Plasminogen Is a Complement Inhibitor*

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Background: Plasminogen regulates the coagulation cascade.

Results: Plasminogen binds to complement C3 and to C5. Activated plasmin inhibited complement as demonstrated by hemolytic assays.

Conclusion: Plasmin is a potent complement inhibitor that inactivates complement at the level of C3 and C5.

Significance: This complement-inhibitory activity of plasmin provides a new explanation why pathogenic microbes utilize plasmin(ogen) for immune evasion and tissue penetration.

Plasminogen is a 92-kDa single chain glycoprotein that circulates in plasma as a zymogen and when converted to proteolytically active plasmin dissolves preformed fibrin clots and extracellular matrix components. Here, we characterize the role of plasmin(ogen) in the complement cascade. Plasminogen binds the central complement protein C3, the C3 cleavage products C3b and C3d, and C5. Plasminogen binds to C3, C3b, C3d, and C5 via lysine residues, and the interaction is ionic strength-dependent. Plasminogen and Factor H bind C3b; however, the two proteins bind to different sites and do not compete for binding. Plasminogen affects complement action in multiple ways. Plasminogen enhanced Factor I-mediated C3b degradation in the presence of the cofactor Factor H. Plasminogen when activated to plasmin inhibited complement as demonstrated by hemolytic assays using either rabbit or sheep erythrocytes. Similarly, plasmin either in the fluid phase or attached to surfaces inhibited complement that was activated via the alternative and classical pathways and cleaved C3b to fragments of 68, 40, 30, and 17 kDa. The C3b fragments generated by plasmin differ in size from those generated by the complement protease Factor I, suggesting that plasmin-mediated C3b cleavage fragments lack effector function. Plasmin also cleaved C5 to products of 65, 50, 30, and 25 kDa. Thus, plasmin(ogen) regulates both complement and coagulation, the two central cascade systems of a vertebrate organism. This complement-inhibitory activity of plasmin provides a new explanation why pathogenic microbes utilize plasmin(ogen) for immune evasion and tissue penetration.
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exposed on bacterial surfaces. Each of the three pathways generates a C3 convertase that cleaves the central protein C3 (14, 15). This cleavage generates the anaphylactic and antimicrobial peptide C3a and forms C3b, which is deposited onto any nearby surfaces and acts as an opsonin (18). Binding of a second C3b protein to the C3 convertase (C3bBb) generates a C5 convertase, which cleaves C5 to generate the anaphylatoxin C5a and C5b (19). The complement system is tightly regulated by soluble and membrane-bound proteins. Factor H, FHL-1, and the C4b-binding protein are the main soluble complement regulators that act at the level of the C3 convertase (20, 21). The AP regulator Factor H serves as a cofactor for the serine protease Factor I, which cleaves and inactivates C3b, whereas C4b-binding protein, which is also a cofactor for Factor I, binds C4b and inhibits the CP C3 convertase (22, 23).

Both the coagulation and the complement cascades of the human organism are tightly connected (24). The cross-talk of these two major cascade systems is a focus of ongoing research, and proteases that act in both systems are of special interest. Over 50 years ago, Pillemer et al. (25) showed that plasminogen when activated by the staphylococcal staphylokinase cleaves the complement proteins C1, C2, C3, and C4 and inhibits complement activation. Other groups have reported that plasmin by cleaving C3 and C5 generated biologically active anaphylatoxins (7, 26–28). Similarly, plasma treated with the human plasminogen activator uPA generated a protease that interfered with fibrinolysis (29). In addition, thrombin, the central coagulation protease, cleaves C5 and initiates a fourth complement activation pathway that bypasses C3 (30). So far, an activating role of the two coagulation proteases for the complement cascade was proposed as thrombin degrades the complement proteins C3 and C5 and generates the anaphylatoxins C3a and C5a (28). However, whether these coagulation proteases also allow further processing and even inactivation of the two complement components remains unclear.

Both the coagulation and the complement cascades maintain homeostasis, and components of both cascades recognize and eliminate infectious microbes. However, pathogenic microbes, which survive in an immunocompetent host, have found special means to control both host cascades and block the action of effector molecules. Plasminogen as a component of the coagulation system and similarly Factor H, FHL-1, CFHR1, and C4b-binding protein as major soluble regulators of the complement cascade are acquired by a large number of human pathogens, including Haemophilus influenzae (31–33), Pseudomonas aeruginosa (34), Borrelia burgdorferi (35–38), Streptococcus pyogenes (39), Streptococcus pneumoniae (40, 41), Staphylococcus aureus (42), and Candida albicans (43, 44). For several pathogens, the corresponding microbial plasminogen-binding proteins have been identified. Plasminogen bound by these microbial proteins is accessible and activated by human as well as microbial activators of plasmin, which cleaves the natural substances fibrinogen and C3b (39, 45).

As several microbial and fungal complement evasion proteins bind Factor H and plasminogen (39, 45), we hypothesized that plasminogen has a complement-regulatory role. Therefore, we analyzed whether plasmin inhibits the complement cascade and whether plasminogen binds to human complement proteins. Here, we characterize plasmin as a complement regulator, modulator, and inhibitor. Plasminogen binds several human complement proteins such as C3, C3b, and C5. When activated, plasmin cleaves the central human complement proteins C3b and C5 and generates cleavage fragments that are distinct from those generated by the complement protease Factor I. In addition, plasminogen enhances the cofactor activity of Factor H. Understanding the role of plasmin for complement regulation also provides a basis for novel therapeutic approaches.

