INFECTION AND IMMUNITY, Sept. 1995, p. 3600–3608 Vol. 63, No. 9
0019-9567/95/$04.00+0

Interaction of Outer Envelope Proteins of *Chlamydia psittaci* GPIC with the HeLa Cell Surface

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Received 27 April 1995/Returned for modification 22 May 1995/Accepted 22 June 1995

The chlamydial life cycle involves the intimate interaction of components of the infectious elementary body (EB) surface with receptors on the susceptible eukaryotic cell plasma membrane. We have developed an in vitro ligand binding assay system for the identification and characterization of detergent-extracted EB envelope proteins capable of binding to glutaraldehyde-fixed HeLa cell surfaces. With this assay, the developmentally regulated cysteine-rich envelope protein Omp2 of *Chlamydia psittaci* strain guinea pig inclusion conjunctivitis was shown to bind specifically to HeLa cells. HeLa cells bound Omp2 selectively over other cell wall-associated proteins, including the major outer membrane protein, and the binding of Omp2 was abolished under conditions which alter its conformation. Furthermore, trypsin treatment, which reduces EB adherence, resulted in the proteolytic removal of a small terminal peptide of Omp2 at the EB surface and inactivated Omp2 in the ligand binding assay, while having a negligible effect on the major outer membrane protein. Collectively, our results suggest that Omp2 possesses the capacity to engage in a specific interaction with the host eukaryotic cell. We speculate that, since Omp2 is present only in the infectious EB form, the observed in vitro interaction may be representative of a determining step of the chlamydial pathogenic process.

Chlamydiae are obligate intracellular bacteria which cause diseases in humans and animals worldwide (38, 42). They exist as two morphologically and functionally distinct forms, the elementary body (EB) and the reticulate body. The infectious EB, which survives extracellularly but is metabolically inert, attaches to and enters into a susceptible epithelial cell, where it differentiates into a metabolically active and replicating form, the noninfectious reticulate body. Reticulate bodies eventually differentiate back into infectious EBs, which are released from the infected cell to start a new replication cycle (31).

The earliest and perhaps most crucial event in chlamydial pathogenesis is the attachment of an infectious EB to a susceptible mucosal epithelial cell. This is thought to occur by a receptor-mediated adherence mechanism involving high-affinity adhesins present at the EB surface (22, 23, 35, 40, 46, 50). The specificity and affinity of this binding interaction are, presumably, determining factors for the next step, entry of the bound EB into the host cell. Once internalized, the nascent EB-containing inclusion must still fend off at least one additional line of cellular defense, fusion with bactericidal lysosomes. By a mechanism which remains unknown, vacuoles which contain invading chlamydiae do not fuse with lysosomes (14). This provides the differentiating EB with a uniquely safe haven for growth, offering protection from within and from without, i.e., from host cellular and immune defenses. Remarkably, all determinants required for each of these three early steps of the pathogenic process, i.e., attachment, internalization, and inhibition of phagolysosome fusion, are contained in the EB envelope, since purified cell walls can be used to faithfully reproduce the EB entry pathway (15, 29).

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Without the power of genetic analysis of *Chlamydia* spp., relatively little is firmly defined about the molecular mechanisms mediating EB attachment to eukaryotic cells. The following EB envelope components have been proposed as adhesins: (i) the major outer membrane protein (MOMP), whose role in adherence is based on the inhibitory properties of trypsin and of monoclonal antibodies which target surface-exposed domains of MOMP (43, 44); (ii) a 38-kDa cytadhesin shown to bind specifically to glutaraldehyde-fixed HeLa cells (24, 25); (iii) an unidentified glycosaminoglycan-like molecule implicated in a trimolecular mechanism involving unidentified glycosaminoglycan receptors on EBs and host cells (12, 51); (iv) an unidentified glycan presumed to be covalently linked to MOMP (45); and (v) the heat shock protein Hsp70 or a genetically linked gene product, whose role in attachment and invasion was suggested by use of recombinant methods (34, 39).

A possible role in pathogenesis is also suggested for the cysteine-rich Omp2 protein on the basis of its selective presence in the cell envelope of the infectious EB (19, 20, 32, 37) and the observation that Omp2 is processed differentially and significantly more basic in EBs of the more virulent *Chlamydia trachomatis* LGV biovar (4). Experiments using Omp2-specific antibodies have, however, failed thus far to detect Omp2 at the EB surface (13, 48). Moreover, on the basis of the results of detergent partitioning and hydrophobic affinity labeling experiments and primary structure considerations, Omp2 (EnvB) of *Chlamydia psittaci* 6BC has been proposed recently to be located in the periplasm (16, 17).

In this report, we describe an in vitro ligand binding assay system that permits the identification and characterization of EB envelope proteins, which have the capacity to associate with eukaryotic cell surfaces. Our results are consistent with the occurrence of a specific interaction between Omp2 of *C. psittaci* guinea pig inclusion conjunctivitis (GPIC) and the host HeLa cell plasma membrane and suggest an important function for this protein in chlamydial pathogenesis.

MATERIALS AND METHODS

Cell lines and chlamydiae. HeLa 229 cells (ATCC CCL 2.1) were routinely grown and maintained in Dulbecco's modified minimal essential medium (DMEM; Gibco BRL), without antibiotics, and supplemented with 10% decomplemented fetal bovine serum (Gibco BRL). The *C. psittaci* GPIC strain used in this study was obtained from the laboratory of Roger G. Rank, University of Arkansas for Medical Science, Little Rock. EBs grown in HeLa 229 monolayers were harvested and purified on discontinuous Renografin (Squibb Diagnostics) density gradients as described previously (5).

Antibodies. Hyperimmune anti-Omp2 polyclonal antiserum was generated by subcutaneous immunization of two New Zealand White rabbits (East Acres Biologicals) with recombinant His6-Omp2 fusion protein (rOmp2) affinity purified as described previously (49). Primary immunization of each animal was with 250 μg of purified rOmp2 in Freund's complete adjuvant. Three boosts of 100 μg each of rOmp2 in Freund's incomplete adjuvant followed at days 21, 42, and 63. Hyperimmune serum obtained 14 days after the last boost was characterized as Omp2 monospecific by immunoblot and was used subsequently for specific detection of denatured Omp2 by immunoblot analysis and for that of conformationally native Omp2 by slot blot analysis.

Three GPIC MOMP-specific monoclonal antibodies obtained from You-xun Zhang as ascites were pooled and used at a 1:1,000 dilution in MOMP immunoblots. These antibodies recognize epitopes located between VD2 and VD3, VD4, and a MOMP discontinuous epitope (51a).

A hyperimmune guinea pig anti-GPIC serum obtained from Roger G. Rank and fluorescein isothiocyanate-conjugated rabbit anti-guinea pig antibodies (ICN Biomedicals) were used as primary and secondary reagents in all immunofluorescence experiments.

