Evidence for a Bacterial Lipopolysaccharide-Recognizing G-Protein-Coupled Receptor in the Bacterial Engulfment by Entamoeba histolytica

Matthew T. Brewer, Prince N. Agbedanu, Mostafa Zamanian, Tim A. Day, Steve A. Carlson
Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

Entamoeba histolytica is the causative agent of amoebic dysentery, a worldwide protozoal disease that results in approximately 100,000 deaths annually. The virulence of E. histolytica may be due to interactions with the host bacterial flora, whereby trophozoites engulf colonic bacteria as a nutrient source. The engulfment process depends on trophozoite recognition of bacterial epitopes that activate phagocytosis pathways. E. histolytica GPCR-1 (EhGPCR-1) was previously recognized as a putative G-protein-coupled receptor (GPCR) used by Entamoeba histolytica during phagocytosis. In the present study, we attempted to characterize EhGPCR-1 by using heterologous GPCR expression in Saccharomyces cerevisiae. We discovered that bacterial lipopolysaccharide (LPS) is an activator of EhGPCR-1 and that LPS stimulates EhGPCR-1 in a concentration-dependent manner. Additionally, we demonstrated that Entamoeba histolytica prefers to engulf bacteria with intact LPS and that this engulfment process is sensitive to suramin, which prevents the interactions of GPCRs and G-proteins. Thus, EhGPCR-1 is an LPS-recognizing GPCR that is a potential drug target for treatment of amoebiasis, especially considering the well-established drug targeting to GPCRs.

Entamoeba histolytica is an important cause of amoebic dysentery (1). E. histolytica infection often manifests as colitis, but trophozoites can also gain access to the systemic circulation and result in liver or brain abscesses (2, 3). The majority of infections are asymptomatic (4), and the host factors that determine the pathological severity of infection have not been well characterized.

The virulence of E. histolytica may be due to interactions with the host bacterial flora. Coculture with bacteria can restore amoebic virulence in E. histolytica cell lines attenuated through serial passage (5, 6), and this effect is related to an upregulation of genes associated with enhanced phagocytosis (7). Specifically, E. histolytica exhibits enhanced adherence and cytotoxic capabilities following engulfment of enteropathogenic bacteria (8). E. histolytica is also dependent on colonic bacteria as a nutrient source for trophozoites. Prior to the advent of selective medium, trophozoites could be grown only in culture medium containing bacteria (9). Although trophozoites are now routinely grown in bacteria-free culture medium, exposure to Escherichia coli enhances their growth (8). However, while it is clear that E. histolytica regularly engulfs bacteria, the specific bacterial epitopes recognized by amoebic receptors are unknown.

Phagocytosis is a stepwise process that is initiated by activation of receptors that bind an extracellular target and ultimately activate cytoskeletal rearrangements. The phagocytic ability is essential for the pathogenesis of amoebiasis and is strongly correlated with virulence; Entamoeba dispar, a nonpathogenic species, is less efficient at engulfing bacteria (10, 11). While many studies have established the importance of phagocytosis in amoebic pathogenesis, relatively few receptors mediating this process have been identified. The transmembrane kinase TMK96 is involved in erythropagocytosis, while TMK39 is a cholesterol receptor that may also mediate bacterial engulfment (12, 13). In metazoan phagocytes, G-protein-coupled receptors (GPCRs) have been found to initiate the phagocytosis of bacteria (14). GPCRs are transmembrane receptors that act as guanine nucleotide exchange factors upon binding extracellular ligands. Recent studies by Bosch et al. described G-protein signaling in E. histolytica; however, the receptors that interact with amoebic G-proteins are not well established (15). Picazari et al. described GPCR-1 of E. histolytica (EhGPCR-1), a putative GPCR associated with vesicular trafficking of proteins that localize to phagocytic cups (16). EhGPCR-1 is highly expressed in pathogenic E. histolytica but not in E. dispar (www.amoebadb.org; version 2.0), possibly accounting for the differential engulfment of bacteria by these divergent Entamoeba species (17). The ligand for EhGPCR-1 had not been identified prior to the present study.

