

# A Sialoglycoprotein Complex Linked to the Microvillus Cytoskeleton Acts as a Receptor for Pilus (AF/R1) Mediated Adhesion of Enteropathogenic *Escherichia coli* (RDEC-1) in Rabbit Small Intestine

Parvaneh Rafiee,\* Hakon Leffler,† James C. Byrd,\* Fredrick J. Cassels,§ Edgar C. Boedeker,§ and Young S. Kim\*

\*Gastrointestinal Research Laboratory, Veterans Administration Medical Center, San Francisco, California 94121; Department of Medicine, University of California San Francisco, San Francisco, California 94143; †Department of Psychiatry, University of California San Francisco, San Francisco, California 94143-0984; and §Department of Gastroenterology, Walter Reed Army Institute of Research, Washington, D.C. 20307

**Abstract.** *Escherichia coli* strain RDEC-1 is an entero-adherent, diarrheagenic pathogen in rabbits that utilizes AF/R1 pili for initial (stage 1) adherence, but the host receptors for this adhesion are unknown. Here we demonstrate that RDEC-1 binds, via AF/R1 pili, to a specific rabbit ileal microvillus membrane glycoprotein receptor complex of subunits 130 and 140 kD. The binding involves sialic acid present on oligosaccharide moieties of the glycoprotein receptor. Furthermore, the

microvillus membrane glycoprotein receptor complex appears to be associated with cytoskeletal components via brush border myosin 1. This newly described link between AF/R1 receptor and cytoskeletal components suggests that, in addition to this function in mucosal adherence, the pili may facilitate subsequent (second stage) close effacing attachment of RDEC-1 to the host epithelium by influencing cytoskeletal function.

THE ability of pathogenic bacteria to adhere to host epithelial cells is regarded as a prerequisite for the initial colonization of the host tissue (Svanborg-Eden et al., 1976; Beachey, 1981; Gaastra and deGraaf, 1982). In many cases, the adhesion of *Escherichia coli* and other gram-negative bacteria takes place through the binding of bacterial pili to specific receptors on the host cell surface, some of which have been identified as glycolipids and glycoproteins (Anderson et al., 1980; Mirelman, 1986; Karlsson, 1989; Sharon and Lis, 1989). The specificity of the bacterial adhesions, on the one hand, and the range of receptor structures expressed by particular epithelial cells, on the other, have been suggested to be important determinants of the host range and tissue tropism of each pathogen.

Pilus-mediated adhesion is regarded as a first stage in pathogenesis of many *E. coli* intestinal infections, permitting the production of disease through the second stage operation of other virulence factors. For example, enterotoxigenic *E. coli* attach via their fimbrial colonization factor antigens. Although there is some evidence that pilus-mediated attachment of enterotoxigenic *E. coli* alone can cause a degree of intestinal cell dysfunction (Smith and Linggood, 1971; Schlager et al., 1990), secretory diarrhea is induced through the production of enterotoxins.

Enteropathogenic *E. coli* (EPEC)<sup>1</sup> causes disease through

a different second stage mechanism. In the case of RDEC-1 *E. coli* infection of rabbits with spontaneous enteritis (Cantey and Blake, 1977), AF/R1 pilus-mediated initial (first stage) attachment promotes mucosal colonization and seems to facilitate the subsequent development of the second stage attaching and effacing (AE) lesion (Takeuchi et al., 1978) of the apical surface of the intestinal absorptive cells (Wolf et al., 1988; Cantey et al., 1989) which coincides with diarrhea. In vitro adherence of RDEC-1 to rabbit ileal brush borders, which is mannose resistant and species specific, is mediated by the pili designated AF/R1, expressed on the bacterial surface (Boedeker and Cheney, 1983; Wolf et al., 1988; Wolf and Boedeker, 1990). In studies in vivo, AF/R1 pili have been shown to promote pathogenicity and tissue tropism. However, neither the specific intestinal mucosal cell receptor for AF/R1 pili of RDEC-1 nor the mechanism of binding of pili to its receptor has been characterized.

It is now known that the second stage AE lesions (which are seen in both RDEC-1 and human EPEC infections) require gene products distinct from pili. These well-described lesions include loss of microvilli, close apposition of bacteria to the remaining flat apical membrane of the intestinal epithelial cells, and a dense aggregation of actin filaments (Ulshen and Rollo, 1980; Rothbaum et al., 1982; Robins-Browne, 1987) in the apical cytoplasm directly beneath the attached bacteria (Knutton et al., 1989). Recent immunocytochemical studies of EPEC adherence to Caco-2 cells also demonstrated a typical AE lesion with a dense cytoplasmic condensate of microfilaments consisting of both actin and

1. *Abbreviations used in this paper:* AE, attaching and effacing; BB, brush border; EPEC, enteropathogenic *E. coli*; PAB, Penassay broth; PAS, periodic acid-Schiff; PI-PLC, phosphatidylinositol-specific phospholipase C.

villin (Gates, J. A., M. D. Peterson, L. C. Tyrrell, and A. B. West. 1990. *Gastroenterology*. 98:A450). These studies indicate that both RDEC-1 and classical EPEC activate selective disassembly and localized redistribution of cytoskeletal components.

In this study we have demonstrated that RDEC-1 binds specifically to a 130/140-kD glycoprotein complex of rabbit ileal brush borders via AF/R1 pili, and that this binding involves sialic acid of the glycoprotein receptors. Furthermore, these microvillar membrane glycoprotein receptors appear to be tightly associated with cytoskeletal components via brush border myosin 1.

## Materials and Methods

### Chemical

Standard chemicals were of the highest quality available. Monosaccharides, transferrin, fetuin, ATP, SDS, and prestained SDS molecular weight markers were from Sigma Chemical Co. (St. Louis, MO). Ovine submaxillary mucin was purified as described by Tettamanti and Pigman (1968). Acrylamide and bis-acrylamide were from United States Biochemical Corp. (Cleveland, OH). Antibiotic medium 3 (Penassay broth [PAB]) was from Difco Laboratories Inc. (Detroit, MI). [<sup>3</sup>H]Glucose was from ICN Biomedicals, Inc. (Costa Mesa, CA). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Goat anti-mouse IgG coupled with peroxidase was from Tago, Inc. (Burlingame, CA). A monoclonal antibody against chick myosin 1 was a gift from Dr. Mark S. Mooseker, Department of Biology, Yale University (New Haven, CT).