Metabolic labeling of GPIC EBs. Radiolabeled EBs were generated by metabolic incorporation of ^{35}S-labeled methionine and cysteine (EXPRE^{35}S^{35}S; NEN). HeLa monolayers grown in 75-cm^2 tissue culture flasks (Corning) in DMEM supplemented with 10% fetal bovine serum, 3 mg of glucose per ml, and 1.0 μg of cycloheximide (Sigma) per ml were infected with GPIC (2×10^8 inclusion-forming units) by incubation at room temperature for 3 h. ^{35}S-labeled precursor amino acids (10 μCi/ml final concentration) were added to the cultures at 18 h postinfection, and ^{35}S-labeled EBs were harvested and purified at 42 h postinfection on discontinuous Renografin density gradients. Purified ^{35}S-labeled EBs, resuspended in sucrose-phosphate-glutamate buffer for storage at −70°C, ranged between 2,000 and 10,000 cpm/μg of protein in specific activity and between 1.0 and 3.0 μg/μl in protein concentration in 22 different preparations used in this study.

For determining the number of Omp2 and MOMP putative binding sites, the specific activity of the EXPRE^{35}S^{35}S label was 1.175×10^3 Ci/mmol, corresponding to 8.9×10^{-8} cpm per sulfur-containing amino acid molecule, and the final amounts of labeled cysteine and methionine in medium relative to their unlabeled counterparts were 0.08 and 3.0%, respectively. MOMP and Omp2 contain 7 and 34 cysteine and 6 and 4 methionine residues, respectively (23a, 52).

Estimation of the protein mass of the GPIC EB. Purified GPIC EBs diluted in phosphate-buffered saline (PBS; pH 7.2) were placed onto the wells of frosted 6-mm slides (Cel-line Associates), air dried, fixed with 100% methanol, and stained with guinea pig anti-GPIC hyperimmune serum and fluorescein isothiocyanate-conjugated rabbit anti-guinea pig antibodies. EBs were enumerated in 30 fields per well under a 100× objective of a fluorescence microscope (Olympus BX 40). With this method, there were 2.5×10^7 EB particles per μg of purified EB protein, corresponding to 4×10^{-8} μg of protein per EB particle.

Measurement of EB binding to live HeLa cells. For binding experiments, several preparations of freshly thawed purified ^{35}S-labeled EBs were pooled and, when necessary, diluted with unlabeled purified EBs to provide a base stock with a specific activity of 1,000 cpm/μg of protein. Aliquots containing 1.0 to 300 μg of protein (100 μl) of ^{35}S-labeled EBs resuspended in PBS containing 1% bovine serum albumin (BSA; Boehringer Mannheim Biochemicals) were added to confluent HeLa monolayers (2×10^5 cells) grown in DMEM supplemented with 10% fetal bovine serum (Gibco BRL) in 48-well tissue culture plates (Costar) in duplicate. After incubation for 1 h at 4°C without agitation, the medium was removed, and the monolayers were washed three times with ice-cold PBS (500 μl). HeLa-associated ^{35}S-labeled EBs were solubilized with 2% sodium dodecyl sulfate (SDS; 100 μl), mixed with 4 ml of scintillation solution (Escoscint A; National Diagnostics), and counted with a Beckman LS 1801 scintillation counter.

Measurement of EB infectivity. Purified EBs were diluted in DMEM supplemented with 10% fetal bovine serum, 3 mg of glucose per ml, and 1.0 μg of cycloheximide per ml to a concentration estimated to yield approximately 4×10^5 inclusion-forming units per ml. Aliquots (100 μl) were inoculated onto confluent HeLa monolayers (2×10^5 cells) grown on glass coverslips (10-mm diameter; Dynalab) in duplicate. Following a 3-h incubation at 37°C, the inoculum was removed and the monolayers were incubated with fresh medium (500 μl) for 24 h at 37°C. The monolayers were fixed with 100% methanol for 5 min, and inclusions were detected by immunofluorescence as described previously (5). Fixed monolayers were washed sequentially twice with PBS, incubated with hyperimmune guinea pig anti-GPIC serum at 37°C for 30 min, washed thrice with PBS, incubated with fluorescein isothiocyanate-conjugated rabbit anti-guinea pig antibody at 37°C for 30 min, and washed thrice with PBS. EB infectivity was measured by counting the total number of inclusions in 20 fields under a fluorescence microscope (Olympus BX 40) with a 40× objective.

Detergent extraction of GPIC envelope proteins. EB outer membrane proteins were solubilized as described previously (6) with some modifications. In a typical experiment, ^{35}S-labeled EBs (4.0 mg of protein) were resuspended in PBS containing 1% Sarkosyl (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Boehringer Mannheim Biochemicals) and incubated at 37°C for 30 min with several short sonications (three 10-s bursts at 0, 10, and 20 min) in a bath sonicator (Branson). Sarkosyl-insoluble chlamydial outer membrane complexes (COMCs) were sedimented and washed once in PBS-PMSF by centrifuging for 1 h at 40,000 rpm in a Beckman TLA-100.3 rotor. Purified COMCs (400 μg) were further extracted in the presence of 10 mM dithiothreitol (DTT) (Sigma) in 500 μl of PBS–1 mM PMSF also containing 1% Sarkosyl, 1% SDS (BDH Biochemicals), 1% deoxycholate (Sigma), 1% *n*-octyl β-D-glucopyranoside (OGP; Boehringer Mannheim Biochemicals), or 1% OGP containing 0.1% SDS by incubation at 37°C for 30 min with several short sonications as described above. Solubilized COMC proteins were separated from insoluble residues by centrifugation over a sucrose cushion (10%) in PBS–1 mM PMSF for 1 h at 40,000 rpm in a Beckman TLS-55 rotor. Solubilized COMC proteins were obtained by collecting the sucrose supernatant fraction (500 μl).

Ligand binding assay. Confluent HeLa monolayers grown in 48-well tissue culture plates were fixed with 1% glutaraldehyde for 1 h at room temperature and washed three times with PBS immediately prior to use. ^{35}S-labeled detergent-extracted COMC proteins (between 6.0 and 36.0 μg in 50 μl) mixed with an equal volume of PBS containing 1% detergent (same as that used for extraction), 10 mM DTT, and 2% BSA were added to glutaraldehyde-fixed HeLa monolayers (2×10^5 cells). Following a 1-h incubation period at room temperature, unbound COMC proteins were removed and the monolayers were washed three times with PBS. COMC proteins bound to glutaraldehyde-fixed HeLa cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, and immunoblot.

Other procedures. SDS-PAGE was performed as described by Lugtenberg et al. (30). Immunoblot analysis was performed by the method of Burnette (9), while slot blot analysis was done as described by Westbay et al. (49), except that samples were not boiled prior to spotting onto nitrocellulose filters. For detection, both methods relied on ^{125}I-labeled staphylococcal protein A (ICN Biomedicals) when rabbit anti-Omp2 serum was used as the source of primary antibody or ^{125}I-labeled goat anti-mouse immunoglobulin G (ICN Biomedicals) when anti-MOMP monoclonal antibodies were used as the primary antibodies.

The protein concentration was measured by the method of Bradford (8) with a commercially available kit (Bio-Rad). Phosphorimager quantitative analysis was performed by integrating whole-band areas by use of ImageQuant version 3.3 software (Molecular Dynamics).

All images included in this report (autoradiographs, immunoblots, and slot blots) were generated from scanned originals by sequential processing with Adobe Photoshop 2.5 and Aldus Persuasion 2.1 software.

