Specific Ligand Binding Attributable to Individual Epitopes of Gonococcal Transferrin Binding Protein A

Heather P. Masri and Cynthia Nau Cornelissen

The gonococcal transferrin receptor complex comprises two iron-regulated proteins, TbpA and TbpB. TbpA is essential for transferrin-iron uptake and is a TonB-dependent integral outer membrane protein. TbpB is thought to increase the efficiency of iron uptake from transferrin and is lipid modified and surface exposed. To evaluate the structure-function relationships in one of the components of the receptor, TbpA, we created constructs that fused individual putative loops of TbpA with amino-terminal affinity tags. The recombinant proteins were then overexpressed in Escherichia coli, and the fusions were recovered predominately from inclusion bodies. Inclusion body proteins were solubilized, and the epitope fusions were renatured by slow dialysis. To assess transferrin binding capabilities, the constructs were tested in a solid-phase dot blot assay followed by confirmatory quantitative chemiluminescent enzyme-linked immunosorbent assays. The constructs with only loop 5 and with loops 4 and 5 demonstrated dose-dependent specific ligand binding in spite of being out of the context of the intact receptor. The immunogenicities of individual TbpA-specific epitopes were investigated by generating rabbit polyclonal antisera against the fusion proteins. Most of the fusion proteins were immunogenic under these conditions, and the resulting sera recognized full-length TbpA in immunoblots. These results suggest that individual epitopes of TbpA are both immunogenic and functional with respect to ligand binding capabilities, and the vaccine implications of these findings are discussed.

The gonococcal transferrin receptor is made up of an integral outer membrane protein (TbpA) and a loosely associated surface lipoprotein (TbpB), which work together to bind and internalize iron from transferrin. TbpA, the larger of the two proteins, has sequence homology with the TonB-dependent family of outer membrane protein receptors and is essential for transferrin-iron uptake. TbpA is highly conserved among the pathogenic neisseriae, while TbpB is surface exposed, lipid modified, more variable, and not absolutely required for transferrin-iron acquisition. For these reasons, we have focused on TbpA as a particularly attractive vaccine candidate.

We used our proposed TbpA topology model to guide the design of TbpA-specific epitope fusions, which are the focus of the present study. The hypothetical topology model was constructed using computer predictions for classical siderophores; however, the bacterium is capable of utilizing iron bound to human transferrin, human lactoferrin, hemoglobin, and heme. N. gonorrhoeae expresses an energy-dependent, iron-repressible receptor that binds host transferrin at the bacterial cell surface. This receptor is expressed by all clinical gonococcal isolates, whereas only approximately 50% of isolated strains are capable of lactoferrin-iron utilization. Furthermore, the transferrin receptor is not subject to high-frequency phase or antigenic variation, is relatively well conserved, and is required for initiation of experimental urethral infection in human males.

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the receptor (7, 21), at which point ligand entry is allowed by a shift in the position of the plug within the barrel.

The purpose of the present study was to determine if predicted surface-exposed epitopes of gonococcal TbpA are functional and immunogenic. To explore this, we expressed individual putative loops of TbpA with fusion partners to facilitate purification. These recombinant proteins were overexpressed in *E. coli* and isolated primarily from inclusion bodies. Solubilized and renatured fusion proteins were tested for transferrin binding by solid-phase colorimetric and chemiluminescent transferrin binding assays. By virtue of the fusion tag, the TbpA-specific loops were purified to a limited extent with an S-protein affinity matrix. The loop fusions were ultimately used as immunogens in rabbits in order to generate epitope-specific antisera.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were routinely cultured on Luria-Bertani (LB) agar or in LB broth (23) containing appropriate antibiotics. For immunoblot analysis of whole-cell lysates, gonococci were maintained on Luria-Bertani (LB) agar or in LB broth (23) containing appropriate antibiotics. The bacterial strains and plasmids used in this study are described in Table 1.

