Chlamydia pneumoniae Uses the Mannose 6-Phosphate/Insulin-Like Growth Factor 2 Receptor for Infection of Endothelial Cells

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Several mechanisms for attachment and entry of Chlamydia have been proposed. We previously determined that the major outer membrane protein of Chlamydia trachomatis is glycosylated with a high-mannose oligosaccharide, and a similar structure inhibited the attachment and infectivity of C. trachomatis in epithelial cells. Because insulin-like growth factor 2 (IGF2) was shown to enhance the infectivity of Chlamydia pneumoniae but not C. trachomatis in endothelial cells, a hapten inhibition assay was used to analyze whether the mannose 6-phosphate (M6P)/IGF2 receptor that also binds M6P could be involved in infection of endothelial cells (HMEC-1) by Chlamydia. M6P and mannose 6-phosphate-poly[N-(2-hydroxyethyl)acrylamide] (M6P-PAA) inhibited the infectivity of C. pneumoniae AR-39, but not C. trachomatis serovar UW5 or L2, while mannan inhibited the growth of C. trachomatis, but not C. pneumoniae. Using metabolically labeled organisms incubated with cells at 4°C (organisms attach but do not enter) or at 37°C (organisms attach and are internalized), M6P-PAA was shown to inhibit attachment and internalization of C. pneumoniae in endothelial cells but did not inhibit attachment or internalization of C. trachomatis serovar E or L2. These findings indicate that C. pneumoniae can utilize the M6P/IGF2 receptor and that the use of this receptor for attachment and entry differs between C. pneumoniae and C. trachomatis.

Chlamydia pneumoniae is an intracellular bacterium that causes respiratory infections. C. pneumoniae infections can also become chronic, and chronic infections of the vasculature have been suggested to initiate and/or promote atherogenesis (4). Because chlamydiae multiply only within eukaryotic cells, internalization of the infectious chlamydial elementary bodies (EBs) and the avoidance of fusion of EB-containing phagosomes with cellular lysosomes are critical steps for establishment of infection. Chlamydiae appear to use several mechanisms to enter host cells, including receptor-mediated endocytosis in clathrin-coated pits, pinocytosis in non-clathrin-coated pits, and phagocytosis (reviewed in reference 28). In general, EBs are rapidly internalized by eukaryotic cells, even nonprofessional phagocytes (3). Most likely there are multiple mechanisms for attachment and internalization, which may differ depending on the host cell type. However, the specific chlamydial structures and receptor molecules on eukaryotic cells that mediate chlamydial entry remain undefined.

Several chlamydial ligands have been suggested to mediate attachment, including heparan sulfate, chlamydial hsp70, OmcB, and the major outer membrane protein (MOMP) (28). Previous studies from our laboratory have shown that Chlamydia trachomatis MOMP is glycosylated and contains N-linked high-mannose-type oligosaccharides (16). Also, the mannose residues in the glycan have been shown to mediate internalization, as C. trachomatis can utilize the mannose receptor to enter and establish productive infection in mouse macrophages, while C. pneumoniae appears to use a receptor(s) other than the mannose receptor (15).

Recently, a monocyte-derived soluble factor, insulin-like growth factor 2 (IGF2), was shown to enhance infection of endothelial cells by C. pneumoniae, but not C. trachomatis, suggesting involvement of a IGF2 receptor in C. pneumoniae infection (18, 19). The IGF2 receptor also binds mannose 6-phosphate (M6P) and is called the M6P/IGF2 receptor (23). The majority of this receptor is localized intracellularly, and 10 to 20% is on the cell surface. Functions of the M6P/IGF2 receptor include transport of phosphomannosylated lysosomal enzymes from the Golgi complex into lysosomes and endocytosis of exogenous lysosomal enzymes from clathrin-coated pits (23). A third ligand, which binds to the M6P/IGF2 receptor at a separate site from IGF2 or phosphomannosylated residues, is the antioxidant retinoic acid (RA) (13). Binding of RA to the cellular M6P/IGF2 receptor affects binding of the phosphomannosylated residues and alters intracellular trafficking of the M6P/IGF2 receptor and its ligands (12). The aim of this study was to investigate whether the M6P/IGF2 receptor can act as a cellular receptor for C. pneumoniae.