We hypothesized that since EhGPCR-1 has a putative role in the initiation of phagocytosis, it may recognize bacterial prey that are an essential nutrient source and potential virulence for E. histolytica. In the present study, we used a heterologous Saccharomyces cerevisiae expression system to screen bacterial components for their ability to activate EhGPCR-1. In addition, we tested the ability of E. histolytica trophozoites to selectively engulf bacteria based on the presence of a bacterial component putatively identified as a ligand for EhGPCR-1.

MATERIALS AND METHODS
Creation of the yeast expression vector encoding EhGPCR-1. DNA encoding EhGPCR-1 (accession number AY880672) was synthesized by GeneScript via codon optimization for yeast expression. The gene was cloned into the pUC57 vector, and the cDNA was amplified with forward

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Address correspondence to Steve A. Carlson, stevec@iastate.edu.

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and reverse primers, adding restriction sites for NcoI and BamHI (5′-GC CATACCATGGCATCAATTCGTAATCAA-3′ and 5′-GCCATAGGATCCCTAAGTCAAGTTAATTTCCTTGAAA-3′) to the 5′ and 3′ ends of the ampiclon, respectively. Purified amplicons and the linearized yeast expression vector Cp4258, which contains a leucine auxotrophic marker and encodes ampicillin resistance (18, 19), were codigested with NcoI and BamHI restriction endonucleases. The EhGPCR-1 gene was then ligated into Cp4258 by using T4 DNA ligase (New England BioLabs). The resulting plasmid was transformed into E. coli, and individual clones were selected and aerobically grown overnight at 37°C in Luria-Bertani (LB) broth containing 32 μg/ml ampicillin. Plasmid DNA was purified using a HiSpeed plasmid minikit (Qiagen), and inserts were sequenced to confirm the cDNA orientation and fidelity.

**Transfection of yeast with the EhGPCR-1 expression vector.** *Saccharomyces cerevisiae* strain CY 18043 [J. Broach, Princeton University] was used as the yeast recipient, since this strain is a histidine auxotroph that exhibits histidine prototrophism upon GPCR activation, even for exogenous receptors (18, 19). Nontransfected CY 18043 yeast was grown in yeast extract-peptone-dextrose medium supplemented with all essential amino acids. Cells at mid-log phase (optical density at 600 nm [OD600] of 0.3 to 0.5) were transfected with 1 μg cDNA construct or 1 μg empty vector (mock transfectants) in the presence of 200 μg salmon sperm DNA (Invitrogen) and lithium acetate (100 mM; Sigma-Aldrich). Yeast cells were then incubated at 30°C and heat shocked at 42°C for 15 min. Cells were placed on leucine-deficient medium (1× yeast nitrogen base [YNB; Difco], 1× yeast synthetic dropout medium supplement without leucine [Sigma], 10 mM ammonium sulfate [Sigma], and 50% glucose) to select for transfection of Cp4258 with the EhGPCR-1-carrying plasmid. Transfectants were verified by isolating plasmids (Promega), and colonies expressing EhGPCR-1 were verified by PCR prior to the functional assay.

**Yeast growth assay.** Since the Cp4258 vector encodes leucine prototrophism, leucine-deficient medium was inoculated with yeast cells expressing EhGPCR-1 or mock-transfected yeast cells that were grown at 30°C to an OD600 of 1. Cells were washed three times with leucine/histidine-deficient medium (1× YNB [Difco], 1× yeast synthetic dropout medium supplement lacking leucine and histidine [Sigma], 10 mM ammonium sulfate [Sigma], 30% glucose, 50 mM 4-morpholinepropanesulfonic acid [pH 6.8]) and resuspended in 1 ml leucine/histidine-deficient medium to a density of 15 to 20 cells/μl. Approximately 3,000 cells were added to each well of 96-well plates containing the same medium along with test agonists in a total volume of 200 μl. Cells were grown at 30°C for approximately 24 h. Initial and final OD600 values were determined with a spectrophotometer to determine growth of the yeast cells.

