Intimin Types α, β, and γ Bind to Nucleolin with Equivalent Affinity but Lower avidity than to the Translocated Intimin Receptor*

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The outer membrane adhesins of enteropathogenic Escherichia coli, Citrobacter rodentium, and enterohemorrhagic E. coli (EHEC) O157:H7 that mediate attach and efface intestinal lesions are classified as intimin α, β, and γ, respectively. Each of these intimin types binds to its cognate, bacterially encoded receptor (called Tir for translocated intimin receptor) to promote tight adherence of the organism to the host-cell plasma membrane. We previously reported that γ intimin of EHEC O157:H7 also bound to a eucaryotic receptor that we determined was nucleolin. The objective of this study was to investigate in vitro and in vivo the interactions of intimins α, β, and γ with nucleolin in the presence of Tir from EHEC O157:H7. Protein binding experiments demonstrated that intimin of types α, β, and γ bound nucleolin with similar avidity. Moreover, all three intimin types co-localized with regions of nucleolin expressed on the surface of HEp-2 cells. When intimin α, β, or γ bound to Tir in vitro, the intimin interaction with nucleolin was blocked. Both Tir and nucleolin accumulated beneath intimin-presenting bacteria that had attached to the surface of HEp-2 cells. Taken together, these findings suggest that nucleolin is involved in bacterial adherence promoted by all intimin types and that Tir and nucleolin compete for intimin during adherence.

Bacteria that produce characteristic attach and efface (A/E) lesions during adherence to the host intestinal epithelium include enterohemorrhagic Escherichia coli (EHEC), enteropathogenic E. coli (EPEC), and Citrobacter rodentium (1–3). EPEC and EHEC cause acute diarrhea in humans, whereas C. rodentium infection of mice results in colonic hyperplasia. The induction of A/E lesions by these organisms is dependent on the expression of the outer-membrane protein intimin. Each intimin molecule has an amino-terminal periplasmic tail, an extracellular domain. The intimin binding site for nucleolin is unique. Reece et al. (13) reported that mutations in intimin-α engineered by site-directed mutagenesis of the eae locus in the region encoding the C-type lectin domain influenced EPEC colonization but had no affect on Tir binding. This finding suggests that the eucaryotic receptor-binding site also resides in the lectin domain (36). To better understand the role of each type of receptor in bacterial adherence, we used in vitro methods as well as a tissue culture model to investigate the inter-

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The abbreviations used are: A/E, attach and efface; EPEC, enteropathogenic E. coli; EHEC, enterohemorrhagic E. coli; Tir, translocated intimin receptor; LEE, locus of enterocyte effacement; TMB, 3,3',5,5'-tetramethylbenzidine; PBS, phosphate-buffered saline; EMEM, Eagle’s minimal essential medium; MAP, mitogen-activated protein.

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All intimin types studied to date bind to an associated translocated intimin receptor, Tir (15–18), that is produced by the bacteria and inserted into the host-cell membrane through a type III secretion system (19, 20). The interaction between intimin and Tir triggers actin condensation beneath the bacterium and permits attachment to the host-cell cytoskeleton (21, 22). The genes for intimin (eae) and Tir (tir) are contained in a pathogenicity island called the locus of enterocyte effacement (LEE) (23–25). All bacteria that display the A/E phenotype possess the LEE genes.

Various intimin types bind to protein receptors expressed by eucaryotic cells (26–28), and the extracellular domains of different intimin types can bind to tissue culture cells in the absence of Tir (29). Frankel et al. (30) demonstrated that intimin-α binds to β, integrin, as does invasin, a closely related adhesin produced by some Yersinia species (31). We recently reported that intimin-γ from E. coli O157:H7 binds to nucleolin that is localized to the plasma membrane of HEp-2 cells (32). Nucleolin is a ubiquitous, conserved protein that is involved in cell proliferation and is produced by all vertebrate species (33). The role in bacterial adherence of the interaction of intimin with these eucaryotic receptors in animals remains unclear. However, we have demonstrated that antiserum raised against nucleolin can inhibit the adherence of enterohemorrhagic E. coli O157:H7 to HEp-2 cells. This result suggests that bacterial interaction with nucleolin may contribute to the pathogenic process initiated by EHEC O157.

