The Journal of Experimental Medicine

Potentiation of C1 Esterase Inhibitor by StcE, a Metalloprotease Secreted by *Escherichia coli* O157:H7

Wyndham W. Lathem, Tessa Bergsbaken, and Rodney A. Welch

Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706

Abstract

The complement system is an essential component of host defense against pathogens. Previous research in our laboratory identified StcE, a metalloprotease secreted by *Escherichia coli* O157:H7 that cleaves the serpin C1 esterase inhibitor (C1-INH), a major regulator of the classical complement cascade. Analyses of StcE-treated C1-INH activity revealed that surprisingly, StcE enhanced the ability of C1-INH to inhibit the classical complement-mediated lysis of sheep erythrocytes. StcE directly interacts with both cells and C1-INH, thereby binding C1-INH to the cell surface. This suggests that the augmented activity of StcE-treated C1-INH is due to the increased concentration of C1-INH at the sites of potential lytic complex formation. Indeed, removal of StcE abolishes the ability of C1-INH to bind erythrocyte surfaces, whereas the proteolysis of C1-INH is unnecessary to potentiate its inhibitory activity. Physical analyses showed that StcE interacts with C1-INH within its aminoterminal domain, allowing the unaffected serpin domain to interact with its targets. In addition, StcE-treated C1-INH provides significantly increased serum resistance to *E. coli* K-12 over native C1-INH. These data suggest that by recruiting C1-INH to cell surfaces, StcE may protect both *E. coli* O157:H7 and the host cells to which the bacterium adheres from complement-mediated lysis and potentially damaging inflammatory events.

Key words: complement • serpins • serum resistance • contact activation • reactive center loop

Introduction

Innate mechanisms of immunity are fundamental components of host defense and are essential for the detection and clearance of many invading pathogens. Among them, the complement system serves multiple functions, including opsonization and lysis of organisms, production of chemoattractant molecules, mediation of inflammation, and augmentation of humoral immunity (for review see reference 1). Acting in a series of interlinked proteolytic cascades comprised of multiple serine proteases, complement pathways are rapidly up-regulated and the components are amplified upon activation. To protect the host from the adverse inflammatory and cytolytic effects resulting from complement activation, multiple factors exist to prevent unregulated complement activity (2–5).

One of these factors is C1 esterase inhibitor (C1-INH), an essential regulator of classical complement, intrinsic coagulation, and contact activation (for review see reference 6). C1-INH is a member of the serine protease inhibitor (serpin) family of molecules, which includes antithrombin, α-1-antitrypsin, and plasminogen activator type-1 (7). Serpins control multiple proteolytic cascades in the body by nonenzymatically inactivating target serine proteases in a 1:1 stoichiometric ratio. Serpins interact with their targets via the serpin reactive center loop (RCL) which, when cleaved by the serine protease, inserts into the target molecule. This insertion irreversibly displaces the active serine of the protease from its catalytic partners (8).

C1-INH is the only known inhibitor of activated C1r and C1s of the classical complement cascade and the MASP 1 and 2 proteases of the mannose-binding ligand pathway, and is a major inhibitor of Factor XIIa (Hageman Factor), kallikrein, and Factor XIa of the inflammation, fibrinolysis, and the intrinsic coagulation pathways (9–14). Additionally, C1-INH competes with Factor B for binding to C3b to

Address correspondence to Rodney A. Welch, Department of Medical Microbiology and Immunology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706. Phone: (608) 263-2700; Fax: (608) 262-8418; email: rawelch@wisc.edu

W.W. Lathem's present address is Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Ave., Saint Louis, MO 63110.

T. Bergsbaken's present address is Department of Microbiology, University of Washington, 1959 NE Pacific St., Seattle, WA 98195.

Abbreviations used in this paper: C1-INH, C1 esterase inhibitor; LB, Luria-Bertani; RCL, reactive center loop.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/04/1077/11 $8.00
Volume 199, Number 8, April 19, 2004 1077–1087
http://www.jem.org/cgi/doi/10.1084/jem.20030255

inhibit the activation of the alternative complement pathway (15). C1-INH is a heavily glycosylated protein and the majority of the posttranslational modifications occur within the aminoterminal domain unique to this serpin. Interestingly, removal of this domain does not affect the ability of C1-INH to interact with or inhibit its targets (16, 17). As with other serpins, glycosaminoglycans can potentiate the inhibitory capacity of C1-INH, although the effect of potentiation differs depending on the substrate (18, 19). C1-INH forms large SDS-insoluble complexes with its target proteases that are subsequently removed from circulation (6). In addition to its soluble form, cell-associated C1-INH may protect cells from proinflammatory events at their surface (6, 20, 21). However, the mechanism by which this form of C1-INH is bound to the cell surface is unknown.

Escherichia coli O157:H7 is an enteric human pathogen responsible for diarrhea, hemorrhagic colitis, and the potentially lethal hemolytic uremic syndrome. Multiple virulence determinants have been recognized in *E. coli* O157:H7, including the Shiga toxin, an ADP-ribosylating enzyme that inhibits protein synthesis, and the locus of enterocyte effacement, responsible for conferring the attaching and effacing phenotype on intestinal epithelial cells (for review see references 22 and 23). Our laboratory has identified an additional potential virulence factor, StcE, a metalloprotease that cleaves C1-INH from its apparent full-length M_r of 105 kD to produce unique ~60–65-kD species (24). StcE is encoded on pO157, a large plasmid carried by strains of *E. coli* O157:H7, and is ubiquitous among isolates of the enterohemorrhagic *E. coli* 1 complex (25). StcE is secreted by a functional type II secretion apparatus also encoded on pO157, and its expression has been detected in vivo (24, 26).

In this report, we investigate the functional consequence of the interaction between StcE and C1-INH. We show that instead of inactivating C1-INH, StcE potentiates the ability of C1-INH to inhibit classical complement activity by recruiting the inhibitor to cell surfaces. Additionally, physical analysis indicates that StcE cleaves C1-INH not in the RCL, but within its heavily glycosylated NH_2-terminal domain. Finally, we show that StcE-treated C1-INH provides increased serum resistance to *E. coli*, suggesting that StcE may aid the protection of *E. coli* O157:H7 from complement activity during infection.

Materials and Methods

Bacterial Strains, Buffers, and Materials. All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Buffers used were PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4), TBS (20 mM Tris, 150 mM NaCl, pH 7.5), VBS (5 mM veronal, 145 mM NaCl, pH 7.4), VBS^{2+} (VBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and VBS containing 10 mM EDTA. Recombinant DNA manipulations were performed by standard methods. pTB4 was constructed by amplifying bases 207–2795 of the *stcE* gene from pO157 by PCR with the primers *stcE*Sac5′207 (5′-CCGAGCTCCGGCTGATAATAATTCAGCCATTTATTTC-3′) and *stcE*3′Xba2795 (5′-CCTCGAGTTTATATACAACCCTGATTG-3′), and cloning the product into the SacI-XhoI sites of pET24d(+) (Novagen). WAM2751 is *E. coli* strain BL21(DE3) (Novagen) transformed with pTB4. pTB5 was constructed in the same manner as pTB4 by amplifying the equivalent bases of the *stcE* E435D mutant from pTEG1 (24). WAM2804 is *E. coli* strain BL21(DE3) transformed with pTB5. StcE′-His, lacking the StcE NH_2-terminal signal sequence and containing a 6xHis tag at the COOH terminus, and StcE′ E435D-His, a similar protein with a single amino acid change at residue 435 (glutamic to aspartic acid) that disrupts the catalytic activity of the protease, were purified from WAM2751 and WAM2804, respectively, as previously described (24) and dialyzed into VBS, pH 6.5. Purified C1-INH was obtained from Advanced Research Technologies and purified kallikrein and C1s were from Calbiochem. All proteins were stored at −80°C. mAbs 3C7 and 4C3 were provided by P.A. Patston (University of Illinois at Chicago, Chicago, IL).

Hemolytic Assays. Sheep erythrocytes were prepared according to Mayer (27). Erythrocytes were opsonized with an anti–sheep red blood cell Ab for 10 min before use. Human serum (0.5%) was mixed with opsonized erythrocytes (10^7 in 50 μl) in VBS^{2+} to a total volume of 200 μl for 1 h at 37°C before the addition of 1 ml VBS plus 10 mM EDTA to stop complement activity. To measure the amount of hemoglobin released by lysed cells, erythrocytes were pelleted and the OD_{412} of the supernatant was measured in a spectrophotometer. The percent lysis was determined by subtracting the OD_{412} in the absence of serum and dividing by the maximum possible OD_{412} obtained by lysis of erythrocytes in water. Where indicated, increasing concentrations of StcE′-His or BSA were incubated with serum overnight at room temperature before the start of the assay. To determine the effect of StcE-treated C1-INH on erythrocyte lysis, increasing concentrations of StcE′-His or BSA were mixed with 16 μg C1-INH, or increasing concentrations of C1-INH were mixed with 1 μg StcE′-His or 1 μg StcE′ E435D-His in a total volume of 149 μl VBS^{2+} overnight at room temperature before the start of the assay. Statistical analyses were performed by the unpaired *t* test.

