Attachment of Chlamydia trachomatis L2 to host cells requires sulfation

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Attachment of *Chlamydia trachomatis* L2 to host cells requires sulfation

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*Chlamydia trachomatis* is a pathogen responsible for a prevalent sexually transmitted disease. It is also the most common cause of infectious blindness in the developing world. We performed a loss-of-function genetic screen in human haploid cells to identify host factors important in *C. trachomatis* L2 infection. We identified and confirmed B3GAT3, B4GALT7, and SLC35B2, which encode glucuronosyltransferase I, galactosyltransferase I, and the 3'-phosphohexosamine 5'-phosphosulfate transporter 1, respectively, as important in facilitating *Chlamydia* infection. Knockout of any of these three genes inhibits *Chlamydia* attachment. In complementation studies, we found that the introduction of functional copies of these three genes into the null clones restored full susceptibility to *Chlamydia* infection. The degree of attachment of *Chlamydia* strongly correlates with the level of sulfation of the host cell, not simply with the amount of heparan sulfate. Thus, other, as-yet unidentified sulfated macromolecules must contribute to infection. These results demonstrate the utility of screens in haploid cells to study interactions of human cells with bacteria. Furthermore, the human null clones generated can be used to investigate the role of heparan sulfate and sulfation in other settings not limited to infectious disease.

Often leading to pelvic inflammatory disease and infertility, *Chlamydia trachomatis* is the most common bacterial sexually transmitted disease (1). It is also the leading cause of infectious blindness worldwide (2). *Chlamydia* is an obligate intracellular pathogen with a biphasic developmental cycle. The infectious agent is the elementary body (EB). On invasion, the EB differentiates into a metabolically active reticulate body. *Chlamydia* resides entirely within a protected membrane-bound inclusion. Because of this biphasic lifestyle, there is currently no straightforward genetic system with which to manipulate the *Chlamydia* genome. Without the ability to easily knock out, mutate, alter, or add to the *Chlamydia* genome, our knowledge of how *Chlamydia* co-opts host functions remains limited.

Although *Chlamydia* can infect most cultured mammalian cells, the host receptors involved in the uptake of the pathogen have not been definitively identified. An initial reversible interaction, followed by irreversible binding of the EB to the host cell, has been hypothesized (3). The possible role of heparan sulfate as a host factor that contributes to adhesion in *Chlamydia* infection is controversial. Heparan sulfate proteoglycans may be important in the initial reversible interaction of the EB with the host cell (4–6); however, *Chlamydia* infection independent of heparan sulfate has been reported (7). Our genetic screen in a human cell line not only addresses this controversy, but also shows the more general utility of this tool in examining the role of sulfation beyond heparan sulfate.

Past genetic screens for host factors that affect *Chlamydia* infection of mammalian cells have met with limited success. Using chemical mutagenesis, Carabeo et al. (8) isolated a CHO cell clone resistant to *Chlamydia*, and Fudyk et al. (9) found 12 CHO clones that are resistant to *Chlamydia*, but had difficulty identifying the mutations that impart resistance. Only one of the 12 clones initially identified was subsequently characterized as carrying a mutation in protein disulfide isomerase (10). Chemical mutagenesis screens suffer from the fact that a great many genes will be altered because of the dose of mutagen required to obtain a pool of mutants that can be screened. This makes it difficult to connect mutant phenotypes with any particular affected gene.

RNA interference screens also have been used to identify host factors important for *Chlamydia* infection, such as PDGFβR (11) and the MEK-ERK signaling pathway (12). Despite their widespread use, siRNA screens suffer from off-target effects, and do not always succeed in completely eliminating gene expression. This is of particular relevance when the products of the target genes have enzymatic activity, the complete suppression of which may be required to elicit a phenotype.

