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Immune Evasion of the Human Pathogen

*Pseudomonas aeruginosa*: Elongation Factor Tuf Is a Factor H and Plasminogen Binding Protein

Anja Kunert,* Josephine Losse,* Christin Gruszin,* Michael Hühn,* Kerstin Kaendler,* Stefan Mikkat,† Daniela Volke,‡ Ralf Hoffmann,‡ T. Sakari Jokiranta,§ Harald Seeberger,* Ute Moellmann,* Jens Hellwage,* and Peter F. Zipfel1*¶

*Pseudomonas aeruginosa* is an opportunistic human pathogen that can cause a wide range of clinical symptoms and infections that are frequent in immunocompromised patients. In this study, we show that *P. aeruginosa* evades human complement attack by binding the human plasma regulators Factor H and Factor H-related protein-1 (FHR-1) to its surface. Factor H binds to intact bacteria via two sites that are located within short consensus repeat (SCR) domains 6–7 and 19–20, and FHR-1 binds within SCR domain 3–5. A *P. aeruginosa* Factor H binding protein was isolated using a Factor H affinity matrix, and was identified by mass spectrometry as the elongation factor Tuf. Factor H uses the same domains for binding to recombinant Tuf and to intact bacteria. Factor H bound to recombinant Tuf displayed cofactor activity for degradation of C3b. Similarly Factor H bound to intact *P. aeruginosa* showed complement regulatory activity and mediated C3b degradation. This acquired complement control was rather effective and acted in concert with endogenous proteases. Immunolocalization identified Tuf as a surface protein of *P. aeruginosa*. Tuf also bound plasminogen, and Tuf-bound plasminogen was converted by urokinase plasminogen activator to active plasmin. Thus, at the bacterial surface Tuf acts as a virulence factor and binds the human complement regulator Factor H and plasminogen. Acquisition of host effector proteins to the surface of the pathogen allows complement control and may facilitate tissue invasion. *The Journal of Immunology*, 2007, 179: 2979–2988.

The opportunistic pathogen *Pseudomonas aeruginosa* causes life-threatening infections in humans, including pneumonia and bloodstream infections. This Gram-negative bacterium is a major cause of hospital-acquired infections especially in immunocompromised patients (1). In a recent report of the Infectious Diseases Society of America, *P. aeruginosa* is listed as one of the most dangerous human pathogens (2). Due to antibiotic resistance, *P. aeruginosa* infections are difficult to treat and suitable drugs controlling *P. aeruginosa* infections are currently limited (3). Lung colonization of cystic fibrosis patients by *P. aeruginosa* is an important cause of chronic infections and results in high morbidity and mortality. Several virulence factors such as exotoxin A, hemolysin, and phospholipase C have been identified, which enable the bacterium to evade the host immune attack (4). However, it is unclear how this bacterium controls innate immunity and evades complement attack.

*P. aeruginosa* isolates and strains differ in resistance to complement-mediated lysis (5). The human complement system plays an important role in clearance of early pulmonary *P. aeruginosa* infection (6). Deposition of the complement component C3b at the bacterial surface is important for induction of host responses and elimination of the pathogen. Complement-deficient mice when challenged with *P. aeruginosa* showed an amplified inflammatory response and did not clear the pathogen (7). *P. aeruginosa* exploits multiple strategies for immune evasion (4). The pathogen secretes catalytic enzymes like alkaline protease and elastase, which degrade the complement activation product C3b deposited at the bacterial surface (8, 9). In addition, *P. aeruginosa* expresses LPS variants that interfere with C3b deposition at the surface (5, 10). Complement evasion is particularly important during the initial phase of infection, when individual bacteria are in contact with the body fluids of the host. In this phase, the alternative pathway of complement activation is central for immune recognition (11). Similarly, the pathogen forms an alginate layer, which limits accessibility and the action of host plasma factors like complement (12). During late infection, *P. aeruginosa* can form biofilms, which protect the bacteria from complement attack and prevent complement-mediated lysis, phagocytosis, and access of antibiotics (13).

Factor H is the major host fluid-phase complement regulator that controls alternative pathway activation and the amplification reaction at the level of C3 (14). Factor H promotes cleavage of surface-bound C3b by acting as a cofactor for the plasma serine protease Factor I. In addition, Factor H accelerates the decay of the alternative pathway C3 convertase, C3bBb, and competes with Factor B for binding to C3b (15). Factor H is a member of a protein family that includes the Factor H-like protein (FHL)-1,2 which is an alternatively spliced product of the Factor H gene, and five

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2 Abbreviations used in this paper: FHL, Factor H-like protein; FHR, Factor H-related protein; SCR, short consensus repeat; HiNHS, heat-inactivated normal human serum; uPA, urokinase plasminogen activator.

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additional Factor H-related proteins (FHR) (specifically, FHR-1 to FHR-5), which are encoded by distinct genes (14). All members of the Factor H protein family represent structurally and immunologically related proteins that are exclusively composed of repetitive structural domains, termed short consensus repeat (SCR) domains (16).