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MATERIALS AND METHODS

Chlamydial organisms. C. pneumoniae AR-39 and C. trachomatis UW-5 (E/ UW-5/Cx) and L2 (L2/434/Bu) were propagated in cell culture in HL (human line) cells and HeLa cells, respectively, and purified as described earlier (18). Cell lines. A human transformed arterial endothelial cell line (HMEC-1) was maintained as described previously (18). Mouse L cells (D9), which lack the M6P/IGF2 receptor, and mouse L cells transfected with bovine M6P/IGF2 receptor (CC2) were generously provided by Stuart Kornfeld, Washington University, St. Louis, Mo. (14, 20). D9 cells are not transfected. Mouse L cells were grown in a minimal essential medium containing 10% heat-inactivated fetal calf serum.
serum and 100 μg/ml of streptomycin (D9 cells) or 500 μg/ml of G418 (CC2 cells). To ascertain expression of the M6P/IGF2 receptor, HMEC-1, D9, and CC2 cells were stained with a monoclonal antibody that recognizes an epitope in the extracellular domain of M6P/IGF2 receptor (Affinity Bioreagents, Golden, CO). As expected, intense staining was observed on the surface and cytoplasm of CC2 cells, whereas no staining was seen in D9 cells (not shown). In HMEC-1 cells, most of the staining was seen in the cytoplasm, which is in accordance with the observation that approximately 10 to 20% of the receptor is localized on the cell surface.

**Infection of cells with chlamydiae.** Confluent HL, HMEC-1, and mouse L cell monolayers growing on glass coverslips in 24-well plates were inoculated with *C. pneumoniae* or *C. trachomatis* at a multiplicity of infection of 10. The plates were incubated for 2 h at 37°C on a rocking platform. The optimal infectious dose was predetermined to achieve 100 to 300 chlamydial inclusions in 30 ×1,000 fields. After 2 (*C. trachomatis*) or 3 (*C. pneumoniae*) days of incubation, the cell monolayers were fixed and stained with Chlamydia genus-specific monoclonal antibody (CF-2) conjugated to fluorescein isothiocyanate or with *C. pneumoniae*-specific monoclonal antibody TT-401 conjugated to fluorescein isothiocyanate.

**Inhibition of infectivity by selected ligands.** Inoculating HMEC-1 cells in the presence of 1 to 10 mM M6P, 1 to 10 mM glucose 6-phosphate (G6P), yeast mannose (1 mg/ml), or heparin (5 to 500 μg/ml) (all from Sigma, St. Louis, MO) or 1 to 65.5 μg/ml M6P-PAA (mannose 6-phosphate-poly-N-(2-hydroxyethyl)-acrylamide) (GlycoTech, Rockville, MD) at 37°C. In some experiments, the ligands were also present during the culture period of 2 to 3 days (Fig. 1; see also Fig. 3). In other experiments using M6P, M6P-PAA, and heparin, ligands were present only in the inoculum (Fig. 2). No differences were observed in the effect on *C. pneumoniae* growth regardless of whether the hapten was present in the inoculum or in the inoculum and growth medium. Control cultures were inoculated and grown in media without ligands.

**The retinoids used in hapten inhibition experiments were RA (0 to 25 μM) and 4-(E)-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenylbenzoic acid (TTNPB) (0 to 12.5 μM) (both from Sigma, St. Louis, MO). In addition to binding to the M6P receptor, RA can also bind to the nuclear retinoic acid receptor (RAR). TTNPB is an RA analog that binds to the RAR but not to the IGF-2/M6P receptor and thus can be used to differentiate effects of RA that are due to binding to the IGF-2/M6P receptor or the RAR (12). For these hapten inhibition experiments, two methods were used. Either cells were incubated for 1 h prior to inoculation with RA or TTNPB and washed with phosphate-buffered saline (PBS) prior to inoculation or the retinoid was added to the inoculum as described above.

**For binding assays, cells were metabolically labeled with 15N-methionine as described previously (25) and incubated with cells for 1 h at 4°C (organisms attach but do not enter host cells) or at 37°C (organisms attach and enter) in the presence of M6P-PAA or buffer. After being washed to remove unbound label, cell lysates were air dried on glass fiber filter papers. Filters and scintillation fluid (Formula-989; Packard Instrument Co., Meriden, CT) were added to scintillation vials, and the radioactivity of bound or internalized EBs was counted on the scintillation counter.

