Exposure to Host Ligands Correlates with Colocalization of Gal/GalNAc Lectin Subunits in Lipid Rafts and Phosphatidylinositol (4,5)-Bisphosphate Signaling in Entamoeba histolytica

Amanda M. Goldston
Clemson University

Rhonda R. Powell
Clemson University

Amrita B. Koushik
Clemson University

Lesly A. Temesvari
Clemson University, itemesv@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/bio_pubs

Part of the Microbiology Commons

Recommended Citation
Please use publisher's recommended citation: http://ec.asm.org/content/11/6/743.full?sid=62033a9c-6009-4997-a6a3-317e4ac51447

This Article is brought to you for free and open access by the Biological Sciences at TigerPrints. It has been accepted for inclusion in Publications by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
Exposure to Host Ligands Correlates with Colocalization of Gal/GalNAc Lectin Subunits in Lipid Rafts and Phosphatidylinositol (4,5)-Bisphosphate Signaling in *Entamoeba histolytica*

Amanda M. Goldston,a Rhonda R. Powell,b Amrita B. Koushik,a and Lesly A. Temesvari b

Department of Genetics and Biochemistry, Clemson University, Clemson, South Carolina, USA,a and Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA b

*Entamoeba histolytica* is an intestinal parasite that causes dysentery and liver abscess. Parasite cell surface receptors, such as the Gal/GalNAc lectin, facilitate attachment to host cells and extracellular matrix. The Gal/GalNAc lectin binds to galactose or N-acetylgalactosamine residues on host components and is composed of heavy (Hgl), intermediate (Igl), and light (Lgl) subunits. Although Igl is constitutively localized to lipid rafts (cholesterol-rich membrane domains), Hgl and Lgl transiently associate with this compartment in a cholesterol-dependent fashion. In this study, trophozoites were exposed to biologically relevant ligands to determine if ligand binding influences the submembrane distribution of the subunits. Exposure to human red blood cells (hRBCs) or collagen, which are bona fide Gal/GalNAc lectin ligands, was correlated with enrichment of Hgl and Lgl in rafts. This enrichment was abrogated in the presence of galactose, suggesting that direct lectin-ligand interactions are necessary to influence subunit location. Using a cell line that is able to attach to, but not phagocytose, hRBCs, it was shown that physical attachment to ligands was not sufficient to induce the enrichment of lectin subunits in rafts. Additionally, the mutant had lower levels of phosphatidylinositol (4,5)-bisphosphate (PIP₂); PIP₂ loading restored the ability of this mutant to respond to ligands with enrichment of subunits in rafts. Finally, intracellular calcium levels increased upon attachment to collagen; this increase was essential for the enrichment of lectin subunits in rafts. Together, these data provide evidence that ligand-induced enrichment of lectin subunits in rafts may be the first step in a signaling pathway that involves both PIP₂ and calcium signaling.
adhesion of *E. histolytica* trophozoites to host cells (23) and collagen (33) but only slightly inhibits the adhesion of trophozoites to fibronectin (33). This suggests that *E. histolytica* lipid rafts play a significant role in binding to host cells and collagen and a lesser role in binding to host fibronectin. The parallel roles of the Gal/GalNAc lectin and lipid rafts in collagen and fibronectin, suggest that these membrane domains regulate the function of the lectin.

In addition to protein receptors, lipids also participate in signaling pathways that emanate from lipid rafts. One such family of signaling lipids are the phosphoinositides. Two phosphorylated members of the phosphoinositide family are phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Both of these lipids play important roles in cellular processes such as phagocytosis, protein kinase activation, and actin polymerization (9, 18). PIP2 also regulates calcium signaling (8, 19, 28). For example, signal transduction can lead to hydrolysis of PIP2, resulting in the production of second-messenger molecules, inositol trisphosphate (IP3) and diacylglycerol (DAG) (20). These, in turn, facilitate the release of calcium into the cytoplasm from intracellular calcium stores and from the extracellular space through channels in the plasma membrane (16, 40).