Pathogens use a common strategy for immune evasion and acquire the fluid-phase complement regulators Factor H, FHL-1, and C4BP from host serum. When bound to the surface of the pathogen, each host regulator maintains complement regulatory activity, which aids in survival of the pathogen (17). This type of complement evasion was shown for Gram-negative bacteria, e.g., *Borrelia burgdorferi* (18, 19) or *Neisseria gonorrhoeae* (20), for Gram-positive bacteria, e.g., *Streptococcus pneumoniae* (21, 22) or *S. pyogenes* (23), for the eukaryotic yeast *Candida albicans* (24), for the multicellular parasites *Onchocerca volvulus* (25) or *Echinococcus granulosus* (26), and for viruses (27, 28). In several cases the corresponding surface proteins were identified and cloned (14).

Pathogens like *P. aeruginosa* require proteolytic activity for tissue invasion, which is provided by endogenous or host acquired proteases. *P. aeruginosa* bind host plasminogen, and at the bacterial surface, the inactive precursor is converted to proteolytically active plasmin, which causes fibrin degradation and aids in, for example, tissue invasion. So far plasminogen receptors of *P. aeruginosa* were postulated but not identified (29, 30).

In this study, we identify the elongation factor Tuf as a surface protein of *P. aeruginosa* that binds the host plasma molecules Factor H and plasminogen. Factor H and plasminogen bound to Tuf, both maintain their functional activity. *P. aeruginosa* inhibits host complement activity by endogenous and acquired mechanisms: the bacterium uses endogenous proteases for complement control and also binds host regulators to its surface. For the analyzed *P. aeruginosa* strains, the acquired C3b degrading activity mediated by Factor H was stronger and more efficient than the endogenous activity.

**Materials and Methods**

### P. aeruginosa strains and growth conditions

*P. aeruginosa* strains 27853 (American Type Culture Collection (ATCC)), 10602, SG137, PAO1 (National Collection of Type Cultures (NCTC)), the PAO1 derivative AH177 (31), and various clinical isolates were cultivated overnight at 37°C in enriched Nutrient Broth (Sera). Eight clinical isolates (Is1-Is8) were derived from patients with different diseases including pylonephritis, leukopenia, T cell lymphoma, urosepsis, or myeloma. All cultures were grown to an OD_{600} of 1. Cells were collected by centrifugation and washed three times with PBS supplemented with 20 mM EDTA.

**Expression of recombinant proteins and generation of antisera**

Factor H deletion constructs were expressed in Sf9 insect cells following infection with recombinant baculovirus coding for Factor H SCRs 1–5, 1–6, 1–7, 15–18, 15–19, and 15–20 (15, 32, 33). The Factor H construct SCR1–20, the cDNA of FHR-1, and its C-terminal deletion construct FHR-1/SCR3–5 were cloned in pPICZaB (Invitrogen Life Technologies) and expressed in the yeast *Pichia pastoris* strain X-33 according to standard protocols.

The *tufB* gene from *P. aeruginosa* was amplified from genomic DNA of strain PAO1 using primers tuf (forward) 5’-GGGATACCCATGCGCTAAA GAAAAATTGAAACCAGG (and reverse) 5’-TTCTGCGATTTCGGAT GATCTTGGCAAAC-3’. The amplicon was cloned into expression vector pQE-9 (Qiagen) and transformed into *Escherichia coli* strain SG13009 (pREP4; Qiagen), and protein expression was induced by isopropyl b-D-thiogalactoside. His-tagged Tuf was purified by chromatography using a zinc-loaded HiTrap chelating column (GE Healthcare) and dialyzed against PBS using Slide-A-Lyzer (Pierce). All other recombinant proteins were His-tagged and were purified by HisTrap columns (GE Healthcare).

**Serum and protein adsorption experiments with live**

*P. aeruginosa*

*P. aeruginosa* (2 × 10^8) cells was resuspended in 150 μl of PBS supplemented with 75 mM NaCl, 100 mM MgSO_4_, and 1% BSA and incubated with 750 μl of 50% heat-inactivated normal human serum (HINHS) or PBS for 1 h at room temperature. For mapping of the Factor H or FHR-1 binding domains, bacteria were incubated in culture supernatant containing Factor H deletion (i.e., SCR 1–5, 1–6, 1–7, 15–18, 15–19, and 15–20) or purified proteins (i.e., SCR 19–20, FHR-1, FHR-1/SCR 3–5). Following incubation, bacteria were washed four times with PBS, and bound proteins were eluted using 3 M potassium thiocyanate. The last wash and the elution fractions were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Proteins were identified by Western blotting using a polyclonal anti-Factor H antibody (Roth).

For flow cytometry bacteria were incubated in 50% HINHS or PBS. Cells were resuspended in 100 μl of PBS and incubated for 1 h with the monoclonal Factor H Ab C18 (1/200) (34). After washing three times in PBS, bacteria were incubated with 1/100 Alexa Fluor 488-labeled rabbit anti-mouse antibody (Molecular Probes) for 1 h at 4°C in the dark. The cells were washed again in PBS, diluted to a concentration of 1 × 10^7 cells/ml, and analyzed using a FACSCalibur (BD Biosciences). Some 10,000 events were routinely counted.

