Production and Characterization of Chimeric Transferrins for the Determination of the Binding Domains for Bacterial Transferrin Receptors*

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Pathogenic bacteria in the Neisseriaceae and Pasteurellaceae possess outer membrane proteins that specifically bind transferrin from the host as the first step in the iron acquisition process. As a logical progression from prior studies of the ligand-receptor interaction using biochemical approaches, we have initiated an approach involving the production of recombinant chimeric transferrins to further identify the regions of transferrin involved in receptor binding. In order to prepare bovine/human hybrids, the bovine transferrin gene was cloned, sequenced, and compared with the existing human transferrin gene sequence. After identification of potential splice sites, hybrid transferrin genes were constructed using the polymerase chain reaction-based approach of splicing by overlap extension. Five hybrid genes containing sequences from both bovine and human transferrin were constructed. Recombinant transferrins were produced in a baculovirus expression vector system and affinity-purified using concanavalin A-Sepharose. The recombinant proteins were analyzed for reactivity against polyclonal and monoclonal antibodies and assessed for binding to Neisseria meningitidis transferrin receptor proteins in solid-phase binding assays and affinity isolation experiments. These experiments enabled us to localize the regions of human transferrin predominantly involved in binding to the N. meningitidis receptor to amino acid residues 346–888. The construction of these chimeras provides unique tools for the investigation of transferrin binding to receptors from both human and bovine bacterial pathogens.

The requirement for iron is ubiquitous among living cells due to its essential role in a large number of enzymes and redox proteins involved in metabolic processes (1). Despite an abundance of iron in the mammalian host, it affords a hostile iron-restricted environment to potential bacterial pathogens. This is manifested by the binding of iron to the glycoproteins lactoferrin, found in mucosal secretions, and transferrin (Tf) in the serum and interstitial compartments. These modes of iron binding lower the concentration of free aqueous iron to only $10^{-18}$ M (2), which is far below that required to support microbial growth. Thus the ability of microbial organisms to acquire iron in vivo from these reservoirs is considered to be an essential characteristic of bacteria that can successfully cause disease (1, 3).

Bacteria have evolved high affinity iron acquisition mechanisms designed to scavenge this essential nutrient from the external environment. One strategy involves the direct binding and removal of iron from the host's iron binding proteins by bacterial cell surface receptors. This receptor complex typically consists of two transferrin binding proteins, (Tbp1 and Tbp2) (4, 5). Studies of isogenic mutants that lack the expression of Tbp1 and/or Tbp2 have shown that both proteins play an important role in the effective binding of transferrin (6–9). Based on comparative amino acid sequence analysis, the Tbp1 polypeptide is predicted to be an integral outer membrane protein, while Tbp2 appears to be anchored to the outer membrane via an N-terminal lipid anchor (8, 10, 11). The specificity of these receptors for iron binding proteins of only their hosts is a characteristic feature of this mode of iron acquisition (12).

Transferrin is a bilobed, monomeric glycoprotein of approximately 80 kDa (13). The N-terminal and C-terminal lobes of Tf, which are similar in amino acid sequence and tertiary structure, are connected by an interdomain bridge that varies in length between different transferrin species. Native human transferrin (hTf) N-lobe occupies the initial 333 amino acids in the polypeptide; the bridge region from amino acid 333 to 341; and the C-lobe from amino acid 342 to the C terminus. Phylogenetic studies of different transferrin species have demonstrated highly conserved internal regions as well as conservation of three of the four iron-binding ligands (14).

Previous studies have indicated that the N-linked oligosaccharide side chains of hTf are not required for binding to the bacterial transferrin binding proteins (Tbps), suggesting that the amino acids at the surface of human transferrin mediate receptor binding (15). The observation that the human pathogens Neisseria meningitidis, Haemophilus influenzae, and Moraxella catarrhalis recognize the same spectrum of primate transferrins, and that transferrin binding to these species is inhibited by the same mononodal antibody, suggests that the transferrin-receptor interaction involves a similar region of transferrin in these species (16). As well, the primary region of interaction has been localized by binding and affinity isolation experiments using proteolytically derived C-lobe and N-lobe fragments (17). Using this approach both Tbp1 and Tbp2 of N. meningitidis were shown to interact exclusively with the C-lobe of human transferrin.

Although the use of proteolytically derived fragments has provided significant insight into the interaction of transferrin and its bacterial receptors, there are several inherent limitations to this biochemical approach. The pattern of proteolytic cleavage is a function of the properties of the protein substrate...
and protease involved and does not allow for selection of a particular peptide to be analyzed. In addition, protease cleavage may not necessarily result in dissociation of the proteolytic subfragments, and subsequent attempts at separating and isolating the resultant peptides (involving denaturing conditions and cleavage of disulfide bridges) may result in irreversible loss of conformation that is essential for the binding interaction. These factors may be responsible for the lack of success in preliminary attempts at obtaining further subfragments of hTf that retain sufficient binding avidity for detection in the binding and affinity isolation. Binding studies have demonstrated that the Tf receptors of certain bacteria interact with both Tf lobes (18), indicating that an intact Tf molecule would provide more insight into these binding interactions.