EXPERIMENTAL PROCEDURES

Proteins and Antiserum—Human plasminogen, uPA (Hemochrom Diagnostica), and the complement proteins C3, C3b, C3c, C3d, C3a, C5, and Factor H (CompTech) were used for binding studies. Plasminogen was generated by incubation of plasminogen and uPA for 15 min at 37 °C. Proteins were identified with goat antiserum specific for plasminogen (Acris Antibodies); complement protein C3, C3d, C5, or Factor H (CompTech); or rabbit antiserum specific for C3a (CompTech). Corresponding horseradish peroxidase (HRP)-conjugated antiserum was purchased from Dako, and the protease inhibitors aprotinin, α2-antiplasmin, from Calbiochem and e-amino caproic acid (eACA) Sigma, respectively. Sheep and rabbit erythrocytes were from Rockland.

Protein Binding Assays—For ligand affinity assays, individual complement proteins were separated by SDS-PAGE and transferred to a membrane (PVDF; Millipore). The membrane was blocked with blocking buffer (1% BSA, 4% milk powder in PBS, and 0.1% Tween 20) overnight at 4 °C following incubation with plasminogen (20 μg/ml) for 1.5 h at room temperature. After washing three times with PBS-T (PBS + 0.05% Tween 20), bound plasminogen was identified by Western blotting with appropriate antiserum and corresponding HRP-conjugated antiserum (46).

For ELISA, the various proteins (each at 0.5 μg in 50 μl of carbonate-bicarbonate buffer; Sigma) were immobilized onto a 96-well microtiter plate (MaxiSorb, Nunc) overnight at 4 °C and the various C3 activation fragments (C3b, C3c, C3d, and C3a) were immobilized at equal molar concentrations. After washing three times with PBS-T, wells were blocked with Blocking Buffer I (AppliChem) for 2 h at 37 °C. The appropriate ligand (1 μg in 100 μl of PBS) was added either alone or with the lysine analog eACA or NaCl. The various C3 activation fragments were used at equal molar concentrations; i.e., 1 μg was added. After incubation for 1.5 h at room temperature, unbound protein was removed by washing three times with PBS-T, and bound proteins were identified with appropriate antiserum and corresponding HRP-conjugated antiserum.

After three additional washing steps, orthophenylendiamine (DakoCytomation) was added, the reaction was stopped by addition of 2 M H2SO4, and the absorption was measured at 492 nm in a microtiter plate reader.

For surface plasmon resonance assays, a Biacore 3000 instrument was used. Complement proteins C3b, C3d, and C5 were immobilized via standard amine coupling to the flow cells of a CMD 500m sensor chip as described (34, 47). The surface of the flow cells was activated, and the analyte diluted in coupling
buffer (10 mM acetate buffer, pH 4.0) was injected until an appropriate level of coupling was reached (~5000 resonance units). Plasminogen in the absence or presence of 10 mM eACA was used as an analyte at the indicated concentrations at a flow rate of 20 µl/min at 25 °C in 150 mM PBS.

In addition, the effect of plasminogen on Factor H binding to immobilized C3b was analyzed by ELISA. Factor H (used at 1 µg/well) and plasminogen were combined at different molar ratios, and then the mixtures were added to immobilized C3b (0.5 µg/well). Following incubation and extensive washing, each serum protein was detected separately with specific antiserum.

**Cofactor Assay**—Cofactor activity of plasminogen was analyzed by measuring Factor I-mediated degradation of C3b by Western blotting. Plasminogen at increasing concentrations (1–10 µg) was added to Factor H (30 ng), Factor I (30 ng), and C3b (0.3 µg). The reaction mixture was separated by SDS-PAGE, transferred to a membrane, and C3 degradation products were detected using goat C3 antiserum. The intensities of degradation products (β-chain and α'43 band) were analyzed by densitometry (19).

**Complement Cleavage Assay**—Cleavage of C3b and C5 was assayed in fluid and solid phases. For fluid phase activity, plasminogen (0.5 µg), uPA (0.25 µg), and C3b or C5 (0.5 µg) were mixed in PBS, and the mixture was incubated for 1 h at 37 °C. The protein degradation was assayed in the absence or presence of 5 µg/ml aprotinin, 5 µg/ml α2-antiplasmin, or 1 mM eACA.

In control experiments, C3b was incubated with Factor H and Factor I and with plasminogen or uPA. Samples were separated by SDS-PAGE and transferred to a membrane, and cleavage products were detected with appropriate antiserum and corresponding HRP-conjugated antiserum.

For solid phase activity, plasminogen (0.5 µg in 50 µl of carbonate-bicarbonate buffer) was immobilized onto a microtiter plate overnight at 4 °C. After washing with PBS-T, blocking buffer (PBS + 0.2% gelatin) was added and incubated overnight at 4 °C. Following washing, uPA and C3b (0.5 µg in PBS) were added, and the mixture was incubated at 37 °C. At the indicated times (0.5, 1, 5, and 24 h), samples were separated by SDS-PAGE under reducing conditions, transferred to a membrane, and assayed by Western blotting using goat C3 antiserum.

**Wieslab ELISA**—To assay the effect of plasmin on each of the three complement pathways, a Wieslab (Wieslab) was used according to the instructions of the supplier. Plasmin at increasing concentrations (0.5–2 µM) was added to NHS. After incubation for 30 min at 37 °C, NHS was added to the Wieslab microtiter plate. Terminal complement complex (C5b-9) formation was measured with a specific alkaline phosphatase-labeled antiserum to a neoantigen expressed during membrane attack complex formation.

**Hemolysis Assay**—Hemolysis was assayed in the absence or presence of plasminogen or uPA alone or the serine protease inhibitor aprotinin. After incubation, each sample was centrifuged at 5000 rpm for 2 min, the supernatants were added to a microtiter plate, and the optical density was measured at 414 nm (42).