To study the role of the M6P/IGF2 receptor in *C. pneumoniae* infection, mouse L cells and the same cells transfected with bovine M6П/IGF2 receptor were first treated with β-glucuronidase (1 to 1,000 U/ml), a substrate for the receptor (14, 17, 22, 24), for 30 min prior to inoculation at 4°C to allow binding to the receptor but not internalization of the ligand-receptor complex. Subsequently, the cells were inoculated with *C. pneumoniae* and *C. trachomatis* in the presence of β-glucuronidase for 1 h at room temperature to allow both attachment and internalization. In some experiments, mouse L cells were pretreated with Wortmannin (40 to 1,000 nM; Sigma, St. Louis MO) for 30 min at 37°C before inoculation. Wortmannin was also present during inoculation. It has been shown that one of the effects of Wortmannin is to decrease the number of M6P/IGF2 receptors on the cell surface (14). Triplicate coverslips were counted for each experiment, and each experiment was repeated twice. Inclusions were counted in 30 fields at a magnification of ×400. Infectivity titers expressed as inclusion forming units (IFU)/ml were based on inclusion counts and standardized by adjusting for the dilution factor and inoculum volume.

**BAP treatment.** Prior to inoculation of cells, the organisms were incubated for various periods (0.5 to 3 h) with 20 U bacterial alkaline phosphatase (BAP; Sigma) in potassium phosphate buffer at pH 7.4 to 7.8 and 37°C. The control inoculum was treated in the same manner, except BAP was not added. Subsequently, HMEC-1 and mouse L cells were inoculated as described above.

**Statistical analysis.** Statistical analysis was done using Student’s t test.
RESULTS

Hapten inhibition of C. pneumoniae and C. trachomatis infections in endothelial cells. M6P (1 to 10 mM) significantly inhibited the growth of \( C.\ pneumoniae \) in HMVEC-1 cells in a dose-dependent manner at 5 and 10 mM (Fig. 1A). G6P (10 mM), a phosphorylated sugar that does not bind to the M6P/IGF2 receptor, or mannan (1 mg/ml), a ligand of the mannose receptor, did not affect the growth of \( C.\ pneumoniae \). In contrast, M6P did not affect growth of \( C.\ trachomatis \) serovar E, whereas mannan did inhibit infectivity (Fig. 1A). In addition, treatment of cells with 10 mM M6P had no effect on \( C.\ trachomatis \) serovar L2 infectivity of endothelial cells (data not shown). M6P-PAA, a water-soluble polyvalent glycoconjugate, inhibited the infectivity of \( C.\ pneumoniae \) (Fig. 2A) but not \( C.\ trachomatis \) serovar L2 (data not shown).

To determine whether inhibition of infectivity occurred at attachment, internalization, or neither, binding assays were done at 4°C (organisms attach but are not internalized) or 37°C (organisms attach and enter the host cell). M6P-PAA inhibited attachment and entry of radiolabeled \( C.\ pneumoniae \) AR-39 (Fig. 2A). In contrast, M6P-PAA had no effect on the attachment of \( C.\ trachomatis \) serovar E (UW5) (Fig. 2B) or \( C.\ trachomatis \) serovar L2 (data not shown). As a positive control, heparin, a polyanionic compound that competitively inhibits infection of epithelial cells by preventing attachment (6, 7, 27), was also tested at 5 to 500 μg/ml. A dose-dependent inhibition of \( C.\ pneumoniae \) infection of endothelial cells was observed (Fig. 1B). Heparin, which was tested at 500 U/ml, was also shown to inhibit attachment (73% reduction at 4°C; \( P < 0.01 \)) and entry (57% reduction at 37°C; \( P < 0.05 \)) of radiolabeled \( C.\ pneumoniae \) EBs.

