The Staphylococcal Transferrin-Binding Protein Is a Cell Wall Glyceraldehyde-3-Phosphate Dehydrogenase

BELINDA MODUN1,2,* AND PAUL WILLIAMS1,2,3

Institute of Infections and Immunity1 and School of Clinical Laboratory Sciences,3 Queen’s Medical Centre, Nottingham NG7 2UH, and School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom

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Staphylococcus aureus and Staphylococcus epidermidis possess a 42-kDa cell wall transferrin-binding protein (Tpn) which is involved in the acquisition of transferrin-bound iron. To characterize this protein further, cell wall fractions were subjected to two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis blotted, and the N-terminus of Tpn was sequenced. Comparison of the first 20 amino acid residues of Tpn with the protein databases revealed a high degree of homology to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis of staphylococcal cell wall fractions for GAPDH activity confirmed the presence of a functional enzyme which, like Tpn, is regulated by the availability of iron in the growth medium. To determine whether Tpn is responsible for this GAPDH activity, it was affinity purified with NAD+ agarose. Both S. epidermidis and S. aureus Tpn catalyzed the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. In contrast, Staphylococcus saprophyticus, which lacks a Tpn, has no cell wall-associated GAPDH activity. Native polyacrylamide gel electrophoresis of the affinity-purified Tpn revealed that it was present in the cell wall as a tetramer, consistent with the structures of all known cytoplasmic GAPDHs. Furthermore, the affinity-purified Tpn retained its ability to bind human transferrin both in its native tetrameric and SDS-denatured monomeric forms. Apart from interacting with human transferrin, Tpn, in common with the group A streptococcal cell wall GAPDH, binds human plasmin. Tpn-bound plasmin is enzymatically active and therefore may contribute to the ability of staphylococci to penetrate tissues during infections. These studies demonstrate that the staphylococcal transferrin receptor protein, Tpn, is a multifunctional cell wall GAPDH.

The capacity of an invading bacterial pathogen to colonize tissues and proliferate is a prerequisite for the establishment of infection in any host. This in turn depends on the availability of nutrients such as iron, an essential cofactor for diverse biochemical reactions. Although iron is abundant in extracellular mammalian body fluids, the level of free ionic iron is far too low to sustain bacterial growth because the iron is predominantly bound to carrier proteins such as transferrin and lactoferrin (4, 39). To overcome this in vivo iron restriction, bacteria have evolved high-affinity iron-scavenging mechanisms. These depend on one of two mechanisms. The first is the secretion of siderophores, low-molecular-mass ferri-specific ligands which remove iron from transferrin and transport it back to the cell via specific surface receptors (4, 13, 39). The other iron-scavenging mechanism employed by pathogens such as Neisseria meningitidis, Haemophilus influenzae, and Actinobacillus pleuropneumoniae (4, 13, 39), which do not secrete siderophores, involves direct contact between the host iron-binding glycoprotein and specific bacterial surface receptors. This transferrin receptor-mediated iron acquisition is distinct from siderophore-mediated iron transport in that there is a high degree of host transferrin species specificity (12, 39). In gram-negative bacteria, the receptors for transferrin generally consist of two iron-regulated outer membrane proteins termed TbpA and TbpB (4, 13, 39). Iron is removed from receptor-bound transferrin via an energy-dependent process which, in contrast to mammalian transferrin receptors, does not involve internalization of the iron-binding glycoprotein (4, 5, 39). In contrast to the gram-negative bacteria, much less information is available on the molecular basis of iron transport in gram-positive bacteria. In the staphylococci, a number of iron chelators are capable of stimulating growth (22). Staphylococci have been reported to utilize ferric iron-enterochelin complexes (23) and to produce their own siderophores (6, 7, 17, 22, 24). Although diferric human transferrin, when supplied as the sole iron source, promotes staphylococcal growth (27), the role of staphylococcal siderophores in this process has not been fully elucidated. Modun et al. (27) have shown that the purified staphylococcal siderophore staphyloferon A (17, 24) in vitro can remove iron from dfferic human transferrin. However, both S. aureus and S. epidermidis bind both transferrin (25) and lactoferrin (31). For transferrin, the staphylococcal transferrin receptor has been identified as an iron-regulated 42-kDa transferrin-binding protein (Tpn) located within the cell wall and common to both S. aureus and a number of coagulase-negative staphylococcal species including S. epidermidis, S. capitis, S. hemolyticus, and S. hominis (25). This protein is absent from S. saprophyticus and S. warneri, which are consequently unable to bind human transferrin (25). In common with gram-negative bacterial transferrin receptors, the staphylococcal receptor exhibits a preference for certain mammalian transferrins. For example, human, rabbit, and rat serum transferrins, but not bovine or porcine serum transferrins or hen ovotransferrin, compete efficiently with human transferrin for the S. aureus and S. epidermidis transferrin receptors (25).

