Transferrin-binding protein B isolated from *Neisseria meningitidis* discriminates between apo and diferric human transferrin

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Neisseria meningitidis utilization of human serum transferrin (hTF)-bound iron is an important pathogenicity determinant. The efficiency of this system would clearly be increased through preferential binding of diferric hTF over the iron-free form. To characterize this process, functionally active meningococcal transferrin-binding protein A (TbpA) and TbpB have been purified from *N. meningitidis* using a novel purification procedure. The association of isolated Tbps and Tbps in the presence of hTF was investigated by gel filtration. Co-purified TbpA + B formed a complex of molecular mass 300 kDa which bound 1–2 molecules of hTF. Purified TbpA formed a complex of 200 kDa, indicating association as a dimer, whereas TbpB aggregated to form multimers of variable sizes. On recombining TbpA and TbpB, a stable complex of equivalent size to co-purified TbpA + B was formed. This complex may be composed of a single TbpA dimer and 1 molecule of TbpB. The technique of surface plasmon resonance (SPR) was used to demonstrate

clearly that TbpB of either high (85 kDa) or low (68 kDa) molecular-mass preferentially bound diferric hTF in comparison with iron-free hTF. This selectivity was not observed with TbpA, but was found at low levels with co-purified TbpA + B. Individual TbpA and TbpB, recombined in a 1:1 molecular ratio, showed iron-mediated discriminatory binding at an intermediate level. SPR was also used to show that TbpA and TbpB bound to distinct regions of hTF, and that prior saturation with TbpB reduced subsequent TbpA binding. The results demonstrated that hTF bound more TbpA than TbpB, with an approximate ratio of 2:1. We have demonstrated that *in vitro*, TbpA + B exists as a receptor complex composed of a TbpA dimer and one molecule of TbpB, and that TbpB selectively binds diferric hTF. We propose that, *in vivo*, TbpA and TbpB also exist as a receptor complex, with TbpB selectively binding diferric hTF, bringing it close to TbpA, the transmembrane component, where the ferric iron can be transported to the periplasm.

INTRODUCTION

Iron is an essential nutrient for almost all microorganisms [1]. However, free iron is rare in the human body, generally being complexed by a number of high-affinity binding proteins, including the homologous glycoproteins transferrin (hTF), in plasma, and lactoferrin (hLF), in secretions and milk [2]. Both hTF and hLF have a molecular mass of approx. 80 kDa and are composed of two homologous lobes each subdivided into two domains. These regions enclose a site capable of binding a single ferric ion in synergy with an anion, usually carbonate. Thus, hTF and hLF are each able to bind two ferric ions, although *in vivo*, serum transferrin iron-saturation rarely exceeds 30 % [3].

Neisseria meningitidis has a siderophore-independent iron-uptake system which requires direct binding of host iron-containing proteins [4]. Sequestration of hTF-bound iron is reliant upon two iron-repressible transferrin-binding proteins: TbpA (formerly Tbp1) of molecular mass about 100 kDa and TbpB (formerly Tbp2) of molecular mass 68–85 kDa, depending on strain [4]. TbpA is highly conserved between meningococcal strains and is thought to be a porin-like integral membrane protein, whereas TbpB is more variable in size, lipidated and fully surface exposed [4,5]. The study of meningococcal and

gonococcal mutants expressing either TbpA or TbpB has shown that both proteins are required for the optimal uptake of hTF iron [5,6]. The conservation of two structurally distinct proteins, both capable of independently binding hTF, implies that they play different but related roles. The requirement for both Tbps further suggests that these proteins may interact, forming the functional meningococcal hTF receptor. Transferrin binding by the Neisseriaceae shows distinct species specificity, and as meningococcal disease is unique to man, utilization of hTF-bound iron is likely to be an important pathogenicity determinant [7].