RA has been shown to bind to the M6P/IGF2 receptor at a site distinct from IGF-2 and M6P (13). Binding of RA to the cellular M6P/IGF2 receptor affects binding of the phosphomannosylated residues and alters intracellular trafficking of the M6P/IGF2 receptor and its ligands (12). Thus, whether RA would also affect the infectivity of \( C.\ pneumoniae \) was determined. Retinoids can elicit a variety of cellular responses (5, 21) and interfere with glycosylation of viral proteins (11). The classical mechanism of action is mediated by binding of retinoids to the nuclear RAR and retinoid X receptor. In order to differentiate effects that are due to binding to the M6P/IGF2 receptor from those due to binding to the nuclear receptors, the RA analog TTNPB, which binds to the nuclear receptor but not to the M6P/IGF2 receptor, was also tested in hapten inhibition experiments. As shown in Fig. 1C, RA inhibited infection of endothelial cells by \( C.\ pneumoniae \) regardless of whether the cells were preincubated with RA before inoculation or RA was added at the time of inoculation of cells with \( C.\ pneumoniae \). In contrast, TTNPB had no effect on \( C.\ pneumoniae \) infection of endothelial cells, indicating that the effect of RA on infectivity occurred at the level of the M6P/IGF2 receptor.

\( C.\ pneumoniae \) infection in mouse L cells. To study whether \( C.\ pneumoniae \) also uses the M6P/IGF2 receptor for internalization into other eukaryotic cell types, mouse L cells (D9) and L cells that had been transfected with the bovine M6P/IGF2 receptor (CC2) were infected with \( C.\ pneumoniae \). \( C.\ pneumoniae \) infected both D9 and CC2 cells, but the infectivity titers were 1.5 times higher in CC2 cells than in D9 cells (2.6 ⋅ 10^6 ± 1.2 ⋅ 10^6 IFU/ml versus 1.7 ⋅ 10^6 ± 0.7 ⋅ 10^6 IFU/ml, respectively; \( P < 0.05 \); \( n = 28 \)). To further confirm that the increased infectivity of CC2 cells was due to utilization of the M6P/IGF2 receptor, mouse L cells were treated with β-glucuronidase, an acid hydrolase whose uptake is known to be mediated by M6P/IGF2 receptor (14, 22, 24). β-Glucuronidase treatment decreased the infectivity of \( C.\ pneumoniae \) for CC2 cells, whereas the infectivity for D9 cells was unaffected (Fig. 3A). In contrast, the growth of \( C.\ trachomatis \) L2 was not affected by β-glucuronidase in either CC2 cells (Fig. 3B) or D9 cells (data not shown). To confirm these findings, mouse L cells were treated with wortmannin to reduce the number of M6P/IGF2 receptors on the cell surface (14) and subsequently infected with \( C.\ pneumoniae \). Wortmannin treatment reduced

FIG. 2. Effects of M6P-PAA treatment on growth of \( C.\ pneumoniae \) and \( C.\ trachomatis \) and attachment to and internalization by endothelial cells. For hapten inhibition studies, endothelial cells were inoculated with \( C.\ pneumoniae \) (AR-39) or \( C.\ trachomatis \) (UW5) in the presence of different concentrations of M6P-PAA. Growth of \( C.\ pneumoniae \) and \( C.\ trachomatis \) is expressed as mean inclusion counts per well (as a percentage of the control). For binding assays, endothelial cells were inoculated with [35S]methionine-labeled purified \( C.\ pneumoniae \) organisms in the presence of M6P-PAA for 2 h at 4°C or 37°C and washed. The radioactivity in cell lysates was counted and expressed as the mean of cpm per well (as a percentage of the control). The error bars indicate 1 standard deviation from the means of cpm from triplicate wells. (A) Effect of M6P-PAA treatment on \( C.\ pneumoniae \). \( P < 0.05 \) (Student’s t test) for treated cultures versus untreated cultures (control) at all time points for growth (infectivity), attachment, and internalization of \( C.\ pneumoniae \). (B) Effect of M6P-PAA treatment on \( C.\ trachomatis \). There were no statistically significant differences observed in treated cells versus untreated controls.
the growth of \textit{C. pneumoniae} in CC2 cells but not in D9 cells (Fig. 3C).