**Biotinylation and isolation of *P. aeruginosa* surface proteins**

Surface biotinylation of intact *P. aeruginosa* was performed as described (34). In short, bacteria (2 × 10^11 cells) were washed in buffer A (PBS, 1 mM CaCl_2, 0.5 mM MgCl_2) and resuspended in buffer B (buffer A supplemented with 1.6 mM D-biotin). Bacteria were pelleted and surface proteins were labeled with biotin by incubation with 500 μl of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min on ice. Cells were washed extensively in buffer C (50 mM Tris (pH 7.4), 100 mM NaCl, 27 mM KCl, 1 mM CaCl_2, 0.5 mM MgCl_2) and resuspended in buffer C supplemented with Protease Inhibitor Cocktail Complete (Roche). Bacteria were lysed by sonication and biotinylated envelope proteins were purified by affinity chromatography using Immunopure Immobilized Monomeric Avidin (Pierce). Proteins were eluted with D-Biotin (2 mM in PBS) according to the manufacturer’s recommendations. The elute fractions were separated by SDS-PAGE and transferred to a membrane, and biotinylated proteins were identified by HRP-conjugated avidin (Roche). Positive fractions were pooled.

**Isolation of Factor H ligands with magnetic beads**

Factor H SCR 8–20 (40 μg) was covalently coupled to magnetic beads (100 μl of suspension) according to the manufacturer’s instructions (Invitrogen Life Technologies). These beads were incubated with purified biotinylated *P. aeruginosa* surface proteins for 2 h at 37°C. After washing three times (50 μl HEPES (pH 7.5), 1% Nonidet P-40, 1 mM dithioerythritol, 1 mM MnCl_2, 1 mM CaCl_2), proteins were eluted with 40 μl of 1 M NaCl for 10 min at 37°C, separated by SDS-PAGE, and visualized by Coomassie staining. Individual bands were excised from the gel and submitted to mass spectrometry.

**Protein identification by peptide mass fingerprinting**

Silver-stained bands were destained using the ProteoSilver Plus silver stain kit (Sigma-Aldrich) according to the manufacturer’s protocol. Subsequently, the proteins were reduced and alkylated using 10 mM DTT and 100 mM iodoacetamide in 25 mM NH_4HCO_3, washed with 25 mM NH_4HCO_3, and dehydrated with acetonitrile. Digestion with trypsin, MALDI-TOF-MS, and protein identification by peptide mass fingerprinting were performed as described (35).

**Protein interaction assays**

For binding studies human Factor H (Merck), recombinant Factor H deletion constructs, recombinant FHR-1 and plasminogen (Chromogenix) were used. Bound proteins were identified with goat antisera specific for Factor H (Merck), goat antisera recognizing plasminogen (Acris Antibodies). Tuf antisera generated in rabbit against recombinant Tuf, or mouse anti-Tuf MaAb (36).

For ligand affinity blotting recombinant Tuf was either separated by SDS-PAGE and transferred to a membrane or directly blotted onto a membrane under native conditions. Membranes were blocked with RotiBlock (Roth) supplemented with 2.5% BSA overnight and incubated with the corresponding binding proteins. Bound proteins were identified with specific antisera and visualized by ECL.

For ELISA recombinant Tuf (0.25 μg in carbonate buffer) was coated to the wells of Costar half-area plates (Corning) overnight at 4°C. Wells were
blocked with PBS containing 1% BSA for 2 h at room temperature. After washing with PBS, the indicated ligands (10 μg/ml in 75 μl) were added and the mixture was incubated for 1 h at room temperature. Bound proteins were identified with appropriate antisera. After addition of orthophenylenediamine (DakoCytomation) the absorbation at 490 nm was measured.

Competition of Factor H and plasminogen to immobilized Tuf was analyzed by ELISA. A total of 0.2 μg of plasminogen was mixed with different quantities of Factor H resulting in various molar ratios and added to each well containing immobilized Tuf. Both serum proteins were detected individually using specific antisera.

For whole cell ELISA, intact bacteria (10^5/well) were resuspended in carbonate buffer and immobilized to Maxisorp plates (Nunc). ELISA was performed as described. HiNHS was used at a 20-fold dilution. The amino acid analogs e-aminoacproic acid and imidazole were added to a final concentration of 1 mM. Incubations were performed in a volume of 150 μl.

For surface plasmon resonance assays a Biacore 3000 instrument was used. Briefly, Factor H SCR 8–20, or recombinant Tuf, was immobilized via standard amine coupling to the flow cells of a CM5 sensor chip as described (37). The surface of the flow cells was activated and the analyte diluted in coupling buffer (10 mM acetate buffer (pH 5.0)) was injected until an appropriate level of coupling was reached (~3400 resonance units). Recombinant Tuf or plasminogen was used as analytes at the indicated concentrations at a flow rate of 5 μl/min.

**Immunofluorescence studies**

Bacteria of *P. aeruginosa* strain AH777 (2 x 10^9) were washed three times in PBSB (PBS supplemented with 0.2% BSA and 100 μl) and incubated for 1 h at room temperature with 1/100 diluted primary Ab (polyclonal goat antiserum raised against Factor H). Following washing in PBSB, bacteria were resuspended in 20 μl of buffer, applied to microscopic slides and dried overnight. After fixation with 100 μl of aceton for 15 min, bacteria were incubated with an Alexa Fluor 647-labeled anti-mouse or anti-goat immune serum (1/500 in PBSB; Molecular Probes) for 1 h at room temperature. After extensive washing in PBSB, bacteria were embedded in ProTaq Mount Fluor (Biocyt) and fluorescence was analyzed using an Olympus BX51 microscope. For detection of plasminogen binding, intact bacteria were incubated with 0.5 μg of plasminogen in 100 μl of PBS for 1.5 h at room temperature and then treated with goat plasminogen anti-serum (Acris Antibodies).