**Isolation of TbpA.** Six regions of TbpA (Fig. 1), were cloned individually into the expression vector pET-34LIC. The oligonucleotides oVCU-3 and oVCU-4 (Table 2) were used to PCR amplify the *tbpA* region that encodes the putative L5 (Fig. 1). These primers, like the others listed in Table 2, contain a 3' region that anneals to *tbpA* and a 5' segment that anneals to the vector. After PCR amplification, the product was treated with T4 DNA polymerase and dATP according to the manufacturer's recommendations. In this way, 13- to 14-base “sticky ends” which were compatible with the ends of the commercially prepared vector were created. Annealed vector and template mixtures were transformed directly into *E. coli* strain NovaBlue (Novagen) (Table 1) according to the manufacturer's recommendations; transformants were selected in the presence of kanamycin. Recombinant plasmid pVCU301 (Table 1) expressed a translational fusion between amino-terminal fusion tags and the domain encoding putative L5 of TbpA (Fig. 1). Plasmids pVCU302 through pVCU306 (Table 1) were created similarly, using the *tbpA*-specific oligonucleotides listed in Table 2. A negative control

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td><em>E. coli</em> BL21(DE3) F&lt;sup&gt;−&lt;/sup&gt; ompT hsdS&lt;sub&gt;2+&lt;/sub&gt; (rB&lt;sub&gt;−&lt;/sub&gt; m&lt;sub&gt;−&lt;/sub&gt;) gal dcm (DE3)</td>
<td>Novagen</td>
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<td><em>N. gonorrhoeae</em> FA19</td>
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<td>C. Kenney and C. N. Cornelissen, unpublished data</td>
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<td>pUNCH412</td>
<td><em>HindIII</em>-to-<em>XbaI</em> fragment of <em>tbpA</em> from pUNCH411 cloned into pET-11</td>
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<tr>
<td>pVCU301</td>
<td>PCR product encoding L5 sequence cloned into pET-34b (+)</td>
<td>This study</td>
</tr>
<tr>
<td>pVCU302</td>
<td>PCR product encoding L4-L5 sequence cloned into pET-34b (+)</td>
<td>This study</td>
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<tr>
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<td>PCR product encoding amino-terminal plug region cloned into pET-34b (+)</td>
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<td>PCR product encoding L2 sequence cloned into pET-34b (+)</td>
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<td>PCR product encoding L3 sequence cloned into pET-34b (+)</td>
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<td>pVCU306</td>
<td>PCR product encoding L9-L10 sequence cloned into pET-34b (+)</td>
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<tr>
<td>pVCU307</td>
<td>PCR product encoding LacZ control cloned into pET-34b (+)</td>
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**Plasmids**

- pET-34b (+) Kan<sup>′</sup>
- pUNCH412
- pVCU301
- pVCU302
- pVCU303
- pVCU304
- pVCU305
- pVCU306
- pVCU307

**Bacterial strains and plasmids**

- *E. coli* BL21(DE3) pLysS
- *E. coli* NovaBlue

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**Standard recombinant DNA techniques and DNA sequencing.** Isolation of plasmid DNA, digestion with restriction endonucleases, and ligation were carried out according to manufacturers' recommendations. For amplification of fragments encoding TbpA-specific epitopes, PCR conditions were as suggested by the supplier of the ligation-independent cloning expression vector, pET-34LIC (Novagen), with annealing conditions optimized for each primer pair. The sequence of inserts in the pET-34 recombinants was identical to the corresponding regions of *tbpA* from gonococcal strain FA19, with the exception of the construct consisting of loops 9 and 10 (L9-L10) construct, pVCU306 (Table 1), which contained two single-base changes. The first exchanged a glutamic acid for a glycine, and the second was a silent mutation. Due to the conservative nature of the base changes, no corrective mutagenesis was undertaken.