**Cofactor Assay**—Cofactor activity of plasminogen was analyzed by measuring Factor I-mediated degradation of C3b by Western blotting. Plasminogen in the absence or presence of 10 mM eACA was used as an analyte at the indicated concentrations at a flow rate of 20 µl/min at 25 °C in 150 mM PBS.

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**Complement Cleavage Assay**—Cleavage of C3b and C5 was assayed in fluid and solid phases. For fluid phase activity, plasminogen (0.5 µg), uPA (0.25 µg), and C3b or C5 (0.5 µg) were mixed in PBS, and the mixture was incubated for 1 h at 37 °C. The protein degradation was assayed in the absence or presence of 5 µg/ml aprotinin, 5 µg/ml α2-antiplasmin, or 1 mM eACA.

In control experiments, C3b was incubated with Factor H and Factor I and with plasminogen or uPA. Samples were separated by SDS-PAGE and transferred to a membrane, and cleavage products were detected with appropriate antiserum and corresponding HRP-conjugated antiserum.

For solid phase activity, plasminogen (0.5 µg in 50 µl of carbonate-bicarbonate buffer) was immobilized onto a microtiter plate overnight at 4 °C. After washing with PBS-T, blocking buffer (PBS + 0.2% gelatin) was added and incubated overnight at 4 °C. Following washing, uPA and C3b (0.5 µg in PBS) were added, and the mixture was incubated at 37 °C. At the indicated times (0.5, 1, 5, and 24 h), samples were separated by SDS-PAGE under reducing conditions, transferred to a membrane, and assayed by Western blotting using goat C3 antiserum.

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Plasminogen used as an analyte showed strong association to both C3b and C3d. At concentrations from 50 to 400 nM, binding was dose-dependent (Fig. 1, E and F). The complexes were rather stable, and the dissociation profile was rather slow. In this setup, plasminogen bound to both C3b and C3d with similar intensity.

Plasminogen-C3 Interaction Is Mediated by Lysine Residues and Is Affected by Ionic Strength—C3 has about 7% lysine residues, and therefore, we hypothesized that the lysine analog eACA would affect the interaction (4). eACA inhibited plasminogen binding to C3 and C3b and more efficiently to C3d. The effect was dose-dependent. eACA at a concentration of 1 mM inhibited plasminogen binding to C3d by 75%, C3b by 60%, and C3 by 40% (Fig. 2A). In addition, the effect of ionic strength was assayed. NaCl influenced plasminogen binding to all three C3 variants to a rather similar extent, and again, the effect was dose-dependent. NaCl at the physiological level of 150 mM decreased plasminogen binding to C3 and the C3 variants by about 40%, and at a concentration at 600 mM, NaCl inhibited plasminogen binding to C3 and the C3 variants by 80% (Fig. 2B). The inhibitory effect of eACA for the plasminogen-C3b or -C3d interaction was confirmed by surface plasmon resonance. eACA inhibited plasminogen binding to the complement proteins C3b and C3d. Again, the effect was dose-dependent, and eACA at a concentration of 1 mM inhibited plasminogen binding to C3b and C3d almost completely (Fig. 2C and D). Thus, plasminogen binding to C3 and to the C3b and C3d activation fragments is mediated by lysine residues and is ionic strength-dependent.

FIGURE 1. Plasminogen binds to central human complement proteins C3 and C5. A, plasminogen binding to complement proteins C3 and C5 was analyzed by ELISA. C3 or C5 was bound to immobilized plasminogen, and bound proteins were detected using goat C3 or C5 antiserum and HRP-conjugated goat antiserum. Binding of plasminogen to immobilized C3 and to the C3 activation fragments C3b, C3c, C3d, and C3a at equal molar concentrations was analyzed. Plasminogen bound to immobilized C3, C3b, C3c, and C3d but not to C3a. C, plasminogen at concentrations ranging from 0.25 to 1.0 μg was bound to immobilized C3, C3b, and C3d at equal molar concentrations, and bound plasminogen was detected with goat plasminogen antiserum and HRP-conjugated goat antiserum; binding was dose-dependent. The bars represent the means of three independent experiments ± S.D. ***, p ≤ 0.001. D, plasminogen binding to C3 and C3 fragments was analyzed by Western blotting. Purified C3 and the C3 fragments C3b and C3c were separated by SDS-PAGE and transferred to a membrane, and the membrane was incubated with plasminogen. Bound plasminogen was detected with goat plasminogen antiserum. Plasminogen binding to C3b (E) and C3d (F) was analyzed by surface plasmon resonance. C3b or C3d was immobilized on the chip surface, and plasminogen was used as the analyte at concentrations ranging from 50 to 400 nM in the fluid phase. The results show a representative result of three independent experiments. w/o, without.
Plasminogen and Factor H Bind C3b at Separate Sites and Do Not Compete for Binding—As both plasminogen and Factor H bind to C3b, we asked whether the two human plasma proteins bind to identical, overlapping or to distinct sites of the C3b protein and whether the two proteins compete for binding. Therefore, the effect of plasminogen on Factor H binding to immobilized C3b was analyzed. Plasminogen did not compete with Factor H for binding to C3b, and even at a molar ratio of 1:10, plasminogen did not affect Factor H binding (Fig. 2E). In the presence of Factor H, plasminogen bound to C3b, and binding was dose-dependent. The molar ratio of plasminogen:Factor H in plasma is ~1:2.5, and at this concentration, both proteins bind to C3b. Thus, plasminogen and Factor H bind simultaneously to C3b, and the two human plasma proteins do not compete for binding.