**Fibrinogen degradation of *P. aeruginosa***

*P. aeruginosa* strain PAO1 (10^5 cells) were thoroughly washed with PBS and incubated in reaction buffer (64 mM Tris, 350 mM NaCl, 0.15% (v/v) Triton X-100 (pH 7.5)) supplemented with plasminogen (2 μg) and urokinase plasminogen activator (uPA, 20 ng) for 1 h at room temperature. After extensive washing fibrinogen (0.5 μg plasminogen-depleted; Calbiochem) was added and cells were incubated at 37°C. At the indicated times (4 and 16 h), aliquots were removed and separated by SDS-PAGE. Fibrinogen degradation was analyzed after transfer to a membrane by Western blotting using a polyclonal fibrinogen Ab (Calbiochem) and an HRP-conjugated anti-rabbit antisera.

**C3b cleavage assay**

The C3b cleavage capacity of *P. aeruginosa* was assayed after incubation of the bacteria in 50% HINHS or PBS for 1 h. After washing with PBS cells were resuspended in 40 μl PBS, supplemented with 10 μg/ml C3b and 20 μg/ml Factor I (Merck), and incubated at room temperature either for 2 h or overnight. Bacteria were pelleted, lysed, and separated by SDS-PAGE. After transfer onto a nitrocellulose membrane, C3b degradation products were identified by Western blotting using an anti-C3c antisera raised in rabbits (Merck).

To assay cofactor activity of Tuf-bound Factor H, recombinant Tuf was coated to microtiter plates and Factor H (0.2 μg/well) was added. After 1 h incubation, C3b and Factor I were applied and the mixture was incubated for 15 min at 37°C. The fluid phase was removed and C3b degradation products were analyzed as described.

**Plasminogen activation and plasmin activity**

Recombinant Tuf was immobilized onto Costar half-area plates (Corning). After blocking with PBS containing 2% BSA, plasminogen (0.6 μg/well) was added for 2 h at room temperature. Unbound plasminogen was removed by washing with PBS. The activator uPA (4 ng per well; Millipore) and the chromogenic substrate S-2251 (4-valyl-leucyl-lysine-p-nitroanilide dihydrochloride, 150 μg; Sigma-Aldrich) dissolved in reaction buffer (64 mM Tris, 350 mM NaCl, 0.15% Triton X-100 (pH 7.5)) were added. Plasmin activity was measured as described (38). The activity of the generated plasmin was measured at 37°C over an incubation period of 15 h by recording the absorbance at 405 nm.

**Serum sensitivity assay**

*P. aeruginosa* strain SG137 (10^7 cells) were incubated at room temperature in 50% complement active normal human plasma or 50% Factor H-depleted human plasma (39) in reaction buffer (20 mM HEPES, 144 mM NaCl, 10 mM EGTA, 7 mM MgCl2, (pH 7.4), final volume 160 μl). After 1 h bacteria were harvested and thoroughly washed, and serial dilutions were plated on Nutrient Broth agar. After 24 h incubation at 37°C, colonies were counted and CFUs were calculated. In additional experiments, Factor H-depleted human plasma was reconstituted with purified Factor H (Calbiochem).

**Statistical analysis**

Where appropriate, data points were subjected to the unpaired Student’s t test.

**Results**

**Binding of Factor H and FHR-1 to *P. aeruginosa***

Binding of Factor H to intact *P. aeruginosa* was assayed by serum absorption experiments. Bacteria were incubated in HINHS and after extensive washing bound proteins were eluted, separated by SDS-PAGE and analyzed by Western blotting. A band of 150 kDa representing Factor H and a doublet of 37 and 43 kDa representing the two forms of FHR-1 (40) were identified in the elution fractions of all tested strains: i.e., serum-resistant PAO1, ATCC 27853, NCTC 10662, and serum-sensitive SG137 (Fig. 1A). For strains PAO1 and ATCC 27853, Factor H binding was further assayed by flow cytometry. Following incubation in human serum Factor H binding was visualized by an increase in fluorescence (Fig. 1B).
In addition, binding of host immune regulators to eight clinical
P. aeruginosa isolates was tested, which differ in respect to pigment
production, morphology, and mucosity. All isolates (Is1 to Is8) bound Factor H and FHR-1 (Fig. 2). Additional bands were
detected with weaker intensity for Is1, Is3, Is5, and Is6. These
nonspecific bands were not detectable when bacteria were incubated
in PBS instead of HiNHS. Thus, binding of Factor H and
FHR-1 seems to be a general feature of P. aeruginosa.

Mapping of Factor H and FHR-1 binding domains
For the two human immune regulators Factor H and FHR-1, the
binding regions for interaction with live P. aeruginosa were locali-
zed using recombinant deletion mutants. N-terminal (SCR do-
 mains 1–6 and 1–7) and C-terminal deletion constructs of Factor
H (SCR domains 15–19, 15–20, and 19–20) bound to intact bac-
teria (strain ATCC 27853), whereas constructs SCRs 1–5 and
15–18 did not bind (Fig. 3, A and B). In addition, complete FHR-1
and a deletion construct representing the three C-terminal SCR
domains (FHR-1/SCRs 3–5) did bind to P. aeruginosa (Fig. 3C).
The same binding profile was identified for Factor H and FHR-1
binding to P. aeruginosa strain PAO1 (data not shown). The ad-
tional bands observed for several recombinant Factor H frag-
ments most likely represent dimeric or even multimeric forms and
are also detectable with purified recombinant fragments. Thus,
Factor H binds with two domains to the surface of P. aeruginosa,
i.e., SCRs 6–7 and 19–20 and FHR-1 binds with one domain,
which is contained in SCRs 3–5.