The goal of this research was to examine the association of different intimin types with both Tir and nucleolin. Molecular structures obtained by nuclear magnetic resonance and x-ray crystallography have been used to map the interaction of intimin-α with Tir (34, 35). Tir binds to a C-type lectin motif of intimin that is located at the carboxyl terminus of the extracellular domain. The intimin binding site for nucleolin is unknown. Reece et al. (13) reported that mutations in intimin-α engineered by site-directed mutagenesis of the eae locus in the region encoding the C-type lectin domain influenced EPEC colonization but had no affect on Tir binding. This finding suggests that the eucaryotic receptor-binding site also resides in the lectin domain (36). To better understand the role of each type of receptor in bacterial adherence, we used in vitro methods as well as a tissue culture model to investigate the inter-
actions of three intimin types (α, β, and γ) with human nucleolin and Tir from EHEC O157:H7.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Bacterial strains and protein constructs used in this study are listed in Table I. EPEC O127:H6 strain E2348/69 that expresses intimin-α, and plasmid pCVD438 that contains the eae gene from E2348/69 were provided by Dr. Ann Jerse. Construction of pCVD438 has been previously described (32). Plasmid pIntg380. Plasmid pIntb936. Plasmid pEB310 (intimin-β) was previously described (32).

| EPEC O127:H6 strain E2348/69: |
| Isolated from a patient in Taunton, England (55). Produces intimin type α |
| C. rodentium: |
| Isolated from laboratory mice (56). Produces intimin type β |
| EHEC O157:H7 strain 86-24: |
| Isolated in 1986 from a patient in Seattle, WA (57). Produces intimin type γ |
| BL21(DE3): |
| E. coli K12 strain used for the expression of recombinant intimin intimin-αβ γ: full-length intimin-α intimin-β γ: full-length intimin-β intimin-γ γ: full-length intimin-γ intimin-αβ γ: histidine-tagged extracellular domain of intimin-α intimin-β γ: histidine-tagged extracellular domain of intimin-β intimin-γ γ: histidine-tagged extracellular domain of intimin-γ |
| TiR157: full-length Tir from EHEC O157:H7 |
| 6H-nucleolin: histidine-tagged human nucleolin |

Nucleolin—Production of the recombinant nucleolin protein was induced by growth of the S. cerevisiae strain YPH500 transformed with p6his-ncl at 30 °C for 36 h in yeast synthetic minimal medium with 2% galactose and without tryptophan (Bio 101, Inc., Vista, CA). Western blot analysis of yeast whole cell lysates showed that the induced culture produced a protein with an apparent size of 114 kDa that was recognized by rabbit anti-nucleolin antiserum (Santa Cruz Biotechnology) as well as mouse anti-his tag antibody (Qiagen). We refer to this recombinant 6-histidine-tagged human nucleolin protein as 6H-nucleolin in the text. Full-length Intimin Extracellular Domains

E. coli XL1-Blue-competent cells were transformed with plasmids p6his-Inta385, p6his-Intb385, or p6his-Intg380. Transformed cells were selected for ampicillin resistance. Mid-log phase LB cultures of the transformed cells grown at 37 °C were induced with 2 mM isopropyl-β-D-thiogalactopyranoside. These cells produced truncated intimin with an amino-terminal 6-histidine tag and the carboxyl-terminal 385 amino acids of intimin-α or intimin-β, or 380 amino acids of intimin-γ. We refer to these molecules in the text as intimin-α385, intimin-β385, and intimin-γ380, respectively. Purification of the His-tagged intimins by nickel-affinity chromatography was accomplished as described above for 6H-nucleolin.