We performed a loss-of-function genetic screen in human haploid cells to identify mutant cell lines resistant to *Chlamydia* (13–16). This system avoids the potential off-target effects and incomplete knockdowns of an siRNA screen. It has the advantage over a chemical mutagenesis screen in causing only a limited number of disrupted genes per cell, which can be readily identified by deep sequencing and restored to WT by provision of the corresponding cDNA. An obvious drawback is the false-negatives resulting from the exclusion of genes essential to cell survival. Genes that function redundantly would be equally difficult to isolate as null mutants. We identified three host genes, all of which are involved in the sulfation of host proteins that facilitate *Chlamydia* attachment. By isolating null alleles from the screen, our results demonstrate the importance in *Chlamydia* infection of B3GAT3, B4GALT7, and SLC35B2, which encode glucuronosyltransferase I, galactosyltransferase I, and the 3'-phosphohexosamine 5'-phosphosulfate (PAPS) transporter 1 (PAPST1), respectively. The clone deficient in PAPST1 retains ~5% of its heparan sulfate level, yet is more resistant to *Chlamydia* than the two clones that have no detectable heparan sulfate. Thus, there must be a role for sulfated proteins beyond heparan sulfate as critical mediators in *Chlamydia* attachment.

Sulfation is important for many diverse processes. Lacrimal gland development (17), viability and neural development in *C. elegans* (18), FGFR-induced signaling (19), and cartilage homeostasis (20) all depend on sulfation. Sulfation of tyrosine residues is important for the interaction of chemokines and their receptors (21–23) and for binding and entry of HIV (24). Beyond studying interactions of bacteria with human host cells, the tools generated here can be used to investigate in tissue culture other processes


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in which sulfation may be important. Attempts to reduce expression of SLC35B2 with such methods as RNAi have led to only modest reductions in function, hindering study of the role of sulfation (25). Armed with the isolated null clones, we can now study and better understand many other processes that involve sulfation.

**Results**

**Haploid Genetic Screen Yields Three Gene Candidates Important in Chlamydia Infection.** To identify host genes important in *Chlamydia* infection, we used a genetic screen based on the human haploid cell line HAP1 (26) (Fig. 1A). The HAP1 cell line is a derivative of the near-haploid leukemia cell line KBM7 generated by transduction with the four reprogramming factors: OCT4, Sox-2, c-MYC, and KLF4. Unlike the KBM7 parental cell line, the majority of HAP1 cells contain a single copy of chromosome 8. The cells grow adherently, and are susceptible to infection with and killed by *C. trachomatis* L2. To find genes important for *Chlamydia* cytotoxicity, we mutagenized HAP1 cells with a retroviral gene-trap vector, yielding a heterogeneous cell population with inactivating insertions in nonessential genes containing on average one insertion event per cell (13, 15). Approximately 10<sup>6</sup> mutagenized HAP1 cells were inoculated with *C. trachomatis* L2 at a multiplicity of infection (MOI) of ~6 and incubated for 5 d, after which 99.99% of the starting pool of mutagenized cells died. After 5 d, antibiotics were added to eliminate any remaining *Chlamydia*. We recovered the surviving cells, which we estimated to total ~500 clones.

To identify the inactivated genes that permitted survival, we extracted genomic DNA from the resistant clones, performed inverse PCR, and used parallel sequencing with primers flanking the retroviral integration site to map these sites onto the human genome. We compared the frequency of genes enriched for the gene-trap insertions to the frequency of such insertions in unselected but mutagenized control cells (Fig. 1B). In the figure, circles represent individual genes; the area of these circles is proportional to the number of independent insertions identified in the resistant mutagenized HAP1 population. Three genes that, when disrupted, provide resistance to *Chlamydia* infection were significantly enriched. *B3GAT3*, *B4GALT7*, and *SLC35B2* were represented with 26, 9, and 8 independent insertions, respectively (respective *P* values of 2.97 × 10<sup>-6</sup>, 1.77 × 10<sup>-21</sup>, and 7.5 × 10<sup>-21</sup>). Although these genes are enriched to a highly significant degree, the screen will not detect some false-negatives or genes important in *Chlamydia* infection, because of the stringency applied and the possibility of redundancy of host proteins. *B3GAT3*, *B4GALT7*, and *SLC35B2* are all involved in the synthesis of glycosaminoglycans such as heparan sulfate. The active site residues of these enzymes are known, allowing the design of vectors that encode catalytically active and inactive variants.