More recently, the contribution of the staphylococcal transferrin receptor to the acquisition of transferrin-bound iron has been established (27). S. aureus and S. epidermidis, but not S. saprophyticus, converted human diferric transferrin but not...
porcine deferred transferrin into its apo form via an energy-dependent process. During conversion, iron was removed sequentially from the N-lobe and then from the C-lobe transferrin iron-binding site. Iron was also removed from the single-site iron-containing N-lobe fragment of human transferrin, which also competed efficiently with the intact iron-binding glycoprotein for the staphylococcal receptor (27). Thus, S. aureus and S. epidermidis are capable of efficiently removing iron from transferrin via a receptor-mediated process involving a primary receptor recognition site on the N-lobe of human transferrin.

After thorough purification of the staphylococcal transferrin receptor by ion exchange and affinity chromatography, the iron-binding capability of this receptor has not yet been established, supporting evidence for its likely importance in vivo has been obtained from experiments with implanted peritoneal chambers in rats, where staphylococci recovered without subculture are coated with surface-bonded transferrin and express the 42-kDa Tpn (26). In addition, both serum and dialysate samples from patients undergoing continuous ambulatory peritoneal dialysis and suffering from staphylococcal peritonitis contained antibodies to Tpn which were capable of inhibiting transferrin binding (26). In the present study, we sought to gain further insights into the nature of the staphylococcal Tpn as a necessary prelude to cloning the gene coding for Tpn and constructing defined receptor-negative mutants. N-terminal protein sequence analysis of Tpn revealed that it belongs to the newly emerging family of multifunctional cell wall-associated glycer- aldehyde-3-phosphate dehydrogenases (GAPDHs) which retain the ability to catalyze the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and incorporate binding sites for both transferrin and the serine protease plasmin.

MATERIALS AND METHODS

Bacterial growth conditions. S. aureus BB, S. epidermidis 138, and S. saprophyticus 907 have been described previously (3, 25, 27). 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 (3, 27). Iron was removed from the medium by batch incubation with 6% (wt/vol) Chelex 100 (Sigma) for 18 h as described previously (27). After removal of the resin, calcium chloride (10 M) and magnesium sulfate (100 M) were added and the medium was sterilized by addition of 0.1% of the antimicrobial agent chloramphenicol.

Extraction of cell wall proteins. Staphylococcal cell wall proteins were prepared as described by Smith et al. (36). Briefly, staphylococci grown in either iron-replete or iron-deprived RPMI 1640 were prepared as described above, washed three times with phosphate-buffered saline (PBS; 120 mM NaCl, 10 mM sodium phosphate [pH 7.4]), and their cell content was adjusted to the same optical density at 600 nm before they were resuspended in 0.6 ml of digestion buffer (30% [wt/vol] raffinose, 0.1% [vol/vol] of nonradioactive cell wall--associated glycer- aldehyde-3-phosphate dehydrogenases (GAPDHs) which retain the ability to catalyze the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and incorporate binding sites for both transferrin and the serine protease plasmin.

Materials and methods

Native PAGE. Cell wall proteins or affinity-purified Tpn were separated by two-dimensional SDS-PAGE with a 12.5% polyacrylamide resolving gel in the second dimension and electrophoretically transferred to a polyvinylidene difluoride membrane by using CAPS (3-[cyclohexylaminol]-1-propanesulfonic acid) buffer (pH 10.5; Sigma) containing 20% (vol/vol) methanol (38). The 42-kDa Tpn was identified by staining the blot with Coomasie brilliant blue and probing the blot with a human transferrin-HRP conjugate. Tpn was excised from the polyvinylidene difluoride membrane for NH₂-terminal amino acid sequence determination by solid-phase direct protein sequencing at the Kreb's Institute, Sheffield University, Sheffield, United Kingdom. Amino acid sequences were compared with known proteins in the Swiss-Prot database (University of Geneva, Geneva, Switzerland).

