Identification of Two Protein-binding and Functional Regions of Curli, a Surface Organelle and Virulence Determinant of Escherichia coli*

Curli are surface organelles of Escherichia coli. These fibrous proteins, formed by polymerization of a 15-kDa subunit, are expressed by E. coli strains associated with severe infections in humans. A remarkable property of curli is their ability to interact with a wide range of human proteins, interactions that contribute to the enhanced virulence of curli-expressing E. coli. To define the protein-binding region(s) of curli, we investigated the binding properties of overlapping synthetic peptides covering the curli subunit. Two peptides, one covering a 24-amino acid residue sequence in the NH2-terminal half of the subunit (NNS24) and one corresponding to the 26 COOH-terminal residues (VDQ26), were found to bind a number of human proteins. Physiochemical analysis revealed that NNS24 adopts a thermally stable β-structure, and in solution the peptide forms soluble multimers, predominantly octamers. Intact curli are known to activate the proinflammatory and procoagulant contact system, and when added to human plasma, the NNS24 and VDQ26 peptides induced the release of the potent vasoactive peptide bradykinin. The results map important curli functions to the regions corresponding to the NNS24 and VDQ26 sequences.

Some strains of Escherichia coli and Salmonella express fibrous surface proteins that aggregate into matrix-like structures attached to the bacterial surface. In E. coli these fibrous proteins are called curli (1) and in Salmonella, thin aggregative fimbriae (2). They are closely related structurally (1, 3), genetically (4, 5), and functionally (6, 7). Genes encoding a homologous structure were recently described also in Shigella spp (8). Curli in E. coli are composed of a major 15-kDa subunit protein, CsgA (4), and a minor component, CsgB (9), acting as a nucleator in the formation of insoluble curli aggregates at the bacterial surface (10). Temperature and various stress conditions, such as low osmolarity and starvation, regulate the expression of curli (11). A recent report describes the complex network of regulatory factors that control the production of both curli and thin aggregative fimbriae (12). Among clinical E. coli isolates, curli are expressed by most enterohemorrhagic, enterotoxicogenic, and sepsis strains, whereas enteroinvasive and enteropathogenic strains do not express curli (4, 13). This difference in expression suggests a role for curli in E. coli pathogenicity, a notion that is further supported by findings that curli induce proinflammatory cytokines and that anti-CsgA antibodies are found in serum samples from patients with E. coli sepsis (14). Recent studies have also shown that curliated E. coli when injected into mice activate the NO/NOS2 arm of the innate immune system, resulting in blood pressure fall (14). Finally, curliated E. coli, but not a non-curliated mutant strain, has the ability to enter eukaryotic cells (15).

A noteworthy property of curli is their broad protein-binding capacity. A large number of human proteins have been shown to interact with curli, including many plasma proteins and MHC1 class I antigens (4, 7, 13, 16). Available data indicate that the CsgA subunit is responsible for most of these interactions, which in some cases have been shown to contribute to E. coli virulence. Thus, the components of the contact system bind and assemble on curli fibers, and as a consequence the proinflammatory peptide bradykinin is generated and a hypocoagulative state is induced (17). Moreover, blocking of contact system activation prevents curliated bacteria from inducing severe lung lesions in rats (18). The connection between bacterial virulence and the protein-binding properties of curli prompted us to investigate whether the binding and functional properties of curli could be mapped more precisely. In the present study we identify two regions in the CsgA subunit that have broad protein binding activity and the capacity to activate the contact system.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—A series of overlapping peptides (see Fig. 2A), based on the sequence of the curli subunit CsgA, were synthesized at Innovagen, Lund, Sweden. HPLC and mass spectrometry were used to control the quality of the peptides.

Antibodies and Proteins—Anti-fibrinogen antibodies were from DAKO A/S, Glostrup, Denmark, and antibodies against high molecular weight kininogen (H-kininogen) were raised in sheep as described (19). Human fibrinogen, fibronectin, and serum albumin (HSA) were purchased from Innovagen, Lund, Sweden, Dako, and Sigma.