Identification of a Factor H binding protein in P. aeruginosa
To isolate Factor H binding proteins from P. aeruginosa, a matrix
representing Factor H/SCRs 8–20 was immobilized to magnetic
beads and incubated with purified biotinylated surface proteins of
strain PAO1. Bacterial proteins that bind to the Factor H/SCRs
8–20 matrix were eluted, separated by SDS-PAGE and visualized
by silver staining (data not shown). Three bands were excised from
the gel and analyzed by peptide mass fingerprinting, and the band
of 43 kDa was positively identified. Of 19 ion signals labeled in the
assigned mass spectrum, 10 signals matched to the amino acid
sequence of the E. coli lysate. washed (w) and elution (e) fraction were
separated by SDS-PAGE and visualized by silver staining (data not shown). Three bands were excised from
the gel and analyzed by peptide mass fingerprinting, and the band
of 43 kDa was positively identified. Of 19 ion signals labeled in the
assigned mass spectrum, 10 signals matched to the amino acid

FIGURE 2. Factor H and FHR-1 bind to clinical iso-
lates of P. aeruginosa. Clinical isolates of P. aeruginosa
derived from patients with pyelonephritis, leukopenia, T
cell lymphoma, urosepsis, or myeloma (Is1–Is8) were incu-
bated with human serum. Bound proteins were eluted,
separated by SDS-PAGE, and analyzed by Western Blot-
ting. Factor H was detected in the elution fraction as a
150-kDa protein, and FHR-1 was identified as the doublet
of 37 and 43 kDa. The mobility of the marker proteins is
indicated on the right side. A representative experiment of
five is shown. e, elution fraction; w, final wash fraction.

FIGURE 3. Mapping of the binding domains within Factor H and
FHR-1. P. aeruginosa strain ATCC 27853 was incubated with recombinant
deletion constructs of Factor H and FHR-1. N-terminal (A) and C-terminal
fragments of Factor H (B), or recombinant FHR-1 and the C-terminal frag-
ment SCRs 3–5 (C) were used. Bound proteins were eluted, separated by
SDS-PAGE, and analyzed by Western Blotting. Arrows indicate the posi-
tion of the recombinant deletion constructs, which differ in mobility as they
include a variable number of SCR domains. The mobility of the marker
proteins is indicated on the right side. A representative experiment of five
is shown. e, elution fraction; w, final wash fraction.

FIGURE 4. Elongation Factor Tuf of P. aeruginosa is a surface protein.
A, Tuf of P. aeruginosa strain PAO1 was recombinantly expressed in E.
coli as a His-tagged protein and purified by affinity chromatography. The
E. coli lysate, flow through (FT), wash (w), and elution (e) fraction were
separated by SDS-PAGE and analyzed by silver staining (lanes 1–4). The
elution fraction was analyzed by Western blotting using a mAb detecting
the N-terminal histidine tag (lane 5). The band of ~57 kDa is visualized by
silver staining but did not react with the histidine antisera (C). B, Tuf is
detected at the surface of immobilized P. aeruginosa (strain PAO1) using
whole cell ELISA. Tuf was detected using a polyclonal antisera, which
was raised against recombinant Tuf. Data show the mean of four experi-
ments with SD indicated with error bars. *, p < 0.001. C, Surface expres-
sion of Tuf as identified by immunofluorescence microscopy. P. aerugi-
nosa (strain PAO1) was incubated with a monoclonal anti-Tuf Ab and
stained with a Alexa Fluor 647-labeled antisera (left). Tuf is detected in the
absence of the mAb served as control (right). A representative experiment of
three is shown. D, Tuf is identified in the surface protein fraction of P.
aeruginosa. Purified biotinylated surface proteins of strains PAO1 (lanes 1
and 3) and SG137 (lanes 2 and 4) were separated by SDS-PAGE and
analyzed by Western blotting using a monoclonal anti-Tuf Ab or a poly-
clonal anti-GroEL antisera. The mobility of the marker proteins is indi-
cated on the right. A representative experiment of three is shown.
sequence of the elongation factor Tuf (EF-Tu) from *P. aeruginosa*, resulting in sequence coverage of 24%. Tuf is 397 aa long and has a predicted molecular mass of 43.4 kDa (39).

*Tuf* is a surface protein of *P. aeruginosa*

The gene coding for Tuf was amplified using DNA of *P. aeruginosa* strain PAO1, cloned into an appropriate expression vector, and expressed as a His-tagged protein in *E. coli*. Recombinant Tuf was purified almost to homogeneity as indicated by silver staining of SDS-PAGE-separated purified protein (Fig. 4A, lane 4). Recombinant Tuf reacted with a polyhistidine antiserum (Fig. 4A, lane 5). In addition, a specific polyclonal antiserum was raised against recombinant Tuf. With this antiserum, Tuf was detected on the surface of *P. aeruginosa*, using a whole cell ELISA (Fig. 4B). Surface-exposed Tuf was also identified by immunofluorescence microscopy with *P. aeruginosa* (Fig. 4C). Surface expression of Tuf was further confirmed analyzing surface protein fractions of *P. aeruginosa* strains PAO1 and SG137. Surface proteins were labeled with biotin, isolated by avidin affinity chromatography, and separated by SDS-PAGE. After Western blotting Tuf was identified among the surface proteins. GroEL (heat shock protein 60), which was used as a marker for cytoplasmic proteins, was not detected in these fractions (Fig. 4D, lanes 3 and 4).