Flow Cytometry. 8 μg C1-INH was untreated or treated with 1 μg StcE′-His in a total volume of 149 μl VBS^{2+} as described above before the addition of opsonized sheep erythrocytes and human serum deficient in complement component C5 (Quidel Corp.). Erythrocytes were incubated for 10 min at 37°C before the addition of VBS plus 10 mM EDTA. Cells were washed with VBS^{2+} and incubated on ice for 30 min with polyclonal goat anti–human IgG against C1-INH (Cedarlane Laboratories). Erythrocytes were washed, incubated on ice for 30 min with FITC-conjugated rabbit anti–goat IgG, and resuspended in VBS^{2+} for analysis by flow cytometry using a fluorescence-activated cell sorter (FACSCalibur™; Becton Dickinson). Where indicated, StcE′-His was removed from the assay mixture by adsorption to Ni-NTA agarose beads (QIAGEN) in the presence of 50 mM imidazole before the addition of sheep erythrocytes. To measure StcE-treated C1-INH saturation kinetics of erythrocytes, increasing concentrations of C1-INH were mixed with 1 μg StcE′ E435D-His before the addition of 10^7 sheep erythrocytes as described above in the absence of human serum and analyzed by flow cytometry.

To determine if StcE binds sheep erythrocytes, StcE′-His was labeled via its primary amines with the Alexa Fluor 488 dye as described by the manufacturer (Molecular Probes). 5×10^6 sheep erythrocytes were opsonized as described above, pelleted, and resuspended in 500 μl VBS^{2+} before the addition of 250 ng StcE′-His or Alexa-labeled StcE′-His for 10 min at 37°C. Erythrocytes

were pelleted and washed with VBS^{2+} before analysis by flow cytometry. To measure the point at which erythrocytes become saturated with StcE, increasing concentrations of Alexa-labeled StcE′-His were added to 10^7 sheep erythrocytes for 10 min at 37°C before analysis by flow cytometry as described above.

Immunoblot Analyses. 1.5 μg purified, activated C1s were untreated, treated with 100 ng C1-INH, or treated with C1-INH in the presence of 50 ng StcE′-His or StcE′ E435D-His for 1 h at 37°C in a total volume of 30 μl VBS^{2+}. An equal volume of nonreducing sample buffer was then added, the samples were heated to 95–100°C for 5 min, and the proteins were separated on an 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose and analyzed by immunoblot as previously described (24) using a polyclonal goat anti–C1s Ab (Calbiochem).

In other experiments, mAb 4C3 was coupled to protein A–Sepharose beads as previously described (28) and used to remove trace amounts of RCL-cleaved C1-INH from the purified C1-INH preparation. 1 μg virgin C1-INH was then incubated with or without 1 μg StcE′-His or 2 μg kallikrein for 18 h at room temperature before electrophoresis on an 8% reducing SDS-PAGE gel. Separated proteins were transferred to nitrocellulose and analyzed by immunoblot as described using a polyclonal anti–human C1-INH Ab (Serotec), mAb 3C7, or mAb 4C3.

Cell Culture. COS-7 cells (provided by D. Paulnock, University of Wisconsin, Madison, WI) were cultured in DMEM (Invitrogen) with 10% heat-inactivated FCS (Mediatech), nonessential amino acids, and penicillin/streptomycin/amphotericin B (Invitrogen). Cells were transfected with either hC1-INH/pcDNA3.1(−) or C-serp(98)/pcDNA3.1(−) (provided by A.E. Davis, Harvard University, Cambridge, MA) using cationic lipids (Lipofectamine PLUS; Invitrogen). After transfection, cells were cultured in the presence of G418 (Invitrogen). Recombinant proteins were metabolically labeled with [^{35}S]methionine (Amersham Biosciences) for 24 h before immunoprecipitation.

Immunoprecipitation. 100 μl culture supernatants from C1-INH–transfected COS-7 cells were treated with 10 μg StcE′-His overnight at room temperature before incubation with polyclonal goat anti–human C1-INH IgG (Cedarlane Laboratories) and protein A–Sepharose for 2 h at room temperature. Pellets were washed three times with TBS, resuspended in sample buffer, and electrophoresed on 10% reducing SDS-PAGE gels. Gels were fixed, dried, and visualized with a phosphorimager (Typhoon 8600; Amersham Biosciences). In other experiments, 5 μg C1-INH was untreated or treated with 5 μg StcE′-His or StcE′ E435D-His for 10 min at 37°C in 500 μl buffer (100 mM Tris, pH 8.0) before the addition of polyclonal goat anti–human C1-INH IgG. The mixture was rotated for 30 min at 4°C, after which 20 μl of a protein A–Sepharose slurry was added for 2 h. The protein A–Sepharose beads were subsequently washed three times in buffer before immunoblot analysis with an anti-StcE Ab.

Kallikrein Activity Assay. Increasing concentrations of C1-INH were mixed with 250 ng StcE′-His in a total volume of 100 μl assay buffer (50 mM Tris, pH 8.0, 100 mM NaCl) at room temperature overnight, after which EDTA was added to 5 mM to stop the reaction. Purified kallikrein was diluted to 100 ng in 50 μl assay buffer and mixed with C1-INH for 1 h at 37°C before adding the chromogenic substrate S-2302 (Chromogenix) to each tube. Tubes were incubated at room temperature for 30 min before determining the absorbance of the substrate at 410 nm in a spectrophotometer. Percent kallikrein activity was determined by subtracting the OD_{410} in the absence of kallikrein and dividing by the maximum possible OD_{410} obtained by kallikrein activity in the absence of C1-INH.

Serum Resistance. 8 μg C1-INH was untreated or treated with 1 μg StcE′-His in a total volume of 176 μl VBS^{2+} overnight at room temperature, after which human serum was added to 2%. *E. coli* K-12 strain C600 was grown to an OD_{595} of 0.5 in Luria-Bertani (LB) broth at 37°C with aeration before being washed once and resuspended with an equivalent volume of VBS^{2+}. 20 μl bacteria were added to the reactions, incubated at 37°C for 1 h, and 10 μl aliquots were mixed with VBS plus 10 mM EDTA to stop complement activity. 10-fold serial dilutions of bacteria were plated on LB agar and percent survival was determined by dividing CFUs by the total number of bacteria after 1 h in the absence of serum. Statistical analysis was performed by the unpaired *t* test.

Results

Inhibition of Classical Complement-mediated Erythrocyte Lysis by StcE. Previous research from our laboratory demonstrated that StcE, a metalloprotease secreted by *E. coli* O157:H7, cleaves the serum protein C1-INH from its apparent M_r of 105 kD to produce ~60–65-kD species (24). As C1-INH is an essential regulator of the classical complement pathway, we examined the effect of StcE on the classical complement-mediated lysis of sheep erythrocytes. Human serum was mixed overnight with increasing concentrations of StcE or the control protein BSA before adding to opsonized sheep erythrocytes for 1 h at 37°C. The reaction was stopped with EDTA and the amount of hemoglobin released by lysed erythrocytes into the supernatant was measured. Serum alone lysed 75.0% (±2.7% SEM) of erythrocytes and BSA had no effect on the ability of serum to lyse erythrocytes (Fig. 1). At higher concentrations, though, StcE significantly reduced classical complement-mediated erythrocyte lysis compared with equivalent amounts of BSA (2 μg, $P < 0.01$; 4 μg, $P < 0.005$).