RESULTS

Binding of EBs to live and glutaraldehyde-fixed HeLa cells. We have directly measured the binding of metabolically labeled, Renografin-purified, *C. psittaci* GPIC EBs to confluent HeLa cell monolayers over a broad range of EB concentrations (Fig. 1). In view of the utilization of centrifugation to enhance adherence, internalization, or infectivity in previous related experiments (10, 28, 35, 36, 41, 47), we first tested the effect of centrifugation at $800 \times g$ for 5 min prior to the incubation period. Although binding was significantly enhanced (approximately 50% of input EBs were bound in the initial linear range), dose-dependent attachment of EBs to live, chilled HeLa cells reproducibly obeyed a complex reverse sigmoid curve, never reaching saturation, even when up to 300 μg of input EBs was used in the experiment (data not shown). Furthermore, direct observation of bound EBs by immunofluorescence methods revealed large EB aggregates (data not shown), suggesting that centrifugation promotes EB binding to itself and to HeLa cells. Because of the experimental difficulty in separating these phenomena and the lack of physiological relevance of centrifugation, we opted to use assay conditions excluding centrifugation.

Under static conditions, the observed binding was linear up to 100 μg of input EBs, and a reproducible saturation plateau was reached beyond 100 to 150 μg of input EB proteins (Fig. 1). Assuming a protein mass of 4×10^{-8} μg per EB (see Materials and Methods) and a pure EB suspension, saturation

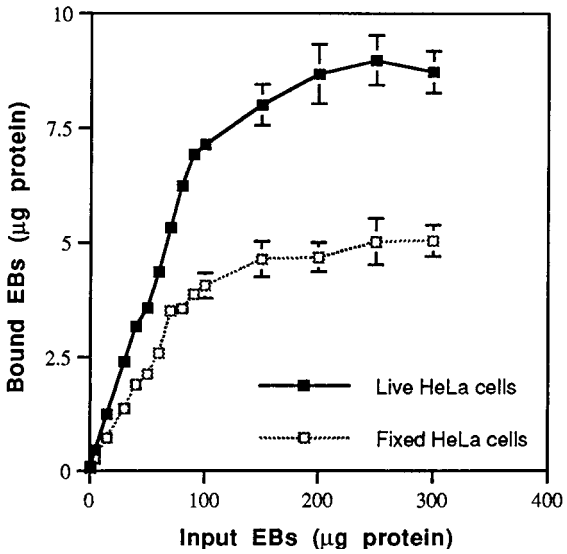

FIG. 1. Binding of purified EBs to live and glutaraldehyde-fixed HeLa cells. Confluent HeLa monolayers (2×10^5 cells) were incubated with purified ^{35}S-labeled EBs (10^3 cpm/μg) at 4°C as described in Materials and Methods, and the ^{35}S radioactivity associated with the monolayers was determined by scintillation counting. EBs bound to live and glutaraldehyde-fixed HeLa cells are expressed as micrograms of EB proteins per 2×10^5 HeLa cells. This experiment was repeated four times with identical results. Error bars represent the standard deviations from duplicate samples.

represents approximately 1,000 EB particles bound per live HeLa cell, corresponding to an input of 1.2×10^4 to 1.9×10^4 EBs per HeLa cell. The former number is consistent with estimates obtained by direct observations of immunofluorescently stained bound EBs (data not shown). When EBs were incubated with glutaraldehyde-fixed HeLa monolayers, EB binding was also dose dependent and reached saturation at approximately the same input concentration. However, probably as a result of alterations of putative binding sites incurred during fixation, EBs bound to fixed HeLa cells about 40% less efficiently than they did to live HeLa cells, saturating at about 580 EB particles per cell (Fig. 1). Similar results were obtained when glutaraldehyde treatment was followed by treatment with excess NH_4Cl to saturate potential remaining aldehyde groups or when glutaraldehyde was substituted for paraformaldehyde or formaldehyde as a fixative (data not shown). Thus, the observed binding to fixed cells appears to be independent of the method of fixation and is not caused by residual reactive aldehyde groups. Specificity of the binding assays was demonstrated by preincubating live or fixed HeLa cells with excess (>200 μg of protein) unlabeled purified EBs prior to labeled EB binding. Under these conditions, the binding of ^{35}S-labeled EBs (50 μg) was reduced by approximately 80% (data not shown).

Binding of detergent-extracted envelope proteins to glutaraldehyde-fixed HeLa cells. Complementary to the whole-cell assay described above, we have developed an in vitro ligand binding assay to directly evaluate the binding of detergent-extracted EB envelope proteins to the glutaraldehyde-fixed HeLa cell surface. COMCs, the raw material for preparation of the extracts, were first obtained from Renografin-purified ^{35}S-labeled EBs by differential extraction with 1% Sarkosyl as described previously (6, 11). The Sarkosyl-insoluble COMCs were further extracted with various detergents in the presence of the reducing agent DTT to break the outer membrane disulfide linkages. Figure 2A shows a typical result of such an extraction. In the presence of DTT, Sarkosyl and deoxycholate were nearly as efficient as SDS in extracting COMC proteins, including MOMP and Omp2 (Fig. 2A and B, lanes 3, 5, and 4, respectively). However, the efficiency of solubilization was severely diminished when the nonionic detergent OGP was used under reducing conditions (Fig. 2A and B, lanes 6) with, in particular, relatively little solubilization of Omp2 by OGP. When OGP extraction was assisted by the inclusion of 0.1% SDS in the extraction mixture, the efficiency of extraction was raised to a level similar to that of the other stronger detergents (Fig. 2A and B, lanes 7).

To identify HeLa-binding proteins, radiolabeled COMC detergent extracts, which had been supplemented with excess BSA (1%) to block nonspecific sites, were incubated statically with freshly glutaraldehyde-fixed HeLa monolayers (see Materials and Methods). Following washes to remove unbound proteins, the residual HeLa-associated fraction was analyzed by SDS-PAGE and autoradiography. A representative result of such an experiment is shown in Fig. 2C. Both the Omp2 and MOMP proteins are observed to bind to fixed HeLa cells in this assay, albeit with noticeable differences in bound yield as a function of the extraction condition. In spite of the different levels of MOMP found in the extracts, the amount of extracted MOMP which is bound to HeLa cells appears relatively constant (Fig. 2C). In contrast, the binding of detergent-extracted Omp2 is variable, with the highest relative yields obtained when the nonionic OGP was used in the extraction (Fig. 2, lanes 6 and 7). Moreover, solubilization of Omp2 in the strongly denaturing anionic detergent SDS nearly abolished its binding capacity (Fig. 2C and D, lanes 4), while solubilization in the less-chaotropic anionic reagents Sarkosyl and deoxycholate resulted in intermediate levels of binding. Since COMC solubilization with OGP-SDS in the presence of DTT yielded the combined highest levels of extraction and binding,