Recombinant Tuf binds human complement regulators

Ligand affinity blotting with recombinant Tuf was used to show binding to Factor H, recombinant FHL-1 (Fig. 5A), and FHR-1. Binding was further assayed by ELISA. Tuf was immobilized, and binding of Factor H, FHL-1, and FHR-1 was tested. Factor H and FHL-1 binding was prominent. Binding of FHR-1 was further assayed using ligand affinity blotting. When Tuf was subjected to SDS-PAGE and blotted onto a membrane, binding of FHR-1 was observed (Fig. 5B). FHR-1 binding was detected, but the signal showed variations (Fig. 5C). Factor H interaction with Tuf was further confirmed by surface plasmon resonance. A C-terminal fragment of Factor H, i.e., SCRs 8–20, was immobilized to the chip surface and Tuf was applied as analyte. Tuf binding was concluded based on the association curve and the pronounced dissociation profile following removal of the ligand. Binding was dose-dependent as shown for two Tuf concentrations (Fig. 5D). To localize the binding regions within Factor H, binding of recombinant Factor H deletion mutants to Tuf was analyzed by ELISA. The N-terminal constructs SCRs 1–6 and SCRs 1–7 bound to immobilized Tuf, and SCRs 1–5 showed weak binding. Similarly, the C-terminal constructs SCRs 15–18 and SCRs 15–20 did bind, but not the deletion constructs that represent the middle region, i.e., SCRs 8–11 and SCRs 11–15 (Fig. 5E). These results show that the human complement regulator Factor H binds to recombinant Tuf of *P. aeruginosa*. Factor H has two binding regions for Tuf, one is shared with FHL-1 and the second region is located in the C terminus, i.e., SCRs 18–20.

*Tuf* is a plasminogen-binding protein of *P. aeruginosa*

Interaction of Tuf with other human serum proteins was tested, and plasminogen was identified as an additional Tuf binding protein. Plasminogen bound strongly to Tuf as demonstrated by ligand affinity blotting and by ELISA (Figs. 6, A and B). As Tuf interacts with the two human plasma proteins Factor H and plasminogen, we analyzed whether the two proteins bind simultaneously and compete for binding. Immobilized Tuf was incubated with a mixture of Factor H and plasminogen in different molar ratios. Bound human proteins were detected individually. Factor H affected the interaction between plasminogen and Tuf, as increasing the concentration of Factor H resulted in decreased plasminogen binding. When both human proteins were applied at a molar ratio that corresponds to the plasma situation, concurrent binding of both plasma proteins was observed (Fig. 6C). Thus plasminogen and Factor H bind simultaneously to bacterial Tuf and apparently use similar or even overlapping binding sites. Binding of plasminogen to Tuf was further characterized by surface plasmon resonance.
Tuf was immobilized to the surface of a sensor chip and binding of plasminogen was followed. Plasminogen binding was dose-dependent over the range from 12.5 to 400 nM (Fig. 6D).

**Binding of human plasminogen to intact P. aeruginosa**

Binding of plasminogen to live *P. aeruginosa* of strain PAO1 was assayed. Plasminogen binding was first demonstrated using immunofluorescence microscopy. Bacteria were incubated with plasminogen and binding was visualized with the specific antiserum (Fig. 7A). Binding of purified plasminogen and serum-derived plasminogen to *P. aeruginosa* was also observed using a whole-cell ELISA. Apparently, lysine residues are relevant for the interaction as the lysine analog /H9255-aminocaproic acid inhibited plasminogen binding, but the histidine analog imidazol had no effect (Fig. 7B).
To characterize the function of bound plasminogen, plasminogen was attached to the surface of intact bacteria and activated with uPA. Following extensive washing, the cells with the attached protease were incubated with fibrinogen. In the presence of plasminogen, fibrinogen was completely degraded after 16 h (Fig. 7C). This response shows that surface bound plasminogen is proteolytically active and aids in fibrinolysis and degradation of the extracellular matrix components.

**Factor H attached to the surface of *P. aeruginosa* displays cofactor activity**

To determine the regulatory role of surface-attached Factor H, degradation of C3b was analyzed directly at the surface of *P. aeruginosa* after incubation in human serum. C3b processing was visualized following separation by SDS-PAGE and Western blotting. Incubation of *P. aeruginosa* strain PAO1 in human serum resulted in acquisition of complement regulatory activity as demonstrated by the disappearance of the 110 kDa α′ chain of C3b and the appearance of the cleavage products α′ 68, α′ 46, and α′ 43. As *P. aeruginosa* uses endogenous proteases to inactivate complement (8), the C3b degradation was compared following incubation of the bacteria in PBS in the absence of human serum. Endogenous C3b cleavage was revealed for strain PAO1 by the appearance of the α′ 43, α′ 41, and α′[prime] 35 fragments of C3b, which were detected after 18 h of incubation (Fig. 8A, lane 3). A comparison of the intensities of the degradation products showed that the acquired C3b cleavage activity from human serum is stronger than the endogenous activity (Fig. 8A, compare lane 3 with lane 4). Similar results were observed for the *P. aeruginosa* strains ATCC 27853 and NCTC 10662 (Fig. 8, B and C). The three analyzed strains showed endogenous C3b cleaving activity as well as strain-specific C3b degradation products. For all analyzed strains the acquired activity was more pronounced.