Full-length Intimin

Plasmids pnta939, pntb936, or pntg934 were transformed into E. coli strain BL21(DE3), and cells with the plasmids were selected for ampicillin resistance. These cells produced full-length intimin molecule extracellular domain containing a 539, 596, and 934 amino acids of type α, β, or γ, respectively. In the text we refer to these recombinant molecules as intimin-α539, intimin-β596, and intimin-γ934. Full-length intimin was expressed in the outer-membrane of transformed bacteria as demonstrated by reactivity of whole cells with rabbit anti-intimin serum and by the capacity of

CAGTCAAACACACAGC that incorporated restriction sites for BamHI and PsiI, respectively. After cutting the amplified product with these enzymes, the resultant DNA fragment was ligated into the 6-histidine tag expression vector pQE32 (Qiagen) in an attempt to produce a recombinant histidine-tagged version of the nucleolin protein. This construct was transformed into E. coli strain BL21 (Novagen), but no expression of the recombinant human protein was observed. Therefore, the ncl gene, along with the sequence that encoded the amino-terminal 6-histidine tag, was cut from the pQE32 vector with the restriction enzymes XhoI and NheI. This linear fragment was cloned into the yeast episomal vector pESC-Trp (Stratagene) that had been restricted with the enzymes SalI and NheI. This construct, designated p6his-ncl, contained the tagged nucleolin gene behind a galactose-inducible promoter, as well as the trp1 gene from S. cerevisiae (pESC-Trp). The plasmid was electroporated into Saccharomyces cerevisiae strain YPH500 (Stratagene) that is auxotropic for several amino acids, including tryptophan. Yeast were selected and maintained on synthetic dextrose minimal medium without tryptophan.

Protein Expression and Purification

Tir—All salts and chemicals used for protein purification were of reagent grade and were purchased from Sigma-Aldrich unless otherwise noted. Plasmid pTir was transformed into the E. coli strain BL21, and expression of the recombinant Tir protein was induced from mid-log cultures with 2 mM isopropyl-β-D-thiogalactopyranoside. Induced cells were lysed by sonication, and the cell lysate was subjected to 20% ammonium sulfate precipitation. The lysate supernatant was extensively dialyzed against phosphate-buffered saline (PBS). The dialyzed protein was centrifuged at 10,000 rpm to remove insoluble material. Tir protein in the supernatant was purified from contaminating proteins by passage over a column of DEAE-Sepharose (Amersham Biosciences) and eluted with a linear sodium chloride gradient. Column fractions were assessed by Western blot with rabbit anti-Tir serum (52), and fractions with the greatest reactivity were pooled. We refer to this recombinant Tir protein as Tir157, in the text.

Histidine-tagged Intimin Extracellular Domains

E. coli XL1-Blue-competent cells were transformed with plasmids p6his-Inta385, p6his-Intb385, or p6his-Intg380. Transformed cells were selected for ampicillin resistance. Mid-log phase LB cultures of the transformed cells grown at 37 °C were induced with 2 mM isopropyl-β-D-thiogalactopyranoside. These cells produced truncated intimin with an amino-terminal 6-histidine tag and the carboxyl-terminal 385 amino acids of intimin-α or intimin-β, or 380 amino acids of intimin-γ. We refer to these molecules in the text as intimin-α385, intimin-β385, and intimin-γ380, respectively. Purification of the His-tagged intimins by nickel-affinity chromatography was accomplished as described above for 6H-nucleolin.

Table I

| Bacterial strains and recombinant proteins used for this study |
| EPEC O127:H6 strain E2348/69: |
| Isolated from a patient in Taunton, England (55). Produces intimin type α |
| C. rodentium: |
| Isolated from laboratory mice (56). Produces intimin type β |
| EHEC O157:H7 strain 86-24: |
| Isolated in 1986 from a patient in Seattle, WA (57). Produces intimin type γ |
| BL21(DE3): |
| E. coli K12 strain used for the expression of recombinant intimin intimin-αβ γ: full-length intimin-α intimin-β γ: full-length intimin-β intimin-γ γ: full-length intimin-γ intimin-αβ γ: histidine-tagged extracellular domain of intimin-α intimin-β γ: histidine-tagged extracellular domain of intimin-β intimin-γ γ: histidine-tagged extracellular domain of intimin-γ |
| TiR157: full-length Tir from EHEC O157:H7 |
| 6H-nucleolin: histidine-tagged human nucleolin |

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**Bacterial Adherence Assay**

