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Localisation to lipid rafts correlates with increased function of the Gal/GalNAc lectin in the human protozoan parasite, *Entamoeba histolytica*

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ABSTRACT

Entamoeba histolytica is the causative agent of dysentery and liver abscess and is prevalent in developing countries. Adhesion to the host is critical to infection and is mediated by amoebic surface receptors. One such receptor, the Gal/GalNAc lectin, binds to galactose or *N*-acetylgalactosamine residues on host components and consists of heavy (Hgl), light (Lgl) and intermediate (Igl) subunits. The mechanism by which the lectin assembles into a functional complex is not known. The parasite also relies on cholesterol-rich domains (lipid rafts) for adhesion. Therefore, it is conceivable that rafts regulate the assembly or function of the lectin. To test this, amoebae were loaded with cholesterol and lipid rafts were purified and characterised. Western blotting showed that cholesterol loading resulted in co-compartmentalisation of all three subunits in rafts. This co-compartmentalisation was accompanied by an increase in the ability of the amoebae to bind to host cells in a galactose-specific manner, suggesting that there is a correlation between location and function of the Gal/GalNAc lectin. Cholesterol loading did not increase the surface levels of the lectin subunits. Therefore, the cholesterol-induced increase in adhesion was not the result of externalisation of an internal pool of subunits. A mutant cell line that modestly responded to cholesterol with a slight increase in adhesion exhibited only a slight enrichment of Hgl and Lgl in rafts. This supports the connection between location and function of the Gal/GalNAc lectin. Actin can also influence the interaction of proteins with rafts. Therefore, the sub-membrane distribution of the lectin subunits was also assessed after treatment with an actin depolymerising agent, cytochalasin D. Cytochalasin D-treatment had no effect on the submembrane distribution of the subunits, suggesting that actin does not prevent the association of lectin subunits with rafts in this system. Together, these data provide insight into the molecular mechanisms regulating the location and function of this adhesin.

1. Introduction

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebic dysentery and amoebic liver abscess (ALA; reviewed in Laughlin and Temesvari, 2005). Infection is acquired by the ingestion of the cyst form of the parasite. Excystation occurs in the small intestine after which the newly emerged amoeboid trophozoites translocate to and colonise the bowel lumen. Colonisation can result in two non-mutually exclusive outcomes: non-invasive disease or invasive disease. In non-invasive disease, the production and release of newly formed cysts facilitate disease spread. In invasive disease, amoebae invade the gut mucous barrier and bind to and damage colonic epithelial cells. Invasion may allow the parasite to cross the epithelial layer, enter the circulatory system and establish extraintestinal infections, the most common of which is ALA.

Adhesion of the parasite to host cells and host extracellular matrix components is considered an important virulence function, especially during invasive disease, and several amoebic cell surface receptors that participate in parasite–host interactions have been identified (reviewed in Laughlin and Temesvari, 2005; Boettner et al., 2008; Buss et al., 2010). The best characterised of these receptors is a multimeric galactose/*N*-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin (reviewed in Petri et al., 1989, 2002). This adhesin binds to galactose and *N*-acetylgalactosamine residues on host components and is proposed to be a major virulence factor. It includes a transmembrane heavy subunit (Hgl) which may be disulphide-linked to a glycosylphosphatidylinisotol (GPI)-anchored light subunit (Lgl). The extracellular portion of Hgl possesses the carbohydrate recognition domain and the cytoplasmic tail of Hgl interacts with actin (McCoy and Mann, 2005) and exhibits sequence homology to signalling domains of β2 and β7 integrins (Vines et al., 1998; Dodson et al., 1999). This suggests that the

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Gal/GalNAc lectin might also have a signalling function. Overexpression of the cytoplasmic domain of Hgl has a dominant negative effect on parasite–host adhesion, supporting the importance of this domain as a signalling platform (Vines et al., 1998).

Immunoprecipitation studies suggest that the heterodimer associates non-covalently with a GPI-anchored intermediate subunit (Igl) (Cheng et al., 1998, 2001). Immunofluorescence microscopy demonstrates that all three subunits co-localise at the plasma membranes of trophozoites adhered to laminin-coated surfaces (Cheng et al., 2001). These findings suggest that the heterotrimer is the functional adhesin; however, little is known about what regulates the assembly of the subunits into an active complex.

In other systems membrane microdomains, known as lipid rafts, can regulate the assembly of adhesion and signalling molecules into functional multimers (reviewed in Brown, 2006). These membrane domains are characterised by high levels of cholesterol and sphingolipid. Biochemical purification of these domains is initiated by extraction with cold non-ionic detergents, which results in the isolation of detergent-resistant rafts and detergent-resistant actin-rich membrane. Further separation of these two membrane types can be achieved by sucrose gradient density centrifugation. Lipid rafts may be enriched in GPI-anchored and transmembrane proteins; changes in membrane cholesterol (Reversi et al., 2006), binding to ligand (Valensin et al., 2002), and/or interaction with the cellular cytoskeleton (Hao and August, 2005), can influence the association of such proteins with raft microdomains.