Characterization of the interaction between meningococcal Tbps and hTF is fundamental in understanding this pathogenicity process. We have previously demonstrated that TbpA and TbpB form a complex in solution using photon correlation spectroscopy [8], but characterization of hTF binding by Tbps *in vitro* has previously not been possible, due to difficulties in purifying functionally active proteins. In this study, we demonstrate a novel method for the purification of these proteins, which maintains their ability to bind hTF, and we have characterized the association between Tbps and hTF using gel filtration and surface plasmon resonance (SPR) biosensors. The efficiency of hTF iron scavenging would clearly be increased through pref-

Abbreviations used: hLF, human lactoferrin; hTF, human serum transferrin; HRP, horseradish peroxidase; SPR, surface plasmon resonance; TbpA, meningococcal transferrin-binding protein A; TbpB, meningococcal transferrin-binding protein B; TbpA + B, co-purified transferrin-binding proteins A + B.

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erential binding of iron-loaded hTF over the iron-free apo form. We have therefore examined the effects of iron-saturation on the binding of hTF to purified meningococcal TbpA, TbpB and co-purified transferrin-binding proteins A + B (TbpA + B).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Group B *N. meningitidis* strains SD [9] and B16B6 were grown under iron-limited conditions as described previously [10]. Cells were sub-cultured on to agar plates containing 5% (v/v) horse blood and grown overnight at 37 °C in an atmosphere of 5% CO₂/95% air. Colonies from these plates were used to inoculate 100 ml aliquots of Mueller Hinton broth in 250 ml conical flasks which were incubated with shaking at 37 °C for 6 h. A 5 ml volume of this culture was introduced into 750 ml of Mueller Hinton broth in 2-litre conical flasks, made iron-limited by the addition of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (5 µg/ml) and incubated with shaking at 37 °C for 18 h.

Purification of the TbpA + B

TbpA + B was solubilized from iron-limited *N. meningitidis* whole cells using Elugent detergent (Calbiochem) and hTF–Sepharose affinity chromatography [10]. Extracts were prepared from strains SD and B16B6, which express high- and low-molecular-mass forms of TbpB respectively. The purity of TbpA + B in each preparation was assessed by SDS/PAGE [11] and hTF-binding ability was assessed by dot blotting, probed using hTF–horse-radish peroxidase (hTF–HRP).

Separation of TbpA and TbpB by ion-exchange chromatography

TbpA and TbpB were separated by ion-exchange chromatography on a Mono S HR 5/5 column (Pharmacia Biotechnology) using an FPLC system (Pharmacia Biotechnology). Strain-specific conditions were required for the purification of TbpA and TbpB from co-purified mixtures. In the case of strain SD, optimal separation was achieved using 50 mM succinic acid, pH 4.5, +1% (v/v) Elugent with an NaCl gradient of 0.15–0.5 M. For strain B16B6, 50 mM Mes, pH 5.0, +1% (v/v) Elugent with a gradient of 0.1–0.4 M NaCl was used.

Purification of hTF

A crude protein extract was prepared by ammonium sulphate precipitation of human serum. hTF was purified by ion-exchange chromatography involving a Q Sepharose FF HiLoad 16/10 column (Pharmacia Biotechnology) on FPLC and subsequent gel filtration using an S200 gel-filtration column (Pharmacia Biotechnology) [12]. Bound iron was removed from purified hTF by overnight dialysis against multiple changes of 0.1 M sodium citrate/citric acid buffer (pH 4.7) [13].

Gel filtration

The molecular size of purified Tbp in the presence and absence of hTF was estimated by gel filtration on a Superose 12 column (Pharmacia Biotechnology) in PBS, pH 7.4, +0.3% (v/v) Elugent, at a flow rate of 0.5 ml/min. The column was calibrated using low- and high-molecular-mass gel-filtration calibration kits (Pharmacia Biotechnology).