The abbreviations used are: MHC, major histocompatibility complex; BK, bradykinin; CsgA, curli subunit protein A; CsgB, curli subunit protein B; DOSY, diffusion-ordered spectroscopy; H-kininogen, high molecular weight kininogen; HPLC, high-pressure liquid chromatography; HSA, human serum albumin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; t-PA, tissue plasminogen activator.

* This work was supported by grants from the Swedish Research Council (Projects 7480, 13413, and 14272), the Crafoord, Bergvall, Kock, Nilson, and Österlund Foundations, the Royal Physiographic Society in Lund, the Medical Faculty, Lund University, and Hansa Medical AB. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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chased from Sigma. The preparations of human MHC class I antigen, β₂-microglobulin, factor XII, H-kininogen, plasminogen, and tissue plasminogen activator (t-PA) were used as described previously (7, 16, 17).

Slot Binding Experiments—Synthetic peptides were dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.06 M phosphate, pH 7.2) and applied to PVDF membranes (Immobilon, Millipore, Bedford, MA) using a Milliblot-D system (Millipore). Membranes were blocked at room temperature for 1 h with PBSAT (PBS + 0.02% sodium azide, 0.25% bovine serum albumin + 0.25% Tween 20). Various proteins were labeled with 125I using the Bolton-Hunter reagent (20). Membranes were incubated with radiolabeled protein for 3 h at room temperature, washed in PBSAT, dried, and exposed to Kodak X-Omat AR film using regular intensifying screens (Kodak, Rochester, NY).

Bacterial Binding Assay—Binding of radiolabeled proteins to intact bacteria was done as described (7). Basically, 2 × 10⁸ bacteria were resuspended in PBS containing 0.1% Tween 20. The bacteria were incubated with 125I-labeled protein (50 ng) for 3 h at room temperature, washed once in 2 ml of PBS containing 0.1% Tween 20, and the radioactivity associated with the pellet was determined after centrifugation. Binding was expressed as the percentage of the added radioactivity.

Plasma Absorption Experiments—The NNS24 and VDQ26 peptides (10 μg) were coupled separately to 1 ml of CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions. To suspensions of 200 μl of NNS24- or VDQ26-coupled Sepharose, 0.5 ml of citrated fresh human plasma was added and incubated overnight at 4 °C. After incubation the Sepharose beads were washed in PBS, and bound proteins were eluted with 100 μl of 0.1 M glycine-HCl, pH 2.0. As a control, plasma was simultaneously absorbed with glycine-Sepharose. After elution, the pH was adjusted to 7.4 by adding 1 M Tris to the samples.

SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and Immunoprinting—Proteins were separated by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 1% SDS (21). After electrophoresis proteins were transferred onto nitrocellulose membranes for electrophoresis (PAGE) in the presence of 1% SDS (21). After electrophoresis proteins were transferred onto nitrocellulose membranes for immunoprinting. The transferred proteins were probed with 50 ng protein for 3 h. After incubation, the bacteria were washed once in 2 ml of PBS containing 0.1% Tween 20, and the radioactivity associated with the pellet was determined after centrifugation. Binding was expressed as the percentage of the added radioactivity.

Secondary Structure Analysis of the NNS24 Peptide—Circular dichroism (CD) spectra were recorded on a Jasco J-720 spectropolarimeter equipped with a thermostatted cell holder. The spectra were recorded in the far-UV region (260–190 nm) in a cell with a path length of 0.1 cm. The experiments were recorded in H₂O at 20 °C at concentrations of 50, 24, and 12 μM. Spectra were acquired at a scan speed of 20 nm/min and a 2-s response time. Four scans were accumulated for each experiment. The solvent dichroic absorbance was subtracted using conjugated secondary antibodies against rabbit or sheep IgG (Sigma) diluted 1:3,000 followed by a chemiluminescence detection method as described by Nesbitt and Horton (24).

Diffusion-ordered Spectroscopy (DOSY)—DOSY experiments on NNS24 (50 μM in D₂O) were carried out on a Varian INOVA 500 (500 MHz) NMR spectrometer at 27 °C using bipolar paired stimulation echo (BPPSTE) pulse sequence (25, 26). The duration of pulsed field gradients was 1 ms; the amplitudes were increased in 11 steps from 18.59 gauss/cm to 74.35 gauss/cm, and 8192 transients (with 8 dummy scans) were acquired for each increment. Data analysis was carried out using DOSY processing software (27).

