Identification of Putative Cytoskeletal Protein Homologues in the Protozoan Host *Hartmannella vermiformis* as Substrates for Induced Tyrosine Phosphatase Activity upon Attachment to the Legionnaires' Disease Bacterium, *Legionella pneumophila*

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Summary

The Legionnaires' disease bacterium, *Legionella pneumophila,* is a facultative intracellular pathogen that invades and replicates within two evolutionarily distant hosts, free living protozoa and mammalian cells. Invasion and intracellular replication within protozoa are thought to be major factors in the transmission of Legionnaires' disease. We have recently reported the identification of a galactose/*N*-acetyl-D-galactosamine (Gal/GalNAc) lectin in the protozoan host *Hartmannella vermiformis* as a receptor for attachment and invasion by *L. pneumophila* (Venkataraman, C., B.J. Haack, S. Bondada, and Y.A. Kwaik. 1997. *J. Exp. Med.* 186:537–547). In this report, we extended our studies to the effects of bacterial attachment and invasion on the cytoskeletal proteins of *H. vermiformis*. We first identified the presence of many protozoan cytoskeletal proteins that were putative homologues to their mammalian counterparts, including actin, pp125^{FAK}, paxillin, and vinculin, all of which were basally tyrosine phosphorylated in resting *H. vermiformis*. In addition to *L. pneumophila*–induced tyrosine dephosphorylation of the lectin, bacterial attachment and invasion was associated with tyrosine dephosphorylation of paxillin, pp125^{FAK}, and vinculin, whereas actin was minimally affected. Inhibition of bacterial attachment to *H. vermiformis* by Gal or GalNAc monomers blocked bacteria-induced tyrosine dephosphorylation of detergent-insoluble proteins. In contrast, inhibition of bacterial invasion but not attachment failed to block bacteria-induced tyrosine dephosphorylation of *H. vermiformis* proteins. This was further supported by the observation that 10 mutants of *L. pneumophila* that were defective in invasion of *H. vermiformis* were capable of inducing tyrosine dephosphorylation of *H. vermiformis* proteins. Entry of *L. pneumophila* into *H. vermiformis* was predominantly mediated by noncoated receptor-mediated endocytosis (93%) but coiling phagocytosis was infrequently observed (7%). We conclude that attachment but not invasion by *L. pneumophila* into *H. vermiformis* was sufficient and essential to induce protein tyrosine dephosphorylation in *H. vermiformis*. These manipulations of host cell processes were associated with, or followed by, entry of the bacteria by a noncoated receptor-mediated endocytosis. A model for attachment and entry of *L. pneumophila* into *H. vermiformis* is proposed.

Key words: intracellular • bacteria • protozoa • cytoskeleton • lectin

Entry of intracellular bacteria into host cells involves interaction with the host cell receptor and manipulation of host signaling and cytoskeletal processes to induce bacterial uptake (1, 2). Invasion of epithelial cells by *Shigella flexneri* and *Yersinia enterocolitica* requires contact with the β1 integrins present on host cell surfaces (1). Integrins are heterodimeric transmembrane receptors comprising of α and β subunits that serve as a link between the extracellular matrix and the actin cytoskeleton at sites of close cell–substratum contact (3). Clustering of integrins upon binding to their ligands induces tyrosine phosphorylation of integrins and recruitment of several cytoskeletal and regulatory proteins forming focal adhesions at contact sites (3–5). Protein tyrosine phosphatases are involved in the regulation of focal contacts and their stability (6–9).

The Legionnaires' disease bacterium, *Legionella pneumophila*, is a facultative intracellular pathogen that invades and replicates within a rough endoplasmic reticulum (RER)[1]-surrounded phagosome in human macrophages and epithelial cells (10). In the aquatic environment, *L. pneumophila* is a parasite of protozoa. The bacteria are transmitted by aerosols generated in the environment. Intracellular replication within protozoa probably play major factors in bacterial amplification and transmission to humans (10–13). Interestingly, similar to the intracellular infection of macrophages, the bacteria are also targeted within protozoa into a replicative phagosome surrounded by the RER (14). Although *L. pneumophila* has loci that are required for survival within both macrophages and protozoa, the bacterium has mammalian-specific infectivity loci (*mil*) that are not required for infectivity of protozoa (15, 16).

Initial attachment of *L. pneumophila* to protozoa is mediated by the bacterial type IV pili, but the receptor for these pili is not known (17). Recently, we have demonstrated that attachment and invasion of *L. pneumophila* into its protozoan host, *H. vermiformis*, is mediated by bacterial attachment to a 170-kD galactose/*N*-acetyl-D-galactosamine (Gal/GalNAc) inhibitable lectin, a homologue of a β_2 integrin–like Gal/GalNAc lectin of the human pathogen, *Entamoeba histolytica* (18–20). Bacterial uptake by *H. vermiformis* is insensitive to the inhibitory effects of cytochalasin D (21). In addition, many genes are induced upon bacterial attachment and invasion, and inhibition of protein synthesis of *H. vermiformis* blocks bacterial entry (22).

Attachment of *L. pneumophila* to the Gal/GalNAc lectin of *H. vermiformis* is associated with a time-dependent and reversible tyrosine dephosphorylation of several host cell proteins, including the 170-kD lectin (18). This bacteria-induced host tyrosine dephosphorylation is blocked by prior treatment of *H. vermiformis* with tyrosine phosphatase inhibitors, indicating a bacterial attachment-mediated induction of a tyrosine phosphatase activity (18). It is not known whether protein tyrosine dephosphorylation in *H. vermiformis* plays any role in modulating host cytoskeletal events upon attachment and entry of *L. pneumophila* into the protozoan host.

Focal adhesions have been recognized in *Entamoeba histolytica* and *Acanthamoebae*, and have been shown to be involved in attachment to surfaces, interaction with the receptor upon engagement to ligands, and locomotion of the parasite (23–28). The protozoan focal adhesions are also disrupted by cytochalasin D, similar to the mammalian focal adhesions (26). Protozoan focal adhesions are composed of cytoskeletal and regulatory proteins that are antigenically and functionally homologous to their mammalian counterparts, including actin, α-spectrin, myosin II, pp125[FAK], vinculin, α-actinin, protein kinase C, and MAP kinase (25, 27, 28). Many of these protozoan proteins, similar to their mammalian homologues, have been also shown to undergo tyrosine phosphorylation upon receptor engagement (25, 27, 28). These observations indicate that many aspects of receptor-mediated signal transduction are highly conserved through evolution.

