Purified meningococcal transferrin-binding protein B interacts with a secondary, strain-specific, binding site in the N-terminal lobe of human transferrin

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INTRODUCTION

Iron is an essential nutrient for bacterial growth and has a critical role in many biochemical processes [1]. Human serum is rendered bacteriostatic by the high-affinity iron-binding protein transferrin (hTF); lactoferrin performs a similar function in milk and other secretions [2]. These homologous proteins are monomeric with a molecular mass of approx. 80 kDa and comprise an N-terminal lobe (residues 1–333 in hTF) and a C-terminal lobe (residues 342–679 in hTF) linked by a short inter-domain bridge (residues 334–341 in hTF). The N- and C-terminal lobes show distinct homology in terms of their sequence, structure and function, each being further subdivided into two domains that enclose a site capable of binding a single molecule of ferric iron in coordination with an anion, usually carbonate in vivo [3].

The ability to sequester iron from the host is essential for invasive pathogenic bacteria such as Neisseria meningitidis, and is likely to confer an ecological advantage on such species [4]. The pathogenic Neisseriaceae have a siderophore-independent iron uptake system reliant on a direct interaction between the bacterial cell and hTF [5]. In the meningococcus this system shows a high degree of specificity for hTF and is dependent on two surface-exposed transferrin-binding proteins (TbpA and TbpB), both of which are essential for the optimal uptake of iron from this source [6]. We have previously described a procedure for the purification of functionally active TbpA and TbpB capable of binding hTF and by using the technique of surface plasmon resonance (SPR) were able to show that TbpA and TbpB exist as a 2:1 complex in vitro and that TbpB discriminates between apo-hTF and diferric hTF [7].

The region on hTF involved in binding to the Tbp had previously been localized solely to the hTF C-terminal lobe [8]. However, these studies involved proteolytically derived hTF fragments and, by definition, the effect of inter-lobe and inter-domain interactions, known to influence the conformation of hTF, would not be apparent in such studies [3,9,10]. In addition, proteolysis might not produce entirely consistent degradation products, rendering the resultant results open to further question. However, by using chimaeric transferrins constructed from hTF and bovine transferrin (bTF), which shares 70% amino acid identity with hTF but is not bound by meningococcal Tbps, it was confirmed that the primary binding site to co-purified transferrin-binding proteins A and B (TbpA + B) was located within the hTF C-terminal lobe [11]. These chimaeric transferrins (hbTF-1, 2, 3, 4 and 7; Figure 1) were expressed in Spodoptera frugiperda and have the same iron-binding properties and molecular masses as native hTF. In this study we have used these chimaeras to quantify the level of interaction with Tbps by SPR, using this information to determine the hTF regions required for binding by isolated TbpA and TbpB. In addition, the effect of the

Abbreviations used: bTF, bovine serum transferrin; hbTF, chimaeric human–bovine serum transferrin; hTF, human serum transferrin; SPR, surface plasmon resonance; Tbp, meningococcal transferrin-binding protein; TbpA + B, co-purified transferrin-binding proteins A and B.

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iron status of hbTF on Tbp binding has been established. The results indicate distinct differences between the behaviours of isolated and co-purified Tbps, strongly suggesting both association and possible functional co-operativity between these proteins. TbpA from different N. meningitidis strains is conserved [12], whereas TbpB shows considerable variation [13,14]; in this study we identify a secondary TbpB-binding region on hTF that is dependent on the meningococcal strain. The implications of these results for pathogenesis are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Group B N. meningitidis strains SD and B16B6 were cultured under iron-limited growth conditions, as described previously [7].

Purification of TbpA + B and TbpA and TbpB

TbpA + B and TbpA and TbpB were purified by hTF–Sepharose-affinity chromatography and ion-exchange chromatography, by the method described previously [7].

Production of chimaeric transferrins

Chimaeric transferrins were expressed in Spodoptera frugiperda and were previously characterized by Retzer et al. [11] (Figure 1). Protein concentrations were estimated throughout with a Genequant spectrophotometer (Pharmacia Biotechnology).

