Several Carcinoembryonic Antigens (CD66) Serve as Receptors for Gonococcal Opacity Proteins

By Tie Chen,* Fritz Grunert,‡ Andrew Medina-Marino,* and Emil C. Gotschlich*

Summary

Neisseria gonorrhoeae (GC) is a human pathogen that adheres to and invades genital surfaces. Although pili are required for the initial adherence, the interaction of GC with epithelial cells is also promoted by a family of outer membrane proteins, the opacity (Opa) proteins such as OpaA protein from strain MS11. Studies have demonstrated that the interaction of the OpaA GC with epithelial cells involves binding to heparan sulfate attached to syndecan receptors. However, other Opa proteins interact with CEA gene family member 1 (CGM1) or biliary glycoprotein (BGP), members of the CD66 antigen family. In this study, we demonstrate that, in addition, the 180-kD carcinoembryonic antigen (CEA) is a receptor for Opa proteins. This conclusion was based on the following observations. First, transfected HeLa cells expressing CEA (HeLa-CEA) and the CEA-expressing colon cancer cell line (LS 174T) bound and subsequently engulfed the Opa1 bacteria. These interactions were inhibited by anti-CEA antibody, but could not be inhibited by addition of heparin. Furthermore, OpaE coli directly bound purified CEA. We also compared the adherence and invasion by Opa1 bacteria of CD66 transfected HeLa cells: HeLa-BGPa, HeLa-CGM6, HeLa-NCA, HeLa-CGM1a, HeLa-CEA, and HeLa-Neo serving as negative control. Using Opa1 as the prototype, the relative ability of the transfected HeLa cell lines to support adherence was (CEA > BGPa > CGM1a > NCA > CGM6 = Neo). The ability to mediate invasion of the transfectant cells was (CGM1a > CEA > BGPa > NCA > CGM6 = Neo). Among the Opa proteins tested, OpaC proved to be bifunctional, able to mediate adherence to both syndecan receptors and to CD66 antigens.
entry of Listeria monocytogenes into epithelial cells (14). In GC, a variety of components participate in the interaction with the eukaryotic cells. These include pili (3), LPS (15), a 36-kDa glycolipid-binding adhesin (16), interaction with carbohydrate structures on epithelial cells (17), and the Opa proteins (18). In gonococcal strain MS11, the Opa protein family consists of 11 unlinked opa genes whose sequences are known (19). One distinct Opa protein, the OpaA, has been correlated with adherence and subsequent internalization of GC by Chang conjunctival cells (20–22). The other members of Opa family are able to stimulate PMN adherence and phagocytosis (22, 23). It has been demonstrated that the interaction of the OpaA GC with epithelial cells involves binding to heparan sulfate syndecan receptors that are situated on the cell surface (24, 25). Moreover, recent studies indicated that gene family member (CGM1) (26) and biliary glycoprotein (BGP) (27), members of the carci-

noembryonic antigen (CEA) or CD66 family, serve as receptors for OpaC bacteria for promoting adherence and internalization. In the instance of BGP or CD66a, it has been shown that the portion of the molecule that interacted with the OpaC protein is the NH2-terminal domain that is homologous to the IgG variable domain (IgV-like; 28). This is supported by the ability of CGM1 to interact with OpaC proteins since its surface-exposed portion consists solely of one IgV-like domain (26).

However, in addition to CD66a (BGP) and CD66d (CGM1), the CEA family also includes CD66b (CGM6), CD66e (NCA), and CD66f, which is called the classical tumor associated CEA. We have investigated the activity of the different CD66 antigens with OpaC proteins by expressing these five members of the CD66 family in HeLa cells and demonstrated that the transfectants differ in their ability to support adherence and internalization. Finally, OpaC protein from MS11 demonstrated a dual function; it interacted with both heparan sulfate syndecan receptors and members of the CEA family.