Transferrins have a significant degree of conservation in amino acid sequence (>70%); thus, they are predicted to have a very similar overall tertiary structure. The conserved structure of transferrins, coupled with the specific interactions between a host transferrin and bacterial pathogen, has prompted us to investigate the potential of chimeric transferrins. This approach utilizes the intact transferrin molecule as a framework on which heterologous sequences can be exchanged in order to identify those sequences necessary for the interaction of transferrin with bacterial receptor complexes. By combining domains of a host transferrin with domains from an alternate species, the regions required for interacting with the bacterial receptor complex can be identified.

**EXPERIMENTAL PROCEDURES**

**Materials**—The MAXBAC baculovirus expression system, which includes the pBlueBacII expression vector, insect cells, and transfection components, was obtained from Invitrogen (San Diego, CA). Insect cell culture, including Grace’s Insect Culture Medium (GIM), was purchased from Invitrogen. Human alpha phosphatase was from Life Technologies, Inc. Restriction enzymes, T4 DNA ligase, and calf intestinal phosphatase were also purchased from Life Technologies, Inc. Pfu DNA polymerase was from Stratagene Cloning Systems (La Jolla, CA). DNA fragments were purified using Prep-A-Gene matrix from Bio-Rad Laboratories (Richmond, CA). Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems, Lincoln City, CA). PCR was carried out using a DNA thermocycler (Perkin-Elmer Corp., Norwalk, CT).

**Bacterial Strains and Growth Conditions**—*E. coli K-12* strain M982 was originally obtained from C. Frasch. Cells stored frozen at −70°C in 30% glycerol were streaked onto brain-heart infusion agar and incubated at 37°C for 24 h. Cells used during experiments, an additional 10 min incubation at 72°C was used to ensure that all amplified fragments were completely extended to the 3′-termini. The PCR products were purified by preparative agarose gel electrophoresis and quantitated prior to the subsequent PCR to generate hybrid transferrin cDNAs. Equal molar amounts of appropriate PCR fragments (0.2 pmol) were combined in the PCR mixture to generate a hybrid product defined by terminal oligonucleotide primers, which allowed amplification of the hybrid product. The PCR profile used for SOEing PCR fragments included an initial thermocycle file with a 5-min incubation at 94°C, a 5-min ramp to 37°C, a 5-min incubation at 37°C, and a 5-min incubation at 72°C. This was linked to a step file consisting of 30 cycles of 1 min at 94°C, a 2-min incubation at 50°C, and a 2-min incubation at 72°C. Following the final cycle, an additional 10-min incubation at 72°C was used to ensure that all amplified fragments were completely extended to the 3′-termini. The hybrid genes with splice sites defined by the oligo primers shown in Table I were amplified with terminal primers that had a blunt 5′-end for directional cloning in the vector pBlueBacIIIS-N. Chimeric HBTf1 and HBTf2 were obtained using the primer pair HBTF5–5/HBTF3–3; chimeric hTfT2 was obtained by primer pair HBTF5–4/HBTF3–3; chimeric hTfT3 by HBTF5–1/HBTF3–3; and chimeric hTfT4 by HBTF5–1/HBTF3–3 and HBTF5–5/HBTF3–3 (Table I). PCR fragments were purified by agarose gel electrophoresis, digested with BamHI, and ligated into the pBlueBacIIIS-N vector at the BamHI site.

**Construction of Hybrid Transferrin Genes**—The human transferrin cDNA cloned in the pUC13 plasmid was obtained from Ross MacGillivray (University of British Columbia). To facilitate production of recombinant hTf, the hTf gene was PCR-amplified from pUC13 with primers introducing a BamHI site at the 5′-end and a PsI site at the 3′-end of the hTf gene. After subcloning into the pCR1 vector this fragment was partially digested with BamHI and PsI, cloned into the pBlueBacIIS vector and hereafter referred to as pBhTf.