**Cloning domains that encode individual epitopes of TbpA.** Six regions of *tbpA*, most encoding putative loops of TbpA (Fig. 1), were cloned individually into the expression vector pET-34LIC. The oligonucleotides oVCU-3 and oVCU-4 (Table 2) were used to PCR amplify the *tbpA* region that encodes the putative L5 (Fig. 1). These primers, like the others listed in Table 2, contain a 3' region that anneals to *tbpA* and a 5' segment that anneals to the vector. After PCR amplification, the product was treated with T4 DNA polymerase and dATP according to the manufacturer's recommendations. In this way, 13- to 14-base “sticky ends” which were compatible with the ends of the commercially prepared vector were created. Annealed vector and template mixtures were transformed directly into *E. coli* strain NovaBlue (Novagen) (Table 1) according to the manufacturer's recommendations; transformants were selected in the presence of kanamycin. Recombinant plasmid pVCU301 (Table 1) expressed a translational fusion between amino-terminal fusion tags and the domain encoding putative L5 of TbpA (Fig. 1). Plasmids pVCU302 through pVCU306 (Table 1) were created similarly, using the *tbpA*-specific oligonucleotides listed in Table 2. A negative control

**FIG. 1.** Schematic representation of proposed topology model for gonococcal TbpA (5). The horizontal dashed lines represent the boundaries of the gonococcal outer membrane. Twenty-two amphipathic β-strands are predicted to cross the outer membrane to form a β-barrel. Putative surface-exposed loops (numbered L1 to L11) are located above the membrane plane. Epitopes that were overexpressed as fusion proteins in the present study are indicated by thick black lines, the end points of which are shown by short bars.
TABLE 2. Oligonucleotides used in this study

<table>
<thead>
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<th>Oligonucleotide</th>
<th>Sequencea</th>
<th>Amplifyingb</th>
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<tr>
<td>oVCU-2</td>
<td>5'-GACGAGCAGAAGTGCAAGCAGGACATGGTTCGCC3'</td>
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<td>oVCU-3</td>
<td>5'-GACGAGCAGAAGTGCAAGGACATGGTTCGCC3'</td>
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</tr>
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<td>L9-L10</td>
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<td>oVCU-15</td>
<td>5'-GACGAGCAGAAGTGCAAGGACATGGTTCGCC3'</td>
<td>L9-L10</td>
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</table>

a Underlined residues represent those that anneal to the pET-34 vector sequence.
b Denotes putative loop designation, corresponding to Fig. 1 and reference 5.

plasmid, pVCU307 (Table 1), in which the lacZ gene was translationally fused to the fusion tags in pET-34LIC, was constructed. The primers for this reaction were supplied by the manufacturer of the vector (Novagen). Because the CBD (cellulose-binding domain)-LacZ fusion protein was poorly expressed by recombinant E. coli (data not shown), subsequent experiments included CBD, expressed by pET-34 without an insert, as a negative control. The plasmid constructs (Table 1) were transformed into E. coli strain BL21(DE3) (Novagen) for expression of fusion proteins. This strain expresses an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T7 polymerase, which recognizes the promoter upstream of the fusion tags in the expression vector.

Heterologous protein expression and refolding. Cultures of recombinant E. coli strains were prepared by growing cells overnight in LB medium containing kanamycin (25 μg ml−1), ampicillin (50 μg ml−1), or kanamycin (30 μg ml−1) with chloramphenicol (12.5 μg ml−1). After 1:25 dilution in fresh medium containing the appropriate antibiotic, the cells were grown at 35°C to an A600nm of 0.7, which corresponds to approximately 3 × 10^8 to 5 × 10^8 cells ml−1. IPTG was added to a concentration of 1 mM, and the cells were grown for 4 h. Whole-cell lysates were prepared from samples collected before and after IPTG induction. Whole-cell proteins were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gels (20).

