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Vitronectin Mediates Internalization of *Neisseria gonorrhoeae* by Chinese Hamster Ovary Cells

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Gonococci producing a distinct opacity protein (OpaA in strain MS11) adhere to and are efficiently internalized by cultured epithelial cells such as the Chang conjunctiva cell line. Both adherence and uptake require interactions between OpaA and heparan sulfate proteoglycans on the mammalian cell surface. Chinese hamster ovary (CHO) cells also support adherence of gonococci through interactions of OpaA with cell surface heparan sulfate proteoglycans. However, despite this similarity in the requirements for adherence, CHO cells are not capable of internalizing gonococci. In this report, we characterized this apparent deficiency and identified a factor in fetal calf serum (FCS) which is capable of mediating uptake of gonococci by CHO cells. In the absence of FCS, OpaA$^+$ gonococci adhered to but were not internalized by CHO cells, whereas in the presence of up to 15% FCS, the bacteria were efficiently internalized by the cells. Preincubation of bacteria, but not cells, with FCS also stimulated internalization, suggesting that a factor present in FCS was binding to the surface of gonococci and subsequently stimulating entry. Using a combination of chromatographic purification procedures, we identified the adhesive glycoprotein vitronectin as the serum factor which mediates the internalization of gonococci by CHO cells. Vitronectin-depleted serum did not support gonococcal entry, and this deficiency was restored by the addition of purified vitronectin. Further experiments using a set of gonococcal recombinants, each expressing a single member of the family of Opa outer membrane proteins, demonstrated that vitronectin bound to the surface of OpaA-producing gonococci only and that the vitronectin-mediated uptake by the CHO cells was limited to this bacterial phenotype. To our knowledge, our data are the first example that vitronectin can serve as a molecule that drives bacterial entry into epithelial cells.

Neisseria gonorrhoeae is a human-specific pathogen that usually colonizes the mucosal epithelium of the genitourinary tract and often gives rise to a potent local inflammatory response and destruction of the epithelial barrier. At the molecular level, gonococcal infection of human mucosal cells is thought to involve a sequential interaction between adhesins on the surface of the bacteria (including pili and opacity [Opa] proteins) and receptors on the surface of host epithelial cells (for reviews, see references 19 and 20). In vitro experiments suggest that after initial pilus-mediated anchoring of the bacteria to the surface of the host cells (26, 27), additional bacterial surface constituents facilitate a tight association between the bacteria and the mammalian cells and that the bacteria may even induce their own uptake by the epithelial cells (1, 3, 5, 28, 31, 34, 36). The latter event may be critical to the establishment of an infection, since gonococci have been detected within epithelial cells from infected individuals (1, 10, 35).

Gonococci may exploit different strategies to gain access to the epithelial cell interior (5, 17, 23). One mechanism of bacterial entry into host cells requires expression of a distinct opacity outer membrane protein (17, 36). In vitro assays using the Chang conjunctiva epithelial cell line have demonstrated that this protein (OpaA in strain MS11) binds to heparan sulfate proteoglycans that are present on the surface of epithelial cells and that this interaction results in adherence and internalization of gonococci by the Chang cells (6, 30). This uptake is mediated by a cellular mechanism resembling conventional phagocytosis; it is accompanied by the accumulation of F-actin, it does not involve the formation of clathrin-coated pits, and in mixed infections it is highly specific for gonococci producing the appropriate Opa protein (11). At this time, the molecular events that follow binding of OpaA-producing gonococci to the proteoglycans at the surface of the host cells and that lead to internalization of the bacteria are unknown.

In the present study, we sought to further unravel the Opa-dependent uptake pathway in epithelial cells by studying the interaction of gonococci with Chinese hamster ovary (CHO) cells. Similar to adherence to Chang cells, gonococcal adherence to CHO cells occurs through in the interaction of OpaA with cell surface heparan sulfate proteoglycans (6, 30). However, unlike that in the Chang cell line, this adherence is not followed by internalization of the bacteria. Thus, there appears to be a specific defect which renders CHO cells incapable of gonococcal phagocytosis. In this report, we show that gonococcal internalization by CHO cells can be effected by the addition of fetal calf serum (FCS) and that the adhesive glycoprotein vitronectin is the factor in FCS that mediates uptake of OpaA$^+$ gonococci by CHO cells.