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eACA inhibited plasminogen binding to C5, and the effect was dose-dependent. At 1 mM, eACA inhibited binding to C5 by 50% (Fig. 3D). Similarly, NaCl affected plasminogen binding. NaCl at the physiological level of 150 mM reduced C5 binding by 50%, and at 600 mM, NaCl inhibited binding by 70% (Fig. 3D). Thus, plasminogen binds to C5, binding is mediated by lysine residues, and the interaction is ionic strength-dependent.

Plasminogen Enhances Cofactor Activity of Factor H—As both plasminogen and Factor H bind to C3b simultaneously, we asked whether plasminogen influences cofactor activity of Factor H for Factor I-mediated C3b degradation. Plasminogen, Factor H, and Factor I were added to C3b. After incubation, the reaction mixture was separated by SDS-PAGE and transferred to a membrane, and cleavage products were identified by Western blotting. Intact C3b was detected as α’- and β-chains (Fig. 4A). Factor I in the presence of the cofactor Factor H cleaved C3b as demonstrated by the appearance of the α’68, α’43, and α’41 bands. In the presence of the zymogen plasminogen, C3b cleavage was enhanced as revealed by the higher intensity of the α’-chain cleavage fragments of 43 and 41 kDa. The intensity of α’43 increased by 17% as revealed by densitometry (ratio of β75:α’43; Fig. 4B). Thus, as a zymogen, plasminogen enhanced Factor I-mediated cleavage of C3b and thereby enhanced complement inactivation.

Plasmin Cleave C3b Protein—As plasminogen binds C3b and enhances Factor H-mediated cofactor activity, we asked whether plasminogen when converted to plasmin cleaves C3b. First, C3b cleavage by plasmin was assayed in the fluid phase.

Plasminogen was converted by uPA to the active protease plasmin, and then C3b was added. After incubation, the reaction mixture was separated by SDS-PAGE and transferred to a membrane, and C3b cleavage products were identified by Western blotting. Intact C3b was identified by the α’- and
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β-chains (Fig. 5A). Plasmin cleaved C3b, and cleavage fragments were identified as α’68, α’40, α’30, and α’17 bands. Except for the α’68 fragment, the fragments generated by plasmin were distinct from those generated by the complement protease Factor I in the presence of Factor H. The 68-kDa fragments generated by both proteases have comparable mobilities, thus indicating that plasmin and Factor I cleave the α’-chain at the same site. However, the additional three fragments, α’40, α’30, and α’17, are unique in size and are only generated by plasmin. Thus, plasmin cleaves C3b and generates unique C3b fragments.

As eACA affected plasminogen binding to C3b, we therefore asked whether eACA influences the cleavage by plasmin. eACA used at 1 mM inhibited plasmin-mediated C3b cleavage weakly (Fig. 5A). In contrast, the serine protease inhibitor aprotinin and the specific plasmin inhibitor α2-antiplasmin inhibited C3b degradation by plasmin almost completely.

As plasminogen binds to extracellular matrix and to exposed subendothelial matrices and is acquired by pathogenic microbes, we next asked whether surface-bound plasminogen when activated to plasmin also inactivates C3b. Plasminogen was immobilized, then uPa and C3b were added, plasmin was generated, and proteolytic cleavage of C3b was assayed. Immobilized plasminogen when converted to plasmin cleaved C3b, cleavage was time-dependent, and the same cleavage fragments appeared (Fig. 5B). As a single protein, plasminogen did not cleave C3b; however, in the presence of uPa, an additional 55-kDa band was generated. Thus, both surface-bound plasmin and plasmin in solution cleaved C3b and generated the same fragments.

Plasmin Cleave C5 Protein—In addition, the effect of plasmin on C5 cleavage was assayed. In the fluid phase, plasminogen activated by uPa cleaved C5, and four major cleavage fragments appeared as bands of 65, 50, 30, and 25 kDa (Fig. 6A).

eACA and aprotinin reduced this plasmin-mediated cleavage by about 50%, and the specific plasmin inhibitor α2-antiplasmin almost completely blocked C5 cleavage.

As complement regulation on surfaces is central for tissue homeostasis, we next assessed whether surface-bound plasminogen also cleaved C5. Surface-attached plasmin cleaved C5 in a time-dependent manner, and after 30 min, bands of 65 and 45 kDa likely representing intermediate products appeared. Over time, both the pattern of bands and the intensity of individual cleavage products increased. After 5 and 24 h, two additional bands of 50 and 25 kDa were detectable (Fig. 6B). After 24 h, four major degradation fragments of 65, 50, 30, and 25 kDa were detected. Plasminogen alone had no effect, and uPA alone had a minor effect on C5 cleavage. Thus, surface-bound plasmin also cleaves the complement protein C5.

Plasmin Inhibits Complement Activation—To confirm that plasminogen activated to plasmin influences complement activation, we first analyzed whether plasmin inhibits complement action in general. Active plasmin used at increasing concentrations was added to NHS in which each complement pathway was activated specifically. Complement activation was measured by assaying terminal complement complex formation. Plasmin at the physiological level (2 μM; arrow) inhibited AP and CP activation by 40% (Fig. 7, A and B) and inhibited LP activation by 60% (Fig. 7C). This activity was specific for plasmin as plasminogen used alone inhibited AP and CP activation to a low extent, i.e. by 17 and 32%, and inhibited LP activation by 45%. Similarly, when added separately to NHS, plasminogen or uPA inhibited AP, CP, and LP activation by 30%. The explanation of this effect by the single proteins is that these individual proteins when added to NHS disturb the endogenous balance between plasminogen activators and inhibitors.