Previously, it was shown that *E. histolytica* trophozoites possess lipid raft-like domains (Laughlin et al., 2004). Further, it was shown that treatment with raft-disrupting agents inhibits adhesion of the parasite to host cells (Laughlin et al., 2004) and to host extracellular matrix (Mittal et al., 2008). Biochemical purification of detergent-resistant rafts from trophozoites revealed that the subunits of the Gal/GalNAc lectin localise to both lipid rafts and actin rich-membranes (Laughlin et al., 2004). Thus, it is conceivable that the assembly and function of the Gal/GalNAc lectin subunits may be regulated by their association with various membrane microdomains.

The aim of this study was to explore the relationship between rafts and Gal/GalNAc lectin function by loading trophozoites with cholesterol and then examining the sub-membrane distribution of Hgl, Igl and Lgl, and measuring the parasite's ability to bind to host cells. We further explored the relationship between lipid rafts and Gal/GalNAc function by characterising rafts and adhesion in an *E. histolytica* mutant cell line with a known defect in Gal/GalNAc function. Finally, given that actin can influence the association of proteins with various microdomains (Hao and August, 2005), we explored the role of the actin cytoskeleton in Gal/GalNAc lectin-membrane microdomain interactions.

2. Materials and methods

2.1. Strains and culture conditions

Entamoeba histolytica trophozoites (strain HM-1:IMSS) were cultured axenically in TYI-S-33 media (Diamond et al., 1978) in 15 ml glass screw cap tubes at 37 °C. The generation of an *E. histolytica* cell line conditionally over-expressing a mutant version of EhRabA is described elsewhere (Welter and Temesvari, 2009). This mutant cell line was maintained in TYI-S-33 supplemented with 5 μg/ml of G418 and 15 μg/ml of hygromycin in the culture medium. Mutant EhRabA expression was induced by the addition of 5 μg/ml of tetracycline to the culture medium 24 h prior to performing assays. Chinese hamster ovary (CHO) cells were grown at 37 °C in DMEM supplemented with 10% (v/v) FBS, 1 mM of HEPES and a PenStrep mixture (50 units/ml of penicillin/50 μg/ml of streptomycin) in 96-well plates.

2.2. Cholesterol loading and depolymerisation of actin

Trophozoites (3.5×10^6) were incubated in serum-free (TYI-33) medium at 37 °C for 30 min and then incubated with 3 mg/ml of cholesterol as lipoprotein–cholesterol concentrate (LCC) (MP Biomedicals, Solon, OH, USA) in the presence or absence of a sterol carrier, 50 mM methyl-β-cyclodextrin (MβCD). Exposure to the lipid and carrier was carried out for 60 min at 37 °C.

To disrupt the actin cytoskeleton, 3×10^5 trophozoites were pre-treated with an actin disrupting agent, cytochalasin D (CytoD, Sigma–Aldrich, St. Louis, MO, USA 10 μM), or an equivalent volume of CytoD diluent (DMSO), for 60 min at 37 °C prior to isolation and characterisation of detergent-resistant membrane (DRM).

2.3. Measurement of adhesion to host epithelial cells

To assess parasite–host adhesion we used a previously developed fluorescence-based assay (Powell et al., 2006). Briefly, CHO cells were grown to confluency in 96-well plates and fixed with 4% paraformaldehyde (to prevent parasite-mediated lysis of the epithelial cells during the assay). The fixed monolayers were washed twice with PBS, incubated with 250 mM glycine (to inactivate residual paraformaldehyde) and washed twice with PBS. Control and treated *E. histolytica* cells were stained with the fluorescent vital dye, calcein AM (5 μg/ml, Invitrogen, Carlsbad, CA, USA) in serum-free medium (37 °C, 30 min). Stained cells were added to the wells containing fixed CHO monolayers and incubated (37 °C, 30 min). The wells were then washed twice with warm PBS and the number of parasites adhering to CHO cells was determined by measuring fluorescence with a fluorimeter/plate reader (Model FLX800, BioTek Instruments, Winooski, VT, USA).

2.4. Lipid raft isolation and characterisation

For control and treated cells, isolation of Triton-X-100-resistant membrane, resolution of DRM by sucrose gradient density centrifugation and characterisation of gradient fractions by SDS–PAGE and western blot were carried out as previously described (Laughlin et al., 2004). Densitometric analyses of immunoblots were conducted using ImageJ Software (Version 1.42q; U.S. National Institutes of Health, Bethesda, MD, USA).

2.5. Measurement of cholesterol

Whole cells were lysed by incubation in 1% (v/v) Triton-X-100 in PBS at room temperature. Alternatively, DRM was isolated by cold-Triton-X-100 extraction as described above for lipid raft isolation followed by treatment with 1% (v/v) Triton-X-100 in PBS at room temperature. Cholesterol was quantified using the fluorescence-based Amplex Red Cholesterol Assay Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol.

2.6. Cell surface biotinylation

Control and treated cells (3.5×10^6) were surface biotinylated and modified proteins were purified by avidin affinity chromatography using the Pierce Cell Surface Protein Isolation Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's specifications. Whole cell lysates and biotinylated surface proteins that were captured by avidin affinity chromatography were resolved by SDS–PAGE and analyzed for Hgl, Lgl, Igl and actin by western blotting and densitometry.