For protein fractionation, induced cultures were harvested by centrifugation (6,500 × g; 4°C; 15 min) and resuspended in inclusion body wash buffer (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 1% Triton X-100) (Novagen). Lysozyme (Sigma-Aldrich) was added to a concentration of 100 μg ml−1, and the resuspended pellets were incubated at 30°C for 15 min. Samples were sonicated on ice with a Branson sonifier. MgSO4, DNase (Promega), and RNase A (Sigma Aldrich) were added to the lysates at final concentrations of 12 mM, 1 U ml−1, and 10 μg ml−1, respectively, and the mixtures were incubated at room temperature for 20 min. The insoluble fraction was collected by centrifugation (10,000 × g; 10 min), and the inclusion body pellets were washed three times in the buffer described above. Dialyzed inclusion body preparations were processed as indicated above and developed by addition of 150 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) per well with incubation at room temperature for 1 min. Luminescence was detected by a Tropix TR717 microplate luminometer (FE Applied Biosystems). All assays were performed in triplicate.

**Purification of epitope fusions.** The epitope fusion proteins were purified with S-protein-agarose (Novagen) according to the manufacturer’s instructions. Brieﬂy, resuspended or dialyzed inclusion body preparations were added to S-protein slurry and incubated at 4°C overnight with gentle rocking. Following incubation, the entire volume was spun down at 500 × g for 10 min, and the unbound fraction was removed. The matrix was then washed three times with wash buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Triton X-100) and dialyzed as described above. The purified fusion proteins were eluted by addition of 3 M MgCl2 to the matrix. This mixture was allowed to incubate for 1 h at room temperature, and the eluate was collected. The eluates were then dialyzed in Slide-A-Lyzer cassettes (3,500-MW cutoff [Pierce]) against PBS and concentrated by evaporation. The starting material and subsequent purified product were analyzed by SDS-PAGE on 12% acrylamide gels and transferred to nitrocellulose for immunoblot analysis.

**Production of epitope-specific antiserum.** Two New Zealand White Elite female rabbits were immunized (Covance Research Products, Denver, Pa.) with each recombinant protein preparation by intradermal and subcutaneous routes. One injection of 250 μg of the dialyzed inclusion body preparation in Freund’s complete adjuvant was followed by five subsequent injections of 125 μg each in Freund’s incomplete adjuvant. These five boosts occurred on days 21, 42, 63, 84, and 105 after the initial immunization. The rabbits were exsanguinated 18 weeks and 5 days (131 days total) after the first immunization. Sera from individual rabbits were maintained separately and screened individually.

**Evaluating the immunogenicity of short epitopes of TbpA.** Whole-cell proteins from iron-stressed gonococcal strains FA6839 (TbpA- Lbp) [Table 1] and MCV9602 (TbpA- Lbp) [Table 1] were separated by SDS-PAGE and transferred to nitrocellulose (36). These lysates were probed with individual production bleed sera (10 weeks and 4 days after the first immunization) by immunoblotting as previously described (15). For these experiments, antiserum raised against full-length gonococcal TbpA (15) was used as a positive control. Bound polyclonal antibodies were detected by addition of a secondary goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Aldrich) followed by...
by development with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

RESULTS

Choice of TbpA-specific epitopes for overexpression. To assess the function and immunogenicity of putative loops of gonococcal TbpA, we created epitope fusions with two aminoterminal fusion tags, the CBD from Clostridium cellulovorans and the S tag (Novagen). We focused our initial efforts on designing constructs in which L5 or L4-L5 (Fig. 1) were translationally fused to the affinity tags. This focus was due to several compelling findings. First, to test our hypothetical topology model of gonococcal TbpA, we previously generated polyclonal anti-peptide sera in mice that were directed at putative surface-exposed, antigenic regions of gonococcal TbpA (5, 11). Out of the seven anti-peptide sera generated, only four recognized full-length TbpA in immunoblots, and the one that was specific for putative L5 sequence reacted with whole iron-stressed gonococci in a dot blot assay (11). This result suggested that at least part of the putative L5 was surface exposed. In another study, we individually deleted loops 4 and 5 from TbpA (5) and found that with the loss of either of these predicted loops, the resultant mutants were incapable of binding transferrin in solid- or liquid-phase transferrin binding assays. Together, these findings suggested that L5 was in fact surface exposed and that both L4 and L5 were inherently important for ligand binding. Finally, we did a pairwise comparison (5) between TbpA and the E. coli TonB-dependent receptor, FepA, whose crystal structure had recently been elucidated (7). From this comparison, we found that putative L5 fell within the same region as the FepA ligand binding domain (11, 28).

