteins 1 (lanes B and C; upper band) and 2 (lanes B and C; lower band) to the same degree as does the native apo-hTf (lane A). Thus, the hTf oligosaccharides do not appear to play a role in binding to either of the two bacterial receptor proteins.

While our findings indicate that carbohydrate chains are not required for binding of hTf to its bacterial receptors, they do not exclude the possibility that surface regions of hTf where oligosaccharides are attached may be quite important in some other aspect(s). Further work is necessary to identify regions of hTf involved in binding to the receptor.

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