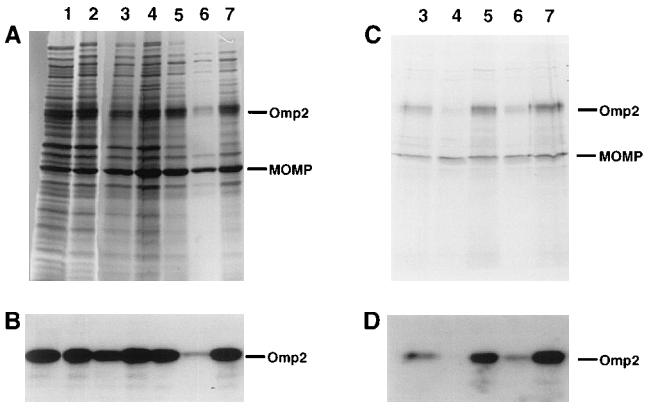

FIG. 2. Extraction of chlamydial envelope proteins and binding to glutaraldehyde-fixed HeLa cells. Sarkosyl-insoluble COMCs were extracted in the presence of 10 mM DTT with 1% Sarkosyl, 1% SDS, 1% deoxycholate, 1% OGP, or a combination of 1% OGP and 0.1% SDS as described in Materials and Methods. (A) Equivalent aliquots (25 μl) of each extract were analyzed directly by SDS-PAGE and autoradiography or used in binding experiments as described in Materials and Methods. (C) HeLa-bound proteins were analyzed by SDS-PAGE and autoradiography. (B and D) To distinguish Omp2-specific signal from comigrating bands in panels A and C, identical gels were transferred to nitrocellulose and analyzed by immunoblot with anti-Omp2 antiserum at a 1:20,000 dilution. The specificity of the MOMP band identified in panels A and C was similarly confirmed by immunoblot analysis (data not shown). Lanes: 1, purified EBs (35 μg of protein); 2, COMCs (20 μg of protein); 3 to 7, proteins extracted with Sarkosyl-DTT (lane 3), SDS-DTT (lane 4) deoxycholate-DTT (lane 5) OGP-DTT (lane 6), and OGP-SDS-DTT (lane 7) (extract input amounts for binding containing 15, 18, 12, 3, and 15 μg of protein are shown in panel A, lanes 3 to 7, respectively). The positions of Omp2 and MOMP are indicated.

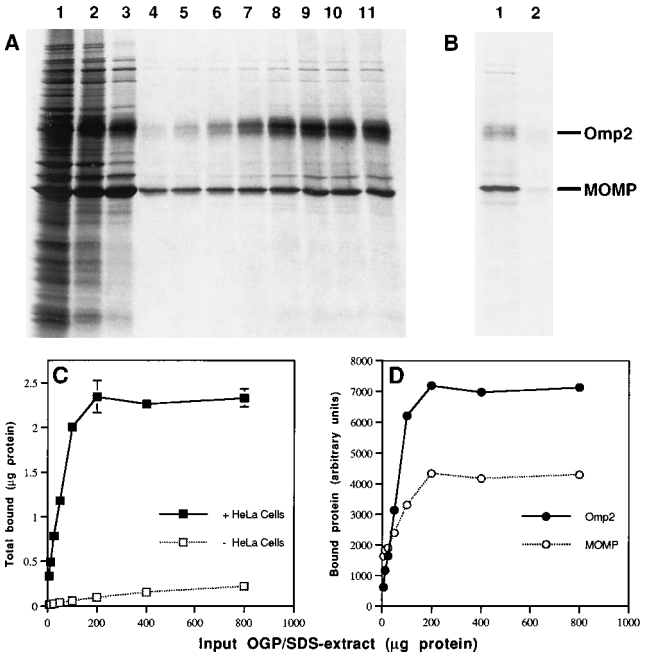

FIG. 3. Specificity of the binding of detergent-extracted outer membrane protein to HeLa cell surfaces. (A) Purified ^{35}S-labeled EBs were extracted sequentially with Sarkosyl and OGP-SDS in the presence of DTT as described in Materials and Methods. Increasing amounts of the extract were then applied in the ligand binding assay, and the bound proteins were analyzed by SDS-PAGE and autoradiography. Lanes: 1, purified EBs (55 μg of protein); 2, COMCs (32 μg of protein); 3, OGP-SDS-DTT extract (25 μg of protein); 4 to 11, HeLa-bound proteins corresponding to inputs of 6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg of protein in the assay. The positions of Omp2 and MOMP are indicated. (B) Inhibition of labeled extract binding by prior binding of excess unlabeled extract. Lanes: 1, HeLa-bound proteins corresponding to an input of 25 μg of protein in the assay (identical to lane 6 of panel A); 2, same as lane 1, except that the fixed HeLa monolayer was saturated with excess unlabeled extract (400 μg of protein) prior to the binding assay with labeled extract. The positions of Omp2 and MOMP are indicated. (C) Binding of ^{35}S-labeled extract proteins to fixed HeLa cells and to the plastic support as measured by scintillation counting. (D) Quantitative analysis of Omp2 and MOMP binding to fixed HeLa cells. Arbitrary units represent integrates of peak areas generated from whole bands by phosphorimaging.

we have used OGP-SDS-DTT extracts exclusively in further experiments.

Specificity and selectivity of COMC protein binding to glutaraldehyde-fixed HeLa cells. When the input of OGP-SDS-DTT-extracted labeled COMC proteins was increased gradually in the binding assay, analysis of the bound fractions by SDS-PAGE and autoradiography (Fig. 3A) or measurement of total bound ^{35}S (Fig. 3C) revealed a linear increase up to about 100 μg of input, at which point binding was saturated. When larger amounts were applied, minor bands present in the original extract were also observed to bind to HeLa cells (Fig. 3A). Since the binding of these minor components was near the background level, never reaching more than a few percent of the total bound, further evaluation of their individual binding properties was not possible under our assay conditions. However, since the binding properties of all minor components paralleled those of MOMP, measurements of MOMP binding were used to represent that of all other extracted COMC proteins for the purpose of quantitative accuracy.

To further demonstrate the specificity of the COMC protein binding assay, excess (e.g., 400 μg) unlabeled OGP-SDS-DTT-extracted COMC proteins were bound to fixed cells prior to the binding of labeled proteins. Under these conditions, total ^{35}S-labeled bound counts were reduced by 85% (data not shown). Further analysis of the residual bound proteins by SDS-PAGE and autoradiography (Fig. 3B) revealed significant reductions in the levels of bound Omp2 and MOMP (60 and 95%, respectively, as measured by phosphorimager quantitation of whole peak areas). Moreover, binding of extracted proteins to the plastic support was negligible (Fig. 3C).

Omp2 binding to HeLa cells was compared quantitatively with that of other extracted COMC proteins, by use of phosphorimager measurements of SDS-polyacrylamide gels such as that from which the autoradiogram in Fig. 3A was obtained. (MOMP was chosen for this computation since it is the most abundant bound protein apart from Omp2; computation of the data on the basis of the ratio of Omp2 to other minor bound polypeptides yielded results similar to those obtained with the bound Omp2/bound MOMP ratio.) By this analysis, the binding of Omp2 and that of other COMC proteins including MOMP were saturated similarly at 100 μg of input (Fig. 3D). In the linear portion of the dose-response curve (i.e., for extract inputs of 6.25, 12.5, 25, 50, and 100 μg of protein), the calculated ratio of bound Omp2 to bound MOMP gradually increases to reach a value fivefold higher than that of the original extract (0.38, 0.64, 0.87, 1.31, and 1.88, respectively, versus 0.31). Thus, the binding of Omp2 to HeLa cells is selective relative to that of other COMC proteins.