### Bacteria

*E. coli* RDEC-1, isolated from rabbits (Cantey and Blake, 1977), was inoculated into 10 ml PAB containing 100  $\mu$ Ci of [<sup>3</sup>H]glucose (30 Ci/mmol) and incubated overnight at 37°C with adequate aeration to promote expression of mannose-resistant AF/R1 pili (Berendson et al., 1983). The metabolically labeled bacteria were washed with PBS and resuspended in PBS containing 1% BSA and 0.15 M mannose before use. M34 is a variant of RDEC-1 in which the plasmid-encoded gene for AF/R1 pili has been inactivated by transposon mutagenesis (Wolf et al., 1988).

### Purification of AF/R1 Pili

RDEC-1 were grown in PAB and harvested, and the pili were sheared from cells in a blender (Waring, New Hartford, CT) and precipitated by addition of ammonium sulfate (Issacson, 1977). The partially purified pili were resuspended in deoxycholate-containing buffer (50 mM Tris, 0.1% sodium deoxycholate, and 5 mM EDTA, pH 8.2), passed through a Sepharose CL-4B column matrix (90  $\times$  2.4 cm), equilibrated in the same buffer, and collected from the void volume of the column (Klemm, 1982). Purified pilus subunits were obtained by dissociation of the intact pili in saturated guanidinium chloride (8.6 M) and further purified on Superose 12 FPLC (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) in 8.0 M guanidinium chloride, 50 mM Tris, pH 8.0 (Sjoberg et al., 1988). These pilus subunits were purified from other bacterial proteins to a high degree as evidenced by SDS-PAGE isoelectric focusing, and electron microscopic examination (data not shown). Antisera to purified pilus subunits were raised in rabbits by subcutaneous injection of 500  $\mu$ g of antigen in complete Freund's adjuvant (day 0), followed by additional subcutaneous injections of 100  $\mu$ g of antigen in incomplete Freund's adjuvant at days 21 and 35. Rabbits were bled on days 45 and 55.

### Rabbit Brush Borders and Extracts of Intact Brush Borders

Intact apical brush borders were prepared as described (Cheney et al., 1979). For extraction with different solutions, the brush borders were washed with PBS and pelleted at 2,000 g and the pellet was sonicated for 10 s on ice in a Branson Sonifier 200 (Branson Ultrasonics Corp., Danbury, CT) at setting 2 in the appropriate extraction solution and incubated as described under Results. The sample was centrifuged at 100,000 g and the supernatant and pellet were recovered.

### Preparation of Microvilli, Demembrated Microvilli, and ATP Extract

Microvilli from rabbit brush borders were isolated as described by Mooseker et al. (1989). The brush border pellets were homogenized in 10 ml of buffer A (75 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM imidazole Cl (pH 7.2) 0.4 mM DTT, and 0.2 mM PMSF) by 50–100 strokes in a tight-fitting Dounce homogenizer (Kontex, Vineland, NJ). Separation of microvilli from the terminal web was achieved by differential centrifugation. The microvilli were collected in the supernatant from centrifugation at 5,000 g for 10 min, then in the pellet after centrifugation at 40,000 g for 15 min. The terminal web was collected in the 5,000 g pellet. The microvilli were resuspended in 10 vol of buffer A and the sedimentation protocol was repeated three times. At this stage, microvilli were observed by phase-contrast light microscopy. To remove most of the membrane proteins, the pellets of microvilli were resuspended in 20 ml of buffer B (same as buffer A with 2 mM MgCl<sub>2</sub>) containing 1.5% Triton X-100 (wt/vol) and centrifuged at 40,000 g for 15 min. The pellet, containing the microvillar core, was washed three times in 20 vol of buffer B to remove residual detergent. To extract cytoskeleton-associated glycoproteins, the microvillar core was resuspended in 4 ml of buffer B containing 0.1% Triton X-100 and 8 mM ATP and solubilized material was recovered after 15 min of centrifugation at 40,000 g.

### Chromatography and Electrophoreses

A model 332 HPLC apparatus (Beckman Instruments, Inc., Fullerton, CA) was used. Superose 6 and 12 columns were from Pharmacia LKB Biotechnology Inc. DEAE-cellulose and agarose-bound wheat germ agglutinin were purchased from Sigma Chemical Co.

Preparative isoelectric focusing was performed in a Rotofor apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

SDS-PAGE was performed on 10% gels by the method of Laemmli (1970) and electroblotted to nitrocellulose membrane by the method of Towbin et al. (1979). Gels were stained with Coomassie blue or periodic acid Schiff (Kapitany and Zebrowki, 1973). For development of the nitrocellulose blots the membrane was blocked (6 h at 4°C with 3% BSA and 1% nonfat dry milk in PBS) and incubated with primary antibody (monoclonal anti-myosin 1) overnight at 4°C. The secondary antibody used was peroxidase-conjugated goat anti-mouse IgG.

### Binding Assay

Brush borders or other particulate fractions, resuspended in PBS and sonicated, or soluble fractions, diluted with PBS, were immobilized on 24-well polystyrene plates (Costar Corp., Cambridge, MA) by incubating 0.3 ml/well overnight at 4°C. Then the wells were rinsed briefly with PBS to remove unbound proteins and blocked with 0.5 ml of 1% BSA in PBS for 6 h at 4°C. <sup>3</sup>H-labeled *E. coli* (typically  $9 \times 10^5$  cells with  $3 \times 10^5$  cpm <sup>3</sup>H) in 0.3 ml/well PBS containing 0.15 M mannose and 1% BSA were added to the wells and the plates were incubated overnight at 4°C. After incubation the wells were washed three times with PBS to remove unbound bacteria. Adherent bacteria were solubilized in 1% SDS (0.5 ml/well) for 3 h at 37°C and radioactivity was measured in a scintillation counter. All results are the median values of triplicate wells. In three separate experiments where four, five, and six triplicates of a single concentration of brush borders were assayed to assess plate-to-plate variation, there was a 15.6, 10, and 14% standard deviation among the median values.