StcE Potentiates C1-INH–mediated Inhibition of Classical Complement. Although StcE cleaves the serum component C1-INH, the data represented in Fig. 1 do not implicate the serpin directly in StcE-mediated classical comple-

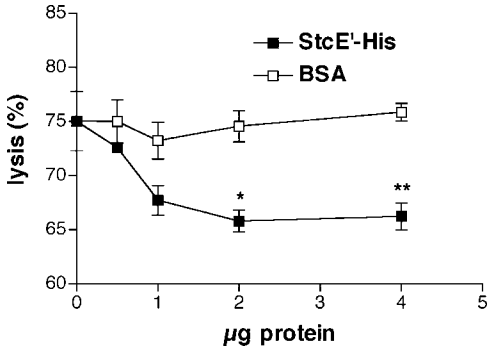

Figure 1. StcE reduces the classical complement-mediated erythrocyte lysis by human serum. 1 μl human serum was untreated or treated with increasing concentrations of StcE′-His or BSA overnight before the addition of 10^7 opsonized sheep erythrocytes for 1 h at 37°C, in triplicate. Unlysed cells were pelleted and the OD_{412} of the supernatant was measured in a spectrophotometer (*, $P = 0.0077$; **, $P = 0.0028$; unpaired *t* test). Suspension in distilled water was used to determine the absorbance of 100% lysis of erythrocytes.

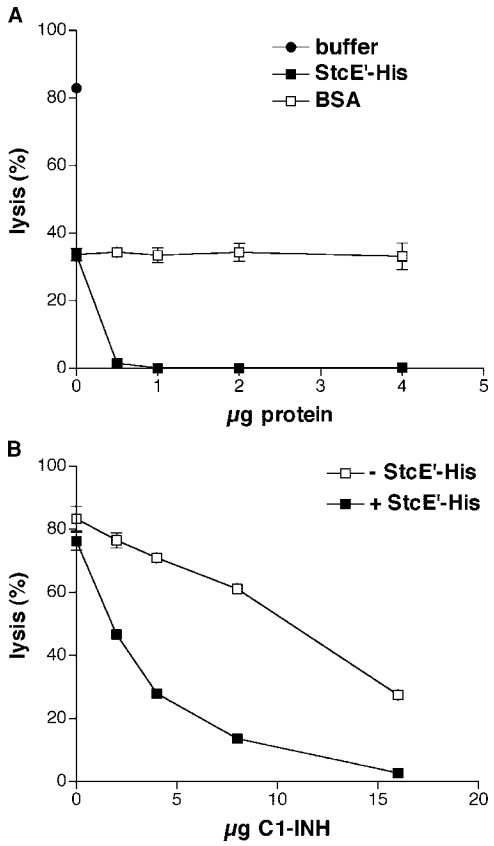

Figure 2. StcE potentiates C1-INH–mediated inhibition of classical complement. (A) 16 μg C1-INH was untreated or treated with increasing concentrations of StcE′-His or BSA overnight at room temperature before the addition of 1 μl human serum and 10^7 opsonized sheep erythrocytes, in triplicate. Erythrocytes were incubated at 37°C for 1 h, pelleted, and the OD_{412} of the supernatant was measured in a spectrophotometer. The point indicated by ● lacked C1-INH. (B) Increasing amounts of C1-INH were untreated or treated with 1 μg StcE′-His overnight at room temperature before the addition of human serum and opsonized sheep erythrocytes as described in A, in triplicate. Suspension in distilled water was used to determine the absorbance of 100% lysis of erythrocytes (all p-values <0.0005; unpaired *t* test).

ment inhibition. To examine if StcE-treated C1-INH plays a role in this process, we mixed purified C1-INH with increasing concentrations of StcE or BSA overnight and added this mixture to human serum and opsonized sheep erythrocytes for 1 h at 37°C. As before, the reaction was stopped with EDTA and the amount of hemoglobin released by lysed erythrocytes into the supernatant was measured. The addition of 0.4 IU untreated C1-INH to the assay decreased erythrocyte lysis from 82.9 (±1.1% SEM) to 33.7% (±1.8% SEM; Fig. 2 A), demonstrating the effective role of C1-INH in the inhibition of classical complement activity. Although BSA-treated C1-INH was unchanged in inhibitory activity, 0.4 IU StcE-treated C1-INH reduced erythrocyte lysis to between 1.5 (±0.5% SEM) and 0.1% (±0.1% SEM) of total.

To confirm the effect of StcE on C1-INH–mediated inhibition of erythrocyte lysis, increasing concentrations of C1-INH were untreated or treated with StcE′-His overnight before the addition of human serum and opsonized sheep erythrocytes. Increasing concentrations of untreated C1-INH (0.05–0.4 IU) resulted in a dose-dependent decrease in erythrocyte lysis, ranging from 76.5 (±2.4% SEM) to 27.5% (±1.1% SEM; Fig. 2 B). However, in the presence of 1 μg StcE′-His, the same concentrations of C1-INH significantly reduced lysis below that of untreated C1-INH (ranging from 46.7 [±1.5% SEM] to 2.8% lysis [±1.3% SEM], all p-values <0.0005). These results demonstrate the direct role of StcE-treated C1-INH in the decrease of classical complement-mediated erythrocyte lysis. Additional experiments confirm that StcE-treated C1-INH continues to react with its natural targets C1r and/or C1s to mediate the inhibition of classical complement, maintaining the target specificity of the serpin (unpublished data).

StcE Binds Erythrocyte Surfaces. To understand how StcE might potentiate C1-INH, we asked if StcE could interact with erythrocytes, thereby acting as a binding protein for C1-INH on cell surfaces. 250 ng unlabeled StcE′-His or a form of StcE′-His fluorescently labeled via its primary amines with the Alexa Fluor 488 dye (Alexa-StcE′-His) were added to opsonized sheep erythrocytes for 10 min at

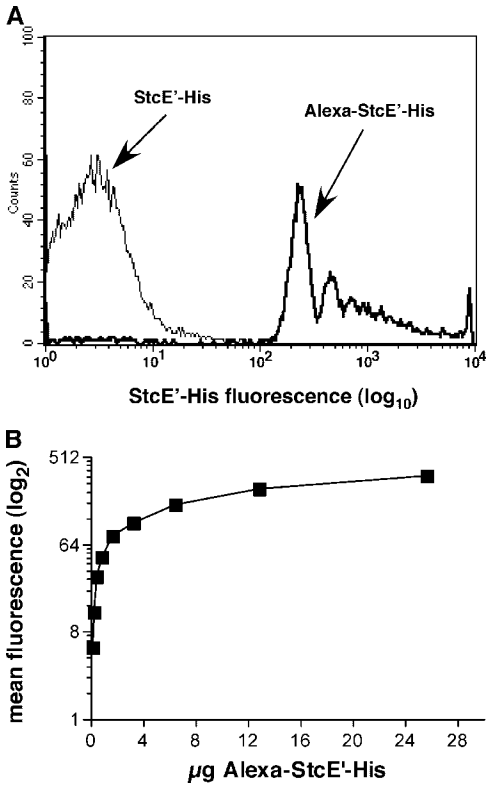

Figure 3. StcE binds erythrocyte surfaces. (A) 250 ng StcE′-His or Alexa-StcE′-His was incubated with opsonized sheep erythrocytes for 10 min at 37°C, after which the cells were washed and the binding of StcE was detected in a flow cytometer. (B) Increasing concentrations of Alexa-StcE′-His (0.1–25.6 μg) were added to sheep erythrocytes. StcE binding was detected by flow cytometry as described above, and the geometric mean fluorescence for each sample was plotted against the corresponding concentration of Alexa-StcE′His.