Materials and Methods

Bacterial Strains, Monoclonal Antibodies, and Cell Lines. GC strain MS11 was cultured and maintained as previously described (29). Only pilus GC and LOS (lacto-N-neotetraose) phenotype were used (30). Recombinant opa genes from GC MS11 were constructed and expressed in E. coli HB101 as described previously (23). The designations of Opa proteins of both GC and E. coli are based on papers of Swanson et al. (11) and Belland et al. (23). E. coli HB101 containing the vector pGEM-3Z is designated as pGEM. E. coli HB101 expressing OpaA, OpaB, OpaC, OpaH, and OpaI genes were designated as pEXA, pEXB, pEXC, pEXH, and pEXI, respectively. Bacterial suspensions were from Luria-Bertani plates containing 50 μg/ml carbenicillin after growth for 16–20 h at 37°C. COL-1 mAb, specific for CGM1 and CEA only, was donated by Z. Shi (Zymed Laboratories Inc., San Francisco, CA) and mAb IB4 reactive with CD18 was provided by S. Wright (Merck Inc., Rahway, NJ).

The human colon adenocarcinoma cell line LS 174T was purchased from American Type Culture Collection (Rockville, MD). HeLa-CEA, HeLa-CGM1a, HeLa-BGPa, HeLa-NCA, and HeLa-CGM6 cells were constructed by transfecting HeLa cells with CEA, CGM1a, BGPa, NCA, and CGM6 cDNAs, and selected for surface antigen expression (31). HeLa-Neo cells are HeLa cells that were transfected with neomycin-resistance gene only (31).

Wild-type Chinese hamster ovary (CHO)-K1 and isogenic mutants 745 and 677 that have specific defects in proteoglycan biosynthesis were provided by J.D. Esko (University of Alabama, Birmingham, AL) (32–34). Mutant CHO cell 745 lacks xylosyltransferase and expresses no heparan sulfate and chondroitin sulfate, while mutant 677 which has defective N-acetylgalosaminyl and glucoronosyltransferases, produces no heparan sulfate, but 2–3 times increased chondroitin sulfate.

A adherence and Internalization Assays. All cell lines were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) with 10% FCS (HyClone Labs., Logan, UT). For adherence assays, cells were grown to confluence (1–2 × 108 cells/well) in 24-well culture plates (Falcon, Lincoln Park, NJ), and washed twice with serum-free RPMI. E. coli were suspended in RPMI 1 at OD540 of 0.04, and 0.5 ml of the bacterial suspensions was added to each well. The plates were incubated at 37°C with 5% CO2 for 4 h. The incubation period was limited to 2 h when mAb were included to avoid their inactivation. Experiments were terminated by washing three times with 1 ml of serum-free RPMI. Adherent bacteria were counted by suspending the cells in PBS containing 0.5% saponin (Calbiochem Corp., La Jolla, CA) and plating dilutions on Luria-Bertani agar medium containing 50 μg/ml of carbenicillin or on GC plates. The level of adherence of GC and E. coli to cells was calculated by determining the CFU associated with the host cell monolayers. Internalization assays were done in a similar fashion to adherence assays, but after the period of bacte-

rial interaction to cell lines, the monolayers were washed twice and then incubated for 90 min with 1.5 ml of RPMI supplemented with 100 μg/ml gentamicin (GIBCO BRL). For adherence inhibition assays, the bacteria were added as a suspension in RPMI containing heparin at 30 μg/ml or 25 μg/ml mAb. The experiments were performed in duplicate or triplicate.

Binding of CEA to Opa + E. coli. pGEM or pEXI were sus-
pended in 1 ml of serum-free RPMI 1 at OD540 of 0.8. 4 μg of puri-
fied CEA antigen (Calbiochem Corp.) was added to each bacterial suspension and incubated at room temperature for 60 min with gentle shaking. The bacteria were pelleted and washed once with RPMI and subjected to SDS-PAGE. The CEA bound to the bacteria was detected by Western blotting with COL-1 mAb after electrophoretic transfer to Immobilon-P membrane (Millipore Corp., Bedford, MA) (35). The bound COL-1 mAb was detected with HRP-conjugated protein A by chemiluminescence (Amersham Life Science, Arlington Heights, IL).