### Calculation of Total Binding Activity of a Fraction

For quantitative comparison of the binding activity of different fractions, the following calculation was done. First, different sized aliquots of a fraction, diluted to 300  $\mu$ l with PBS, were tested for binding activity as described above. An aliquot size giving between 20 and 50% of maximum binding was selected for the calculation. The radioactivity bound by this aliquot, in counts per minute, was multiplied by the total volume of the fraction and divided by the aliquot volume to give total binding activity, expressed as total counts per minute.

### Inhibition Experiments

Inhibition by soluble sugars and glycoproteins was tested as follows: Brush borders were coated and blocked according to the standard procedures.

Then inhibitors (0.1 ml), mixed with <sup>3</sup>H-labeled RDEC-1 (0.2 ml), were added to the wells, incubated, and solubilized as described for the standard binding assay.

For periodate oxidation, brush borders (3 µg protein in PBS) were immobilized on 24-well tissue culture plates as described for the standard binding assay, then blocked with 1% BSA for 1 h at room temperature. NaIO<sub>4</sub> in the range of 1–30 mM in 50 mM sodium acetate, pH 5.5, was added to the wells and incubated for 1 h at room temperature. Then the wells were incubated with 1% glycine (pH 5.5) for 1 h at room temperature. After blocking again with 1% BSA for 6 h at 4°C, labeled *E. coli* RDEC-1 (0.3 ml) was added to the wells and binding was measured by the standard procedures, using brush borders treated with acetate buffer and 1% glycine as control.

For neuraminidase treatment, brush borders were immobilized and blocked with 1% BSA in PBS for 1 h at room temperature as above. 300 µl of neuraminidase (up to 0.2 U/ml) from *Clostridium perfringens* (Sigma Chemical Co.) or from *Arthrobacter ureafaciens* (EY Labs, San Mateo, CA) in 50 mM sodium acetate, pH 5.5, was added to the wells and incubated for 60 min at room temperature. After blocking again with 1% BSA in PBS for 6 h at 4°C, binding of *E. coli* was assayed by the standard method. The neuraminidase inhibitor 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid was purchased from Sigma Chemical Co.

### Electron Microscopy

An aliquot of the ATP supernatant of demembrated microvilli was mixed with an equal volume of aqueous 0.1% silicotungstic acid and pipetted onto Formvar-coated nickel grids. After standing for 30 s at room temperature, excess solution was wicked off with a filter paper and the grid was allowed to dry and then examined in a transmission electron microscope (Carl Zeiss, Inc., Thornwood, NY).

## Results

### Binding of *E. coli* RDEC-1 to Brush Borders Mediated by AF/R1 Pili

We first developed an assay suitable for measuring binding of *E. coli* RDEC-1 to brush borders or fractions thereof. In this assay the binding of radiolabeled bacteria to receptors immobilized in tissue culture wells was measured (Fig. 1). The degree of binding of *E. coli* RDEC-1 was dependent on the concentration of brush border protein. At the maximum level ~10% of the total activity was bound, while background binding to albumin was typically <0.1%.

Next we examined the role of AF/R1 pili in the binding measured by this assay. An RDEC-1 variant (M34) with the AF/R1 gene inactivated and therefore lacking AF/R1 (Wolf et al., 1988) pili, gave no binding above background. When the *E. coli* RDEC-1 were cultured under the standard conditions (aerated PAB), promoting expression of AF/R1 pili, the binding, as expected, was not inhibited by mannose. However, when bacteria were grown in static culture, conditions that favor expression of type 1 pili (Sherman et al., 1985), the binding was partially inhibited by 0.15 M mannose (not shown). The binding of bacteria grown in aerated PAB was also prevented by addition of rabbit anti-AF/R1 antiserum (1:30 dilution) or purified AF/R1 pili (3 µg/ml) to the incubation medium (not shown). These data indicate that the binding measured as in Fig. 1 requires the expression of AF/R1 pili.

### Role of Carbohydrate for the Bacterial Binding

Since host cell surface carbohydrates are receptors for many other bacterial adhesins, we explored this possibility for the AF/R1-mediated binding. First, we tested whether bacterial

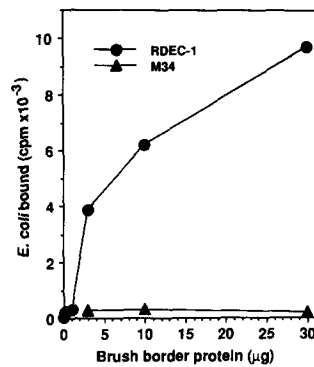


Figure 1. Tissue culture wells were coated with various amounts of sonicated brush borders (abscissa), nonspecific sites were blocked with albumin, and the coated wells were incubated with radiolabeled bacteria (~2 × 10<sup>5</sup> cpm). After unbound bacteria were removed by washing, the bound bacteria were released by buffer containing SDS and their radioactivity was counted in a scintillation counter (ordinate). Wild-type RDEC-1 and a mutant strain, M34, lacking AF/R1 pili were used.

binding was inhibited in the presence of various saccharides and glycoproteins. Of the free saccharides, only N-acetylneuraminic acid was strongly inhibitory (Table I). Glucose-6-phosphate inhibited by 80% at 300 mM and by 65% at 30 mM, but other acidic carbohydrates, glucose-6-sulfate, galactose-6-phosphate, and glucosamine-6-phosphate, gave only partial inhibition (50% or less) at the highest concentration tested (300 mM). A variety of other carbohydrates, including mannose, galactose, fucose, glucose, N-acetylglucosamine, N-acetyl-galactosamine, sucrose, lactose, raffinose, myoinositol, and glucuronic acid did not inhibit at a concentration of 300 mM.