In this report, we examined the events leading to cytoskeletal changes during the uptake of *L. pneumophila* by *H. vermiformis*. Attachment of *L. pneumophila* to *H. vermiformis* is associated with a time-dependent and reversible tyrosine dephosphorylation of many putative homologues of mammalian cytoskeletal proteins, including, paxillin, vinculin, and pp125[FAK] whereas actin was minimally affected. These alterations in host cell processes were associated with or followed by a noncoated receptor-mediated endocytic uptake of the bacteria.

Materials and Methods

Bacterial and Protozoan Strains and Culture. *Legionella pneumophila* AA100 is a virulent clinical isolate that has been described previously (29). The construction of a mini Tn*10*::kan transposon insertion library in *L. pneumophila* AA100 has been described previously (15). The mini Tn*10*::kan insertion mutants of *L. pneumophila* (GF162, GG104, GB112, GM128, GO128, GP65, GQ262, and GT251) were selected according to their defects in invasion of *H. vermiformis*, using gentamicin protection assays, as we described previously (18). In brief, after 1 h of infection, extracellular bacteria were killed with gentamicin, and intracellular bacteria were plated for colony enumeration. The percentage of invasion by the mutants was obtained relative to the wild-type strain. Through Southern hybridizations, these mutants have been confirmed to contain distinct insertions in their chromosomes (15). *L. pneumophila* was grown on buffered charcoal yeast extract agar plates at 37°C, or in the presence of 50 μg/ml of kanamycin for the mutants. For infections, bacteria grown for 48 h on agar plates were resuspended in serum-free axenic medium to a desired concentration.

H. vermiformis strain CDC-19 (American Type Culture Collection [ATCC] 50237) is an established model for the study of pathogenesis of *L. pneumophila*, and was isolated from a water source of an outbreak of nosocomial Legionnaires' disease (14, 30). The amebae were maintained in ATCC culture medium 1034 (30).

Reagents and Antibodies. Biotinylated and horseradish peroxidase (HRP)-conjugated recombinant antiphosphotyrosine antibodies were purchased from Transduction Laboratories (Lexington, KY). D(+)-Galactose, *N*-acetyl-D-galactosamine (GalNAc), D(+)-mannose, and antibodies to vinculin (monoclonal mouse anti–human vinculin, IgG1, clone hVIN-1), and actin (monoclonal mouse anti–amebae actin, IgG1, clone KJ43A) were obtained from Sigma Chemical Co. (St. Louis, MO). Antipaxillin (monoclonal mouse anti–chicken paxillin antibody, IgG1, clone Z035) and anti-pp125[FAK] (polyclonal rabbit anti–human pp125[FAK] antibody) antibodies were from Zymed Laboratories (San Francisco, CA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Methylamine was purchased from Sigma Chemical Co.

Detection of Tyrosine-phosphorylated Proteins in H. vermiformis upon Contact with L. pneumophila. *H. vermiformis* were harvested and infected with *L. pneumophila* as we described previously (18). At several time intervals of coincubation at 37°C, amebal cell lysates were prepared for immunoblot and immunoprecipitation analysis as described below.

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[1]*Abbreviations used in this paper:* GalNAc, D(+)-Galactose, *N*-acetyl-D-galactosamine; HRP, horseradish peroxidase; *mil*, mammalian-specific infectivity; moi, multiplicity of infection; RER, rough endoplasmic reticulum.

To examine the ability of some sugars to block tyrosine dephosphorylation of amebal proteins upon contact with *L. pneumophila*, *H. vermiformis* were preincubated before the infection in the presence of different sugars. Preincubation of amebae was performed for 15 min on ice followed by coincubation with the bacteria at 37°C. At the end of the coincubation period, amebal cell lysates were prepared as described below.

Preparation of Cell Lysates. After incubation of *H. vermiformis* with *L. pneumophila*, infections were stopped using cold stop buffer containing protease and phosphatase inhibitors (18). Cells were washed three times with stop buffer and pelleted by low speed centrifugation at 735 *g* for 2 min. The amebae were lysed using cold 0.1% Triton X-100 lysis buffer, and the detergent-soluble and -insoluble fractions were separated by centrifugation, as described previously (18). Proteins from detergent-insoluble fraction (pellet) were resuspended in 50 μl of lysis buffer and sonicated for 5 s.

Western Blotting and Immunoprecipitation Studies. Proteins from detergent-soluble and -insoluble fractions were resolved on 10% SDS-PAGE under reducing conditions. After transfer onto Immobilon-P (Millipore, Bedford, MA), membranes were incubated in a blocking buffer containing 1.5% BSA for 30 min. Membranes were probed with antiphosphotyrosine antibody (RC-20; Transduction Laboratories). After extensive washing, blots were developed using the enhanced chemiluminescence kit (DuPont NEN, Boston, MA) according to the manufacturer's instructions.

For immunoprecipitations, cell extracts were incubated with biotinylated anti-phosphotyrosine antibodies and immune complexes were collected using avidin-agarose beads (Pierce Chemical Co., Rockford, IL). The beads were washed extensively with lysis buffer and eluted proteins were resolved under SDS-PAGE conditions. After transfer onto Immobilon-P membranes, the blots were probed with antibodies to pp125[FAK], paxillin, or vinculin. Antiphosphotyrosine immunoprecipitates were also analyzed by immunoblotting with antiactin antibody in the same experiment. This was followed by incubation with either HRP-conjugated goat anti–mouse or goat anti–rabbit antibody (Santa Cruz Biotechnology Inc.) and proteins were visualized as described above. The relative intensities of the protein bands were quantitated using the NIH Image program (version 1.6). The results were expressed as percentages of uninfected controls. The percent tyrosine phosphorylation of each of the cytoskeletal proteins compared with uninfected controls (PY%) was calculated as a ratio of tyrosine-phosphorylated paxillin, pp125[FAK] or vinculin to the tyrosine-phosphorylated actin for that time point during infection in the same experiment.

