Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from in vitro FimH binding

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SUMMARY

The binding of uropathogenic *Escherichia coli* to the urothelial surface is a crucial initial event for establishing urinary tract infection because it allows the bacteria to gain a foothold on the urothelial surface, thus preventing them from being removed by micturition. In addition, it triggers bacterial invasion as well as host urothelial defense. This binding is mediated by the FimH adhesin located at the tip of the bacterial type 1-fimbrium, a filamentous attachment apparatus, and its urothelial receptor. We have prepared a biotinylated, recombinant FimH-FimC adhesin:chaperone complex and used it to identify its mouse urothelial receptor. The FimH-FimC complex binds specifically to a single 24 kDa major mouse urothelial plaque protein, which we identified as uroplakin Ia by mass spectrometry, cDNA cloning and immunoreactivity. The terminal mannosyl moieties on Asn-169 of uroplakin Ia are responsible for FimH as well as concanavalin A binding. Although FimH binds to uroplakin Ia with only moderate strength (K_d ~100 nM between pH 4 and 9), the binding between multiple fimbriae of a bacterium and the crystalline array of polymerized uroplakin receptors should achieve high avidity and stable bacterial attachment. The FimH-FimC complex binds preferentially to the mouse urothelial umbrella cells in a pattern similar to uroplakin staining. Our results indicate that the structurally related uroplakins Ia and Ib are glycosylated differently, that uroplakin Ia serves as the urothelial receptor for the type 1-fimbriated *E. coli*, and that the binding of uropathogenic bacteria to uroplakin Ia may play a key role in mediating the urothelial responses to bacterial attachment.

Key words: Urothelial cells, Bacterial adhesin, Urinary tract infection

INTRODUCTION

Urinary tract infection is one of the most common infectious diseases, causing over 7 million patient visits annually and costing more than a billion US dollars (Hooton et al., 1996; Johnson and Stamm, 1987). Over 80% of these infections are caused by *Escherichia coli* strains that are equipped with filamentous adhesive organelles known as type 1 fimbriae, or pili (Hooton and Stamm, 1997; Hultgren et al., 1993; Langermann et al., 1997). These fimbriae consist of polymerized pilin subunits capped with the lectin FimH that can bind to the terminal mannose moieties on the surface receptors of the superficial urothelial umbrella cells (Abraham et al., 1987; Connell et al., 1996; Jones et al., 1995; Krogfelt et al., 1990; Svanborg Eden and Hansson, 1978). This binding is required to initiate the infection, as it allows the bacteria to gain a foothold on the urothelial surface, thus preventing them from being swept away during micturition (Beachey, 1981). Moreover, the FimH-mediated bacterial binding to urothelial surface receptors can trigger major host cell responses including the activation of various kinases and recruitment of adaptor proteins, culminating in the local reorganization of the actin cytoskeleton and engulfment of the bacteria (Fukushi et al., 1979; Martinez et al., 2000; McTaggart et al., 1990; Mulvey et al., 1998). Such a bacterial invasion into the host cells insulates the pathogens from a wide battery of extracellular host defense mechanisms, allows the pathogen to undergo intracellular propagation or to persist in a quiescent state in the host cells, and provides a bacterial reservoir for recurrent infection (Hopkins et al., 1998; Hvidberg et al., 2000; Mulvey et al., 1998; Mulvey et al., 2000). To understand how this bacterial invasion occurs so that one can devise new therapeutic measures, it is crucial that we study the detailed molecular mechanism of adhesin:host receptor interaction. A prerequisite of this is the precise identification of the urothelial receptors for the type 1 fimbriated *E. coli*.

In a recent in vivo study of the bacterial infection of mouse urothelium, high resolution scanning electron microscopy showed that the tip of the type 1 pili can mediate direct bacterial contact with the uroplakin particles of the apical cell plasma membrane (Mulvey et al., 1998; Mulvey et al., 2000). Uroplakins are protein subunits of the characteristic 16 nm particles forming prominent two-dimensional crystals (0.2-1.0 μm in diameter), known as urothelial plaques, that cover >90%

of the umbrella cell apical surface (Hicks, 1975; Kachar et al., 1999). Four major uroplakins have been initially identified in bovine urothelium: uroplakins Ia (27 kDa), Ib (28 kDa), II (15 kDa) and III (47 kDa) (Kallin, 1991; Lin et al., 1994; Wu et al., 1990; Wu and Sun, 1993; Yu et al., 1994; Yu et al., 1990). These four uroplakins are highly conserved in mammalian urothelia, although their molecular weights vary slightly from species to species (Wu et al., 1994). Although uroplakins II and III have a single transmembrane domain, uroplakins Ia and Ib have four transmembrane domains and belong to the 'tetraspanin' superfamily (Maecker et al., 1997; Yu et al., 1994). Wu et al. (Wu et al., 1996) showed that radiolabeled type 1-fimbriated *E. coli* bound to two bovine urothelial plaque proteins, thought to represent uroplakins Ia and Ib, in the molecular range of 25-28 kDa (Wu et al., 1996). However, due to the unavailability of soluble FimH protein, no investigation was performed to study the direct interaction between FimH and uroplakins at the molecular level.

