Chlamydial Infection Induces Pathobiotype-Specific Protein Tyrosine Phosphorylation in Epithelial Cells

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Members of the genus Chlamydia are strict obligate intracellular pathogens that exhibit marked differences in host range and tissue tropism despite sharing a remarkable level of genomic synteny. These pathobiotype differences among chlamydiae are also mirrored in their early interactions with cultured mammalian host cells. Chlamydial attachment and entry is known to trigger protein tyrosine phosphorylation. In this study, we examined the kinetics and pattern of protein tyrosine phosphorylation induced by infection with a comprehensive collection of chlamydial strains exhibiting diversity in host, tissue, and disease tropisms. We report new findings showing that protein tyrosine phosphorylation patterns induced by infection directly correlate with the pathobiotype of the infecting organism. Patterns of protein tyrosine phosphorylation were induced following early infection that unambiguously categorized chlamydial pathobiotypes into four distinct groups: (i) Chlamydia trachomatis trachoma biovars (serovars A to H), (ii) C. trachomatis lymphogranuloma venereum biovars (serovars L1 to L3), (iii) C. muridarum, and (iv) C. pneumoniae and C. caviae. Notably, chlamydia-infected murine and human epithelial cells exhibited the same protein tyrosine phosphorylation patterns; this is indirect evidence suggesting that the phosphorylated protein(s) is of chlamydial origin. If our hypothesis is correct, these heretofore-uncharacterized proteins may represent a novel class of bacterial molecules that influence pathogen-host range or tissue tropism.

Chlamydiae are obligate intracellular bacteria that exhibit remarkable diversity in host species, tissue tropism and disease pathogenicity (Table 1). Chlamydiae have two distinct developmental forms: the extracellular, infectious, metabolically inactive elementary body (EB) and the intracellular, noninfectious, metabolically active reticulate body (RB) (20). Early attachment of the EB and commencement of infection is mediated by binding of the chlamydial major outer membrane protein (MOMP) to heparin or heparin sulfate glycosaminoglycan moieties on the host cell surface (1, 4, 5, 22, 24–26). After attachment, a second uncharacterized step is required for entry of the EB into host cells (7, 28). Upon completion of the attachment and entry steps, chlamydiae promote actin cytoskeletal rearrangement during entry into nonlyosomal fusogenic phagosomes where the pathogen replicates (6, 9, 20). Attachment and/or entry of chlamydiae into host cells induces protein tyrosine phosphorylation (2, 9, 10, 14). The identity and source of these phosphorylated proteins remains controversial. Birkeland et al. (2) first reported that infection of host cells with Chlamydia trachomatis serovar L2 induces protein tyrosine phosphorylation of several proteins: a triplet of ~64, 66, and 68 kDa and polypeptides with masses of 97 and 140 kDa, respectively; however, in a subsequent study, C. pneumoniae VR1310 failed to induce tyrosine phosphorylation in either HeLa 229 or Henle 407 host cells (14). Fawaz et al. (10) later extended these findings by demonstrating that L2 and the mouse strain C. muridarum (MoPn) induced different patterns of phosphorylation in HeLa cells. More recently, Clifton et al. reported that a protein tyrosine-phosphorylated during chlamydial infection (Tarp) was of chlamydial origin (9). They suggested that Tarp was translated into the host cytosol by a chlamydial type III secretion mechanism and that secreted Tarp facilitated actin rearrangement during entry into host cells. Altogether, these results, using a limited number of strains suggested that tyrosine phosphorylation may be critical to early chlamydial-host interaction and might vary among chlamydial strains of differing host and tissue tropism in vivo.

In the present study, we determined the relationship between chlamydial pathobiotype and the pattern of protein tyrosine phosphorylation induced by attachment and entry into cultured human and murine epithelial cells. We compared protein tyrosine phosphorylation profiles induced by 15 Chlamydia reference serovars, comprising trachoma and lymphogranuloma venereum (LGV) biovars, the human respiratory pathogen C. pneumoniae strain AR-39, and two rodent chlamydial pathogens, C. muridarum and C. caviae. We report that protein tyrosine phosphorylation induced by chlamydial infection in vitro differs with respect to infecting strain and that these differences directly correlate with chlamydial pathobiotype, suggesting that they may play an important functional role in the pathogenesis of infection.

MATERIALS AND METHODS

EB were purified by density gradient centrifugation, and infection-forming units (IFU) were determined as described previously (5).