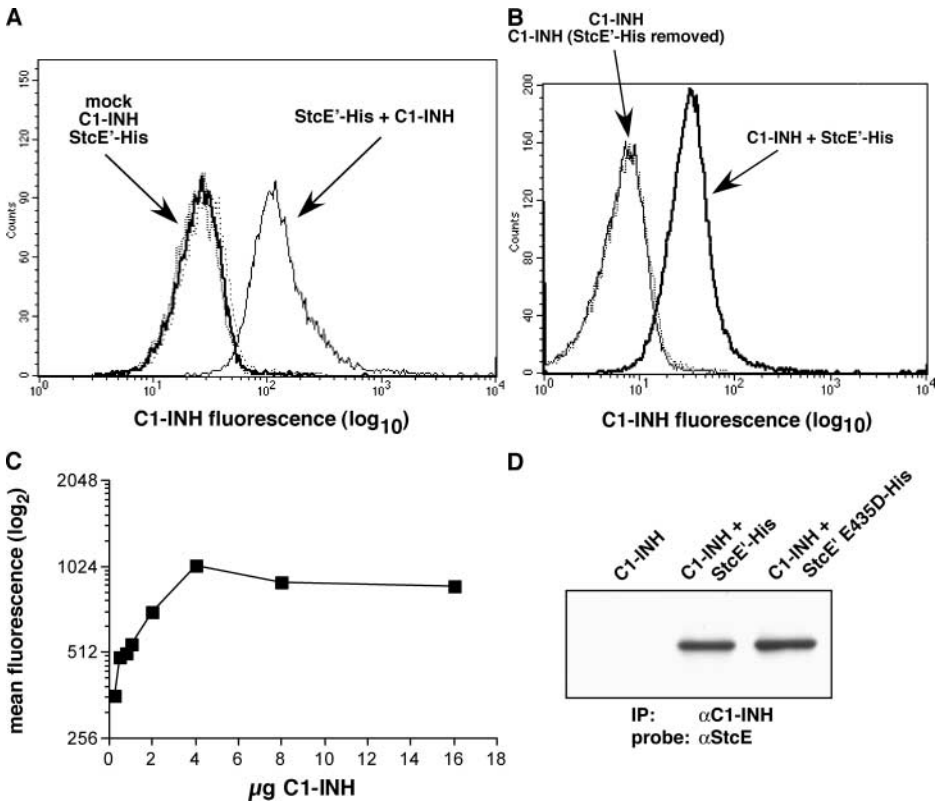

Figure 4. C1-INH binds erythrocyte surfaces in the presence of StcE. (A) 8 μg C1-INH was untreated or treated with 1 μg StcE′-His overnight at room temperature. C5-deficient human serum was added to opsonized sheep erythrocytes in the presence of 8 μg C1-INH, 1 μg StcE′-His, C1-INH, and StcE′-His, or buffer alone (mock) for 10 min at 37°C. Erythrocytes were washed and polyclonal goat anti–human C1-INH IgG was added to cells. C1-INH binding was detected with FITC-conjugated anti–goat IgG in a flow cytometer. (B) 8 μg C1-INH was incubated with or without 1 μg StcE′-His overnight at room temperature before the addition of 50 mM imidazole and Ni-NTA agarose beads to remove StcE′-His from the sample. Opsonized sheep erythrocytes were added for 10 min at 37°C and C1-INH binding was detected as described. As a control for StcE-treated C1-INH binding, StcE′-His was not removed from one sample before analysis. (C) Increasing concentrations of C1-INH (0.25–16 μg) were mixed with 1 μg StcE′ E435D-His overnight at room temperature before the addition of opsonized erythrocytes. C1-INH binding was detected in a flow cytometer as described above, and the geometric mean fluorescence for each sample was plotted against the corresponding concentration of C1-INH. (D) 5 μg C1-INH was untreated or treated with 5 μg StcE′-His or StcE′ E435D-His for 10 min before being immunoprecipitated with an anti–C1-INH Ab. Precipitated proteins were separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with an anti-StcE Ab as described in Materials and Methods.

37°C. Erythrocytes were pelleted and washed with VBS^{2+} before analysis by flow cytometry. Erythrocytes treated with Alexa-labeled StcE′-His showed 80-fold greater mean fluorescence compared with cells treated with unlabeled StcE′-His (Fig. 3 A), demonstrating a direct interaction between these cells and the protease. Furthermore, we observed that StcE continues to bind sheep erythrocytes even at lower temperatures (0–4°C), suggesting that this interaction is not mediated by an active cellular process (not depicted). To determine if the interaction between erythrocytes and StcE is specific and therefore saturable, we mixed increasing concentrations of Alexa-labeled StcE′-His with 10^7 sheep erythrocytes as described above. We observed that this number of erythrocytes becomes saturated with StcE′-His at 3.2 μg of the protease in 500 μl (Fig. 3 B). Based on the calculated molecular weight of StcE′-His, at this concentration we estimate $\sim 1.8 \times 10^6$ molecules of StcE are bound per erythrocyte.

StcE Localizes C1-INH to Erythrocyte Surfaces. Based on the ability of StcE to directly bind erythrocytes, we asked if StcE could localize C1-INH to erythrocyte surfaces, thereby possibly increasing the local concentration of inhibitor at the site of potential lytic complex formation. C1-INH was untreated or treated with StcE′-His overnight before the addition of opsonized sheep erythrocytes and C5-deficient human serum (to prevent formation of the membrane attack complex and lysis of the cells) for 10 min at 37°C. Erythrocytes were washed, treated with an Ab against C1-INH, and washed again before the addition of an FITC-conjugated secondary Ab. Deposition of C1-INH was subsequently analyzed by flow cytometry. Little to no C1-INH binding was measured on erythrocytes treated with only StcE′-His or native C1-INH compared with mock-treated cells (Fig. 4 A). However, increased deposition of C1-INH was detected on erythrocytes mixed with 0.2 IU StcE-treated C1-INH. Similar results were observed in the absence of human serum, indicating that serum components or complement activation are not required for the localization of StcE-treated C1-INH to erythrocyte surfaces. As we observed with the interaction between StcE and sheep erythrocytes, StcE-treated C1-INH continues to bind the cells at 0–4°C (not depicted).

The results suggest that StcE may directly mediate the binding of C1-INH to the cell surface. To test this possibility, C1-INH was incubated with or without StcE′-His overnight as described earlier, after which Ni-NTA agarose beads were added to the samples in the presence of 50 mM imidazole to specifically remove the 6xHis-tagged StcE protein from the assay. The agarose beads were pelleted and the supernatants were mixed with sheep erythrocytes and analyzed by flow cytometry as described above. In the absence of the protease, StcE-treated C1-INH no longer binds to erythrocyte surfaces (Fig. 4 B), indicating that StcE itself is required to sequester C1-INH to erythrocytes. The absence

of StcE′-His from the Ni-NTA agarose-treated samples and the presence of equivalent amounts of C1-INH between Ni-NTA agarose-treated and untreated samples were confirmed by immunoblot analyses (unpublished data).

To determine the level at which erythrocytes become saturated with StcE-treated C1-INH, we mixed StcE′ E435D-His (a mutant form of the protein containing a single amino acid change from glutamic to aspartic acid at position 435 that is unable to cleave C1-INH; reference 24) with increasing concentrations of C1-INH (from 0.25 to 16 μg) before the addition of opsonized sheep erythrocytes as described above. We chose to use StcE′ E435D-His in this experiment so as to measure the saturation of sheep erythrocytes with C1-INH without the creation of StcE-cleaved C1-INH, which might reduce the levels of the serpin bound to the cell surface, thereby increasing the amount needed to saturate the cells. We observed that in the presence of 1 μg StcE′ E435D-His, this number of erythrocytes becomes saturated with C1-INH at 4 μg, or 0.1 IU, of the serpin (Fig. 4 C). Based on the observed molecular weight of mature C1-INH and assuming uniform binding of the primary and secondary antibodies to their antigens, at this concentration we estimate $\sim 2.25 \times 10^6$ molecules of C1-INH are bound per erythrocyte. Finally, to determine if C1-INH and StcE can interact in solution (before binding erythrocytes), C1-INH was mixed with either StcE′-His or StcE′ E435D-His for 10 min at 37°C before immunoprecipitating the mixture with an anti–C1-INH Ab. After separating the immunoprecipitated proteins by SDS-PAGE and transferring them to nitrocellulose, both StcE′-His and StcE′ E435D-His were detected with an anti–StcE′-His Ab, demonstrating that a complex of StcE and C1-INH can be formed in solution (Fig. 4 D).

Cleavage of C1-INH by StcE Is Not Necessary to Protect Cells against Complement Activity. To test if the proteolysis of C1-INH by StcE is necessary to provide erythrocytes increased protection against classical complement activity over that of untreated C1-INH, we mixed C1-INH with either StcE′-His or the proteolytically inactive StcE′ E435D-His. After overnight incubation, the samples were added to human serum and opsonized sheep erythrocytes for 1 h at 37°C before determining the amount of hemoglobin released into the supernatant by the lysed cells as described above. In the presence of 0.2 IU C1-INH, human serum lysed 75.8% (±1.5% SEM) of the erythrocytes, whereas StcE′-His–treated C1-INH significantly decreased erythrocyte lysis to 25.4% (±3.5% SEM, $P < 0.005$; Fig. 5 A). Interestingly, the cleavage of C1-INH by StcE is not required for the protection of erythrocytes from complement activity, as StcE′ E435D-His–treated C1-INH was able to significantly reduce the lysis of the cells to 16.7% (±1.3% SEM, $P < 0.005$). The difference in the amount of erythrocyte lysis between the StcE′-His– and StcE′ E435D-His–treated C1-INH samples was not significant ($P > 0.05$).