We used various criteria to design the other four fusion constructs (Fig. 1). We concentrated on regions of TbpA that had predicted hydrophilicity, antigenicity, and surface exposure, because these were the most likely to be involved in ligand interaction. First, we chose regions that contained most of the peptide sequences used in the previous immunizations (11); however, we added length. Whereas the peptides ranged from 11 to 14 residues, the TbpA sequences in the fusion constructs ranged from 62 to 140 amino acids in length. Additionally, we previously evaluated the sequence diversity of TbpA proteins from strains of pathogenic neisseriae, as well as from other species, including Haemophilus influenzae, Actinobacillus pleuropneumoniae, Pasteurella haemolytica, and Moraxella catarrhalis (11). From this analysis, we identified four hypervariable domains within the TbpA sequence and suggested that these areas were surface accessible (11). Accordingly, L2 represents a stretch of TbpA that is predicted to be hydrophilic and encodes a hypervariable domain (11). L2 also contains one of the three pairs of cysteine residues found within the protein that may form a surface-exposed disulfide bond (7, 11, 21). The L3 sequence also contains a hypervariable domain and is predicted to be on the surface (11). We also focused on the other end of the protein and designed a construct consisting of loops 9 and 10 (L9-L10), whose sequence is somewhat divergent among the neisseriae and which is predicted to be surface accessible (11). Finally, we chose to use sequence from the putative plug region, which has homology with the FepA globular plug domain (7). The TbpA-specific epitopes chosen for subsequent analysis are graphically depicted in Fig. 1.

Expression and localization of epitope fusion proteins. The fusion clones were produced by PCR amplification of tbpA-specific regions, followed by cloning into the expression vector, pET-34 (Table 1), to allow production of recombinant proteins in E. coli. Recombinant plasmids (Table 1) were used to transform E. coli strain BL21(DE3). The recombinants were grown in liquid culture and induced with IPTG, which resulted in expression of proteins ranging in mass from approximately 33 to 43 kDa after 4 h (Fig. 2A). To determine if the recombinant proteins were expressed in soluble or insoluble form, the induced cells were disrupted by sonication and solubilized in Triton X-100, and subsequent fractions were analyzed by SDS-PAGE. All of the overexpressed proteins, with the exception of the plug construct (which was localized to the cytosolic fraction), were enriched in inclusion body fractions (Fig. 2B and data not shown).