**Ectopic Expression of Proteins Encoded by B3GAT3, B4GALT7, or SLC35B2 in Their Respectively Deficient Hap1 Clones Restores Susceptibility to Chlamydia.** We verified the results of the genetic screen as follows. Three independent clones were isolated that carry gene disruptions for the *B3GAT3*, *B4GALT7*, and *SLC35B2* genes. Analysis by gene-specific PCR confirmed that the gene trap indeed interrupted the respective gene in each of the clonal isolates (Fig. S1A). We confirmed that no contaminating WT cells remained in our clonal isolates. To further demonstrate that the clones lacked the WT transcripts of the respective genes, we performed RT-PCR and failed to detect the corresponding mRNAs (Fig. 2A). We then transduced the null clones with an empty vector; these lines are designated B3GAT3<sup>mut</sup>−, B4GALT7<sup>mut</sup>−, and SLC35B2<sup>mut</sup>−. We restored expression in the null clones with full-length cDNAs encoding the WT products equipped with a C-terminal HA tag: these lines are designated B3GAT3<sup>WT</sup>−HA, B4GALT7<sup>WT</sup>−HA, and SLC35B2<sup>WT</sup>−HA. Finally, we transduced the individual mutant clones with enzymatically inactive versions, likewise equipped with a C-terminal HA tag; these lines are designated B3GAT3<sup>mut</sup>−HA, B4GALT7<sup>mut</sup>−HA, and SLC35B2<sup>mut</sup>−HA (Fig. S1B).

Retroviral expression of C-terminally HA-tagged B3GAT3, B4GALT7, and SLC35B2 fully restored susceptibility to killing by *Chlamydia*. In contradistinction, HA-tagged enzymatically inactive forms of the encoded gene products did not restore susceptibility to *Chlamydia*, and these clones readily survived beyond 4 d after inoculation with *Chlamydia* (Fig. 2B), long after their susceptible counterparts had succumbed. The striking difference in survival between the reconstituted and null clones validates the results of the screen and underscores the importance of *B3GAT3*, *B4GALT7*, and *SLC35B2* in facilitating *Chlamydia* infection.

**Fig. 1.** Loss-of-function haploid genetic screen with *C. trachomatis* L2. (A) Outline of the loss-of-function haploid genetic screen with *C. trachomatis* L2; see the text for details. (1) KBM7 cells are reprogrammed to adherent cells by OCT4, Sox-2, c-MYC, and KLF4. (2) HAP1 cells are transduced with the gene trap virus. (3) The mutated cells are inoculated with *C. trachomatis* L2. (4) Virtually all (99.99%) of the mutant cells die from *Chlamydia* infection. (5) Survivors are expanded, the DNA is extracted, and the DNA is sequenced. (B) B-Ranking plot of loss-of-function haploid genetic screen results. The x axis shows genes with at least one inactivating mutation ranked in order of chromosomal position. The y axis shows the −log of the *P* value of the enrichment of gene-trap insertions in the indicated gene compared with an unselected control dataset. The size of the bubble is correlated with the number of independent insertions. Genes with a *P* value < 0.0001 are labeled, and the number of independent insertions is given in brackets.
Infection in Clones. and into high molecular weight (MW) are resistant to death.

