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Bacterial lactoferrin-binding protein A binds to both domains of the human lactoferrin C-lobe

Henry Wong and Anthony B. Schryvers

Bacterial Pathogenesis Research Group, Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, Rm 274, Heritage Medical Research Building, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

Correspondence
Anthony B. Schryvers
schryver@ucalgary.ca

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Pathogenic bacteria in the family *Neisseriaceae* express surface receptors to acquire iron from the mammalian iron-binding proteins. Transferrins and lactoferrins constitute a family of iron-binding proteins highly related in both sequence and structure, yet the bacterial receptors are able to distinguish between these proteins and uphold a strict binding specificity. In order to understand the molecular basis for this specificity, the interaction between human lactoferrin (hLf) and the lactoferrin-binding protein A (LbpA) from *Moraxella catarrhalis* was studied. A periplasmic expression system was designed for the heterologous expression of LbpA, which enabled the investigation of its binding activity in the absence of lactoferrin-binding protein B (LbpB). To facilitate delineation of the LbpA-binding regions of hLf, chimeric proteins composed of hLf and bovine transferrin were made. Binding studies performed with the chimeric proteins and recombinant LbpA identified two binding regions within the C-terminus of hLf. Furthermore, native LbpA from *Moraxella* and *Neisseria* spp. bound the identical spectrum of hybrid proteins as the recombinant receptor, demonstrating a conserved binding interaction with the C-lobe of hLf.

INTRODUCTION

Iron is an essential element for the growth of most organisms (Bullen & Griffiths, 1987). Due to its relative insolubility and potential toxicity under aerobic conditions, specific systems for transport, utilization and storage of this element have been developed. The majority of iron in mammalian hosts is sequestered within intracellular compartments where it is bound to ferritin or, in erythrocytes, complexed with haemoglobin. Trace amounts of extracellular iron are seized by the host's glycoproteins, transferrin and lactoferrin, reducing the effective concentration of iron to 10^{-18} M (Weinberg, 1978). Transferrin is the predominant iron-binding protein in serum, while its homologue, lactoferrin, is found in milk, on mucosal surfaces, and at sites of infection.

Iron scavenging by secretion of low-molecular-mass compounds, siderophores, has been well documented in a variety of micro-organisms (Winkelmann, 2002). Gram-negative bacteria from the family *Neisseriaceae* do not synthesize siderophores, although pathways for utilization of exogenous siderophores appear to be present in some species (Carson *et al.*, 1999). In contrast, members of the *Neisseriaceae* express a myriad of surface receptors that directly bind the host proteins haemoglobin, transferrin and lactoferrin and mediate iron uptake from these sources (Schryvers & Stojiljkovic, 1999). The bacterial receptors for transferrin and lactoferrin are distinct receptor complexes. Furthermore, there is a host specificity associated with these receptors in that human-specific pathogens cannot utilize ligands from other mammalian species (Schryvers & Gonzalez, 1990).

Apart from the difference in the ligand, transferrin- and lactoferrin-mediated iron acquisition in these pathogenic bacteria are considered functional homologues with a conserved mechanism of action (Gray-Owen & Schryvers, 1996). The bacterial transferrin or lactoferrin receptor complex consists of two outer-membrane proteins: a TonB-dependent integral membrane protein (TbpA or LbpA) and a peripheral lipidated protein (TbpB or LbpB) (Gray-Owen & Schryvers, 1996; Schryvers & Wong, 2000). Inactivation of TbpA or LbpA abrogates iron uptake from transferrin and lactoferrin, respectively, confirming their essential and central role in the uptake process (Cornelissen *et al.*, 1992; Pettersson *et al.*, 1994). The lipoprotein component of the transferrin receptor complex, TbpB, is capable of independently binding ligand and undoubtedly participates in the iron-uptake process, yet utilization of transferrin iron can occur in its absence (Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995).

The ligand-binding properties of LbpB and its contribution to utilization of lactoferrin-bound iron have not been convincingly demonstrated (Bonnah & Schryvers, 1998; Bonnah *et al.*, 1999). Initial attempts at characterizing the interaction between lactoferrin and its receptor demonstrated that

Abbreviations: bTf, bovine transferrin; hLf, human lactoferrin; HRP, horseradish peroxidase.

deglycosylated human lactoferrin retained its binding properties (Alcantara *et al.*, 1992). This established that the *N*-linked oligosaccharide side chains are not required for binding and the interaction is mediated primarily by the surface polypeptide regions. Subsequent experiments demonstrated that proteolytically derived N- and C-lobe halves of hLf were capable of binding to the lactoferrin receptor protein from *Moraxella catarrhalis* (Yu & Schryvers, 1993). In contrast, TbpAs were shown to primarily bind to the C-lobe of transferrins (Yu & Schryvers, 1994). However, the experiments with lactoferrin receptors were performed when it was believed that the lactoferrin receptor from *M. catarrhalis* only consisted of a single protein. Subsequent realization that LbpA and LbpB from *M. catarrhalis* are very similar in molecular mass, and that they were masked as a single protein band on SDS-PAGE (Bonnah *et al.*, 1998), dictated the need to re-examine the receptor–ligand interaction.

Utilizing the high degree of sequence and structural homologies between transferrins and lactoferrins (Baker *et al.*, 1987), we have generated a series of hybrid human lactoferrin/bovine transferrin (hLf/bTf) proteins. In this study we used these hybrid proteins, and a novel recombinant form of LbpA, to investigate the interaction between hLf and LbpA.