We have investigated here the in vitro interactions between FimH and mouse urothelial plaques. The use of purified, recombinant FimH, in the form of a soluble FimH-FimC complex, allowed us to better define the molecular characteristics of the binding. We used FimH-FimC complex because FimC, a periplasmic chaperone, is required to stabilize the otherwise easily degradable FimH (Choudhury et al., 1999; Jones et al., 1993; Pellecchia et al., 1999). Mouse, instead of bovine, urothelial plaques were used in the present study because the former can be purified in a fresher state, mouse uroplakins Ia (25 kDa) and Ib (29 kDa) are much better resolved electrophoretically than their bovine counterparts (27 and 28 kDa), and mouse bladder is an important animal model for studying urinary tract infection (Hagberg et al., 1983; Johnson and Manivel, 1991; Mulvey et al., 1998). The greatly improved electrophoretic resolution between the uroplakins Ia and Ib of mouse plaques enabled us to clearly demonstrate that FimH selectively recognized uroplakin Ia, without detectable binding to its structurally related uroplakin Ib. The affinity between FimH and mouse urothelial plaques was moderate, with a dissociation constant of ~100 nM, which remained constant over a wide pH range of 4-9 indicating that urine pH does not appreciably affect bacterial binding. The realization that uroplakin Ia is the unique bacterial receptor has major implications for the mechanisms of bacterial invasion.

MATERIALS AND METHODS

Biotinylation of recombinant FimH-FimC

The FimH-FimC complex was expressed in bacteria and purified as described (Pellecchia et al., 1999). For biotinylation, 1 mg of FimH-FimC in 1 ml 50 mM Na_2CO_3-$NaHCO_3$ (pH 9.0) was mixed with 50 μl 1 mg/ml sulfo-NSH-biotin (Pierce Chemical, Rockford, IL) freshly solubilized in water. The mixture was left on ice for 2 hours, and its buffer was changed to Tris-buffered saline (TBS) (150 mM NaCl, 25 mM Tris-HCl, pH 7.5) through ultrafiltration (Centricon, 10 kDa cut-off; Millipore, Bedford, MA) to remove free biotin. Plates with immobilized α-D-mannose were purchased from NNI BioTech, Lund, Sweden. Other biotin-labeled lectins (kits containing 21 lectins) were purchased from Vector Laboratories, Burlingame, CA.

Bacterial and protein overlay assays

Bovine and mouse uroplakin plaques were isolated by sucrose density gradient and differential detergent wash (Liang et al., 1999; Wu et al., 1994). Bacterial overlay assay was performed as described (Wu et al., 1996). For overlay with biotin-labeled proteins, purified urothelial plaques was dissolved in loading buffer (0.2% SDS, 0.2% 2-mercapathanol, 100 mM Tris-HCl, pH 6.8), resolved electrophoretically by 17% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was first incubated at 37ºC for 45 minutes with 3% bovine serum albumin (BSA; Sigma) in TBS and 0.05% Tween-20, followed by adding the biotinylated protein to a final concentration of 1 μg/ml. Horseradish peroxidase (HRP)-labeled streptavidin (Sigma) was used to localize the bound biotinylated protein probe. SuperSignal enhanced chemiluminescent substrate (Pierce Chemical) was used to enhance the signals (Fuji, Stanford, CT). Immunoblots were performed similarly except that the membrane-bound proteins were incubated with rabbit antibodies against uroplakin peptides (Wu et al., 1994) and subsequently with a secondary antibody of HRP-conjugated goat anti-rabbit IgG.

Protein characterization by mass spectrometry

Coomassie-stained protein bands on an SDS-PAGE were excised, washed, reductive alkylated and digested with trypsin (Boehringer Mannheim, Indianapolis, IN) (Shevchenko et al., 1996). The resulting peptide mixture was extracted, dried under vacuum, resuspended in 0.1% trifluoroacetic acid and desalted. The peptides were eluted with 2-3 μl of 70% acetonitrile, brought to 1% acetic acid and analyzed by nanoelectrospray tandem mass spectrometry in a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Beverly, MA).

The *N*-glycosylation site was identified by mass spectrometry using partial ^{18}O labeling (Kuster and Mann, 1999). After reductive alkylation, the protein in an SDS-gel slice was digested with peptide N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) in a buffer containing 50% $H_2{}^{18}O$ (Aldrich, Milwaukee, WI). Residual PNGase F was removed by washing the gel with 0.1% SDS in 0.1 M ammonium bicarbonate; the deglycosylated protein was digested with trypsin and the resulting peptide mixture was extracted from the gel slice. In the mass spectrometry survey scan, the peptide containing a partially ^{18}O-labeled site, which resulted from the enzymatic hydrolysis of an *N*-glycan in buffer containing 50% $H_2{}^{18}O$, could be identified by a characteristic distribution of isotopic peaks.

Cloning and sequencing of mouse uroplakin Ia

Five μg of total mouse urothelial RNA were used to synthesize the first strand cDNA, using a RACE kit (Gibco BRL). PCR primers were designed based on mass spectrometric data and a mouse EST sequence (GenBank # AI225453). The appropriate PCR fragments were then cloned and sequenced.

Protein deglycosylation

Uroplakins were incubated for 1 hour at 37ºC with 5000 units/ml PNGase F (New England Biolabs, Beverly, MA) in 50 mM sodium phosphate (pH 7.5), 0.5% SDS, 0.2% β-mercaptoethanol and 1% NP-40. The reaction was terminated by adding equal amount of 2× SDS-PAGE sample buffer. Deglycosylated proteins were separated by SDS-PAGE and immunoblotted.

Enzyme-linked lectin binding assay

The mannose-containing target proteins were dissolved at a concentration of 100 μg/ml in 50 mM bicarbonate buffer (pH 8.5), and 50 μl aliquots were dispensed into every well of a 96-well plate and left at 4ºC overnight. The wells were incubated with 3% BSA/TBS at 37ºC for 2 hours. Biotinylated FimH-FimC in 3% BSA/TBS and 0.02% Tween-20 was added to the wells, incubated at 37ºC for 1 hour, and the wells were then washed three times with TBS. Streptavidin HRP conjugate was used to detect the FimH-FimC binding using 3,3′,5,5′-tetramenthylbenzidine (tablets from Sigma) as the substrate. After 5 minutes, the reaction was terminated by adding

an equal volume of 3.6 N H_2SO_4, and the absorbance of the reaction product was read at 450 nm in a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The data were analyzed with Sigmaplot (Jandel Scientific, San Rafael, CA). All measurements were done in quadruplicate and repeated more than three times (Perlmann and Perlmann, 1994).