To determine if the subsequent cleavage of C1-INH by StcE affects the binding of the serpin to erythrocytes, we incubated 2 μg C1-INH with 1 μg StcE′-His or StcE′ E435D-His overnight before assessing the levels of surface-associated C1-INH by flow cytometry as described above. Indeed, the levels of C1-INH in the presence of StcE′ E435D-His on erythrocyte surfaces are 22-fold higher than in the presence of StcE′-His (Fig. 5 B), suggesting that once cleaved by StcE, C1-INH no longer binds to erythrocytes. In total, the data presented in Figs. 4 and 5 demonstrate that the increased protection of erythrocytes by StcE-treated C1-INH is dependent upon the physical presence of StcE and not the cleavage of C1-INH by the protease.

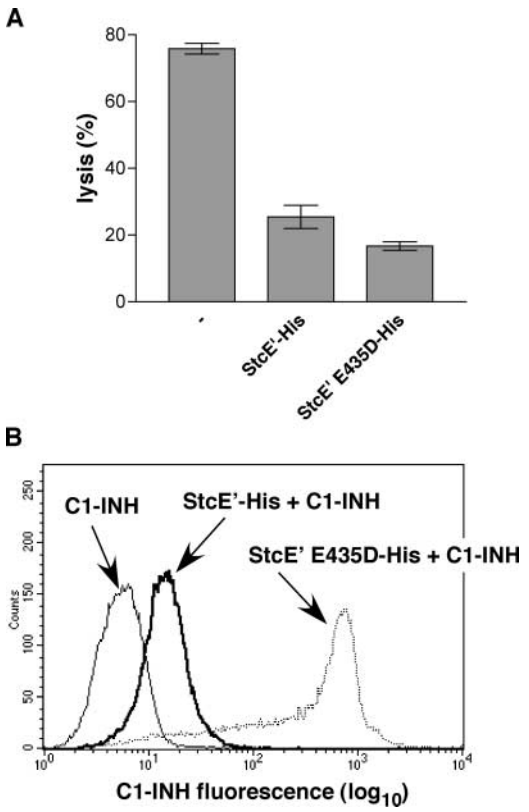

Figure 5. Cleavage of C1-INH by StcE is not necessary to bind erythrocytes or provide protection against classical complement. (A) Classical complement-mediated erythrocyte lysis was determined as described in Materials and Methods in the presence of 8 μg C1-INH, C1-INH and 1 μg StcE′-His, or C1-INH and an enzymatic point mutant of StcE, 1 μg StcE′ E435D-His (*, $P < 0.005$; unpaired *t* test). (B) 2 μg C1-INH was untreated or treated with 1 μg StcE′-His or StcE′ E435D-His before the addition of sheep erythrocytes as described in Materials and Methods. Erythrocytes were washed and polyclonal goat anti–human C1-INH IgG was added to cells. C1-INH binding was detected with FITC-conjugated anti–goat IgG in a flow cytometer.

StcE Is Unable to Potentiate C1-INH in the Absence of Cells. Data presented so far demonstrate the ability of StcE to localize C1-INH to erythrocytes, thereby providing increased complement-inhibiting activity at the cell surface. To determine if this potentiation can occur in solution (i.e., in the absence of cells), we measured whether StcE affects the ability of C1-INH to inhibit kallikrein, another C1-INH–regulated molecule, by monitoring the cleavage of a chromogenic substrate of kallikrein, S-2302 (*H*-D-Prolyl-L-phenylalanyl-L-arginine-*p*-nitroaniline dihydrochloride).

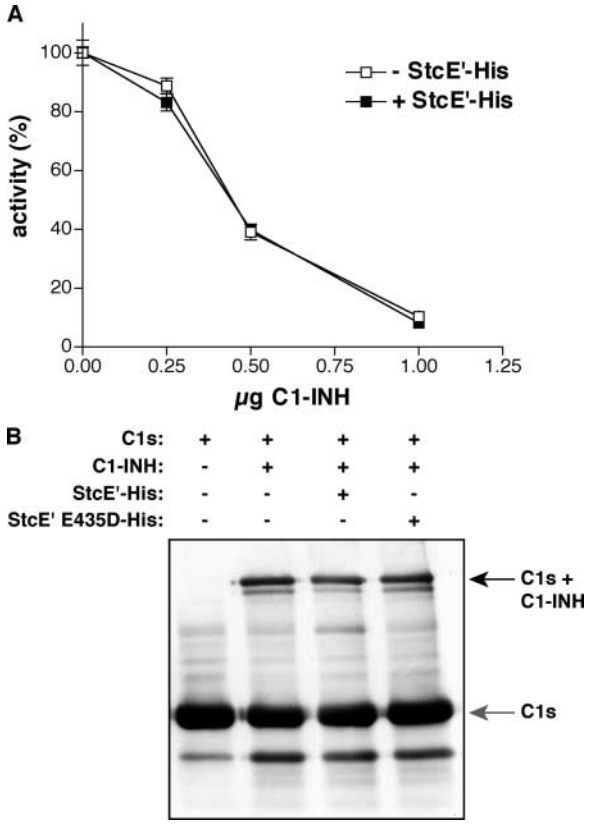

Figure 6. StcE is unable to potentiate C1-INH in the absence of cells. (A) Increasing concentrations of C1-INH (0–1.0 μg) were mixed with 250 ng StcE′-His overnight at room temperature and incubated with 100 ng kallikrein for 1 h at 37°C before the addition of S-2302, in triplicate. Absorbance of the chromogenic substrate was measured at 410 nm in a spectrophotometer after 30 min at room temperature. 100% kallikrein activity was determined in the absence of C1-INH. (B) An excess of purified, activated C1s (1.5 μg) was untreated or treated with 100 ng C1-INH in the absence or presence of 50 ng StcE′-His or StcE′ E435D-His for 1 h at 37°C before being separated by nonreducing SDS-PAGE and analyzed by immunoblot with an anti-C1s Ab. The gray arrow indicates the presence of uncomplexed C1s and the black arrow indicates the presence of C1s bound to C1-INH in an SDS-insoluble complex.

Increasing concentrations of C1-INH were untreated or treated with StcE′-His overnight at room temperature, after which the samples were allowed to react with kallikrein for 1 h at 37°C. The C1-INH/kallikrein mixtures were subsequently incubated with S-2302 for 30 min at room temperature before determining total kallikrein activity by measuring the change in absorbance of the samples in a spectrophotometer. For the purpose of this assay, kallikrein in the absence of C1-INH was considered to be 100% active. As expected, increasing concentrations of C1-INH resulted in a dose-dependent decrease in kallikrein activity, ranging from 88.8 (±2.6% SEM) to 10.3% (±1.3% SEM) activity (Fig. 6 A). The addition of StcE-treated C1-INH to the assay did not significantly alter the inactivation of kallikrein compared with untreated C1-INH.

We also examined the ability of StcE-treated C1-INH to interact with an excess of C1s in solution, thereby forming an SDS-insoluble complex. 1.5 μg purified, activated C1s was mixed with 100 ng C1-INH and 50 ng StcE′-His or StcE′ E435D-His for 1 h at 37°C before separating the proteins by nonreducing SDS-PAGE and analyzing the mixture by immunoblot with an anti-C1s Ab. The high molecular weight band in samples containing C1-INH that are absent from the sample containing C1s alone are indicative of the C1s–C1-INH interaction. If StcE were able to potentiate C1-INH in solution, an increase in the intensity of the C1s–C1-INH band would be visible. However, this does not appear to be the case (Fig. 6 B). Thus, the mechanism of StcE-mediated potentiation of C1-INH is dependent on the presence of cell surfaces upon which the protease–serpin complex can bind.

Interaction of StcE with the NH_2 Terminus of C1-INH. The ability of StcE to interact with C1-INH while maintaining the inhibitory activity of the molecule suggests that

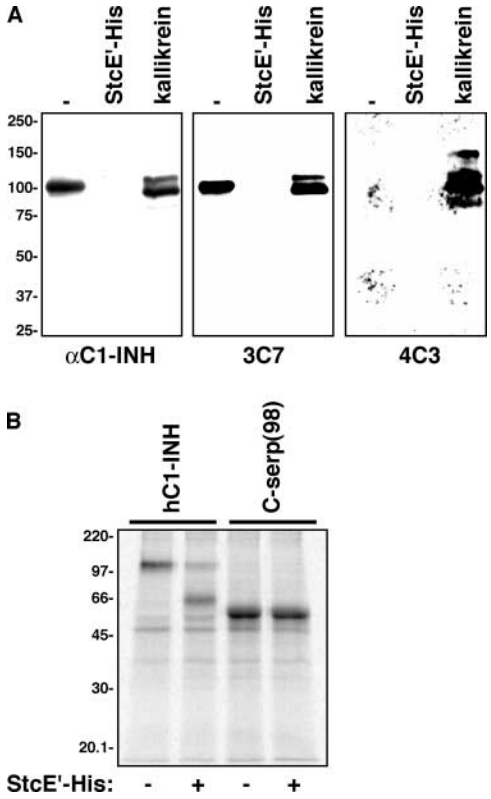

Figure 7. StcE interacts with the aminoterminal domain of C1-INH. (A) StcE-treated C1-INH is nonreactive with mAbs against the NH_2 terminus of and RCL-inserted C1-INH. 1 μg virgin C1-INH was untreated or treated with 1 μg StcE′-His or 2 μg kallikrein overnight at room temperature, separated by electrophoresis on 8% reducing SDS-PAGE gels, transferred to nitrocellulose, and analyzed with a polyclonal anti–human C1-INH Ab (left), mAb 3C7 (middle), or mAb 4C3 (right). (B) StcE does not cleave C-serp(98), a recombinant C1-INH molecule truncated at amino acid 98. COS-7 cells were transfected with hC1-INH/pcDNA3.1(−) or C-serp(98)/pcDNA3.1(−) and metabolically labeled with [^{35}S]methionine. 100 μl supernatants were untreated or treated with 10 μg StcE′-His overnight, immunoprecipitated with polyclonal anti–human C1-INH IgG protein A–Sepharose, and separated by electrophoresis on a 10% reducing SDS-PAGE gel.