Transferrin binding by L5 and L4-L5. After overexpressing all of the epitope fusions, we assessed their ligand binding functions in a solid-phase dot blot assay. As shown in Fig. 3, the L5 fusion expressed by BL21(DE3)(pVCU301) bound HRP-Tf, as did the L4-L5 fusion expressed by BL21(DE3) (pVCU302), although the latter appeared to do so with more intensity. The other fusion constructs demonstrated virtually no binding of HRP-Tf above background levels attributable to the negative control (β-galactosidase [Fig. 3]). The L9-L10 fusion expressed by BL21(DE3)(pVCU306) demonstrated low-level binding at the highest concentration tested but showed no activity with lower concentrations. This preliminary evidence suggested that L5 and L4-L5, out of the context of the intact receptor, retained transferrin binding capabilities.
Specificity of transferrin binding by L5 and L4-L5. To determine if this binding phenomenon was dose dependent and specific, we analyzed the L5 and L4-L5 fusions in solubilized and in renatured forms by solid-phase dot blot analysis. Dialyzed inclusion body preparations expressed by BL21(DE3) containing vector alone (CBD) were used as a negative control (Fig. 4). Total membrane preparations from gonococcal strain FA19 were used as a positive control (Fig. 4). In the case of L5, the solubilized inclusion body preparations demonstrated concentration-dependent binding that was specific for human transferrin (Fig. 4). In the presence of excess unlabeled human transferrin, binding of HRP-Tf was eliminated or greatly diminished. The dialyzed, renatured inclusion body preparations demonstrated somewhat-increased ligand binding relative to the undialyzed preparations, which was likewise dose dependent and specific. The L4-L5 construct exhibited some binding activity that was dose dependent and specific (Fig. 4), but the results were more variable and less reproducible with both the solubilized and renatured forms. In total, these results indicated that L5, and possibly L4 as well, contained specific transferrin binding epitopes that are potentially critical for ligand binding to full-length TbpA.

Ligand binding by L5 and L4-L5 in a chemiluminescent solid-phase binding assay. The dot blot assays allowed us to demonstrate specific binding, but not in a quantitative fashion. We subsequently developed and utilized a chemiluminescent solid-phase assay to measure the transferrin binding activity of each epitope fusion. Dialyzed inclusion body preparations were bound to microtiter plates and processed as described in Materials and Methods. The results from three independent assays are shown in Fig. 5 and were consistent with the results of the screening dot blot shown in Fig. 3. The L5 fusion (Fig. 5A) displayed specific, dose-dependent ligand binding; however, the L4-L5 preparation (Fig. 5B) was more variable and required the addition of 2.5-fold more protein. The other epitope fusions (Fig. 5C to F) did not display any significant binding compared to the negative control, in which we used inclusion body preparations containing CBD alone (the fusion partner) (Fig. 5G). As a positive control, we used inclusion body preparations containing overexpressed, full-length rTbpA (Fig. 5H).

Specificity of ligand binding by L5 and L4-L5 in a chemiluminescent solid-phase assay. Specific transferrin binding in the chemiluminescent assay was calculated by subtracting the non-specific binding (detected in the presence of competitor) from total binding for each of the two fusion constructs that bound ligand, L5 and L4-L5 (Fig. 5A and B). The specific binding attributable to each of these fusion proteins is displayed graphically in Fig. 6. The L5 construct demonstrated saturable, specific transferrin binding with a capacity that was between two- and threefold higher than that of the L4-L5 fusion.

Purification of epitope fusions using S protein-agarose. We initially planned to purify the epitope fusions via their affinity tags for use in subsequent immunizations. We achieved limited purification of all fusions using the S-tag fusion partner. The purified products also contained some contaminating proteins, as analyzed by SDS-PAGE, and some breakdown products that reacted with anti-CBD tag polyclonal sera (data not shown). However, the yield of purified protein (ranging from 0.04 to 1.96 mg of purified protein ml⁻¹ per 250 ml of induced culture) was major deterrent. We therefore decided to use...
dialyzed inclusion body preparations as immunogens, since contaminating *E. coli*-reactive antibodies could be absorbed out postproduction.

**Immunogenicity of TbpA-specific epitopes.** To determine if the sera generated against defined epitopes of gonococcal TbpA recognized denatured full-length TbpA, we performed immunoblots probing whole-cell lysates of strains FA6839 (TbpA⁺ Lbp⁺ [Table 1]) and MCV602 (TbpA⁻ Lbp⁻) with the epitope-specific sera. We used Lbp mutants to eliminate cross-reactivity between the anti-TbpA sera and gonococcal
LbpA (15). All of the fusion sera reacted with TbpA to different degrees, with the exception of the anti-L9-L10 serum and the anti-CBD serum (Fig. 7A). The plug and L2 constructs appeared to have generated more-vigorous immune responses than did the other fusions. The sera reacted minimally with other proteins expressed by the isogenic TbpA/H11002 mutant (Fig. 7B), indicating that each was specific for its target antigen, TbpA. All of the sera were capable of recognizing their respective immunizing epitope fusions in immunoblots (data not shown).