HEp-2 cell monolayers were grown in 8-well chamber slides as described for the cell binding assay. EHEC O157:H7 strain 86–24, strain 86–24encA10, and EPEC strain E2348/69 were grown overnight at 37 °C in static cultures of EMEM. Cultures of E. coli strain BL21(DE3) that expressed intimin-γ936, intimin-β954, or intimin-γ934 were grown overnight with agitation in LB supplemented with 0.1 mg/ml ampicillin to maintain selection for the intimin plasmids. HEp-2 cells were infected with 8 μl of overnight cultures inoculated into 300 μl of EMEM and incubated at 37 °C. After a 4-h infection period, the liquid on the cells was gently aspirated with a pipette to minimize the disturbance of bacteria that had adhered to the cell surface. The infected cells were fixed for 20 min in 2% formaldehyde, washed with PBS, permeabilized with 0.1% Tween 20 for 4 min, and then washed again with PBS. Nucleolin and Tir were stained by primary antiserum labeled with Alexa Fluor 488 and Alexa Fluor 350, respectively, as described. Polymerized actin was visualized with Alexa Fluor 594 phalloidin (Molecular Probes). All immunostaining procedures were carried out in PBS that contained 3% bovine serum albumin.

**Deconvolution Microscopy**

All images were obtained with an Olympus BX60 system microscope with a BX-FLA reflected light fluorescence attachment. Images were grabbed with a SPOT RT charge-coupled device camera (Diagnostic Instrument, Inc.) in 8-bit grayscale format. For each microscopic field shown, four images were obtained with red, green, and blue filters through sequential focal planes separated by 1-μm increments. These images were processed into stacks by the public domain program ImageJ (developed at the National Institutes of Health and available from http://rsb.info.nih.gov/ij). The deconvolved image was then normalized with TEB Gadient, and the analyzed images were saved at the highest image quality. Each image was then deconvolved using the radial symmetric equation, $A^\ast \exp(-r^2/2k_1^2)\cos(2\varphi/\theta/t) + B^\ast \exp(-r^2/2k_2^2)\sin(2\varphi/\theta/t) = 1/2$, where the variables $A$ and $B$ represent the contributions of the cos and sin functions, $k_1$ and $k_2$ represent the decay of the functions, and $\theta$ and $\varphi$ represent the period of the functions at distance r from the center. The ImageJ module DeconvolutionJ, written by N. Linnenbrugger, accomplished deconvolution of image stacks. DeconvolutionJ uses a regularized Wiener filter to deconvolve in-focus light from low frequency out-of-focus light. One or two image stack sections were selected for presentation, with the maximal intensity from each slice added to the final z-section. For each image, a background value was subtracted, and the signal intensity was normalized between the values of 0 and 255. Images were colored red, green, or blue, and overlaid and annotated in the final figures with the program Adobe Photoshop.

**Colocalization Correlation Determination**

To evaluate the colocalization between intimin and nucleolin on the surface of HEp-2 cells a cross-correlation analysis was performed. Intimin and nucleolin were immunostained with the fluorescent dyes Alexa Fluor 555 and Alexa Fluor 480, respectively. Images obtained through the red and green filter sets were deconvolved and normalized as described above. Numerical values of the pixel intensities for the separate images were converted to text strings by the program ImageJ. The intensity of staining was compared at each X-Y coordinate pair of the separate images. For the intimin-stained images, pixels with intensity values greater than 50 were considered valid for inclusion in the correlation analysis. The Pearson correlation coefficient, $r = n(\SigmaXY) - (\SigmaX)(\SigmaY)/(n\SigmaX^2 - (\SigmaX^2))^1/2(\SigmaY^2 - (\SigmaY^2))^1/2$, was evaluated by the program Microsoft Excel from valid X-Y intensity pairs. The significance of the correlation value was determined from a two-tailed paired t test.

**RESULTS**

**Intimin Types α, β, and γ Bound to Nucleolin with Similar Affinity**—Previously we demonstrated that intimin-γ of EHEC O157:H7 binds to nucleolin isolated from HEp-2 cells (32). One purpose of the present study was to determine if other intimin types interact with nucleolin. To answer this question, recombinant versions of nucleolin and various intimin extracellular domains were purified and used in an *in vitro* binding assay. Recombinant 6H-nucleolin was absorbed onto the wells of a microtiter plate. To measure the affinity of intimin for nucleolin, a series of 2-fold dilutions of biotinylated intimin-α945, intimin-β954, and intimin-γ936 were incubated with the immo-