METHODS

Bacterial strains, plasmids, insect cell line and growth conditions. All subcloning steps and plasmid isolations were carried out with *Escherichia coli* strain DH5αF′. The hybrid genes for generating chimeric hLf/bTf are described in Fig. 1 and Fig. 2. *E. coli* cultures were propagated on Miller's LB agar at 37 °C or in Miller's LB broth base. *Moraxella catarrhalis* strain N141 was obtained from Dr S. Ainsworth, Veterans' Administration Hospital, Shreveport, LA, USA. *M. catarrhalis* strain Q8 (N157) was provided by Dr M. G. Bergeron, Department of Microbiology, University of Laval, Quebec, Canada. The Q8 *lbpB* mutant (N182) was derived from N157 by natural transformation as described by Bonnah *et al.* (1999). The *Neisseria meningitidis* B16B6 *tbpA*::*erm tbpB*::*kan lbpB*::*gent* mutant (N193) used for this study has been characterized previously (Bonnah & Schryvers, 1998).

hLf cDNA, as a pGEMT construct, was kindly provided by Dr J. Tweedie, Massey University, New Zealand, and bTf cDNA was amplified from pBBbTf1, which has been previously described (Retzer *et al.*, 1996). *Spodoptera frugiperda* cells (SF21) and their culturing medium, SF900 II SFM, were purchased from Gibco-BRL.

M. catarrhalis and *N. meningitidis* cells were propagated on chocolate agar plates at 37 °C with 5 % CO_2 prior to being grown in suspension using O'Reilly-Niven broth (ORN: 0·1 M NaCl, 10 mM KCl, 10 mM Na_2HPO_4, 20 g tryptone l^{-1}, 5 g yeast extract l^{-1}), supplemented with 5 mM β-nicotinamide adenine dinucleotide (β-NAD) and 0·2 % glucose. All media were supplemented with the appropriate antibiotics prior to inoculation, and liquid cultures were incubated at 37 °C with aeration by shaking at 200 r.p.m.

For iron starvation of *Neisseriaceae*, fresh colonies (cultured as described above) were used to inoculate 15 ml ORN broth. Once the OD_{600} reached 0·1, the culture was transferred into a 50 ml flask of pre-warmed broth. When the OD_{600} reached 1·0, the culture was used to inoculate a 1 l flask of pre-warmed broth. The initial OD_{600} was recorded, and when cells had divided twice, ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA, Sigma) was added to a final concentration of 100 μM. Iron-starved bacterial cultures were then allowed to grow until stationary phase was reached.

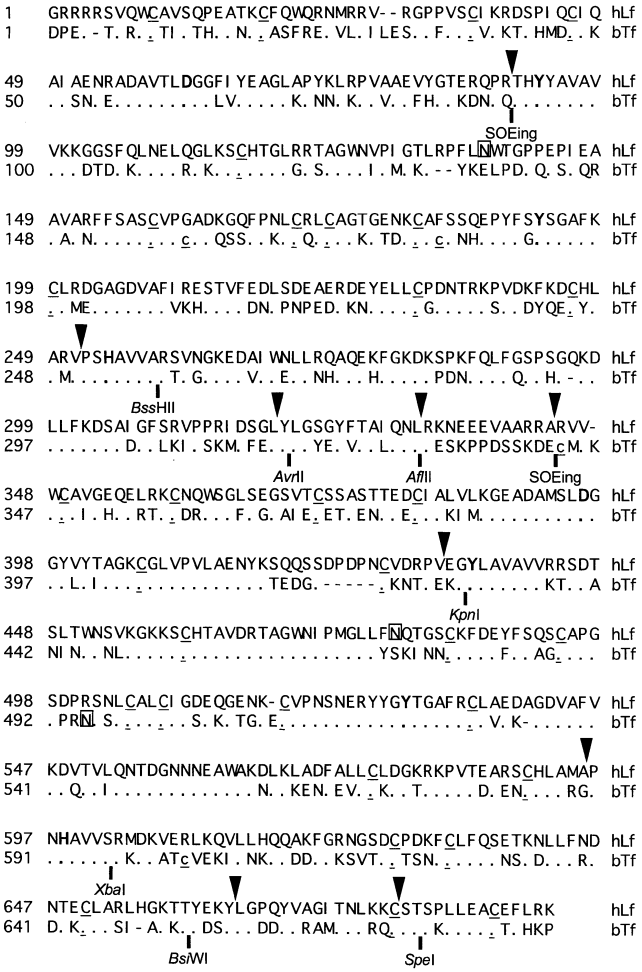

Fig. 1. Sequence alignment between hLf and bTf. The mature hLf and bTf amino acid sequences are shown in single-letter code, with the residue position and sequence name indicated to the left and right, respectively. Conserved residues in the bTf sequence are represented as periods. Gaps inserted into either the hLf or the bTf in order to achieve the optimum alignment of the sequences are indicated by hyphens. The iron-binding residues are in bold, the cysteines are underlined and the native glycosylation sites are boxed. The bTf cysteines forming disulfide bridges N11 and C12 are indicated in lower-case lettering. Solid triangles indicate the junction sites (Anderson *et al.*, 1989). The unique restriction sites and SOEing positions incorporated into both cDNAs are as illustrated.

