Receptor-Mediated Recognition and Uptake of Iron from Human Transferrin by *Staphylococcus aureus* and *Staphylococcus epidermidis*  

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*Staphylococcus aureus* and *Staphylococcus epidermidis* both recognize and bind the human iron-transporting glycoprotein, transferrin, via a 42-kDa cell surface protein receptor. In an iron-deficient medium, staphylococcal growth can be promoted by the addition of human diferric transferrin but not human apotransferrin. To determine whether the staphylococcal transferrin receptor is involved in the removal of iron from transferrin, we employed 6 M urea–polyacrylamide gel electrophoresis, which separates human transferrin into four forms (diferric, monoferric N-lobe, and monoferric C-lobe transferrin and apotransferrin). *S. aureus* and *S. epidermidis* but not *Staphylococcus saprophyticus* (which lacks the transferrin receptor) converted diferric human transferrin into its apotransferrin form within 30 min. During conversion, iron was removed sequentially from the N lobe and then from the C lobe. Metabolic poisons such as sodium azide and nigericin inhibited the release of iron from human transferrin, indicating that it is an energy-requiring process. To demonstrate that this process is receptor rather than siderophore mediated, we incubated (i) washed staphylococcal cells and (ii) the staphylococcal siderophore, staphyloferin A, with porcine transferrin, a transferrin species which does not bind to the staphylococcal receptor. While staphyloferin A removed iron from both human and porcine transferrins, neither *S. aureus* nor *S. epidermidis* cells could promote the release of iron from porcine transferrin. In competition binding assays, both native and recombinant N-lobe fragments of human transferrin as well as a naturally occurring human transferrin variant with a mutation in the C-lobe blocked binding of 125I-labelled transferrin. Furthermore, the staphylococci removed iron efficiently from the iron-loaded N-lobe fragment of human transferrin. These data demonstrate that the staphylococci efficiently remove iron from transferrin via a receptor-mediated process and provide evidence to suggest that there is a primary receptor recognition site on the N-lobe of human transferrin.

One common factor among the complex interactions which occur between a bacterial pathogen and its host is the ability of the invading pathogen to multiply in host tissues. In extracellular mammalian body fluids, the iron transport proteins transferrin and lactoferrin maintain the level of free ionic iron at a level (about 10^{-18} M) which is far too low to sustain bacterial growth (6, 43). In spite of this, pathogenic bacteria clearly multiply successfully in vivo to establish an infection. Since all known bacterial pathogens need iron to multiply, it can be argued that they must be able to adapt to the severely iron-restricted extracellular environment usually found in vivo and develop mechanisms for assimilating transferrin- or lactoferrin-bound iron. Such high-affinity iron-scavenging mechanisms capable of removing iron from transferrins have been intensively investigated in gram-negative bacteria, where they depend either on the synthesis and secretion of low-molecular-mass iron chelators (siderophores) or, alternatively, on direct contact between the host transferrin and a surface receptor (for reviews, see references 9, 18, and 43). While siderophores remove iron from transferrin irrespective of its origin, bacterial transferrin receptors exhibit significant specificity for the transferrins of their natural hosts. For example, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* exhibit a marked preference for human transferrin (18, 43) while the porcine pathogen *Actinobacillus (Haemophilus) pleuropneumoniae* is able to bind and use pig but not human transferrin as an iron source (17, 32, 33). Furthermore, gonococcal transferrin receptor mutants are incapable of causing experimental urethritis in human male volunteers, demonstrating for the first time that an iron acquisition system is an essential virulence factor for human infection (11).

In contrast, the mechanism(s) by which the gram-positive staphylococci acquire iron from transferrin has not been fully elucidated. Early work by Schade (37) indicated that *Staphylococcus aureus* grows in human serum which contains transferrin, implying that staphylococci are able to utilize glycoprotein-bound iron. Staphylococci have been shown to secrete siderophores such as staphyloferin A and staphyloferin B which, chemically, are carboxylate-type siderophores (20, 25, 29). They have also been reported to employ primary metabolites such as a-ketoacids and a-hydroxyacids as siderophores (23). More recently, a new *S. aureus* siderophore termed “au-reochelin” has been identified and partially characterized (12). Whether these siderophores are capable of removing iron from transferrin has not been determined. However, Lindsay et al. (27) reported that *S. aureus* but not *Staphylococcus epidermidis* could remove iron from 55Fe-labelled transferrin via a process...
which did not require transferrin-staphylococcal cell surface contact and which therefore was assumed to be siderophore-mediated.

