Defining Potential Vaccine Targets of *Haemophilus ducreyi* Trimeric Autotransporter Adhesin DsrA

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Haemophilus ducreyi is the causative agent of the sexually transmitted genital ulcer disease chancroid. Strains of *H. ducreyi* are grouped in two classes (I and II) based on genotypic and phenotypic differences, including those found in DsrA, an outer membrane protein belonging to the family of multifunctional trimeric autotransporter adhesins. DsrA is a key serum resistance factor of *H. ducreyi* that prevents binding of natural IgM at the bacterial surface and functions as an adhesin to fibronectin, fibrinogen, vitronectin, and human keratinocytes. Monoclonal antibodies (MAbs) were developed to recombinant DsrA (DsrA_I) from prototypical class I strain 35000HP to define targets for vaccine and/or therapeutics. Two anti-DsrA_I MAbs bound monomers and multimers of DsrA from genital and non-genital/cutaneous *H. ducreyi* strains in a Western blot and reacted to the surface of the genital strains; however, these MAbs did not recognize denatured or native DsrA from class II strains. In a modified extracellular matrix protein binding assay using viable *H. ducreyi*, one of the MAbs partially inhibited binding of fibronectin, fibrinogen, and vitronectin to class I *H. ducreyi* strain 35000HP, suggesting a role for anti-DsrA antibodies in preventing binding of *H. ducreyi* to extracellular matrix proteins. Standard ELISA and surface plasmon resonance using a peptide library representing full-length, mature DsrA_I revealed the smallest nominal epitope bound by one of the MAbs to be MEQNTHNINKLS. Taken together, our findings suggest that this epitope is a potential target for an *H. ducreyi* vaccine.

Introduction

HAEMOPHILUS DUCREYI IS A GRAM-NEGATIVE bacterial pathogen that causes the sexually transmitted genital ulcer disease chancroid.^(1–3) Chancroid is an important co-factor in the acquisition and transmission of HIV.^(4–8) *H. ducreyi* has also been shown to cause chronic, non-genital, lower limb cutaneous ulcers in patients from countries in the South Pacific, including New Zealand, Samoa, Papua New Guinea, and Vanuatu.^(9–12) Although the relationship between cutaneous (non-genital) and genital strains is undefined, genital isolates of *H. ducreyi* are grouped in classes, termed class I and class II, according to multiple phenotypic and genomic variations, including those found in the major outer membrane protein DsrA (Ducreyi serum resistance A).^(13–15) Class I *H. ducreyi* strains therefore express the variant form of DsrA called DsrA_I, while class II isolates express the DsrA_{II} protein.⁽¹³⁾

The DsrA protein of *H. ducreyi* belongs to a large family of protein ubiquitous in Gram-negative bacteria, the trimeric autotransporter adhesins (TAAs), involved in serum resistance, agglutination, and adherence.^(16,17) TAAs share a highly conserved C-terminal translocator domain⁽¹³⁾ and

a variable N-terminal passenger domain, wherein the functional binding regions are located (Fig. 1A). Despite dissimilarity of the passenger domains, both classes of DsrA proteins confer resistance to complement and act as adhesins to the extracellular matrix (ECM) proteins fibronectin (Fn),⁽¹⁸⁾ vitronectin (Vn),^(18,19) and fibrinogen (Fg).⁽²⁰⁾ Using a panel of class I DsrA proteins truncated in the passenger domain, our laboratory showed that the C-terminal portion of this domain is involved in Fn, Fg, and Vn binding and serum resistance in *H. ducreyi* (Fig. 1A).^(20,21) DsrA is an important determinant of pathogenesis during early steps of experimental human infection.^(19,22)

Adherence of pathogenic bacteria to eukaryotic cells or to host ECM proteins is thought to be the first step in most infections. Blocking the interaction between pathogen and host may therefore be an effective approach in preventing infection. Preventative strategies may be developed based on the identification of bacterial proteins and amino acid sequences involved in adherence to ECM proteins and eukaryotic cells. We previously showed that monoclonal antibody (MAb) 1.82 developed against a recombinant form of class I DsrA (DsrA_I) blocked Fn binding to viable

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FIG. 1. Variability of *H. ducreyi* DsrA proteins. (A) Schematic depiction of DsrA₁ protein showing typical architecture of TAAs. Also indicated are the shortest truncated DsrA₁ proteins to render a *dsrA* mutant strain capable of serum resistance (Sr) and binding to Fn and Vn (Fn/Vn⁺) and the amino acid sequence involved in binding to Fg (Fg⁺). The numbers below refer to the last amino acid of the particular domain, in reference to immature DsrA₁. (B) Amino acid alignment of predicted amino acid sequences of DsrA protein from selected *H. ducreyi* strains, including representative isolates from both classes of *H. ducreyi* strains and two non-genital/cutaneous chancroid isolates (BE3145 and SB5756). Sequences were aligned using ClustalW (<http://workbench.sdsc.edu>) using default settings. Boxes indicate identical residues, while underlined amino acids share homology.