**Human and bovine transferrin genes** were spliced using the PCR technique of splicing by overlap extension (SOEing) (22). Splice sites were selected at conserved regions in transferrins to reduce the possibility of perturbing the tertiary structure of transferrin in the chimeric transferrins and to also delineate possible binding regions to bacterial Tf receptors. Either human or bovine cDNA fragments were amplified by PCR using Pfu DNA polymerase to reduce the possibility of misincorporation mutations in the PCR products. Standard PCR conditions were used for the amplification of template DNA fragments to be used as the subsequent SOEing reactions (initial cycle of 5 min at 95°C, 5 min at 50°C, and 5 min at 72°C linked to 30 cycles of 1 min at 94°C, a 2-min incubation at 50°C, and an additional 10-min incubation at 72°C was used to ensure that all amplified fragments were completely extended to the 3′-termini). The PCR products were purified by preparative agarose gel electrophoresis and quantitated prior to the subsequent PCR to generate hybrid transferrin cDNAs. Equal molar amounts of appropriate PCR fragments (0.2 pmol) were combined in the PCR mixture to generate a hybrid product defined by terminal oligonucleotide primers, which allowed amplification of the hybrid product. The PCR profile used for SOEing PCR fragments included an initial thermocycle file with a 5-min incubation at 94°C, a 5-min ramp to 37°C, a 5-min incubation at 37°C, and a 5-min incubation at 72°C. This was linked to a step file consisting of 30 cycles of 1 min at 94°C, 1 min at 37°C, and 5 min at 72°C. Following the final cycle, an additional 10-min incubation at 72°C was used to ensure that all amplified fragments were completely extended to the 3′-termini. The hybrid genes with splice sites defined by the oligo primers shown in Table I were amplified with terminal primers that had a blunt 5′-end and a BamHI site at the 3′-end for directional cloning in the vector pBlueBacIIIS-N. Chimeric HBTf1 was obtained using the primer pair HBTF5–5/HBTF3–3; chimeric hTfT2 was obtained by primer pair HBTF5–4/HBTF3–3; chimeric hTfT3 by HBTF5–1/HBTF3–3 and HBTF5–5/HBTF3–3 (Table I). PCR fragments were purified by agarose gel electrophoresis, digested with BamHI and ligated into the pBlueBacIIIS vector at the BamHI site.

**Expression of Recombinant Transferrins in the Baculovirus Expression System**—The baculovirus expression vector system was described previously (MaxBac, Invitrogen). Recombinant baculovirus cDNAs were identified by plaque assay with the ex-length bovine transferrin cDNA was generated by splicing at a unique BglII site the 5′-end of the bovine transferrin cDNA in a given clone with the remainder of the bovine transferrin cDNA in an alternate bovine transferrin cDNA done. The 2.1-kilobase full-length bTf cDNA was amplified by PCR with the terminal primers for the bTf cDNA. Following digestion of a unique BglII site in the 3′-primer region of the bTf gene, the 2.1-kilobase full-length bTf cDNA was directed at the Small and SmalI sites of a modified pBlueBacIIS-N vector in which the Smal, Smal, and BglII sites had been inserted between the BamHI and PsI sites to generate the plasmid pBBbTf. The DNA sequences for the BglI site of the 5′- and 3′-bTf cDNA fragments as well as the junctions between the pBlueBacIIS-N vector and the bTf cDNA insert fragment were determined to confirm the construction of the expression plasmid clone for bovine transferrin.

The DNA sequence for bovine transferrin cDNA was established using the T7 DNA polymerase system (Pharmacia Biotech Inc.) for manual sequencing and the Taq-DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) for automated sequencing.
Pression of β-galactosidase and the absence of polyhedron expression by recombinant baculovirus. The putative recombinant baculovirus clones were further purified by repeating the plaque assay. S. frugiperda Sf21 cells grown in serum-free medium (SF9001-1-SFM) were infected with recombinant Autographa californica nuclear polyhedrosis virus baculovirus and the wild type AdNPV control to generate high titer viral stocks and recombinant protein. Culture medium containing the budded virus and the secreted recombinant protein was collected 4–5 days post-infection.

The expression of transferrin by putative recombinant baculovirus clones was determined by Western blot analysis of the secreted protein fraction for insect cells (Sf21) grown in serum-free medium. Control Sf21 cells that were noninfected, infected with wild type Autographa californica nuclear polyhedrosis virus, or infected with recombinant virus constructed with only the pBlueBacIII vector had no detectable expression of transferrin protein as determined by Western blot analysis with rabbit antiserum prepared against human transferrin followed by horseradish peroxidase-labeled goat anti-rabbit Ab (data not shown).

Expressed recombinant Tf protein was isolated from cell culture supernatant using a concanavalin A-Sepharose (Sigma) affinity resin. Recombinant Tf protein was dialyzed against a sample application buffer (50 mM sodium acetate, 1 mM NaCl, 1 mM MgCl2, CaCl2, 1 mM MnCl2) and added to the concanavalin A column, and the column was washed with 3–5× bed volume of application buffer. Specifically bound Tf was eluted using application buffer containing 200 mM methyl-α-D-mannopyranoside, dialyzed against a 20 mM ammonium bicarbonate buffer, lyophilized, and resuspended in bicarbonate/citrate buffer (100 mM sodium bicarbonate, 100 mM sodium citrate, pH 8.6). Recombinant Tf recovered at this step typically was at 400–800 µg/ml concentration.