Treatment with bacterial alkaline phosphatase decreases infectivity of \textit{C. pneumoniae} for CC2 and endothelial cells, but not D9 cells. As an alternative approach, \textit{C. pneumoniae} organisms were incubated for 0.5 to 3 h with BAP to remove phosphorylated residues prior to infection of host cells. Both endothelial cells and CC2 cells infected with \textit{C. pneumoniae} treated with BAP for 3 h demonstrated statistically significant decreases in inclusion counts in comparison to cells infected with \textit{C. pneumoniae} incubated in PBS for 3 h prior to inoculation (88% reduction and 85% reduction, respectively; \( P < 0.05 \)). In contrast, no effect of BAP treatment was observed on the infectivity of \textit{C. pneumoniae} for D9 cells.

**DISCUSSION**

The IGF2-mediated enhancement of \textit{C. pneumoniae} infection of human arterial endothelial cells suggested that infection could be affected by mechanisms involving an IGF2 cell surface receptor (18). Because binding of IGF2 to the M6P/IGF2 receptor inhibits uptake of phosphomannosylated lysosomal enzymes into the endocytic vacuoles, which are ultimately delivered to the lysosomes (23, 26), Lin et al. hypothesized that binding of IGF2 or simultaneous binding of IGF2 and the glycan moiety of \textit{C. pneumoniae} to the M6P/IGF2 receptor could promote intracellular survival of \textit{C. pneumoniae} in the phagosome (19). The corollary to this hypothesis was that if the \textit{C. pneumoniae} high-mannose oligosaccharide bound to the M6P binding site on the M6P/IGF2 receptor, which is distinct from the IGF2 binding site (23), phosphomannosylated residues that bind to this receptor should decrease the infectivity of \textit{C. pneumoniae}. Accordingly, differences were observed between \textit{C. pneumoniae} and \textit{C. trachomatis} in the hapten inhibition assay, as both M6P and M6P-PAA, but not G6P, competitively inhibited infection of human arterial endothelial cells by \textit{C. pneumoniae} but the growth of \textit{C. trachomatis} serovars E and L2 was not affected by any of the haptens. \textit{C. pneumoniae} infectivity was also inhibited by RA, which binds to a site distinct from that of phosphomannosylated residues on the M6P/IGF2. That this effect on infectivity was not mediated through the RAR was shown by the lack of any effect of the RA agonist TTNPB, which binds only to the RAR.

These results suggest that the M6P/IGF2 receptor serves as a receptor for \textit{C. pneumoniae}. This usage was further supported by binding assays showing that attachment and internalization of endothelial cells by \textit{C. pneumoniae} was inhibited by M6P-PAA, while M6P-PAA did not affect attachment or internalization by \textit{C. trachomatis}. Collectively, these results suggest that \textit{C. pneumoniae} competes with phosphomannosylated residues for the same binding site on the M6P/IGF2 receptor.

M6P receptors have been reported to act as cell surface receptors for other pathogens, namely, human herpesviruses (1, 2, 29). Herpes simplex virus glycoprotein D contains M6P residues and binds to the M6P receptor (1, 2). At least four envelope glycoproteins of varicella-zoster virus (10) contain N-linked complex oligosaccharides with M6P groups, which serve as ligands for binding to the M6P/IGF2 receptor, and M6P competitively inhibits viral infection of host cells (29).
a recent study using antisense cDNA or small interfering RNA-like transcripts to generate human cell lines deficient in the M6P receptor, the deficient lines were resistant to infection by varicella-zoster virions (8). It remains to be determined which glycoprotein on C. pneumoniae is modified with 6-phosphate residues; however, a prime candidate is the high-mannose oligosaccharide moiety on the MOMP (16). Interestingly, treatment of C. pneumoniae with BAP prevented infectivity of mouse CC2 cells expressing the M6P receptor. While BAP treatment would also affect any other phosphorylated entity on the chlamydial cell surface, the finding that neither C. pneumoniae in infected mouse macrophages, and neither mannan nor mannose-PAA treatment of infected mouse macrophages from envelope glycoproteins was found to inhibit infection of host cells (29).