In the same inhibition assay, intact brush borders, when mixed with the bacteria, also inhibited binding of <sup>3</sup>H-labeled *E. coli* to immobilized brush borders, with 50% inhibition by 0.1–0.3 mg/ml protein (0.4–1.1 µg/ml NeuAc). In contrast, there was no inhibition of binding by three well-characterized sialoglycoproteins: ovine submaxillary mucin (tested at up to 10 mg/ml, 4,000 µg/ml NeuAc), transferrin (6 mg/ml, 50 µg/ml NeuAc), and fetuin (6 mg/ml, 300 µg/ml NeuAc).

Next, we examined the effect of pretreatment of the brush borders with agents that perturb carbohydrate structure (Table I). Treatment in situ (Woodward et al., 1985) with 30

Table I. Reduction of Binding Activity by Periodate, Neuraminidase, and Sialic Acid

	Binding activity (% of control)
Periodate in NaAc, pH 5.5	
1 mM	79
3 mM	29
10 mM	29
30 mM	21
Neuraminidase*	
<i>C. perfringens</i>	38
<i>A. ureafaciens</i>	35
NeuAc	
30 mM	16.3
100 mM	5.7
300 mM	4.4

\* Treated for 60 min with 0.1 U/ml. For the *C. perfringens* enzyme, increasing enzyme concentration twofold or increasing incubation time fourfold gave no further decrease in binding.

mM periodic acid abolished >75% of the bacterial binding. Between 1 and 3 mM was required for 50% reduction in activity. Neuraminidase treatment of the immobilized brush borders also prevented the bacterial binding.

These data show clearly that brush border carbohydrates are necessary for bacterial binding and suggest that sialic acid residues are involved. However, the fact that rabbit brush borders were much more potent than *N*-acetylneuraminic acid for inhibition and the lack of inhibition by known sialoglycoproteins suggest that additional structures are necessary for optimal binding.

### ***Evidence That the Receptor Is an Integral Membrane Protein Linked to the Cytoskeleton***

To learn more about the properties of the bacterial receptor and to facilitate its purification, we extracted intact brush borders under various conditions. The extracts and the remaining insoluble material were coated on tissue culture wells and their bacterial binding activity was examined (Table II).

When the brush borders were extracted with detergent under conditions known to solubilize integral membrane glycoproteins, most of the receptor activity remained in the pellet. In contrast, when agents that disrupt the cytoskeleton were added, i.e., high salt or ATP, >90% of the active component could be extracted with detergent. These conditions are typical for an integral membrane protein linked to the cytoskeleton. Buffer containing high salt or ATP but no detergent failed to extract the active component.

We also considered the possibility that the receptor protein may be linked to the membrane via a phospholipid anchor, since some such proteins are relatively resistant to detergent extraction (Low, 1987). Pretreatment of the brush borders with PI-PLC solubilized 55–70% of alkaline phosphatase activity, but did not solubilize the receptor activity (Table II). Papain treatment, which will release many brush border membrane hydrolases (Maestracci, 1976), was also ineffective in solubilizing receptor activity.

Since many bacterial adhesins are known to bind glycolipids, the brush borders were also extracted with chlo-

roform/methanol. The extract was dried, redissolved in methanol, and used to coat the tissue culture wells under conditions suitable for glycolipids (Karlsson and Stromberg, 1987). No binding to the glycolipid-coated wells was observed. The pellet after lipid extraction, sonicated and absorbed to the wells, was also inactive, suggesting that the receptor is denatured by organic solvents.

### ***Association of the Receptor Protein with Microvilli***

Next, we examined to which part of the brush border the bacteria bound. The brush borders were homogenized and intact microvilli were separated from the terminal web by differential centrifugation. The quality of the preparation was assessed by phase contrast light microscopy and SDS-PAGE (not shown). The microvillus fraction was highly enriched in rod-like structures similar to those found by others (Bretscher and Weber, 1978). The terminal web fraction was enriched in 200 kD myosin as detected by silver staining of SDS-PAGE, while this protein was not detected in the microvilli. The microvillus fraction contained >80 times the bacterial binding activity of the terminal web fraction (Table III). There was more binding activity in the microvilli than in the brush borders, suggesting that some receptor sites are not accessible to bacteria in intact brush borders.

When the microvilli were extracted with detergent, >90% of the binding activity remained with the pellet. When the demembrated microvilli were subsequently extracted with ATP, most of the binding activity could be released.

Microvillus membrane vesicles isolated directly from mucosal scraping in the presence of Ca<sup>2+</sup> also supported bacterial binding in the same assay as used for brush border (BB). As for microvilli, the bacterial binding activity was not extracted by buffer containing detergent alone, but was completely solubilized after the addition of ATP (data not shown).

These data indicate that the RDEC-1 receptor is a component of the microvillus membrane and that it interacts with the microvillus cytoskeleton.

### ***Purification and Characterization of Receptor Glycoprotein Complex Containing Myosin I***

The ATP extract of demembrated microvilli was passed

**Table II. Solubilization of RDEC-1 Receptor from Intact Brush Borders**

Treatment*	% Activity in supernatant <sup>§</sup>
1% Triton X-100 n PBS	20
1% Triton X-100 + 0.6 M KCl	100
0.6 M KCl	0
1% Triton X-100 + 8 mM ATP	100
8 mM ATP	0
Papain	0
PI-PLC	10

\* For extraction with buffer, 1 mg/ml of BB were sonicated or homogenized in each buffer, then centrifuged at 100,000 *g* for 30 min. For digestion with papain, 1 mg/ml of BB in PBS was incubated 30 min at 37°C with 0.1 mg/ml of papain (activated for 30 min at room temperature), then centrifuged at 100,000 *g* for 30 min. For digestion with PI-PLC, 1 mg/ml of BB in PBS was incubated 5 h at 37°C with 1 U/ml of PI-PLC, then centrifuged at 100,000 *g* for 30 min.