Effects of heat treatment on EB binding and infectivity. Mild heating of EBs, typically 5 min at 60°C (10) or 30 min at 56°C (26), has been shown to curtail association with (7, 10, 21, 26), binding to (21, 43, 46), and infectivity toward (7, 10, 43) cultured cells. In this study, we have examined the effect of heating over a range of temperatures and for two different durations (5 and 30 min) on the binding, infectivity, and aggregative properties of whole EBs. The results presented in Fig. 4 indicate that both EB binding and infectivity are inhibited by heat treatment. However, while the loss of binding is gradual over a wide range of temperatures, the loss of infectivity occurs over a much narrower range. Infectivity is affected to a greater degree than binding at all temperatures tested, since even under the harshest conditions tested (65°C for 30 min), 25% residual binding was observed. In contrast, infectivity was nearly abolished after relatively milder treatments, e.g., 45°C for 30 min or 56°C for 5 min. Finally, loss of both binding and infectivity is seen at lower temperatures when a longer heat treatment is applied. Only 5 min of treatment at 56°C was required to reach more than 25% loss of binding (Fig. 4A), while 30 min at 45°C was required for a similar effect (Fig. 4B).

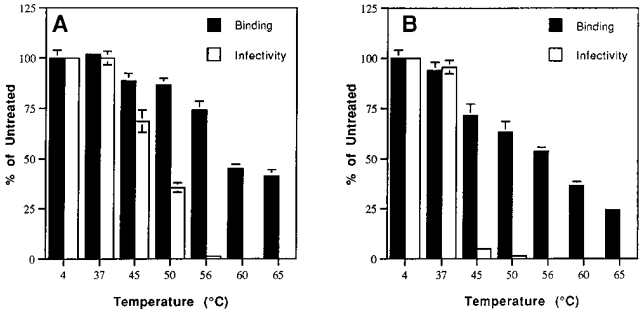

FIG. 4. Effect of heat pretreatment of EBs on binding and infectivity. Purified ^{35}S-labeled EBs resuspended in PBS were incubated for 5 (A) or 30 (B) min at the indicated temperatures, chilled, and diluted at 4°C prior to binding experiments. Untreated (4°C) and heated EBs were then assayed for binding and infectivity. Results are expressed as the percentage of the binding or infectivity by untreated EBs. This experiment was repeated three times with similar results. Error bars represent the standard deviations from duplicate samples.

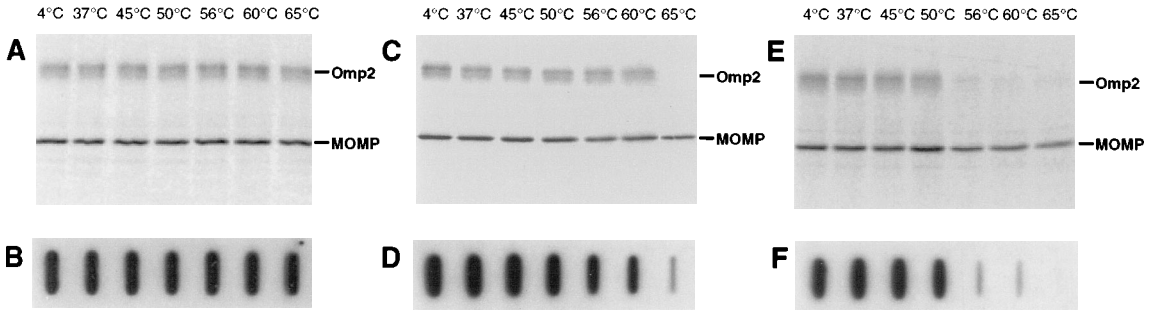

FIG. 5. Effect of heat treatment on the binding of detergent-extracted COMC proteins to glutaraldehyde-fixed HeLa cells. (A and B) ^{35}S-labeled EBs were heated (samples are identical to those used in Fig. 4B), and OGP-SDS-DTT COMC extracts were prepared. (C to F) Extracts were prepared from unheated ^{35}S-labeled EBs and then heated for 5 (C and D) or 30 (E and F) min at the temperatures indicated. All extracts (25 μg of protein each) were then tested for binding of solubilized COMC proteins to glutaraldehyde-fixed HeLa monolayers. (A, C, and E) SDS-PAGE and autoradiography of HeLa-bound proteins; (B, D, and F) slot immunoblot analysis of extracted COMC proteins (0.0625 μg per slot) with anti-Omp2 rabbit antiserum at a 1:12,500 dilution. The positions of Omp2 and MOMP are indicated.

Heat treatment was also observed to cause spontaneous aggregation of EBs, which could be observed microscopically by immunofluorescence with GPIC convalescent-phase guinea pig antibodies. In these experiments, large EB aggregates were observed upon heat treatment for 30 min at 50, 56, 60, and 65°C (data not shown). Thus, the heat-induced denaturation of EB surfaces promotes EB aggregation. This phenomenon may be a contributing factor to the observed loss of binding properties of EBs at these temperatures.

Effects of heat treatment on the binding of detergent-extracted outer membrane proteins to HeLa cells. To further correlate the losses of adherence and virulence properties of heat-treated EBs to the adherence properties of detergent-extracted outer membrane proteins, heat-treated EBs were used as substrates for the extraction of COMC proteins. The binding of extracted proteins to glutaraldehyde-fixed HeLa monolayers was then tested as described above. As seen in Fig. 5A, neither binding of MOMP nor that of Omp2 was affected by heat treatment for 30 min, even at the highest temperatures used here. However, when the heat treatment was performed following prior reduction and detergent solubilization of the outer membrane proteins, i.e., on OGP-SDS-DTT extracts from untreated EBs, a temperature-dependent loss of Omp2 binding was observed (Fig. 5C and E). Omp2 binding was nearly abolished after heating at 65°C for 5 min or above 56°C for 30 min, even though similar amounts of Omp2 were present in the heat-treated extracts (data not shown). Again, an extended heat treatment lowered the temperature threshold of Omp2 activity in the ligand binding assay; i.e., temperatures of 65°C for 5 min (Fig. 5C) or 56°C for 30 min (Fig. 5E) yielded comparable decreases in binding. In contrast, heat treatment of OGP-SDS-DTT-extracted COMCs had a relatively minor effect on MOMP binding, i.e., a maximum reduction of 20% following a 30-min treatment at 65°C as measured densitometrically (Fig. 5E).

The loss of Omp2 binding to HeLa cell surfaces correlates with the loss of Omp2 conformation. A high-titer rabbit antiserum raised against the affinity-purified recombinant Omp2 protein (49) was generated. Immunoblot analysis confirmed the specificity of this serum for Omp2 with no detectable cross-reactivity to other COMC proteins (data not shown). Slot blot analysis of heat-treated extracts using this antibody revealed parallel losses of Omp2 immunoreactivity and Omp2 binding capacity (Fig. 5C to F). In contrast, when EBs were heat treated prior to extraction, neither a loss of Omp2 binding nor a decrease in Omp2 reactivity with the antibody was detected (Fig. 5A and B). These data are consistent with the antibodies recognizing one or several immunodominant conformational Omp2 epitopes, whose assembly in the native protein is disrupted upon heat treatment only when reduced and/or dissociated (i.e., solubilized) from the EB wall.