Iron Saturation of Transferrins—Prior to analysis, the recombinant transferrins were converted to an iron-saturated form. Briefly, recombinant transferrin was added to 2–3 times molar excess of ferric chloride dissolved in bicarbonate/citrate buffer and incubated for 30 min at 22 °C. Unbound excess iron was removed using a G-25 gel filtration column (1.5 × 6 cm) equilibrated in 50 mM Tris (pH 8.0). Iron-saturated transferrin was initially detected by its characteristic red color in column fractions and confirmed by spectral analysis (23).

SDS-PAGE and Western Blot Assays—Methods for SDS-PAGE and Western blot assays to detect the expression of recombinant transferrins by the baculovirus clones are essentially as described previously (16). Protein samples for Western blot assays were not reduced or heat-denatured prior to fractionation on SDS-PAGE (10% polyacrylamide) (16). Polyclonal rabbit antibodies for hTf or bTf were passed through hTf-Sepharose or bTf-Sepharose columns, respectively, in an attempt to remove cross-reacting antibody. N-lobe and C-lobe preparations of hTf were obtained by chymotryptic digestion as described previously (17).

Solid Phase Binding Assays—The interaction between the transferrin receptors on human pathogens and the recombinant wild type or chimeric transferrins was investigated using a competitive solid-phase binding assay as described previously (16). In brief, 1 µl aliquots of crude membranes prepared from the human pathogen N. meningitidis M982 (1 mg/ml) were spotted onto Immobilon paper (Millipore, Bedford, MA). Unlabeled transferrins were prepared as 1 mg/ml solutions in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5), and 1.3 serial dilutions were prepared in blocking solution (TBS containing 0.5% skim milk). The transferrin solutions (150 µl) were combined with horseradish peroxidase-conjugated transferrin (50 µl of a 1:4000 dilution of a 1.5 mg/ml stock for hTf-horseradish peroxidase or a 1:1000 dilution of a 0.5 mg/ml for bTf-horseradish peroxidase) and exposed to immobilized membranes, followed by washing, labeling, and detection steps as described previously (16, 17).

Affinity Isolation Assay—hTf-Sepharose was prepared from CNBr-activated Sepharose essentially as described previously for bTf-Sepharose (18). The affinity isolation of Tbps of N. meningitidis by hTf-Sepharose and competitive inhibition of this isolation by various transferrins are essentially from procedures also described previously (18).

Molecular Modeling—Three-dimensional models of recombinant transferrin structures were constructed using the homology module of InsightII (BioSym Technologies) using the known structures of human lactoferrin (11If.pdb) and N-lobe of rabbit transferrin (1lfd.pdb) from the protein data base.

RESULTS

Cloning and Sequencing of the Bovine Transferrin Gene—To facilitate the production of chimeric human/bovine transferrin proteins, it was necessary to first clone and sequence the bovine transferrin gene so that homologous regions of the human and bovine genes could be identified and specific sites for constructing hybrid transferrin genes could be chosen. A probe prepared from cloned hTf cDNA was used to screen a λ phage library constructed with bovine cDNA insert fragments. Attempts with a conventional library yielded recombinant phage with inserts containing most of the bTf cDNA but lacking the 5'-end of the coding sequence. The partial bTf cDNA was therefore utilized as a probe for screening an alternate library specifically constructed to include the 5'-end of cDNAs. Although this did not result in isolation of recombinant phage containing the intact bTf cDNA sequence, it did provide phage with inserts containing the 5'-end of the gene and overlapping the previously incomplete gene. The individual inserts were subcloned and sequenced, and the intact bTf cDNA was subsequently assembled by ligation of the appropriate subfragments.

The predicted protein sequence for bTf was obtained from the DNA sequence and compared with the hTf sequence (Fig. 1). As shown in Fig. 1, there is an overall 70% amino acid sequence identity between hTf and bTf, with most sequence differences occurring in small clusters that were found throughout the length of the protein.

Comparison of bTf with the cysteines that form the known disulfide bridges of hTf (24) revealed that all cysteines were present except those predicted to form disulfide bridge 10 in the N-lobe (corresponding to amino acids 137 and 336 in hTf). The predicted absence of disulfide bridge 10 in bTf may result in certain conformational differences between human and bovine transferrin and particularly in chimeras generated from these two proteins.

Asn611 and Asn613, which are N-linked glycosylation sites in human transferrin, are not conserved in bovine transferrin. Evidence for glycosylation of porcine transferrin at Asn497 has been recently presented (25), and since there is a corresponding residue in bTf (Asn996), this may also be the site of glycosylation.