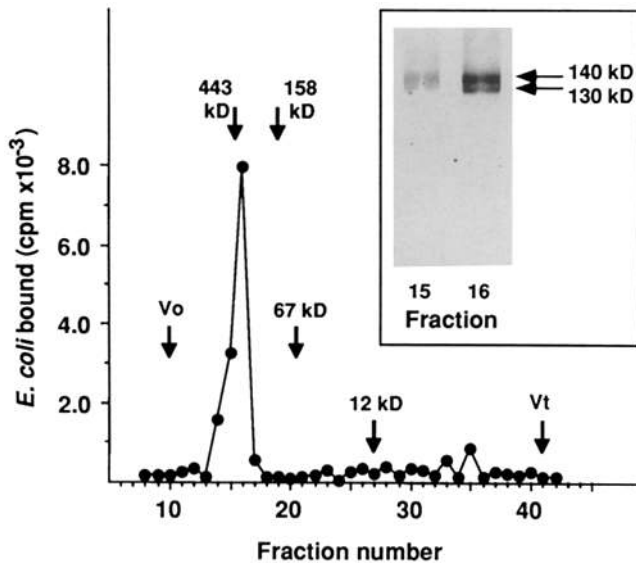
<sup>§</sup> The activity of the supernatant and pellet from each experiment was measured and calculated as described under Materials and Methods. The activity in the supernatant is given as the percentage of the activity in supernatant plus activity in pellet.

**Table III. Binding Activity of Brush Border Components**

Fraction*	Activity <sup>§</sup>
	<i>cpm</i> × 10 <sup>6</sup>
Brush borders	65
Terminal web	4
Microvilli	350
Wash of microvilli	0.3
Triton X-100 supernatant	26
Wash of Triton-extracted residue	0.5
Triton pellet	290
ATP supernatant	260
ATP-insoluble pellet	6

\* Microvilli were prepared, demembrated with Triton X-100, and extracted with ATP as slightly modified from Mooseker et al. (1989) and described under Materials and Methods.

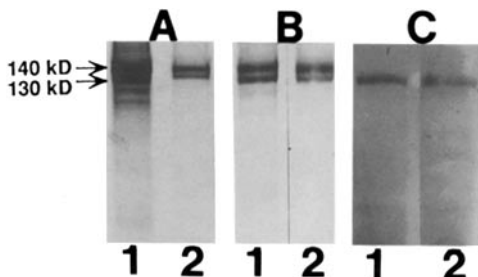
<sup>§</sup> The activities of aliquots of the fractions were measured as in Fig. 1. The total activity of each fraction was then calculated as described under Materials and Methods.



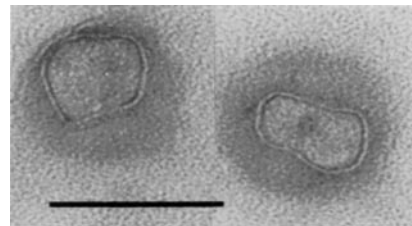
**Figure 2.** Gel filtration of ATP extract from demembrated microvilli. The extract (1.0 ml) was passed over a Superose 12 column equilibrated in buffer B (Materials and Methods) and 0.1% Triton, 0.5 ml/min. 0.5-ml fractions were collected. The bacterial binding activity of each fraction was measured using 20  $\mu$ l/well. The arrows indicate the retention volume of the following standards of known molecular weight: ferritin, 443 kD; rabbit IgG, 158 kD; BSA, 67 kD; and cytochrome *c*, 12.4 kD.  $V_o$  is void volume and  $V_t$  is total volume. The inset is SDS-PAGE of fractions 15 and 16 that were active for RDEC-1 binding.

over a size exclusion column (Superose 12). Fractions were collected and assayed for bacterial binding activity. A sharp peak of activity was detected corresponding to a molecular weight of  $\sim$ 450,000 (Fig. 2). After SDS-PAGE under reducing conditions, Coomassie blue staining showed two major bands of  $\sim$ 130,000 and 140,000 apparent molecular weight (Fig. 3 A). The periodic acid-Schiff (PAS) reagent stained both of the major bands, indicating that these are glycoproteins (Fig. 3 B).

Since a major component of ATP extracts of demembrated chick microvilli is myosin I (Mooseker and Coleman, 1989), we examined whether our extracts also contained my-



**Figure 3.** SDS-PAGE of active fractions. The ATP extract of demembrated microvilli (lane 1) and the most active fraction isolated by gel filtration (lane 2) were separated by SDS-PAGE and stained with Coomassie (A), PAS (B), or by an anti-myosin I mAb after blotting onto nitrocellulose (C). The arrows indicate molecular weights calculated by comparison with known standards.



**Figure 4.** Negative staining of ATP extract from detergent-treated microvillar cores. The disks are morphologically similar to those of chick intestine visualized by Mooseker et al. (1989). Bar, 0.1  $\mu$ m.

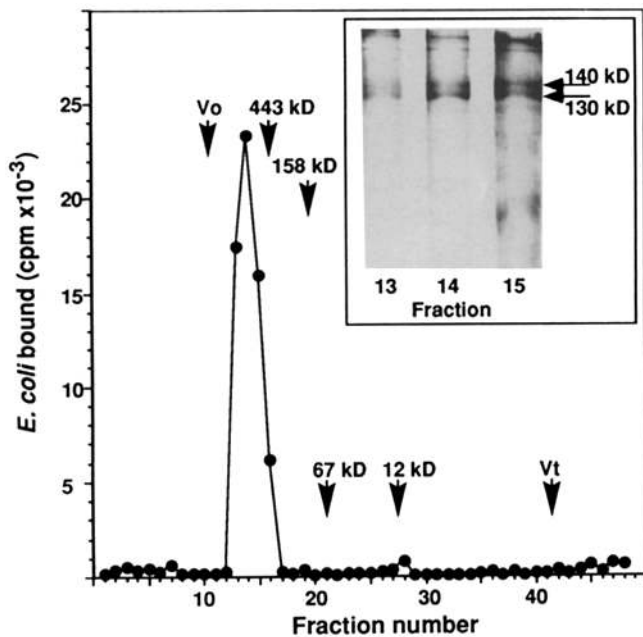
osin I, using a monoclonal antibody raised against chick myosin I that crossreacts with mammalian brush border myosin I (Carboni et al., 1987). Western blots of the ATP extracts and of the active fractions after gel filtration showed a band of  $\sim$ 130,000 apparent molecular weight (Fig. 3 C). This was clearly higher than the 110,000 observed for a reference of chick myosin I run on the same gel (not shown).