Results

A adherence and Invasion of Opa+ Bacteria into HeLa-C66 Transfectants. The HeLa-Neo, HeLa-BGPa (CD66a), HeLa-CGM6 (CD66b), HeLa-NCA (CD66c), HeLa-CGM1a (CD66d), and HeLa-CEA (CD66e) were examined for their ability to support adherence and invasion of Opa+ bacteria. The interactional of Opa+ bacteria into HeLa-C66 transfectants was measured by gentamicin killing assay and the results were confirmed by electron microscopy. pEXI adhered poorly to HeLa-Neo and CGM6, but attached very well to HeLa-CEA and HeLa-BGP (Fig. 1 A). The
adherence of pEXI to HeLa-CGM1a and HeLa-NCA was intermediate.

The ability of these cell lines to promote invasion showed a different pattern. Although the adherence of pEXI to HeLa-BGPa was high, invasion was limited (Fig. 1 B). Electron microscopy demonstrated that HeLa-CEA cells were also able to engulf pEXI (Fig. 1 B) and OpaI GC (Fig. 2). However, compared to HeLa-CGM1a (Fig. 1 B, 2E), HeLa-CEA cells demonstrated lower ability to internalize pEXI or OpaI GC (Figs. 1 B and 2 B). Virtually all HeLa-CGM1a cells contained large numbers of GC, but a lower degree of cellular invasion by OpaI bacteria was observed with HeLa-CEA cells. HeLa-NCA and HeLa-CGM6 were hardly invaded at all.

OpaI E. coli Bind the CEA Antigen. The HeLa-CEA cells supported the highest level of adherence with pEXI (Fig. 1 A), indicating that the CEA protein is a receptor for Opa protein. To demonstrate a direct binding of Opa+ bacteria with CEA, purified CEA was mixed with equal amounts of Opa negative pGEM or positive pEXI bacteria. The bacteria-CEA mixture was incubated for 1 h, pelleted, and solubilized. The amount of CEA antigen bound to the bacteria was detected with COL-1 antibody by Western blotting. As seen in Fig. 3, pEXI bound much more CEA than pGEM.

Multiple Opa Proteins Interact with HeLa-CEA Cells, and Are Inhibited by Anti-CEA Antibody. Since strain MS11 can express several Opa proteins, we examined whether other Opa proteins possess the same function, as illustrated in Fig. 4 A. HeLa-CEA cells also bound OpaA, OpaB, OpaC, and OpaH E. coli with similar efficiency, but only OpaA-mediated adherence was strongly inhibited by soluble heparin.
The adherence mediated by Opa pal and OpaC was inhibited by anti-CEA antibody (COL-1), but not by IB4 directed to CD18 antigen (Fig. 4 B). These results demonstrate that CEA antigen serves as receptor for several Opa proteins.

Opa pal Bacteria Bind HeLa-CEA and CEA-expressing Colon Cancer Cells in a Heparin-independent Manner. To further substantiate that the binding of Opa pal GC with CEA is due to ligand–receptor interaction, we compared the well-characterized CEA-expressing colon cancer cell line LS 174T with HeLa-CEA in adhesion assays. As seen in Fig. 5 both HeLa-CEA and LS 174T bound the Opa pal GC in a heparin-independent fashion, in sharp contrast to the OpaA-mediated interaction. The colon cancer cell line supported adherence about threefold less efficiently than HeLa-CEA, but the overall pattern was identical.