Enzymatic activity of bound plasmin. Plasmin binding to the affinity-purified Tpn was examined by a modification of the method described by Lottenberg et al. (19). The affinity-purified Tpn (50 µg per well) in carbonate coating buffer (15 mM Na₂CO₃, 30 mM NaHCO₃, [pH 9.1]) was immobilized onto a 96-well microtiter plate and incubated for 1 h at 37°C. The wells were washed three times with PBS containing Tween 20 (0.005% [wt/vol]), and nonspecific binding was blocked by incubation at 37°C for 1 h with PBS-Tween. After incubation, the plate was again washed three times with PBS-Tween and incubated with human plasmin (10 nM) in PBS-Tween for 1 h at 37°C. Unbound plasmin was removed by washing three times with PBS-Tween. To detect plasmin bound to Tpn, the plate was incubated with the synthetic substrate N-tosyl-Gly-Pro-Lys-parani- tronilide (400 mM; Sigma) for 30 min and the reaction was stopped by adding 10% (vol/vol) glacial acetic acid (100 µl). The release of para-nitroanilide from the synthetic peptide substrate was measured at A₄₀₅ and represents a measure of the enzymic activity of bound plasmin. As controls, plates in which either plasmin or Tpn was omitted were used.

RESULTS

Staphylococcal Tpn is related to the GAPDH family of proteins. A comparison of the first 20 NH₂-terminal amino acid residues derived from the S. aureus Tpn with known sequences...
in the Swiss-Prot protein database indicated a high degree of homology to the glycolytic enzyme GAPDH (Fig. 1A). The best match was with the group A streptococcal GAPDH, where 17 of the first 20 amino acid residues are identical. The N terminus of the *S. epidermidis* Tpn has the same sequence as *S. aureus* (data not shown).

GAPDH activity is associated with the staphylococcal cell surface. The location of Tpn on the staphylococcal cell surface suggests that whole staphylococcal cells and cell wall preparations containing this protein should catalyze the conversion of G-3-P to 1,3-diphosphoglycerate and the formation of NADH from NAD\(^+\). Whole cells and cell wall fractions were prepared from *S. aureus* grown under iron-depleted or iron-replete conditions and assayed for their GAPDH activity by monitoring the formation of NADH at A\(^{340}\) nm (Fig. 1B). GAPDH activity associated with whole *S. aureus* cells (cell concentrations ranging from 0 to 8 x 10\(^8\) CFU/ml) as determined by the conversion of NAD\(^+\) to NADH in the presence (open bars) or absence (solid bars) of G-3-P as described in Materials and Methods. (C) GAPDH activity associated with the cell wall fractions of *S. aureus* as measured following the catalytic conversion of G-3-P to 1,3-diphosphoglycerate and the formation of NADH from NAD\(^+\). Staphylococci grown to stationary phase in iron-replete RPMI 1640 (C) or iron-depleted RPMI 1640 (C) were harvested, washed in PBS, and resuspended to the same optical density at 600 nm prior to fractionation as described in Materials and Methods.

**Tpn has GAPDH activity and is a tetramer.** To confirm that the cell wall GAPDH activity was due to the 42-kDa Tpn, we exploited the affinity of GAPDHs for NAD\(^+\). Using NAD\(^+\)-agarose beads, we were able to affinity purify Tpn from cell wall fractions prepared from iron-depleted *S. aureus* (Fig. 2) and *S. epidermidis* but not *S. saprophyticus* (data not shown). The affinity-purified *S. aureus* and *S. epidermidis* proteins both exhibited GAPDH activity (Fig. 3). No activity was observed with *S. saprophyticus* (Fig. 3).

On nondenaturing PAGE, the affinity-purified *S. aureus* and *S. epidermidis* proteins both exhibited GAPDH activity (Fig. 3). Similar results were obtained with *S. epidermidis* (data not shown).

**Transferrin binding to the native and denatured cell wall GAPDH.** To determine whether the purified *S. aureus* cell wall GAPDH binds human transferrin in both its native and denatured forms, the NAD\(^+\) affinity-purified Tpn was subjected to Western blotting. Figures 2 and 4 show that the purified Tpn is able to bind human transferrin irrespective of whether it is in the native tetrameric conformation or in its monomeric form. Similar results were obtained on dot blots with the affinity-purified Tpn from both *S. aureus* and *S. epidermidis* (data not shown). The *B. stearothermophilus* GAPDH, however, was unable to bind human transferrin as either the tetramer or monomer (Fig. 4).