Histochemical staining

Thin sections (5 μm) of mouse bladder fixed with 4% paraformaldehyde were incubated at 37ºC for 1 hour with an antibody to uroplakins or with biotinylated FimH-FimC, followed by three washes with TBS (150 mM NaCl, 25 mM Tris-HCl, pH 7.5). The antibodies and FimH-FimC were then detected with FITC-conjugated goat anti-rabbit IgG (Vector Laboratories) and FITC-labeled streptavidin (FITC-SP; Sigma), respectively. The substrate Hoechst 33258 (Sigma) was used to visualize the nuclei of the cells.

RESULTS

Biotinylation of FimH-FimC and urothelial plaque purification

The recombinant FimH-FimC complex was extracted from the periplasmic fraction of an overproducing *E. coli* strain and purified by ion exchange chromatography (Pellecchia et al., 1999). The amino acid sequence of this FimH was identical to that of *E. coli* strain SH48 used in the previous studies by Wu et al. (Wu et al., 1996) (data not shown). Treating this protein complex with the water-soluble sulfo-NHS-biotin (Fig. 1A) resulted in the biotinylation of FimH and FimC with a labeling ratio of ~1:3, which was consistent with the number of amino groups in these two protein subunits (Fig. 1A). Diluting the biotinylated FimH-FimC with an equal amount of unmodified protein complex resulted in a 50% reduction in its ability to bind to immobilized α-D-mannose, thus demonstrating that biotinylation did not reduce the ability of the FimH-FimC complex to bind α-D-mannose (Fig. 1B).

Previous studies indicated that urothelial plaques can be isolated from nine mammalian species including bovine, human and mouse, using a combination of sucrose density gradient centrifugation and detergent wash (Wu et al., 1994). Using this procedure, we have purified urothelial plaques from both bovine and mouse (in the latter case with a yield of ~0.1 mg of urothelial plaques from five mouse bladders). The purified mouse urothelial plaques were quite large in size (up to 1 μm; Fig. 1C) and, when visualized by high resolution electron microscopy, yielded a higher resolution electron diffraction than the bovine urothelial plaques (G.M., G.Z. and X.-P.K., unpublished).

Identification of mouse uroplakin Ia as the major FimH binding protein

Using ^{35}S-labeled type 1-fimbriated *E. coli* as a probe, it was found previously that these bacteria bound to two bovine urothelial plaque proteins with apparent molecular weights of approximately 25 and 28 kDa (Wu et al., 1996). We have confirmed this finding (Fig. 2A, lane 5), and noted that the higher molecular weight band could usually account for over 80% of the total binding. The purified FimH-FimC complex was found, however, to recognize only to the upper 27/28 kDa band (Fig. 2A, lane 2). These binding reactions were prevented by α-D-mannose (lanes 3 and 6), but not by galactose (lane 4). Because bovine uroplakins Ia and Ib are very similar in size and were only partially resolved by SDS-PAGE (Fig. 2A, lane 1), we repeated these binding experiments using purified mouse urothelial plaques. As shown in Fig. 2B, mouse urothelial plaques contained four major proteins with apparent molecular weights of 47 kDa, 29 kDa, 24 kDa and 15 kDa; the two intermediate-sized proteins, presumably the uroplakin Is, were therefore well separated. Both radiolabeled *E. coli* and the biotinylated FimH-FimC complex were found to recognize exclusively a single protein band of 24 kDa (Fig. 2B), which was shown to be uroplakin Ia by mass spectrometry and cDNA cloning (Fig. 3), as well as by immunoblotting (Fig. 4A). These results indicate that uropathogenic *E. coli* recognizes mainly, if not exclusively, the uroplakin Ia subunit of mouse urothelial plaques (see Discussion).

The selective binding of FimH to mouse uroplakin Ia was unexpected, and it indicated that uroplakin Ia's sugar moieties

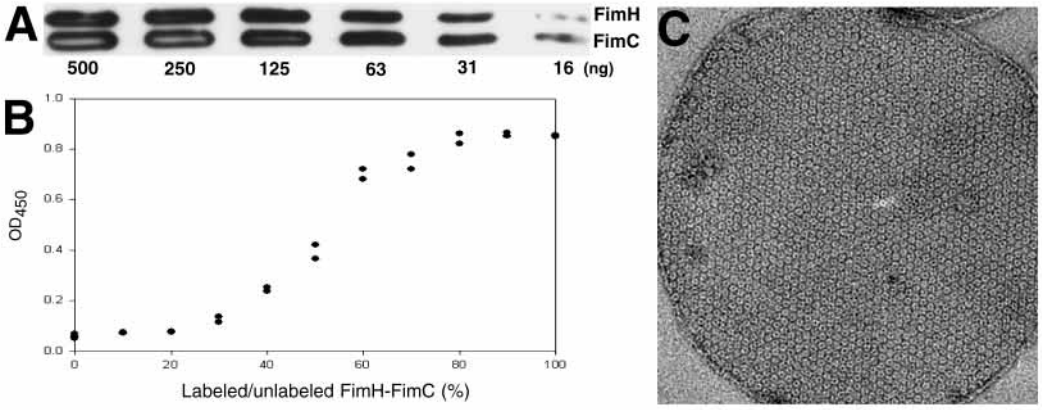

Fig. 1. Biotinylation of recombinant FimH-FimC complex. (A) Detection of the biotinylated FimH-FimC complex after the proteins were electrophoretically separated and transferred onto a nitrocellulose membrane. Note the detectability of as little as 16 ng of the complex per lane. (B) Competitive binding between the biotinylated and unlabeled FimH-FimC to immobilized α-D-mannose. Note that a one-to-one dilution of the biotinylated FimH-FimC with unlabeled protein resulted in 50% reduction in the binding of the labeled protein to immobilized mannose, indicating that the mannose-binding activity of the recombinant protein was not affected by biotinylation. (C) Typical ultrastructure of negatively stained mouse urothelial plaques showing two-dimensional crystals of 16 nm uroplakin particles.