Transmission Electron Microscopy. *H. vermiformis* were infected with *L. pneumophila* using a multiplicity of infection (moi) of 100 at 4°C to allow attachment, followed by a temperature shift to 37°C for 8 min to allow uptake. Preparation of ultrathin sections was performed as described previously (15). In brief, infected amebae were extensively washed with tissue culture medium to remove extracellular bacteria, fixed with 3.5% glutaraldehyde followed by 1% OsO$_4$, dehydrated by ethanol, and embedded in Eponate 12 resin (Ted Pella, Redding, CA). Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined by a Hitachi H-7000/STEM electron microscope (Hitachi Inc., Tokyo, Japan) at 75 KV. Multiple sections of different samples were examined.

Results

Attachment and Invasion of H. vermiformis by L. pneumophila Is Associated with Tyrosine Dephosphorylation of Several Detergent-insoluble Proteins. Previously, we have shown that attachment and entry of *L. pneumophila* into its protozoan host, *H. vermiformis*, was associated with a time-dependent and reversible tyrosine dephosphorylation of multiple host proteins in the detergent-soluble fraction (18). To further investigate this, we examined the changes in the tyrosine phosphorylation status of proteins in the detergent-insoluble fractions, which constitute most of the cytoskeletal proteins. *H. vermiformis* was infected with *L. pneumophila* for several time intervals and detergent-insoluble fractions of *H. vermiformis* were subjected to antiphosphotyrosine immunoblot analysis. In contrast to uninfected *H. vermiformis* at time zero (Fig. 1 *A*, lane *1*) or 30 min incubated under identical conditions of infection (Fig. 1 *A*, lane *6*), tyrosine dephosphorylation of many proteins was seen in infected *H. vermiformis* (Fig. 1). Prominent tyrosine dephosphorylation of many proteins with apparent molecular mass of 170, 150, and 120–125 kD indicated by arrowheads was detectable as early as 1 min, and remained dephosphorylated during the 30-min infection period (Fig. 1 *A*, lanes *2–5*). Although the degree of protein tyrosine dephosphorylation varied slightly between experiments, similar to our observations with the detergent-soluble proteins (18), the observations of tyrosine dephosphorylation of the 170-, 150-, and 120–125-kD was always consistent in several experiments (data not shown). Electrotransfer of the proteins >200-kD species was inconsistent but when it occurred the 200-kD species was dephosphorylated upon infection (data not shown). Tyrosine phosphorylation of the proteins of molecular masses <69 kD was variable, and their dephosphorylation upon infection was inconsistent in multiple experiments (data not shown). The molecular masses of the major tyrosine-dephosphorylated proteins corresponded to some known cytoskeletal proteins such as vinculin (120 kD) and myosin II (200 kD). This process of protein tyrosine dephosphorylation was highly reversible. When *H. vermiformis* was infected for 30 min with *L. pneumophila* and the cells were washed to remove extracellular bacteria and further incubated in medium for 15 min, the tyrosine phosphorylation pattern of proteins returned to normal levels similar to uninfected cells (Fig. 1 *A*, compare lane *1* with *7*). Protein tyrosine dephosphorylation in *H. vermiformis* in response to invasion by *L. pneumophila* was not induced by supernatant from *Legionella* culture (Fig. 1 *B*, lane *3*) or equivalent numbers of the *Escherichia coli* strain HB101 (lane *4*).

Cytoskeletal Proteins Are Basally Tyrosine Phosphorylated in H. vermiformis. Since many detergent-insoluble proteins underwent tyrosine dephosphorylation, we asked whether the increase in tyrosine phosphatase activity upon infection by *L. pneumophila* (18) altered the phosphorylation status of host cytoskeletal proteins. Many protozoan cytoskeletal and regulatory proteins involved in formation of focal adhesions have been shown to be homologous to their mammalian counterparts, including actin, myosin II, pp125[FAK], vinculin, α-actinin, protein kinase C, and MAP kinase (25, 27, 28). In addition, some of these protozoan proteins have been also shown to be tyrosine phosphorylated, such as pp125[FAK] (28). Many of the protozoan cytoskeletal proteins are antigenically similar to higher eukaryotic homologues,
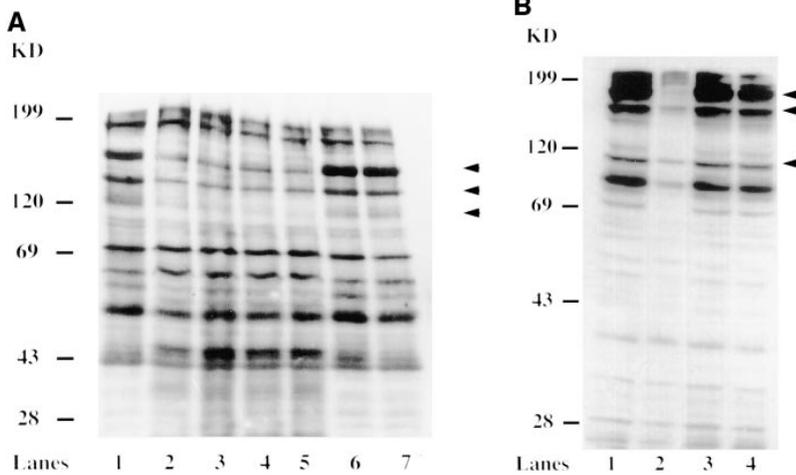
Figure 1. Attachment to and invasion of *H. vermiformis* by *L. pneumophila* is associated with a time-dependent and reversible tyrosine dephosphorylation of detergent-insoluble proteins. (*A*) Detergent-insoluble protein extracts were prepared from resting *H. vermiformis* (lane *1*) or after infection by *L. pneumophila* for 1, 5, 15, and 30 min (lanes *2–5*, respectively) and subjected to immunoblot analysis with antiphosphotyrosine antibody. Lane *6* represents amebal proteins from uninfected cells incubated for 30 min under identical conditions. Lane *7* contains proteins from *H. vermiformis* infected for 30 min, after which extracellular bacteria were washed away and the cells were further incubated at 37°C for 15 min. (*B*) Detergent-insoluble extracts were prepared from resting *H. vermiformis* (lane *1*), or after infection with *L. pneumophila* for 20 min (lane *2*) and subjected to antiphosphotyrosine analysis. Lanes *3* and *4* represent cell extracts prepared from *H. vermiformis* incubated with supernatant from *Legionella* culture and equivalent numbers of *E. coli* strain, HB101 for 20 min, respectively. Arrowheads represent the prominent proteins that underwent tyrosine dephosphorylation upon infection. Results are representative of three independent experiments.

since they are recognized by antibodies raised against the mammalian or avian homologous proteins, including pp125^{FAK}, actin, vinculin, and α-actinin (25, 27, 28, 28).