MATERIALS AND METHODS

Cell lines and microorganisms. CHO cells (ATCC CCL 61) were grown in RPMI 1640 plus 5% FCS (Life Technologies, Gaithersburg, Md.). The characteristics of *N. gonorrhoeae* MS11 and its recombinants, each expressing a different recombinant opacity protein (generously provided by T. F. Meyer), have been described previously (13). All gonococcal strains were nonpiliated and expressed lipooligosaccharide (LOS) type b (lacto-*N*-neotetraose$^+$). Bacteria were routinely maintained on GC phosphate agar plates (composition per liter, 3.75 g of Trypticase peptone [BBL, Becton-Dickinson, Cockeysville, Md.], 7.5 g of Thiotone E [BBL], 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch

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[BBL], and 1% Bacto Agar [Difco Laboratories, Detroit, Mich.] [pH 8]) containing 1% IsoVitaleX (BBL) at 37°C in 5% CO_2.

Infection assays. For use in infection experiments, CHO cells were plated onto 12-mm-diameter circular glass coverslips in 24-well plates and grown to near confluence in RPMI 1640 plus 5% FCS. Before addition of the bacteria, the monolayers were washed three times with 1 ml of RPMI 1640 without FCS ($RPMI^-$). In the assay, we used bacteria that had been grown to the mid-logarithmic phase in 10 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM glucose [pH 7.2]) enriched with 1.5% proteose peptone no. 3 (Difco) in a gyratory water bath shaker (2 h, 125 rpm, 37°C). Bacteria were collected by centrifugation (2,000 × *g*, 6 min, 20°C) and resuspended in HEPES buffer. Bacteria (10^7) were added to CHO cells (approximately 2×10^5 cells per well) in 1 ml of $RPMI^-$ along with additives as indicated for individual experiments. After the plates were incubated for 2 h at 37°C, the wells were washed three times with 1 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 2.5 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ [pH 7.4]), fixed with 2% paraformaldehyde in PBS, and stained by immunogold silver staining, which differentially stains intracellular and extracellular bacteria as described previously (31). Under light microscopy, 50 cells per well were chosen at random, and the numbers of adherent and intracellular bacteria per cell were determined. The data represent the means and standard errors of at least three experiments.

Column chromatography. Initial fractionation of FCS was accomplished by diluting 10 ml of FCS serum 1:10 in 0.1 M sodium phosphate buffer (pH 5.4; PO_4 buffer) and passing it over a 2-ml DEAE-Sephacel (Pharmacia, Piscataway, N.J.) column that had been equilibrated with PO_4 buffer. Fractions were collected after elution with a sodium chloride step gradient (0.025 to 0.4 M NaCl in PO_4 buffer, 10 times, 1 ml each step). The fractions were diluted 1:20 in distilled water to reduce the salt concentration and analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% gels (14) and silver stain (4). For analysis of internalization activity, 50-μl volumes of undiluted samples from each fraction were added to 1 ml (final concentration, 5% [vol/vol]) of $RPMI^-$ in the infection assay. Further purification of the factor mediating internalization was achieved by passing the pooled active fractions (18-ml total volume, diluted 1:10 in distilled water to reduce the salt concentration) from the DEAE column over a column containing 2.5 ml of Affi-Gel heparin gel (Bio-Rad) that had been equilibrated with PO_4 buffer. All material bound to the heparin column was eluted with 10 ml of 0.5 M NaCl in PO_4 buffer, diluted 1:5 in distilled water, and analyzed for protein content and internalization activity.

Biotinylation of heparin-binding proteins. To determine gonococcal binding proteins in the heparin column eluate, 200 μl of the eluate was diluted in 1 ml of PBS, and NHS-LC-biotin (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 5 mM. After incubation on ice for 2 h, the unincorporated biotin was removed by ultrafiltration with three washes (0.5 ml of HEPES buffer for each wash, 1,800 × *g*, 30 min, 20°C) through a 10,000-kDa-molecular-mass-cutoff Ultrafree-MC unit (Millipore, Bedford, Mass.). The biotinylated eluate (final concentration, 5%) was incubated with 10^8 $OpaA^+$ gonococci in 200 μl of HEPES buffer for 15 min at 37°C. The bacteria were collected by centrifugation (2,000 × *g*, 6 min, 20°C), washed three times with 200 μl of HEPES buffer, and lysed by boiling in 100 μl of SDS-PAGE sample buffer. Samples were electrophoresed and immunoblotted (9), and bacterium-associated biotinylated proteins were detected with horseradish peroxidase (HRP)-streptavidin (Amersham, Arlington Heights, Ill.) at a dilution of 1:1,500 in the enhanced chemiluminescence (ECL) protocol (Amersham).