Phosphoinositides can also facilitate signaling by recruiting downstream proteins that have specific phosphoinositide binding domains. For example, FYVE-finger domains, which were originally observed in Ebf1p, YOTB, Vac1p, and EFA1 proteins, bind specifically to phosphatidylinositol 3-phosphate (44). Additionally, certain pleckstrin homology (PH) domains, such as that from Bruton’s tyrosine kinase (PH2), have been shown to specifically bind PIP2 (42). Overexpression of green fluorescent protein (GFP)-FYVE-finger domains or GFP-PHHTK domains has been used to localize phosphoinositides in real time in *E. histolytica* (4, 38).

Previously, we demonstrated that cholesterol loading of parasite membranes induced the enrichment of the Ga/GalNAc lectin subunits in lipid rafts, which in turn increased the activity of the Ga/GalNAc lectin (4,14). The localization of Ga/GalNAc lectin subunits after attachment to biological relevant extracellular ligands. We showed that binding to human red blood cells (hRBCs) and collagen results in the enrichment of Hgl and Lgl in lipid rafts, while attachment to fibronectin does not change the localization of the subunits. We also demonstrated that cells expressing GFP-PHHTK exhibit reduced PIP2 levels. In these cells, attachment to ligand is not correlated with enrichment of Hgl and Lgl in lipid rafts; the phenotype is reversible upon the addition of exogenous PIP2, indicating that the role of PIP2 in regulating the submembrane position of the Ga/GalNAc lectin. Finally, intracellular calcium levels increase upon attachment to collagen; increased intracellular calcium levels appear to be essential for the enrichment of lectin subunits in rafts. Together, our data suggest that colocalization of Ga/GalNAc lectin subunits in rafts may be the first step in the activation of a signaling pathway and that PIP2 and calcium may be involved in this pathway.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *E. histolytica* trophozoites (strain HM1: IMSS) were grown axenically in TYI-S-33 medium supplemented with 10% (vol/vol) bovine serum albumin (G418) and 15 µg/ml hygromycin. The expression of GFP-PHHTK was induced with 5 µg/ml tetracycline for 24 h prior to use in assays. Prior to performance of assays, cells were incubated on ice for 10 or 20 min in order to release them from tube or flask surfaces, respectively.

**Exposure to ligands.** Wild-type or GFP-PHHTK-expressing cells (3.5 × 106) were incubated in serum-free medium for 30 min and then exposed to various ligands prior to lipid raft extraction. For hRBC exposure, trophozoites were incubated in the presence of 3.5 × 108 hRBCs (U.S. Biological, Swampscoot, MA) for 5 min at 37°C. For exposure to collagen and fibronectin, cells were incubated on ECM-coated flasks (BD Biosciences, Bedford, MA) or uncoated flasks (Sarstedt) for 15 min at 37°C.

**Lipid raft extraction.** After exposure to ligands, isolation and characterization of lipid rafts were carried out as previously described (23). Extracted raft-associated proteins were characterized by SDS-PAGE and Western blotting as described previously (23). Primary antibodies included a mixture of monoclonal anti-Lgl antibodies (3C2, IC8, IA9, and ID4) (1:4,000 dilution), polyvalent anti-Hgl antibodies (1:5,000 dilution), monoclonal anti-Hgl antibodies (1G7) (1:1,000 dilution), or a mixture of monoclonal anti-Igl antibodies (3G5-A3-G3, 5H1-F11-D11, and 4G2-D8-H1) (1:4,000 dilution) (antibodies were kind gifts from William A. Petri, Jr., University of Virginia School of Medicine, Charlottesville, VA). Western blots were analyzed by densitometry using ImageJ software (version 1.42q; U.S. National Institutes of Health, Bethesda, MD).