StcE may bind C1-INH in the heavily glycosylated NH_2-terminal domain, leaving the serpin domain unaffected. Therefore, to further characterize the site(s) of cleavage by StcE, we examined StcE-treated C1-INH with the mAbs 3C7 and 4C3, directed against the amino terminus of C1-INH (Patston, P.A., personal communication) and the RCL-inserted form of C1-INH (29), respectively. As most preparations of purified C1-INH contain trace amounts of RCL-cleaved C1-INH, we removed this species of C1-INH from the mixture by immunoprecipitation with mAb 4C3 before analysis. Virgin C1-INH was treated with StcE or kallikrein, a serine protease inactivated by C1-INH via its interaction with and cleavage of the RCL, before analysis by immunoblot with a polyclonal anti–human C1-INH Ab (Fig. 7 A, left), 3C7 (Fig. 7 A, middle), or 4C3 (Fig. 7 A, right). As expected, analysis with 3C7 detected both virgin C1-INH and kallikrein-reacted C1-INH, but did not detect StcE-cleaved C1-INH, indicating a modification of the C1-INH NH_2 terminus by StcE. Additionally, analysis with 4C3 detected RCL-inserted C1-INH produced upon interaction with kallikrein, but not virgin C1-INH or StcE-treated C1-INH.

To confirm that StcE interacts with the NH_2-terminal domain of C1-INH, we examined the ability of StcE to cleave a recombinant C1-INH protein lacking this region. Coutinho et al. (16) demonstrated that C-serp(98), a recombinant C1-INH molecule lacking the NH_2-terminal amino acids 1–97 and containing only the serpin domain, binds its serine protease substrates identically to wild-type C1-INH and is effective in inhibiting C1 activity in hemolytic assays. We expressed recombinant, full-length human C1-INH (hC1-INH) and C-serp(98) in COS-7 cells and harvested the culture media after 24 h in the presence of [^{35}S]methionine. Samples were untreated or treated with StcE′-His overnight before immunoprecipitating metabolically labeled protein with polyclonal anti–human C1-INH IgG. Both hC1-INH and C-serp(98) migrated at the appropriate molecular weights on a reducing SDS-PAGE gel, however, only hC1-INH was cleaved by StcE′-His. C-serp(98) was unaffected by the protease (Fig. 7 B). These analyses further support the evidence that StcE does not inactivate C1-INH, but instead interacts with the heavily glycosylated NH_2-terminal domain of C1-INH, leaving the serpin domain available for interaction with C1-INH targets.

Increased Bacterial Serum Resistance in the Presence of StcE-treated C1-INH. StcE is secreted by *E. coli* O157:H7 (24), a human pathogen that may come in contact with blood or blood products during the course of an infection. Based on its ability to enhance C1-INH–mediated inhibition of classical complement, we examined if StcE could provide serum resistance to *E. coli*. As *E. coli* O157:H7 is naturally serum resistant and contains a variety of factors that could contribute to its protection from complement (30), we chose to assess the role of StcE-treated C1-INH in the survival of a serum-sensitive strain of *E. coli*. *E. coli* K-12 strain C600 was grown to mid-log phase, pelleted, and resuspended in an equivalent amount of VBS^{2+} before the addition of human serum and 0.2 IU C1-INH or StcE-treated C1-INH. Bacteria were incubated at 37°C for 1 h, serially diluted, and plated onto LB agar to determine the numbers of surviving CFUs. In the presence of human serum alone, 0.07% (±0.06% SEM) of bacteria survived, demonstrating the exquisite serum sensitivity of *E. coli* strain C600 (Fig. 8). The addition of StcE′-His to bacteria at the beginning of the assay had no significant effect on survival (0.04% survival, ±0.03% SEM). As expected, the addition of untreated C1-INH increased survival of bacteria to 3.9% (±0.9% SEM). The addition of StcE-treated C1-INH to the assay, however, caused a significant increase in bacterial survival over untreated C1-INH (16.5% survival, ±1.9% SEM; $P < 0.005$), indicating a contribution to complement resistance by StcE.

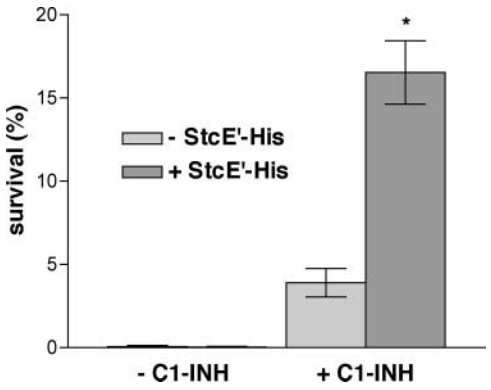

Figure 8. StcE provides increased serum resistance to *E. coli* K-12 by the potentiation of C1-INH. 8 μg C1-INH was untreated or treated with 1 μg StcE′-His overnight. *E. coli* strain C600 was grown in LB broth at 37°C to midlogarithmic phase and diluted at 1:10 into a buffer containing 2% human serum and either 8 μg C1-INH, 1 μg StcE′-His, C1-INH, and StcE′-His, or no additional protein, in triplicate. Bacteria were incubated at 37°C for 1 h, serially diluted, and plated onto LB agar. Percent survival was determined by comparing the number of CFUs to bacteria incubated under the same conditions without serum (*, $P = 0.0037$; unpaired *t* test).

Discussion

In this report, we define the outcome of the interaction between StcE, a metalloprotease secreted by the enteric pathogen *E. coli* O157:H7, and its apparent target, the serpin C1-INH. Rather than reducing or destroying its serpin activity, this interaction in effect enhances the ability of C1-INH to down-regulate the classical complement cascade, thereby protecting cells from the lytic effects of complement activity. Specifically, we found that the addition of StcE-treated C1-INH to opsonized sheep erythrocytes and human serum significantly decreased erythrocyte lysis below that of equivalent amounts of native C1-INH (Fig. 2). The decrease is specific for StcE and C1-INH, as BSA had no effect on erythrocyte lysis. We also observed a significant decrease in complement activity when human serum was treated with StcE before the addition of sheep erythrocytes (Fig. 1). This is likely due to the activity of StcE on

the endogenous C1-INH found in serum, although other potential targets cannot be ruled out. Furthermore, analysis of complement component deposition on erythrocyte surfaces indicates that StcE-treated C1-INH continues to act on its natural targets C1r and/or C1s.

Interestingly, we observed that StcE was unable to potentiate C1-INH activity in the absence of cells (Fig. 6). These data suggest that rather than modifying C1-INH directly to increase its inhibitory ability, StcE might be tethering the serpin to cell surfaces, thereby increasing the local concentration of the inhibitor at the sites of potential lytic complex formation. Functional C1-INH has been identified on the surface of multiple cell types including endothelial cells and platelets (20, 21). Moreover, work by Patston and Schapira (31) demonstrated that C1-INH binds extracellular matrix proteins such as collagen IV and laminin while retaining inhibitory activity against C1s. C1-INH cross-linked by tissue transglutaminase to the extracellular matrix may influence the local regulation of C1-INH–controlled proteolytic pathways (32) and recombinant, GPI-anchored C1-INH–expressing porcine endothelial cells are protected from human complement-mediated lysis (33). Indeed, ex vivo reperfusion of pig liver with C1-INH showed immunoreactive C1-INH localized to the sinusoidal endothelium and reduced plasma C3 activation (34).