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**Protein Binding Experiments**

Purified intimin-α945, intimin-β954, and intimin-γ936 (1 μg/ml) were biotin-labeled by reaction with 2 μg of biotinamidocaproate-N-hydroxysuccinimide ester (Sigma) in 0.1 M sodium borate buffer, pH 9.0, for 4 h at room temperature. Unreacted ester was quenched by the addition of 5 mM ammonium chloride, and the samples were dialyzed against PBS. Polyvinylchloride microtiter plates (96-well format) were coated for 3 h at room temperature with 50 μl of a 20 μg/ml solution of either TirO157 or 6H-nucleolin. Nonspecific binding sites in the wells were blocked with 1% bovine serum albumin (Sigma). Purified samples of histidine-tagged intimin incubated in the wells overnight at 4 °C. A 2-fold dilution series was made for each biotinylated intimin sample. These dilution series were incubated with the immobilized receptor proteins in a binding buffer that contained PBS with 3% bovine serum albumin and 0.2% Tween 20. After 1-h incubation at room temperature, intimin that had not bound to the immobilized receptors was rinsed from the wells with three washes of PBS. The amount of intimin bound nonspecifically to the wells was estimated from 2-fold dilutions added to wells that contained only bovine serum albumin. Bound intimin was incubated with streptavidin-conjugated horseradish peroxidase (Amersham Biosciences) diluted 1:1000 in binding buffer. The intimin-peroxidase complexes were detected by the enzyme-catalyzed color change of 3, 3', 5'-tetramethylbenzidine (TMB) using the TMB Peroxidase EIA Substrate Kit (Bio-Rad). The bound intimin concentration was estimated from the change in absorbance at 450 nm of the solution in each well, measured with an Elx800 Universal Microplate reader (Bio-Tek Instruments). These values were corrected for nonspecific binding by subtraction of the absorbance reading in wells that contained bovine serum albumin only. In a separate experiment designed to test the effect of Tir on the intimin interaction with nucleolin, various concentrations of TirO157 were mixed with an intimin-γ936 dilution series in binding buffer. The intimin-Tir mixtures were incubated with nucleolin that had been immobilized on a microtiter plate. After incubation for 1 h at room temperature, intimin-γ936 that had bound to 6H-nucleolin, was detected as described.

**HEp-2 Cell Binding Assay**

HEp-2 (ATCC CCL23) human laryngeal epithelial cell cultures were maintained in EMEM (BioWhitaker) supplemented with 10% fetal calf serum, 20 mM L-glutamine, 100 μg/ml gentamicin, 10 units/ml penicillin, 10 μg/ml streptomycin, and 10 μg/ml L-sodium boroate. Confluent cells grown at 37 °C in an atmosphere of 5% CO2 were released from the culture flasks by treatment with trypsin, and then 0.3 ml of cell suspension was seeded into 8-well chamber slides (Lab-Tek) at a cell density of 6 × 104 cells/ml. The chamber slides were then incubated for 24 h at 37 °C in an atmosphere of 5% CO2. Protein binding buffer consisted of RPMI 1640 (BioWhitaker), 10% fetal calf serum, 50 μg/ml gentamicin, 10 units/ml penicillin, 10 μg/ml streptomycin, and 20% bovine serum albumin. Purified intimin and nucleolin were immunostained with the fluorescent dyes Alexa Fluor 488 and Alexa Fluor 350, respectively, as described. Poly-
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**Fig. 1.** Protein titration of the intimin extracellular domain bound to 6H-nucleolin or Tir<sub>O157</sub>. A range of concentrations of purified intimin-<i>α</i>, intimin-<i>β</i>, or intimin-γ (circles) were added to microtiter plate wells that contained purified Tir<sub>O157</sub> (open symbols) or 6H-nucleolin (closed symbols). The fraction of receptor with intimin bound (γ-axis) was determined for each intimin concentration (α-axis) as described under “Experimental Procedures.”

The solid line through each data set is a theoretical binding curve calculated from the equilibrium constants listed in Table II. Each data point represents the average of three repeats. The error bars that bracket each symbol approximate the standard error of the mean of three replicate determinations. Each data set was corrected for nonspecific background binding.