Preparation of hybrid hLf/bTf proteins. Unique restriction sites were introduced by designing oligonucleotide primers with the restriction recognition sequence at the 5′ terminus. Using pGEM-hLf and pBlueBacIII-bTf as templates, the various regions from the two coding sequences were amplified with *Pfu* polymerase (Stratagene) and the mutagenic primers. Three regions (α.1, α.2 and tail) were

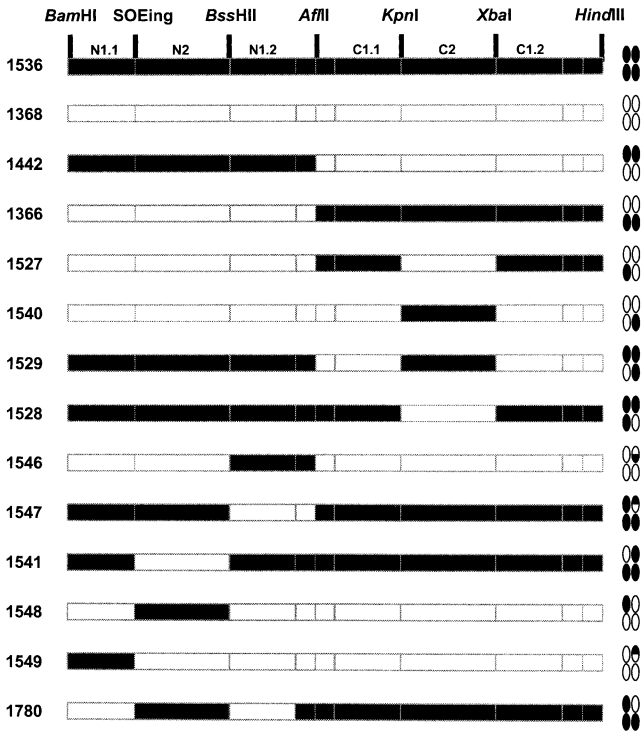

Fig. 2. Schematics of hybrid hLf and bTf constructs. The domain structure of lactoferrin is illustrated as a linear polypeptide schematic with the unique restriction sites engineered in both cDNAs indicated at the top. The composition of the chimeric constructs is shown with hLf sequence in black and bTf sequence in white. The construct numbers are indicated on the left and the cartoon representation on the right. The N-lobe of hLf encompasses residues 1–320, which is subdivided into two domains. Domain 1 (N1) is non-contiguous; it consists of residues 1–90 (N1.1) and 252–320 (N1.2). The intervening domain 2 (N2) encompasses residues 91–251. The N-lobe is connected to the C-lobe by an interdomain/bridge region, which is designated as α.1 (residues 321–332) and bridge (333–344). Similarly, the C-lobe is also composed of two domains, with domain 1 being non-contiguous (residues 345–433 [C1.1]; 596–663 [C1.2]) and domain 2 representing residues 434–595 (C2). The C-tail region following the C-lobe sequences is a homologue of the interdomain bridge region. It is also subdivided into two regions: α.2 (residues 664–678) and tail (residues 679–691). For simplicity, the N1, N2, C1 and C2 domains are represented by the four ovals in a counterclockwise direction, respectively. The top and bottom halves of the top right oval represent the N1.1 and N1.2 regions, respectively in constructs 1546, 1547, 1549.

too small to reliably amplify and subclone, so they were initially amplified with the adjoining region. Standard amplification reactions were as follows: 30 cycles of 60 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C. The amplified products were purified with PCR Clean-up Kit (Qiagen) according to the manufacturer's instruction. The purified product was A-tailed by incubation with *Taq* polymerase (Gibco-BRL) for 15 min at 72 °C with dATP as the only nucleotide to facilitate subcloning into pGEM-T (Promega). Reassembly of the resulting gene subfragments was achieved by ligating contiguous fragments via the newly incorporated restriction sites. Once the cDNAs were reassembled, they were re-amplified in three segments (N1.1–α.1, bridge–α.2 and tail–part of the vector) in order to incorporate the *Avr*II and *Spe*I sites, which are located in between N1.2 and α.1, α.2 and tail, respectively.

The hybrid constructs were expressed as secreted proteins using the Bac-to-Bac Baculovirus Expression System (Gibco-BRL) according to the manufacturer's instructions. SF21 cells were maintained in SF900 II SFM medium as suspension cultures at an initial cell density of 3×10^5 cells ml^{-1}. Insect cells were cultured at 27 °C with gentle agitation (140 r.p.m), and were allowed to reach $2–3 \times 10^6$ cells ml^{-1} (72–96 h) before they were re-seeded at a density of 3×10^5 cells ml^{-1}. SF21 cells were infected at an m.o.i. of 10 and incubated for 96 h for protein expression.

The recombinant proteins were purified by concentrating the clarified culture supernatant 10-fold with PEG 8000 (Fisher Scientific). The concentrate was dialysed with three changes of concanavalin A buffer (0·1 M sodium acetate, 1 M NaCl, 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$, pH 6·0) at 4 °C. Subsequently, the glycosylated protein was purified from the concentrate with concanavalin A Sepharose (Sigma). Non-specifically bound proteins were removed by washing with 5–10 column volumes of concanavalin A buffer. The bound protein was eluted with 2 column volumes of concanavalin A buffer containing 0·2 M methyl α-D-mannopyranoside (Sigma). The eluate was concentrated with PEG 8000 to approximately 5 ml, and then dialysed with four changes of citrate/bicarbonate buffer (0·1 M sodium citrate, 0·1 M sodium hydrogen carbonate, pH 8·4) at 4 °C. The hybrid proteins were iron-loaded in the presence of ferric chloride (20 mg $FeCl_3$ ml^{-1} prepared in 0·1 M sodium citrate, 0·1 M sodium hydrogen carbonate, pH 8·4). The excess iron was removed by repeated dialysis in citrate/bicarbonate buffer.

Labelling and immobilization of lactoferrin. hLf (Sigma) was conjugated to horseradish peroxidase (HRP, Sigma) to detect binding to the bacterial receptors by a previously described method (Schryvers & Lee, 1993). Covalent linkage of transferrin and lactoferrin to CNBr-activated Sepharose 4B (Pharmacia) was performed as described by the manufacturer's instructions and prior publication (Schryvers & Lee, 1993).