**RESULTS**

**EhGPCR-1 activation by bacterial lysates.** To determine if EhGPCR-1 recognizes bacterial components, we monitored the response of the receptor to bacterial lysates in a histidine-auxotrophic yeast heterologous expression assay (18, 19). In this assay, the GPCR of interest is expressed in histidine-auxotrophic *S. cerevisiae* cells engineered to lack their native GPCRs. When the expressed GPCR of interest is stimulated by its cognate ligand or agonist, a promiscuous GPCR-inducible pheromone pathway is activated, which leads to downstream expression of the His3 reporter gene. His3 encodes de novo histidine synthesis enzymes, and thus His3 expression provides the ability to grow on histidine-deficient media. As a result, heterologous receptor activation can be quantified by spectrophotometric analysis of yeast growth. Receptor activation is calculated by comparing ligand-induced yeast growth to growth of yeast cells transfected with empty vector and exposed to the same ligand or agonist.

Application of *E. coli* K-12 lysates to EhGPCR-1-expressing yeast produced a significant increase in yeast growth; specifically, the EhGPCR-1-expressing yeast cells were stimulated more than 1,400% compared to mock-transfected yeast cells (Fig. 1). This growth was markedly attenuated by the addition of anti-LPS antibodies, and the effect of the anti-LPS antibodies was abrogated by proteinase K. Addition of a rough strain of *E. coli* K-12 that lacks the outer O-antigen of LPS (21) stimulated EhGPCR-1 to a significantly lesser extent. Histidine prototrophism was not observed when EhGPCR-1-expressing yeast cells were exposed to a
panel of biogenic amines (histamine, serotonin, dopamine, epinephrine, and norepinephrine; 0 to 100 mM) that are classic GPCR agonists or antagonists in mammals (data not shown). Specifically, these biogenic amines were used in early deorphanization attempts, since they are important GPCR ligands in a variety of organisms.

Concentration-dependent activation of EhGPCR-1 by LPS. Since EhGPCR-1 was putatively activated by LPS, we examined the ability of LPS to stimulate EhGPCR-1 in a concentration-dependent manner. EhGPCR-1-expressing yeast cells were incubated with various concentrations of purified LPS isolated from E. coli O111:B4 and, again, yeast growth in histidine-free medium was measured as an indicator of EhGPCR-1 activation. LPS activated EhGPCR-1 in a concentration-dependent manner, with a 50% effective concentration (EC$_{50}$) of 15 nM (Fig. 2).

Preferential engulfment of LPS-expressing E. coli by Entamoeba histolytica trophozoites. To determine if E. histolytica trophozoites selectively engulf bacteria based on the presence of LPS, we compared the engulfment of E. coli K-12 and a rough isostrain of E. coli that lacks intact LPS. Bacteria were fluorescently labeled with FITC and coincubated with E. histolytica HM1 trophozoites. Trophozoites were washed to remove bacteria that were not engulfed or attached, and the number of E. histolytica trophozoites containing bacteria was quantitated by flow cytometry. Phagocytosis assays revealed that 23.2% of trophozoites contained E. coli K-12, while only 3.8% of trophozoites engulfed the rough strain. This represented a $>80\%$ reduction in the bacterial engulfment capability of E. histolytica (Fig. 3). Because no EhGPCR-1-specific drugs are known, we confirmed the role of G-protein signaling in engulfment of bacteria by conducting phagocytosis assays in the presence of suramin, which uncouples GPCR and G-proteins (22). Suramin blocked phagocytosis in a concentration-dependent manner, with a 50% inhibitory concentration (IC$_{50}$) of approximately 80 $\mu$M (Fig. 4).

Identification of GPCR-like sequences in the E. histolytica genome. In order to assess the potential role of other GPCRs in the suramin-mediated effects on phagocytosis of bacteria, we investigated the possible existence of other E. histolytica GPCRs. Using a search algorithm that mines occult GPCR-encoding sequences from nonmammalian genomes by targeting transmembrane signatures (20), we uncovered eight sequences encoding putative GPCRs. As summarized in Fig. 5, EST and microarray studies (www.amoebadb.org) revealed that none of these receptors appeared to be expressed in E. histolytica. EhGPCR-1 is expressed in E. histolytica but not in E. dispar.

**DISCUSSION**

Previous work indicated that EhGPCR-1 is linked to phagocytic pathways in E. histolytica (16). The goal of the present study was to characterize EhGPCR-1 by determining its cognate ligand in a
heterologous expression system and to confirm the functional activity of the ligand in *E. histolytica* trophozoites.