The complement-inhibitory effect of plasmin on AP and CP was confirmed in hemolytic assays. First, the effect of plasmin
on NHS-mediated lysis of rabbit erythrocytes was assayed. Plasminogen and uPa were added to NHS, and then rabbit erythrocytes were added. Activated plasmin inhibited lysis of erythrocytes, and the effect was dose-dependent. Plasmin(ogen) used at a physiological level (i.e. 2 nM) inhibited erythrocyte lysis by 30%, and at a concentration of 4 nM, plasmin(ogen) inhibited erythrocyte lysis by 80% (Fig. 8A, ●). In the presence of the serine inhibitor aprotinin, which inhibited plasmin activity, erythrocytes remained intact and were not lysed. This effect was dose-dependent (Fig. 8A, ○). In this assay, neither plasminogen nor uPa alone significantly degraded C5 (lanes 6 and 7). A representative experiment of three is shown.

The inhibitory effect of activated plasmin was also assayed for CP. The CP was activated by antiserum specific for sheep erythrocytes, and again, complement activation was assayed by erythrocyte lysis. When uPa-treated plasminogen was added to NHS with sheep erythrocytes, plasmin, which inhibited erythrocyte lysis, was generated. The inhibitory effect was dose-dependent, and plasmin used at the physiological concentration of 2 μM (arrow) inhibited erythrocyte lysis by about 40% (Fig. 8B). The specific plasmin inhibitor α2-antiplasmin blocked plasmin-mediated lysis in a dose-dependent manner (Fig. 8C). Thus, plasmin inhibits complement, which is activated either by the AP or the CP.

As plasmin(ogen) binds to C3b and inhibits complement activation, we next asked whether plasmin directly blocks C3 convertase activity. Therefore, the inhibitory effect of plasmin on C3 generation was characterized using zymosan, which activates complement in NHS. Following incubation, the reaction mixtures were separated by SDS-PAGE and transferred to a membrane, and C3a generation was followed by Western blotting. Addition of zymosan to NHS activated complement as shown.
inactivates the two major complement proteins C3b and C5 and thereby blocks complement cascade progression and effector function. Plasmin cleaves C3b at multiple sites. The first cleavage site is shared by plasmin and the complement protease Factor I. However, the next two cleavage reactions occur at specific sites in the C3b protein, generating fragments of unique sizes. Thus, plasmin by cleaving C3b and C5 inhibits complement activation, blocks formation of C3 effector fragments, and consequently inhibits cascade progression. Plasmin blocks complement activation in the fluid phase and on surfaces. Thereby, plasmin can protect the surface of damaged self-cells or of the exposed subendothelial matrix. In addition, this powerful inhibitory effect on complement provides a new explanation why pathogenic microbes attach human plasminogen to their surface and use surface-bound plasmin(ogen) for immune evasion (50).

Plasminogen, which cleaves the complement proteins C3b and C5 and dissolves fibrin clots, links the two important human effector cascades. A regulatory role of plasmin(ogen) for the complement and coagulation cascades has been proposed (7, 25, 29), and recently an activating role for plasmin, i.e. generation of chemotactic C3a and C5a activation fragments, was reported (28). Here, we demonstrate for plasmin an additional inhibitory effect in the complement cascade and show that the zymogen plasminogen enhances cofactor-mediated inactiva-

DISCUSSION

Here, we identify human plasminogen as a C3- and C5-binding protein and demonstrate that active plasmin degrades and revealed by the generation of C3a (Fig. 9A). Plasmin inhibited this C3a generation in a dose-dependent manner. When added alone, plasminogen did not inhibit C3a generation. In contrast, uPA inhibited C3a generation, but the effect was lesser as compared with plasmin. Factor H as an inhibitor of the C3 convertase blocked C3a generation completely.

The effect of plasmin on C3a generation was also quantitated by ELISA. C3a generated by zymosan-activated NHS was set as 100% (Fig. 9B). Plasminogen with uPA decreased C3a levels by 40%. Factor H as the C3 convertase inhibitor blocked complement activation by 75%. Thus, plasmin inhibits the C3 convertase of the AP and consequently inhibits complement cascade progression.

FIGURE 9. Plasmin inhibited C3a generation. A, the blocking effect of plasmin on complement activation in NHS was assayed by following C3a generation. Complement was activated via the AP by adding zymosan to NHS. Then plasminogen (5–10 μg), plasminogen (10 μg), uPA (0.2 μg), or Factor H (5 μg) was added. Samples were separated by SDS-PAGE and transferred to a membrane, and C3a generation was analyzed. No C3a was generated in the absence of zymosan (lane 1). Zymosan activated the AP, and C3a was generated (lane 2). uPA-treated plasminogen inhibited C3a generation (lanes 3 and 4). Plasminogen used alone did not inhibit C3a generation, uPA added alone had a minor blocking effect on C3a generation (lanes 5 and 6), and Factor H inhibited C3a generation (lane 7). C3a was used as a control (lane 8).

B, C3a generation was also followed by ELISA. NHS in the absence of zymosan lacked C3a (column 1). Activation of NHS with zymosan resulted in the generation of C3a (black column 2). Again, plasmin used at 5 μg inhibited C3a generation (white striped column 3). Factor H also used at 5 μg inhibited C3a generation (gray striped column 4). Buffer represents the antibody control (column 5), and C3a was used as a positive control (column 6). The results are representative data of four independent experiments. **, p < 0.01.
tion of C3b by the complement protease Factor I. In addition, plasmin blocks the C3 and C5 convertases, favors C3b inactivation, degrades complement effector components, and blocks effector functions.