Previously we identified a saturable specific receptor for transferrin on the surface of S. aureus and a number of different species of coagulase-negative staphylococci and S. epidermidis (30). This receptor involves a 42-kDa cell wall transferrin-binding protein which, in common with the gram-negative bacterial transferrin binding proteins, exhibits consider- able transferrin species specificity. Human, rabbit, and rat serum transferrins but not bovine or porcine serum transferrins or hen ovotransferrin compete efficiently with the 125I-labelled human transferrin for the S. aureus and S. epidermidis transferrin receptor (30). Furthermore, staphylococci recovered without subculture from an implanted peritoneal chamber in rats are coated with surface-bound transferrin and express the 42-kDa transferrin-binding protein (31). The presence of a cell surface transferrin-binding protein, which, in many staphylococci, is iron regulated (30) suggests that this receptor may contribute to virulence by facilitating the acquisition of transferrin-bound iron.

Human transferrin is an approximately 80-kDa monomeric protein consisting of 679 amino acid residues with two N-linked glycan chains and can be divided into two homologous domains, referred to as the N and C lobes, each of which contains an iron-binding site (2, 42). Each site binds one ferric ion atom coordinated with a bicarbonate anion. Amino acid sequencing of the two transferrin lobes has revealed that they have a high degree of homology and may have arisen by gene duplication (42). Approximately 42% of amino acids in the N lobe have an identical counterpart in the C lobe which also contains both glycan chains (21). It is therefore possible that, since both human apotransferrin and dfferic human transferrin have similar affinities for the staphylococcal receptor (30), the primary receptor-binding site on transferrin is located within a single domain, as the two domains within a given lobe undergo substantial conformational changes upon iron binding (19).

In the present work, we explore the contribution of the staphylococcal receptor to the acquisition of iron from transferrin and provide evidence that there is a primary receptor recognition site on the N lobe of human transferrin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. S. aureus BB was obtained from J. P. Aebertshnth (University of Strathclyde, Glasgow, United Kingdom); S. epidermi-
dis 138 and Staphylococcus saprophyticus 907 were isolated from the peritoneal dialysis fluid of infected patients undergoing continuous ambulatory peritoneal dialysis (41). Staphylococci were grown in an iron-depleted, serum-free tissue culture medium (RPMI 1640 Sigma) statically for 18 h at 37°C in air enriched with 5% CO2. Iron was removed from the RPMI by batch incubation with 6% (wt/vol) Chelex 100 (Sigma) for 18 h as described before (22). After removal of the resin, calcium chloride (10 μM) and magnesium sulfate (100 μM) were added and the medium was filter sterilized. For some experiments, iron-depleted RPMI was supplemented with either dfferic human transferrin (250 μM protein) or with ferric chloride (25 μM). Growth of staphylococci was followed by measuring the optical density at 600 nm at 1-h intervals for 24 h.

Preparation of transferrin. Human transferrin was isolated from outdated plasma by ammonium sulphate precipitation and ion-exchange chromatography. Porcine transferrin was purchased from First Link, Briarley Hill, West Midlands, United Kingdom. Transferrins were made iron saturated by the addition of iron(III) nitrolitratetracetate to transferrin dissolved in 1 M NaHCO3. Unbound iron was removed by gel filtration on Sephadex G-25 equilibrated with 0.05 M NH4HCO3, and the protein-containing fractions were collected and lyophilized (15). The iron saturation of the protein was confirmed by electrophoresis on a 6% urea-polyacrylamide gel (see below). The C-lobe human transferrin variant from a subject heterozygous for an abnormal transferrin (14) was isolated by immunoaffinity chromatography and ion-exchange chromatography on a Pharmacia LKB (Uppsala, Sweden) HP Q-Sepharose column as previously described (15). The N-terminal lobe of human transferrin was prepared as described by Evans et al. (15) by digestion of the diferic protein in 0.1 M NaHCO3, pH 8.3, with subtilisin (Sigma) for 6 h at 37°C at an enzyme/protein ratio of 1:30 (wt/wt). The digest was fractionated by gel filtration on a Sephadex S-200 column (2.4 by 120 cm) equilibrated with 0.1 M NH4HCO3. The 36-kDa fragment obtained had the characteristic absorption band of iron transferrin at 470 nm and lacked carbohydrate. The recombinant N lobe of human transferrin was expressed in baby hamster kidney cells and purified from culture fluid by immunoaffinity and ion-exchange chromatography as described by Zak et al. (47).