H. ducreyi.⁽¹⁸⁾ Based on these data, we report here on the characterization of two anti-DsrA MAbs, including 1.82, using cellular binding assays, an epitope library, and surface plasmon resonance (SPR). We document a key DsrA epitope critical for binding of class I *H. ducreyi* strains to host ECM proteins.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are described in Table 1. The *dsrA* gene sequence from *H. ducreyi* strains NZS4

(BE3145) and NZS2 (SB5756) is highly homologous to the *dsrA* from class I *H. ducreyi* strains (predicted amino acid sequence shown in Fig. 1B). We therefore describe herein these strains as expressing a class I-like DsrA protein. Strains were routinely passaged on chocolate agar plates (CAP) supplemented with 5% FetalPlex (Gemini, Bio-Products, West Sacramento, CA) and 1x GGC (0.1% glucose, 0.001% glutamine, 0.026% cysteine)⁽²³⁾ at 34.5°C in a water-saturated atmosphere containing 5% CO₂. *H. ducreyi* were also grown on heme plates containing gonococcal (GC) medium supplemented with 1x GGC and 50 µg/mL heme (MP Biomedicals, Santa Ana, CA).

TABLE 1. STRAINS USED IN THIS STUDY

Strain	Origin	DsrA	# of NTHNINK repeats	Ref.
35000HP	Winnipeg, Canada	I	1	(30,31)
FX517 (35000HPΔdsrA)	35000HPdsrA::CAT**	None	None	(32)
HMC50	Jackson, MS	I	2	(20, 33)
HMC18	Seattle, WA	I	3	(20)
HMC112	—	II	N/A	(13)
DMC111	Bangladesh	II	N/A	(34)
NZS4* (BE3145)	New Zealand/Samoa	I	2	(9)
NZS2* (SB5756)	New Zealand/Samoa	I	2	(9)

*Isolated from a non-genital, lower limb ulcer.

**Chloramphenicol Acetyl Transferase.

Polymerase chain reaction and sequencing

The nucleotide sequences of the *dsrA* gene from strains NZS4 (BE3145) and NZS2 (SB5756) were amplified using primers *dsrA*14 and *dsrA*24,⁽¹³⁾ whole cells as templates, and illustra PuReTaq Ready-To-GoTM PCR beads (GE Healthcare, Buckinghamshire, United Kingdom). Polymerase chain reaction (PCR) conditions are as follows: a single 5-min cycle at 95°C for initial denaturation; 30 cycles including a 1-min denaturation step at 95°C, a 1-min annealing step at 52°C, a 1.5-min elongation step at 72°C, and a final single 5-min polishing step at 72°C. Purified PCR products were sequenced using primers *dsrA*14 and *dsrA*24 at the Genome Analysis Facility (UNC-CH, Chapel Hill, NC). Sequences were annotated using Sequencher (v. 4.8, Gene Codes, Ann Arbor, MI).

Polyclonal sera and monoclonal antibodies

The generation of murine MAb 1.82 using recombinant full-length DsrA (rDsrA) as the immunogen is described elsewhere.⁽¹⁸⁾ MAb 6.95 was obtained in the same manner and timeframe as MAb 1.82. Hybridomas were maintained in serum-free culture medium at the Tissue Culture Facility (UNC-CH, Chapel Hill, NC). Polyclonal DsrA_I and DsrA_{II} antisera were developed in rabbits to recombinant N-terminal DsrA_I or DsrA_{II}, respectively.⁽¹³⁾ Rabbit polyclonal antisera to recombinant TdX, an *H. ducreyi* TonB-dependent transporter,⁽²⁴⁾ served as an irrelevant/negative control in the Fn/Fg/Vn binding assays described below.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis

Bacterial proteins (from $\sim 1 \times 10^7$ CFU) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% NuPage gels (Invitrogen, Carlsbad, CA) at 150 volts. Proteins from the gel were transferred to a nitrocellulose membrane (Thermo Fisher Scientific, Pittsburgh, PA) at 200 mA for 1 h. The membrane, stained with Ponceau S to evaluate protein loading, was blocked overnight in 0.5% Tween-20 (Tw20) in phosphate-buffered saline (PBS) before incubation with primary antibodies (Abs) (1 µg/mL for MAb, 1:10,000 to 1:20,000 dilution for polyclonal antisera). After three washes with 0.05% Tw20 in PBS (Western wash buffer), membranes were incubated with anti-rabbit or anti-mouse alkaline phosphatase-conjugated secondary Abs (Sigma-Aldrich, St-Louis, MO), washed three times, and developed with a chemiluminescence substrate (Lumi-Phos^{WB}, Thermo Fisher Scientific, Rockford, IL). Bands were visual-

ized on film or using the FluorChem E imager (Protein Simple, Santa Clara, CA) to enable quantitation by densitometry.