**Whole-cell PIP2 extraction and lipid dot blots.** Total lipid was extracted from wild-type and GFP-PHHTK-expressing trophozoites according to the methods of Gray et al. (14). Briefly, 1 × 106 cells were washed twice with phosphate-buffered saline (PBS). Lipids were precipitated by the addition of 5 ml of 0.5 M trichloroacetic acid (TCA) and centrifuged at 500 × g for 5 min at 4°C. The pellets were washed with 3 ml of 5% (wt/vol) TCA–1 mM EDTA and centrifuged at 500 × g for 5 min. To the pellets, 3 ml of methanol-chloroform (2:1) was added, and the mixture was vortexed 3 times over a period of 10 min at room temperature to facilitate neutral lipid extraction. The extracted lipids were centrifuged at 500 × g for 5 min at 4°C. To the pellet, 2.25 ml methanol-chloroform-12.1 N HCl (80:80:1) was added, and the mixture was vortexed 4 times over 15 min at room temperature and centrifuged at 500 × g. The resulting supernatant was subjected to phase split by the addition of 750 µl chloroform and 1.35 ml 0.1N HCl. The solution was centrifuged at 500 × g for 5 min at 4°C. After centrifugation, the organic phase was collected and dried using a MiVac Duo Sample Concentrator Speed Vac centrifuge (GeneVac, Gardiner, NY).

The vacuum-dried lipid pellets were resuspended in a methanol-chloroform–water mixture (2:1:0.8) and vortexed for 30 s, followed by sonication in a cold water bath for 10 min. The lipids were then spotted onto a nitrocellulose membrane. The membrane was blocked with 1.5% fatty acid-free bovine serum albumin (BSA) for 1 h at room temperature and probed with mouse anti-PIP2 (Abcam, Cambridge, MA) or mouse anti-PH (Echelon Biosciences, Salt Lake City, UT) antibodies. Densitometric analysis was performed using Image J software.

**PIP2 loading.** GFP-PHHTK-expressing cells were loaded with PIP2 using a shuttle PIP2 kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer’s guidelines. Concentrations of 25 µM PIP2 and 12.5 µM PIP2 carrier histone (H1) were used. Loading was carried out for 30 min at 37°C. PIP2 loading was confirmed using fluorescence microscopy of a BODIPY-labeled PIP2 (Nikon Eclipse TI-E spectral confocal microscope; Nikon Instruments Inc., Lewisville, TX). After PIP2 loading, cells were exposed to hRBCs and lipid rafts were extracted as described above.

**Calcium assay.** Relative intracellular calcium levels were assessed using the calcium indicator fluo-4/AM according to the manufacturer’s instructions. Fluo-4/AM is fluorescent when bound to calcium. Wild-type cells were washed twice with calcium stock loading buffer (CSB) (30) and then incubated in CSB supplemented with 5 µM fluo-4/AM (Invitrogen, Carlsbad, CA) for 20 min at 37°C.
Carlsbad, CA) or an equivalent volume of dimethyl sulfoxide (DMSO) (diluent control) for 30 min at 37°C. After staining, cells were washed twice with CSB, and 1 \times 10^6 cells (stained or control) were added to the wells of a 12-well plate which contained 1 mM CaCl\(_2\) (5) and a glass coverslip coated with collagen or fibronectin (BD Biosciences). After 3 min, plates were transferred to a BioTek Flx800-I microplate reader (BioTek, Winooski, VT) and incubated at 37°C, and fluorescence (excitation, 485 nM; emission, 525 nM) was monitored at 5-min intervals for 10 min. To account for background fluorescence, the fluorescence value of control cells (DMSO) was subtracted from the fluorescence value of fluo-4/AM-stained cells.

**Calcium chelation.** To chelate intracellular calcium, cells were incubated in the presence of 50 \mu M 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) (EMD Chemicals Group, Darmstadt, Germany) in serum-free medium for 30 min at 37°C. Cells were then exposed to collagen-coated coverslips, and the calcium assay was performed as described above. Cells were also exposed to collagen-coated flasks, and lipid rafts were isolated and characterized as described above.

**Adhesion assay.** To determine the effect of intracellular calcium chelation on adhesion, we used a previously described adhesion assay (33, 38). Cells were preexposed to serum-free medium with or without 50 \mu M BAPTA/AM for 30 min at 37°C in the presence of the fluorescent vital stain calcine-AM (5 \mu g/ml). Cells (3 \times 10^6) were seeded in the wells of a 96-well collagen-coated plate (BD Biosciences) (in triplicate) for each condition and incubated at 37°C for 15 min. The wells were then washed with warm PBS to remove nonadherent cells. Fluorescence was measured using a BioTek Flx800-I microplate reader (excitation, 485 nM; emission, 525 nM). Values were reported as percentages of the control value, which was arbitrarily set to 100%.