Effect of trypsin treatment on EB surface proteins and on EB binding and infectivity. EBs were treated with increasing concentrations of trypsin (0.1 to 250 μg/ml) at 37°C for 30 min prior to incubation with HeLa cells. Both the binding and infectivity of trypsinized EBs were reduced, with a maximal 50% decrease at the higher trypsin concentrations (Fig. 6). To identify putative differential trypsin susceptibilities of outer membrane proteins, trypsinized EBs and untreated controls were subjected to SDS-PAGE, autoradiography, and immunoblot with Omp2- and MOMP-specific antibodies. The protein profiles of the trypsin-treated EBs that were tested for binding and infectivity in Fig. 6 are shown in Fig. 7. While both Omp2 and MOMP were susceptible to trypsin treatment (Fig. 7B and C), the patterns of susceptibility are distinct for the two proteins. Trypsin susceptibility of Omp2 was quantitative and de-

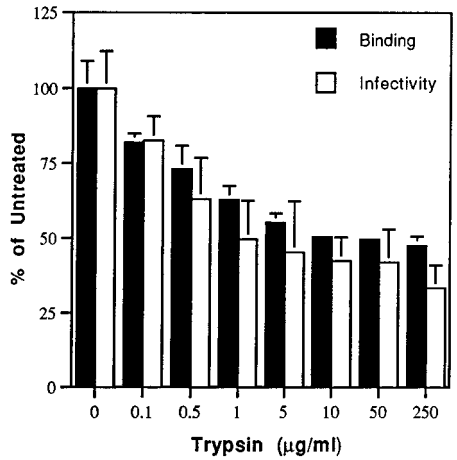

FIG. 6. Effect of trypsin pretreatment of EBs on binding to and infectivity toward HeLa cells. Purified ^{35}S-labeled EBs (100 μg/100 ml) resuspended in PBS were incubated with various concentrations of trypsin (Sigma type XIII; catalog no. T8642) for 30 min at 37°C prior to binding experiments. Trypsin activity was stopped by the addition of 0.67 μg of soybean trypsin inhibitor (Sigma type II-S) per μg of trypsin to the suspension. Untreated and trypsin-treated EBs were then assayed for binding and infectivity. Results are expressed as the percentage of the binding or infectivity by untreated EBs. This experiment was repeated three times with similar results. Error bars represent the standard deviations from duplicate samples.

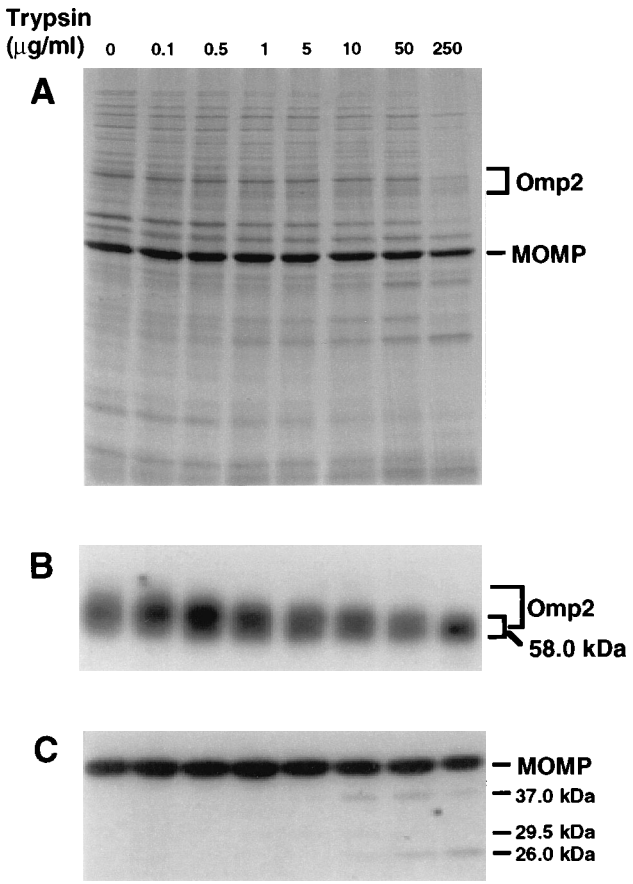

FIG. 7. Effect of trypsin treatment on EB surface proteins. Trypsin-treated ^{35}S-labeled EBs identical to those in Fig. 6 were analyzed by SDS-PAGE and autoradiography (A), immunoblot with anti-Omp2 antiserum at a 1:20,000 dilution (B), and immunoblot with a pool of anti-MOMP monoclonal antibodies (C). The positions of Omp2 and MOMP and Omp2 and MOMP proteolytic fragments are indicated.

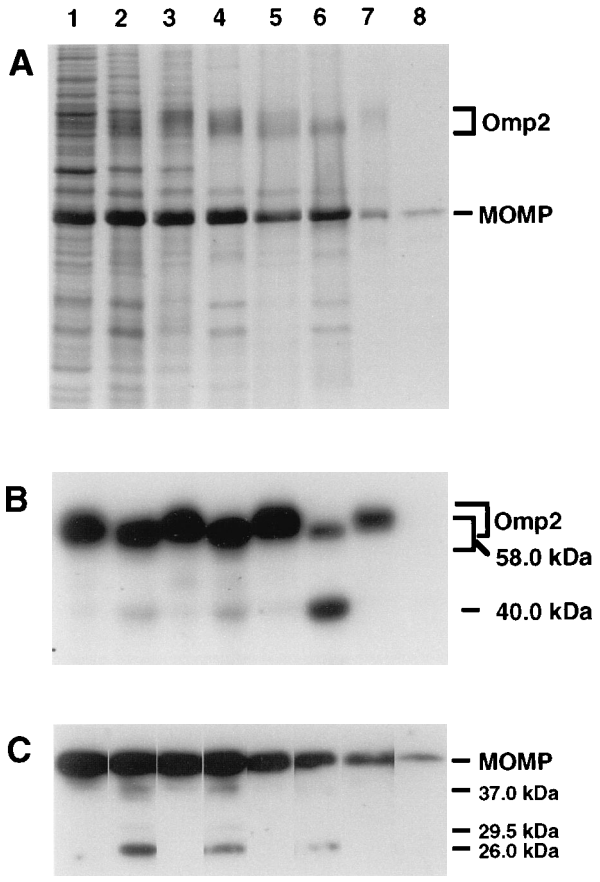

FIG. 8. Effect of EB trypsin treatment on the binding of detergent-extracted COMC proteins to glutaraldehyde-fixed HeLa cells. OGP-SDS-DTT COMC extracts (25 μg of protein) generated from trypsin-treated (100 μg of trypsin per ml) and untreated ^{35}S-labeled EBs were tested for binding of solubilized COMC proteins to glutaraldehyde-fixed HeLa monolayers. (A) SDS-PAGE and autoradiography of EB (lane 1), COMC (lane 3), OGP-SDS-DTT extract (lane 5), and HeLa-bound proteins (lane 7) and corresponding samples obtained from trypsin-treated EBs (lanes 2, 4, 6, and 8). (B) Immunoblot analysis of the SDS-polyacrylamide gel in panel A with anti-Omp2 rabbit-raised antiserum at a 1:20,000 dilution. (C) Immunoblot analysis of SDS-PAGE in panel A with a pool of anti-MOMP monoclonal antibodies (see Materials and Methods). The positions of MOMP and Omp2 and of MOMP and Omp2 fragments are indicated.

tectable even at low trypsin concentrations (Fig. 7B). In contrast, relatively small amounts of MOMP antigenic fragments were detected, and this was the case only at the higher trypsin concentrations, i.e., above 10 μg/ml (Fig. 7C). At these concentrations of the protease, other EB proteins were also susceptible to digestion as evidenced by the concomitant disappearance or reduction of several bands (Fig. 7A).