**Functional activity of Tuf-bound human serum proteins**

To assay whether Tuf-bound host effector proteins are functionally active, plasminogen was bound to immobilized Tuf and treated with the activator uPA, and plasmin activity was assayed. Tuf-bound plasminogen was converted to proteolytically active plasmin as evidenced by cleavage of the chromogenic substrate S-2251. The proteolytic activity was dose-dependent and correlated with the amount of immobilized Tuf (Fig. 8D). Thus, plasminogen attached to Tuf is accessible for the activator uPA, and the activated Tuf-bound plasmin has proteolytic activity.

To assay whether Factor H attached to Tuf maintains complement-regulating activity, Factor I-mediated cleavage of C3b was analyzed. Following binding of Factor H to immobilized Tuf, C3b and Factor I were added. After incubation the mixture was separated by SDS-PAGE and C3b cleavage products were identified by Western blotting. Tuf-bound Factor H displayed cofactor activity as demonstrated by the appearance of the α′ 46 and α′ 43 bands of C3b (Fig. 8B, lanes 1–3). The cofactor activity of Tuf-bound Factor H was comparable to that of Factor H immobilized (Fig. 8E, compare lane 3 with lane 5).

**Attachment of Factor H affects survival in human plasma**

To assay the protective role of surface attached Factor H, survival of bacterial was compared following incubation in Factor...
CONCENTRATION OF FACTOR H IS ABOUT 500

The plasma concentration of Factor H is about 500 mg/ml. CFU obtained under physiologically relevant concentrations of purified Factor H. The plasma concentration of Factor H is about 500 μg/ml. CFU obtained under physiological Factor H concentrations was set at 100%. This experiment was repeated four times and data indicate mean values and SD as error bars.

H-deficient and -sufficient complement-active human plasma. Incubation in Factor H-depleted plasma results in poor survival of P. aeruginosa strain SG137. However in Factor H-containing plasma bacterial survival was observed (Fig. 9A). The damaging effect of plasma was dependent on Factor H, as addition of this regulator to depleted plasma resulted in a dose-dependent increase in survival (Fig. 9B). Thus demonstrating that acquisition of Factor H to the bacterial surface has a protective effect.

Discussion

The Gram-negative bacterium P. aeruginosa exploits host plasma proteins like Factor H and plasminogen for immune evasion and tissue destruction. Binding of the host proteins Factor H and FHR-1 was observed for four laboratory strains (i.e., P. aeruginosa PAO1, ATCC 27853, NCTC 10662, and SG137) and eight clinical isolates. Factor H bound to P. aeruginosa strain SG137 was incubated for 1 h in Factor H-depleted human plasma, which was supplemented with the indicated concentrations of purified Factor H. The plasma concentration of Factor H is about 500 μg/ml. CFU obtained under physiological Factor H concentrations was set at 100%. This experiment was repeated four times and data indicate mean values and SD as error bars.

FIGURE 9. Survival of P. aeruginosa in Factor H-depleted human plasma. A, P. aeruginosa strain SG137 was incubated for 1 h either in Factor H-depleted complement active human plasma (DHP) or in complement active normal human plasma (NHP). Afterward, bacteria were plated onto agar plates at various dilutions, and CFUs were determined. This experiment was repeated three times, and the mean data and error bars for SD are indicated. *, p < 0.005. B, P. aeruginosa strain SG137 was incubated for 1 h in Factor H-depleted human plasma, which was supplemented with the indicated concentrations of purified Factor H. The plasma concentration of Factor H is about 500 μg/ml. CFU obtained under physiological Factor H concentrations was set at 100%. This experiment was repeated four times and data indicate mean values and SD as error bars.

The elongation factor Tuf was identified as a Factor H binding protein. A newly generated antiserum identified Tuf at the surface of P. aeruginosa. Factor H binds to recombinant Tuf, which in its plasmic location, but was identified at the surface of P. aeruginosa, which lacks classical signal and transport sequences, has a cytoplasmic domain. The translation elongation factor Tuf (EF-Tu) was initially identified as a GTP binding protein (39). Tuf, factor Tuf (EF-Tu) was initially identified as a GTP binding protein. The translational elongation factor Tuf binds to recombinant Tuf, which in its plasmic location, but was identified at the surface of P. aeruginosa, which lacks classical signal and transport sequences, has a cytoplasmic domain. The translation elongation factor Tuf was identified as a Factor H binding protein of P. aeruginosa using a combination of surface protein extraction, affinity chromatography, and mass spectrometry. A second independent approach, which was based on cross-linking of P. aeruginosa surface proteins to Factor H, immunoprecipitation and mass spectrometry also identified Tuf as a Factor H binding protein (data not shown). The translational elongation factor Tuf (EF-Tu) was initially identified as a GTP binding protein, which is associated with the 50 S ribosomal subunit (39). Tuf, which lacks classical signal and transport sequences, has a cytoplasmic location, but was identified at the surface of P. aeruginosa (Fig. 4, B–D). Surface localization of Tuf is also reported for Mycobacterium leprae and Mycoplasma pneumoniae (46, 47). Recombinant Tuf of P. aeruginosa binds the host plasma proteins Factor H, FHL-1, FHR-1, and plasminogen (Figs. 5 and 6). In summary, the pathogen P. aeruginosa acquires human plasma proteins Factor H and plasminogen via the surface protein Tuf and uses the attached host proteins for immune evasion and tissue interaction.