SPR

The stoichiometry of complex formation and the effects of hTF iron loading on Tbp–hTF binding were investigated by SPR

using BIAcore X and BIAcore 2000 biosensors (BIAcore). hTF was immobilized on a CM5 sensor chip (BIAcore) by covalent linkage between activated carboxyl groups on the chip matrix and lysine residues in the ligand protein. Immobilized hTF was saturated with iron by exposure to 20 µl of 25 mM FeSO₄ diluted 1:100 in 15 mM ammonium bicarbonate buffer, pH 8.0, at a flow rate of 5 µl/min. Analyte proteins (Tbps) were eluted from immobilized hTF using 20 µl of Gentle Ab/Ag Elution bufferTM, pH 6.5 (Pierce). Residual iron was then removed using a 10 µl wash of 20 mM glycine, pH 2.0. This treatment reduced the resonance response to its original level and did not inhibit subsequent hTF–Tbp binding. Immobilized hTF was exposed to 20 µl aliquots of purified TbpA, TbpB and co-purified TbpA + B at concentrations of 20 µg/ml and at a flow rate of 10 µl/min. These aliquots were injected into a continuous flow of 0.01 M Hepes buffered saline, pH 7.4, containing 160 mM NaCl, 3.4 mM EDTA and 0.005% (v/v) P20 surfactant (BIAcore) and the increase in resonance response was noted. For iron discrimination studies two parallel flow cells were coated with approx. 4.5 ng of apo hTF. One cell in each pair was exposed to 20 µl of 15 mM ammonium bicarbonate buffer, pH 8.0, while the other was washed with the same volume of iron-saturation buffer. A 30 µl volume of approximately 300 µg/ml TbpA, TbpB or TbpA + B from strains SD and B16B6 was injected over each surface in consecutive experiments and the resonance response noted. Injection of each Tbp was followed by surface regeneration and a low-pH wash of each cell in order to remove residual iron from the immobilized hTF. In each case, the Tbp solution was simultaneously injected over both cells, allowing direct comparison of results.

RESULTS

Purification of TbpA and TbpB

TbpA + B were co-purified by affinity chromatography at concentrations up to 1 mg/ml. Proteins were separated using the strain-specific conditions as described, with TbpB present in the initial fractions and TbpA eluted during the ionic gradient. The purity of TbpA and TbpB from both meningococcal strains was confirmed using SDS/PAGE (Figure 1). Both proteins maintained their ability to bind hTF, as determined by dot blotting (Figures 2a and 2b). Dot blots prepared using equal protein concentration of TbpA and TbpB indicated that TbpA bound less hTF–HRP than TbpB (Figure 2a). Equivalent blots in which the concentration of TbpA was twice that of TbpB bound comparable amounts of hTF–HRP (Figure 2b).

Figure 1 Composite SDS/PAGE of co-purified TbpA + B, TbpA and TbpB separated by ion-exchange chromatography (*N. meningitidis* strains SD and B16B6) and hTF purified from human serum

Lanes, numbered from the left, were: 1, co-purified TbpA + B (strain SD); 2, TbpA (strain SD); 3, TbpB (strain SD); 4, co-purified TbpA + B (strain B16B6); 5, TbpA (strain B16B6); 6, TbpB (strain B16B6); 7, hTF.

Figure 2 Assessment of relative hTF-binding ability of purified TbpA and TbpB by dot blotting

(a) Dot blots of purified TbpA and TbpB at equal protein concentration, and (b) 2:1 protein concentration probed with hTF-HRP. The protein content of each Tbp sample was estimated using a GENEQUANT spectrophotometer (Pharmacia Biotechnology).

Table 1 Molecular mass of proteins determined by gel filtration on Superose 12

Protein	Apparent molecular mass (kDa)	Apparent molecular mass in the presence of hTF (kDa)
hTF	80	—
TbpA	200	300
TbpB	400–600	400–600
TbpA + B	300	400–500
TbpA + B (R)*	300	400–500

* Recombined individual TbpA and TbpB.

Characterization of co-purified Tbp A + B complex, individual TbpA and TbpB and their complexes with hTF

Purified TbpA had an apparent molecular mass of approx. 200 kDa, as determined by gel-filtration (Table 1), indicating that it may form a dimer in solution. In the presence of hTF, TbpA eluted as a complex of approx. 300 kDa (Table 1). Purified TbpB, in both the absence and presence of hTF, appeared to form multimers of variable molecular mass as it eluted as a broad band over the molecular-mass range 400–600 kDa (Table 1). Consequently, the hTF-TbpB complex was difficult to demonstrate on gel filtration and no stoichiometric estimation was attempted. Affinity-purified TbpA + B had an apparent molecular mass of approx. 300 kDa (Table 1), and a complex of comparable size was observed following the recombination of separate TbpA and TbpB.