Chlamydial infection of HeLa 229, BM12.4 cells. HeLa 229 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (DMEM-10) at 37°C in 5% CO2. Cells were seeded into 24-well tissue culture plates at a density of 5 × 10^5 cells/ml in DMEM-10, DMEM-10 containing rifampin (1 μg/ml), or emetin (1 μg/ml) and then incubated for 24 h at 37°C. BM12.4 primary murine oviduct epithelial cells were seeded identically except that they were propagated in 1:1 DMEM-10:F12K (Sigma Chemical Co.) medium supplemented with human recombinant FGF-7, as described previously (15). Cell monolayers were incubated on ice for 15 min, washed twice with 4°C Hanks balanced salt solution supplemented with 10 mM HEPES (HBSS), and incubated on ice in 45 μg of DEAE-dextran/ml in HBSS solution for 15 min. After dextran treatment, the EBs suspended in sucrose phosphate glutamic acid buffer were added to the cell layers at various multiplicities of infection (MOI), and the cells were incubated at 4°C for an additional 60 min. To initiate EB entry, the inoculum was aspirated, the cells were washed twice with cold HBSS, and the temperature was shifted to 37°C by adding prewarmed DMEM-0% fetal bovine serum (DMEM-0). After temperature shift (designated time zero in experimental time course infections), the infected and mock-treated cells were incubated at 37°C in 5% CO2 until protein harvest.

Western blot detection of chlamydia-induced tyrosine, serine, and threonine phosphorylation in HeLa 229 and BM12.4 cells. Total cellular proteins from 2 × 10^6 infected HeLa 229 or BM12.4 cells were extracted in 200 μl of 2× Laemmli sample buffer supplemented with 5% (vol/vol) 14.2 M β-mercaptoethanol and denatured at 95°C for 5 min. Proteins were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted (100 V, 30 min) onto 0.2-μm pore-size nitrocellulose membranes (Bio-Rad, Inc.). Membranes were pre-blocked in TBSTB (1× Tris buffered saline [TBS], 0.1% Tween, 1% bovine serum albumin) solution for 1 h at room temperature. Blots were incubated with primary antiphosphotyrosine antibody 4G10 (1:1,000; Upstate, Lake Placid, N.Y.), antiphosphoserine antibody ab9334-100 (1:200; Abcam, Cambridge, Mass.), or antiphosphothreonine antibody ab9338-50 (1:200; Abcam) in TBSTB overnight at 4°C. Membranes were washed three times (20 min each wash) in TBS supplemented with 0.1% Tween and 5% powdered milk. Washed membranes were incubated with secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies for 2 h at room temperature in TBS supplemented with 0.1% Tween and 5% powdered milk and then washed as described above. Proteins were detected by using the Phototope-HRP Western blot detection system (Cell Signaling Technology, Boston, Mass.) according to the manufacturer’s instructions.

Direct inclusion staining and determination of recoverable IFU. HeLa 229 monolayers were infected with C. trachomatis serovars (B, D, H, and L2), C. muridarum strain mouse pneumonitis (MoPn), C. pneumoniae AR-39, or C. caviae strain GPIC, in quadruplicate, at MOI ranging from 0.025 to 1.0, as described above for protein harvest infections and then grown in DMEM-10 at 37°C in 5% CO2. After inclusion maturation, two wells of each infection condition were methanol fixed at 42 h after infection (MoPn, GPIC, and L2), 50 h after infection (D, H, and B), and 70 h after infection (C. pneumoniae); labeled using primary antichlamydial lipopolysaccharide monoclonal antibody EVI-H1 and secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and photographed at ×100 magnification. To determine recoverable IFU, the two remaining wells from each experimental infection were scrapped into 0.5 ml of sucrose phosphate-glutamic acid buffer; the cells were then mechanically disrupted with glass beads, and the lysates were passed onto HeLa 229 monolayers and counted as previously described (5).