Whole-cell binding enzyme-linked immunosorbent assay

rDsrA_I (490) and rDsrA_{II} (456) antisera⁽¹³⁾ were first adsorbed with 100 µL of packed 35000HPΔ*dsrA* (FX517) bacteria grown on CAP three times for 30 min at room temperature. Antisera were filter-sterilized (Costar Spin-X® Centrifuge Tube Filters, Corning, Tewksbury, MA) after adsorption. Bacterial suspensions (200 µL of *H. ducreyi* grown for 14 to 16 h on CAP or heme plates suspended in GC broth [GCB] at OD₆₀₀ = 0.2 or about 1×10^8 CFU/mL) were mixed with 50 µL of antisera (MAbs at 1 µg/mL or adsorbed polyclonal anti-rNTDsrA_I and anti-rNTDsrA_{II} at 1:1000) in wells of MultiScreen® HTS filter plates (Millipore, Billerica MA) and incubated at room temperature for 1.5 h with shaking. The wells were thereafter washed four times with 0.01% Tw20 in GCB. One hundred microliters of anti-mouse (1:5000) or anti-rabbit (1:2000–1:5000) horseradish peroxidase (HRP)-conjugated secondary Abs (Sigma-Aldrich) were then added to the wells and the plate was incubated for 1 h with shaking. After four washes with 200 µL of 0.01% Tw20/GCB, HRP substrate ECLTM Western Blotting Detection Reagent (GE, Buckinghamshire, UK) was added to the wells and developed using the luminescence setting on the Wallac 1420 Victor² plate reader (Perkin Elmer, Waltham, MA).

Preparation of IgG and Fab fragments

IgG were purified from hybridoma culture supernatants (Tissue Culture Facility, UNC-CH) using protein A/G agarose (Exalpha Biologicals, Shirley, MA). Briefly, 1 mL of a 50% slurry of protein A/G agarose was incubated at 4°C with either 1 mL of polyclonal serum (1 h) or 50 mL of MAb culture supernatant (overnight) and eluted from the protein A/G agarose with 70 mM glycine-HCl (pH 2.5). Fab fragments were subsequently generated and purified from IgG using a Fab preparation kit (Thermo Scientific Pierce, Rockford, IL) according to the manufacturer's instructions. The amount of Fab present in the preparations was quantified for SPR using the IgG setting on the ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific).

Fibronectin, fibrinogen, and vitronectin binding assays

A modified extracellular matrix protein binding assay⁽¹⁸⁾ was used to determine binding of *H. ducreyi* to Fn (Sigma),

Fg (FIB 3, Enzyme Research Laboratories, South Bend, IN), and Vn (Advanced Biomatrix, San Diego, CA). Vn was labeled with digoxigenin (DIG-Vn) following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). One milliliter of a viable bacterial suspension ($OD_{600}=0.5$; grown on CAP [for Fg and Vn] or heme plates [Fn] for 16–18 h) was mixed with purified IgG at room temperature for 30 min. The Fn binding assay used bacteria grown on heme plates because the hemoglobin in CAP prevents binding of Fn by *H. ducreyi*.⁽¹⁸⁾ After incubation with IgG, Fn (2 μ g), Fg (2.5 μ g), or 1 μ g of DIG-Vn was added to the suspension, which was incubated for 30 min at 34.5°C. The cell suspension was washed three times with 1 mL GCB, moved to a new microcentrifuge tube, and washed a fourth time. Bacterial pellets were suspended in 1x Laemmli loading buffer and subjected to SDS-PAGE and Western blotting with anti-Fn (Sigma-Aldrich), anti-Fg (Sigma-Aldrich), or anti-DIG (Roche Diagnostics). Nitrocellulose membranes were stained with Ponceau S and incubated with an Ab to recombinant D15⁽²⁵⁾ to ensure equal loading. Membranes were developed as described above and densitometry of bands was determined using the Alpha-View program (v. 3.2.4.0, Protein Simple, Santa Clara, CA).