**Table II**

| Intimin fragment | 6H-nucleolin | Tir<sub>O157</sub> |
|------------------|--------------|-------------------|
| Intimin-<i>α</i>  | 120.3 (±/−10.0) | 22.1 (±/−1.4) |
| Intimin-<i>β</i>  | 88.0 (±/−10.0) | 17.5 (±/−1.1) |
| Intimin-γ       | 100.0 (±/−3.3) | 17.0 (±/−1.2) |

*The reported constants represent the average of three separate determinations obtained on three different occasions. The values in parentheses are the average of the standard error values from the three determinations.

**Tir Blocks the Association between Intimin and Nucleolin**—One objective of this research was to observe the simultaneous interactions between intimin, Tir, and nucleolin. If intimin has separate, distinct binding sites for Tir and nucleolin, then both molecules should bind concurrently to intimin. On the other hand, if the binding sites for the receptors are overlapping or in close proximity, then steric hindrance would prevent both molecules from binding to intimin simultaneously. To test these possibilities we examined the competition between Tir<sub>O157</sub> and 6H-nucleolin for a limited number of intimin binding sites. The recombinant protein 6H-nucleolin was absorbed onto the wells of a microtiter plate. Intimin-γ was incubated with immobilized nucleolin in a solution containing various concentrations of Tir<sub>O157</sub>. After equilibrium had been established, intimin not bound to nucleolin was washed from the wells, and bound intimin was detected as described. The results of this experiment are shown in Fig. 2A. With increasing concentrations of Tir<sub>O157</sub>, the amount of intimin-γ bound to 6H-nucleolin was diminished. There was no association between Tir<sub>O157</sub> and immobilized 6H-nucleolin as measured by anti-Tir enzyme-linked immunoassay (data not shown). This observation ruled out the possibility that intimin adherence was blocked by Tir<sub>O157</sub> that had bound nucleolin. The fraction of intimin-γ that was bound to 6H-nucleolin as a function of Tir<sub>O157</sub> concentration is presented in Fig. 2B. These data were consistent with a simple competition between the receptors in which one molecule of Tir was able to block one molecule of intimin from binding to nucleolin. Tir<sub>O157</sub> was also able to block the interaction between 6H-nucleolin and intimin-<i>α</i>, intimin-<i>β</i>, and intimin-γ (data not shown).

Intimin of Types α, β, and γ Associated with Endogenous Nucleolin and Tir on the Surface of HEp-2 Cells—In an earlier report, we showed by immunofluorescence staining that recombinant intimin-γ of EHEC O157 binds to the surface of HEp-2 cells in regions that contain nucleolin (32). In the present study, we investigated the association of the three different intimin types with native nucleolin in the presence or absence of translocated Tir. Intimin-<i>α</i>, intimin-<i>β</i>, and intimin-γ were added to the supernatant of HEp-2 cell monolayers. Intimin bound to the cell surface was detected with a labeled antibody directed against the amino-terminal histidine tag.

Nucleolin expressed by the cells and localized to the plasma membrane was detected with the appropriate primary and secondary anti-sera. Fig. 3A shows that the staining pattern for each intimin type (stained red) demonstrated overlap with the staining pattern for cell surface-localized nucleolin (stained green) as revealed by the orange/yellow color in the merged images. A typical cross-correlation plot of the staining intensity for nucleolin compared with that of intimin is presented in Fig. 3A. Values of the Pearson product moment correlation coefficient were determined to be 0.38, 0.42, and 0.40, all with <i>p</i> < 0.0005, for intimin-<i>α</i>, intimin-<i>β</i>, and intimin-γ, respectively. These results demonstrate that there was a statistically significant co-localization between intimin and
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The plasma membrane, there was an increase in the amount of intimin binding (x-axis) were performed in the presence of TirO157 at concentrations of 1 nM (+), 500 nM (triangle), 250 nM (-), 125 nM (diamond), 62 nM (square), 16 nM (X), and without TirO157 (circle). The fraction of immobilized 6H-nucleolin with intimin bound (y-axis) was measured as described under “Experimental Procedures.” TirO157 in solution with the recombinant intimin blocked binding to 6H-nucleolin in a concentration-dependent manner. The data in A were corrected for nonspecific binding of intimin-γ280 to control wells coated with bovine serum albumin. The solid line through each data set is calculated from a simple two-state binding equilibrium. B, the fraction of immobilized nucleolin with intimin-γ280 bound (y-axis) is shown for one intimin-γ280 concentration of 250 nM and various TirO157 concentrations (x-axis). The solid line is a theoretical fit to the data for a competitive inhibition model for two ligands with equilibrium dissociation constants of 20 and 100 nM. The error bars approximate the average standard error of the mean between replicate determinations.