ATP extracts of demembrated chick microvilli have been reported to contain myosin I in soluble and aggregated forms (Mooseker et al., 1989). The latter had the form of 0.1- $\mu$ m disks and contained a 140-kD glycoprotein. When we used an identical protocol for ATP extraction of demembrated rabbit microvilli, most of the RDEC-1 receptor remained in the supernatant after centrifugation for 5 min at 40,000 *g*, but could be pelleted by centrifugation for 15 min at 100,000 *g*. Electron microscopy of the ATP supernatant revealed structures of a size and appearance similar to the disks reported by Mooseker et al. (1989) (Fig. 4). Thus, a portion of the receptor activity can be extracted in an aggregated form. However, the receptor activity can also be obtained in a soluble form that is not sedimented by centrifugation for 60 min at 100,000 *g* and is not excluded on gel filtration columns (Fig. 2). The yield of the soluble form was increased by inclusion of detergent at the ATP extraction step as well as in gel filtration. In the absence of detergent, the receptor activity was eluted at the void volume of Superose 6 and Superose 12 (not shown), suggesting that it aggregated.

#### Further Characterization of Receptor Glycoprotein Complex

Since the active fractions isolated by gel filtration contained two glycoproteins and myosin I, we examined whether the components could be dissociated under mild conditions. When the high salt extract of brush borders was chromatographed on Superose 12 in a buffer containing 0.6 M KCl, receptor activity was eluted as a single peak (Fig. 5). The active fractions contained both glycoproteins. Upon chromatography of these active fractions on Superose 12 in a low salt buffer (25 mM Tris, pH 7.4, 0.1% Triton X-100), the elution pattern was unchanged and the two glycoproteins were not separated (data not shown).

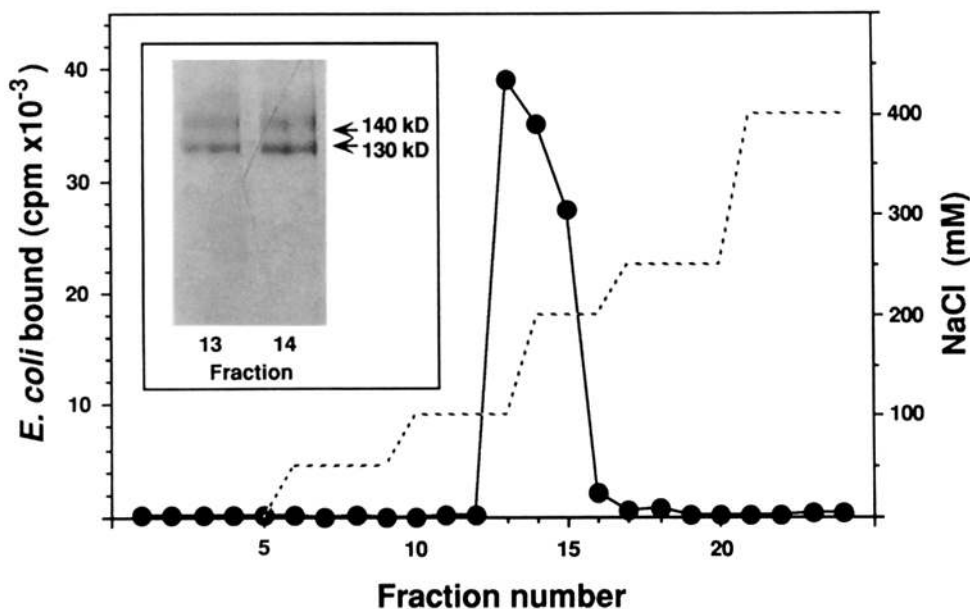
When the active fraction obtained by gel filtration of the high salt extract was subjected to ion exchange chromatography on DEAE-cellulose, a single peak of receptor activity, eluted by 200 mM NaCl, was obtained. Both glycoproteins were present in these DEAE-cellulose fractions that exhib-



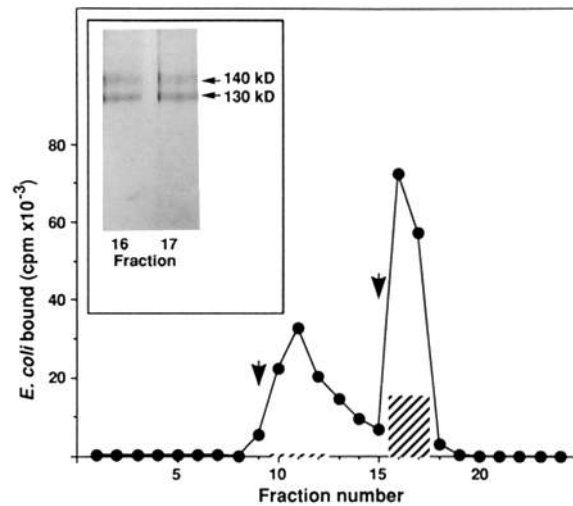
**Figure 5.** Gel filtration of a high salt detergent extract of brush borders. The BB were extracted with buffer containing detergent and 0.6 M KCl as described in Table II. The extract (0.25 ml) was passed over a Superose 12 column equilibrated in high salt buffer, 0.1% Triton. Elution was done at 0.5 ml/min and 0.5-ml fractions were collected. 50- $\mu$ l aliquots of each fraction were immobilized in wells and tested for activity. The inset shows SDS-PAGE of the most active fraction with Coomassie blue staining.

ited receptor activity (Fig. 6) and the fractions also stained with anti-myosin 1 monoclonal antibody (not shown).