TABLE I

Oligonucleotide primers for the SOEing reactions

| Site  | Splice junction sequence | Template | Primer name | Primer sequence |
|-------|-------------------------|----------|-------------|-----------------|
| a     | VPSHAVARTVGGK           | hTf (coding strand) | HBTF3-2    | GCCACAGGCGCATGGAAAGCGAC |
| b     | DECMVKWCA               | hTf (coding strand) | HBTF5-3    | GTCTGGGCTGAACCCTGGCCAGGCAAGG |
| c     | DECMVKWCAl             | hTf (coding strand) | HBTF3-4    | GCACACATCTTCCATGCACATC |
| d     | PNHAV5SRK              | hTf (coding strand) | HBTF3-5    | ATGAGCTATGGTAAGGTTGTC |

a Amino acids from the hTf sequence are underlined, while those from the bTf sequence are in italics. Amino acids not encoded by sequences within the oligonucleotide primers are indicated by lowercase letters.

b Regions of the oligonucleotide primers designed for the overlap required for the second phase of the SOEing reactions are indicated by italics.
Construction of Hybrid Transferrin Genes—To obtain hybrid genes for production of the desired chimeric proteins, the technique of SOEing was used. The advantage of this technique is that the splice site is not dependent on preexisting restriction enzyme recognition sites and does not involve the inclusion or alteration of any amino acids. Splice sites for creating the chimeric transferrins were chosen to investigate the binding potential of certain domains while attempting to maintain a native conformation of the molecule. The splice sites for the chimeric transferrins are indicated by the signposts (a, b, c, and d) on the aligned amino acid sequences (Fig. 1), and the oligonucleotide primers that were used to obtain the splice sites are listed in Table I. Essentially the N- and C-lobes, the bridge region, and the C terminus of transferrin were selected as regions to be recombined from either human or bovine sources (Fig. 2).

Analysis of the amino acid sequence composition of the chimeric transferrins revealed potential structural differences from the native transferrins based on disulfide bridge locations. The absence of Cys137 and Cys336 (forming cystine 10) in bovine transferrin N-lobe (Fig. 1) creates an absence of this disulfide bridge in hbTf3, hbTf4, and hbTf7. As the N-lobes of hbTf3 and hbTf4 are comprised of human transferrin up to amino acid 254, they both contain a free cysteine residue at amino acid 137.

Expression of Recombinant Transferrin Proteins—To obtain recombinant transferrin protein for analysis, the chimeric Tf genes were cloned and expressed in a baculovirus expression vector system by subcloning into the pBlueBac vector and subsequent introduction into recombinant baculovirus by coinfection with viral vector DNA (MaxBac, Invitrogen). S. frugiperda insect cells were infected with the resulting recombinant baculovirus containing Tf genes and isolated plaques were assayed for their relative ability to express recombinant transferrin (rTf). Recombinant virus expressing the highest levels of rTf were propagated for each rTf construct for scaled up production of rTf protein. Levels of protein product typically were...
Production and Analysis of Chimeric Transferrins

Three monoclonal antibodies, raised against hTf (16), were utilized to probe the structure of the recombinant transferrins. Proteolytically-derived N-lobe and C-lobe subfragments of hTf were included in the analysis to assist in identification of the epitopes recognized by the anti-hTf monoclonal antibodies (Table II). mAb 44-63 reacted with proteolytically derived C-lobe but not N-lobe, indicating that the epitope recognized by this mAb is located in the C-lobe. This mAb reacted with hTf, hbTf2, and hbTf3, which all contain C-lobe or a large portion thereof as well as most or all of the N-lobe. In contrast, hbTf7, which only contains the hTf C-lobe, did not react with mAb 44-63. mAb 44-33 reacted with proteolytically derived N-lobe but not C-lobe, indicating that the epitope recognized by this antibody is located in the N-lobe. This mAb reacted with rhTf, hbTf1, hbTf2, hbTf3, and hbTf4, which all contain intact hTf N-lobe or large portions thereof. rbTf and hbTf7 did not react with mAb 44-33. mAb 39-49, which blocks binding of hTf to the Tbps of N. meningitidis (16), recognized epitopes on rhTf, hbTf2, and hbTf3. These chimeras all contain portions of both hTf N-lobe and C-lobe, hbTf1, hbTf4, and hbTf7, which contain regions of hTf but lack regions common to both lobes of hTf, did not react with mAb 39-49.