To analyze blocking of Fg binding to the surface of viable *H. ducreyi* by IgG using flow cytometry, FITC-Fg (5 μ g labeled, according to the manufacturer's instructions; Fluor-reporter® FITC Protein labeling kit, Life Technologies, Grand Island, NY) was incubated with *H. ducreyi* following IgG incubation as described above. Cells were washed with GCB as indicated above and subjected to flow cytometry as previously described using the Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA), recording 200,000 events with a 15,000 threshold.⁽²⁶⁾

Polyclonal Abs to the *H. ducreyi* TonB-dependent transporter TdX or from swine immunized with Freund's adjuvant were used as negative/irrelevant control Abs for blocking assays in Western blots and flow cytometry, respectively, because neither one interferes non-specifically with DsrA. The TdX antiserum reacts to an outer membrane protein of *H. ducreyi* that does not interact with DsrA at the cell surface as opposed to HgbA or NOMP (unpublished observations). Polyclonal Abs were used instead of irrelevant MAb controls because Abs unreactive with MOMP or HgbA were not available in the quantities necessary for this study.

DsrA₁ peptide library and ELISA screening

A peptide library representing the mature, full-length, 236-residue DsrA₁ protein was synthesized using SynPhase™ Lanterns (Mimotopes, Clayton, Australia), using this unique proprietary parallel array platform. Each peptide was labeled at its N-terminus with biotin through a SGSG linker incorporated in the peptide. This library consisted of 15-mers overlapping by 12 amino acids, totaling 75 peptides. Each peptide was initially solubilized in 40% acetonitrile at a dilution of 5 mg/mL (2.4 nmole/ μ L) and stored at –20°C. From the 2.4 nmole/ μ L (0.5 μ g/mL) stock solution, peptides were diluted to a concentration of 50 pmol/ μ L in 1% BSA/PBS prior to use. Reacti-Bind NeutrAvidin High-Binding plates (Pierce Biotechnology, Rockford, IL) were incubated with 300 μ L of a blocking solution (1% BSA in PBS) for 1 h at room temperature. Thereafter, 100 μ L of the blocking solution containing 500 pmole of

each peptide were incubated in triplicate well at room temperature with agitation for 1 h. Wells were washed three times with 0.05% Tw20 in PBS. One hundred microliters of MAb culture supernatant, diluted at 1:4000 in 1% BSA/PBS, were added to the wells and the plate was incubated for 1 h at room temperature with agitation. After three washes with 0.05% Tw20/PBS, 100 μ L of anti-mouse HRP-conjugated secondary Abs (Sigma-Aldrich) were added to the wells at a dilution of 1:10,000 and incubated for 1 h at room temperature with agitation. After three additional washes, 100 μ L of 1 Step Ultra TMB (Pierce Biotechnology) were added to each well, which were incubated for 15 min at room temperature prior to the addition of stop solution (50 μ L of 0.18 M H₂SO₄). Optical density was measured at 405 nm on the VICTOR² plate reader.

Surface plasmon resonance kinetic measurements

Fab binding K_d and rate constant measurements were carried out on a BIAcore 3000 instrument (GE Healthcare, Piscataway, NJ). Biotinylated peptides were coupled to streptavidin sensor chips to about 50–100 response unit (RU). At a flow rate of 50 μ L/min, Fabs were injected for 300 s and dissociation was monitored for 600 s. Fab 1.82 was titrated in a range of 10–100 μ g/mL on peptide 48 and a range from 1–10 μ g/mL on peptides 49 and 50. Surfaces were regenerated with an injection of glycine-HCl (pH 2.0). Non-specific binding of Fab 1.82 to peptide 51 was subtracted for each binding interaction as a negative control surface. All data analysis was performed using BIAevaluation 4.1 software (GE Healthcare). All curve fitting were performed using a global fit to the 1:1 Langmuir model.

Accession numbers for nucleotide sequences

The Genbank accession numbers for the *dsrA* genes of the different strains discussed in this report are as follows: 35000HP, AAP95674; HMC50, KF880962; HCM18, AAF37815; BE3145, KF880963; DMC111, AAU12596; and HMC112, AAU12588.

Statistical analyses

A paired *t*-test was used to assess differences between binding of the different MAbs to the surface of viable *H. ducreyi* (Prism, GraphPad Software, La Jolla, CA). The relationship between the amount of Fn, Fg, or Vn bound to *H. ducreyi* and the number of pixels obtained from densitometry analyses of the bands was determined by a standard curve of purified Fn, Fg, or Vn subjected to Western blotting. By plotting in a graph using amounts of ECM proteins and pixel numbers, a linear relationship was found between pixel number and amount of loaded protein. A single sample *t*-test (www.vassarstats.net) was used to compare mean band density between treatments in this modified ECM protein binding assay and in the Fg blocking assay using flow cytometry. A *p* value ≤ 0.05 was chosen as statistically significant.