**Statistical analysis.** All data are reported as a mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Instat V.3. Comparisons were carried out using a one-way analysis of variance (ANOVA) with posttest. \( P \) values of less than 0.05 (*) were considered statistically significant, and values of less than 0.01 (**) or 0.001 (***) were considered highly statistically significant.

**RESULTS**

**Exposure to hRBCs correlates with enrichment of Hgl and Lgl subunits in lipid raft fractions.** In mammalian cells, binding to ligand induces clustering of integrins in lipid raft domains (17). To determine if ligand engagement also influences the submembrane distribution of the subunits of the Gal/GalNAc lectin, we exposed trophozoites to hRBCs and isolated and characterized lipid rafts as described previously (23). The composition of lipid rafts confers detergent resistance to these membrane domains. Therefore, purification of lipid rafts was initiated by extraction with cold Triton X-100. This resulted in the isolation of detergent-resistant membrane (DRM), which consists of both lipid raft and actin-rich membrane. Since the buoyant density of lipid rafts is less than that of actin-rich membrane, these two membrane domains were further separated by sucrose density gradient centrifugation. To address possible contamination of DRM from hRBCs, whole-cell lysates from hRBCs were tested by Western blotting with antibodies for Hgl, Lgl, and Igl and were shown to have no cross-reacting proteins (data not shown).

Western blot analysis of gradient fractions revealed that the majority of Igl was found in a low-density region (fractions 9 to 14) (Fig. 1). Previously, these fractions were shown to possess the highest levels of cholesterol among other detergent-resistant fractions (23). Thus, these fractions are identified as lipid rafts. The localization of Igl to these low-density rafts was consistent with previous reports (23, 53). In control cells, the majority of Hgl and Lgl was associated with less buoyant, actin-rich fractions (fractions 17 to 20) (Fig. 1). However, after exposure to hRBCs, there was an increase in the proportion of Hgl and Lgl that was localized to lipid raft fractions (fractions 9 to 14), whereas the submembrane distribution of Igl remained unchanged (Fig. 1). This observation suggests that binding to at least one ligand, hRBCs, can induce the enrichment of Hgl and Lgl in lipid rafts.

To determine if enrichment of Hgl and Lgl in lipid rafts was dependent on a physical interaction between the Gal/GalNAc lectin and its ligand, cells were pretreated with galactose, a competitive inhibitor of lectin-ligand binding, or mannose (a control sugar) prior to hRBC exposure. Incubation with galactose prevented the enrichment of Hgl and Lgl in lipid raft fractions after exposure to hRBCs.
hRBC exposure, while incubation with mannose did not inhibit the enrichment of Hgl and Lgl in lipid raft fractions after hRBC exposure (Fig. 2). The localization of Igl in lipid raft domains was unaffected in the presence of galactose or mannose. These data suggest that physical interaction between the Gal/GalNAc lectin and its ligand is necessary for raft enrichment of Hgl and Lgl.

**Exposure to collagen type I correlates with galactose-sensitive enrichment of Hgl and Lgl subunits in lipid rafts.** To determine if another ligand also induces the enrichment of Gal/GalNAc lectin subunits in lipid rafts, we exposed trophozoites to collagen type I, which has been shown to initiate signaling in *E. histolytica* (7, 10, 35). Trophozoites were incubated on collagen-coated flasks or uncoated control flasks. Lipid rafts were extracted and characterized. Similar to the case for incubation with hRBCs, incubation on collagen was accompanied by an increase in the levels of Hgl and Lgl subunits in high-buoyancy lipid raft fractions (Fig. 3). Interestingly, the fractions with the highest levels of Hgl and Lgl (fractions 13 to 16) (Fig. 3) differed from those with the highest