Solid-phase Competitive Dot Binding Assay—The intent of the solid-phase competitive dot assay was to identify the degree of specific binding of recombinant Tfs to Tbps present in isolated total membrane from a representative human pathogen. Serially diluted recombinant transferrins were premixed with horseradish peroxidase-labeled hTf conjugate, and the transferrin mixture was then exposed to immobilized total membrane preparation from iron-starved meningococcal cells. After washing to remove unbound protein, bound hTf conjugate was detected by development with chromogenic horseradish peroxidase substrate. The effective level of competition of recombinant transferrin for Tbps present in isolated membrane is reflected by the absence of bound and detectable conjugate.

In Fig. 4 it can be seen that recombinant rhTf was able to compete for membrane receptor to a similar degree as commercial hTf. The chimeras hbTf2, hbTf3, and hbTf7 also demonstrated blocking of conjugate binding to the membrane preparations. No blocking by commercial bTf, recombinant rbTf, hbTf1, or hbTf4 was evident.

Competitive Affinity Isolation Assay—Competitive inhibition of affinity isolation experiments was performed to determine which chimeric transferrin could specifically inhibit the isolation of Tbps from N. meningitidis. In this approach, the competing ligand is preincubated with solubilized meningococcal membranes prior to exposure to the human transferrin-Sepharose. After subsequent binding and washing steps, the material remaining bound to the affinity resin is eluted in SDS-PAGE sample buffer and analyzed by standard SDS-PAGE gel electrophoresis. As shown in Fig. 5, commercial hTf and recombinant hTf were able to successfully compete with hTf-Sepharose for both Tbp1 and Tbp2 of N. meningitidis strain M982 present in the total membrane preparation. As expected, neither commercial bTf nor recombinant rbTf were able to compete for the Tbps of this human pathogen. The chimeric transferrins hbTf3 and hbTf7 also successfully competed for both Tbp1 and Tbp2. In contrast to the competitive immobilized dot assay, hbTf2 was unable to outcompete hTf-Sepharose for either Tbp. The recombinants hbTf1 and hbTf4 were unsuccessful in competing with hTf-Sepharose for N. meningitidis Tbps. A protein of molecular mass 64,000 daltons is isolated by hTf-Sepharose, but this isolation is not competitively inhibited by soluble hTf (Fig. 5). This suggests that the observed isolation may be due to binding to Sepharose and not to conjugated hTf.

**DISCUSSION**

The transferrins from human and bovine species exhibit conservation in amino acid sequence and three-dimensional structure (14). Despite this, bacterial pathogens' transferrin binding proteins are specific for transferrin from their host species. This distinct interaction has previously been shown to
be dictated by protein structure and not N-linked oligosaccharides (15), indicating that genetic approaches for investigating the ligand-receptor interaction would be appropriate. Thus to take advantage of the properties of the transferrins and bacterial receptors we elected to prepare human/bovine hybrid transferrins for analyzing the ligand-receptor interaction. In this preliminary study we prepared chimeric Tf's that were composites of the respective lobes and bridge regions from these species, as this was a suitable starting point for evaluating the effectiveness of this approach.

There were a number of options for hybrid transferrin gene expression for the production of recombinant transferrins. Mammalian cells should be an appropriate host for expression of these genes, and several studies have demonstrated that recombinant human transferrin and lactoferrin can be effectively produced in a baby hamster kidney cell expression system (23, 26, 27). Analysis of several properties such as estimated molecular mass, glycosylation, and iron binding indicated that the recombinant proteins were essentially identical to the native protein, indicating that the recombinant proteins were properly processed and exported in this system. Significant concentrations of secreted recombinant protein could be obtained in the culture medium, and efficient methods of chromatographic purification have been developed (23).

However, there were several limitations of this expression system that prompted us to consider utilizing a baculovirus expression system. The preparation of cell lines for production of recombinant transferrin is time-consuming and relatively labor-intensive, and the requirement for selection of gene duplication (by methotrexate resistance) for high level production has some inherent potential for instability. In addition, the absolute requirement for fetal bovine serum as a growth supplement can complicate subsequent purification of recombinant Tf due to the presence of fetal bovine Tf.

The affinity isolation of the recombinant Tf's with concanavalin A in this study indicates that they were glycosylated, and according to the results from prior studies with the baculovirus expression system (28, 29), the glycosylation was probably identical to that of the native Tfs. The isolation of hbTf1 and hbTf4 by concanavalin A suggests that previously undetected glycosylation sites for Tf are also in the C-lobe. In addition, when considering properties such as estimated molecular weight, iron binding, the ability to bind to the bacterial receptors (Figs. 4 and 5), and anti-hTf mAbs (Table II), the recombinant hTf could not be differentiated from native hTf. The ability to obtain significant quantities (10–20 mg/liter) of secreted protein in culture media devoid of supplemental fetal calf serum enabled us to use a simple lectin affinity step for obtaining pure preparations of recombinant protein. These results indicate that the baculovirus expression system is an appropriate and effective alternative system for production of recombinant transferrin.