Depletion of vitronectin from FCS. Immunoglobulins from a polyclonal anti-vitronectin antiserum (Calbiochem, La Jolla, Calif.) were purified by protein A-Sepharose chromatography as follows. One gram of protein A-Sepharose (Sigma) swollen in 10 ml of PBS was poured into a column and equilibrated with 50 ml of PBS. One milliliter of the polyclonal serum was diluted in 10 ml of PBS and applied to the protein A column. The column was washed with 200 ml of PBS, and the bound immunoglobulins were eluted with 10 ml of 0.2 M glycine HCl in 1-ml fractions. The fractions containing the eluted material (as measured by absorbance at 280 nm) were pooled (total volume, 10 ml) and exchanged into 0.1 M sodium phosphate buffer (pH 7.0) by ultrafiltration with three phosphate buffer washes (0.5 ml each wash, 1,800 × *g*, 30 min, 20°C) through a 10,000-kDa-molecular-mass-cutoff Ultrafree-MC unit (Millipore).

The eluted immunoglobulins from the protein A column (7.2 mg) were coupled through their carbohydrate moieties to a CarboLink gel (Pierce, Rockford, Ill.) as described in the manufacturer's instructions. Briefly, the carbohydrates of the immunoglobulins were oxidized by incubation in 1 ml of sodium phosphate buffer (pH 7.0) containing 5 mg of sodium metaperiodate for 30 min at room temperature. After removal of the periodate by passing the sample over a desalting column (supplied by Pierce), the immunoglobulins (in 2 ml of sodium phosphate buffer) were allowed to incubate with 2 ml of the CarboLink gel overnight at room temperature on a rotating test tube rack. The gel was packed into a column and washed with 20 ml of PBS.

This column was used to specifically deplete vitronectin from FCS. One milliliter of FCS was diluted 1:10 in RPMI 1640 and passed five times over the vitronectin affinity column described above. The depletion of vitronectin was assessed by SDS-PAGE and silver staining as well as by Western blotting (immunoblotting) (6) the column flow through the use of a vitronectin-specific polyclonal antiserum (Calbiochem) diluted 1:2,000 and HRP-conjugated protein A (Sigma) diluted 1:10,000 in the ECL protocol. Purified vitronectin (bovine and human) as well as fibronectin, tenascin, and thrombospondin were obtained from Life Technologies. The fibronectin did not contain contaminating vitronectin as assayed by immunoblotting (data not shown) with the vitronectin-specific polyclonal antiserum (Calbiochem).

Analysis of vitronectin binding. Mid-log-phase-grown gonococci (10^8) were incubated with 10% FCS in 200 μl of HEPES buffer for 15 min at 37°C. The bacteria were collected by centrifugation (1,800 × *g*, 6 min, 20°C), washed three times with HEPES buffer, and lysed by boiling in 100 μl of SDS-PAGE sample buffer (14). Bacterium-associated vitronectin was detected by SDS-PAGE and Western blotting (9) with a vitronectin-specific polyclonal antiserum (Calbiochem) diluted 1:2,000 and HRP-conjugated protein A (Sigma) diluted 1:10,000 in the ECL protocol.

RESULTS

Uptake of gonococci by CHO cells in the presence of FCS. In our search for the molecular basis of the apparent inability of CHO cells to internalize adherent gonococci, we noticed a few intracellular bacteria when CHO cell monolayers were not washed thoroughly with $RPMI^-$ before the addition of bacteria to the cells, suggesting that perhaps a serum factor was able to compensate for the defect in gonococcal uptake. To test this hypothesis, we added increasing amounts of FCS with $OpaA^+$ or Opa^- gonococci to thoroughly washed (with $RPMI^-$) monolayers of CHO cells and determined the number of adherent and intracellular bacteria by light microscopy after fixation and immunogold-silver staining, a technique which discriminates between intracellular and extracellular bacteria (31). As illustrated in Fig. 1A, in the absence of FCS, $OpaA^+$ gonococci adhered to but were not internalized by CHO cells, in agreement with previous results (30). By contrast, the bacteria were efficiently internalized by the cells in the presence of FCS at concentrations of 1.5% or higher; up to 17 gonococci per cell were observed in the presence of FCS. At higher concentrations of FCS, adherence of $OpaA^+$ gonococci appeared to be inhibited.

Since the CHO cells were grown for 2 days in 5% FCS before the infection assays were performed, it seemed unlikely that the entry-promoting factor in FCS exerted its activity by binding directly to the eukaryotic cells. To determine whether the serum factor was mediating internalization through an effect on the bacteria, we preincubated $OpaA^+$ gonococci in HEPES buffer containing different concentrations of FCS for 15 min, washed them three times with $RPMI^-$, and analyzed their fate when added to CHO cells. Gonococci ($OpaA^+$) preincubated with FCS were efficiently internalized in contrast to bacteria preincubated in buffer alone (Fig. 1B), demonstrating that the factor mediating internalization was exerting its effect by interacting with the bacteria. As expected, preincubation of CHO cells in FCS, followed by washing with $RPMI^-$, did not support entry (data not shown).