Time Course of Chlamydia Infection in Clones. We next determined at what stage the genes disrupted in our clones impart resistance to Chlamydia infection. Using quantitative real-time PCR, we analyzed a time course of replication of Chlamydia within the inclusions. We plotted the increase in DNA in Chlamydia versus a host housekeeping gene. At 2 h postinfection, less Chlamydia DNA was associated with the null clones, as expected. For the fraction of bacteria that did succeed in establishing a foothold, even in the null clones, Chlamydia replicated equally well in the null clones and the reconstituted clones, as inferred from the similar slopes of the plotted growth curves (Fig. 3 A–C). The figure shows a downward shift of the curve for the null clones compared with Chlamydia growth in the reconstituted clones. Thus, the mechanism of resistance for the null clones most likely involves the attachment/entry phase. If the Chlamydia cannot attach to the null clones, then it will be unable to infect the host cells and then kill them. The small fraction of the null clones to which Chlamydia attaches are still infected and ultimately die. Chlamydia released from the null clones was fully infectious and equivalent in this regard to the Chlamydia produced from reconstituted mutant cells, showing that a defect in sulfation pathways in the host cell does not affect growth properties of Chlamydia per se. Transmission electron microscopy revealed no obvious ultrastructural differences in the Chlamydia inclusions in WT compared with null clones at 36 h postinfection (Fig. 3D). Whereas there are fewer inclusions in the null clones, the inclusions that do form are of similar size, and apparently similar numbers of elementary and reticulate bodies contained within them, as inclusions in WT cells (Fig. 3E).

Attachment Assay of Chlamydia Infection in Isolated Clones. To confirm that attachment of Chlamydia to host cells is affected by the genes discovered in the screen, we compared the number of Chlamydia that attach to host cells in the null and reconstituted clones. Chlamydia do not attach as well to the null clones compared with their respective reconstituted counterparts (Fig. 4A). There is a significant difference in attachment between the reconstituted and null clones for B3GAT3, B4GALT7, and SLC35B2, as measured by quantitative real-time PCR. This also can be visualized via immunofluorescence for the null clones (Fig. 4B). We found enhanced resistance to Chlamydia attachment in the SLC35B2 null clone compared with the B3GAT3 and B4GALT7 null clones.

Confirmation That Sulfated Proteins Besides Heparan Sulfate Are Involved in Chlamydia Attachment. We performed an attachment assay to verify significant differences between B3GAT3GT and SLC35B2GT, between B3GAT3GT and B4GALT7GT, and between B4GALT7GT and SLC35B2GT (P = 0.0418, P = 0.0232, and P = 0.0139, respectively, one-sided unpaired t test) (Fig. 5A). In addition, we harvested Chlamydia from the null clones and the reconstituted clones and measured the number of infectious-forming units (IFUs) produced by reinoculating dilutions into McCoy cells (Fig. 5B). The differences between B3GAT3GT and SLC35B2GT, between B3GAT3GT and B4GALT7GT, and between B4GALT7GT and SLC35B2GT were significant as well (P < 0.0001, P < 0.0012, and P < 0.0002, respectively, one-sided unpaired t test). Because the mechanism of resistance of the null clones is via attachment, we hypothesized that Chlamydia co-opts a sulfated product to gain entry into the host cell.

We labeled the individual clones with 35SO4 for ~24 h and observed that the SLC35B2GT null clone showed a >90% reduction in incorporation of 35SO4 into high molecular weight (MW) materials as resolved by SDS/PAGE. B3GAT3GT and B4GALT7GT were completely deficient in the production of high MW proteins with heparan sulfate, but showed sulfation of other proteins (Fig. 5C). The inability of B3GAT3GT and B4GALT7GT to produce any heparan sulfate and the ability of SLC35B2GT clone to still yield heparan sulfate at ~5% of WT levels were
confirmed by FACS analysis (Fig. 5D). Thus, other sulfated proteins must contribute to Chlamydia attachment. On autoradiography of $^{35}$SO$_4$-labeled cell-associated materials resolved by SDS/PAGE, the reconstituted SLC35B2 GT + SLC35B2-HA and WT clones both showed smears at $\sim$220 KDa (Fig. S2). This material corresponds to heparan sulfate, being heparitinase-sensitive. The SLC35B2 GT had only $\sim$5–10% of the WT levels of heparan sulfate. Other polypeptides are sulfated as well, some of which are sensitive to PNGase F and thus are N-glycosylated.