The recent demonstration of production of recombinant lactoferrin in an inducible Aspergillus nidulans expression system that was indistinguishable from native human lactoferrin with respect to size, immunoreactivity, and the ability to bind iron (30) indicates that even more heterologous eucaryotic expression systems may be effective. Although this suggests that more convenient yeast expression systems (31) can be considered, the functional attributes of the recombinant proteins, such as receptor binding, will have to be evaluated. Although it is even possible to produce recombinant transferrins in prokaryotic systems such as E. coli (32), there is a lack of suitable post-translational processing (i.e. glycosylation proteolytic processing) (33). The large number of disulfide bridges in these proteins makes it quite unlikely to achieve proper refolding of these proteins using the E. coli expression system.

The rationale for utilization of recombinant chimeric transferrins for delineation of the receptor binding region was based on the assumption that the high degree of identity and predicted tertiary structure of hTf and bTf would ensure that the chimeras attained a proper conformation. The glycosylation of these proteins and their ability to bind iron suggest that the general structure of these recombinant proteins is correct. The fact that all of the chimeras containing portions of the hTf C-lobe (hbTf2, hbTf3, and hbTf7; Fig. 4) were capable of binding to the meningococcal receptor is also consistent with this prediction. Similarly, the reactivity of the chimeras containing portions of the hTf N-lobe (hbTf1, hbTf2, hbTf3, and hbTf4; Table II) with a monoclonal antibody directed against the N-lobe, which loses reactivity when hTf is boiled and treated with reducing agents, suggests that at least some conformational epitopes in this lobe are present in the chimeras. Similarly the reactivity of hbTf2 and hbTf3 with mAb 39-49 and mAb 44-63 (Table II) indicate that these chimeras possess epitopes present in native hTf.

Although hbTf7 appears to be properly glycosylated, is capable of binding iron and is as effective as native hTf in competing for the bacterial receptor proteins in the binding and affinity isolation assays (Figs. 4 and 5), the inability to bind mAbs
39-49 and 44-63 (Table II) indicates that there are subtle structural differences from native hTf. This failure to bind the specific mAbs cannot be solely attributed to a lack of stability of this chimeric protein under conditions of SDS-PAGE and electrophoresing, as the lack of binding to mAb 44-63 is also observed in solid phase bindings using purified preparations of the protein directly (data not shown). This might be attributable to conformations that are dependent upon interactions with portions of the hTf N-lobe (see results with mAb 39-49, Table II) that are present in hbTf2 and hbTf3. Significant N-lobe and C-lobe association has been demonstrated in human lactoferrin, where cleavage of lactoferrin by trypsin at amino acids 283–284 did not result in fragment dissociation until strong denaturing conditions were used despite a lack of covalent linkage between the two fragments (34). A strong lobe-lobe interaction is likely also occurring in transferrin species, and this might be expected to affect some conformational epitopes in the C-lobe region. This scenario for hbTf2 could result in a steric hindrance of the bTf N-lobe on mAb 44-63 binding to its C-lobe epitope.

However, it is probably more likely that the lack of mAb 44-63 binding to hbTf7 is due to a mutational event that occurred during the preparation of this recombinant virus. This possibility cannot be excluded by sequencing of the hybrid gene in the expression vector, as the mutational event could have occurred during the subsequent steps of coinfection or isolation and propagation of the recombinant virus. We are planning to prepare a set of additional constructs only containing segments of the hTf C-lobe and will include repetition of the construction of hbTf7 to address this issue. In spite of the subtle structural differences that were revealed by analysis with mAbs, hbTf7 was nevertheless useful for the originally intended purpose, identification of the receptor binding domain.

mAb 39–49 binds the same spectrum of primate transferrins as do the Tbps of selected human pathogens N. meningitidis, H. influenzae, and M. catarrhalis (16). Furthermore, receptors from these pathogens are inhibited from binding hTf by mAb 39-49. These results imply that the mAb 39-49 epitope is in a region that may be involved in binding the receptor complex. However, hbTf7 was still able to bind immobilized Tbps of N. meningitidis M982 and competitively inhibit their affinity isolation (Figs. 4 and 5), yet it lacked the epitope for mAb 39-49, indicating that the epitope for mAb 39-49 is distinct from the binding domain. Furthermore, mAb 39–49 binds hbTf2, yet hbTf2 cannot competitively inhibit affinity isolation of the Tbps of M982. The C-lobe of hbTf7 has been shown to mediate binding to the Tbps of human bacterial pathogens, and the lack of the mAb 39-49 epitope on biochemically derived hTf C-lobe does not appear to affect its Tbp binding, providing further evidence that the Tbp binding domain and the mAb 39-49 epitope are distinct.