Expression of LbpA. The *lbp*A region was originally amplified from a cloned *M. catarrhalis* Q8 *lbp* operon insert in the pBlueScript plasmid (McLDW1) (Du *et al.*, 1998). The gene was amplified to introduce a *Xmn*I site at the predicted signal cleavage site of LbpA. The new construct was sequenced and then subcloned downstream of the β-lactamase (*bla*) gene in a pT7-7 vector (Novagen) that was modified to facilitate production of a fusion protein. A polyhistidine region and a consensus biotinylation sequence (Schatz, 1993) were introduced between the *bla* and *lbp*A genes to create a functional fusion protein (unpublished data). The *lbp*A gene with preceding polyhistidine and consensus biotinylation sequences was subsequently subcloned into the pMal-p2 vector (New England Biolabs) to generate a fusion with the periplasmic maltose-binding protein. Bacterial membranes containing recombinant LbpA or the native lactoferrin receptors were prepared as previously described (Schryvers & Morris, 1988).

Binding assays. For solid-phase binding assays, total membrane preparations were diluted to approximately 1 mg ml^{-1} total protein concentration and 2 μl spots were applied onto a mixed cellulose ester membrane support (HA paper, Millipore). The spots were air-dried and blocked with blocking buffer [0·5% non-fat dry milk (Bio-Rad) in Tris-buffered saline (TBS: 50 mM Tris, pH 7·5; 150 mM NaCl)] for 30 min. Serial dilutions of a 100 μg ml^{-1} control or chimeric protein solution were prepared by dilution with an equal volume of blocking buffer. Samples (100 μl) of the serially

diluted protein solutions were incubated with the immobilized total membrane for 1 h at 37 °C. Subsequently, HRP-hLf was added at a dilution of 1 : 500 in blocking buffer, and incubated for 1 h. The blot was washed with TBS and developed with 4-chloro-1-naphthol (Bio-Rad) according to the manufacturer's instructions.

For the isolation of LbpA, the standard high-stringency isolation procedure involved solubilization of membrane proteins from approximately 2 mg of total membrane in 2 ml solubilization buffer [50 mM Tris pH 8·0, 1 M NaCl, 10 mM EDTA, 0·75 % *N*-lauroyl sarcosine (Sigma)] for 1 h with gentle agitation. The insoluble debris was removed by centrifugation at 10 000 ***g*** for 10 min. The receptor-containing supernatant was incubated with excess amounts of chimeric proteins for 1 h with gentle agitation. An equivalent amount of lactoferrin-Sepharose was then added to the mixture to capture the unbound receptor protein. Subsequently, the resin was collected by centrifugation at 1000 ***g*** for 3 min and washed three times with 1 ml wash buffer (50 mM Tris pH 8·0, 1 M NaCl, 10 mM EDTA, 0·25 % Sarkosyl and 250 mM guanidine.HCl). The resin was washed with 1 ml water prior to protein elution by boiling in a small volume of Laemmli sample buffer (Laemmli, 1970) containing 0·2 M DTT. The isolation procedure was carried out at room temperature.

RESULTS

Generation of chimeric transferrins

In order to systematically define the regions of hLf that interact with LbpA, we prepared a series of chimeric proteins containing sequences derived from hLf (which binds LbpA) and bTf (which does not bind LbpA). Amino acid sequence alignments between hLf and bTf were made in order to identify the consensus interdomain and interlobe junctions (Anderson *et al.*, 1989). Alignment of the cysteines and iron-binding residues (Fig. 1) suggested that proper protein folding and iron-binding properties would be maintained in these chimeras. Once the junction sequences were aligned, silent mutations were designed to introduce unique restriction sites within these regions, thereby facilitating subsequent gene splicing. There were two junctions (between region 1.1 and region 2 in the N-lobe, and between the bridge and region 1.1 in the C-lobe) in which no unique restriction sites could be incorporated. Splicing in these regions was accomplished by the SOEing approach (Horton *et al.*, 1990). bTf has two additional disulfide bridges designated N11 and C12 (Metz-Boutigue *et al.*, 1984), which bridge within the N2 domain and across the C1.1 and C1.2 domains (Fig. 2, lower-case letters in Fig. 1). These disulfide pairings were maintained in all the constructs such that the disulfide network was not compromised.

A PCR-based mutagenesis approach was used in which the restriction enzyme recognition sequence was incorporated at the 5′ end of the oligonucleotide primers. The resulting products were sequenced and confirmed to be essentially identical to the original cDNA templates. However, several non-conservative changes were identified in the original template DNAs by comparison with GenBank sequences [U07643 (hLf) and U02564 (bTf)]. Mutations K29R, S335N and N539D were found in the hLf sequence, and mutations A132G, G419D, E420G and D450G were found in the bTf sequence. Since these templates had been used to produce fully functional recombinant protein, it was deemed unnecessary to rectify these changes. The fragments were assembled into full-length cDNAs with the newly incorporated restriction sites for domain swapping.

Once the two full-length cDNAs were assembled, hybrid genes were generated by exchanging individual lobe- or domain-coding sequences. Fourteen hybrid genes were made; their compositions are illustrated in Fig. 2. The chimeric proteins were expressed by the Bac-to-Bac Baculovirus Expression System (Gibco-BRL). In order to confirm the chimeric nature of these constructs, the recombinant proteins were tested for reactivity against polyclonal antisera for hLf and bTf. As illustrated in Fig. 3, the recombinant proteins are similar in molecular mass to the commercially available hLf and bTf, and all the constructs can be detected by one or both of the antisera. As expected, the commercial preparation of bTf resolved as two bands on SDS-PAGE due to a maturational proteolytic cleavage event (Maeda *et al.*, 1980).