With this in mind, we investigated whether StcE could localize C1-INH to erythrocyte surfaces, thereby increasing the local concentration of the inhibitor at sites of potential lytic complex formation. We found that StcE-treated C1-INH, but not native C1-INH, binds erythrocyte surfaces (Fig. 4 A), providing a plausible explanation for the reduced classical complement activity observed. The StcE-mediated binding of C1-INH to cell surfaces would also explain why StcE had no effect on C1-INH–mediated inhibition of kallikrein (Fig. 6) because this assay was performed in the absence of cells. However, as the contact activation pathway is initiated upon the interaction of Factor XII and prekallikrein with negatively charged surfaces (6), it is feasible that StcE-treated C1-INH may down-regulate this cascade in a manner similar to that observed with classical complement.

How does StcE-treated C1-INH obtain the capacity to bind to cells? In some cases, a change in the structure of C1-INH allows the altered molecule to bind to receptor(s) on the cell surface. For instance, desialylated C1-INH is thought to interact with hepatic asialoglycoprotein receptors (35), whereas C1-INH–C1s complexes bind to the low density lipoprotein receptor–related protein, LRP (36). Rather than modifying C1-INH in this way, however, our data indicate that StcE sequesters C1-INH to erythrocytes by itself binding to cells (Fig. 3) and acting as a "bridge" between the serpin and the cell surface (Fig. 5). Indeed, the cleavage of C1-INH by StcE is unnecessary to provide increased protection against complement activity, as a point mutant of StcE defective in proteolytic activity against C1-INH protects erythrocytes to similar levels as wild-type StcE in vitro (Fig. 5 A). The interaction between StcE and erythrocytes is specific, saturating the cells at $\sim 1.8 \times 10^6$ molecules of StcE per cell (Fig. 3 B). In turn, this allows a high affinity interaction between C1-INH and erythrocytes, reaching 2.25×10^6 molecules of C1-INH per cell in the presence of 1 μg StcE. This amount of C1-INH (0.1 IU) is well within the physiological concentration of C1-INH found in serum, suggesting that this interaction might be biologically relevant in vivo. Although it appears that the ratio of C1-INH to StcE is >1:1, this might be due to the heterogeneous nature of C1-INH (native, inactivated, and variably glycosylated forms of the molecule), leaving only a fraction of the population of C1-INH able to interact with StcE. Alternatively, StcE may act as a coligand to a multimeric molecule capable of binding C1-INH and other components. Future analyses of the binding requirements of StcE and C1-INH will address this possibility.

In our model, the initial interaction between StcE and the NH_2-terminal region of C1-INH at the cell surface allows the serpin to inactivate its targets via the exposed RCL at the site of C1 complex formation. This occurs before the eventual cleavage of C1-INH by StcE and the subsequent release of the serpin from the cell surface, thereby freeing StcE to interact with another molecule of C1-INH (Fig. 9). Indeed, we observed increased levels of C1-INH bound to erythrocytes in the presence of the proteolytically inactive StcE mutant compared with StcE′-His, supporting the notion of C1-INH turnover at the cell surface (Fig. 5 B). Thus, the relatively slow cleavage rate of C1-INH by StcE (24) may actually be beneficial to the potentiation of the serpin, as a more rapid rate of catalysis might not allow enough time for the inactivation of the C1 complex to occur. Additionally, the interaction of StcE with the NH_2-terminal region of C1-INH (Fig. 7) while bound to the cell surface likely permits continued access of the serpin domain to its targets without compromising its activity. The binding of StcE to host cells might also allow the protease to be carried to sites distal to *E. coli* O157:H7 colonization in a manner similar to the Shiga toxin (37, 38), thereby affecting C1-INH-regulated processes outside the local environment of

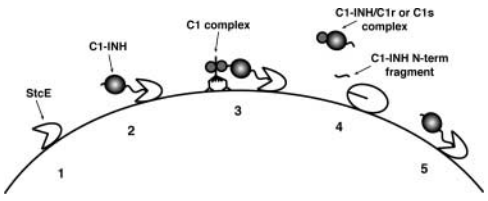

Figure 9. Model of the mechanism by which StcE potentiates C1-INH-mediated inhibition of classical complement. (1) StcE interacts with the cell surface. (2) StcE binds the NH_2-terminal domain of C1-INH, sequestering the serpin to the cell surface. Alternatively, StcE may interact with C1-INH before binding to the cell surface. (3) Cell-bound C1-INH binds to C1r and/or C1s of the C1 complex via the RCL, inactivating the serine protease. (4) StcE cleaves within the NH_2 terminus of C1-INH, releasing the serpin/serine protease complex and a smaller, aminoterminal cleavage fragment of C1-INH from the cell surface. (5) StcE binds another C1-INH molecule and repeats the cycle as described above. Note that the figure is not drawn to scale.

bacterial infection. Finally, the observation that cell-bound C1-INH might affect leukocyte adhesion (39) suggests StcE could influence the migration of inflammatory mediators to the sites of enterohemorrhagic *E. coli* colonization.

The complement system is an essential means of host defense against invading organisms, however, pathogenic bacteria have developed multiple ways to subvert complement activity. Capsules and long *O*-polysaccharide side chains of LPS can effectively prevent the formation of the membrane attack complex at bacterial cell surfaces (for review see references 40 and 41), and general metalloproteases such as the *Serratia marcescens* 56-kD protease (42, 43) and the *Pseudomonas aeruginosa* elastase (44) can proteolytically inactivate complement components. Additionally, the sequestration of complement inhibitors to bacterial surfaces can reduce complement activity and promote serum resistance. Examples include the binding of Factor H by the outer membrane protein OspE to *Borrelia burgdorferi* surfaces (45) and C4b-binding protein by the well-studied M protein of group A streptococci (46, 47). In this report we demonstrate that StcE-treated C1-INH acts in a similar manner by providing increased serum resistance to *E. coli* over native C1-INH (Fig. 8). By preventing complement activity at such an early stage in the pathway via the recruitment of C1-INH to cells, StcE may also reduce the opsonization of *E. coli* O157:H7 by C3b and the production of the chemoattractant anaphylatoxins C3a and C5a. Because StcE is a secreted protein (24), the protection against complement activation may extend to the colonic epithelial cells colonized by *E. coli* O157:H7.

The genome of *E. coli* O157:H7 contains other potential serum-resistance loci, including homologues of *traT* and the λ *bor* gene encoded on the 933W prophage (30, 48). As such, StcE likely acts in concert with other factors to prevent complement activation. In addition, the StcE-cleaved aminoterminal domain of C1-INH may contain unidentified functions not previously associated with native C1-INH, much like the smaller, cleaved "a" fragments of various complement components. The effect of StcE on the control of other C1-INH–regulated targets remains to be investigated, however, based on our data, StcE may well have profound consequences on multiple aspects of *E. coli* O157:H7 pathogenesis.

We thank members of the University of Wisconsin Clinical Science Center Flow Cytometry Facility for their assistance with the analyses of complement deposition. We also thank Dr. Philip A. Patson for his gift of mAbs against C1-INH and Dr. Alvin E. Davis for the C1-INH cDNAs.

This work was supported by Public Health Service grants AI051735 and AI20323 and the University of Wisconsin School of Medicine.