Plasmin(ogen) blocks all three complement pathways but to different extents. Used at physiological levels, plasmin(ogen) blocked AP and CP by about 40% and inhibited LP activation by 60%. Similarly, in hemolytic assays with either rabbit or sheep erythrocytes, uPa-treated plasminogen blocked AP by about 30% and CP by about 40%, thus demonstrating that even in human serum in the presence of additional inhibitory proteins plasmin inhibits complement. Plasminogen, the proteolytically inactive zymogen, enhances C3b processing by the complement protease Factor I in the presence of the cofactor Factor H. Plasminogen binds to the central complement components C3b and C5 and when activated to plasmin cleaves and degrades C3b and C5. Plasminogen when activated to plasmin blocks all three major complement pathways; thereby, both plasminogen and in particular the active protease plasmin influence the activity of the complement cascade in terms of activation and inhibition.

The coagulation system plays an important role in host-pathogen interactions and host responses to infections. Both cascades, the coagulation and the complement systems, start immediately and simultaneously in response to tissue injury (54) or during systemic inflammation (48). During the process of coagulation, new surfaces are formed, e.g., platelet thrombi. These thrombi stop bleeding and provide a barrier for invading microbes. In addition, the complement system is also triggered to protect against microbes and mediate inflammation. To protect these new host surfaces, both processes must be highly controlled. Interestingly, a single serine protease, i.e., plasminogen, regulates complement cascade progression and complement-mediated inflammation.

A large number of pathogenic microbes bind plasminogen to their surface, and several microbial plasminogen-binding proteins have been identified. This kind of interaction mimicked by pathogen-encoded plasmin(ogen)-binding proteins is similar to the plasminogen-C3 and -C5 interactions, which are mediated by lysine residues and which are ionic strength-dependent. Examples include protein E from *H. influenzae*, Tuf from *P. aeruginosa*, PspC from *S. pneumoniae*, CRASP1 from *B. burgdorferi*, and Pra1 and Gmp1 from *C. albicans* (33, 34, 40, 44, 51).

The majority of microbial plasminogen-binding proteins bind additional human plasma proteins and complement regulators, including Factor H, FHL-1, CFHR1, and C4b-binding protein (34, 43, 44, 51–53). Plasminogen-bound host proteins are either functionally active or in the case of plasminogen are accessible for activators. As the attached human regulators control complement activation, degrade complement activation fragments, enhance cofactor-assisted C3 inactivation, or degrade extracellular matrix proteins, these multifunctional bacterial surface proteins assist in bacterial survival and contribute to virulence by controlling and blocking host immune reactions. Surface-attached and activated plasmin allows the
pathogen to control host complement attack, to cross host immune barriers, and to invade into deeper human tissues. Such acquisition of multiple host-derived complement inhibitors by one single pathogen-encoded protein is an efficient defense strategy that apparently is used by many pathogenic microbes. The powerful inhibitory effect of acquired plasminogen for the immune and complement defense apparently explains why so many pathogenic microbes attach human plasminogen to their surface and how they exploit surface-bound plasminogen for immune evasion (23).

Plasminogen binds to C3b, the central complement activation fragment, which also binds the complement regulator Factor H. The two human regulators bind simultaneously to C3b, they bind to independent sites, and they do not compete for binding. Factor H acts as a regulator of the C3 convertase and as a cofactor for the serine protease Factor I, which cleaves and inactivates C3b. Interestingly, plasminogen in the proteolytically inactive form enhances cofactor-assisted inactivation of C3b by Factor I. Thus, even plasminogen prior to conversion and activation contributes to C3b inactivation.

Plasminogen also binds to C5, and plasmin cleaves the α-chain of the C5 protein in a time-dependent manner, ultimately generating four fragments. The first plasmin-mediated cleavage of C5 generates an intermediate fragment of 45 kDa and C5a (28) and then four fragments of 65, 50, 30, and 25 kDa. Thus, plasminogen regulates complement and blocks complement activation on multiple levels. (i) Plasminogen, the proteolytically inactive zymogen, enhances C3b processing by the complement protease Factor I in the presence of the cofactor Factor H. (ii) Plasminogen binds to the central complement component C3; to C3 activation fragments C3b, C3c, and C3d; and to C5 and when activated to plasmin cleaves and degrades C3b and C5. (iii) Plasminogen when activated to plasmin blocks all three major complement pathways (Fig. 10).

Here, we identify plasminogen as a multifunctional protease that controls two major cascade systems of a vertebrate organism and that acts on multiple levels. Plasmin in addition to regulating fibrin clot formation and extracellular matrix protein degradation has an inhibitory role in complement. These multiple controls are consequently utilized by the host for protection of newly formed thrombi and by microbial pathogens for inactivating C3b and C5 and for immune evasion.

Acknowledgment—We thank Steffi Hälbich for excellent assistance with surface plasmon resonance analysis.