Preliminary characterization of recombinant hLf and bTf

Total membranes were prepared from iron-starved *M. catarrhalis*, a human pathogen, and *Moraxella bovis*, a bovine pathogen. Both of these pathogens have transferrin and lactoferrin receptors. In competitive solid-phase binding experiments, recombinant hLf (1536) was able to specifically block the binding of labelled commercial hLf, but not labelled human transferrin, to *M. catarrhalis* membranes and had no effect on binding of bTf or bovine Lf by *M. bovis* membranes. Similarly, recombinant bTf (1368) blocked binding of labelled bTf but not labelled bovine Lf to *M. bovis* and had no effect on binding experiments with *M. catarrhalis* (data not shown). This indicates that the recombinant hLf and bTf attained sufficient structural integrity to be distinguished by the bacterial receptors.

Expression of recombinant lactoferrin-binding protein A

Difficulties in expression of the native *lbpA* gene in *E. coli* and the reduced expression of LbpA in *lbp*B isogenic mutants (Bonnah *et al.*, 1999; Lewis *et al.*, 1998) prompted us to explore alternative sources of LbpA for our experiments. Initially we chose the TEM-1 β-lactamase (*bla*) gene as an N-terminal fusion partner so that we could select for export to the periplasm. We included a polyhistidine region and a consensus biotinylation sequence in the linker region between the β-lactamase and LbpA for subsequent purification by metal-chelate chromatography (Hochuli *et al.*, 1987) and labelling by the *E. coli* BirA enzyme (Cull & Schatz, 2000). The expression of functional LbpA was confirmed by Western blot analysis and binding of horseradish-peroxidase-conjugated hLf (HRP–hLf) by intact cells (data not shown). Cell fractionation experiments confirmed that LbpA was exclusively in the outer-membrane fraction. To

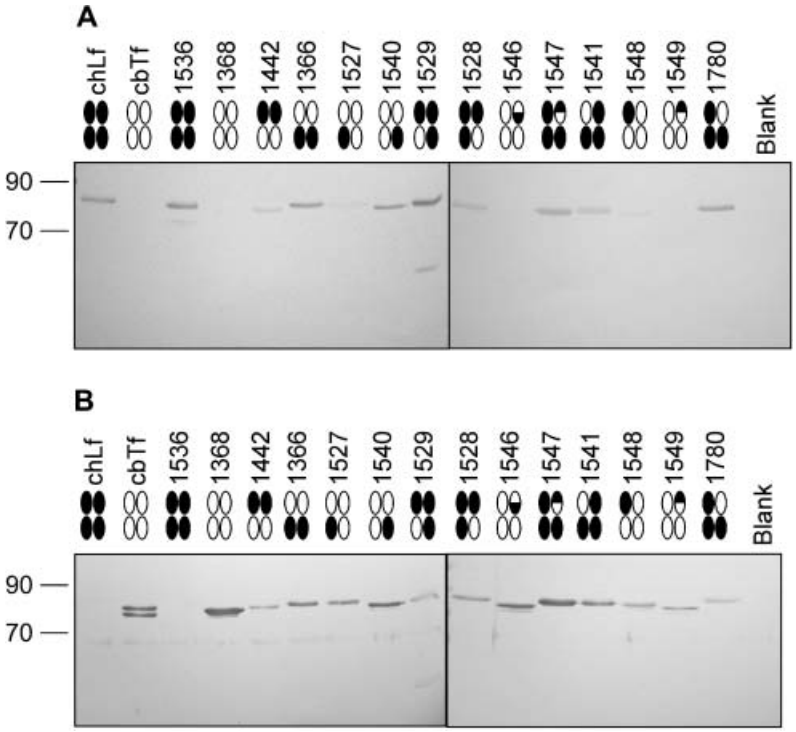

Fig. 3. Reactivity of chimeric proteins with anti-hLf and anti-bTf antisera. Aliquots (1 μg) of the various chimeric proteins or control commercial hLf (chLf) or commercial bTf (cbTf) were mixed with Laemmli sample buffer containing 0·2M DTT and denatured by boiling. Samples were resolved, in duplicate, on 10% SDS-PAGE gels and electroblotted. (A) Electroblot probed with polyclonal serum against hLf; (B) electroblot probed with polyclonal serum against bTf. The numbers and the cartoon of the protein above each lane are as illustrated in Fig. 2. Molecular mass in kDa is indicated on the left.

increase the levels of cell surface binding activity, the tagged receptor was subcloned into the pMAL-p2 vector (New England Biolabs) and expression was induced by addition of IPTG.

LbpA-binding regions on hLf

The receptor-binding regions of hLf were examined with the Mbp–LbpA fusion protein in solid-phase binding experiments where equal amounts of the chimeric proteins were allowed to bind the immobilized Mbp–LbpA prior to the addition of HRP–hLf (Fig. 4). This inhibition binding assay revealed that the recombinant LbpA bound the intact lactoferrin (chLf and 1536) as well as the chimeric proteins containing the C-lobe of hLf (1366), but there was no observable binding by proteins containing the hLf N-lobe (1442). Moreover, chimeric proteins containing either the C1 (1527, 1528) or the C2 (1540, 1529) domains of lactoferrin were capable of blocking the binding of HRP–hLf to the receptor, albeit somewhat less effectively than intact hLf. In other binding studies, chimeric proteins that contained portions of the hLf C-lobe (1547, 1541 and 1780) blocked binding of labelled hLf to the recombinant LbpA while chimeric proteins only containing portions of the hLf N-lobe (1546, 1548 and 1549) did not (data not shown).