As an initial step, we sought to identify some of the cytoskeletal proteins that may be tyrosine phosphorylated in uninfected cells to monitor changes after infection. As a screening procedure, phosphotyrosine immunoprecipitates of amebal cell lysates (PY/IP) were immunoblotted with appropriate antibodies that recognized cytoskeletal proteins.

Actin, paxillin, vinculin, and pp125^{FAK} appeared as 43-, 69-, 116–120-, and 100–105-kD proteins, respectively (Fig. 2, *A–D*). All four proteins were basally tyrosine phosphorylated in uninfected *H. vermiformis* (Fig. 2, *A–D, PY/IP*). The anti pp125^{FAK}, and antivinculin antibodies were generated against the respective mammalian proteins, while the antiactin was raised against protozoan actin (see Materials and Methods). Antibodies to paxillin, vinculin, and pp125^{FAK} identified more than one protein band in Western blot analysis but the most prominent band comigrated with their mammalian homologues from murine B (Fig. 2, *B–D*, lane *M*) and T lymphocytes (data not shown). Tyrosine-phosphorylated FAK migrated with slightly higher mobility when compared with its mammalian counterpart (Fig. 2 *D*). Antibodies to several other cytoskeletal proteins failed to recognize *H. vermiformis* proteins in Western blot analysis (data not shown). These experiments identified four of the cytoskeletal proteins, actin, paxillin, vinculin, and pp125^{FAK} as basally tyrosine-phosphorylated proteins in *H. vermiformis*. These data are the first demonstration of the presence of cytoskeletal protein homologues in the protozoan *H. vermiformis* that were immunologically cross-reactive with their mammalian counterparts.

Attachment and Invasion of H. vermiformis by L. pneumophila Is Associated with Tyrosine Dephosphorylation of Paxillin, Vinculin, and pp125^{FAK}. Changes in tyrosine phosphorylation status of cytoskeletal proteins are important in the formation of focal contacts, which are regulated in part by tyrosine phosphatases (3, 6–9). Since attachment and invasion of *H. vermiformis* by *L. pneumophila* resulted in the tyrosine dephosphorylation of multiple detergent-insoluble host proteins, we decided to determine whether any of the cytoskeletal protein homologues were substrates for this increased tyrosine phosphatase activity mediated by attachment and invasion by *L. pneumophila* (18). *H. vermiformis* was left uninfected or infected with *L. pneumophila* for 5 or 20 min, and tyrosine-phosphorylated proteins were immunoprecipitated with antiphosphotyrosine antibody, and subsequently subjected to immunoblotting with antibodies to actin, paxillin, vinculin, or pp125^{FAK}. A time-dependent tyrosine dephosphorylation of paxillin, vinculin, and pp125^{FAK} was observed upon attachment and invasion by *L. pneumophila* (Fig. 3, *A–C*). Tyrosine phosphorylation of actin was minimally affected in these experiments (data not shown), and was used as an internal control in these experiments. The percent tyrosine phosphorylation of each of the cytoskeletal protein compared with uninfected controls (PY%) was calculated as a ratio of tyrosine-phosphorylated paxillin, pp125^{FAK}, or vinculin to the tyrosine-phosphorylated actin for that time point in the same experiment. Thus, it is possible that the degree of phosphorylation of vinculin, paxillin, or pp125^{FAK} may be slightly underestimated due to a minimal decrease in actin tyrosine phosphorylation upon infection. Despite this potential underestimate, we consistently observed tyrosine dephosphorylation of paxillin, pp125^{FAK}, and vinculin upon infection when compared with actin in the same experiment for the same time point of infection (data not shown). After 20 min of infection, the relative tyrosine phosphorylation of paxillin, vinculin, and pp125^{FAK} was 60, 50, and 23%, respectively, compared with their levels in uninfected cells. The relative tyrosine phosphorylation of pp125^{FAK} was ~20% within 5 min of the infection (data not shown). Specificity of these *L. pneumophila*–induced host cell pro-
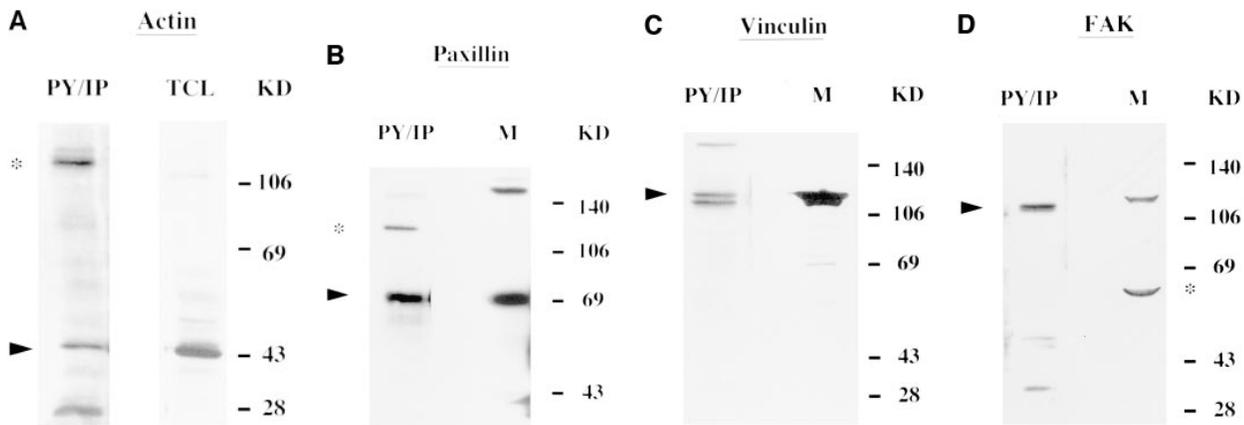
Figure 2. Presence of tyrosine-phosphorylated cytoskeletal proteins in *H. vermiformis*. Phosphotyrosine immunoprecipitates (*PY/IP*) of resting *H. vermiformis* were resolved under SDS-PAGE conditions and immunoblotted with antibodies to actin, paxillin, vinculin, or pp125^{FAK}, followed by appropriate HRP-conjugated secondary antibodies (*A–D*). Total cell lysates from 10^7 murine splenic B lymphocytes (*M*) was used as a positive control for comparison, except in *A* where total cell lysate from *H. vermiformis* was used (*TCL*), since antiactin was amebae specific. Arrowheads indicate the relative positions of the cytoskeletal proteins and asterisks represent other cross-reactive protein bands under these immunoblotting conditions. Results are representative of two independent experiments.