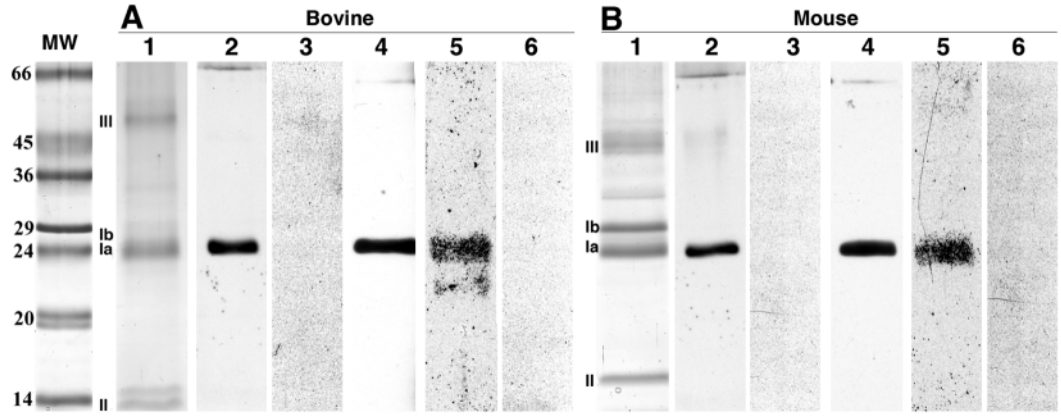

Fig. 2. In vitro binding of type 1-fimbriated *E. coli* and FimH-FimC protein complex to mouse and bovine uroplakins. Purified bovine (A) and mouse (B) urothelial plaque proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Lane 1: coomassie blue staining showing the positions of uroplakins Ia, Ib, II and III; note the excellent resolution of the mouse 24 kDa uroplakin Ia and 29 kDa uroplakin Ib, in comparison with the poorly resolved bovine 27 kDa uroplakin Ia and 28 kDa uroplakin Ib. Lane 2: binding of biotinylated FimH-FimC to uroplakins; note the selective binding to mouse uroplakin Ia with no detectable binding to uroplakin Ib. Lane 3: same as in lane 2 but the binding was carried out in the presence of 1 mM α-D-mannose; note the complete inhibition of the FimH-FimC binding to uroplakin Ia. Lane 4: same as in lane 2 but done in the presence of 1 mM galactose; note the lack of inhibition. Lane 5: binding of ^{35}S-labeled, type 1-piliated *E. coli* (strain SH48) to urothelial plaque proteins; note again the selective binding to mouse uroplakin Ia. Lane 6: binding of a similarly labeled, but nonfimbriated *E. coli* (strain P678) to urothelial proteins; note the complete lack of binding. Positions of uroplakins Ia, Ib, II and III are marked. MW: molecular weight standards.

must be different from those of the structurally closely related uroplakin Ib. Probing the sugars of mouse uroplakins Ia and Ib using a panel of ~20 lectins with different sugar specificities showed that several mannose-binding lectins, including concanavalin A (ConA), selectively recognized uroplakin Ia, thus confirming the FimH binding results (Fig. 4A, lane 3). We have also demonstrated that the binding of FimH to mouse uroplakin Ia was sugar-dependent because deglycosylation of uroplakin Ia abolished the binding (Fig. 4A, lane 2). Mass spectrometry confirmed that the single, potential *N*-glycosylation site of mouse uroplakin Ia was occupied by high mannose sugars that were responsible for the FimH-mediated bacterial binding (Fig. 4B). The FimH-FimC complex bound mainly to the umbrella cells of mouse urothelium in frozen bladder sections (Fig. 5A), with a staining/distribution pattern similar to that produced by antibodies to uroplakins (Fig. 5B).

Determination of the FimH-uroplakin Ia binding affinity

To assess the strength of FimH-uroplakin Ia binding, we measured the amounts of the biotinylated FimH-FimC complex that were bound by immobilized mouse urothelial plaques using an ELISA-like, enzyme-linked lectin binding assay (Fig. 6). The binding was found to be saturable with a dissociation constant of ~111±7 nM. This binding was clearly mediated through the mannose moieties of the uroplakin receptor, because under the same experimental conditions FimH bound to α-D-mannose-BSA with an almost identical affinity (K_d=107±5 nM; r=0.9758), and because the binding could be