Despite its apparent absence on the N-lobe or C-lobe fragments, it is possible to assign the mAb 39-49 epitope to a particular region on hTf using the results obtained in this study. mAb 39-49 binds to recombinant transferrins, which contain regions of both lobes of hTf. Furthermore, fragmenting of hTf into isolated N-lobes and C-lobes destroys mAb 39-49 binding. These observations imply that the mAb 39-49 epitope is in a region comprised of both lobes or an epitope that is dependent upon the interlobe interaction. The blocking of receptor binding by this mAb suggests that it is blocking by steric hindrance and implies that this epitope may occupy the same face of Tf as the Tbp binding determinants.

A solid-phase competitive binding assay was utilized to determine the relative effectiveness of the recombinant transferrins to compete with horseradish peroxidase-hTf for available receptor in an immobilized membrane preparation. The most obvious characteristic of those recombinants that could compete effectively in this assay is the presence of the C-lobe of hTf or, in the case of hbTf2, a substantial portion of the hTf C-lobe. The apparent requirement of hTf C-lobe for success in this assay containing meningococcal membranes, is consistent with previous studies involving proteolytically derived hTf C-lobe (17). Furthermore these results with hbTf2 further localize the domain required for binding in the solid-phase binding assay to amino acid residues 346–588.

The affinity isolation assay provides two additional features by analyzing the interaction between ligand and the individual receptor proteins and the requiring greater avidity and stability of binding than can be assessed in the solid-phase binding assays. The competitive inhibition of the isolation of both Tbp1 and Tbp2 of N. meningitidis M982 by chimeras hbTf3 and hbTf7 (Fig. 5) is consistent with previous results, which indicated that regions of hTf C-lobe are involved in binding to both receptor proteins (17). The inability of hbTf2 to effectively inhibit isolation of the receptor proteins, while resulting in effective inhibition in the solid-phase binding assay, suggests that it may lack regions that are necessary for the more avid binding required in the affinity isolation procedure. Thus the “C-tail” either participates in the binding interaction or is important for maintaining a conformation required for avid binding. Since no disulfide bond changes are expected by substituting the terminal amino acids of hTf with bTf (as in hbTf2), the different avidity of binding during affinity isolation may be due to localized sequence differences between hTf and bTf in the C-terminal region. These results are also consistent with the hypothesis that transferrin binding involves a number of amino acid sequences that are oriented in proximity to facilitate binding, and the loss of a single region with the preservation of the remaining sequences reduces binding in a graded rather than all-or-none process.

The prospect of multiple binding domains participating in receptor interaction was further investigated by comparative sequence analysis and molecular modeling of the chimeric Tf structures. Inspection of the aligned hTf and bTf amino acid sequences revealed significant stretches of homology and small clusters of divergence throughout the polypeptide. It is likely that the homologous regions are structural determinants that provide for the conserved three-dimensional structure of transferrins. Analysis of transferrin sequences from a number of different species (14) revealed that conserved regions are in the internal β-sheets of domains 1 and 2. Using the known structure of human lactoferrin (35), three-dimensional representations of the recombinant transferrins were generated. A preliminary comparison revealed that certain divergent sequences were localized to the surface regions of the protein. For instance the model of hbTf2 structure shows divergent bTf residues of the C-tail that are located at exposed surface loops. Amino acids Ser640, Lys644 are localized to a face of the C-lobe that transverses the two domains in a random coil fashion as the polypeptide winds its way toward the bridge region before terminating. This region is located near Asn813, Asp820 due to disulfide C8 (Cys521–Cys637); thus, the presence of a divergent bTf sequence in one surface loop could be responsible for the lower avidity of hbTf2 binding to meningococcal Tbps in the affinity isolation experiment. Two additional surface loops containing divergent sequences are present in the Tf C-lobe, raising the possibility that effective binding to the receptor may involve these four regions. The putative surface exposure of these divergent regions implies that they may provide the species-specific binding regions for bacterial Tbps and species-specific epitopes for anti-hTf and anti-bTf antibody.
In this study we are able to exploit two characteristics of transferrins and transferrin binding to bacterial receptors in order to facilitate our understanding of this interaction. In particular we were able to localize the regions of hTf primarily responsible for binding the Tbps of N. meningitidis M982 to amino acid residues 346–588. The development of chimeric transferrins as more accurate tools of study utilizes the inter-species conservation of transferrin structure as well as the transferrin species-specific binding by particular bacterial pathogens. Although subtle conformational differences could be detected in one chimeric construct, these studies clearly demonstrate that hybrid human/bovine transferrins can be constructed that reflect these two properties. By creating chimeric transferrins from human and bovine sources, it is now possible to investigate the binding characteristics of both human and bovine pathogens that utilize a Tbp-receptor complex to acquire iron during the infection process.

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