The above analyses were carried out using TbpA and TbpB derived from *N. meningitidis* strains SD and B16B6, which express high- and low-molecular-mass forms of TbpB respectively, and results were consistent irrespective of strain.

Table 2 Mean saturation data and implied stoichiometry of diferric hTF-Tbp interactions (strain B16B6)

Binding ratio refers to the implied number of TbpA or TbpB molecules binding to immobilized hTF. Quantification of these interactions was carried out by reference to previous calibration studies (see Materials and methods section, SPR). Values of standard deviation are given in parentheses; $n = 3$.

Saturation sequence	Binding ratio TbpA:hTF	Binding ratio TbpB:hTF	Total Tbp binding (%)
TbpA followed by TbpB	1.5 (0.2)	0.4 (0.1)	100
TbpB followed by TbpA	1.9 (0.3)	0.4 (0.1)	72

Stoichiometry of diferric hTF-Tbp interactions

Using the technique of SPR, it was observed that hTF immobilized on the dextran matrix sensor surface was able to bind TbpA, TbpB and TbpA + B. Bound Tbps could be eluted from the surface at near neutral pH, with restoration of the resonance response to baseline levels and without reduction of the level of subsequent binding. In a control experiment, immobilized duck ovo-transferrin bound neither individual nor co-purified Tbps, suggesting that the specificity of meningococcal hTF binding is maintained under these conditions. Separate flow cells were coated with hTF at levels ranging from 3.48 ng to 10.41 ng and were used in consecutive duplicate experiments. hTF on each surface was iron loaded and exposed to sequential injections of TbpA until no further significant binding increases were observed. The surface was then exposed to TbpB and injections were again repeated until saturation was achieved. The hTF surface was regenerated, loaded with iron and the baseline re-established at the original level. Tbp injections were then repeated, but the order of interaction was reversed, the surface being initially saturated with TbpB and subsequently with TbpA. The stoichiometry of these interactions was then calculated, based on a linear relationship between resonance response (measured in resonance units) and the weight of bound protein [14]. In duplicate analyses, the order of initial saturation was again reversed to determine the effect of intervening surface regeneration on Tbp-binding levels.

Saturation of immobilized hTF with TbpA had little inhibitory effect on subsequent TbpB binding. In contrast, TbpA binding was reduced following prior exposure of hTF to TbpB, as was the overall level of Tbp association (Table 2). Resonance data indicated that hTF binds more TbpA than TbpB, with a ratio of approx. 2:1, irrespective of saturation order, hTF immobilization level and repeated surface regeneration (Tables 2 and 3).

In an additional procedure, approximately 5.12 ng of hTF, corresponding to 3.84×10^{10} molecules was immobilized. This surface was iron-loaded and exposed to sequential additions of co-purified TbpA + B (strain B16B6). At the point where no further resonance increases were observed, approximately 7.91 ng of the TbpA + B preparation had been bound. Assuming that the native meningococcal hTF receptor is composed of a TbpA dimer and a single molecule of TbpB, this quantity of protein is the equivalent of approximately 1.79×10^{10} intact receptors. This indicated that TbpA + B bound hTF with an apparent stoichiometry of 1:2 (Table 3). Similar calculations carried out following sequential saturation by purified TbpA and TbpB yielded results consistent with this hypothesis, irrespective of saturation order and hTF immobilization level (Table 3).

Following Tbp saturation of immobilized hTF, 20 μ l of 250 μ g/ml diferric hTF was injected over the chip surface. No

Table 3 Molecular ratio of holo hTF–Tbp binding at saturation levels (Strain B16B6)

In each case, the second saturations were performed following regeneration of the original hTF surface. The implied number of TbpA + B complexes bound by the surface was calculated assuming a molecular mass of 265 kDa.