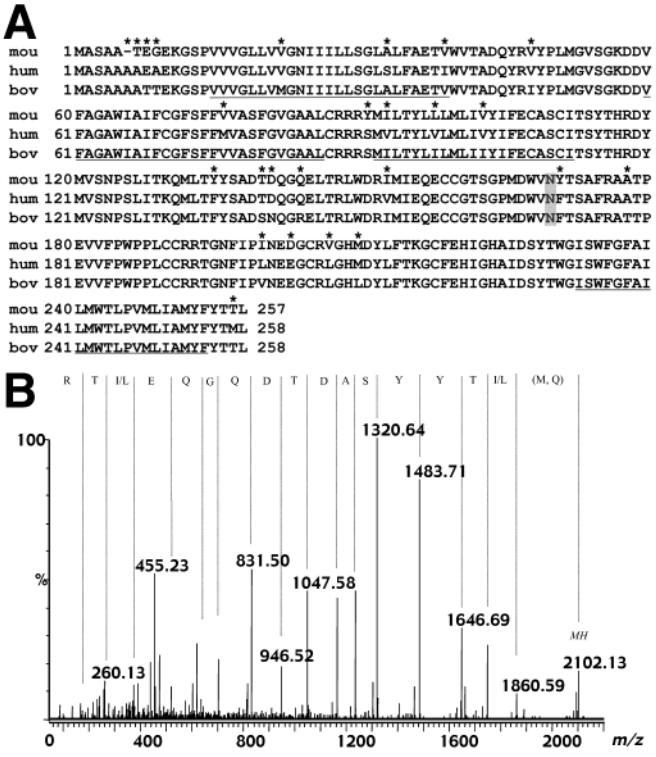

Fig. 3. Identification of the major mouse FimH binding urothelial plaque protein by mass spectrometry and cDNA cloning. The 24 kDa protein identified as the major FimH and bacterial binding protein of purified mouse urothelial plaque was gel-purified, microsequenced and cDNA-cloned. (A) Identification of the major mouse FimH-binding protein as uroplakin Ia. The cDNA-deduced amino acid sequence of the mouse (mou) FimH binding protein is aligned here with those of human (hum) and bovine (bov) uroplakin Ias. The predicted *N*-glycosylation sites and the putative transmembrane domains are shaded in gray and underlined, respectively. Asterisks indicate amino acids that vary among the species. (B) Sequencing of a major tryptic peptide using mass spectrometry. The sequence of this peptide was derived from the y series ions and is indicated on the top from right (N-terminal) to left (C-terminal).

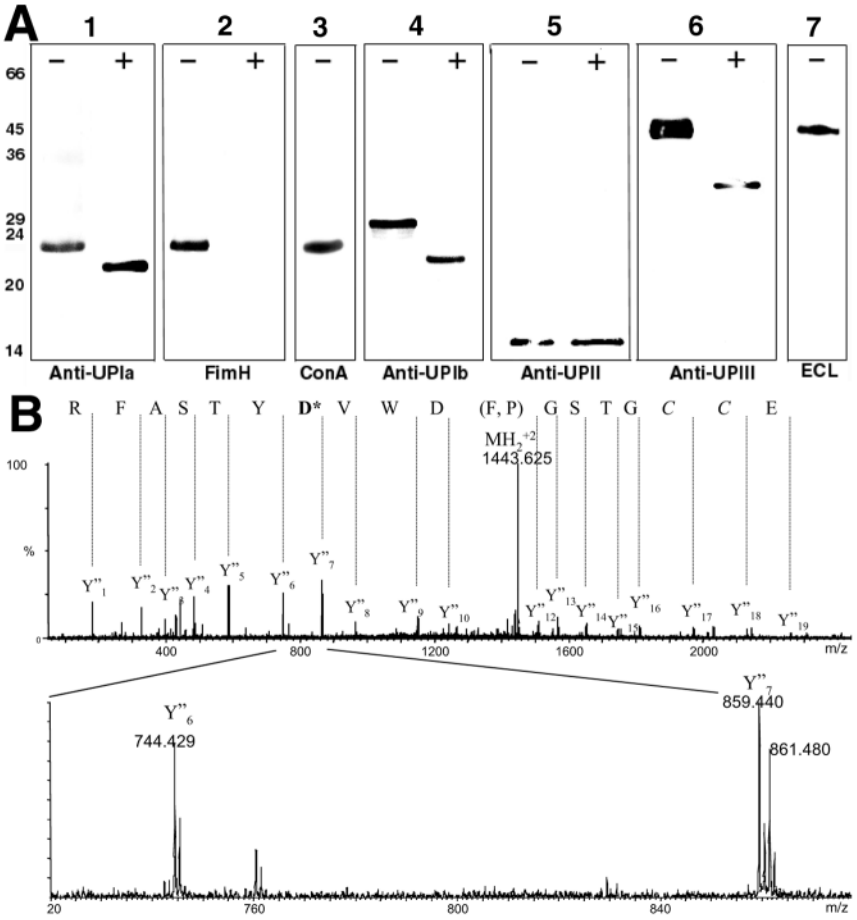

Fig. 4. Carbohydrate heterogeneity among mouse uroplakins and the identification of the mouse uroplakin Ia glycosylation site. (A) Carbohydrate heterogeneity among mouse uroplakins as assessed by deglycosylation and lectin binding. Mouse uroplakins dissolved in SDS were incubated in the absence (–) or presence (+) of PNGase F before they were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies to specific uroplakins (lanes 1, 4, 5 and 6 as indicated), FimH (lane 2), ConA (lane 3), or erythrina cristagalli lectin (ECL), a lectin that has specificity for glucosamine (lane 7). Note the reduction in the apparent sizes of mouse uroplakins Ia, Ib and III after deglycosylation (lanes 1, 4 and 6); the recognition of uroplakin Ia, but not uroplakin Ib, by FimH (lane 2) and ConA (lane 3); and the ablation of the uroplakin Ia binding by deglycosylation. The molecular weights of standard proteins are indicated on the left. (B) Mapping of the glycosylation site of mouse uroplakin Ia utilizing the fact that the glycosylated asparagine can be derivatized to a partially ^{18}O-labeled aspartic acid (D*) during deglycosylation. The upper panel shows the full MS/MS spectrum of the doubly charged precursor ion (m/z 1443.625 centroid monoisotopic) with the peptide sequence indicated by y series ions. The lower panel is an expanded segment of the upper panel. Upon partial ^{18}O-labeling, fragment ions containing the ^{18}O-label appeared as doublets that were readily differentiated from the unlabeled ones. UP, uroplakin.