Results

MAbs 1.82 and 6.95 bind multimers of class I DsrA proteins

To determine the specificity of two anti-rDsrA₁ MAbs, named 1.82 and 6.95, reactivity to denatured DsrA from

whole cell lysates was measured using Western blot. For this analysis, select *H. ducreyi* strains were chosen based on the diversity of the amino acid sequence of their DsrA protein (Fig. 1B and Table 1), the class of *H. ducreyi* strain (Table 1), and the type of ulcer the strain was isolated from (genital or cutaneous/non-genital) (Table 1). Four blots with total bacterial protein preparations from the indicated *H. ducreyi* strains were simultaneously reacted with MAbs 1.82 and 6.95, as well as with rabbit polyclonal antisera to full-length rDsrA_I (rFL-DsrA_I)⁽¹³⁾ which binds both DsrA_I and DsrA_{II} proteins. Both MAbs recognized the DsrA protein expressed by class I and non-genital/cutaneous strains, which express a class I-like DsrA, but not class II strains expressing DsrA_{II} proteins. These findings indicate that these MAbs are DsrA_I-specific (Fig. 2A).

TAA are expressed at the bacterial surface in the form of trimers, which are required for proper function of the protein.⁽²⁷⁾ The ability of both MAbs to recognize multimers of DsrA was therefore studied in a Western blot where total cellular proteins were solubilized at 37°C to retain multimeric structure. Figure 2B shows that MAbs 1.82 and 6.95 bind dimers and trimers of the DsrA protein in all class I and non-genital/cutaneous *H. ducreyi* strains tested. The trimer form of DsrA of strain HMC118 was not recognized by either MAb (Fig. 2B). These data suggest that these anti-rDsrA_I MAbs also recognized multimers of DsrA_I and class I-like DsrA proteins from strains of genital and non-genital origins.

Anti-rDsrA_I MAbs 1.82 and 6.95 bind the surface of class I *H. ducreyi* strains

For Abs to possess biological function, such as bactericidal activity, it is required to bind to the bacterial surface. We therefore sought to determine if MAbs 1.82 and 6.95 recognized native DsrA_I on the surface of viable, *H. ducreyi* strains grown on CAP (Fig. 3A) and heme agar plates (Fig. 3B). The rationale for testing surface binding of MAbs to bacteria grown under two different culture conditions is to ensure that this ability is not restricted to bacteria only grown on CAP. Prior experiments in our laboratory indicate that hemoglobin binding at the surface of *H. ducreyi* interferes with Fn binding.⁽¹⁸⁾ It is therefore possible that the presence of hemoglobin in CAP would interfere with MAb binding at the bacterial surface. MAb 1.82 bound with similar intensity to all class I *H. ducreyi* strains tested, regardless of growth conditions (Fig. 3). Relative to MAb 1.82, MAb 6.95 bound well to class I strain 35000HP regardless of culture conditions, but binding to the two other class I strains tested was significantly reduced (Fig. 3). Both MAbs bound poorly to the surface of class II *H. ducreyi* strains and class I-like DsrA expressing strains recently isolated from non-genital lesions. Binding of the anti-DsrA_I MAbs to the isogenic *dsrA* mutant strain 35000HPΔ*dsrA* was either reduced or non-existent (Fig. 3). Of note, reactivity of both MAbs to the bacterial surface of heme-grown bacteria was double that of *H. ducreyi* grown on CAP, even though the amount of DsrA_I or DsrA_{II} expressed at the bacterial surface was not different in bacteria

FIG. 2. Anti-DsrA MAbs 1.82 and 6.95 recognize monomers and multimers of class I and class I-like DsrA proteins, but not DsrA_{II}. (A) Reactivity of MAbs to DsrA proteins in Western blots under reducing and denaturing conditions. Rabbit polyclonal anti-rFL-DsrA_I serves as a control for DsrA expression since it recognizes both classes of DsrA proteins.⁽¹³⁾ Anti-rD15 polyclonal Ab serves as a control for well loading. FX517 is the isogenic *dsrA* mutant of prototypical class I *H. ducreyi* strain 35000HP (35000HPΔ*dsrA*). (B) Reactivity of anti-DsrA_I MAbs to multimers of DsrA was assessed using concurrently developed Western blots. Shown are representative blots from two experiments with identical results.