Effect of trypsin treatment on the binding of detergent-extracted outer membrane proteins to HeLa cells. To examine the effects of trypsin treatment on the binding of detergent-extracted outer membrane proteins to glutaraldehyde-fixed HeLa cells, trypsin-treated EBs were used as substrates for sequential Sarkosyl and OGP-SDS-DTT extractions (Fig. 8A). Both trypsin-treated whole EBs and their corresponding COMC extracts contained a major 58-kDa tryptic Omp2 fragment and a relatively less abundant immunoreactive 40-kDa fragment (Fig. 8B, lanes 2 and 4). Upon OGP-SDS-DTT extraction of the COMCs, further degradation of the larger Omp2 fragment was increased, resulting in a comparatively higher level of the smaller fragment (Fig. 8B, lane 6). When tested in the ligand binding assay, neither one of the two major immunoreactive Omp2 fragments bound to the HeLa cell surface (Fig. 8B, lane 8). This result was confirmed upon extended exposure of the immunoblots to X-ray film (data not shown). In contrast, the binding of MOMP solubilized from trypsin-treated EBs to HeLa cell surfaces was not significantly different from that of the untreated control (Fig. 8C).

DISCUSSION

We report the identification of chlamydial surface-associated proteins that are capable of interacting with sites on the surface of the eukaryotic cell plasma membrane and consequently may play an important function in chlamydial pathogenesis. We have developed an in vitro ligand binding assay system for the identification of detergent-extracted GPIC EB outer membrane proteins which bind efficiently and specifically to glutaraldehyde-fixed HeLa cells. Since the properties of isolated binding proteins should mirror the adherence properties of purified EBs, we have compared the susceptibilities of EBs and of isolated binding proteins to two treatments reported to be negative effectors of chlamydial adherence and infectivity, mild heating (7, 10, 21, 26, 46), and trypsin treatment (44, 46).

The in vitro assay system described here for the identification of detergent-extracted chlamydial binding proteins is de-

rived from similar experimental approaches developed for the identification of adhesins from *Treponema pallidum* (3) and *Trichomonas vaginalis* (1). In our system, two major polypeptide species identified as Omp2 and MOMP appear to bind selectively to fixed HeLa surfaces (Fig. 2). However, while the amount of bound MOMP appears relatively constant, that of bound Omp2 varies significantly with respect to the detergent used in the extraction. In particular, the binding of Omp2 but not that of MOMP is greatly reduced when the denaturing detergent SDS is used (Fig. 2C and D, lanes 4). Conversely, Omp2 binding, and not that of MOMP, is comparatively enhanced when the milder nonionic OGP is in the extract (Fig. 2C and D, lanes 7). These results imply that SDS-mediated conformational alteration of Omp2 causes the loss of binding and suggest that the Omp2 interaction with the HeLa cell surface requires Omp2 native conformation.

Significantly, HeLa cells bound Omp2 selectively over other outer membrane proteins present in the extract, including MOMP. Indeed, the bound Omp2/bound MOMP ratio was more than sixfold higher than the Omp2/MOMP ratio in the original extract. Thus, the ligand binding assay appears to function as an enrichment procedure for Omp2. On the basis of saturated binding levels of Omp2 and MOMP (6.3×10^3 and 3.8×10^3 cpm, respectively) and temporarily considering both proteins as monomers in the assay, the estimated numbers of Omp2 and MOMP binding sites would be approximately 2.4×10^6 and 1.1×10^6 per HeLa cell, respectively. These high values would be consistent with abundant eukaryotic receptor sites such as glycan polymers found in surface-associated glycoproteins. More probably, native Omp2 and MOMP homo- and/or hetero-oligomeric structures are partially conserved in the extracts, even under reducing conditions. Preferential binding of such oligomeric structures in the assay will artificially elevate the bound levels, hence the calculated number of binding sites. Support for the latter interpretation exists in that stable outer membrane protein oligomers and multimers are observed frequently when reducing SDS-PAGE is used, provided that the samples are not boiled (45a). Moreover, the existence of MOMP and Omp2 homo- and heteropolymers has been documented by various biochemical and immunological methods in both *C. trachomatis* and *C. psittaci* (2, 6, 19, 32, 33, 49). Nevertheless, our present results do not allow the precise evaluation of differential affinities and numbers of binding sites. Further fractionation and biochemical characterization of the OGP-SDS-DTT extract may contribute to answer these questions.

Heat lability of the association of EBs with susceptible eukaryotic cells was first reported for *C. trachomatis* and for avian *C. psittaci* by Kuo and Grayston (26) and Byrne (10), respectively. Sensitivity to mild heat treatments has since been used frequently as a telltale property of candidate adhesins (24, 43, 45, 51). We have examined the effects of temperature on infectivity and binding for a range of temperatures (37 to 65°C) and for two durations (5 and 30 min). Our results generally confirm those already described for other systems (Fig. 4). The contrast observed between the apparent narrow range required to abolish infectivity and the broader range required to reduce binding suggests that the two mechanisms are distinct at the molecular level. Thus, it is most likely that the limiting factor for heat-mediated disruption of infectivity is a single critical determinant with a narrow heat sensitivity threshold. In contrast, the observed loss of binding may be mediated by the gradual disruption of several determinants, each of which contributes to EB adherence. Alternatively, the heat-mediated disruption of a single adherence determinant may occur, but the process is inherently slower, causing a gradual and incomplete loss of adherence. Consistent with the latter interpretation, Zhang and Stephens have suggested that, on the basis of the heat-induced loss of tritiated heparin from heparatinase-treated *C. trachomatis* EBs, heat treatment provokes a loss of a surface-associated glycosaminoglycan, itself a ligand involved in EB adherence (51). Regardless, it is clear that a heat-induced loss of EB binding is not solely responsible for the loss of EB infectivity and that the two processes are readily discernible by their differential susceptibilities to heat. We also observed that the most-severe heat treatments used here were sufficient to promote significant aggregation of EBs (data not shown). This phenomenon, predictably caused by the nonspecific heat denaturation of EB surface proteins, may generally affect the reliability of measurements of in vitro adherence for heat-treated EBs. Overall, our results suggest that heat sensitivity of chlamydial adherence is potentially a more broadly acting (i.e., less-specific) phenomenon than has collectively been implied by earlier reports (24, 43, 45, 51). Indeed, heat treatments for 3 min at 60°C or 30 min at 56°C may have multiple effects on EB structure and function and therefore should not be considered mild, at least where EB adherence is concerned.