Identification of the serum factor that mediates internalization. FCS is relatively well characterized in terms of its protein content. We therefore sought to identify the internalization factor from FCS by purification through the use of column chromatography. Preliminary experiments (data not shown) suggested that the internalization-mediating activity in FCS bound to an anion-exchange column at low pH and could be eluted with sodium chloride. Therefore, we diluted 50 ml of FCS 1:10 in PO_4 buffer and applied it to a DEAE-Sephacel column which had been equilibrated with PO_4 buffer. Fractions were collected after elution with a sodium chloride step gradient (0.025 to 0.4 M) in PO_4 buffer. Analysis of the fractions (final concentration, 5%) by infection assay indicated that most of the internalization activity eluted with fractions 33 through 51 (Fig. 2A). SDS-PAGE and silver staining of these fractions

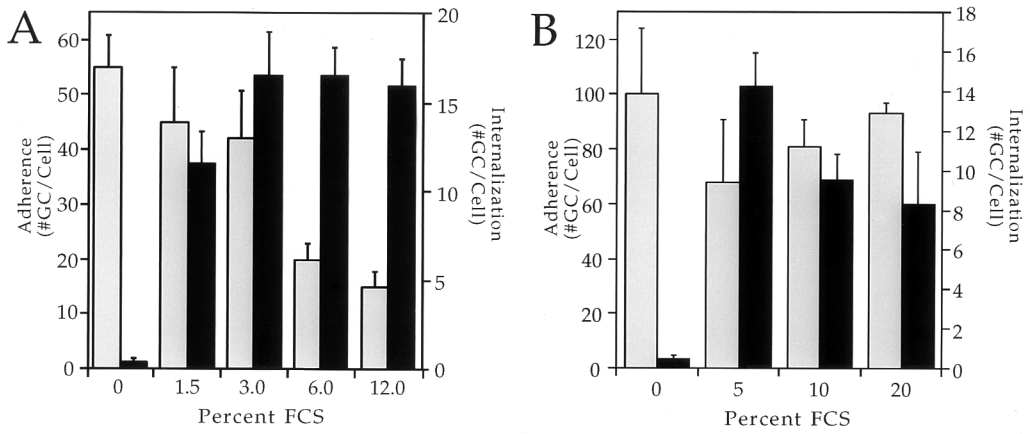

FIG. 1. Internalization of OpaA$^+$ gonococci in the presence of FCS. (A) CHO cells were washed with RPMI$^-$ and infected with gonococci (GC) producing OpaA in the absence and presence of increasing concentrations of FCS. (B) Gonococci were preincubated with 10% FCS and washed before addition to CHO cells. Adherence (gray bars) and internalization (black bars) were scored microscopically after differential immunogold silver staining of infected cells as described in Materials and Methods. Data in this and all subsequent infection experiments represent the average numbers of gonococci per cell ± standard errors from at least three experiments.

showed that chromatographic separation of serum proteins was achieved but that there was no obvious correlation between protein bands and activity (Fig. 2B).

Further characterization of the internalization-promoting activity in FCS was achieved by passing the active fractions (33 through 51) from the DEAE column over a heparin-Sepharose affinity column. FCS contains a number of heparin-binding proteins, and analysis of the eluted heparin-bound material (with 0.5 M NaCl) in the infection assay showed that the entry-promoting activity was capable of binding to the heparin column (Fig. 2A, lane HP). Analysis of the eluate from the heparin column by SDS-PAGE and silver staining revealed several major protein bands with molecular masses of approximately 250, 150, 70 to 80, 67, and 50 kDa (Fig. 2B, lane HP). Thus, the serum factor mediating internalization of gonococci by CHO cells was likely a heparin-binding protein with a molecular mass in the range described above.