Fig. 1. Binding of various radiolabeled human proteins to curliated and non-curliated E. coli bacteria. The binding of labeled protein to the bacteria was expressed as percentage of the total amount of added radiolabeled protein. The bars indicate mean values from at least two experiments.

A. GVV24 GGVPOYGGCGNHGGGNNSPNSE

NNS24 NNSGPNSLHYNYGGGNNSALQG

ALQ26 ALQTJARNBOLTQDHGGGNNSGPSE

ADV28 ADVGGGSGDSSFLTGFGF

FGF24 FFGNSATLDDWAGKNSMTVKQFG

KQF22 KGFGGGINGAAVDQTASNSNVV

VDQ26 VDQTSNBSSNVTQVFQGNNATAHQY

Fig. 2. Binding of fibrinogen to synthetic peptides covering the CsgA curli subunit. Various amounts, 0.5–5 μg as indicated, of synthetic peptides were applied to a PVDF membrane. The membrane was blocked with bovine serum albumin and Tween 20 and probed with 5 × 10⁵ cpm of 125I-labeled fibrinogen (20–50 ng protein). After 3 h of incubation the membrane was washed extensively in PBSAT and exposed to autoradiography. A, the sequences of the seven synthetic overlapping peptides with their overlapping areas indicated in bold. The peptides are designated after their three NH₂-terminal amino acid residues and the number of residues they contain. B, a PVDF membrane, containing the synthetic peptides depicted in A, probed with 125I-labeled fibrinogen.
Jasco software. The thermal unfolding curve was run at a single wavelength (216 nm) characteristic for β-sheet structures at a concentration of 50 μM. The temperature was increased from 5 to 90 °C at a scan rate of 50 °C/h.

**Analysis of Bradykinin Release in Human Plasma following Addition of Curli-derived Peptides**—Peptides NNS24, ALQ26, GFG24, and VDQ26 were added separately to human citrated plasma from healthy individuals. The peptides were dissolved in 100% Me₂SO and diluted 10 times in water. 1, 10, or 100 μg of the peptides in 100 μl of Me₂SO/water was added to 300 μl of plasma. The samples were incubated under rotation for 15 min at room temperature and analyzed for bradykinin content in an enzyme-linked immunosorbent assay (Markit-M-Bradykinin, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) according to the manufacturer’s instructions. The bradykinin concentration was determined from the enzyme activity of peroxidase-labeled BK bound to the anti-BK antibodies; detection limit is 4.9 pg/well. A standard curve was prepared with five BK concentrations (4.9, 19.6, 78, and 313, 1250 pg/well). Samples were analyzed in duplicate and the results expressed as ng/ml plasma.

**RESULTS AND DISCUSSION**

A number of different human proteins have been shown to interact with curli. In most of these experiments, radiolabeled proteins were used in binding experiments with curli-expressing bacteria, whereas isogenic curli-deficient mutants served as controls (4, 7, 13, 16). In some cases, purified curli organelles and the CsgA subunit alone were demonstrated to bind the tested proteins (4, 7, 16). In the present study, initial experiments were performed to confirm and expand these previous data. ¹²⁵I-labeled human fibronectin, fibrinogen, plasminogen, t-PA, H-kininogen, factor XII, MHC class I antigens, β₂-microglobulin, and HSA, were tested for binding to curli-expressing *E. coli* and an isogenic curli-deficient mutant. These results (Fig. 1) show that all proteins except HSA interact with curli-expressing bacteria, whereas none of the tested proteins had affinity for the mutant.