OpaC Bacteria Interact with Both Heparan Sulfate and Members of the CEA Family. Previously published studies demonstrated that OpaA-mediated adherence to Chang conjunctival and CHO cells is dependent on binding to heparan sulfate syndecan receptors (24, 25). These studies also showed that OpaC had this activity, and that OpaC GC were able to bind [tritiated] heparin. However, the data shown in Fig. 4, A and B indicated that pEXC was able to bind the HeLa-CEA, and that this binding was inhibited by treatment with CD66 antibody, suggesting that OpaC was able to mediate both adherence to syndecans and CD66 antigens. To provide further evidence that OpaC-mediated adherence to CHO epithelial cells is heparan sulfate dependent, we examined the adherence to heparan sulfate-deficient CHO mutants 745 and 677. Compared to wild-type CHO-K1, OpaC GC adhered to mutant 745 and 677 at a very low level (Fig. 6 A). Further proof for reactivity with CD66 antigens is provided in Fig. 6 B showing that pEXC, but not pEXA, is internalized by HeLa-CGM1a.

Discussion

GC isolated either from the male urethra or from the cervix of infected females are most often Opa pal GC (36–38). Swanson et al. infected male volunteers intraurethrally with an Opa pal strain of GC, and found that isolates recovered from the infection were predominantly Opa pal (11). Similar findings were also obtained with different challenge strains in further volunteer studies (12). Furthermore, high levels of Opa pal-specific antibody after gonococcal infection have been reported (39). Taken together, these data strongly suggest in vivo expression of Opa pal proteins plays an important role in gonococcal pathogenesis.

The present studies demonstrate that one of the mechanisms for Opa pal protein-mediated adherence was binding to CEA and CEA-related proteins. To provide this evidence, we chose to use a highly defined system, namely the use of E. coli expressing recombinant Opa pal proteins and a single cell line, HeLa transfected with various CD66 antigens. This strategy circumvents the unavoidable tendency of GC to alter their Opa phenotype by antigenic variation and allows certainty that the observed effects are strictly attributable to the expression of specific Opa pal proteins. To confirm that the results were reflective of gonococcal pathogenesis, we included experiments using Opa pal GC and also extended them to a well-characterized CEA-expressing colon cancer cell line, LS 174T. The CEA HeLa cell transfectants (HeLa-CEA) and the CEA-expressing colon cancer line bound and engulfed Opa pal bacteria and this interaction...
was inhibited by anti-CEA mAb. In addition, Opa E. coli bound purified CEA antigen more effectively than Opa E. coli.

CEA, a 180-kD tumor-associated cell-surface glycoprotein, was first described in 1965 (40). Later, it was found that antibodies to CEA reacted at low level with normal cells. Also, a number of closely related, cross-reacting antigens were identified that were initially termed nonspecific cross-reacting antigens (NCA; 41–43). The gene encoding CEA is located on human chromosome 19 (44), and is a member of a family of >20 expressible closely related genes (45) that belong to the Ig gene superfamily (46). The human CEA family consists of CEA, NCA, BGP (47), CGM1 including splicing variants CGM1a, CGM1b, and CGM1c (31, 48), CGM2, CGM6, CGM7, and pregnancy-specific glycoproteins (49–51). Although the in vivo function of CEA has not been determined, evidence shows that CEA functions as an intercellular adhesion molecule (52–54). The binding of CEA to E. coli has been reported (55–57), and CEA may also play a role as an accessory molecule in binding tumor cells to collagen type I (58).

We investigated the relative ability of the individual CD66 antigens to mediate adherence and internalization of Opa+ bacteria using HeLa cell transfectant cell lines. HeLa-CEA and HeLa-BGPa showed the highest level of adherence to Opa+, but BGP internalized the bacteria poorly. On the other hand, HeLa-CEA 1a and HeLa-NCA showed intermediate levels of adherence, and only CGM 1a promoted aggressive phagocytosis of Opa+ E. coli (26) and GC (Fig. 2 E). HeLa-CGM 6 (CD66b) could neither bind or engulf Opa+ bacteria (Fig. 1 A), although CGM 6 is highly expressed on this HeLa cell line (59). Since CEA is not expressed in neutrophils (60), the most likely candidate for promoting phagocytosis of Opa+ bacteria is CGM 1a. These results indicated that CD66 family showed variable ability in promoting adherence and internalization of Opa+ bacteria although they share homology in their NH2 termini (60).