FIG. 3. Surface binding of MAb 1.82 and 6.95 to viable bacteria varies among different classes of *H. ducreyi* strains. Whole cell binding of anti-rDsrA_I MABs to bacteria grown either in CAP (A) or heme plates (B). Whole, viable *H. ducreyi* were probed with 1 μg/mL of MABs. Isogenic *dsrA* mutant strain FX517 (35000HPΔ*dsrA*) serves as negative control for expression of DsrA. Shown are means ± standard deviations of at least five independent experiments. * $p < 0.05$ using a paired *t*-test.

grown on CAP and heme plates, as assessed by binding of polyclonal rNt-DsrA_I or rNT-DsrA_{II} antisera⁽¹³⁾ (data not shown). Taken together, these findings indicate that both MABs bind specifically to DsrA at the surface of class I *H. ducreyi* isolates that cause genital ulcerations. Furthermore, MAb 6.95 binding to DsrA appears to be strain specific as reactivity was reduced in two out of three class I strains tested.

Differential inhibition of extracellular matrix proteins binding by anti-DsrA MABs

Our laboratory previously showed that MAB 1.82 partially inhibits the binding of Fn to the surface of viable *H. ducreyi*.⁽¹⁸⁾ Since both anti-DsrA_I MAB bind to the surface of class I *H. ducreyi* strain 35000HP, we wanted to extend these findings to MAB 6.95 by investigating the ability of the MABs to block the interaction between *H. ducreyi* DsrA and the

ECM proteins Fn, Vn, and Fg. In this assay, viable class I *H. ducreyi* strain 35000HP was incubated with anti-DsrA Abs prior to the addition of purified Fn, Fg, or DIG-labeled Vn (DIG-Vn) to bacterial suspensions.

Fibronectin. MAB 1.82 significantly reduced binding of strain 35000HP to Fn by 57% compared to a no treatment control (Fig. 4A; $p = 0.013$). Polyclonal anti-rNT-DsrA_I IgG also significantly decreased binding of *H. ducreyi* to Fn by 61% (Fig. 4A, $p = 0.009$). Neither MAB 6.95 nor an irrelevant Ab control reduced binding to Fn.

Vitronectin. Pre-incubation of *H. ducreyi* with MAB 1.82 significantly reduced Vn binding by 53% (Fig. 4B; $p = 0.01$), while MAB 6.95 or an irrelevant IgG control did not significantly reduce Vn binding at the surface of viable *H. ducreyi* (Fig. 4B).

Fibrinogen. Only MAB 1.82 significantly reduced the amount of Fg binding to strain 35000HP (Fig. 4C; 21%, $p = 0.002$). None of the other monoclonal or polyclonal Abs impacted Fg binding (Fig. 4C). To confirm these results, we used a recently developed flow cytometry assay.⁽²⁰⁾ Pre-incubation of viable *H. ducreyi* with MAB 1.82 significantly reduced binding of FITC-Fg to the bacterial surface (Fig. 4D, E). Conversely, the presence of MAB 6.95 or an irrelevant Ab control did not affect Fg binding. In all experiments, DsrA expression was not affected by incubation with the Abs (Fig. 4). Taken together, these data indicate that MAB 1.82 IgG partially, but significantly, reduced binding of *H. ducreyi* to Fn, Vn, and Fg. These findings suggest that MAB 1.82 binds to a key DsrA epitope that mediates binding of *H. ducreyi* to ECM proteins.

Anti-DsrA MAB 1.82 recognizes sequence MEQNTHNINKLS in the N-terminal section of translocator domain of DsrA

To define the epitope of DsrA recognized by MAB 1.82, we first tested the reactivity of the MAB to a panel of DsrA passenger domain deletion mutants.⁽²¹⁾ MAB 1.82 bound all of the truncated proteins tested, including the smallest construct expressing only the translocator domain of DsrA (Fig. 5A), suggesting that the epitope bound by MAB 1.82 is present in this section of the DsrA protein. We then screened a peptide library representing the mature, full-length class I DsrA protein (15-mers with 12 residue overlap) with MAB 1.82. Three consecutive peptides (48-50) in the translocator domain of DsrA reacted with MAB 1.82, suggesting that the smallest nominal epitope for MAB 1.82 is MEQNTHNINKLS (Fig. 5B). We further tested the capacity of anti-DsrA_I MAB 1.82 to bind specific peptides by measuring binding constants using surface plasmon resonance (BIAcore). Four peptides (48-51) were immobilized onto flow cells on two BIAcore chips (see Fig. 5 for sequence of peptides). Controls and MAB Fabs were then flowed over the chips for SPR measurements (Fig. 5C). On-and-off binding rates were calculated from these measurements and are shown in Table 2. Based on these binding kinetics, binding constants (K_d) were determined (Table 2). BIAcore/SPR data for MAB 1.82 suggest that peptides 49 and 50 contained the smallest nominal epitope since the binding constant was in