SPR

The capacity of isolated TbpA, TbpB and co-purified TbpA + B to bind hTF and hbTF chimaeras was investigated by SPR with a BIAcore X biosensor (BIAcore). Samples of hTF and individual hbTF chimaeras were prepared in 10 mM acetate buffer, pH 3.0, and immobilized by amine coupling on the separate, parallel surfaces of a single CM5 sensor chip (BIAcore). Experimental conditions were as described previously [7]. Amine coupling occurs through lysine residues distributed throughout the N- and C-terminal lobes of hTF and bTF. Immobilization is therefore likely to produce a heterogeneous population in the highly permeable pseudo-gel matrix. The ‘aqueous’ nature of this matrix minimizes the steric inhibition of interactions between ligand (hTF or hbTF) and injected analyte (Tbps). In addition, the extensive similarity between hTF and bTF (69.5% identity at the amino acid level) indicates that immobilization would have similar effects on chimaeric proteins and hTF, with regard to both Tbp association and iron loading. SPR calibration studies on a number of different proteins, including transferrin, have established the near-linear relationship between immobilized protein and resonance response units [15]. The amount of each protein immobilized could then be estimated because an increase of 1000 resonance units corresponds to 1 ng of immobilized protein [15].

Immobilization levels were carefully controlled by altering the concentration of injected ligand and the degree of chip surface activation, thus ensuring that comparable amounts of protein were immobilized on each pair of surfaces. Immobilized hTF and hbTF were saturated with iron by exposure of the apoprotein to 20 μl of freshly prepared 25 mM FeSO₄ diluted 1:100 in 15 mM ammonium bicarbonate buffer, pH 8.0, at a flow rate of 5 μl/min. Immobilized hTF and hbTF in their apo and diferric states were exposed to 10 μl samples containing approx. 30 μg/ml TbpA + B and approx. 50 μg/ml TbpA and TbpB, purified from N. meningitidis strains SD and B16B6. Individual Tbp samples were injected simultaneously over both surfaces in a continuous flow of 0.01 M Heps-buffered saline, pH 7.4, containing 160 mM NaCl, 3.4 mM EDTA and 0.005% (v/v) P2O surfactant (BIAcore). Bound Tbps were eluted from immobilized hTF with 25 μl of Gentle Ab/Ag Elution buffer™, pH 6.5 (Pierce). Residual hTF-iron was then removed by being washed with 25 μl of 20 mM glycine, pH 2.0. This treatment typically decreased the resonance response to its original level, corresponding to the baseline achieved after immobilization of hTF/hbTF and did not inhibit subsequent hTF–Tbp binding. Each interaction experiment was performed a minimum of four times.

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Figure 1 Schematic diagram of hbTFs

The hbTFs are shown as bars representing linear amino acid sequences of hTF (filled) and bTF (open). Domains and lobes are indicated at the top of the diagram. hbTF-2: human N-lobe, human bridge region, 70% human C-lobe (spliced between residues 597 and 598). hbTF-3: 80% human N-lobe, 20% bovine N-lobe, bovine bridge region, human C-lobe (splice sites, residues 254/255 and 350/351). hbTF-7: bovine N-lobe and bridge domain, human C-lobe (spliced between residues 350 and 351). hbTF-1: human N-lobe, bovine bridge domain and C-lobe (spliced between residues 344 and 345). hbTF-4: 80% human N-lobe, 20% bovine N-lobe, bovine bridge domain and C-lobe (spliced between residues 254 and 255).
RESULTS

Tbp binding to transferrins

TbpA, TbpB and TbpA+B bound to recombinant diferric bTF at a residual level, typically less than 10% of that observed with diferric hTF; no significant strain-determined variation was evident with Tbps isolated from strains SD and B16B6. Binding of TbpB from strains SD and B16B6 to hTF, bTF or any chimera in their apo form was similar to that observed for diferric bTF (results not shown).

Tbp binding to hbTF-2

The replacement of the hTF C-terminus (residues 598–679) by their bovine equivalent decreased binding by purified TbpA and TbpB, but had less impact on binding by co-purified TbpA+B (Figure 2). The association between apo-hbTF-2 and co-purified TbpA+B (SD and B16B6) was statistically indistinguishable from binding to apo-hTF, although saturation with iron rendered such differences significant in strain SD (P = 0.050). Individual TbpA and TbpB from either strain bound hbTF-2 at significantly lower levels than hTF, irrespective of iron saturation (0.005 ≤ P ≤ 0.050). The difference between the binding of hTF and hbTF-2 by TbpA (B16B6) increased 3-fold after saturation with iron (P = 0.025), but no similar trend was demonstrated by TbpA purified from strain SD (Figure 2). TbpA (B16B6) bound diferric hTF at a higher level than diferric hbTF-2. However, TbpA (B16B6) bound apo-hbTF-2 at higher levels than the diferric form of this chimera, in contrast with all previous and subsequent Tbp-binding events analysed in this study. This finding strongly implied that saturation with iron significantly altered regions of hbTF-2 recognized specifically by TbpA (B16B6) and rendered this chimera distinct from hTF in iron-replete conditions. Furthermore, these results clearly demonstrate previously unreported, strain-dependent, differences in the behaviour of TbpA, dependent on conditions of saturation with iron. This suggests that TbpA from N. meningitidis strains SD and B16B6 might recognize wholly or partly distinctive regions of hTF that are influenced by the iron-mediated conformational changes in this protein.