**FIG 2** Enrichment of Hgl and Lgl in lipid rafts upon exposure to hRBCs is inhibited in the presence of galactose. Trophozoites (3.5 × 10⁶) were serum starved and exposed to 10 mM galactose (gal) or 10 mM mannose (man) prior to exposure to hRBCs. DRM was isolated and fractionated using sucrose gradient density centrifugation. Nineteen fractions and a pellet (20P) were collected and subjected to Western blot analysis using antibodies specific for Hgl (A), Lgl (B), or Igl (C). Average values and standard deviations for densitometric scans (n = 2) are reported as the percentage of total detergent-resistant protein for each subunit. The localization of Igl remained unchanged after exposure to galactose or mannose followed by hRBCs. The enrichment of Hgl and Lgl in lipid rafts after exposure to hRBCs was inhibited in the presence of galactose but not mannose.

**FIG 3** Exposure to collagen is correlated with a calcium-dependent enrichment of Hgl and Lgl in lipid rafts. Trophozoites (3.5 × 10⁶) were serum starved or serum starved and incubated in the presence of BAPTA/AM and incubated on collagen-coated flasks (coll) or uncoated control flasks. DRM was isolated and fractionated by sucrose gradient density centrifugation. Nineteen fractions and a pellet (20P) were collected and subjected to Western blot analysis using antibodies specific for Hgl (A), Lgl (B), or Igl (C). Average values and standard deviations for densitometric scans (n = 2) are reported as the percentage of total detergent-resistant protein for each subunit. In cells treated with collagen, the distribution of Igl was not different from that in control cells. Hgl and Lgl subunits were enriched in fractions 13 to 16 upon exposure to collagen. Enrichment of Hgl and Lgl in lipid rafts was inhibited in the presence of BAPTA/AM.
levels of Hgl and Lgl after exposure to hRBCs (fractions 9 to 14) (Fig. 2). This suggests that the molecular mechanism governing the submembrane distribution of the Gal/GalNAc lectin subunits differs in a ligand-specific manner. This enrichment was prevented by the addition of galactose but not by the addition of mannose (Fig. 4). Therefore, physical interaction of trophozoites with collagen also appears to be necessary for enrichment of Hgl and Lgl in lipid rafts.

**Exposure to fibronectin does not correlate with an enrichment of Hgl and Lgl subunits in lipid rafts.** Because galactose and raft-disrupting agents have little effect on trophozoite-fibronectin interaction (33), it is likely that neither the Gal/GalNAc lectin nor lipid rafts play a primary role in the interaction between the parasite and this ECM component. Therefore, as a control, we incubated trophozoites on fibronectin-coated flasks and isolated and characterized lipid rafts. In both control cells and cells exposed to fibronectin, Hgl and Lgl were concentrated in the actin-rich fractions (fractions 17 to 20), while Igl was concentrated in lipid raft fractions (fractions 9 to 14) (Fig. 5). Therefore, exposure to fibronectin did not affect the localization of any of the Gal/GalNAc lectin subunits and in particular did not induce the enrichment of Hgl and Lgl in lipid raft domains of *E. histolytica*. This supports the authenticity of our finding that binding to a bona fide ligand of the Gal/GalNAc lectin (e.g., hRBCs and collagen) can influence the submembrane localization of this adhesin.
Attachment to hRBCs is not sufficient for enrichment of Hgl and Lgl in lipid rafts. Previously, an *E. histolytica* cell line expressing GFP-labeled PH domain derived from Bruton’s tyrosine kinase (GFP-PHBTK) was developed (4). The GFP-PHBTK-expressing cell line exhibited interesting phenotypes, including enhanced motility and a phagocytic defect characterized by the ability to bind to, but not internalize, hRBCs (4). The latter characteristic provided the opportunity to test the sufficiency of ligand binding in the regulation of Gal/GalNAc localization. GFP-PHBTK-expressing cells were exposed to hRBCs, and lipid rafts were purified and characterized. In this cell line, attachment to hRBCs was not correlated with the enrichment of Hgl and Lgl in lipid rafts (Fig. 6), suggesting that while necessary (Fig. 2 and 4), ligand binding is not sufficient to induce enrichment of Hgl and Lgl in lipid rafts.