Saturation protocol	Molecules of hTF ($\times 10^{10}$)	Equivalent TbpA + B complexes ($\times 10^{10}$)	Ratio (TbpA + B:hTF)
TbpA followed by TbpB	2.61	1.63	1.60
	5.06	3.87	1.31
	7.81	5.66	1.38
TbpB followed by TbpA	2.61	1.12	2.31
	5.06	3.08	1.64
	7.81	3.98	1.96
TbpA + B (co-purified)	3.48	1.79	1.94

Figure 3 Binding of Tbps to apo hTF expressed as a percentage of binding to diferric hTF determined by SPR

■, B16B6; □, SD. Bars indicate ± 1 standard deviation; $n = 4$.

additional binding was observed, suggesting that the meningococcal TbpA + B complex had no secondary accessible hTF-binding sites.

Ability of TbpA + B and individual TbpA and TbpB to discriminate between diferric and apo hTF

Iron discrimination was determined by SPR on parallel flow cell surfaces coated with hTF in the apo and diferric forms. Exposure of immobilized hTF to iron resulted in greatly enhanced binding of TbpB from strains SD and B16B6. TbpB from strain B16B6 showed lower levels of iron-mediated discriminatory binding than the equivalent protein from strain SD. Iron-saturation of immobilized hTF resulted in a slight increase in binding of TbpA and co-purified TbpA + B (Figure 3). Approximately equal amounts of separated TbpA and TbpB were combined and showed discriminatory binding, intermediate between co-purified TbpA + B and TbpB (Figure 3).

DISCUSSION

The interaction between isolated meningococcal Tbps and hTF has been quantitatively characterized for the first time. This has been possible for individual TbpA and TbpB due to the development of a novel purification procedure which preserved the

hTF-binding properties of both Tbps, in contrast with previously reported methods [15]. TbpA, TbpB and TbpA + B each bound hTF in solid- and liquid-phase assays. Dot blots, probed with hTF–HRP showed that TbpA bound less hTF than TbpB (w/w). Comparable dot blots, in which the concentration of TbpA was twice that of TbpB, bound similar levels of hTF–HRP. This suggested that TbpA forms a dimer with a single accessible hTF-binding site and that monomeric TbpB binds hTF with a 1:1 ratio. This was further supported by gel-filtration results which indicated that purified TbpA exists as a dimer with molecular mass of approx. 200 kDa. TbpB formed higher molecular-mass multimers of approx. 400–600 kDa, which were absent from the co-purified TbpA + B complex or recombinant Tbp mixtures. The variation in size exhibited by these aggregations suggests a non-specific association, possibly due to the potential adhesiveness of lipidated TbpB. Co-purified TbpA + B produced a single complex with molecular mass of approx. 300 kDa. A species of similar size was demonstrated upon recombination of individual TbpA and TbpB, indicating a specific interaction and stoichiometry between these proteins. The addition of hTF resulted in alterations in elution volume consistent with the association of 1–2 molecules of hTF with the putative TbpA + B complex. Separation by ion-exchange chromatography may reduce the hTF-binding capabilities of TbpA. However, TbpA purified in this way was shown to bind hTF in solid-phase assays. The possibility of reduced TbpA–hTF binding could be examined further using individual TbpA, affinity purified from mutant strains lacking TbpB, thus avoiding the ion-exchange procedure. These results are consistent with the results of previous fluid-phase analyses carried out using photon correlation spectroscopy [8].