On preparative isoelectric focusing in free solution all the receptor material in ATP extracts of brush borders precipitated in one fraction at pH 4.7. SDS-PAGE of this precipitate again revealed the two glycoproteins (not shown). From these data we conclude that the two glycoproteins are tightly associated under a variety of conditions.

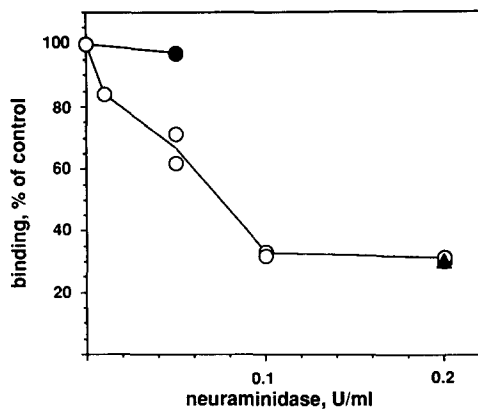


**Figure 6.** Ion exchange chromatography of active fractions. The active fractions obtained by gel filtration as described in Fig. 2 were applied to a DEAE-cellulose column equilibrated in 25 mM Tris, pH 7.4, 0.1% Triton. The column was eluted with a stepwise gradient of NaCl in 25 mM Tris, pH 7.4, and 0.1% Triton X-100. 2-ml fractions were collected and bacterial binding activity was measured on 50- $\mu$ l aliquots. The inset shows SDS-PAGE, stained with a PAS of fraction 18-20.



**Figure 7.** Affinity chromatography on wheat germ agglutinin-agarose. Active fractions after gel filtration, obtained as in Fig. 2, were chromatographed on a column of 3.0 ml agarose-bound wheat germ agglutinin, equilibrated in 25 mM Tris, pH 7.4, and 0.1% Triton X-100. The column was washed with the same buffer, then with 0.5 M NaCl (fraction 9), then with 0.5 M *N*-acetyl-glucosamine (fraction 15), in the same buffer. 1.5-ml fractions were collected and 50- $\mu$ l aliquots were assayed for bacterial binding. Bars represent reassay of 3  $\mu$ l of pooled fractions. *Inset*, SDS-PAGE, stained with PAS, of fractions 16 and 17. No protein bands were detected in fraction 9-12 either by Coomassie blue, silver, or PAS staining.

The periodate and neuraminidase susceptibility of receptor activity suggested that the AF/R1 receptor is a sialoglycoprotein. Therefore, we tested whether receptor activity would bind to immobilized wheat germ agglutinin, a lectin that is specific for sialic acid and terminal GlcNAc. The active fractions obtained by gel filtration of the high salt extract of brush borders bound to wheat germ agglutinin (Fig. 7). Relatively little receptor activity was eluted by NaCl (500 mM). After dilution and reassay, the fraction specifically



**Figure 8.** Effect of neuraminidase digestion on binding activity of purified receptor. The active fractions after affinity chromatography on wheat germ agglutinin-agarose were incubated with varying concentrations of *C. perfringens* neuraminidase for 60 min (○, ●) or 4 h (▲) with (●) or without (○, ▲) 10 mM 2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid, then assayed for binding of RDEC-1 bacteria.

eluted by *N*-acetyl-glucosamine had 25-fold more binding activity than the NaCl eluate. After elution by 500 mM *N*-acetyl-glucosamine, the fractions that had receptor activity also had both the 130- and the 140-kD glycoprotein (Fig. 7) and stained with anti-myosin 1 (not shown). Binding activity of the purified receptor, like that of intact brush borders, was sensitive to neuraminidase digestion (Fig. 8). Inclusion of a neuraminidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid, prevented the inactivation of binding activity in the purified receptor.

## Discussion

In this study we have characterized the molecular mechanism for initial (stage 1) adhesion of the enteropathogenic *E. coli* strain RDEC-1 to rabbit small intestinal brush borders. The adhesion is mediated by binding of bacterial AF/R1 pili to a host microvillus membrane sialoglycoprotein, which is of particular interest among the microvillus membrane glycoproteins in that it is tightly associated with the cytoskeleton.

The involvement of AF/R1 pili in the binding of RDEC-1 organisms is supported by three lines of evidence. First, RDEC-1 expressing AF/R1 pili bind to intestinal brush borders while the M34 strain of RDEC-1, lacking AF/R1 pili, does not. Second, RDEC-1 grown under conditions that do not allow the expression of AF/R1 pili do not bind to the intestinal brush borders in the presence of mannose. Third, RDEC-1 binding to intestinal brush borders is almost completely inhibited by the addition of excess purified pili or antibody to AF/R1 pili in the binding assay mixture.

The host receptor is a sialoglycoprotein complex containing 130- and 140-kD components. The glycoprotein nature of the receptor is demonstrated by PAS staining and by specific binding to wheat germ agglutinin affinity columns. Sialic acid-containing oligosaccharides of the receptor glycoprotein appear to be involved in the binding of RDEC-1, since neuraminidase or mild periodate treatment of the brush borders greatly decreased binding activity, and free sialic

acid caused a significant inhibition of binding. However, the partial resistance of binding activity to neuraminidase, as well as the facts that rabbit brush borders were much more potent than NeuAc as inhibitors and that other sialylated glycoproteins were not inhibitory, suggest that structural features other than sialic acid are necessary for optimal binding. The important role of carbohydrates in the binding of gram-negative bacteria to receptors on epithelial cells has previously been reported (Mireleman, 1986; Karlsson, 1989; Sharon and Liss, 1989; Warner and Kim, 1989).

If the binding specificity for the cytoskeleton-linked sialoglycoprotein represents an adaptation of *E. coli* to adhere specifically to intestinal brush borders, particularly those from small intestine, a wider array of intestinal pathogens may have a similar specificity. In this regard it is interesting that, like RDEC-1, many *E. coli* strains that cause infection of the small intestine have adhesins binding sialic acid (Sjoberg et al., 1988). Sialic acid represents a minor constituent of the glycoproteins on intestinal epithelial cells (Finne et al., 1989). The major saccharide chains instead have fucosylated blood group antigenic determinants (Finne et al., 1989). Therefore, the specificity for sialic acid-containing saccharides may represent an adaptation to allow binding to a special class of brush border glycoproteins as exemplified by that identified here.