Since the immobilization process on HA paper could have affected the receptor's binding activity (Cornelissen & Sparling, 1996), an alternative assay format was chosen in which the hybrids were allowed to bind the receptor in solution. In this experiment, the Mbp–LbpA fusion protein was solubilized from the *E. coli* total membrane and incubated with excess amounts of the chimeric proteins. Lactoferrin-Sepharose was added subsequently to capture any unbound receptor. Receptors captured on the lactoferrin-Sepharose were resolved on SDS-PAGE and revealed in Western analyses (Fig. 5A). The liquid-phase assay showed that Mbp–LbpA can be isolated by lactoferrin-Sepharose in the presence of either the commercial (cbTf) or recombinant bTf (1368), but not when commercial (chLf) or recombinant hLf (1536) was preincubated with the solubilized receptor. The chimeric proteins capable of binding LbpA were the ones containing the entire C-lobe of hLf (1366, 1547, 1541 and 1780). Chimeric proteins containing only the C1 or C2 domain of hLf (1527, 1540, 1528, 1529) did not inhibit isolation of recombinant LbpA by hLf-Sepharose.

In order to determine whether the binding data obtained with the Mbp–LbpA fusion protein were consistent with the native receptor, assays were performed using total membranes from various *Neisseria* strains. Remarkably, an identical binding profile to that observed with the Mbp–LbpA fusion protein was observed with solubilized total membranes prepared from iron-deficient wild-type *M. catarrhalis* strain Q8 (Fig. 5B), an isogenic Q8 mutant which does not express LbpB (N182, Fig. 5C), *M. catarrhalis* strain N141 (Fig. 5D), as well as a previously characterized *N. meningitidis* B16B6 mutant which does not express TbpA, TbpB or LbpB (N193, Fig. 5E) (Bonnah & Schryvers, 1998). These results strongly support a conserved mechanism of lactoferrin binding by bacterial LbpA.

DISCUSSION

The transferrin and lactoferrin receptors play a critical role *in vivo* (Cornelissen *et al.*, 1998) and thus may serve as useful

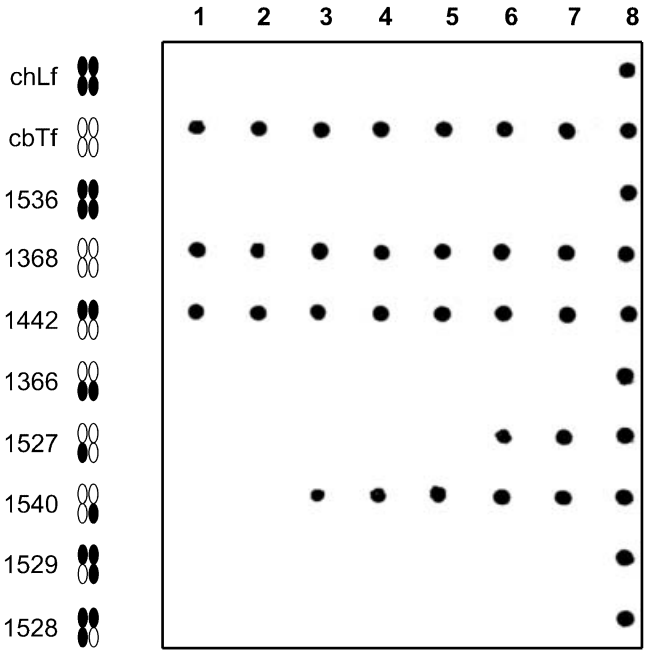

Fig. 4. Solid-phase binding assay with the Mbp–LbpA fusion protein. Aliquots (2 μl) of a total membrane preparation (1 mg protein ml^{-1}) from *E. coli* expressing the Mbp–LbpA fusion were immobilized onto the membrane. One hundred microlitres of different solutions containing control or chimeric proteins were applied to the individual wells to facilitate receptor binding. The concentration of protein in the solution ranged from 100 μg ml^{-1} (column 1) to 1·5 μg ml^{-1} (column 7) by serial 1 : 1 dilutions. Blocking solution was applied to the control wells (column 8). HRP-hLf was added to the incubation mixtures, and after removal and washing, bound HRP-hLf was detected by addition of the substrate (4-chloro-1-naphthol). The numbers and the cartoon of the protein to the left of each row are as illustrated in Fig. 2.

targets for the development of vaccines or therapeutic agents. A greater understanding of the receptors, how they interact with ligand and how they mediate the iron removal and uptake process would provide a more solid basis for targeting these proteins. The TonB-dependent receptors, TbpA and LbpA, are large complex proteins that make this a challenging task. There has been some recent progress at localizing the functional binding domains of TbpA (Boulton *et al.*, 2000; Masri & Cornelissen, 2002) and hopefully additional studies will provide further insights into the ligand–receptor interaction. The current study was designed to provide complementary information by attempting to define the receptor-binding regions on the ligand.

Previous attempts at delineating the receptor-binding regions involved production of individual lobes of transferrin and lactoferrin by proteolytic cleavage and testing them in a variety of direct and competitive binding and affinity isolation assays (Alcantara *et al.*, 1993; Yu & Schryvers, 1994). This approach was not suitable for further localization of the binding regions because of the inability to readily isolate the desired spectrum of proteolytic subfragments. The proteolytic cleavage pattern cannot be controlled and the requirement for reduction of the numerous disulfide bridges to facilitate isolation of individual peptides would compromise the ability to detect conformational epitopes. To overcome these limitations, we generated a panel of full-length chimeric proteins, in which individual domains were grafted onto a homologous protein to evaluate its contribution to receptor binding. In this manner, we preserved the overall structure of the proteins, thereby avoiding alteration of the conformation of the binding peptides. Thus, binding domains were identified with greater confidence and accuracy.