Tbp binding to hbTF-3

The bovine sequence present in hbTF-3 (residues 255–350) does not affect binding to TbpB (B16B6), whereas binding by TbpB (SD) was decreased (Figure 3). This difference was statistically

Figure 2 Binding of Tbps to apo and diferric hbTF-2 expressed as a percentage of binding to apo-hTF and diferric hTF determined by SPR

See Figure 1 for the structure of hbTF-2. Filled bars, strain SD; open bars, strain B16B6. Error bars represent ± 1 S.D.

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

See Figure 1 for the structure of hbTF-3. Filled bars, strain SD; open bars, strain B16B6. Error bars represent ± 1 S.D.
Figure 4 Binding of Tbps to apo and diferric hbTF-7 expressed as a percentage of binding to apo-hTF and diferric hTF determined by SPR

See Figure 1 for the structure of hbTF-7. Filled bars, strain SD; open bars, strain B16B6. Error bars represent ± 1 S.D.

Tbp binding to hbTF-7

The capacity of Tbps to bind hbTF-7 was different from that of native hTF (Figure 4), despite its containing the entire hTF C-terminal lobe. With the exception of TbpA (B16B6), there was a significant decrease in binding of each purified Tbp sample (0.005 ≤ P ≤ 0.100) irrespective of hTF/chimaera saturation with iron. The most pronounced binding difference was shown by TbpB, which recognized diferric hbTF-7 at significantly lower levels than diferric hTF (P = 0.005 and P = 0.010 for strains SD and B16B6 respectively). On this occasion the highest level of chimaeric binding was achieved by TbpB purified from strain B16B6, whereas the equivalent SD-derived protein bound hbTF-7 at a significantly lower level (P = 0.005) (Figure 4). Saturation with iron also increased the difference between hTF and hbTF-7 binding by TbpA+B from strain SD (P = 0.005) and B16B6 (P = 0.010).

Tbp binding to hbTF-1

Significant levels of binding were detected with each Tbp sample (0.005 ≤ P ≤ 0.050). Tbps purified from strain SD bound at consistently higher levels, with or without saturation of the immobilized chimaera with iron. These strain-determined differences were significant for TbpA binding to apo-hbTF-1 (P = 0.010) and TbpB binding to the chimaera in its diferric form (P = 0.050). TbpA+B showed distinct strain-determined binding differences in iron-free and iron-replete conditions (P = 0.025 and P = 0.005 respectively) (Figure 5).
been observed with meningococcal Tbps. It is pertinent to note that the primary receptor recognition site on hTF for the human transferrin receptor was localized to the C-terminal lobe of the protein in a study with proteolytically prepared N- and C-lobes [17]. However, recent work with recombinant forms of the two lobes indicates that the human transferrin receptor recognizes regions in both lobes of hTF [18].

There were minor variations in binding of TbpA isolated from different meningococcal strains to hTF and chimaeric proteins, which might reflect the high degree of inter-strain conservation of TbpA [5,12]. In contrast, TbpB demonstrated significant differences in hTF binding, showing strain-dependent recognition of a secondary site, which we have located, between residues 255 and 350 of the hTF N-terminal lobe. Given the well-documented heterogeneity in TbpB molecular mass [13,14], the demonstration of this strain-specific difference was not surprising. However, despite such variability, it is accepted that the hTF-binding capacity of TbpB is dependent on a large (approx. 270–290-residue) highly structured domain present in divergent forms of this protein [19]. The size of this region, coupled with its critical role in binding of hTF, suggests that several exposed epitopes are involved in ligand recognition by this protein. Given that no similar strain-determined differences were apparent in the interaction of TbpB with either hTF or hbTF-2, we conclude that this specificity is a characteristic of the proposed binding site in the hTF N-terminal lobe. The presence of multiple TbpB recognition sites is supported by recently published results that suggest that the immunogenicity of meningococcal TbpB [20] and the hTF-binding capacity of the equivalent protein in Neisseria gonorrhoeae are influenced by at least two conserved regions [21]. In addition, it has been suggested that meningococcal TbpB has a bilobal structure [22] and might interact simultaneously with regions in the N- and C-terminal lobes of hTF [16] in a manner related to that exhibited by Tbps from several ruminant pathogens capable of sequestering transferrin-bound iron [23].