**DISCUSSION**

In this study, we assessed the possibility that specific regions of gonococcal TbpA participate in transferrin binding and that these limited domains could elicit a defined, epitope-specific immune response. Our recently described TbpA topology model (5) aided us in predicting putatively immunogenic ligand binding domains. The regions chosen for the present study had the following characteristics in common. With the exception of the plug construct, all fusions included regions of TbpA predicted to form extracellular loops. These regions were rich in antigenic and sequence variability and thus are likely to be surface exposed and potentially involved in ligand binding. Several fusions contained paired cysteine residues, which might serve to stabilize or constrain the loop structure. All fusion constructs contained regions of TbpA with high antigenicity index values (18). Finally, all of the fusions contained peptide sequences for which we previously generated peptide-specific antisera (11). Peptide sequence located within putative L5 (in the fusion protein expressed by pVCU301) was previously shown to generate antibodies that react with intact gonococci, implicating this region as a surface-exposed epitope (5, 11). Thus, we focused our early efforts on determining whether L5 was also capable of ligand binding.

Diverse methods have been used to define the ligand binding domains of TbpB proteins expressed by several species, with various degrees of success. For example, we (12) constructed amino- and carboxy-terminal deletions of gonococcal TbpB which were translationally fused to the α-peptide and overexpressed in E. coli. These truncated TbpB proteins were then tested for transferrin binding capability in ligand binding Western blots. This method identified an amino-terminal domain of approximately 400 residues within gonococcal TbpB that retained transferrin binding capabilities. Vonder Haar et al. identified a similarly sized stable transferrin binding domain in meningococcal TbpB (40). Strutzberg et al. (34) used TpfaΔ mutagenesis and a peptide spot approach to map functional regions of Tfa (a TbpB homologue) of A. pleuropneumoniae to three different regions of the amino-terminal half of the protein. The group subsequently generated multiple antigenic peptides containing these binding domains and raised sera against them. Ultimately, they found that the peptide constructs inhibited binding of transferrin to recombinant Tfa but the anti-peptide antibodies did not (33). In another approach, Renault-Montgenie et al. identified binding domains within meningococcal TbpB with both a peptide spot approach and a translational fusion expression system (29). With these methods, they isolated and characterized binding sites in both the amino and carboxy termini of the TbpB protein.

![FIG. 6. Specific transferrin binding to L5 and L4-L5 fusions in solid-phase chemiluminescent transferrin binding assay. (A) Specific transferrin binding to L5 fusion (Loop 5) compared to CBD alone (CBD control). (B) Specific transferrin binding to L4-L5 fusion (Loops 4 + 5) compared to CBD alone. RLU, relative luminescence units.](http://iai.asm.org/)