The species specificity of AF/R1 pili has been demonstrated by their binding to rabbit but not rat, guinea pig, and human brush borders. This implies that some structural features of the sialoglycoprotein must vary among species. The carbohydrate chain structures of other intestinal glycoconjugates are known to vary among species, e.g., glycolipids (Hansson, 1988) and mucins (Karlsson et al., 1989).

Subfractionation of the brush borders showed that the bacterial receptor was present in microvilli but not in the terminal web (Table III). Since it could not be solubilized by papain, PI-PLC, or nonionic detergent (except in the presence of high salt; Table II), we reasoned that it must be attached to the cytoskeleton. Work by others has demonstrated microvillus membrane 140-kD glycoproteins in chicken and pig intestinal microvillus membranes (Coudrier et al., 1982; Mooseker et al., 1989). These are associated with the cytoskeleton by an interaction with brush border myosin 1, which in turn is linked to the actin filament core. The complex of myosin 1 and glycoprotein can be solubilized from demembrated microvilli by extraction with ATP (Mooseker et al., 1989). In the present study, when we treated brush borders or demembrated microvilli with ATP, we achieved successful extraction of most of the bacterial binding activity, confirming the association of the RDEC-1 receptor with the microvillus cytoskeleton.

Further chromatographic and electrophoretic analysis demonstrated a receptor glycoprotein complex of ~450,000 mol wt containing 130- and 140-kD glycoprotein components and myosin 1. The receptor activity chromatographed with both glycoproteins under different conditions, suggesting that glycoproteins are responsible for bacterial binding. Some properties were similar to those of myosin 1-associated glycoproteins found by others. Thus, the salt concentration necessary for elution from anion exchange chromatography (Fig. 6) was similar to that of the pig glycoprotein (Coudrier et al., 1982). The aggregating "disk"-like form (Fig. 5) was similar to that found by Mooseker et al. (1989).



Some properties differed slightly from previous reports. The rabbit myosin 1 heavy chain ran in SDS-PAGE as ~120-130 kD compared with 110 kD reported for chick. The molecular weight of bovine myosin 1 heavy chain calculated from the amino acid sequence is ~119 kD (Hoshimaru et al., 1989). Another difference is our finding of 130- and 140-kD glycoproteins instead of only one, or 140 kD, found by others.

Thus, our data are consistent with the model of the intestinal microvillus cytoskeleton proposed by Mooseker (1983). In this model, each microvillus contains a core of actin filaments and villin, fimbrin, and other proteins that help organize the actin filaments. Myosin 1 projects as side arms between the actin core and the plasma membrane. One possible function of BB myosin 1 is to tether the plasma membrane to the actin core. The interaction between BB myosin 1 and actin has the characteristics of other actin-myosin interaction; e.g., it can promote motility and it is broken by ATP. The nature of the binding of BB myosin 1 to the plasma membrane is less clear. Binding of BB myosin 1 to phospholipid vesicles has been reported (Adams and Pollard, 1989; Hayden et al., 1990). In pig intestine, the 140-kD membrane glycoprotein has been shown to interact directly with BB myosin 1 (Coudrier et al., 1982). Our data are consistent with this latter observation in that the microvillar RDEC-1 receptor glycoprotein complex of ~140- and 130-kD subunits copurify with BB myosin 1. Further experiments are necessary to clarify the mechanism of interaction of receptor glycoprotein complex with BB myosin 1.

At least two bacterial factors appear to be necessary for full pathogenicity of RDEC-1 in rabbits, each accounting for a distinct stage of adhesion (Wolf et al., 1988). One is the AF/R1 pilus, encoded on a large plasmid, that mediates *in vitro* adhesion of RDEC-1 to rabbit small intestinal brush borders. The binding specificity of the AF/R1 pili is clearly important for the host range (rabbit specific) and tissue tropism (intestine) or *E. coli* RDEC-1. It is proposed that the plasmid-encoded pili mediate "initial adhesion" to the rabbit intestinal mucosa, while other bacterial factors cause the second stage AE lesion *per se* in subsequent events. In experimental *in vivo* infection of rabbits, the AF/R1 pili facilitated the development of the AE lesion in both the small and large intestines, while a mutant strain lacking AF/R1 pili still caused the AE lesion, but predominantly in the large intestine.

Experimental infections and *in vitro* studies support a similar paradigm for human and pig EPEC (Levine et al., 1985; Tzipori et al., 1989). These *E. coli* also contain large plasmids necessary for bacterial adhesion and fully symptomatic infection. A chromosomal element, designated *eae*, was recently shown to be necessary for the AE lesion (Jerse et al., 1990). Furthermore, RDEC-1 hybridizes with an *eae* DNA probe from human EPEC.

The protein complex we now have identified as the AF/R1 receptor represents a novel type of receptor for a bacterial adhesin: an integral membrane glycoprotein complex linked to the cytoskeleton. This link suggests a potential role for the AF/R1 pili in interacting with the cytoskeleton and leading to (although not essential for) the AE lesion. Since it is clear from experiments with the AF/R1 negative strain M34 that RDEC-1 can still cause the AE lesion in the large intestine (although with reduced frequency) even in the absence of AF/R1 pili, it is possible that any bacterial adhesin/host cell

receptor interaction that afforded sufficient proximity between EPEC and the host cell membrane would be sufficient to facilitate the AE lesion as suggested (Wolf et al., 1988). However, AF/R1 pili, because of their ability to interact with the cytoskeletal element BB myosin 1 through a membrane glycoprotein complex, may uniquely facilitate the effect of other bacterial virulence factors which induce subsequent cytoskeletal disruption. Further experiments using genetically defined *E. coli* carrying different combinations of adhesins and other factors (e.g., *eae*) are necessary to distinguish these possibilities.

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