Preparation of 125I-labelled human transferrin. Two hundred microlitres of a 100-kBc solution of Iodogen (Pierce and Warren, Chichester, United Kingdom) in dichloromethane was added to a 4-ml test tube, and the dichloromethane was allowed to evaporate by rotating in a water bath at 37°C. Three hundred micrograms of iron-saturated human transferrin (Sigma) and approximately 6 MBq of carrier-free 125I-labelled sodium iodide (Amersham International plc, Little Chalfont, United Kingdom) were added to each Iodogen-coated tube in 300 μl of phosphate-buffered saline (PBS), pH 7.4. The mixture was incubated with agitation at room temperature for 15 min, and the unincorporated 125I was removed by passing the 125I-transferrin down a Sephadex G-25 column (Pharmacia) preequilibrated with PBS containing 0.25% (wt/vol) transferrin.

Transferrin binding assays. Competitive binding assays were carried out essentially as described by Modun et al. (30). Briefly, iron-depleted staphylococci (106 CFU) were washed, resuspended in 1 ml of PBS and incubated with dfferic human 125I-transferrin (4 nM) in the presence of 700 nM of unlabelled dfferic human (normal or C-lobe variant) or porcine transferrin or the monoferric transferrin N lobe (subtilisin generated or recombinant). After 30 min of incu-
bation, bacteria were pelleted and washed three times in PBS and transferred to a fresh microcentrifuge tube and the amount of cell-associated 125I-transferrin was determined with a LKB 1282 Compugamma counter (Pharmacia LKB). Specific binding was defined as the difference between the amounts of 125I-human transferrin bound in the absence and presence of a 100-fold excess of the unlabelled ligand.

6 M urea–polyacrylamide gel electrophoresis. Iron removal from transferrins by staphylococcal cells and the siderophore staphyloferrin A (kindly provided by G. Winkelmann, Tübingen, Germany) was monitored over time by 6 M urea–polyacrylamide gel electrophoresis (13, 28). S. epidermidis, S. aureus, and S. saprophyticus grown in iron-depleted RPMI were washed and resuspended in PBS before incubation with 250 μg of dfferic human transferrin or the recombi-
nant monoferric N lobe of human transferrin in the presence or absence of glucose (0.2% wt/vol) at 37°C for intervals of 5, 10, 20, 30, and 60 min. Bacterial cells were centrifuged, and the supernatant containing transferrin was loaded onto a 6 M urea-polyacrylamide gel prepared by the method of Williams (13). The pH of the supernatant was monitored during each experiment to ensure that loss of iron from the protein was not a direct result of acidification of the medium through the metabolism of glucose. In addition, supernatants were assayed for the presence of siderophores by using the universal chrome azurul S assay system of Schwyn and Neilands (38). For some experiments, the staphylococcal siderophore staphyloferrin A (250 μg/ml) was used instead of whole cells. Electrophoresis was performed at 100 V for 4 h in Tris-borate-EDTA buffer (pH 7.4), and the gel was stained with Coomassie brilliant blue. To determine whether the release of iron from transferrin by whole staphylococcal cells is an energy-requiring process, the metabolic poison sodium azide or nigerin (both from Sigma) was included in the reaction mixtures at concentra-
tions ranging from 0 to 6 mM (26, 36).

RESULTS

Utilization of iron-bound transferrin. Figure 1A shows that in iron-depleted RPMI, the growth of S. aureus is not supported unless the medium is supplemented with an iron source such as ferric chloride. Growth was also promoted by dfferic human transferrin but not by apotransferrin. Similar results were obtained for S. epidermidis (Fig. 1B).