cesses was confirmed by the observation that no loss of tyrosine phosphorylation of the cytoskeletal proteins was detected when *H. vermiformis* was incubated for 20 min with formalin-killed *L. pneumophila* (Fig. 3, *A* and *B*, lane *4* and *C*, lane *3*). Note that vinculin was the only protein (among the proteins studied) with a molecular mass that corresponded to a protein that was dramatically tyrosine dephosphorylated upon infection (Fig. 1 *A*). It is possible that the other two proteins comigrated with other bands that masked the detection of their tyrosine dephosphorylation, which was detectable after immunoprecipitation (Fig. 3). Alternatively, the proteins underwent minor tyrosine dephosphorylation that was undetectable in simple immunoblots that detected the major dephosphorylated proteins.

Attachment of L. pneumophila to H. vermiformis is Essential to Induce Tyrosine Dephosphorylation of Detergent-insoluble Proteins. We have previously shown that *L. pneumophila*–induced tyrosine dephosphorylation of *H. vermiformis* detergent-soluble proteins is inhibited by the monovalent sugars Gal and GalNAc, by inhibiting bacterial attachment to the lectin (18). Hence, we tested whether tyrosine dephosphorylation of detergent-insoluble proteins was altered in the presence of Gal or GalNAc during infection of *H. vermiformis* by *L. pneumophila*. Another monovalent sugar, mannose, was used as a negative control since it does not block *L. pneumophila*–induced tyrosine dephosphorylation of *H. vermiformis* proteins (18). *H. vermiformis* were preincubated in the presence of Gal, GalNAc, or mannose and later infected
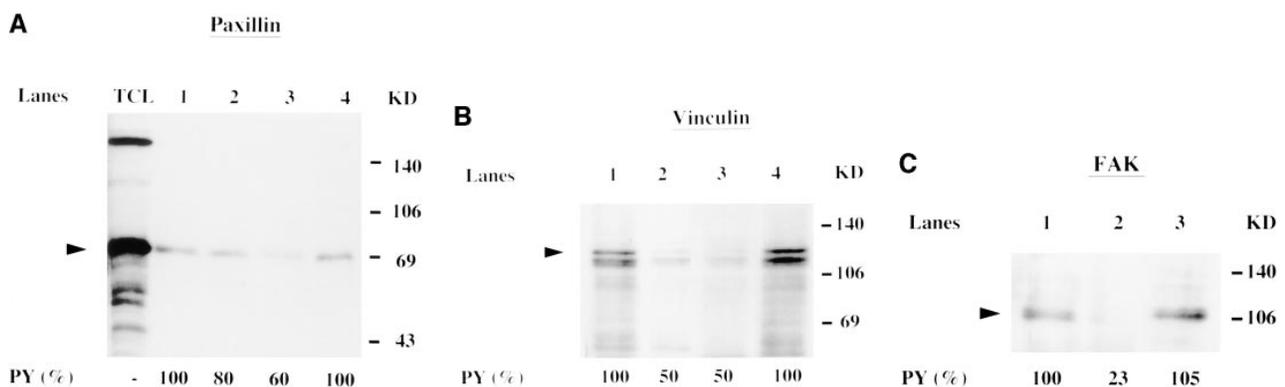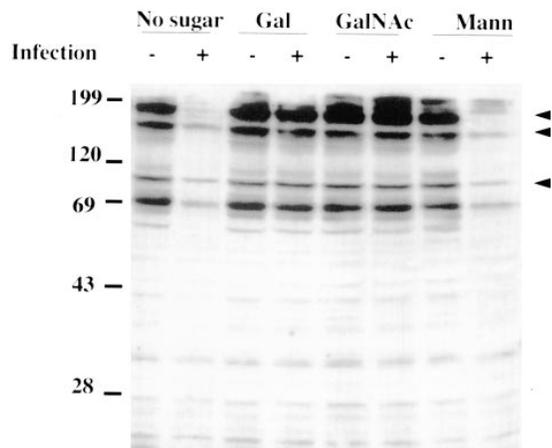

Figure 3. Paxillin, vinculin and pp125^{FAK} are tyrosine dephosphorylated upon attachment and invasion of *H. vermiformis* by *L. pneumophila*. (*A* and *B*) *H. vermiformis* were left uninfected (lane *1*), infected with *L. pneumophila* for 5 min (lane *2*) or 20 min (lane *3*). Lane *4* represents *H. vermiformis* infected with formalin-killed *L. pneumophila* for 20 min. Amebal cell extracts were immunoprecipitated with antiphosphotyrosine antibody followed by immunoblotting with antibodies to paxillin (*A*) or vinculin (*B*). In *C*, cell extracts from *H. vermiformis* left uninfected (lane *1*) or infected with live (lane *2*) or with formaline-killed bacteria (lane *3*) for 20 min were immunoprecipitated with antiphosphotyrosine antibody and probed with antipp125^{FAK} antibody. Tyrosine dephosphorylation of pp125^{FAK} was maximal after 5 min of infection with live *L. pneumophila* (data not shown). The relative intensities of the protein bands were quantitated using the NIH Image program (version 1.6). The results were expressed as percentages of uninfected controls as described in Materials and Methods. Results are representative of two to three independent experiments for each of these proteins. Arrowhead indicates the relative positions of the cytoskeletal proteins.
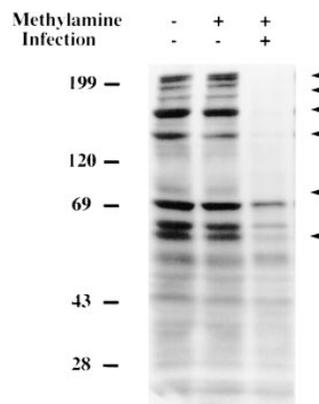
for 40 min by *L. pneumophila*. Cell extracts were prepared and immunoblotted with antiphosphotyrosine antibody. Tyrosine dephosphorylation of detergent-insoluble proteins was completely blocked throughout the 40-min infection period in the presence of Gal and GalNAc but not mannose (Fig. 4). These results indicated that bacterial attachment to the lectin was essential to mediate induction of tyrosine dephosphorylation of the host detergent-insoluble proteins.