**Tpn binds plasmin.** Since the streptococcal cell wall GAPDH was originally identified as a plasmin-binding protein (20, 32), we probed Western blots of the affinity-purified Tpn with biotinylated human plasmin. Figure 5 shows that both the *S. aureus* and *S. epidermidis* Tpn bind human plasmin. To determine
whether plasmin bound to the purified Tpn from *S. aureus* and *S. epidermidis* was enzymatically active, wells of a microtiter assay plate were coated with Tpn and incubated with the synthetic plasmin substrate, N-*p* -tosyl-Gly-Pro-Lys-*p*-paranitroanilide. Figure 6 reveals that Tpn bound plasmin is enzymatically active.

**Inhibition of transferrin binding to Tpn by human plasmin.** Given that Tpn binds both transferrin and plasmin, we used competitive binding assays to determine whether both human serum proteins bound to the same site on Tpn. The data presented in Fig. 7 and 8 show that plasmin blocks the binding of human transferrin but not vice versa.

**DISCUSSION**

In the present study, we demonstrate that the staphylococcal Tpn is related to the glycolytic enzyme GAPDH. Although GAPDHs are generally considered soluble cytoplasmic proteins, a number of cell surface-associated GAPDHs in both prokaryotes and eukaryotes have been described. GAPDHs have, for example, been located in the cell membranes of human erythrocytes (16) and the blood fluke *Schistosoma mansoni* (10). Both *Candida albicans* (9) and group A streptococci also possess cell wall GAPDHs (20, 32). While these glycolytic enzymes have not previously been shown to possess transferrin-binding activity, they are known to possess functions in addition to their capacity to drive the NAD →-dependent formation of 1,3-diphosphoglycerate from G-3-P. These include ADP-ribosylating activity (33) and the ability to bind human proteins such as plasmin, lysozyme, fibronectin, laminin, myosin, and actin (11, 20, 32, 33). GAPDHs have also been report-
ed to be involved in microtubule assembly (15) and in DNA binding (35). Furthermore, other glycolytic enzymes including aldolase and lactate dehydrogenase appear to be capable of binding muscle proteins such as F-actin and myosin (1, 2).

Since both S. aureus and S. epidermidis whole cells and cell wall fractions possessed GAPDH activity, we exploited the affinity of GAPDHs for NAD⁺ to affinity purify the Tpn/GAPDH on NAD⁺-agarose. A single protein which was active as a GAPDH, migrated on SDS-polyacrylamide gels with a mass of 42 kDa, and bound human transferrin was obtained. In the absence of an S. aureus or S. epidermidis GAPDH mutant, we used S. saprophyticus as a negative control since this staphylococcus is unable to bind transferrin and lacks a Tpn. No cell wall GAPDH activity was apparent, and, taken collectively, our data clearly demonstrate that the staphylococcal Tpn and the cell wall GAPDH are indeed the same protein. The relationship between Tpn and the presumably cytoplasmic, glycolytic GAPDH is not yet known. However, in the group A streptococci, there appears to be no difference between the cell wall plasmin-binding protein, Plr, and the cytoplasmic GAPDH, which appear to be products of the same gene (40). Thus, in contrast to conventional gram-positive cell wall proteins, the streptococcal GAPDH lacks a signal sequence and has no apparent cell wall-spanning or membrane-anchoring motifs (20, 21).

Although the cell wall GAPDHs of streptococci are highly expressed in cells grown in iron-rich conventional laboratory media (32, 41), this is not generally the case for the staphylococci. Apart from one S. aureus strain (N100) (25), the S. aureus and S. epidermidis isolates so far examined express Tpn only in iron-deficient growth media (25, 26, 27). N100 appears to produce Tpn constitutively, irrespective of the iron content of the growth medium and may have a defect in an iron-responsive regulatory element such as SirR (14). Recently, a secreted form of the group A streptococcal GAPDH has been reported to be iron regulated, and under conditions of iron starvation, this GAPDH is released into the culture supernatant in a time-dependent manner (8). Whether iron influences the expression of the staphylococcal gene coding for Tpn or the machinery responsible for targeting Tpn to the cell wall is not yet known. However, in group A streptococci, iron depletion does not increase levels of the GAPDH mRNA transcript, suggesting that iron possibly influences the expression of the mechanism responsible for releasing the GAPDH from the streptococcal cell wall (8). We were unable to detect Tpn in cell-free supernatants prepared from staphylococci grown in iron-deficient RPMI 1640, unless the supernatant was concentrated more than 100-fold (28), suggesting that staphylococci, in contrast to the streptococci, do not readily release their cell wall GAPDH.