In an earlier study, a series of chimeric hTf/bTf proteins were constructed in order to identify regions of hTf that mediate binding to the receptors from *N. meningitidis* (Retzer *et al.*, 1996). The hTf/bTf genetic exchanges were accomplished using the SOEing technique. Although SOEing allows splicing between any sequences, it is a laborious method and often requires extensive optimization for each reaction. Since we were generating a comprehensive set of chimeric proteins for this study, we opted for a more convenient gene splicing strategy. The introduction of unique restriction sites at the domain junctions provided a versatile means of exchanging domains for constructing a series of chimeric proteins. This approach has the added advantage of encompassing other transferrins and lactoferrins since the unique restriction sites were selected for their compatibility with the genes encoding these proteins.

Recombinant transferrins and lactoferrins have been expressed using various systems (Ali *et al.*, 1996; Funk *et al.*, 1990; Miyauchi *et al.*, 1997; Ward *et al.*, 1992). Insect cells were chosen because they can be cultured in the absence of serum, which would be a source of contaminating transferrin. The insect-cell-derived oligosaccharide chains, albeit different from native hLf (Salmon *et al.*, 1997), provided a simple means of purification using concanavalin A Sepharose. The *N*-linked oligosaccharide side chains do not participate in ligand binding (Alcantara *et al.*, 1992), and consequently did not compromise our identification of binding regions for LbpA. However, variations in glycosylation may have influenced migration in SDS-PAGE (Fig. 3) and could have had subtle influences on the observed binding activities (Fig. 4). To specifically probe the interaction with LbpA, we required a preparation devoid of LbpB and CopB, both of which will bind hLf under similar conditions (Bonnah *et al.*, 1998). Due to the relatively low level of expression of LbpA in isogenic *lbpB* mutants (Bonnah *et al.*, 1999; Lewis *et al.*, 1998), we opted for the production of recombinant LbpA. The accumulation of a non-functional, non-exported form of TbpA from *Actinobacillus pleuropneumoniae* expressed in *E. coli* (Gonzalez *et al.*, 1995) suggested that export into the periplasm could be critical in obtaining functional receptor protein. We decided to create a fusion between the mature