Attachment but Not Entry of L. pneumophila Is Sufficient to Induce Tyrosine Dephosphorylation of H. vermiformis Proteins. Next, we asked whether entry *of L. pneumophila* was essential to induce tyrosine dephosphorylation of host cell proteins or that bacterial attachment was sufficient to induce these host cell processes. We used two strategies to answer these questions.

First, we used methylamine, an inhibitor of receptor-mediated endocytosis (31) that has been shown to block entry of *L. pneumophila* into protozoa, including *H. vermiformis* (15, 21, 32). *H. vermiformis* was pretreated with 50 mM of methylamine for 30 min and later infected with *L. pneumophila*. Under these conditions, methylamine caused complete inhibition of bacterial entry, as confirmed by gentamicin protection assays (18), but had no effect on the number of attached bacteria compared with untreated cells (data not shown). Phosphotyrosine analysis of *H. vermiformis* total proteins before and after infection showed that inhibition of bacterial entry did not block bacteria-induced tyrosine dephosphorylation of host cell proteins (Fig. 5). Incubation with methylamine did not alter the pattern of protein tyrosine phosphorylation in uninfected cells (Fig. 5, lane *2*). These results indicated that entry of *L. pneumophila* into *H. vermiformis* was not required to induce tyrosine dephosphorylation of host proteins, and that bacterial attachment was

Figure 5. *L. pneumophila*–induced tyrosine dephosphorylation of *H. vermiformis* proteins, is not inhibited by blocking bacterial entry. Tyrosine dephosphorylation of *H. vermiformis* by *L. pneumophila* in the presence of methylamine. Amebae were preincubated for 30 min with 50 mM methylamine and infected with *L. pneumophila* for 20 min. Cell extracts were immunoblotted with antiphosphotyrosine antibody. Results are representative of two independent experiments.

sufficient to induce tyrosine dephosphorylation of *H. vermiformis* proteins. The increased level of bacteria-induced tyrosine dephosphorylation under these conditions (compared with Fig. 1 *A*) may be due to continuous attachment of the bacteria in the absence of uptake throughout the infection period.

Our second strategy was to examine whether mutants of *L. pneumophila* that were defective in entry into *H. vermiformis* were capable of inducing tyrosine dephosphorylation of *H. vermiformis*. Among a bank of 5,200 mini Tn*10*::kan insertion mutants of *L. pneumophila* (15), we isolated 10 mutants that were defective in invasion of *H. vermiformis* using gentamicin protection assays, as we described previously (18). Their invasion level was 3–79% of the parent strain AA100 (Fig. 6 *A*). Interestingly, similar to the parent strain, the mutants were capable of inducing tyrosine dephosphorylation of *H. vermiformis* proteins (Fig. 6 *B*). These data supported our observations that entry of *L. pneumophila* into *H. vermiformis* was not required to induce tyrosine dephosphorylation of host cell proteins. It is important to note that most of the tested mutants are defective in attachment to *H. vermiformis*, indicating that the bacterial ligand that recognizes the lectin is intact in these mutants (32).

Ultrastructural Characteristics of Entry of L. pneumophila into H. vermiformis. Tyrosine dephosphorylation of many cytoskeletal proteins in *H. vermiformis* upon attachment and invasion by *L. pneumophila* suggested disassembly of the cytoskeleton (6–9). In addition, cytoskeletal disruption of *H. vermiformis* by cytochalasin D does not interfere with bacterial entry (21). In contrast, uptake of *L. pneumophila* by human macrophages occurred through a coiling phagocytic mechanism, and the uptake is inhibited by cytochalasin D (15, 21, 33). These observations indicate that uptake of *L. pneumophila* by *H. vermiformis* is different from that of mammalian macrophages. Therefore, we examined the mode of bacterial entry into *H. vermiformis* by transmission electron microscopy. Among 73 internalization events examined, 5 (7%) were manifested as coiling phagocytosis (Fig. 7, *A* and *B*; reference 33). It will be interesting to test whether the coiling phagocytosis seen during bacterial uptake by *H. vermiformis* is affected by cytochalasin D. 68 (93%) uptake events were manifested as a cup-shaped invagination of the