RESULTS

C. trachomatis infection induces rapid, protein synthesis-independent, protein tyrosine phosphorylation in HeLa 229 cells in vitro. To determine whether the human mucosal pathogen C. trachomatis serovar D induces protein tyrosine phosphorylation, HeLa 229 cells were infected at MOI ranging from 0 to 100, and total cellular proteins were harvested 1 h postinfection (p.i.). Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted to nitrocellulose, and probed with the antiphosphotyrosine-specific monoclonal antibody 4G10 (Fig. 1A). Serovar D infection induced protein tyrosine phosphorylation in HeLa 229 cells in an MOI-dependent fashion, with the pattern of phosphorylation being most similar to that previously reported for C. trachomatis.
matis L2. Infection-induced tyrosine-phosphorylated proteins included an intensely labeled complex of proteins migrating with apparent molecular sizes ranging from 70 to 75 kDa (70-kDa complex) induced at MOI of ≥10, presumably corresponding to the 64- to 68-kDa complex of proteins reported by Birkelund et al. (2) in association with C. trachomatis L2 infection. A single tyrosine-phosphorylated protein of ≈110 kDa, corresponding to the 98-kDa L2 protein reported by Birkelund et al. (2), was induced at the same MOI but was less intensely labeled than the 70-kDa complex. Because both the 70-kDa complex and the 110-kDa protein were easily visualized at MOI of 50, this inoculum was used for subsequent kinetic studies.

To determine the kinetics of tyrosine phosphorylation, HeLa 229 cells were infected with C. trachomatis serovar D at an MOI of 50, and total proteins were harvested at various intervals between 0 and 60 min p.i. Tyrosine phosphorylation of the 70-kDa complex was detectable within 10 min of infection (Fig. 1B). The labeling intensity increased progressively during the 1-h time course. As previously described for L2-induced protein tyrosine phosphorylation, the kinetics, the pattern and intensity of tyrosine-phosphorylated proteins were essentially identical in the presence of host (emetin) or bacterial (rifampin) inhibitors of protein synthesis (Fig. 1C and D) (2).

Chlamydial induction of protein tyrosine phosphorylation is pathobiotypic specific. Our investigation into phosphorylation induced by C. trachomatis serovar D suggested the pattern of chlamydia-induced protein tyrosine phosphorylation was similar, but not identical, to that previously reported for L2 (2). Considering the data in toto, we suspected that phosphorylation might correlate with early infection events that differentiate chlamydial biology in vivo. To test this hypothesis, HeLa 229 cells were infected with C. trachomatis trachoma bi variars (A, B, Ba, C, D, E, F, G, and H), C. trachomatis LGV bi variars (L1, L2, and L3), the human respiratory pathogen C. pneumoniae AR-39, and the rodent pathogens C. muridarum MoPn and C. caviae GPIC at MOI of 50 and harvested 1 h PI.

Profiles of protein tyrosine phosphorylation induced by chlamydial infection fell into four distinct groups and, surprisingly, these groups sorted the infecting strains into established in vivo pathobiotypes (Fig. 2A). First, the 110-kDa protein (Fig. 2B, protein numbered 1) was induced by all C. trachomatis strains, including MoPn (formerly designated C. trachomatis strain Nigg). In contrast, neither the more distantly related human pathogen C. pneumoniae AR-39 nor C. caviae strain GPIC induced phosphorylation of any proteins above background (mock infection) levels (Fig. 2A to C). In order to exclude the possibility that the slower growing C. pneumoniae AR-39 induces tyrosine phosphorylation later, we extended our tyrosine phosphorylation screen to 2, 4, and 8 h after infection. Both C. pneumoniae AR-39 and C. caviae GPIC strains failed to induce any detectable tyrosine phosphorylation during this time period (Fig. 2D).

More interestingly, the pattern of tyrosine-phosphorylated proteins in the 70-kDa complex strictly sorted the C. trachomatis strains into trachoma and LGV bi variars. C. trachomatis trachoma bi variars, mucous-tropic pathogens in vivo, induced tyrosine phosphorylation of three major proteins ranging from ca. 75 to 70 kDa (Fig. 2B and C, proteins 4, 5, and 6). In contrast, C. trachomatis LGV bi variars, nonmucosal lymphotropic pathogens in vivo, induced phosphorylation of the two smaller proteins in the 70-kDa complex (Fig. 2B and C, proteins 5 and 6) but failed to phosphorylate the largest of the three proteins (Fig. 2B and C, protein 4) induced by trachoma isolates. C. muridarum MoPn, a more distant relative of the human-adapted strains restricted in vivo to the murine host, triggered phosphorylation of a complex of proteins in the range of 75 to 85 kDa, clearly distinct from all human C. trachomatis strains. Strain-specific protein tyrosine phosphorylation patterns were distinct and conserved between in-
FIG. 2. Induction of protein tyrosine phosphorylation by chlamydial infection. (A) HeLa 229 cells were infected with the C. trachomatis trachoma biovar serovars (A to H), LGV biovar serovars (L1, L2, and L3), C. muridarum strain Nigg (MoPn), C. pneumoniae AR-39 (C. pn.), or C. caviae (GPIC). The most predominant regions of tyrosine phosphorylation are marked with an asterisk. (B) Composite blot from panel A showing examples of each of the four prototypic protein tyrosine profiles induced by C. trachomatis serovar H (trachoma), C. trachomatis LGV biovar L2 (lymphogranuloma), C. muridarum strain Nigg (mouse pneumonitis), and C. pneumoniae AR-39. Major chlamydia-induced tyrosine-phosphorylated proteins are indicated by large arrows and numbers; minor proteins are indicated by small arrows. (C) Magnified view of the 65- to 90-kDa region of the blot in panel B. (D) Tyrosine phosphorylation screen at later time points of chlamydial infection shows that C. pneumoniae and C. caviae (GPIC) failed to induce tyrosine phosphorylation at 2, 4, and 8 h after infection.
dependent experiments performed at multiple MOI (data not shown). Further, differential induction of protein phosphorylation was restricted to tyrosine residues, since all infected samples, as well as mock samples, yielded similar patterns of serine and threonine protein phosphorylation in parallel immunoblots performed with antiphosphothreonine- and antiphosphoserine-specific antibodies (data not shown).