**FIG 6** PIP2 plays a role in Hgl and Lgl enrichment in lipid rafts. GFP-PHBTK-expressing trophozoites (3.5 × 10⁶) were serum starved and exposed to hRBCs. DRM was isolated and fractionated using sucrose gradient density centrifugation. Nineteen fractions and a pellet (20P) were collected and subjected to Western blot analysis using antibodies specific for Hgl (A), Lgl (B), or Igl (C). Average values and standard deviations for densitometric scans (n = 2) are reported as the percentage of total detergent-resistant protein for each subunit. In the mutant, the submembrane distribution of the three subunits remained unchanged upon exposure to hRBCs. PIP2 loading restored the enrichment of Hgl and Lgl in lipid raft fractions.

**FIG 7** GFP-PHBTK-expressing cells have altered PIP2 levels, and can be loaded with PIP2. (A) Phosphoinositides were extracted from whole-cell lysates, and PIP2 levels were measured using dot blots with antibodies specific to PIP2. Levels were analyzed and assigned a value of arbitrary densitometric units. PIP2 levels were lower in GFP-PHBTK-expressing cells than in wild-type cells. (B) PIP2 loading in GFP-PHBTK-expressing cells was confirmed using a BODIPY-labeled PIP2. Both differential interference contrast (DIC) and fluorescence images are shown. Scale bars represent 25 μm.
Lipid Raft Location of Ligand-Bound Gal/GalNAc Lectin

Calcium signaling is necessary for the enrichment of Hgl and Lgl in rafts after ligand binding. In other systems, PIP\(_2\) can be hydrolyzed into IP\(_3\) and DAG, which facilitates calcium signaling (8, 43). Given the importance of PIP\(_2\) in the localization of Hgl and Lgl to lipid rafts, we measured intracellular calcium levels after exposure to collagen and fibronectin using a fluorescence-based calcium assay. We observed a significant increase in intracellular calcium levels after exposure to collagen but not after exposure to fibronectin (Fig. 8).

To determine if the accumulation of intracellular calcium was essential for the localization of Hgl and Lgl in lipid rafts, we exposed trophozoites to BAPTA-AM, an intracellular calcium chelator, prior to exposure to collagen. Reduction of calcium by BAPTA-AM was confirmed using a fluorescence-based calcium assay (Fig. 8). Exposure to BAPTA-AM prior to exposure to collagen prevented the enrichment of Hgl and Lgl in lipid rafts (Fig. 3), suggesting that the accumulation of intracellular calcium is necessary for lipid raft association of Gal/GalNAc lectin subunits. It is possible that the failure of Hgl and Lgl to become enriched in lipid rafts after exposure to BAPTA-AM and collagen was due to decreased adhesion. We measured adhesion to collagen in the presence of BAPTA/AM. Adhesion to collagen was not significantly inhibited in the presence of BAPTA/AM. Adhesion to collagen was measured for wild-type cells that were exposed to serum-free medium with or without BAPTA/AM. Values were averaged, and adhesion is represented as a percentage of the control value, set to 100% (±SD; \(n = 3\)). Adhesion to collagen was not significantly inhibited in the presence of BAPTA/AM or control.

In this study, we have shown that exposure of E. histolytica to bona fide Gal/GalNAc lectin ligands (e.g., HrBCs or collagen) was accompanied by enrichment of the Gal/GalNAc lectin subunits, specifically Hgl and Lgl, in lipid raft domains. Previously, it was shown that cholesterol loading induced colocalization of Gal/GalNAc lectin subunits in rafts and increased activity of the Gal/GalNAc lectin (53). Here, we have provided evidence that another condition, namely, ligand binding, can also influence the submembrane localization of the Gal/GalNAc lectin subunits. We have also shown that binding to ligand was necessary, but not sufficient, to induce enrichment of Hgl and Lgl in lipid rafts after ligand binding. Our data also indicate that PIP\(_2\) and calcium participate in the enrichment of Gal/GalNAc lectin subunits in rafts.