Figure 4. Attachment of *L. pneumophila* to *H. vermiformis* is required to induce tyrosine dephosphorylation of detergent-insoluble proteins. *H. vermiformis* were preincubated for 15 min in the presence of 100 mM of either Gal, GalNAc, or mannose (*mann*) for 15 min and later coincubated with (+) or without (−) *L. pneumophila* for 30 min. Cell extracts were prepared and probed with antiphosphotyrosine antibody. Arrowheads indicate prominent tyrosine-dephosphorylated proteins. Results are representative of three independent experiments.
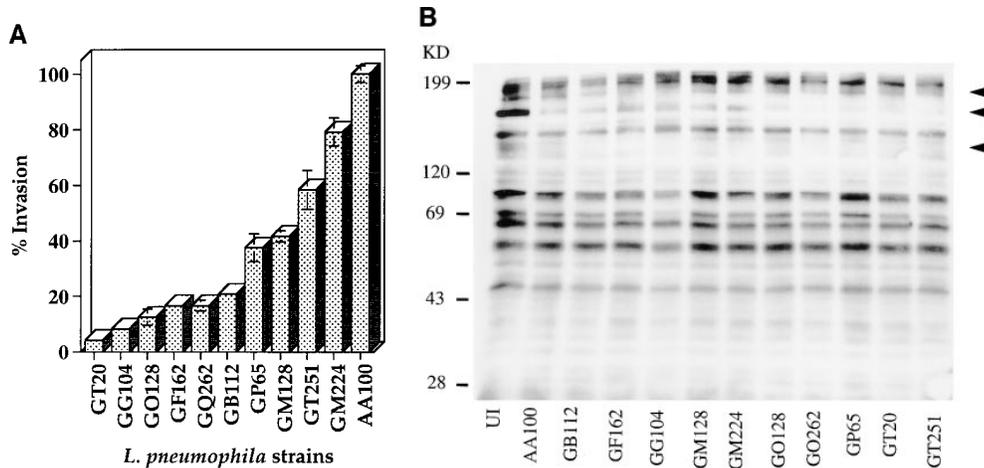
Figure 6. Invasion-defective mutants of *L. pneumophila* are capable of inducing tyrosine dephosphorylation of *H. vermiformis* proteins. (*A*) *H. vermiformis* was infected for 1 h with an equivalent number of bacteria of the parent strain AA100 or each of the 10 mutants. The percentage of invasion was derived by the number of internalized bacteria of each mutant compared with the parent strain. Extracellular bacteria were killed by gentamicin treatment, and the number of intracellular bacteria was determined (18). This is representative of three independent experiments. (*B*) Phosphotyrosine immunoblot of detergent-soluble extracts of uninfected *H. vermiformis* or infected by the parent strain AA100 or the mutants for 20 min. The middle arrowhead indicates the 170-kD Gal/GalNAc lectin, whereas the other arrowheads represent other proteins that underwent tyrosine dephosphorylation upon infection. Results are representative of two independent experiments.
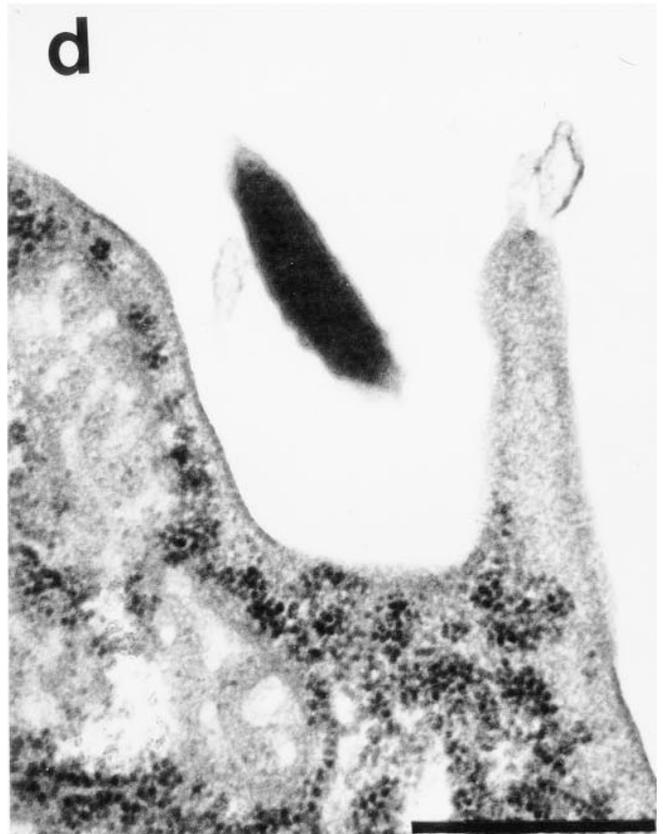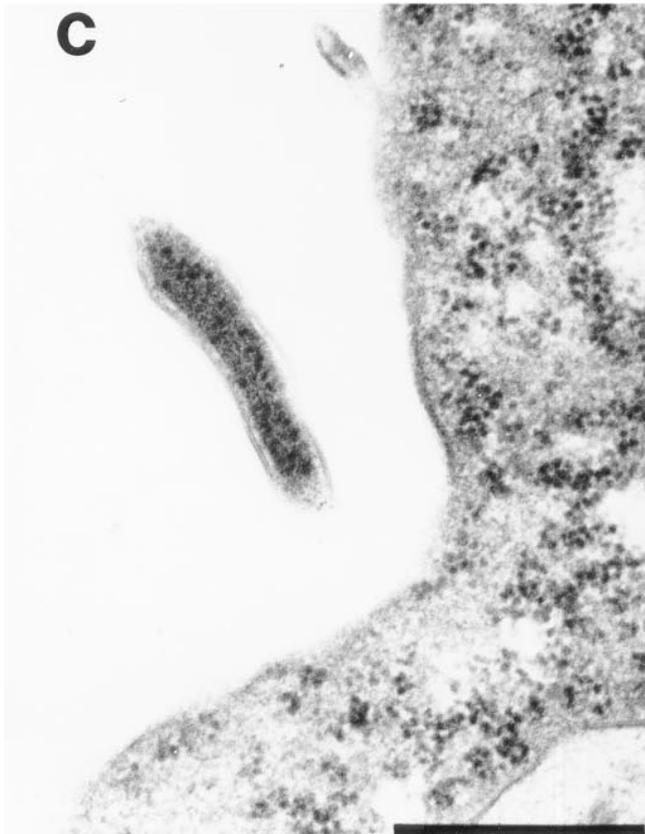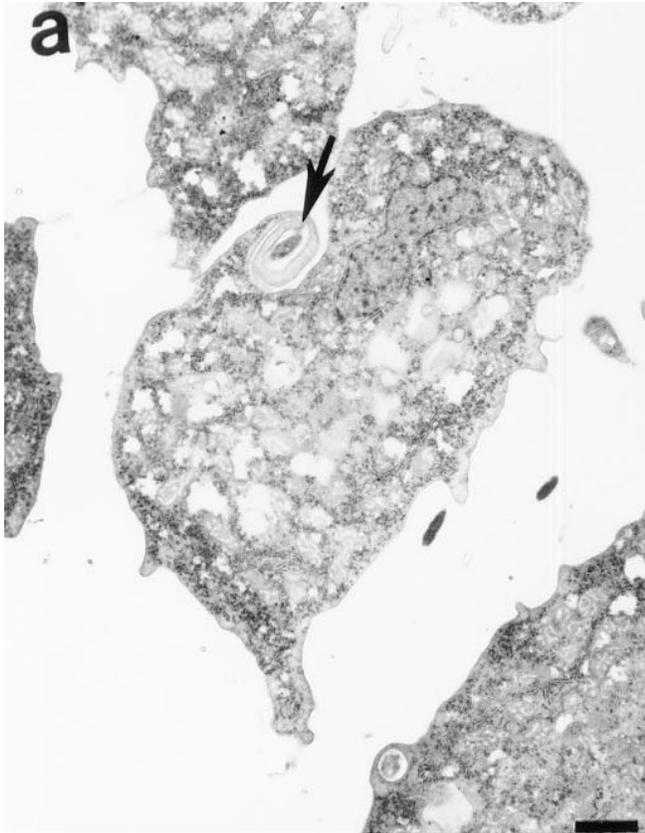
Discussion