In an effort to extend the foregoing results to a different tissue culture model, we created five stable knockdown cell lines each for the B3GAT3, B4GALT7, and SLC35B2 genes, with a GFP knockdown cell line as a control in HeLa cells. The best two knockdowns as determined by RT-PCR are shown in Fig. S3A. We measured heparan sulfate levels by FACS analysis using a heparan sulfate-specific antibody (Fig. S3B). Some reduction in heparan sulfate levels was seen, but to nowhere near the degree seen in the null clones. There was little change in Chlamydia attachment for the knockdown clones, not nearly to the extent seen for the null clones (Fig. S3C). Whether sulfated proteins other than those modified with heparan sulfate contribute to Chlamydia attachment cannot be inferred from these knockdown experiments. Thus, obtaining the null clones by insertion inactivation has a significant advantage over the knockdown approach, yielding complete null mutants.

Taken together, our data indicate that there must be sulfated proteins in addition to those carrying heparan sulfate that are deficient in the B3GAT3 GT, B4GALT7 GT, and SLC35B2 GT null clones, any of which may contribute to Chlamydia attachment.

**Discussion**

We have identified and established by several criteria the involvement of three host genes in the facilitation of Chlamydia infection. The SLC35B2 mutation imparts greater resistance to the attachment of Chlamydia compared with the B3GAT3 or B4GALT7 mutations. Thus, we suggest that the role of sulfated proteins extends beyond the simple provision of heparan sulfate to infection in Chlamydia infection. All of the sulfated proteins present in the reconstituted clones and absent from the null clones are candidates for proteins involved in Chlamydia attachment, but their identity remains to be established. Electrostatic interactions also may play a role, given that negatively charged glycosaminoglycans can block adherence of Chlamydia to host cells (27).

SLC35B2 encodes PAPST1. PAPS, the universal sulfate donor in biological reactions, is synthesized in the cytoplasm or nucleus by PAPS synthetase and transported into the Golgi by the PAPS transporter for sulfation of proteins (28, 29) by the requisite sulfotransferases. The steady-state labeling of the null SLC35B2 and reconstituted SLC35B2 clone with $^{35}$SO$_4$ demonstrates the importance of a functional PAPST1 in allowing sulfation of cellular proteoglycans, such as heparan sulfate (30). The $\sim$5% of heparan sulfate remaining in the SLC35B2 clone may be due to activity of the second PAPS transporter (PAPST2). Chlorate is a competitive inhibitor of PAPS synthetase and blocks sulfation, which partially protects host cells from Chlamydia infection (31). However, the high concentrations of chlorate required to block Chlamydia infection are toxic to the host cells. Furthermore, F-17 cells deficient in 2-O-sulfation of heparan sulfate show no significant reduction in Chlamydia infectivity compared with WT cells (32). Attempts at knockdown of the PAPST1 gene have achieved no better than a 20% reduction in $^{35}$SO$_4$ incorporation, even with a 60% reduction in mRNA levels (25). Even low expression levels of PAPST1 leave the cells largely susceptible to Chlamydia. A small-molecule inhibitor of PAPST1 may be active against Chlamydia infection. The utility of a loss-of-function screen and the null mutants thus identified is self-evident.

Both B3GAT3 and B4GALT7 are important in the synthesis of heparan sulfate and chondroitin sulfate (33–35). CHO cells deficient in enzymes in the heparan sulfate pathway (PgsA-745, PgsD-677, and CHO-761) (4, 36) show decreased infectivity with Chlamydia compared with the parental CHO-K1 line. No reconstitution of chemically mutagenized Chlamydia-resistant CHO cell lines has been reported, presumably because such lines are heavily mutagenized to begin with, complicating identification of the mutations responsible for resistance. No CHO cell line with a mutation in the PAPST1 transporter has been identified to enable more global study of sulfation.

The CHO-18.4 cell line, derived using a retroviral vector with presumably a single insertion and lacking heparan sulfate, showed no difference compared with parental CHO-22 controls, but the location of this insertion remains unknown (7). Heavily mutagenized CHO-224 cells have a presumed glucuronosyltransferase I mutation and, when complemented, show only a twofold difference in both attachment and infectivity. Our results suggest a more important role of glucuronosyltransferase I in Chlamydia...
attachment. Furthermore, FGF2 enhances binding of Chlamydia in an heparan sulfate proteoglycan-dependent manner (37). Candidate Chlamydia proteins involved in these interactions include the Chlamydia OmcB protein, which binds to glycosaminoglycans, such as heparan sulfate (38–40).