Removal of iron from human transferrin. Since transferrin has two binding sites for iron, we used 6 M urea–polyacryl-
amide gel electrophoresis to follow the release of iron from dfferic human transferrin. This technique is capable of resolv-
ing a partially iron-saturated sample of human transferrin into four forms, apotransferrin, C-terminal monoferric transferrin, N-terminal monoferric transferrin, and dfferic transferrin (13, 16, 28, 44). When incubated in PBS supplemented with glu-
cose, S. aureus converted dfferic human transferrin into its apotransferrin form within 30 min (Fig. 2). After 60 min of incu-
bation, the pH of the incubation buffer decreased from pH 7.4 to 6.6 without subculture from an implanted peritoneal cham-
ber. The digest was fractionated by gel filtration on a Sephacryl S-200 column and expressed without subculture from an implanted peritoneal chamber in rats are coated with surface-bound transferrin and express the 42-kDa transferrin-binding protein (31).
the monoferic C lobe (Fig. 2). This monoferic transferrin is then converted to the apoprotein (Fig. 2). Similar results were obtained for S. epidermidis (data not shown). In contrast, S. saprophyticus, which lacks the transferrin receptor and 42-kDa transferrin-binding protein (30), was unable to remove iron from human transferrin (data not shown).

Removal of iron from transferrin is an energy-requiring process. When incubated in PBS without glucose, neither S. aureus (Fig. 3A) nor S. epidermidis (data not shown) was able to remove iron from diferric human transferrin. Similarly, when incubated in PBS plus glucose in the presence of a range of concentrations of sodium azide or nigericin, we observed that S. aureus was unable to promote the release of transferrin-bound iron at a concentration of 6 mM sodium azide (Fig. 3B) or 4 mM nigericin (data not shown). By reducing the azide or nigericin concentration to 2 mM, the removal of iron from transferrin stalled at the monoferic C-lobe form (Fig. 3C).
The acquisition of iron from human transferrin. In an iron-depleted medium, the growth of both *S. aureus* BB and *S. epidermidis* 138 was supported by the addition of human diferric transferrin but not apotransferrin. For *S. aureus* this finding is consistent with the uptake of $^{55}$Fe from radiolabelled transferrin as observed by Lindsay et al. (27). *S. epidermidis*, however, was reported to be unable to acquire $^{55}$Fe from transferrin. In contrast, Brock et al. (5) reported that both *S. aureus* and *S. epidermidis* were able to access $^{55}$Fe from transferrin but only inefficiently. In these experiments, the bacterial cells were separated from the radiolabelled transferrin by a dialysis membrane. As a consequence, no direct interaction between the bacterial cell surface and the iron-binding glycoprotein could occur, which may account for the inefficient uptake of transferrin-bound iron observed by Brock et al. (5).

Since, both *S. aureus* and *S. epidermidis* grew well in RPMI medium containing diferric human transferrin as the sole iron source, we used 6 M urea-polyacrylamide gel electrophoresis to investigate the contribution of the transferrin receptor to the acquisition of iron. Using this approach, we have presented evidence for a receptor-mediated, siderophore-independent iron uptake mechanism in both *S. aureus* and *S. epidermidis*. This receptor-mediated mechanism of iron acquisition is clearly absent in *S. saprophyticus*, which lacks the transferrin receptor (30). Further confirmation was obtained by replacing human transferrin with porcine transferrin, since the latter does not bind to the staphylococcal receptor. While neither *S. aureus* nor *S. epidermidis* was able to remove iron from porcine transferrin, the staphylococcal siderophore staphyloferrin A efficiently removed iron from both mammalian transferrins. These data demonstrate unequivocally that *S. aureus* and *S. epidermidis* are both able to remove transferrin-bound iron either via receptor- or siderophore-mediated processes.

By following the time course of iron removal from human transferrin, we observed that iron was removed first from the N lobe and then from the C lobe. Although the ligands involved in transferrin iron binding are generally assumed to be equiv-
incubated with 125I-transferrin (4 nM) in the presence of 700 nM of one of the following unlabelled transferrins: human (HTT), human C-lobe variant (CVHTT), porcine (POT), subtilisin generated human N lobe (NSHTT), and recombinant human N lobe (NRHTT). After 30 min at 37°C, bacteria were pelleted and the amount of cell-associated 125I-transferrin was determined. Data presented are the means of three independent experiments + standard deviations (error bars).