The TbpA + B complex showed less variation in binding to the hTF chimaeras (e.g. hbTF-2 and hbTF-3), indicating that the complex has binding properties distinct from those of either of its components. However, significant strain-specific binding was also apparent for the TbpA + B complex, despite the absence of an hTF C-terminal lobe (hbTF-1 and hbTF-4), implying that the TbpA + B complex, purified from strain SD, interacts with a previously unreported secondary recognition site located in, or stabilized by, regions within the N-terminal lobe of hTF. We

**DISCUSSION**

The binding of the chimaeric hTFs by meningococcal Tbps is summarized in Figure 7 and Table 1. The results show clearly that the primary binding site for individual TbpA and TbpB is located in the hTF C-terminal lobe, as reported previously for TbpA + B [11]. However, the interaction of each Tbp sample with chimaeras hbTF-1 and hbTF-3 demonstrated that the intact hTF C-terminal lobe is not an absolute prerequisite for binding. It is clear that, although the hTF C-terminal lobe is associated with Tbp binding, other epitopes located in other regions contribute to binding events, either directly or indirectly, by affecting hTF conformation. Tbps from other bacteria have been shown to interact with both N- and C-terminal lobes of transferrin from other host species [16] but this has not previously been observed with meningococcal Tbps. It is pertinent to note
have previously demonstrated that co-purified TbpA + B behaves as a discrete species in solution and that purified TbpA and TbpB bind distinct, separate regions on hTF [7,24]. Collectively these results further strengthen suggestions of co-operative, simultaneous hTF binding by TbpA and TbpB, rendering co-purified TbpA + B a functionally distinct and more avid receptor than either of its purified components. The requirement for a two-component receptor is further explained by the different and complementary properties of TbpA and TbpB. TbpA is thought to form a gated channel in the neisserial outer membrane [5,12], energized by TonB [21], and might contain clusters of negative residues that facilitate the passage of iron through the pore [673, which forms a disulphide bond with Cys-479 in hTF, leaving a unique naturally occurring hTF variant that, although can also affect the recognition of transferrin receptor, as occurs with a unique naturally occurring hTF variant that, although able to bind two ferric ions [30], has a decreased affinity for the human transferrin receptor [31,32]. The lower affinity of the variant has now been attributed to the amino acid substitution in Cys-673, which forms a disulphide bond with Cys-479 in hTF.

In bTF, significant sequence divergence is evident around Cys-673, which forms a disulphide bond with Cys-479 in hTF,
possibly preventing the C-terminal lobe from adopting the fully ‘open’ conformation demonstrated in the apo N-terminal lobe [3]. We speculate that despite the conservation of both Cys residues, subtle variations in sequence could perturb the disulphide bond, thereby influencing the range of C-terminal lobe conformations possible in hTF. In this way the iron-saturat ed chimaeric C-terminal lobe might be rendered distinct from hTF, thus contributing to the observed differences in binding by Tbps. Substitutions in these inter-domain helices are liable to influence the relative orientation of chimaeric N- and C-terminal lobes and thus alter significantly the interactions between these domains. Furthermore, the substitution of Cys-336 in hbTF-3, hbTF-4 and hbTF-7 abolished a single disulphide bridge normally present in hTF. The absence of this potentially stabilizing factor might have structural implications in these chimaeras.

In conclusion, we have described a previously unreported, strain-specific, secondary TbpB-binding site in the hTF N-lobe, localized to residues 255–350. This has improved our understanding of the individual roles of TbpA and TbpB in hTF binding and the need for them to act together to form the functional receptor. We have previously provided evidence that the receptor is composed of a TbpA dimer in association with a single molecule of TbpB [7]. From the results of this study we postulate that a secondary TbpB–hTF binding site, together with the primary binding site in the hTF C-lobe, might act to orientate hTF over the dual pores of the TbpA dimer, permitting the transfer of iron from hTF to the bacteria.

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