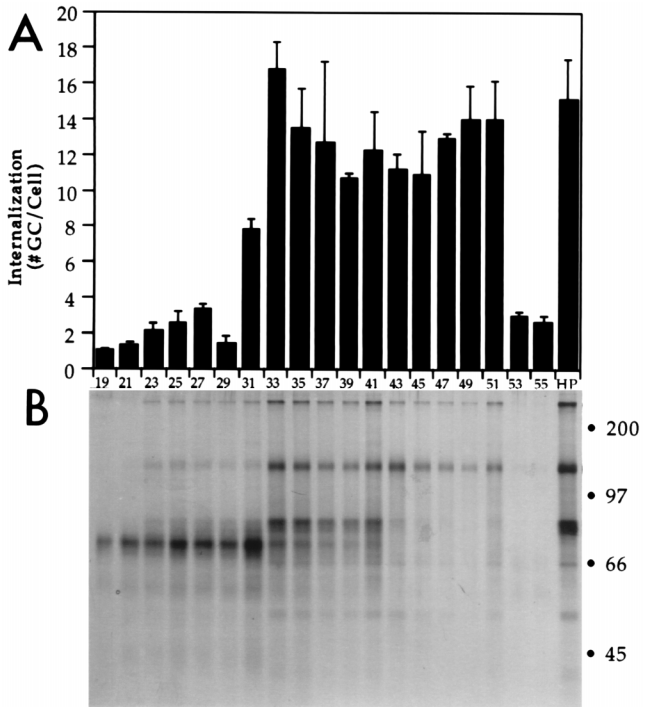

FIG. 2. Partial purification of internalization activity from FCS by anion-exchange and heparin affinity chromatographies. FCS was diluted and passed over a DEAE-Sephacel column, bound proteins were eluted with an NaCl gradient, and the fractions were analyzed by infection assay (A) and SDS-PAGE (B). Fractions 33 to 51 were passed over a heparin affinity column, and eluted proteins were analyzed for protein content and internalization activity (lane HP). The migrations of molecular mass standards are indicated on the right side of the gel in kilodaltons. GC, gonococci.

Vitronectin is the serum factor that promotes gonococcal entry into CHO cells. To further identify the internalization factor in FCS, we took advantage of the apparent binding of this factor to gonococci producing OpaA (Fig. 1B). For this purpose, we biotinylated the proteins present in the eluate from the heparin affinity column, incubated this material with OpaA$^+$ gonococci, removed the unbound material by washing the bacteria in HEPES buffer, and lysed the bacteria in SDS-PAGE sample buffer. Subsequent electrophoresis, blotting, and detection of biotinylated proteins with HRP-streptavidin and ECL showed that several proteins from the heparin column eluate bound to the gonococci. This was indicated by the presence of two major bands with approximate molecular masses of 150 and 67 kDa and a minor band with a molecular mass of 80 kDa (Fig. 3, lane 2). In addition, overexposure of the blot revealed a faint band with a molecular mass of more than 200 kDa (data not shown). Thus, at least one of the factors mediating internalization appeared to be a heparin-binding protein with a molecular mass of >200, 150, 80, or 67 kDa.

To determine the identity of this factor, we obtained several commercially available heparin-binding proteins purified from serum (tenascin, 220 kDa; fibronectin, 220 kDa; thrombospondin, 160 kDa; and vitronectin, 78/68 kDa) with molecular masses that corresponded to those of the proteins binding to the bacteria and visible in lane 2 of Fig. 3. Measurement of the internalization-promoting activity of these proteins (final concentration, 9 μg/ml) in the infection assay demonstrated that only the adhesive glycoprotein vitronectin mediated efficient internalization of OpaA$^+$ gonococci into CHO cells (Fig. 4). This uptake-promoting effect was found for purified bovine vitronectin obtained from several different sources and for

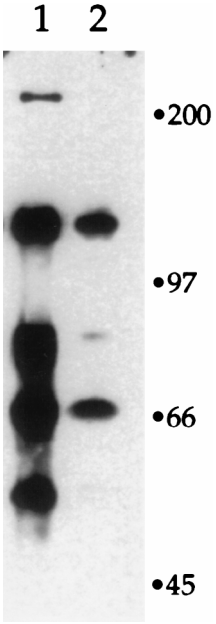

FIG. 3. Binding of proteins eluted from the heparin affinity column to OpaA$^+$ gonococci. The proteins in the eluate from the heparin affinity column (Fig. 2, lane HP) were labeled with biotin and allowed to bind to OpaA$^+$ gonococci. After extensive washing, the bacteria were lysed and subjected to SDS-PAGE and Western blotting. The bands were detected with HRP-streptavidin by ECL. Lanes: 1, biotinylated proteins from the heparin column eluate; 2, biotinylated proteins bound to OpaA$^+$ gonococci. Several proteins bound to the gonococci are apparent in FCS, with approximate molecular masses of 150, 80, and 68 kDa. Overexposure of the blot also revealed a band with a molecular mass of >200 kDa (data not shown).

human vitronectin (data not shown). Moreover, Western blotting with an antivitronectin antibody confirmed the presence of vitronectin in the eluate of the heparin affinity column (Fig. 5A, lane 4) and in the active fractions eluted from the DEAE column (data not shown).