FIG. 4. Anti-DsrA MAb 1.82 partially inhibits binding of DsrA to Fn, Vn, and Fg. Whole, viable, class I *H. ducreyi* strain 35000HP was incubated with the indicated IgG prior to incubation with either Fn (A), DIG-Vn (B), or Fg (C–E), as described in the Materials and Methods section. (A–C) Fn, DIG-Vn, and Fg binding to the surface of viable *H. ducreyi* in the presence of competitor IgG was measured using Western blot. Shown are representative blots from at least three independent experiments performed with each Fn, DIG-Vn, or Fg. Densitometry carried out on appropriate bands from each blot is shown in the graphs below the blots as means $\pm$ standard error of Fn, DIG-Vn, or Fg binding compared to no treatment (no addition of IgG), defined as 100% binding (line). Western blot with anti-rFLDsrA_I⁽¹³⁾ shows equal loading of wells. (D–E) Fg binding to the surface of viable *H. ducreyi*, in the presence or absence of competitor IgG, was measured using flow cytometry. (D) Representative histogram of three independent experiments. (E) Means $\pm$ standard deviations of three independent experiments where FITC-Fg binding to *H. ducreyi* was compared to a no treatment control (no addition of IgG), defined as 100% binding (line). * $p < 0.05$ using a single sample *t*-test.

the nanomolar range (Table 2). These data corroborate the results from the peptide ELISA, confirming the epitope for MAb 1.82 to be MEQNTHNINKLS.

Discussion

The treatment of bacterial infections is becoming more difficult due to increasing antibiotic resistance.^(28,29) Defining novel bacterial targets, such as factors involved in initiating infection, is therefore an important prerequisite in developing therapeutic and/or vaccines to prevent and treat infections with resistant bacteria. TAAs are prospective targets for prophylactic development because proteins belonging to this family are ubiquitous, multifunctional, surface-exposed pro-

teins of Gram-negative pathogenic bacteria involved in many aspects of pathogenesis, including adherence. We therefore sought to define domains or motifs of the *H. ducreyi* TAA DsrA by extensively characterizing two MAbs developed to full-length class I recombinant DsrA, a TAA expressed at the surface and a proven virulence factor of the Gram-negative bacterium *H. ducreyi*. By defining epitopes, binding constants, and functional inhibition of DsrA binding to host ligands, we identified two MAbs that specifically bind multimers of class I DsrA, and one MAb that partially inhibits binding of DsrA to three of its known ligands.

We determined herein that anti-DsrA_I MAbs 1.82 and 6.95 bound monomers and multimers of denatured DsrA_I, including the class I-like DsrA protein expressed by strains

FIG. 5. Shortest nominal peptide of DsrA bound by MAb 1.82 is MEQNTHNINKLS. **(A)** Total cellular proteins (from $\sim 1 \times 10^7$ CFU) from *dsrA* mutant strain expressing truncated DsrA proteins⁽²¹⁾ were subjected to 4–12% gradient SDS-PAGE, followed by Western blot with 0.5 μ g/mL MAb 1.82. Shown is a representative blot from two identical replicate studies. **(B)** Reactivity of anti-rDsrA_I MAb 1.82 to a peptide library representing full-length class I DsrA protein from *H. ducreyi* class I strain 35000HP. Below the chart are amino acid sequences of reactive peptides and their residue numbers in reference to immature DsrA. *Peptide charges were obtained using the online calculator at Innovagen website (www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp). **(C)** Representative titration binding curves of 1.82 Fab with peptides 48 (left), 49 (middle), and 50 (right) from two replicate studies. **(D)** Schematic representation of residues in the N-terminal section of the translocator (grey) domain of DsrA_I. The smallest nominal epitope of MAb 1.82 determined by SPR is shown in bold.

recently isolated from non-genital cutaneous lesions, but not DsrA from class II strains or an isogenic *dsrA* mutant (Fig. 2). However, only DsrA at the surface of class I *H. ducreyi* strains isolated from genital lesions were bound by both MAbs (Fig. 3), not class I-like or class II DsrA proteins

present at the surface of cutaneous or class II *H. ducreyi* strains. A plausible explanation for the inability of anti-DsrA_I MAbs to bind surface-expressed class I-like DsrA from strains that cause non-genital chancroid (NZS4/BE3145 and NZS2/SB5756) might lie in the amino sequence of the DsrA proteins. Our data from peptide ELISA and SPR indicate that the smallest nominal epitope for MAb 1.82 is MEQNTHNINKLS. As shown in Figure 1B, this amino acid sequence is conserved in the recently isolated non-genital/cutaneous chancroid strains compared to the other three class I strains, so a sequence variation in this epitope cannot explain this discrepancy. However, these latter isolates have accumulated mutations in residues 90–97 (Fig. 1B), including a string of four or five prolines, compared to the DsrA protein from strain 35000HP. These differences in amino acid residues may impact the three-dimensional structure of DsrA, thereby shielding the epitope recognized by MAb 1.82 at the surface