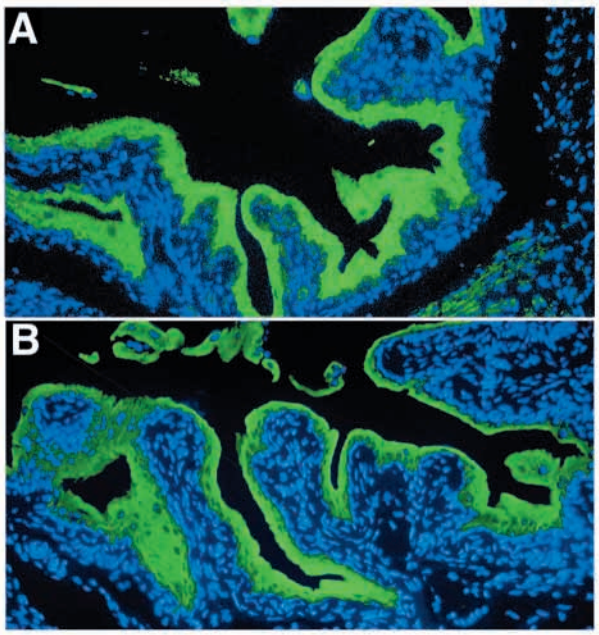

Fig. 5. Selective binding of FimH (A) and antibodies to uroplakins (B) to the umbrella cells of mouse urothelium. Sections of mouse bladder were incubated with biotinylated FimH-FimC (A) or rabbit antibodies to total uroplakins (B), and then with FITC-conjugated streptoavidin or goat anti-rabbit IgG. Nuclei were counterstained with a blue Hoechst dye. Note the similar staining of urothelial umbrella cells by FimH-FimC and antibodies to uroplakins.

blocked by mannose (IC_{50}=38±6 nM) but not by galactose (Fig. 6B). Although it has been suggested that acidic urine pH is unfavorable for bacterial infection, the binding of FimH to its uroplakin receptor was unaffected in the pH range of 4.2-9 (Fig. 6C,D).

DISCUSSION

Uroplakin Ia as the urothelial bacterial receptor

In an earlier study, the in vitro binding of type 1-fimbriated *E. coli* to bovine urothelial plaques was examined (Wu et al., 1996). The present investigation differed from this earlier one in two aspects. First, the previous study utilized bovine urothelial plaques whose uroplakins Ia and Ib (27 kDa and 28 kDa, respectively) were only partially resolved by SDS-PAGE, whereas the current study utilized mouse urothelial plaques whose counterparts (24 kDa and 29 kDa) were well separated. Although the bovine plaque preparation showed two bacterial binding proteins in the molecular weight range of 25-28 kDa, as we have confirmed here (Fig. 2A, lane 5), it is clear from the present analyses of mouse urothelial plaques that uroplakin Ia is the major bacterial binding protein. The lower molecular weight bacterial binding protein band (~25 kDa), which can occasionally be seen in bovine urothelial plaque preparations (Fig. 2), most likely represents a partially degraded form of uroplakin Ia. Second, by using purified uroplakin plaques and recombinant FimH-FimC complexes, we have now