Our data showed that attachment of *L. pneumophila* to the Gal/GalNAc lectin receptor of *H. vermiformis* induces a prominent tyrosine dephosphorylation of many host cell proteins, including the Gal/GalNAc lectin receptor (18) and several putative cytoskeletal protein homologues. Bacterial attachment but not invasion is essential and sufficient to induce protein tyrosine dephosphorylation in *H. vermiformis*. Inhibition of bacterial attachment by Gal or GalNAc effectively blocked bacterial-induced tyrosine dephosphorylation of *H. vermiformis* proteins. Inhibition of bacterial invasion of *H. vermiformis* had no detectable effect on the ability of the bacteria to induce tyrosine dephosphorylation of host cell proteins. Moreover, *L. pneumophila* mutants defective in invasion of *H. vermiformis* were as efficient as the wild-type strain in their ability to induce tyrosine dephosphorylation of host proteins. Tyrosine dephosphorylation of the cytoskeletal protein homologues suggested disruption of the cytoskeleton upon bacterial attachment.

The Gal/GalNAc lectin homologue of *E. histolytica* is a β_2-like integrin (19, 20). In general, binding of a ligand to the extracellular domain of the integrin results in tyrosine phosphorylation of the cytoplasmic domain of the β chain of the integrin, clustering of the integrin receptor, and recruitment of many regulatory and cytoskeletal proteins (3, 35). Tyrosine phosphorylation of the cytoplasmic domain of integrins is important for internalization and targeting of the ligands to the appropriate endosomal compartment (36,

37). Disruption of tyrosine phosphorylation of the cytoplasmic domain of integrins blocks ligand internalization (35, 36, 38), disrupts cytoskeletal rearrangements (3, 35), or alters targeting of the internalized ligand through the endosomal pathway (37). Interestingly, invasion of mammalian cells by the intracellular bacterium, *Yersinia pseudotuberculosis* is mediated by binding to β_1 integrin and is enhanced by mutations in the cytoplasmic domain of the integrin that disrupts its association with the cytoskeleton (39). Similar substitutions of residues in the cytoplasmic tail of the low density lipoprotein receptor results in the rapid endocytosis of the receptor (40). These studies suggest that mutations that disrupt association between the receptor and cytoskeletal components favor efficient internalization of certain ligands (39).

Disassembly of focal contacts and disruption of cytoskeletal rearrangements involves activation of protein tyrosine phosphatases (6, 7). In addition, inhibitors of tyrosine kinases inhibit the formation of focal contacts (3). It is tempting to speculate that tyrosine dephosphorylation of the lectin and the putative cytoskeletal protein homologues are collectively indicative of bacterial attachment-mediated cytoskeletal disruption of the protozoan host. These predictions are supported by the observations that uptake of *L. pneumophila* by *H. vermiformis* is not blocked by cytoskeletal disruption of the protozoan host (21, 32). Similarly, uptake of the intracellular bacterium *Chlamydia* by epithelial cells is also not affected by disruption of the host cytoskeleton (41). In addition, invasion of mammalian cells by many viruses is associated with disruption of the cytoskeleton (42).

Transmission electron microscopy in this study showed that a small proportion of bacterial uptake occurs by coiling phagocytosis. No coated pits are observed in the coil structure. This mode of uptake is morphologically similar to the coiling phagocytosis of complement-opsonized *L. pneumophila* by human monocytes (33). However, most of the up-

take events of *L. pneumophila* by *H. vermiformis* occur by a cup-shaped invagination of the plasma membrane through which the bacterium sinks into the host cell. In addition, there is no direct contact between the bacterium and the plasma membrane in this invagination. We think that initial contacts are made between *L. pneumophila* and *H. vermiformis* followed by signaling of the protozoan host to take up the bacteria by a trigger and not a zipper mode of uptake (43).

Based on our current data and previous studies (18, 21, 22), we propose the following model of attachment and invasion of the protozoan host *H. vermiformis* by the Legionnaires' disease bacterium, *L. pneumophila*. Attachment of *L. pneumophila* to the Gal/GalNAc lectin receptor of *H. vermiformis* is associated with induction of a tyrosine phosphatase activity that results in dephosphorylation of the lectin receptor (18) and several cytoskeletal proteins. We propose that these alterations in the protozoan host are manifested in disruption of the cytoskeleton to avoid shedding of the bacteria. These events are associated with, or followed by, a triggering mechanism of receptor-mediated endocytosis to internalize *L. pneumophila*. In addition, bacterial attachment and invasion is also associated with induced expression of specific protozoan genes and these induced genes may be important for entry of *L. pneumophila* (22). Some of the induced gene products may be the lectin receptor or its associated proteins. The internalized bacteria are targeted into a RER-surrounded phagosome that is excluded from the classical endosomal–lysosomal degradation pathway. Future studies will shed light on the intriguing ability of this intracellular bacterial pathogen to manipulate cell biological processes within two evolutionarily distant hosts, humans and protozoa.

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Figure 7. Transmission electron micrographs of uptake of *L. pneumophila* by *H. vermiformis*. (*a*) Coiling phagocytosis is indicated by the arrow, and the pseudopodia-like coil surrounding *L. pneumophila* is enlarged in *b*. *c* and *d* are different stages of invagination of the plasma membrane of *H. vermiformis* to internalize *L. pneumophila*. Bars: (*a*) 1 μm; (*b*–*d*) 0.5 μm.

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