REFERENCES

1. Søtrup-Jensen, L., Zajdel, M., Claëys, H., Petersen, T. E., and Magnusson, S. (1975) Amino-acid sequence of activation cleavage site in plasminogen: homology with “pro” part of prothrombin. Proc. Natl. Acad. Sci. U.S.A. 72, 2577–2581
2. Castellino, F. J., and McCance, S. G. (1997) The kringle domains of human plasminogen. Ciba Found. Symp. 212, 46–60; discussion 60–45
3. Miyashita, C., Wenzel, E., and Heiden, M. (1988) Plasminogen: a brief introduction into its biochemistry and function. Haemostasis 18, Suppl. 1, 7–13
4. Lääteenmäki, K., Edelman, S., and Korhonen, T. K. (2005) Bacterial metastasis: the host plasminogen system in bacterial invasion. Trends Microbiol. 13, 79–85
5. Lääteenmäki, K., Kuusela, P., and Korhonen, T. K. (2000) Plasminogen activation in degradation and penetration of extracellular matrices and basement membranes by invasive bacteria. Methods 21, 125–132
6. Myöhänen, H., and Vaheri, A. (2004) Regulation and interactions in the activation of cell-associated plasminogen. Cell. Mol. Life Sci. 61, 2840–2858
7. Amara, U., Rittirsch, D., Flierl, M., Bruckner, U., Klos, A., Gebhard, F., Lambris, J. D., and Huber-Lang, M. (2008) Interaction between the coagulation and complement system. Adv. Exp. Med. Biol. 632, 71–79
8. Wolberg, A. S., and Campbell, R. A. (2008) Thrombin generation, fibrin clot formation and hemostasis. Transfus. Apher. Sci. 38, 15–23
9. Wolberg, A. S. (2007) Thrombin generation and fibrin clot structure. Blood Rev. 21, 131–142
10. Cesaran-Maus, G., and Hajjar, K. A. (2005) Molecular mechanisms of fibrinolysis. Br. J. Haematol. 129, 307–321
11. Degen, J. L., Bugge, T. H., and Goguen, J. D. (2007) Fibrin and fibrinolysis in infection and host defense. J. Thromb. Haemost. 5, Suppl. 1, 24–31
12. Moroz, L. A., and Gilmore, N. J. (1976) Fibrinolysis in normal plasma and blood: evidence for significant mechanisms independent of the plasminogen-plasmin system. Blood 48, 531–545
13. Petersen, T. E., Martzen, M. R., Ichinose, A., and Dave, E. W. (1990) Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system. J. Biol. Chem. 265, 6104–6111
14. Walport, M. J. (2001) Complement. First of two parts. N. Engl. J. Med. 344, 1058–1066
15. Walport, M. J. (2001) Complement. Second of two parts. N. Engl. J. Med. 344, 1140–1144
16. Zipfel, P. F., Mihlan, M., and Skerka, C. (2007) The alternative pathway of complement: a pattern recognition system. Adv. Exp. Med. Biol. 598, 80–92
17. Zipfel, P. F., and Skerka, C. (2009) Complement regulators and inhibitory proteins. Nat. Rev. Immunol. 9, 729–740
18. Thurman, J. M., and Holers, V. M. (2006) The central role of the alternative complement pathway in human disease. J. Immunol. 176, 1305–1310
19. Heinen, S., Hartmann, A., Lauer, N., Wiehl, U., Dalhse, H. M., Schirmer, S., Gropp, K., Enghardt, T., Wallich, R., Häßlich, S., Mihlan, M., Schlötzer-Schrehardt, U., Zipfel, P. F., and Skerka, C. (2009) Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. Blood 114, 2439–2447
20. Zipfel, P. F., Skerka, C., Hellwage, J., Jokiranta, S. T., Meri, S., Brade, V., Kraiczky, P., Noris, M., and Remuzzi, G. (2002) Factor H family proteins: on complement, microbes and human diseases. Biochem. Soc. Trans. 30, 971–978
21. Jónsdóttir, G., and Zipfel, P. F. (2008) Factor H family proteins and human diseases. Trends Immunol. 29, 380–387
22. Gigli, I., Fujita, T., and Nussenzweig, V. (1979) Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. Proc. Natl. Acad. Sci. U.S.A. 76, 6596–6600
23. Zipfel, P. F. (2009) Complement and immune defense: from innate immunity to human diseases. Immunol. Lett. 126, 1–7
24. Peerschke, E. I., Yin, W., and Ghebrehiwet, B. (2008) Platelet mediated complement activation. Adv. Exp. Med. Biol. 632, 81–91
25. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H. (1953) The inactivator of complement and its components by plasmin. J. Exp. Med. 97, 573–589
26. Taylor, F. B., Jr., and Ward, P. A. (1967) Generation of chemotactic activity in rabbit serum by plasminogen-streptokinase mixtures. J. Exp. Med. 126, 149–158
27. Ward, P. A. (1967) A plasmin-split fragment of C3 as a new chemotactic factor. J. Exp. Med. 126, 189–206
28. Amara, U., Flierl, M. A., Rittirsch, D., Klos, A., Chen, H., Acker, B., Brückner, U. B., Nilsson, B., Gebhard, F., Lambris, J. D., and Huber-Lang, M. (2010) Molecular intercommunication between the complement and coagulation systems. J. Immunol. 185, 5628–5636
29. Seya, T., Nagasawa, S., Matsukura, M., Hasegawa, H., and Atkinson, J. P. (1985) Generation of C3dg and C3d by urokinase-treated plasma in association with fibrinolysis. Complement 2, 165–174
30. Polley, M. J., and Nachman, R. (1978) The human complement system in
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thrombin-mediated platelet function. J. Exp. Med. 147, 1713–1726
31. Hallström, T., Järve, H., Riesbeck, K., and Blom, A. M. (2007) Interaction with C4b-binding protein contributes to nontypeable Haemophilus influen-zae serum resistance. J. Immunol. 178, 6359–6366