Saturation analysis using SPR allowed estimation of the molecular stoichiometry involved in Tbp–hTF interactions. It was demonstrated that immobilized diferric hTF bound more TbpA than TbpB, with an approximate ratio of 2:1. Prior saturation with TbpA did not appreciably inhibit the subsequent association of TbpB, whereas reversing the order of saturation (TbpB followed by TbpA) partially inhibited subsequent binding and reduced overall Tbp association. In each situation, the inferred stoichiometry was consistent with gel-filtration and dot-blot data, with hTF binding more TbpA than TbpB. The consecutive binding of TbpA and TbpB, irrespective of the injection order, strongly suggests that these proteins recognize independent sites on hTF. Partial binding inhibition may have resulted from the formation of non-specific TbpB clusters, although such large aggregations would have been apparent in SPR data. In the absence of such findings it is suggested that TbpB reduces TbpA binding, possibly through the partial occlusion of hTF regions involved in this interaction. Recent data on the gonococcal hTF receptor indicated that the putative TbpA + B complex adopts at least two conformations and that the physical characteristics of TbpB are influenced by the presence or absence of TbpA [6]. Thus TbpB may exhibit differing properties when independently interacting with hTF, possibly preventing full TbpA saturation. Comparable amounts of co-purified TbpA + B were bound per immobilized hTF, suggesting that molecular stoichiometry is consistent, irrespective of whether co-purified or individual TbpA and TbpB are used for saturation. Calculation of binding ratios, based on the assumption of a 2 TbpA:1 TbpB stoichiometry, again suggested that up to two molecules of hTF are bound by each putative receptor complex.

We have used the technique of SPR to clearly demonstrate that purified TbpB preferentially bound diferric hTF in comparison with apo hTF. This was observed in high- and low-molecular-mass forms of TbpB purified from *N. meningitidis* strains SD and B16B6. Similar results have been demonstrated

using gonococcal mutants lacking TbpA in whole-cell binding assays [6,16]. The strain-determined differences in iron-mediated binding enhancement shown by TbpB may indicate some difference in the behaviour of these proteins, consistent with their heterogeneous molecular mass. Discriminatory binding was absent from TbpA but could be demonstrated at lower levels in co-purified TbpA + B. TbpA and TbpB, recombined at a 1:1 molecular ratio, showed intermediate discrimination between TbpB and co-purified TbpA + B. This indicated that the recombined mixture was functionally dominated by TbpB, with increased iron-discriminatory hTF binding, indicating a numerical excess of TbpB in the 1:1 recombined mixture compared with co-purified TbpA + B. This is consistent with the hypothesis that co-purified TbpA + B form a complex with a ratio of 2:1, composed of a TbpA dimer in association with a single molecule of TbpB. Several outer-membrane proteins, of the TonB-dependent receptor porin type, have been shown to form dimers, trimers and multimers [17,18]. The possibility of TbpA forming a dimer in a similar way has recently been discussed by Pajon et al. [19]. The formation of multimeric complexes has also been suggested as a way to increase the surface area in which collisions with solutes can lead to uptake [18]. Iron is removed at the cell surface as there is no internalization of hTF [4], therefore the mechanism by which, and the molecular form in which, the ferric ion is carried across the membrane remains to be established. Recent topology modelling of TbpA has attempted to address these issues [19]. It seems probable that TbpA and TbpB interact to form the functional hTF-binding complex. As such, it is possible that disruption of this complex could alter the binding characteristics of one or both of its components. In addition, these analyses were carried out in a cell-free system, consequently the energizing TonB protein normally associated with TbpA was absent [4]. This factor could significantly alter the characteristics of TbpA and the putative TbpA + B complex *in vitro* and should be taken into consideration when speculating on the function of these proteins.

In conclusion, it can be inferred from our results that the meningococcal hTF receptor is composed of a TbpA dimer in association with a single molecule of TbpB. This complex binds 1–2 molecules of hTF, with TbpB preferentially binding the diferric form of this protein. We have also determined that TbpA and TbpB bind to distinct separate sites on the hTF molecule.

These results are consistent with the theory that, *in vivo*, TbpB scans the environment, selectively binding diferric hTF and bringing it close to TbpA. TbpA may then bind hTF and

facilitate the removal of iron in an energy-dependent manner involving the TonB system. Ultimately, ferric iron uptake is likely to occur by virtue of a negatively charged transmembrane sequence found in TbpA [4]. hTF, now in the apo form, is bound with reduced affinity and dissociates or is displaced from the putative TbpA + B complex. Further work is in progress to characterize recognition sites for hTF on TbpA and TbpB.

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