Enrichment of Hgl and Lgl in high-buoyancy lipid raft domains after ligand binding is similar to the clustering and activation of mammalian integrins in lipid rafts. For example, in Jurkat T lymphocytes, attachment to collagen type IV or fibronectin induces lipid raft enrichment of \(\alpha\)2\(\beta\)1 and \(\alpha\)4\(\beta\)1 integrins, respectively (17). Furthermore, activation of another integrin in Jurkat T lymphocytes, lymphocyte function-associated antigen 1 (LFA-1), is correlated with its enrichment in lipid rafts (24). Although these signaling pathways are well understood in immune cells, the current study is an important first step toward the understanding of downstream signaling pathways that arise from lipid rafts in a parasite model.

The present study shows that attachment to ligand results in colocalization of the three lectin subunits in lipid raft fractions. Previously, it was shown by immunoprecipitation that Igl associates with Hgl (30). Importantly, we have not shown that Hgl and Lgl physically interact with Igl in lipid rafts. However, it is conceivable that the enrichment of Hgl and Lgl in raft regions, which already contain Igl, facilitates the assembly of the lectin into a functional trimer. This, in turn, may serve to activate subsequent raft-based signaling pathways related to virulence.

Exposure to hRBCs or collagen was correlated with the enrichment of Hgl and Lgl subunits in lipid rafts. Interestingly, these raft populations differed slightly in their buoyant densities. For example, after binding to hRBCs, Hgl and Lgl associated with rafts that

---

FIG 8 Intracellular calcium levels are significantly higher in collagen-exposed cells than in collagen/BAPTA-AM- or fibronectin-exposed cells. Intracellular calcium levels were measured for wild-type trophozoites that were exposed to collagen, with or without BAPTA/AM, or fibronectin. Compared to those in collagen-exposed cells (\(n = 3\)), calcium levels in BAPTA-AM-exposed or in fibronectin-exposed cells (\(n = 4\)) were significantly lower at all tested time points.

FIG 9 Adhesion to collagen is not significantly inhibited in the presence of BAPTA/AM. Adhesion to collagen was measured for wild-type cells that were exposed to serum-free medium with or without BAPTA/AM. Values were averaged, and adhesion is represented as a percentage of the control value, set to 100% (±SD; \(n = 3\)). Adhesion to collagen was not significantly inhibited in the presence of BAPTA/AM or control.
were more buoyant than the rafts harboring these same subunits after collagen binding. It is possible that there are multiple types of lipid rafts within the parasite membrane, and binding to collagen or hRBCs causes the lectin to localize to distinct and separate lipid raft domains. In other systems, there is evidence for distinct raft populations. For example, purification of rafts from Madin-Darby canine kidney cells, using a variety of detergents, resulted in the isolation of distinct lipid raft domains with different protein residents (41). Immunogold labeling and electron microscopy have shown that all lipid raft markers do not colocalize. These data from other systems support the notion that multiple lipid raft domains exist within the plasma membrane (55). Our data suggest that the same is true in *E. histolytica*.

Differences in the buoyant density of rafts containing the lectin may be due to the association of the lectin with a different set of signaling proteins or cytoskeletal proteins in a lipid-specific manner. In neutrophils, heavier detergent-resistant membranes were found to contain more cytoskeletal proteins (34). Adhesion plaques, which contain actin, myosin I and II, α-actinin, vinculin, and tropomyosin (49), have been observed in *E. histolytica* upon attachment to ECM components but have not been observed upon attachment to hRBCs. Thus, the formation of a Gal/GalNAc lectin-containing adhesion plaque after exposure to collagen may explain why the lipid rafts harboring the lectin after collagen exposure are less buoyant than those harboring the lectin after hRBC exposure.

We showed that ligand binding was not correlated with the enrichment of Hgl and Lgl in rafts in a transgenic cell line with reduced levels of PIP<sub>2</sub>. We also showed that addition of exogenous PIP<sub>2</sub> to this cell line partially rescued the phenotype. Together, these data provide strong genetic evidence for a role for PIP<sub>2</sub> in regulating the submembrane distribution of the lectin subunits in *E. histolytica*. To our knowledge, this is the first study, in any system, to use a PIP<sub>2</sub>-deficient mutant to illustrate the role of PIP<sub>2</sub> in protein-lipid raft interactions.