To further investigate the binding properties of curli, we synthesized a set of overlapping peptides covering the entire CsgA curli subunit. The peptides (Fig. 2A) were applied to filters, which were probed with the various radiolabeled proteins tested for binding to intact bacteria (see Fig. 1). Fig. 2B shows the results obtained with ¹²⁵I-labeled fibrinogen. Distinct dose-dependent binding is seen to peptides NNS24, KQF22, and VDQ26. The specificity was tested by incubating the filter with an excess of unlabeled fibrinogen. This completely inhibited the binding of radiolabeled fibrinogen to the peptides (data not shown). Apart from HSA, which did not bind to any of the peptides, and factor XII, which gave weak signals with peptides KQF22 and VDQ26 but a strong signal with peptide NNS24, the other radiolabeled proteins (fibronectin, plasminogen, t-PA, H-kininogen, factor XII, MHC class I antigens, β₂-microglobulin) tested gave rise to the same binding pattern as fibrinogen (data not shown). These results suggest two protein-binding sites in the CsgA subunit, one in the NH₂-terminal region and one in the COOH-terminal region.

**Physicochemical properties of NNS24** were investigated by circular dichroism (CD) analysis and NMR. CD analysis of the peptide revealed that NNS24 is predominantly in a β-sheet conformation as indicated by a single maximum and minimum at 197 and 216 nm, respectively (Fig. 3A). The intensity of the CD signal at 216 nm is correlated directly to the concentration of the peptide indicating that the β-sheet content is invariant within this concentration range (12–50 μM). This conformation is thermally relatively stable against thermal denaturation with a melting point (T_m) of 66 °C (Fig. 3B) compared with the average T_m of proteins, which is 63 °C (28). These data demonstrate that the NNS24 peptide adopts a stable and folded conformation in solution. Amyloid fibrils characteristically are composed of proteins rich in β-sheets, and they bind Congo red. Also curli fibers are stained with this reagent (29).

Interestingly, NNS24 and the two most COOH-terminal peptides, KQF22 and VDQ26, but not the other CsgA-derived peptides (see Fig. 2A), were distinctly stained red when applied to PVDF membranes and incubated with Congo red (data not shown). These observations suggest also that peptides KQF22 and VDQ26 form β-sheets and that the protein-binding regions of curli have this conformation in common. CD analysis of these peptides could not be performed, as they are soluble only in Me₂SO. Although much less pronounced than peptides KQF22 and VDQ26, the NNS24 peptide has a tendency to precipitate, suggesting that it could form soluble multimers. To study the multimerization properties of NNS24, diffusion-ordered NMR spectroscopy experiments were performed, resulting in a diffusion coefficient (D value) of 1.3 × 10⁻¹ (± 0.034) m²/s in D₂O at 2 °C, corresponding to a molecular mass of ~20 kDa. The molecular mass of NNS24 determined from its amino acid sequence is 2460.1 Da, demonstrating that the peptide aggregates and forms multimers, predominantly octamers, in solution.

The multimerization of the protein-binding peptides of curli indicates that these regions could also participate in the polymerization of CsgA subunits and, together with the nucleator protein CsgB (29), contribute to the formation of curli organelles.

An important question raised by the broad protein-binding capacity of curli is to what extent the multitude of possible
protein interactions actually takes place in the complex mixture of proteins surrounding bacteria \textit{in vivo}. Previous experiments (7, 17) in which curli-expressing bacteria were incubated with human plasma showed that a large number of different plasma proteins are indeed simultaneously bound to the bacterial surface through interactions with curli. For instance, incubation with human plasma will result in the binding and assembly of components of the contact system but also in the binding of several other plasma proteins, such as plasminogen and fibrinogen, to curliated bacteria (7, 17). To test whether the NNS24 and VDQ26 peptides, analogous to intact curli, could interact with many different proteins when exposed to an excess of proteins in a complex protein mixture, the peptides were coupled to Sepharose. Following incubation with human plasma and extensive washing, proteins bound to NNS24- or VDQ26-Sepharose were eluted by low pH and subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 4, A and B, \textit{STAIN}, \textit{lanes 1}, several proteins were bound and eluted from NNS24- and VDQ26-Sepharose. Compared with plasma (\textit{lanes 1}) some bands were accentuated, whereas other, such as the HSA bands around 66 kDa, were weaker or missing in the eluates (\textit{lanes 2}). In contrast, no proteins were absorbed from human plasma and eluted from glycine-Sepharose, the negative control (\textit{lanes 3}). These results demonstrate a broad but selective binding of human plasma proteins by the two curli-derived peptides.