FIG. 5. Whole-cell competition binding assay showing the inhibition of binding of human 125I-transferrin to staphylococci by the subtilisin-generated and recombinant N lobe of human transferrin. Staphylococci (10^8 CFU/ml) were incubated with 125I-transferrin (4 nM) in the presence of 700 nM of one of the following unlabelled transferrins: human (HTT), human C-lobe variant (CVHTT), porcine (POT), subtilisin generated human N lobe (NSHTT), and recombinant human N lobe (NRHTT). After 30 min at 37°C, bacteria were pelleted and the amount of cell-associated 125I-transferrin was determined. Data presented are the means of three independent experiments + standard deviations (error bars).

FIG. 6. Gel (6 M urea–polyacrylamide) showing the removal of iron from the N-lobe fragment of human transferrin. S. aureus (10^7 CFU/ml) were incubated in PBS plus glucose and 250 μg of the recombinant monoferric N lobe for intervals of 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 30 (lane 5), and 60 (lane 6) min. Bacterial cells were pelleted, and the supernatant was loaded onto the urea gel. The positions of the iron-loaded N lobe (MS) and apoprotein (AP) are indicated on the left-hand side.

In conclusion, we have now extended our earlier work (30) and demonstrated that both S. aureus and S. epidermidis express a functional transferrin receptor which is involved in the acquisition of transferrin-bound iron. Competitive transferrin

FIG. 6. Gel (6 M urea–polyacrylamide) showing the removal of iron from the N-lobe fragment of human transferrin. S. aureus (10^7 CFU/ml) were incubated in PBS plus glucose and 250 μg of the recombinant monoferric N lobe for intervals of 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 30 (lane 5), and 60 (lane 6) min. Bacterial cells were pelleted, and the supernatant was loaded onto the urea gel. The positions of the iron-loaded N lobe (MS) and apoprotein (AP) are indicated on the left-hand side.

ever, the Neisseria are gram-negative bacteria, and iron released from surface-bound transferrin must cross the outer membrane, the periplasm, and the inner membrane prior to internalization. Iron from receptor-bound transferrin is probably transferred via a gated pore in transferrin binding protein A (TbpA) to the periplasmic iron-binding protein, FbpA (7). Intriguingly, FbpA is structurally and functionally homologous to transferrin itself and reversibly binds one ferric ion per protein molecule (34, 35). In the both Neisseria (1) and H. influenzae (24), FbpA is part of an ATP-dependent transporter necessary for the internalization of iron from receptor-bound transferrin. How iron is released from transferrin bound to the surface of staphylococci is not yet known, but in these gram-positive bacteria it is likely to be mechanistically different. Recently we have cloned and sequenced an iron-regulated staphylococcal ABC transporter incorporating a 32-kDa lipoprotein (8) which could conceivably function in a manner analogous to FbpA by acting as an acceptor for iron released from surface-bound transferrin.

Since the staphylococci exhibit a preference for iron from the N-lobe binding site, we sought to determine whether the binding of the intact transferrin molecule could be blocked by the monoferric N-lobe fragment. Both recombinant and subtilisin-generated N lobes inhibited binding. In addition, a naturally occurring human transferrin variant with a mutation in the C lobe (14, 15) also efficiently blocked the binding of intact normal human transferrin. Taken together these results suggest that the primary receptor-binding site is located within the N-terminal lobe of the protein. Furthermore, when the iron-loaded N lobe of human transferrin was incubated with S. aureus, there was rapid removal of the iron. These results contrast with those obtained for the human transferrin receptor (46) and many gram-negative bacterial transferrin receptors (18), the primary recognition sites for which are located in the transferrin C-terminal lobe. Furthermore, only the C fragment was found capable of donating iron or binding to the transferrin surface receptors of hepatoma-derived HuH-7 cells and leukemic K562 cells (46). Neither the isolated C or N lobes nor a mixture is capable of supporting the growth of Haemophilus paragallinarum (which recognizes both N and C lobe sites on ovotransferrin) or N. meningitidis (which recognizes a C-lobe binding site only) (3, 18).

In conclusion, we have now extended our earlier work (30) and demonstrated that both S. aureus and S. epidermidis express a functional transferrin receptor which is involved in the acquisition of transferrin-bound iron. Competitive transferrin
binding studies and iron-uptake experiments indicate that a primary receptor recognition site lies within the N-terminal lobe. With this information and knowledge of receptor specificity, using site-directed mutagenesis we can now begin to define the precise residues involved in the transferrin-receptor interaction.

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