In the current study, intracellular calcium levels were increased upon exposure to collagen but not fibronectin. Others have shown that calcium levels increase when trophozoites are exposed to fibronectin (5). One explanation for this difference is that we exposed cells to fibronectin-coated coverslips instead of fibronectin in solution (5); adhesion to the solid ECM surface may initiate different signaling pathways. It is currently unknown whether the increased intracellular calcium levels are directly related to PIP<sub>2</sub> hydrolysis in the cell or are attributable to other mechanisms related to calcium influx. In mammalian cells, the physical interaction between α<sub>5</sub>β<sub>3</sub> integrin, sodium-proton exchangers, and sodium-calcium exchangers occurs simultaneously with integrin binding to ligand and results in increased intracellular calcium levels (56). Additionally, in phagocytes, extracellular calcium influx was shown to be essential for movement of an integrin bound to adenylate cyclase toxin from *Bordetella* into lipid rafts (3). Similarly, in the current study, the increase in calcium levels was shown to be necessary for ligand-induced enrichment of Hgl and Lgl in lipid raft domains.

Other studies, with mammalian cells as well as with *E. histolytica*, have supported the connection between calcium, PIP<sub>2</sub> regulation of actin cytoskeleton, regulation of transcription, and virulence. For example, in B cells, calcium signaling has been shown to activate transcriptional regulators, such as NF-κB and NFAT (13). Likewise, attachment to collagen by trophozoites induces an increase in the binding of transcriptional regulators AP-1, STAT1, and STAT3 to DNA (7, 36) and an increase in the expression of several important virulence factors, including amoebapore and cysteine proteases (10). In *E. histolytica*, actin remodeling occurs during attachment to collagen (32) and hRBCs (1), and calcium mobilization can affect actin organization (5). In mammalian cells, calpain, a calcium-dependent protease, has been shown to cleave the cytoskeletal elements talin, filamin, and α-actinin, thereby releasing integrins from the actin cytoskeleton (45). It has been proposed previously that this cleavage of talin may be responsible for freeing proteins to allow their recruitment to lipid raft domains (3). PIP<sub>2</sub> also contributes to actin cytoskeletal reorganization by guiding and activating actin binding proteins (20, 31). PIP<sub>2</sub> plays an important role in mammalian cells by binding to talin, thereby targeting it to focal adhesions, where it can interact with and activate integrins (25). Together with our data, these findings suggest an intriguing link between parasite-host interactions, raft association of the Gal/GalNAc lectin, calcium mobilization, the cytoskeleton, and changes in gene expression.

The data presented here provide insights into signaling pathways in *E. histolytica* and, importantly, add to a developing model of the regulation of Gal/GalNAc lectin function. In the absence of ligand, GPI-anchored Igl subunits reside predominantly in raft-like domains, whereas Hgl-Lgl dimers are localized primarily to a different submembrane compartment. Binding to at least two biologically relevant ligands, hRBCs and collagen, brings all three subunits to the same raft fractions. Interestingly, our data are the first to show a correlation between the submembrane position of the lectin subunits and phosphoinositide-based signaling in this pathogen. In the future, it will be important to identify effectors that act downstream and in parallel with the Gal/GalNAc lectin after ligand binding and enrichment in lipid rafts. Fully understanding the behavior of this receptor after contact with extracellular ligands during invasion is necessary to fully appreciate virulence functions in *E. histolytica*.

**ACKNOWLEDGMENTS**

We thank William A. Petri, Jr. (University of Virginia School of Medicine, Charlottesville, VA), for antibodies specific to the Gal/GalNAc lectin subunits. We thank Brenda Welter for critical reading and editing of the manuscript and Terri Bruce and the Jordan Hall Imaging Facility (JHIF) for microscopy support.

The project described was supported by grant R01AI046414 from the National Institute of Allergy and Infectious Diseases to L.A.T. Additionally, this work was supported by a Grant-In-Aid of Research from Sigma Xi to A.B.K. This material is based upon work supported by NIFA/USDA under project number SC-1700312.

The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the views of the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, or the USDA.

**REFERENCES**