Sepsis and septic shock caused by Gram-negative bacteria is a serious and important clinical condition causing more than 100,000 deaths annually in the United States alone (30). Several observations have indicated that curli play a role in the pathogenesis of sepsis. First, \textit{E. coli} strains isolated from patients with severe sepsis frequently express curli (13), and antibodies to CsgA are found in these patients (14). Second, when curliated bacteria are injected intravenously into mice or rats, they induce bleeding disorders, a fall in blood pressure, and lung lesions (17, 18, 31), symptoms that are common in cases of severe sepsis. The hypocoagulable state of human plasma following exposure to curli is due to the binding of factor XII and fibrinogen to curli (17), and as shown in Fig. 4, \textit{A} and \textit{B}, \textit{BLOT I}, fibrinogen is absorbed from plasma by NNS24- and VDQ26-Sepharose. The contact system consists of the three serine proteinases, factor XI, factor XII, and plasma prekallikrein, and the non-enzymatic cofactor H-kininogen. When the system is assembled and activated, H-kininogen is cleaved by activated kallikrein to generate bradykinin, a highly potent proinflammatory peptide. On bacterial surfaces contact activation will result in the cleavage of H-kininogen into two major 45- and 65-kDa fragments (13), and in sepsis low levels of factor XII and intact H-kininogen correlate with a fatal outcome (32). It is therefore noteworthy that H-kininogen is absorbed from plasma by the Sepharose-coupled curli peptides and cleaved into fragments of 45 and 65 kDa (Fig. 4, \textit{A} and \textit{B}, \textit{BLOT II, lanes 2}). These results suggest that the contact factors are bound and assembled on NNS24- and VDQ26-Sepharose and that the system is activated to generate bradykinin. Bradykinin is a primary mediator of inflammatory processes and induces pain, vasodilatation, and increased vascular permeability due to the local production of prostaglandins and nitric oxide (33). Consequently, a massive release of bradykinin by curliated bacteria could help to explain the hypovolemic hypotension seen in septic shock. To investigate whether the cleavage of H-kininogen shown in Fig. 4, \textit{A} and \textit{B}, \textit{BLOT II}, actually results in the generation of bradykinin, peptides NNS24, GFG24, and VDQ26 were added separately to fresh human plasma. 1, 10, or 100 µg of the peptides in 100 µl of 10% Me₂SO were added to 300 µl of plasma, and following incubation, the amount of bradykinin was determined by enzyme-linked immunosorbent assay. Neither the GFG24 peptide nor 10% Me₂SO alone generated bradykinin above background level (<0.1 ng/ml plasma), whereas even 1 µg of NNS24 induced a significant increase (0.71 ± 0.03 ng/ml plasma). When 10 or 100 µg of NNS24 was added, this resulted in a massive generation of bradykinin (12.8 ± 3.4 and 57.0 ± 13.1 ng/ml plasma, respectively). The VDQ26 peptide did not induce bradykinin release at 1 or 10 µg, but at 100 µg the amount was clearly above background level (2.46 ± 0.15 ng/ml plasma). These results show that the fragmentation of H-kininogen induced by NNS24 and VDQ26 generates bradykinin.
In conclusion, the present work has identified and characterized two protein-binding regions of curli, which are also responsible for the activation of the pro-inflammatory contact system. The important role played by curli in virulence and its unique protein-binding properties should stimulate future investigations of the multipotent structural entities included in the NNS24 and VDG26 peptides.

Acknowledgment—Ingbrit Gustafson is acknowledged for excellent technical assistance.

Addendum—After the initial submission of this manuscript, Chapman et al. (34) reported that purified CsgA subunits polymerize into amyloid curli fibers. It is likely that the NH2- and COOH-terminal CsgA regions defined in this study participate in this assembly process.

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Identification of Two Protein-binding and Functional Regions of Curli, a Surface Organelle and Virulence Determinant of Escherichia coli
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doi: 10.1074/jbc.M206353200 originally published online July 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206353200

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