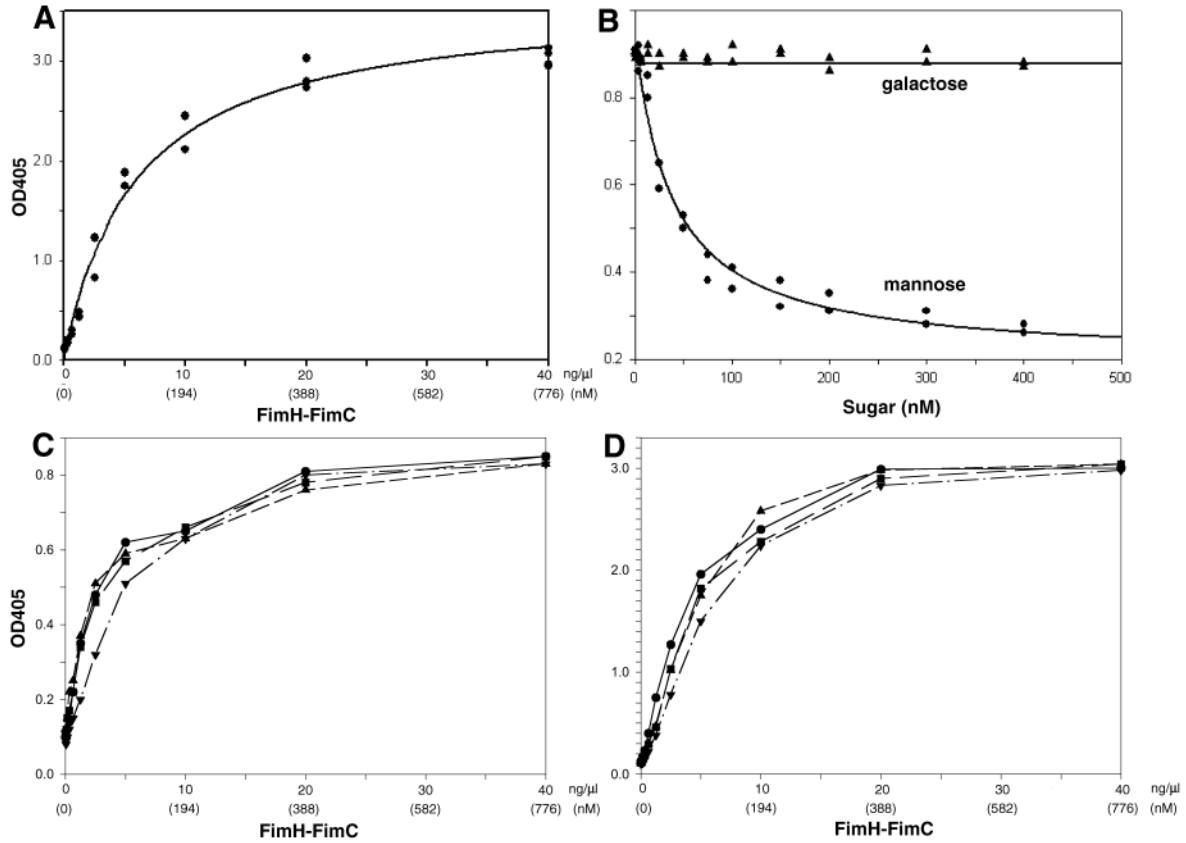

Fig. 6. Effects of sugars and pH on the binding of FimH to mouse urothelial plaques. The wells of 96-well plates were coated with purified mouse urothelial plaques, quenched with bovine serum albumin and incubated with biotinylated FimH-FimC, in the presence or absence of galactose or mannose and at various pHs. (A) Typical saturation curve for the binding of FimH-FimC to mouse urothelial plaques. The solid line represents a best fit from nonlinear regression. This is a typical curve representative of results from ~10 experiments, yielding K_d values in the range of 110-160 nM (correlation coefficient r=0.9948). (B) The binding is completely abolished by α-D-mannose (●, but not by galactose (▲). (C) Binding of the FimH to mouse urothelial plaques at pH 4.2-7.0. pH 4.2 (●), 5.0 (■), 6.0 (▲) and 7.0 (▼). (D) Similar binding at pH 7-9. pH 7.0 (●), 7.5 (■), 8.0 (▲) and 9.0 (▼).

investigated the bacterial-urothelial cell interactions at the protein level (Leffler and Svanborg-Eden, 1990). Our results established that, in mouse, uroplakin Ia is the only receptor for bacterial FimH, and that uroplakin Ia is also most likely the sole FimH receptor for bovine urothelium.

Our analyses of mouse urothelial plaques showed clearly that uroplakins Ia and Ib, despite their 38% identity in amino acid sequence, are glycosylated differently so that the former harbors terminal mannose moieties, whereas the latter does not. The mechanism by which this differential glycosylation occurs between the closely related uroplakins Ia and Ib is unknown, but it is conceivable that the local tertiary structure of uroplakin Ia: (1) makes its high mannose sugars unavailable to further modification by glycosyltransferase; (2) is recognized by a Golgi lectin which then protects the terminal mannose sugars of uroplakin Ia; and/or (3) suppresses the enzymatic activity of the transferases (Rademacher et al., 1988; Trimble et al., 1983).

Accessibility of the uroplakin receptors to the bacteria

Although it has been suggested that the apical surface of the mammalian urothelium is covered by a thick layer of glycosaminoglycans and/or glycocalyx, which has been hypothesized to attach to the urothelial surface, to be as thick as 10 μm, and to play a role in preventing bacterial adherence (Hurst et al., 1997), recent evidence suggests that uroplakins are directly exposed to the urine and are readily accessible to the bacteria (Kachar et al., 1999; Mulvey et al., 1998). Examination of mouse urothelial surface using the quick-freeze deep-etch (QFDE) technique revealed that over 90% of the surface area is covered by 0.2-1.0 μm plaques consisting of hexagonal arrays of 16 nm uroplakin particles (Kachar et al., 1999). Such plaques are interspersed by hinge areas that are relatively particle free. A striking feature of the plaque, as visualized by QFDE, is the lack of an extensive coverage by glycosaminoglycans or glycocalyx. The fact that this technique readily revealed an extensive filamentous glycocalyx network on the surface of frog bladder epithelium (which is equivalent to some mammalian renal cells) indicates that the experimental condition is mild enough to preserve a glycocalyx-like structure. Using the same QFDE technique, Mulvey et al. (Mulvey et al., 1998) showed that the (FimH-containing) tips of the type 1-fimbriae can attach directly to the naked uroplakin particles on the urothelial surface (Mulvey et al., 1998). Taken together, these results indicate that uroplakin Ia receptor is readily accessible for bacterial attachment. In this regard, it

should be noted that there is a battery of urine components that can serve as a defense mechanism to prevent the bacteria from binding to the uroplakin receptor. These components include polysaccharides and glycoproteins such as uromodulin or Tamm-Horsfall protein, a major urinary protein that harbors large amounts of high mannose sugars (Pak et al., 2001; Reinhart et al., 1990; Serafini-Cessi et al., 1984).

Multivalent binding of FimH to the uroplakin receptor

The recombinant FimH binds to its sugar substrate on uroplakin Ia with a dissociation constant of about 100 nM, indicating a moderate strength affinity. Because up to 100-200 fimbriae per bacterium can attach to an urothelial umbrella cell (Brinton, 1965; Pearce and Buchanan, 1980), the likelihood that all the bonds are broken at the same time is exceedingly small. Moreover, excessively tight binding of the bacterial adhesin to phagocytic cells, also through mannosylated receptors (Malaviya et al., 1999), can jeopardize the pathogen's survival (Beachey, 1981). A moderate strength of FimH:receptor binding may therefore be optimal for the bacterium's ability to anchor on urothelium and to escape from the host phagocytic defense. Finally, the FimH-uroplakin Ia binding reaction was practically unchanged over a wide pH range of 4-9, suggesting that lowering the urinary pH is unlikely to hamper bacterial binding to the urothelial cell receptors.