Heparan sulfate is believed to be important for many pathogens (41) to allow the infectious cycle to proceed. In the glucuronosyltransferase-deficient CHO cell line, Toxoplasma replication is retarded (42). In the clones that we obtained, we observed no deficiency in the ability of Chlamydia to replicate in the null clones once access was gained, notwithstanding the deficit in attachment. With the genetic methods as applied to CHO cells and summarized above, it has been difficult to establish functional differences between heparan sulfate and other sulfated proteins as contributors to Chlamydia infection. The unique tools that emerge from the present study should facilitate these investigations.

Genes identified on this screen and the isolated clones harboring the individual disruptions may find application in other areas of biology as well. Given that a mutation in B4GALT7 is linked to a progeroid form of Ehlers–Danlos syndrome (43), it may be worth exploring whether such patients are more resistant to Chlamydia infection. For other infectious diseases, including herpes, Toxoplasma, Neisseria, and HIV, these clones can serve as tools to further examine pathogens’ modes of attachment or other aspects of their infectious cycles, which may involve a role for sulfation beyond heparan sulfate. Given the importance of sulfation in controlling the activity of certain G protein-coupled receptors (21–23, 44), the availability of defined mutant cell lines defective in sulfation creates new opportunities that complement the mutation of putative sulfation sites in the receptors themselves. Should the HAPI cell line prove amenable to differentiation into more specialized cell types (17), it may even be possible to explore tissue-specific aspects of sulfation.

Materials and Methods

Haploid Genetic Screen. The creation of the haploid HAPI cell line (26) and the screening procedure have been described previously (13, 15). In brief, the gene-trap retrovirus was created from transfection of low-passage T293 cells. Approximately 100 million HAPI cells were infected with concentrated gene-trap retrovirus to create a mutagenized library. The mutagenized cells were then inoculated with C. trachomatis L2 at an MOI of ∼6 and incubated for 5 d. Dead cells were removed by exchanging the spent media for fresh media. After 1 wk, approximately 500 visible Chlamydia-resistant colonies were collected. Most of the survivors were purified and grown to 30 million cells after 5 d.

Attachment Assay. The attachment assay was performed by inoculating the clones with an MOI of ∼200 at 24 °C for 1 h without centrifugation. The cells were then washed four times. For microscopy, the cells were then fixed with methanol. The Chlamydia EBs were stained with an FITC-MOMP, and the HAPI-derived cells were counterstained with Evans blue using the MicroTrak Chlamydia trachomatis Kit (Trinity Biotech).

Quantitative PCR. A quantitative PCR assay was performed to quantify attached Chlamydia on host cells (45). The nucleic acids were isolated using the QIamp DNA Mini Kit (Qiagen) or High Pure PCR Template Preparation Kit (Roche). Human β-actin DNA and Chlamydia 16S DNA were quantified on an Applied Biosystems ABI Prism 7000 sequence detection system using primer pairs and dual-labeled probes. The ratio of the weight of Chlamydia to human DNA in the samples was calculated using standard curves from known amounts of Chlamydia and human DNA.
Viability Assay. The Cell-Titer-Glo Luminescent Cell Viability Assay (Promega) was used to quantify the number of cells with a luminescent output. Cells were seeded in a 12-well plate at ~10–30% confluence and inoculated with an MOI of 6. After 4 d of culture, cell viability was determined using the ratio of luminescence from the Chlamydia-infected clone to the luminescence from the uninfected clone.

Sulfate Labeling. Cells were seeded into six-well plates until they achieved ~70% confluence and 0.5 mM [35S]SO4 or 0.3 mM [33]methyl[35]S cysteine (PerkinElmer Life Sciences) was added to the media in each plate, and the cells were left to reach steady state for ~24 h. A portion of each sample was harvested, lysed in Nonidet P-40, and then digested with heparitinase. After the digestion, the samples were subjected to the Viability Assay.

The lysates were resolved on an SDS-PAGE gel, and the polypeptides were visualized by autoradiography.

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