The mechanism of CGM 1a-promoted internalization may be that in CGM 1a there is a cytoplasmic domain containing the YLYL motif, termed the immunoreceptor tyrosine activation motif, which is found in several receptor subunits that associate with cytoplasmic tyrosine kinases (61, 62). Activation of this motif leads to actin polymerization and phagocytosis (for review see reference 63). BGPa, which does not promote internalization of bacteria, does not contain immunoreceptor tyrosine activation motif in its cytoplasmic domain, but rather, two copies of immunoreceptor tyrosine inhibitory motif that are separated by 26

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**Figure 5.** The interaction of Opa+ GC with HeLa-CEA cells and CEA-expressing colon cancer cells LS 174T. Opa+ GC were incubated with HeLa-CEA (A) in RPMI medium 1640 buffer with or without soluble heparin (30 μg/ml). OpaA and OpaI promoted adherence to HeLa-CEA, and Opa+ mediated adherence was inhibited by heparin. Similar results were observed with CEA-expressing colon cancer line LS 174T.

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**Figure 6.** Binding specificity of Opac bacteria for both heparan sulfate and CD66 receptors. Opac GC were incubated with wild-type CHO-K1 cells and the isogenic mutants 745 and 677 lacking surface heparan sulfate. Opac GC adhered to CHO-K1 cells, but not the 745 and 677 mutants (A). (B) pGEM, pEXA, and pEXC were incubated with HeLa-CGM 1a cells in RPMI 1640 medium for 4.5 h. The adherent and intracellular E. coli were distinguished by incubation with gentamicin. Only pEXC was recovered in large numbers after gentamicin treatment, although both pEXA and pEXC adhered to the HeLa-CGM 1a.
amino acids (47). This motif has been found in a number of immunoreceptors that mediate inhibitory effects (64, 65). However, CEA found on LS 174T cells is anchored to the membrane through a glycosyl-phosphatidylinositol anchor (66, 67). The GPI-anchored proteins mediate many functions in cell growth and cell-cell communication, and can initiate signal transduction (68–70). Thus, it is possible that the phagocytosis of Opa proteins by means of CGM1a in neutrophils and CEA in epithelial cells uses the two distinct signaling mechanisms.

Among the Opa proteins tested, only OpaA showed no reactivity with CEA or CGM1a. OpaA has previously been shown to be reactive with heparan sulfate borne by syndecans and to bind radioactive heparin (24, 25). These studies showed that OpaC also had this specificity. Thus, the question of whether OpaC protein can interact with both heparan sulfate syndecan receptors and members of the CEA family arose. The data shown in Fig. 6A demonstrates that OpaC binds to normal CHO cells, but failed to bind to CHO mutant cells defective in heparan sulfate synthesis, which was further proof for this specificity. On the other hand, OpaC promoted heparin-independent adherence to HL-a-CEA cells (Fig. 4A), and induced the phagocytosis of OpaE. coli into Hl-a-CGM1a (Fig. 6B). Thus, MS11 OpaC protein appears to target the two distinct receptors.

Gonorrhea is a genital disease that affects exclusively human beings, and adherence and invasion of epithelial cells is one of the important steps in this disease, as well as bacterial infections in general (71). However, the factors determining the efficient adherence and internalization of GC with or into specific cells are still poorly understood and are very dependent on the specific tissue culture cell line used (72). Identification of CEA and BGP as a receptor for Opa protein is an important step in our understanding of GC pathogenesis. Normal adult human colon epithelium is known to produce small amounts of CEA-like antigens (73). GC, when isolated from rectal infections, express Opa proteins (38). It has also been shown that BGP is expressed on normal epithelia located in many organs including the uterus, cervix, prostate, and colon (74). Thus, it is likely that most of the tissues susceptible to GC infection express one or another member of the CD66 antigen family.

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Address correspondence to Tie Chen, Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Ave., New York, NY 10021-6399.

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