The inhibition of infectivity of C. trachomatis but not C. pneumoniae in endothelial cells by mannan suggests that C. trachomatis uses a different receptor, the mannose receptor, to enter and establish infection in endothelial cells. Microvascular endothelial cells are known to express the mannose receptor. Similar results were observed in our previous studies demonstrating differences in the susceptibilities of mannose receptor-positive mouse macrophages and mannose receptor-negative mouse macrophages to C. trachomatis and C. pneumoniae infection. Specifically, C. trachomatis infected mannose receptor-positive cells better than mannose receptor-negative cells. Furthermore, infection of mannose receptor-positive cells by C. trachomatis could be inhibited with yeast mannan and mannose-PAA. In contrast, C. pneumoniae infected mannose receptor-negative macrophages better than mannose receptor-negative cells. Further, infection of mannose receptor-positive cells by C. trachomatis could be inhibited with yeast mannan and mannose-PAA. In contrast, C. pneumoniae infected mannose receptor-negative macrophages, and neither mannan nor mannose-PAA affected C. pneumoniae infection in mouse macrophages (15).

Taken together, these results suggest that although both C. trachomatis and C. pneumoniae have glycan moieties containing high-mannose oligosaccharide residues, there are differences in phosphorylation of the mannose residues that affect receptor usage. Heparin, a polyanionic compound, competitively inhibits infection of epithelial cells with C. trachomatis (6, 7) and C. pneumoniae (27) by preventing attachment. The current study also showed diminished attachment by and decreased infectivity of C. trachomatis and C. pneumoniae in endothelial cells after heparin treatment. Although the heparin- and heparan sulfate-inhibitable mechanisms are clearly important in the uptake of C. trachomatis into eukaryotic cells (6, 7, 27), others have suggested that heparin- and heparan sulfate-like glycosaminoglycans act only as initial receptors for some organisms by concentrating them on the cell surface, after which more specific receptors mediate entry (1, 27, 29). Specifically, it has been proposed that entry of herpes simplex virus and varicella-zoster virus is a two-step process. The first step is an initial attachment of the virus to cell surface heparan sulfate proteoglycan, followed by the subsequent interaction of the M6P residues on viral glycoproteins with the M6P/IGF2 receptor for viral entry into the host cell (29). As with C. pneumoniae, both heparin and M6P inhibit infection of cells by varicella-zoster virus (29). Thus, it is possible that a similar mechanism is also utilized by C. pneumoniae.

That C. pneumoniae uses the M6P/IGF2 receptor was supported by results from experiments using mouse L cells that had been transfected with bovine M6P/IGF2 receptor (CC2 cells) and noninfected L cells (D9 cells). Although both cell lines were susceptible to infection with C. pneumoniae, demonstrating that C. pneumoniae can also use other potential receptors, infectivity was greater in CC2 cells than D9 cells, suggesting that C. pneumoniae can also use M6P/IGF2 receptor for entry and successfully establish infection. Treatment with β-glucuronidase (a ligand for M6P/IGF2 receptor) and wortmannin (which decreases the number of receptors by 90%) at the plasma membrane by retarding receptor recycling from the endosome to the plasma membrane (14) decreased the infectivity of CC2 cells but had no effect on the infectivity of D9 cells. Because wortmannin is known to inhibit phosphatidylinositol (phosphoinositide) (PI) 3-kinase, it was concluded that this enzyme played a role in regulating trafficking of the M6P/IGF2 receptor (14). Interestingly, inhibitors of PI 3-kinase prevent C. pneumoniae entry into HEP-2 cells (9). The possibility that the effect of wortmannin on the infectivity of endothelial cells is due to another effect resulting from inhibition of PI 3-kinase signaling cannot be ruled out. However, if that is the case, equal effects on infectivity would be expected in both cell types. Because the only difference between CC2 cells and D9 cells is the expression of the M6P/IGF2 receptor, the fact that no effect of wortmannin was observed on infectivity of D9 cells in contrast to CC2 cells suggests that the decreased infectivity of wortmannin-treated CC2 cells reflected its action on the M6P/IGF2 receptor.

In conclusion, M6P and/or M6P-PAA, but not mannan, inhibited the infectivity of C. pneumoniae in human arterial endothelial cells. In contrast, mannan, but not M6P, inhibited the infectivity of C. trachomatis. These findings suggest differences in receptor usage between the two species. Specifically, C. pneumoniae may preferentially use the M6P/IGF2 receptor, while C. trachomatis may use the mannose receptor, for entry and infection of endothelial cells.

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