32. Hallström, T., Zipfel, P. F., Blom, A. M., Lauer, N., Forsgren, A., and Ries-beck, K. (2008) Haemophilus influenzae interacts with the human comple-ment inhibitor factor H. J. Immunol. 181, 537–545
33. Barthel, D., Singh, B., Riesbeck, K., and Zipfel, P. F. (2012) Haemophilus influenzae uses the surface protein E to acquire human plasminogen and to evade innate immunity. J. Immunol. 188, 379–385
34. Kunert, A., Losse, J., Gruszin, C., Hühn, M., Kaendler, K., Mikkat, S., Volke, D., Hoffmann, R., Jokiranta, T. S., Seeberger, H., Moellmann, U., Hell-wage, J., and Zipfel, P. F. (2007) Immune evasion of the human patho-gen Pseudomonas aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein. J. Immunol. 179, 2979–2988
35. Kraiczy, P., Skerka, C., Kirschfink, M., Brade, V., and Zipfel, P. F. (2001) Immune evasion of Borrelia burgdorferi by acquisition of human comple-ment regulators FHL-1/reconectin and Factor H. Eur. J. Immunol. 31, 1674–1684
36. Kraiczy, P., Skerka, C., Kirschfink, M., Zipfel, P. F., and Brade, V. (2001) Mechanism of complement resistance of pathogenic Borrelia burgdorferi isolates. Int. Immunopharmacol. 1, 393–401
37. Seling, A., Siegel, C., Fingerle, V., Jutras, B. L., Brissette, C. A., Skerka, C., Wallich, R., Zipfel, P. F., Stevenson, B., and Kraiczy, P. (2010) Functional characterization of Borrelia spielmanii outer surface proteins that interact with distinct members of the human factor H protein family and with plasminogen. Infect. Immun. 78, 39–48
38. Brissette, C. A., Haupt, K., Barthel, D., Cooley, A. E., Bowman, A., Skerka, C., Wallich, R., Zipfel, P. F., Kraiczy, P., and Stevenson, B. (2009) Borrelia burgdorferi infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. Infect. Immun. 77, 300–306
39. Zipfel, P. F., Hallström, T., Hammerschmidt, S., and Skerka, C. (2008) The complement fitness factor H: role in human diseases and for immune escape of pathogens, like pneumococci. Vaccine 26, Suppl. 8, 167–174
40. Agarwal, V., Asmat, T. M., Luo, S., Jensch, I., Zipfel, P. F., and Ham-merschmidt, S. (2010) Complement regulator Factor H mediates a two-step uptake of Streptococcus pneumoniae by human cells. J. Biol. Chem. 285, 23486–23495
41. Bergmann, S., Rohde, M., Chhatwal, G. S., and Hammerschmidt, S. (2004) Characterization of plasminogen binding to Streptococcus pneumoniae. Indian J. Med. Res. 119, (suppl.) 29–32
42. Haupt, K., Reuter, M., van den Elsen, J., Burman, J., Hölßich, S., Richter, J., Skerka, C., and Zipfel, P. F. (2008) The Staphylococcus aureus protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b. PLoS Pathog. 4, e1000250
43. Poltermann, S., Kunert, A., von der Heide, M., Eck, R., Hartmann, A., and Zipfel, P. F. (2007) Gmp1p is a factor H–FHL-1-, and plasminogen-bind-ing surface protein of Candida albicans. J. Biol. Chem. 282, 37537–37544
44. Luo, S., Poltermann, S., Kunert, A., Rupp, S., and Zipfel, P. F. (2009) Im-mune evasion of the human pathogenic yeast Candida albicans: Pral 1 is a Factor H, FHL-1 and plasminogen binding surface protein. Mol. Immunol. 47, 541–550
45. Sun, H. (2006) The interaction between pathogens and the host coagula-tion system. Physiology 21, 281–288
46. Skerka, C., Hellwage, J., Weber, W., Tillkorn, A., Buck, F., Marti, T., Kam-phen, E., Beisiegel, U., and Zipfel, P. F. (1997) The human factor H-related protein 4 (FHR-4). A novel short consensus repeat-containing protein is associated with human triglyceride-rich lipoproteins. J. Biol. Chem. 272, 5627–5634
47. Weber, S., Lottspeich, F., and Köhl, J. (1995) An epitope of elongation factor Tu is widely distributed within the bacterial and archaeal domains. J. Bacteriol. 177, 11–19
48. Levi, M., van der Poll, T., and Bülter, H. R. (2004) Bidirectional relation between inflammation and coagulation. Circulation 109, 2698–2704
49. Luo, S., Hartmann, A., Dahe, H. M., Skerka, C., and Zipfel, P. F. (2010) Secreted pH-regulated antigen 1 of Candida albicans blocks activation and conversion of complement C3. J. Immunol. 185, 2164–2173
50. Zipfel, P. F., Würzner, R., and Skerka, C. (2007) Complement evasion of pathogens: common strategies are shared by diverse organisms. Mol. Immunol. 44, 3850–3857
51. Hallström, T., Haupt, K., Kraiczy, P., Hortschansky, P., Wallich, R., Skerka, C., and Zipfel, P. F. (2010) Complement regulator-acquiring surface pro-tein 1 of Borrelia burgdorferi binds to human bone morphogenic protein 2, several extracellular matrix proteins, and plasminogen. J. Infect. Dis. 202, 490–498
52. Berge, A., and Sjöbring, U. (1993) PAM, a novel plasminogen-binding protein from Streptococcus pyogenes. J. Biol. Chem. 268, 25417–25424
53. Kraiczy, P., Rossmann, E., Brade, V., Simon, M. M., Skerka, C., Zipfel, P. F., and Walllich, R. (2006) Binding of human complement regulators FHL-1 and factor H to CRASP-1 orthologs of Borrelia burgdorferi. Wien. Klin. Wochenschr. 118, 669–676
54. Lasser, E. C., Slivka, J., Lang, J. H., Kolb, W. P., Lyon, S. G., Hamblin, A. E., and Nazareno, G. (1979) Complement and coagulation: causative consider-ations in contrast catastrophes. AIR Am. J. Roentgenol. 132, 171–176