The intimin deletion mutant EHEC O157:H7 86–24αeΔ10 was used to insert Tir into the HEp-2 cell plasma membrane. Because this mutant bacterium did not express a functional intimin, it was unable to adhere to the infected cells by means of Tir. HEp-2 cells with both nucleolin and Tir on the surface showed a distinctly different pattern for binding of recombinant intimin. Fig. 3B illustrates that in cells with Tir in the plasma membrane, there was an increase in the amount of intimin-α285 bound to the cell surface, and there was not extensive overlap between the intimin- and nucleolin-staining patterns. Tir (stained blue) localization did overlap with regions of increased intimin binding as denoted by the purple color in the merged image. When Tir was present, the association between intimin and nucleolin decreased, and there were clearly regions of intimin staining without nucleolin. Fig. 4B shows a typical cross-correlation plot for the intimin and nucleolin staining intensity on cells with Tir inserted into the plasma membrane. There was no significant correlation between intimin and nucleolin staining intensity on these cells, with the Pearson correlation coefficients calculated to be 0.06, 0.06, and 0.04 (all with p > 0.25) for intimin-α285, intimin-β285, and intimin-γ280, respectively.

Nucleolin and Polymerized Actin Segregate beneath Intimin-Expressing Bacteria—HEp-2 cell monolayers were co-infected with EHEC O157:H7 strain 86–24αeΔ10 (to insert Tir) and E. coli K12 strains that produced intimin-α285, intimin-β285, or intimin-γ280. These K12 transformants were used to analyze bacterial adherence promoted by the various intimin types without the confounding influences of other adherence factors produced by the different wild-type pathogenic strains. Following a 4-h infection period with the intimin-expressing E. coli K12 strains, the HEp2 cells were fluorescently stained for Tir, nucleolin, and actin. Photomicrographs of these stained cells are presented in Fig. 5. Although most adherent bacteria stained for Tir (blue) and actin (red), a subset of bacteria also displayed intense nucleolin immunostaining (green) at the site of adherence. Little to no nucleolin stain was observed in re-
regions with actin polymerized beneath the adherent bacteria. These observations suggest that nucleolin was excluded from the regions of Tir-mediated intimate adherence promoted by all intimin types. To observe the association of nucleolin with wild-type bacteria that express intimin, HEp-2 cells were infected with EPEC O126:H6 or EHEC O157:H7. The cells were stained for Tir, nucleolin, and actin following bacterial infection (Fig. 5). Actin and Tir were evident beneath the wild-type bacteria, but nucleolin appeared to be excluded from those regions directly under the bacteria where polymerization of actin was evident. We surmise that such actin engagement indicates tight bacterial adherence. That nucleolin clustered around the sites of bacterial association with the HEp-2 cells suggests that the intimin-expressing bacteria bound nucleolin at the initial contact of the organism with the host cell but that these intimin-nucleolin interactions were displaced by the Tir-driven formation of the actin-rich pedestal.

**DISCUSSION**

In this investigation, we attempted to analyze the interactions between intimin and two receptors, bacterially encoded Tir and host cell-expressed nucleolin. We found that intimin of types α, β, and γ each bound to nucleolin with similar dissociation constants that were on the order of 100 nM. We previously reported (32) that purified intimin-γ binds to the surface of HEp-2 cells with a dissociation constant of ~90 nM, a value
Various Intimins Bind to Nucleolin and Tir