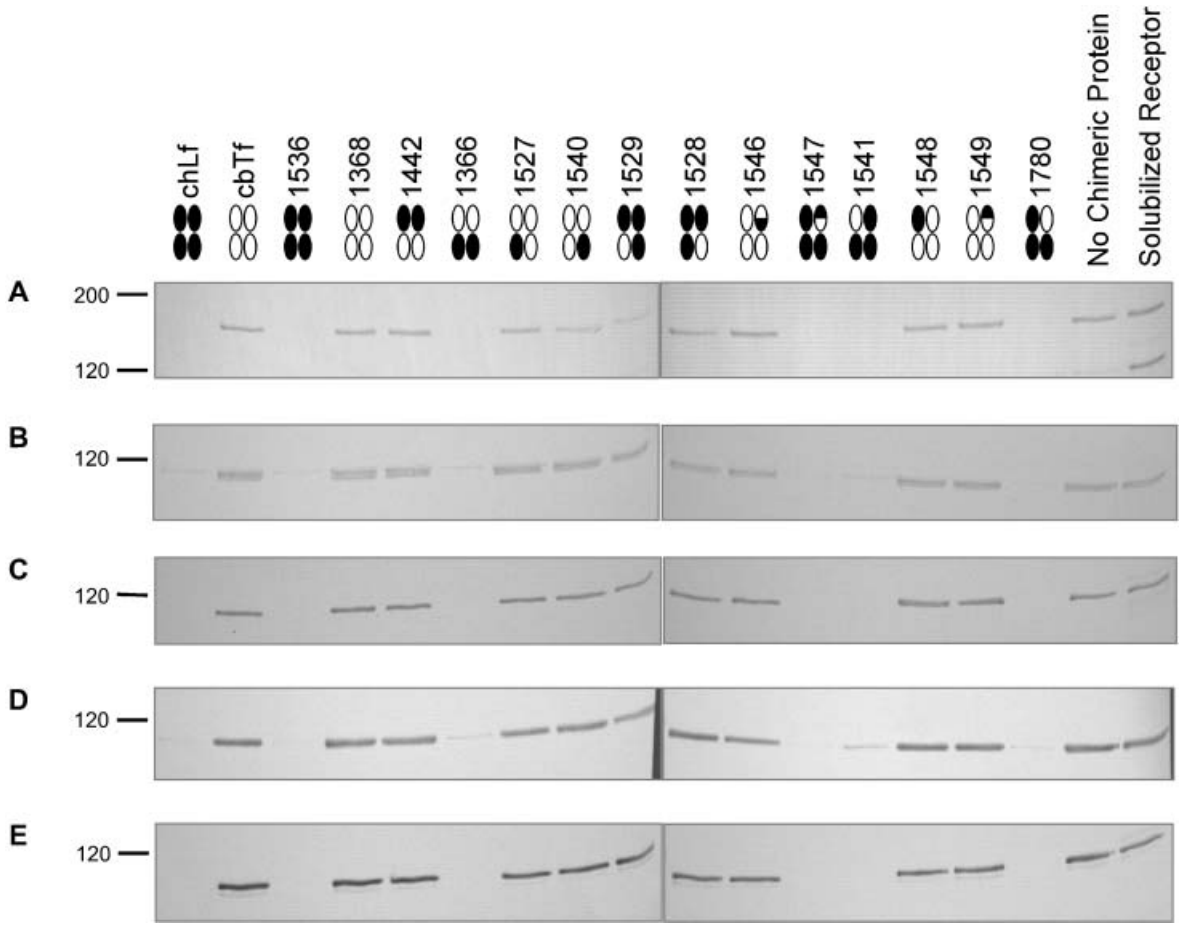

Fig. 5. LbpA affinity isolation experiments. The control or chimeric protein indicated at the top of each lane (number and cartoon from Fig. 2) was mixed with an equal amount of solubilized receptor. A control preparation with no added protein (No Chimeric Protein, second-last lane) was also included. hLf-Sepharose was subsequently added to capture any unbound receptors. The resin was washed and the bound protein was eluted by boiling in Laemmli sample buffer containing 0·2 M DTT. The eluted protein was resolved on an 8% SDS-PAGE gel for Western blot analysis. An aliquot of the solubilized material (last lane) was included in each of the experiments to confirm the presence of the lactoferrin receptor in the starting material. Total membranes were prepared from: (A) the *E. coli* strain expressing Mbp–LbpA; (B), wild-type *M. catarrhalis* strain Q8; (C), isogenic Q8 mutant which does not express LbpB (N182); (D), *M. catarrhalis* strain N141; (E), *N. meningitidis* B16B6 *tbpAB lbpB* triple mutant (N193). The blots illustrated were probed with rabbit polyclonal antiserum against Mbp (A) or rabbit polyclonal antiserum against the lactoferrin receptor from a heterologous *M. catarrhalis* strain (BC4223) (B–D) or rabbit polyclonal antiserum against the lactoferrin receptor from *N. meningitidis* strain B16B6 (E). Molecular mass in kDa is indicated on the left.

LbpA sequence and the TEM-1 β-lactamase (Bla) in order to provide a native *E. coli* signal peptide. A similar strategy was employed in the heterologous expression of the gonococcal P.II protein (Palmer *et al.*, 1989). In addition to utilizing the *E. coli* signal sequence for export, we also relied on Bla's activity as a positive selection system such that only ampicillin-resistant transformants would be analysed for receptor expression. Our strategy was successful in yielding functional LbpA and Bla (unpublished observation), but the lack of an inducible promoter precluded us from obtaining sufficient LbpA for biochemical analyses. Fortunately, by expressing the receptor with the maltose-binding protein system (New England Biolabs) yielded higher levels of functional LbpA for our binding experiments.

The production of a functional fusion between an integral outer-membrane protein and a periplasmic protein is a novel finding. The retention of lactoferrin-binding activity indicates that LbpA was properly inserted into the outer membrane, consistent with the proposal that the required signals are localized to the C-terminal region of integral outer-membrane proteins (Bosch *et al.*, 1989). The expression of ampicillin resistance (up to 200 $\mu g\ ml^{-1}$) indicates that proper folding of the Bla was achieved in spite of the

presence of a large C-terminal fusion. Similarly, the presence of Mbp was demonstrated by the detection of a 160 kDa protein, the combined molecular mass of LbpA and Mbp, on immunoblots by anti-Mbp antiserum (Fig. 5A).

A consensus biotinylation sequence for the *E. coli* BirA enzyme and a polyhistidine region were included at the N-terminus of LbpA to facilitate downstream purification and protein labelling for different assay formats. The single biotin moiety introduced in a region separate from the ligand-binding domain allows labelling or immobilization of the recombinant protein for a variety of different binding and affinity isolation experiments. The polyhistidine peptide provides a means of purification of the recombinant protein using metal-chelate chromatography (Hochuli *et al.*, 1987). Competition assays revealed that LbpA binds to the C-lobe of hLf, since chimeric proteins containing the C-lobe of hLf were able to block the receptor's binding to labelled (Fig. 4) or immobilized hLf (Fig. 5). These results are comparable to what has been observed with TbpA from bovine (Yu & Schryvers, 1994) and human pathogens (Alcantara *et al.*, 1993), supporting the hypothesis that these are functional homologues with similar mechanisms of action. The solid-phase binding experiments depicted in Fig. 4 indicated that there were distinct binding sites in each of the C-lobe domains of hLf capable of binding to LbpA. The failure of chimeric proteins containing only one domain from hLf to block isolation of recombinant or native LbpA in the affinity capture experiments (Fig. 5) does not necessarily contradict this conclusion. The effective local concentration of immobilized hLf on Sepharose beads probably greatly exceeds that of the labelled hLf in the competitive solid-phase binding assays; thus only the chimeric proteins with higher binding affinities could compete with native ligand in the affinity capture assay.

In order to confirm that the lack of LbpA binding in certain chimeric proteins was not due to protein misfolding, parallel binding experiments using TbpB from a bovine pathogen, *Pasteurella haemolytica* were performed (data not shown). Constructs that lacked LbpA-binding activity (e.g. 1368 and 1442) were shown to block the binding of labelled bTf to the bovine pathogen TbpB, indicating that the lack of LbpA-binding activity was not due to protein misfolding, but rather to the absence of binding epitopes.

The demonstration of distinct binding sites on each domain of the C-lobe is consistent with the model for iron removal from Tf/Lf which proposes that TbpA/LbpA promotes the separation of the two domains to alter the iron coordination, and thus promotes iron release (Schryvers & Stojiljkovic, 1999). A similar mechanism has been proposed for the removal of ligand from the periplasmic binding protein HisJ (Liu *et al.*, 1999). Direct evidence for the model of iron-removal may be elusive since the process may be dependent upon conformational changes in LbpA/TbpA induced by interaction with TonB and thus may be difficult to demonstrate *in vitro*. However, our understanding would be substantially enhanced by further localization of the binding determinants on the ligand and receptor combined with a greater appreciation of the structure of LbpA or TbpA. Studies directed at these objectives are under way.

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