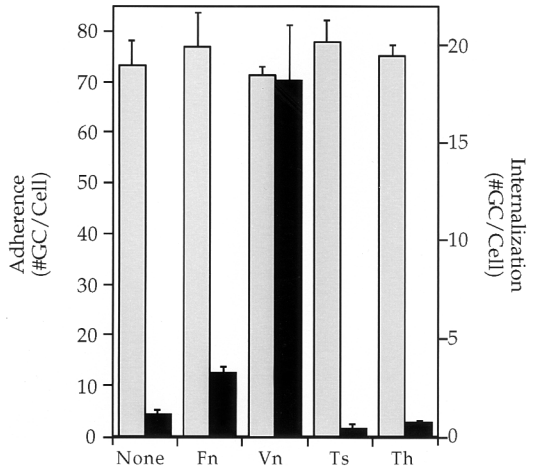

FIG. 4. Vitronectin mediates entry of OpaA$^+$ gonococci into CHO cells. Purified heparin-binding proteins (final concentration, 9 μg/ml) with molecular masses corresponding to those of the bands visible in Fig. 3 were tested for their ability to confer entry of gonococci (GC) into CHO cells. The proteins tested were fibronectin (Fn; 220 kDa), vitronectin (Vn; 75, 65, and 10 kDa), tenascin (Ts; 220 to 230 kDa), and thrombospondin (Th; 160 kDa). Adherence and internalization were quantified as described in the legend to Fig. 1.

Vitronectin is the only factor in FCS that has internalization activity. Although the data mentioned above indicated that vitronectin was capable of mediating internalization, it was possible that other factors present in FCS were involved in this process as well. To address this, we constructed an affinity column with purified antibodies specific for bovine vitronectin and depleted FCS of vitronectin. The depletion was confirmed by a Western blot showing the absence of antivitronectin-reactive bands in the FCS passed through the affinity column (Fig. 5A, lane 2). Testing of the vitronectin-depleted serum in the internalization assay showed that 1% vitronectin-depleted serum was not capable of mediating internalization in contrast to 1% untreated FCS and the heparin affinity column eluate (final concentration, 5%; Fig. 5B). A similar lack of internalization activity was observed for 10% vitronectin-depleted FCS (data not shown). When 1% vitronectin-depleted FCS was reconstituted with 10 μg of purified vitronectin, however, efficient internalization was observed (Fig. 5B). These data strongly suggested that vitronectin mediated the internalization of gonococci by CHO cells and that it was the only factor in FCS with this activity.

Vitronectin-mediated uptake is specific for OpaA$^+$ gonococci. To determine whether vitronectin shows a binding specificity for gonococci producing OpaA similar to that reported for heparan sulfate proteoglycans (6, 30), we tested the ability of vitronectin in FCS to promote the internalization of a panel of 12 MS11 derivatives, each of which produces either no Opa protein or one of the 11 recombinant Opa proteins (Opa_{50} to Opa_{60}) (13). In the presence of 1% FCS, gonococci expressing recombinant OpaA (Opa_{50}) were efficiently internalized by CHO cells, whereas gonococci producing no Opa protein or one of the other 10 Opa proteins (Opa_{51} to Opa_{60}) were not internalized (Fig. 6A). To further establish this apparent specificity of vitronectin for gonococci producing Opa_{50}, we incubated the recombinant strains with 5% FCS, washed them, and measured their vitronectin binding by SDS-PAGE and Western blotting with a vitronectin-specific polyclonal antiserum which recognizes both the 78- and 68-kDa forms of the protein. Vitronectin-specific bands were present only in lysates made from gonococci producing Opa_{50}; these bands were absent in lysates made from gonococci producing no Opa proteins or one of the other 10 Opa proteins (Fig. 6B). Identical results were obtained when purified vitronectin was used in this assay (data not shown). In addition, the bacterial LOS phenotype did not affect vitronectin-mediated uptake by CHO cells, since similar results were obtained with gonococci carrying *galE* mutations (25) or a LOS type a phenotype (lacto-*N*-neotetraose$^-$) (data not shown).