The interaction of intimin–γ with Tir produced by EHEC O157:H7 was reported by DeVinney et al. (17) to have a dissociation constant of -10 nM. We measured intimin-Tir dissociation constants of -20 nM. These values are relatively close, and the discrepancy between them may be attributed to different experimental methods. From these data, we can conclude that the affinity of intimin for Tir is 5 to 10 times stronger than the affinity for nucleolin. When Tir and nucleolin were presented simultaneously, we found that Tir blocks intimin from adhering to nucleolin. This observation indicates that either nucleolin and Tir have overlapping binding sites on the C-type lectin domain of intimin, or that Tir binding induces a conformational change in intimin that precludes nucleolin association. There is no apparent sequence homology between Tir and human nucleolin, so we feel it is unlikely that nucleolin acts as a Tir mimic. Although we do not understand the exact mechanism by which Tir blocks nucleolin binding, the affinity results would suggest that Tir is the preferred receptor for intimin. Nevertheless, when endogenous nucleolin and Tir were presented simultaneously in the milieu of the plasma membrane, we found that recombinant intimin bound to both receptors.

For A/E lesion-forming bacteria, intimin-mediated adherence occurs in conjunction with many other adherence factors (40–42). To focus on potential differences in adherence caused by the different intimin types, the eae genes were cloned from the wild-type bacteria and expressed in an E. coli K12 background. Previous work showed that K12 strains that express intimin, or latex beads coated with intimin can induce pedestal formation when Tir is inserted into the plasma membrane by an intimin-deletion strain (15, 37). Immunofluorescence staining showed nucleolin accumulation beneath adherent bacteria only in regions where there was no Tir-induced actin polymerization. This observation supports the idea that intimin on the bacterial surface can bind to the bacterial receptor or the eucaryotic receptor but can not adhere simultaneously through both. When HEp-2 cells were infected by EPEC or EHEC, only Tir and polymerized actin staining were observed directly beneath the adherent bacteria. Nucleolin staining was observed in proximity to the site of attachment, but significant accumulation did not appear directly beneath the bacteria. The difference in nucleolin staining observed between the E. coli K12 strains that express intimin and the wild-type A/E pathogens may be due to the fact that the K12 strains express significantly more intimin than the pathogens (verified by Western blot analysis).

The data that we have presented suggest that intimin-mediated bacterial adherence involves competition between Tir and nucleolin. How does this competition relate to the process of infection by these pathogenic microorganisms? Our current understanding of the infection process is that the host ingests the bacteria, which then pass through the stomach and into the large intestine. Once in the intestine these bacteria move through the mucus layer and come into contact with the luminal mucosal epithelium where Tir is inserted into host-cell membranes. The role of Tir in linking the bacterium to the host-cell cytoskeleton is well established. The observation that Tir interacts with intimin appeared to dominate the later stages of adherence to tissue culture cells implies that any interactions between intimin and nucleolin must occur prior to the insertion of Tir and the formation of actin-rich pedestals. The interaction between intimin and nucleolin is not sufficient to promote bacterial attachment, and for that reason it is unlikely that nucleolin is acting solely as a passive receptor for intimin. One characteristic of C. rodentium infection in mice is the induction of colonic hyperplasia. Higgins et al. (43) have shown that intimin alone is able to produce a similar hyperplasia in mice and have proposed that intimin interactions with some host-cell receptor produce this response. The interaction between intimin and nucleolin may play role in the pro-inflammatory response induced in host cells during infection by the A/E lesion-forming pathogens. Adherence of these bacteria has been reported to induce the mitogen-activated protein (MAP) kinase cascade, leading to the production of pro-inflammatory cytokines (44–47). Activation of the MAP-kinases has been reported to be both intimin-dependent and -independent (44, 48). Nucleolin is one target of several MAP-kinases, including p38 (49), extracellular-regulated kinase (50), and casein kinase 2 (51, 52). Of particular note is the recent work of Savkovic et al. (53) who showed that protein kinase C-ζ is involved in EPEC-induced inflammatory responses of infected cells. Nucleolin is a protein kinase C-ζ substrate and has been shown to be involved in cell-surface signaling (54). One possible result of the competition between nucleolin and Tir is that the initial contacts between intimin and the host cell may trigger the extracellular signaling cascade that promotes bacterial dissemination while the later contacts with Tir promote tight adherence. Although we do not yet fully understand the role of nucleolin in intimin-mediated bacterial adherence, these data in aggregate support a model in which intimin interactions with nucleolin are distinct from the interactions with Tir that occur during adherence.

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