![FIG. 7. Immunoblots showing specificities of antisera raised against TbpA-specific epitopes. (A) Immunoblot of whole-cell lysates from gonococcal strain FA6839 (TbpA+ Lbp). (B) Immunoblot of whole-cell lysates from gonococcal strain MCV602 (TbpA+ Lbp+). For both blots, the lanes were probed with antisera raised against TbpA-specific epitopes as indicated at the top. The arrowheads indicate the positions of gonococcal TbpA. Lanes −, negative control to which no primary antibody was added; lanes +, positive control, which was probed with an antiserum against holo-TbpA (15). The image was acquired using a Hewlett-Packard Scan Jet 4C and annotated using Adobe PhotoShop version 4.0.](http://iai.asm.org/)
In contrast to the wealth of TbpB ligand-binding studies, the present study is the first to attribute transferrin binding to individual domains of TbpA expressed by any species. These TbpA-specific epitopes were capable of saturable, specific binding of human transferrin, which was surprising, since the excised domains of TbpA were out of the context of the intact receptor. Moreover, we could reconstitute ligand binding to TbpA fragments isolated from inclusion body preparations. Production of recombinant proteins in inclusion body form is generally avoided because of the difficulty in manipulating proteins to their functional states (see references 22 and 32 for reviews). We found, however, that renatured inclusion body preparations containing L5 bound transferrin in a dose-dependent and specific manner. This coincides well with the results of our previous deletion mutagenesis study, which showed that deletion of L5 abolished transferrin binding and transferrin-iron uptake (5). The deletion analysis also implicated L4 as a critical determinant for transferrin binding (5). In the present study, the fusion protein consisting of the L4-L5 construct displayed specific binding, but it was much more variable than the activity attributable to L5 alone. This variability may be due to several compromising factors, such as the increased length of the L4-L5 construct (140 versus 69 residues), the increased number of putative transmembrane domains in the L4-L5 construct (140 versus 69 residues), the increased length in this particular construct did not appear the immune system until the presence of the receptor is sensed. This produces a conformational change in the loop, which exposes the conserved interior (10, 31). Further characterization of L5 in and out of the context of the entire receptor is needed before we can discern the true relevance of the juxtaposition of conservation and diversity observed in this bacterial system.

The TbpA-specific fusion constructs generated in this study were used as immunogens for the generation of epitope-specific antisera. We found that all except one fusion protein elicited antibodies that recognized full-length TbpA on Western blots. It is unclear why the L9-L10 fusion did not elicit a response that was reactive against full-length TbpA; however, it should be noted that the anti-peptide sera directed against L10 generated in our previous study likewise did not recognize full-length TbpA (11). The sequence contained in the L9-L10 fusion is relatively invariant, contains none of the defined hypervariable domains, and is predicted to include two conserved transmembrane strands (11). It is possible that this domain is simply not as immunogenic as predicted; however, the serum elicited against the L9-L10 fusion did recognize the immunizing antigen. We postulated that by adding length, the loop fusions would elicit a more vigorous immune response in animals than did the preceding peptide immunogens. However, the additional length in this particular construct did not appear to enhance the immune response in the present study.

TbpA has been proposed as a vaccine candidate, most recently by West et al. for immunophylaxis against Neisseria meningitidis (42). Earlier, Danve et al. had suggested that TbpA-specific antibodies were not bactericidal against meningococci (17); however, West et al. demonstrated that sera generated against recombinant TbpA, while less bactericidal, were more cross-protective in an intraperitoneal mouse model than those generated against recombinant TbpB (42). Thus, the more conserved of the Tbp proteins was capable of eliciting a cross-reactive, protective immune response. Antigenically variable epitopes have been recognized as immunodominant in neisserial porins (35, 37), in neisserial FpB (38) (now called FetA; [9]), and in meningococcal TbpB (30); however, these immunodominant epitopes have also been shown to elicit strain-specific antibody responses that did not confer broadly cross-reactive biological activities (30, 35, 37, 38). Thus, in the search for a cross-reactive response, we may be more successful identifying immunorecessive epitopes that could be “unmasked” by removal of the immunodominant, antigenically variable residues. Van der Voort et al. (39) found that deleting specific loops of meningococcal porin skewed the immune response toward constant, immunorecessive epitopes against which bactericidal antibodies were generated. Because the targets were more conserved among strains, these authors suggested that the response could be more cross protective (39).

In principle, the Tbp proteins may be similar in that if we could molecularly excise the constant, immunorecessive domains and fuse the epitopes with an appropriate adjuvant, we might be able to generate a cross-reactive immune response that is broadly protective. In light of these possibilities, future studies will be directed at characterizing the immune responses elicited against the TbpA-specific epitopes described in the present study, including determination of bactericidal or op-
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