DISCUSSION

The colonization of *N. gonorrhoeae* on the mucosal epithelium is required for successful infection of the human host. Several factors on the surface of gonococci, including pili, LOS, and opacity proteins, have been implicated in the association of gonococci with human epithelial cells (6, 18, 23, 27, 30). The exact nature of the molecular interactions between gonococci and epithelial cells that are required for adherence and internalization is not fully understood. Gonococci adhere to and are internalized by cultured Chang conjunctiva epithelial cells, events which require the interaction of a specific opacity protein (OpaA in MS11) with cell surface heparan sulfate proteoglycans (6, 30). In this report, we have analyzed the interaction of gonococci with CHO cells, to which the bacteria adhere in an OpaA-dependent fashion but by which

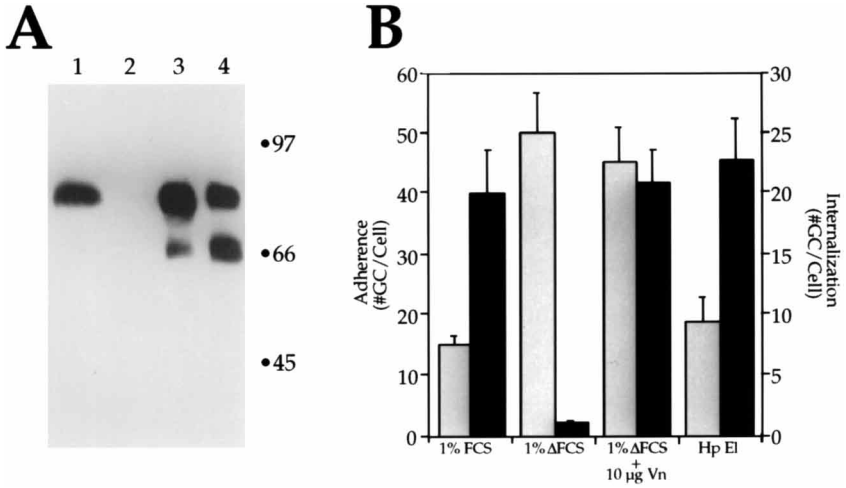

FIG. 5. Vitronectin-depleted FCS does not mediate internalization of gonococci into CHO cells. (A) FCS was passed repeatedly over a vitronectin-specific affinity column. Vitronectin depletion was monitored by Western blotting of the flowthrough from the column by use of a polyclonal antiserum specific for vitronectin. Lanes: 1, 10% FCS, 10 μl loaded; lane 2, 10% FCS passed over vitronectin affinity column, 10 μl loaded; lane 3, purified bovine vitronectin, 150 ng; lane 4, eluate from the heparin affinity column (Fig. 2). The absence of the 68-kDa vitronectin band in lane 1 is most likely due to the large amount of albumin in FCS, which has a molecular weight of 66 kDa, and may interfere with reactivity of the antiserum with the 68-kDa band of vitronectin. (B) FCS (1% FCS), vitronectin-depleted serum (ΔFCS), vitronectin-depleted FCS reconstituted with 10 μg of purified vitronectin (ΔFCS + 10 μg Vn), and the eluate from the heparin affinity column (Hp El) were tested for their ability to mediate internalization of $OpaA^+$ gonococci (GC) by CHO cells. Adherence and internalization were quantified as described in the legend to Fig. 1.

they are not internalized (6, 30). The inability of these cells to internalize gonococci provides a controlled system in which specific factors can be analyzed for their involvement in the uptake of gonococci.

Our results indicate that the adhesive glycoprotein vitronectin promotes gonococcal entry into CHO cells. The successful identification of vitronectin as an internalization factor followed the observations that FCS was capable of mediating uptake of gonococci by CHO cells and that the FCS factor exerted its effect by binding to the surface of the bacteria. Subsequent fractionation of the FCS in combination with internalization assays and binding studies demonstrated that the active factor in serum was associated with a heparin-binding protein with an apparent molecular mass of 67, 80, 150, or >200 kDa. Analysis of several purified serum-derived heparin-binding proteins with molecular masses corresponding to those of proteins found during our isolation procedures identified vitronectin as a protein with the ability to mediate internalization of gonococci by CHO cells. Vitronectin migrates in SDS-PAGE as proteins with apparent molecular masses of 68 and 78 kDa. Unequivocal evidence that vitronectin was mediating the activity was demonstrated by the inability of serum which had been depleted of vitronectin to mediate uptake and the restoration of this deficiency by the reconstitution of the depleted serum with purified vitronectin. In addition, vitronectin purified from a variety of different sources, including fetal bovine serum, adult bovine serum, and human serum, bound to gonococci producing OpaA and stimulated their uptake by CHO cells.