We have also examined the sensitivity of the binding by detergent-extracted outer membrane proteins to similar heat treatments. Interestingly, neither Omp2 binding nor MOMP binding was significantly affected when OGP-SDS-DTT extracts from heated EBs were used in the assay (Fig. 5A). However, when extracts from unheated EBs were then heated and tested in the ligand binding assay, a temperature-dependent quantitative loss of Omp2 binding was observed (Fig. 5C and E). This is in contrast to MOMP binding, which was not severely affected by heat treatment. Thus, it appears that the conformational restriction of Omp2 in the EB cell wall, by disulfide linkages in particular, is sufficient to prevent its denaturation by heat and its inactivation as an HeLa cell ligand. The heat-induced conformational loss of solubilized Omp2 is also fortuitously confirmed by the heat lability of an immunodominant conformational Omp2 epitope recognized by a rabbit monospecific antiserum (Fig. 5). Overall, these data further indicate that the observed interaction of Omp2 with the HeLa cell surface is dependent on the native conformation of Omp2 and is therefore likely to represent a biological activity of the protein.

Trypsin treatment of EBs was first shown to affect *C. trachomatis* adherence by Su and collaborators, who characterized trypsin-sensitive sites on the MOMP of serovars B and L2 of *C. trachomatis* (44). Since differential trypsin activity on MOMP of the two serovars paralleled that on attachment and infectivity to HeLa cells, a functional role for MOMP in EB adherence was suggested. A caveat of this interpretation, however, is that, since MOMP is such an abundant protein at the EB surface, MOMP trypsin cleavage may lead to the differential physical loss or inactivation of an associated adhesin. We have used an experimental design similar to that of Su et al. for GPIC with somewhat different results. First, we confirmed that EB attachment and infectivity was sensitive to trypsin pretreatment of EBs. However, in contrast to the effect of heat treatment (see above), trypsin treatment provokes a similar reduction both in attachment and infectivity over a wide range of trypsin concentrations (Fig. 6). This is supportive of a mechanism of trypsin-mediated inactivation, whereby the loss of infectivity is a direct consequence of the loss of adherence. Second, GPIC MOMP lying on the surface of whole intact EBs is generally not susceptible to trypsin even at high concentrations of the protease, in sharp contrast to *C. trachomatis* MOMP (18, 44). A portion of MOMP nevertheless becomes susceptible to

trypsin action at higher trypsin concentrations, when global loss of many EB protein bands is also visible (Fig. 7). Therefore, MOMP sensitivity to trypsin under these conditions is likely to reflect a loss of EB structural integrity rather than cleavage at surface-exposed sites in MOMP. Third, in contrast to MOMP, GPIC Omp2 is exquisitely sensitive to trypsin, with the loss of a small (1- to 2-kDa) terminal fragment of the protein. Cleavage is complete, indicating that all of EB Omp2 is similarly accessible to trypsin and thus probably possesses a uniform topology within the EB. Since Omp2 is susceptible to trypsin activity even at low concentration of the protease, this provides, for the first time, strong supportive evidence that the protein is exposed at the EB surface. This is contrary to previous reports which failed to reveal surface exposure of Omp2 in *C. trachomatis* strains by the use of immunoelectron microscopy with Omp2-specific monoclonal and polyclonal antibodies (13, 48), a comparatively less-sensitive method than the method used here. Finally, we observed no binding of OGP-SDS-DTT-extracted Omp2 tryptic fragments to glutaraldehyde-fixed HeLa cells in the in vitro ligand binding assay (Fig. 8).

The general architecture of the EB envelope may differ in GPIC and in other chlamydial species and strains. On the basis of available evidence, Everett and Hatch have proposed that Omp2 (EnvB) of *C. psittaci* 6BC is located in the periplasmic space (17). An alternative interpretation based on our results with *C. psittaci* GPIC, however, is that Omp2 is surface exposed uniformly in *Chlamydia* spp. In this case, attempts at antibody surface labeling would have failed if the recognized Omp2 epitopes were not fully accessible to antibody. This might occur if a relatively small portion of Omp2 is selectively immunoaccessible. Interestingly, the most likely trypsin cleavage site occurs at the amino terminus of Omp2, where a segment rich in basic amino acid residues (amino acids 30 to 48 of mature GPIC Omp2) lies. This region is predicted to be hydrophilic, antigenic, and most likely surface exposed (23a). Selective exposure of this segment of mature Omp2 might lead to quantitative trypsin cleavage at this unique site. Because of the observed dependence of isolated Omp2 binding on the presence of this segment and the correlated loss of EB adherence, the molecular characterization of this fragment may be crucial to further our understanding of the role of Omp2 in EB adherence.

In summary, we have established an in vitro assay system which can be exploited for the identification and characterization of chlamydial outer membrane proteins which are capable of specific binding to eukaryotic cell surfaces. We demonstrate that the cell wall-associated protein Omp2 possesses the capacity to engage in a specific and selective interaction with the HeLa cell surface, which is most likely based on the native conformation of Omp2. Since the effects of trypsin on the binding of detergent-extracted Omp2 parallel those on whole EB binding and infectivity, we speculate that the observed in vitro activity of Omp2 is reflective of a biological property of the protein relevant to chlamydial pathogenesis. Since Omp2 is not detected early in the chlamydial developmental life cycle (20, 27, 32, 37), EB-associated Omp2 is probably rapidly diluted or degraded upon internalization and subsequent growth, excluding a possible role in the vegetative growth phase of the organism. Omp2 expression is up-regulated late in the life cycle, approximately when reticulate bodies start to differentiate to initial bodies and eventually EBs (20, 32), thus coinciding with the infectious stage of the organism. In view of the apparent surface exposure of the protein, we further speculate that the binding properties of Omp2 are likely to be important during the late and/or early phases of the chlamydial life cycle, which have in common that they may require direct interaction between an EB surface component and constituents of the eukaryotic plasma membrane.

ACKNOWLEDGMENTS

We thank You-xun Zhang (Boston University) and Roger G. Rank (University of Arkansas for Medical Sciences) for the gift of MOMP-specific monoclonal antibodies and GPIC-hyperimmune guinea pig serum, respectively. We also thank Susan A. Kaye and Aaron Proweller for their help with the immunofluorescence and phosphorimaging experiments, respectively. We are grateful to David Ojcius and Alice Dautry-Varsat (Institut Pasteur, Paris, France) for their critical reading of the manuscript. R.-C.H. and P.M.B. also wish to thank all members of l'Unité de Biologie des Intéractions Cellulaires de l'Institut Pasteur for their hospitality during the later phases of this work.

This work was supported by Public Health Service grant AI26280. R.-C.H. was supported by Public Health Service postdoctoral training grant AI07169, and P.M.B. is recipient of Public Health Service Research Career Development Award AI01057.

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