Chlamydial infection efficiency for HeLa 229 cells. Because chlamydial strain-specific patterns of protein tyrosine phosphorylation were repeatable and observed at different MOI in independent experiments, we reasoned that these differences might reflect differential sensitivity of the host HeLa 229 cells to various chlamydial strains. To test this, HeLa 229 cells were infected at different MOI with the identical chlamydial stocks used in the tyrosine phosphorylation experiments. Infected cells were fixed and chlamydial inclusions visualized by immunofluorescence microscopy (Fig. 3A). In a parallel experiment, inclusions were allowed to mature, and recoverable IFU derived from infections performed at different MOI were determined (Fig. 3B). Both direct inclusion staining and recoverable IFU counts confirmed that essentially equivalent doses of viable chlamydial inoculum were used in determinations of protein tyrosine phosphorylation and that *C. pneumoniae* AR-39 and *C. caviae* GPIC complete their developmental cycles in HeLa 229 host cells. This result strongly argues that the observed differences in phosphorylation profiles reflect fundamental biologic differences between chlamydial strains and that the lack of tyrosine phosphorylation in the case of *C. pneumoniae* and *C. caviae* was not due to the inefficient infection.

Comparison of chlamydia-induced tyrosine phosphorylation in human and mouse epithelial cells. Whether tyrosine-phosphorylated proteins induced by chlamydial infection are of host or bacterial origin remains controversial; data implicating individual *C. trachomatis* serovar L2-induced tyrosine-phosphorylated proteins deriving from both sources have been reported (2, 9, 10, 14). In the absence of direct identification of each of these proteins, we reasoned that infection of different host cells might suggest their origin. To test this, we infected
HeLa 229 and BM12.4 primary murine oviduct epithelial cells (15) with chlamydial strains at an MOI of 50 and immunoblotted whole-cell lysates harvested 1 h p.i. with 4G10 (Fig. 4). Surprisingly, the infection and strain-specific tyrosine-phosphorylated protein profiles induced by chlamydial infection were nearly identical between the human and mouse cells. In contrast to the conserved infection-induced tyrosine-phosphorylated protein profiles in the three cell lines, the pattern of background 4G10 reactivity differed markedly (Fig. 4). Therefore, the results indicate that the trachoma-induced 70-kDa complex proteins and the C. muridarum 75- to 85-kDa complex proteins are of chlamydial origin, however; we cannot exclude the possibility that four different chlamydia strains induced the phosphorylation of a cohort of conserved host cell proteins with very similar sizes in human and murine cells.

DISCUSSION

We report that the profile of protein tyrosine phosphorylation induced by chlamydial infection groups chlamydiae into pathobiotypes differentiated according to host range, tissue infection tropism, and early interactions with cultured host cells (Table 1). Categorically, protein tyrosine phosphorylation profiles sort trachoma mucosa-tropic and invasive LGV macrophage-tropic C. trachomatis human pathogens from one another, as well as from the genetically synteneic C. muridarum murine pathogen (19, 21). Further, C. pneumoniae and the related C. caviae strain GPIC failed to induce phosphorylation in host HeLa 229 cells, even at later times p.i., whereas the infection efficiency of these two strains were similar to the tyrosine phosphorylation inducer strains. To our knowledge, this is the first description of discrete biologic properties that exhibits a precise correlation with chlamydial pathobiotype. This observation implies that chlamydia-induced protein tyrosine phosphorylation reflects early critical events that influence functional outcomes of the host cell response to infection that could affect the pathogen infection tropism and, thus, reflect differences in disease pathogenesis.