Vitronectin has previously been shown to interact with the surface of several bacterial species, including *Escherichia coli*, *Staphylococcus aureus*, streptococci, *Pneumocystis carinii*, and *Neisseria meningitidis* (7, 15, 16, 32), and it has been shown to promote agglutination of gonococci (2). In *N. meningitidis*, the acquisition of vitronectin increases the adherence of this bacterium to human umbilical vein endothelial cells (32). Our data are unique in that they demonstrate that binding of vitronectin by bacteria may not only promote bacterial adherence but also

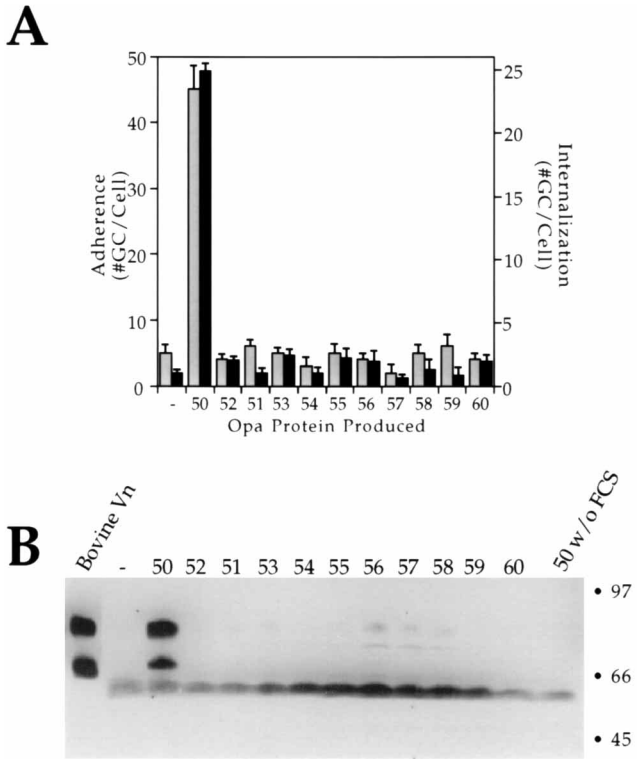

FIG. 6. Internalization and vitronectin binding of gonococci producing various Opa proteins. A panel of MS11 variants, each producing a different recombinant Opa protein (50 through 60) or no Opa protein (−) was tested for internalization by CHO cells (A) and vitronectin binding (B) in the presence of 5% FCS. In panel B, purified bovine vitronectin (Bovine Vn) and a lysate of MS11 Opa_{50}^+ which was not incubated with FCS (50 w/o FCS) were included as controls. The 63-kDa band present in all gonococcal samples in the Western blot represents cross-reactivity of the polyclonal antiserum with a gonococcal protein. Adherence and internalization were quantified as described in the legend to Fig. 1.

drive bacterial internalization by mammalian cells. In mammals, vitronectin is a multifunctional protein with effects on hemostasis, humoral defenses, and cellular adhesion (for reviews, see references 12 and 24), including the removal of protein complexes from the bloodstream. Our findings add to this repertoire the ability of vitronectin to promote a bacterial uptake process in normally nonphagocytic epithelial cells.

The uptake-promoting activity of vitronectin was limited to gonococci that expressed OpaA, suggesting that this protein is the prime binding site at the bacterial surface. Interestingly, this specific interaction with $OpaA^+$ gonococci has also been observed for the binding of heparan sulfate proteoglycans isolated from the surface of CHO cells (30). This raises the possibility that there are cooperative functions for OpaA, vitronectin, and heparan sulfate proteoglycans in the uptake of the bacteria by CHO cells. Many functions of vitronectin require the interaction of the protein with integrin receptors on the surface of mammalian cells. This interaction has been shown to be dependent on a conformational change of vitronectin (21, 22, 29, 37) and appears to require heparan sulfate proteoglycans (8, 21, 33, 37). Thus, one can imagine a scenario for gonococcal uptake in which heparan sulfate proteoglycans and vitronectin bind to the surface of $OpaA^+$ gonococci, which results in a change in conformation of vitronectin, allowing it to interact with integrins on the surface of CHO cells. This may eventually stimulate a signal transduction cascade and cytoskeletal rearrangements, resulting in phagocytosis of the bacteria. Alternatively, internalization of $OpaA^+$ gonococci may require the concomitant binding of OpaA to heparan sulfate proteoglycans and bacterium-associated vitronectin to integrins. Further identification of the host cell components involved in the gonococcal uptake process may further elucidate the mechanism by which vitronectin confers gonococcal entry into CHO cells.

Finally, it should be noted that the gonococcal uptake by CHO cells described here apparently differs from OpaA- and proteoglycan-dependent entry into Chang conjunctiva epithelial cells. In the Chang cell system, FCS is not required for internalization of $OpaA^+$ gonococci, and vitronectin has neither a stimulatory nor inhibitory effect on entry of $OpaA^+$ gonococci into Chang cells (data not shown). Comparison of both forms of bacterial uptake may prove to be useful in the further unravelling of the molecular interactions required for gonococcal uptake in both cell types.

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