The enzymatic activity of GAPDHs as glycolytic enzymes depends on their tetrameric conformation (37). In this respect, and in common with the streptococcal cell wall GAPDH (32), Tpn exists as a tetramer which dissociates in SDS at 37°C in the absence of reducing agents to give a monomer of approximately 42 kDa (25). This indicates that the Tpn subunits are not disulfide bridge linked. Furthermore, the ability of Tpn to bind transferrin is not dependent on the native conformation, since the monomer retains this property and can be renatured to bind transferrin on Western blots after treatment with SDS, provided that the protein is solubilized at a temperature of 37°C or lower (25). In addition, the inability of the B. stearothermophilus GAPDH to bind transferrin suggests that not all...
GAPDHs possess this functionality despite the high degree of N-terminal amino acid sequence similarity. Comparison with the complete Tpn sequence awaits the cloning and sequencing of the staphylococcal gene, which may reveal potential transferrin-binding sites.

The relationship between the GAPDH- and transferrin-binding activities of Tpn is not yet apparent. However, given the contribution of Tpn to the acquisition of iron from transferrin (27), it is possible that the GAPDH activity contributes to the release of iron from bound transferrin. Organic phosphates such as 1,2-diphosphoglycerate are capable of mediating the release of iron from transferrin (29), and it is therefore conceivable that the 1,3-diphosphoglycerate formed from G-3-P performs a similar function. Preliminary experiments suggest that 1,3-diphosphoglycerate can remove iron from dierffer human transferrin (28). Confirmation of a relationship, if any, between GAPDH activity will depend on mutation of the active site of Tpn and the generation of a protein unable to promote the release of iron from bound transferrin.

Although the ability of the streptococcal GAPDH to bind transferrin is not known, it is well established as a multifunctional protein which binds the serine protease, plasmin (19, 20, 32, 33). Previously, Kuusela and Sakesela (18) have shown that staphylococci are capable of binding and activating cell surface-bound plasminogen; however, the staphylococcal receptor protein involved was not identified. In this study, we have shown that Tpn binds enzymatically active plasmin. This suggests that plasmin bound to the staphylococcal cell surface may provide a mechanism for tissue invasion, since plasmin is capable of cleaving extracellular matrix proteins as well as dissolving blood clots (21). This has been suggested to constitute a common mechanism by which invasive pathogens may cross normal tissue barriers (21). Whether this is true for the staphylococci is not yet known. However, although plasmin does not appear to contribute to the turnover of transferrin at the cell surface by degrading the iron-binding glycoprotein (28), it is able to block the binding of transferrin to Tpn. Since transferrin does not block plasmin binding in competitive binding assays, it is possible that there is more than one plasmin-binding site on Tpn, at least one of which is either the same as, or adjacent to, the transferrin-binding site. These assays were, however, carried out with purified proteins in ligand blot competition assays, which may not fully reflect the interactions occurring at the staphylococcal cell surface. Although the relationship between Tpn and the staphylococcal plasmin receptor described by Kuusela and Sakesela (18) is not known, it is conceivable that, in common with the streptococci, staphylococci possess multiple cell surface plasm-in-binding proteins (34, 41). Recently, site-directed mutagenesis of the streptococcal cell wall GAPDH has been shown not to abolish plasmin binding (41) and a novel streptococcal plasin-binding protein, identified as the glycolytic enzyme α-enolase, has been identified (34). Furthermore, site-directed mutagenesis of the group A streptococcal plasmin-binding GAPDH has indicated that there are at least two separate plasm-in-binding sites involving lysine residues, one each in the C terminus and in the N terminus of the protein (41, 42). This may also be the case for Tpn, and the gene coding for the staphylococcal protein is currently being cloned and sequenced to permit structure-function studies and to facilitate detailed mapping of the respective plasmin- and transferrin-binding sites.

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