Submitted: 14 February 2003
Accepted: 2 March 2004

References

1. Frank, M.M., and L.F. Fries. 1989. Complement. *In* Fundamental Immunology. W.E. Paul, editor. Raven Press, New York. 679–701.
2. Pensky, J., and H.G. Schwick. 1969. Human serum inhibitor of C′1 esterase: identity with alpha-2-neuraminoglycoprotein. *Science*. 163:698–699.
3. Weiler, J.M., M.R. Daha, K.F. Austen, and D.T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc. Natl. Acad. Sci. USA*. 73: 3268–3272.
4. Gigli, I., T. Fujita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc. Natl. Acad. Sci. USA*. 76:6596–6600.
5. van den Berg, R.H., M. Faber-Krol, L.A. van Es, and M.R. Daha. 1995. Regulation of the function of the first component of complement by human C1q receptor. *Eur. J. Immunol.* 25:2206–2210.
6. Caliezi, C., W.A. Wuillemin, S. Zeerleder, M. Redondo, B. Eisele, and C.E. Hack. 2000. C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol. Rev.* 52:91–112.
7. Potempa, J., E. Korzus, and J. Travis. 1994. The serpin superfamily of proteinase inhibitors: structure, function and regulation. *J. Biol. Chem.* 269:15957–15960.
8. Huntington, J.A., R.J. Read, and R.W. Carrell. 2000. Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. 407:923–926.
9. Sim, R.B., A. Reboul, G.J. Arlaud, C.L. Villiers, and M.G. Colomb. 1979. Interaction of I125-labelled complement subcomponents C1r and C1s with protease inhibitors in plasma. *FEBS Lett.* 97:111–115.
10. Chan, J.Y., C.E. Burrowes, F.M. Habal, and H.Z. Movat. 1977. The inhibition of activated factor XII (Hageman factor) by antithrombin III: the effect of other plasma proteinase inhibitors. *Biochem. Biophys. Res. Commun.* 74:150–158.
11. Schapira, M., C.F. Scott, and R.W. Colman. 1982. Contribution of plasma protease inhibitors to the inactivation of kallikrein in plasma. *J. Clin. Invest.* 69:462–468.
12. Van den Graaf, F., J.A. Koedam, and B.N. Bouma. 1983. Inactivation of kallikrein in human plasma. *J. Clin. Invest.* 71: 149–158.
13. Pixley, R.A., M. Schapira, and R.W. Colman. 1985. The regulation of human factor XIIa by plasma proteinase inhibitors. *J. Biol. Chem.* 260:1723–1729.
14. Wuillemin, W.A., M. Minnema, J.C. Meijers, D. Roem, A.J.M. Eerenberg, J.H. Nuijens, J.W. ten Cate, and C.E. Hack. 1995. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role of C1-inhibitor. *Blood*. 85:1517–1526.
15. Jiang, H., E. Wagner, H. Zhang, and M.M. Frank. 2001. Complement 1 inhbitor is a regulator of the alternative complement pathway. *J. Exp. Med.* 194:1609–1616.
16. Coutinho, M., K.S. Aulak, and A.E. Davis. 1994. Functional analysis of the serpin domain of C1 inhibitor. *J. Immunol.* 153:3648–3654.
17. Patston, P.A., M. Qi, J.A. Schifferli, and M. Schapira. 1995. The effect of cleavage by a *Crotalus atrox* alpha-proteinase fraction on the properties of C1-inhibitor. *Toxicon*. 33:53–61.
18. Wuillemin, W.A., E. Eldering, F. Citarella, C.P. de Ruig, J.W. ten Cate, and C.E. Hack. 1996. Modulation of contact system proteases by glycosaminoglycans. Selective enhancement of the inhibition of factor XIa. *J. Biol. Chem.* 271: 12913–12918.

19. Caldwell, E.E., A.M. Andreasen, M.A. Blietz, J.N. Serrahn, V. VanderNoot, Y. Park, G. Yu, R.J. Linhardt, and J.M. Weiler. 1999. Heparin binding and augmentation of C1 inhibitor activity. *Arch. Biochem. Biophys.* 361:215–222.
20. Schmaier, A.H., S.C. Murray, G.D. Heda, A. Farber, A. Kuo, K. McCrae, and D.B. Cines. 1989. Synthesis and expression of C1 inhibitor by human umbilical vein endothelial cells. *J. Biol. Chem.* 264:18173–18179.
21. Schmaier, A.H., S. Amenta, T. Xiong, G.D. Heda, and A.M. Gewirtz. 1993. Expression of platelet C1 inhibitor. *Blood.* 82: 465–474.
22. Frankel, G., A.D. Phillips, I. Rosenshine, G. Dougan, J.B. Kaper, and S. Knutton. 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol. Microbiol.* 30:911–921.
23. Nataro, J.P., and J.B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142–201.
24. Lathem, W.W., T.E. Grys, S.E. Witowski, A.G. Torres, J.B. Kaper, P.I. Tarr, and R.A. Welch. 2002. StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. *Mol. Microbiol.* 45:277–288.
25. Lathem, W.W., T. Bergsbaken, S.E. Witowski, N.T. Perna, and R.A. Welch. 2003. Acquisition of *stcE*, a C1 esterase inhibitor-specific metalloprotease, during the evolution of *Escherichia coli* O157:H7. *J. Infect. Dis.* 187:1907–1914.
26. Paton, A.W., and J.C. Paton. 2002. Reactivity of convalescent-phase hemolytic-uremic syndrome patient sera with the megaplasmid-encoded TagA protein of shiga toxigenic *Escherichia coli* O157. *J. Clin. Microbiol.* 40:1395–1399.
27. Mayer, M.M. 1961. Experimental Immunochemistry. Thomas, Springfield, IL. 905 pp.
28. Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 726 pp.
29. de Agostini, A., M. Schapira, Y.T. Wachtfogel, R.W. Colman, and S. Carrel. 1985. Human plasma kallikrein and C1 inhibitor form a complex possessing an epitope that is not detectable on the parent molecules: demonstration using a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 82:5190–5193.
30. Perna, N.T., G. Plunkett III, V. Burland, B. Mau, J.D. Glasner, D.J. Rose, G.F. Mayhew, P.S. Evans, J. Gregor, H.A. Kirkpatrick, et al. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature.* 409:529–533.
31. Patston, P.A., and M. Schapira. 1997. Regulation of C1-inhibitor function by binding to type IV collagen and heparin. *Biochem. Biophys. Res. Commun.* 230:597–601.
32. Hauert, J., P.A. Patston, and M. Schapira. 2000. C1 inhibitor cross-linking by tissue transglutaminase. *J. Biol. Chem.* 275: 14558–14562.
33. Matsunami, K., S. Miyagawa, M. Yamada, M. Yoshitatsu, and R. Shirakura. 2000. A surface-bound form of human C1 esterase inhibitor improves xenograft rejection. *Transplantation.* 69:749–755.
34. Bergamaschini, L., G. Gobbo, S. Gatti, L. Caccamo, P. Prato, M. Maggioni, P. Braidotti, R. Di Stefano, and L.R. Fassati. 2001. Endothelial targeting with C1-inhibitor reduces complement activation in vitro and during ex vivo reperfusion of pig liver. *Clin. Exp. Immunol.* 126:412–420.
35. Minta, J.O. 1981. The role of sialic acid in the functional activity and the hepatic clearance of C1-INH. *J. Immunol.* 126:245–249.
36. Storm, D., J. Herz, P. Trinder, and M. Loos. 1997. C1 inhibitor-C1s complexes are internalized and degraded by the low density lipoprotein receptor-related protein. *J. Biol. Chem.* 272:31043–31050.
37. te Loo, D.M., L.A. Monnens, T.J. van Der Velden, M.A. Vermeer, F. Preyers, P.N. Demacker, L.P. van Den Heuvel, and V.W. van Hinsbergh. 2000. Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood.* 95:3396–3402.
38. te Loo, D.M., V.W. van Hinsbergh, L.P. van Den Heuvel, and L.A. Monnens. 2001. Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 12:800–806.
39. Cai, S., and A.E. Davis III. 2003. Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion. *J. Immunol.* 171:4786–4791.
40. Cooper, N.R. 1991. Complement evasion strategies of microorganisms. *Immunol. Today.* 12:327–331.
41. Frank, M.M. 1992. The mechanism by which microorganisms avoid complement attack. *Curr. Opin. Immunol.* 4:14–19.
42. Molla, A., T. Akaike, and H. Maeda. 1989. Inactivation of various proteinases inhibitors and the complement system in human plasma by the 56-kilodalton proteinase from *Serratia marcescens*. *Infect. Immun.* 57:1868–1871.
43. Oda, T., Y. Kojima, T. Akaike, S. Ijiri, A. Molla, and H. Maeda. 1990. Inactivation of chemotactic activity of C5a by the serratial 56-kilodalton protease. *Infect. Immun.* 58:1269–1272.
44. Hong, Y.Q., and B. Ghebrehiwet. 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin. Immunol. Immunopathol.* 62:133–138.
45. Hellwage, J., T. Meri, T. Heikkila, A. Alitalo, J. Panelius, P. Lahdenne, I.J. Seppala, and S. Meri. 2001. The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. *J. Biol. Chem.* 276:8427–8435.
46. Berggard, K., E. Johnsson, E. Morfeldt, J. Persson, M. Stalhammar-Carlemalm, and G. Lindahl. 2001. Binding of human C4BP to the hypervariable region of M protein: a molecular mechanism of phagocytosis resistance in *Streptococcus pyogenes*. *Mol. Microbiol.* 42:539–551.
47. Thern, A., L. Stenberg, B. Dahlback, and G. Lindahl. 1995. Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. *J. Immunol.* 154:375–386.
48. Plunkett, G., III, D.J. Rose, T.J. Durfee, and F.R. Blattner. 1999. Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. *J. Bacteriol.* 181:1767–1778.