TABLE 2. BINDING CONSTANTS OF MAB 1.82 TO SELECTED DSRA PEPTIDES AS MEASURED BY SURFACE PLASMON RESONANCE

Peptide	K_{on} (1/Ms)	K_{off} (1/s)	K_d (nM)
48	880	0.0115	13100
49	6.99×10^4	4.48×10^{-4}	64
50	5.57×10^{3a}	7.62×10^{-4a}	139 ^a

K_d, binding constants.

^aMean of two measurements.

of *H. ducreyi*. Conversely, the presence of two NTHNINK repeats in the non-genital chancroid strains presumably does not affect binding since MAb 1.82 binds DsrA at the surface of strain HMC50, which has two NTHNINK repeats (Fig. 1B). Taken together, these data indicate that MAbs 1.82 and 6.95 are specific to DsrA_I in its native structure at the surface of viable class I *H. ducreyi* strains that cause genital lesions.

Once we established surface recognition of *H. ducreyi* DsrA by MAbs 1.82 and 6.95, we characterized their functional activity. Preliminary studies suggest that neither MAb 1.82 nor 6.95 was bactericidal for *H. ducreyi* class I strain 35000HP in the presence of 25 or 50% NHS (data not shown). In ECM protein competition assays, MAb 1.82 partially, but significantly, reduced binding of all three ligands tested (Fn, Fg, and Vn) at the bacterial cell surface (Fig. 4). In a previous publication, we found that MAb 1.82 did not interfere with Vn binding at the surface of *H. ducreyi* strain 35000HP.⁽¹⁸⁾ This discrepancy could be explained by the fact that in this earlier report, we used NHS as the source of Vn, while here, we used purified, recombinant Vn. Perhaps recombinant Vn is folded differently and binds a different epitope than native Vn does. Conversely, binding of native Vn in the presence of NHS may be aided through an unknown serum protein. Both of these hypotheses remain to be tested.

Analysis of a panel of truncated DsrA proteins revealed that the translocator domain of DsrA is not sufficient to confer binding to Fn or Vn.⁽²¹⁾ Partial restoration of Fn and Vn binding required 53 amino acids from the C-terminal section of the passenger domain, in addition to the translocator domain.⁽²¹⁾ We show herein that the epitope bound by MAb 1.82 is found in the translocator domain of DsrA (Fig. 5), and that MAb 1.82 only partially blocked Fn, Fg, and Vn binding to the surface of *H. ducreyi*. This can be explained by the fact that as an MAb, 1.82 only recognizes one epitope on the DsrA protein. It is therefore unlikely that the interaction between 1.82 and DsrA would be sufficient to completely prevent binding with its ECM ligands. These ECM proteins are large and presumably have multiple binding sites for DsrA. Since we show that 1.82 recognized an epitope in the translocator domain of DsrA, there are several other unbound epitopes in the passenger domain of DsrA available for interaction with Fn, Fg, and Vn that could permit partial interaction with these ligands. This hypothesis is supported by results from our latest publication in which antisera elicited to the passenger domain of DsrA only partially blocked binding of Fg.⁽²⁶⁾

The fact that MAb 1.82 binds an epitope in the translocator domain, as opposed to the passenger domain where the binding sites of Fn, Fg, and Vn are located, may explain why the inhibition by MAb 1.82 is not specific to one of these ligands. Based on the data obtained herein, we hypothesize that the interaction of MAb 1.82 with the translocator domain of DsrA, situated close to the bacterial membrane, may sterically hinder access of the ECM proteins to the DsrA protein at the surface of *H. ducreyi*. This may also explain the partial inhibition of Fg, Fn, and Vn binding by MAb 1.82 to the surface of *H. ducreyi*.

In conclusion, we have identified two functionally distinct MAbs generated against the TAA DsrA that bound multimers of this protein at the surface of *H. ducreyi*. One of these MAbs (6.95), bound only to homologous DsrA, not heterologous

protein. MAb 1.82, which could recognize DsrA_I at the surface of class I *H. ducreyi* strains that cause genital lesions, partially inhibited the interaction between DsrA and three of its ECM protein ligands. Taken together with previous findings,⁽²⁶⁾ these data indicate that this epitope, combined with the passenger domain of DsrA, could partially abrogate binding of ECM proteins at the bacterial surface.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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