Possible mechanisms of uroplakin Ia-mediated signaling

Relatively little is known about the mechanism by which the uroplakin Ia receptor triggers a cascade of signal transduction events in urothelial cells leading to host cell changes and bacterial invasion. Uroplakin Ia belongs to the tetraspanin superfamily of genes that encode a large number of integral membrane proteins having four transmembrane domains (TMDs). These proteins have two major hydrophilic loops interconnecting TMDs 1 and 2 as well as TMDs 3 and 4, and they share several highly conserved cysteine residues. Members of the tetraspanin family include CD9, which plays a role in cell adhesion, B cell differentiation and sperm-egg fusion; CD53, which serves as a marker for thymocyte selection; CD82, which induces T cell costimulation; CD151, which plays a role in epithelial attachment to the basement membrane; and CD81 (TAPA; target of the antiproliferative antibody), which mediates neuron-astrocyte interaction, plays a role in airway hyper-reactivity and serves as a receptor for the hepatitis C virus (Maecker et al., 1997; Wright and Tomlinson, 1994). Therefore the tetraspanin proteins are quite versatile and are involved in many important cellular functions. How these tetraspanin cell-surface proteins generate the signals that are translated into altered cellular processes is unclear. However, it has recently been shown that many of these tetraspanin proteins can interact with one another, forming complexes that are associated with lipid rafts; they can also recruit integrins, HLA, receptors for EGF, diphtheria toxin and T cell receptor, which may then transduce signals affecting intracellular events (Maecker et al., 1997). The aggregating property of the tetraspanin proteins suggests that they may function as an organizer of cell-surface proteins (Maecker et al., 1997). In the case of uroplakin Ia, it remains to be determined whether it can interact directly or indirectly with, for example, uroplakin III, which has a cytoplasmic domain potentially involved in signal transduction (Wu and Sun, 1993).

We showed here that uroplakin Ia, a tetraspanin protein and a major urothelial differentiation product, can be used by uropathogenic bacteria as a receptor. Other tetraspanin proteins are quite diffusible on the cell surface and their aggregation/association with other cell-surface proteins may bring about a wide variety of cellular responses depending on the exact protein composition and aggregation state of the complex; this process is therefore inherently complex and difficult to analyze. By contrast, the great majority of uroplakin Ia on the apical surface of mature superficial urothelial cells is embedded in well-defined, two-dimensional crystals of uroplakin complexes that have a relatively constant protein composition (Fig. 1C). Moreover, the binding of FimH to the urothelial receptor can induce relatively well-defined host cell changes involving bacterial invasion, and host cell apoptosis and shedding (Martinez et al., 2000; Mulvey et al., 1998). Therefore, the interactions between FimH and its uroplakin Ia receptor provide an excellent model system for studying the signal transduction of a tetraspanin molecule.

Interestingly, uroplakin Ias of bovine, mouse and human contain a C-terminal sequence YTXL, a signature motif for mediating endocytosis (Indik et al., 1995). The last three residues, TXL, when present at the C-terminal cytoplasmic tails of membrane proteins, can also bind to the PDZ motif of submembranous proteins that are involved in signal transduction (Fanning and Anderson, 1999; Gee et al., 1998). It will be interesting to determine whether the C-terminal tail, although short (five to six amino acids), can bind to the urothelial PDZ-containing proteins.

Modulation of the aggregation state of the uroplakin receptor

An important feature of the uroplakin Ia bacterial receptor is that its aggregation state can be greatly modulated depending on the growth or differentiation state of the urothelial cell. Thus, cultured bovine urothelial cells still synthesize large amounts of the uroplakins, although they almost completely lack the cytoplasmic (fusiform) vesicles that are prominent cellular structures in mature in vivo umbrella cells presumably involved in delivering the uroplakins to the apical surface (Surya et al., 1990). Moreover, the apical surface of these cultured urothelial cells completely lack the two-dimensional crystals of 16 nm uroplakin particles (Surya et al., 1990). We have suggested earlier that the cultured urothelial cells mimic the in vivo hyperplastic urothelial cells that are undergoing wound repair (Surya et al., 1990). It has been reported that certain cultured human bladder cancer cell lines that synthesize a low level of uroplakin Ia receptors can still engulf uropathogenic bacteria in a process morphologically similar to in vivo events, suggesting that these cultured cells may serve as a model for studying FimH-urothelial interaction (Lopez-Boado et al., 2000; Martinez et al., 2000). It should be kept in mind, however, that the aggregation state and the specific protein partners of uroplakin Ia receptor in cultured urothelial cells are likely to be different from those of the in vivo normal urothelium, that different or additional mannose-containing FimH receptors may operate in cultured urothelial cells, and that the signal transduction and cellular events of the in vitro

bacterial invasion may not be identical to those of in vivo urothelium (Finlay and Cossart, 1997).

Concluding remarks

Using mouse urothelial plaques whose uroplakins Ia and Ib are much better resolved than those of the bovine, we have shown here that FimH, as well as type 1-fimbriated *E. coli*, binds specifically to uroplakin Ia, but not to uroplakin Ib. The fact that uroplakins Ia and Ib are glycosylated differently raises questions as to how the glycosylation process of the two structurally closely related uroplakin Is is regulated. The identification of the urothelial receptor for the type 1-fimbriated *E. coli* should facilitate studies on the mechanisms by which FimH binding to the urothelial surface triggers intracellular signals for bacterial invasion and for the urothelial defense mechanisms.

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