In addition, we observed that strain-specific chlamydia-induced protein tyrosine phosphorylation patterns are common to human and murine host cells. Induction of identical, strain-specific protein tyrosine phosphorylation profiles in cells from distant host species argues against the interpretation that these proteins are conserved host proteins of mice and humans. Excluding the specific chlamydia-induced tyrosine-phosphorylated proteins, the profile of background 4G10 labeling in murine and human cells was highly variable, strengthening our interpretation that these proteins are likely of chlamydial origin.

If our hypothesis that the 70-kDa chlamydia-induced tyrosine-phosphorylated protein(s) are of chlamydial origin is correct, what might these virulence proteins be? Our experiments establish criteria that this putative protein must fulfill. First, phosphorylation occurs at the time of chlamydial attachment and entry and is insensitive to inhibitors of bacterial and host cell protein synthesis, strongly implicating that the proteins are either located at the EB surface or secreted directly from the EB at the time of infection. Second, the chlamydial protein must be similar between trachoma and LGV strains but significantly variable to distinguish among these groups, as well as C. muridarum, C. pneumoniae and C. caviae. Considering these factors, we narrow our search to a highly polymorphic, pathobiotype-related, surface-exposed chlamydial proteins. One possible candidate is the MOMP, a highly variable surface protein of the EB and RB, which is a primary target of the immune response that functions as a cytoadhesin (25, 26, 28). However, MOMP serotyping and genotyping characteristics fail to differentiate Chlamydia by pathobiotype, MOMP is smaller (40 kDa) than the tyrosine-phosphorylated proteins observed in the present study (which were >70 kDa), and
recombinant MOMP did not induce detectable protein tyrosine phosphorylation in our assay system (data not shown). The 70-kDa family of tyrosine-phosphorylated proteins described here is also not related to the recently described phosphorylated chlamydial Tarp protein (9). This conclusion is based on the following two findings. (i) The mass of the proteins was visibly distinguishable after SDS-PAGE, and Tarp migrates with the following two findings. (i) The mass of the proteins was visible chlamydial Tarp protein (9). This conclusion is based on here is also not related to the recently described phosphorylation.

We speculate that the 70-kDa pathobiotype tyrosine-phosphorylated protein(s) may be member(s) of the polymorphic outer membrane protein gene family (pmp). The pmp are a polymorphic superfamily of six or more genes, depending on the chlamydial strain, encoding large proteins (90 to 187 kDa) with a distinct homology to type V secreted autotransporters (8, 11, 12, 23). Of interest, pmp genes are absent in the genome of the related paracelchlamydial symbiont (UWE-25) of free-living amoeba (13), implying a role for the protein(s) in the infection of mammalian cells. Some polymorphic membrane proteins (PMP) are expressed on the EB surface, are targets of neutralizing antibodies, and induce host-cell signaling and cytokine secretion (8, 18, 23, 27). Moreover, Stothard et al. (23) showed substantial variation among pmp genes E, H, and I by restriction fragment length polymorphism analysis of C. trachomatis serovars. Of particular relevance to the present study was the finding that such polymorphisms among pmp H correlated exactly with the three major C. trachomatis disease groups, prompting Stothard et al. to suggest that the pmp may play a role in pathogenesis (23). Intriguingly, the molecular mass of processed PMP is ~70 kDa, a size consistent with that observed for the major tyrosine-phosphorylated protein complex observed during infection with human-adapted C. trachomatis strains in this study (12, 27).

Although attractive, the role of pmp genes in tyrosine phosphorylation is inconsistent with observations that C. pneumoniae and C. caviae possess full complements of pmp genes but do not induce tyrosine phosphorylation. One possible explanation is that these pmp genes functionally differ; of 21 C. pneumoniae pmp genes, only two can activate NF-κB (17) C. trachomatis and C. muridarum may retain a subset of pmp genes that are stronger inducers of tyrosine phosphorylation. Alternately, C. pneumoniae and its genetically close relative, C. caviae, might also use unique factors, such as the Yersinia YopH protein, to mask this signaling event (3).

The correlations between PMP and the 70-kDa tyrosine-phosphorylated protein(s) described here are significant; however, it is imperative to identify the 70-kDa chlamydia-induced tyrosine-phosphorylated protein(s) to prove this hypothesis, a goal we are currently pursuing.

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