All analyzed P. aeruginosa strains as well as different clinical isolates bind the soluble complement regulator Factor H and the related FHR-1 to their surfaces, suggesting that binding of complement regulators is a general feature of pathogenic P. aeruginosa. The three C-terminal SCR of Factor H and FHR-1, which show near sequence identity, include heparin and glycosaminoglycan binding sites, thus explaining the comparable binding (41, 42). The absence of FHL-1 binding to intact bacteria can be explained either by the different serum concentrations of Factor H and FHL-1 or by the higher binding affinity of the C-terminal SCR 19–20 vs SCR 6–7.

The same two domains of Factor H, which bind to P. aeruginosa, do also interact with virulence factors of other pathogens, like the M protein of S. pyogenes (43), BbCRASP-1 and BbCRASP-2 of B. burgdorferi (44), and CaCRASP-1 of C. albicans (P. Poltermann, A. Kunnert, M. von der Heide, R. Eck, A. Hartmann, and P. F. Zipfel, manuscript in preparation).

Factor H bound to P. aeruginosa is functionally active and together with the serine protease Factor I degrades C3b. P. aeruginosa uses a combination of endogenous and acquired complement-degrading activity. The endogenous C3b-degrading activity generates unique strain-specific cleavage products that differ from those generated by the host protease Factor I (Figs. 8, A and B). The endogenous regulatory activity, which is mediated by elastase and alkaline protease, was initially described for several clinical P. aeruginosa isolates (8, 9, 45). The endogenous C3b cleavage activity varied within the analyzed strains. However, in all strains the acquired C3b-degrading activity was more potent than the endogenous activity.

Complement, in particular the alternative pathway, is essential for clearance of P. aeruginosa in vertebrate hosts. Using a P. aeruginosa pulmonary infection model, C3 knockout mice showed high mortality and increased bacterial loads in lungs and blood. A central role of the alternative pathway is concluded by the high mortality of mice deficient for the alternative complement component Factor B, but survival of mice deficient in the classical pathway protein C4 (11). Thus alternative pathway-induced C3 activation and C3b generation is crucial for immune sensing and host-mediated elimination of P. aeruginosa. As complement is part of innate immunity and is particularly active during the initial phase of the infection, binding of the host complement regulator Factor H represents a potent strategy of P. aeruginosa for innate immune evasion.

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addition, Tuf of *M. pneumoniae* binds fibronectin and Tuf of *Lactobacillus johnsonii* mediates attachment of the bacteria to mucins and to human intestinal cells (47, 48). An additional role of Tuf for immune response was reported for the plant *Arabidopsis thaliana*, which recognizes Tuf at the surface of the pathogen *Pseudomonas syringae*. Treatment of plant leaves with Tuf-derived peptides initiates defense reactions and induces resistance of the plant (49). In summary, Tuf is a multifunctional protein that is located in the cytoplasm and at the bacterial surface. Therefore Tuf is a member of a still expanding family of “moonlighting proteins” (50), which represent virulence factors that are localized in the cytoplasm as well as at the cell surface. The exact export pathway and the surface anchoring mechanisms of these proteins are currently unknown (51). However, for bacteria there is evidence for an additional protein transport mechanism mediated by membrane vesicles (52).

In this study, we identify Tuf as a bacterial plasminogen binding protein. Plasminogen binding to *P. aeruginosa* was recently reported (29), and to our knowledge Tuf is the first plasminogen receptor identified for *P. aeruginosa*. The translational elongation factor Tef1p of the pathogenic yeast *C. albicans*, which shares 29% sequence identity on the amino acid level, was also identified as a plasminogen binding surface protein (53). *P. aeruginosa* Tuf lacks the plasminogen binding motif that was identified for enolase of *S. pneumoniae* (54) and shows no homology to other bacterial plasminogen binding proteins such as GAPDH of *S. pneumoniae* (55), Ospa of *B. burgdorferi* (56), or M protein of *S. pyogenes* (57). Plasminogen binding to recombinant Tuf is accessible for the activator uPA and is converted to proteolytically active plasin (Fig. 8A).

Acquisition of plasminogen has been reported for several pathogens and seems crucial for extracellular matrix interaction, degradation, and systemic dissemination of a pathogen from superficial skin lesions (38, 58–60). The proenzyme plasminogen is converted by the human plasminogen activators uPA and tissue-type plasminogen activator to plasmin, which forms a key protease for intravascular fibrinolysis. However, plasmin also displays extracellular functions like degradation of extracellular matrix components such as laminin, vitronectin, and fibronectin (61). Binding of plasminogen via Tuf to the surface of *P. aeruginosa* may therefore facilitate invasion of host tissue. Thus, Tuf as a bacterial surface protein binds the central host effector proteins plasminogen and the complement inhibitor Factor H, which are relevant for tissue disintegration and complement inactivation by *P. aeruginosa*.

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Disclosures

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References