GPCRs are cell surface receptors that sense the extracellular environment and are activated by a variety of ligands, such as catecholamines, peptides, lipids, carbohydrates, etc. GPCRs are excellent drug targets, reflected by the fact that 30 to 50% of currently marketed drugs target these receptors (23, 24). While GPCRs have been well studied in vertebrates, the study of their role in *E. histolytica* physiology is only in its infancy. *E. histolytica* expresses heterotrimeric G-proteins, which have been shown to modulate pathogenic processes, yet few receptors that interact with these G-proteins have been identified (15). A review by Bosch and Siderovski indicated that *E. histolytica* G-proteins lack homology to mammalian G-proteins (25), suggesting that GPCRs have not been identified in *E. histolytica* because of inadequacies in genomic search tools for divergent receptors. Classic GPCR agonists, such as histamine and serotonin, can modulate *E. histolytica* phagocytosis and virulence in a mouse model, but their receptors remain unidentified (26, 27). EhGPCR-1 did not respond to these ligands in our yeast assay (data not shown). Furthermore, our genomic search tool did not uncover any other GPCRs expressed by *E. histolytica* (Fig. 5). In the absence of another GPCR expressed by *E. histolytica* (that could be targeted as a control, to rule out collateral effects) and our inexperience with hairpin RNA, we were unable to perform the appropriate control experiments via RNA interference-based knockdown (28) of EhGPCR-1 expression.

The present study utilized a novel yeast auxotroph assay for screening GPCRs against potential ligands and agonists. This approach has recently been applied to GPCRs from parasitic helminths (18, 19) and may represent a valuable tool for the study of protozoan receptors, since culture of these microbes is often difficult. By utilizing this approach, we demonstrated that EhGPCR-1 is activated by a bacterial component of *E. coli*. This activation was abrogated in the presence of anti-LPS antibodies or a generic GPCR inhibitor. Purified LPS induced concentration-dependent EhGPCR-1 activation, although this response was not as robust as the response to bacterial lysates. Structural variances between LPS from *E. coli* K-12 and *E. coli* O111:B4 might explain differences in receptor activation. Alternatively, additional bacterial components may be required for maximal

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**FIG 4** Suramin inhibits engulfment of bacteria by *E. histolytica* in a concentration-dependent manner, with an IC50 of approximately 80 μM. Data are the mean percentage (± the standard error of the mean) of trophozoites that contained bacteria after 1 h of coincubation with *E. coli*. Data are from three independent experiments, with each repeated in triplicate. *, P < 0.05 versus suramin-free control.

**FIG 5** Identity of eight putative and novel *E. histolytica* GPCRs uncovered when we used TMf-SVM (20). Expression of each receptor in *E. histolytica* is also shown, as determined from EST and microarray studies (www.amoebadb.org). EhGPCR-1 is presented in the bottom row. The DRY motif (29) is shown in bold and underlined in the sequence for XP_65721.1/EDI_148210.
occupancy of EhGPCR-1. Further research is needed to unveil the unique pharmacologic aspects of EhGPCR-1 in *E. histolytica* with respect to agonist affinity and binding cooperativity. EhGPCR-1 may represent a new GPCR subtype, since an NCBI motif database search revealed that it is most closely related to the MIG-14-Wnt-bd superfamily of proteins and it lacks the E/DRY and NPXXY motifs found in class A-type GPCRs (29). Interestingly, one of the nonexpressed *E. histolytica* GPCRs contains a DRY motif (Fig. 5).

Based on the results of our yeast expression assay, we hypothesized that *E. histolytica* initiates bacterial engulfment after recognizing bacterial LPS. Phagocytosis assays demonstrated a >80% reduction in the number of trophozoites containing bacteria when the bacterial prey lacked O-antigen, the outermost layer of LPS. This result is supported by previous research indicating that *E. histolytica* primarily engulfs Gram-negative pathogens (30). Other investigators have also demonstrated selective engulfment of bacteria by amoebae based upon bacterial O-antigen (31, 32). Therefore, other taxa of protozoa potentially express similar receptors for bacterial recognition. EhGPCR-1-mediated preferential feeding behaviors on bacteria may also lead to disruptions in the intestinal microbiota, which are observed during infection with *E. histolytica* (33). The composition of the intestinal flora is likely to be a factor allowing *E. histolytica* colonization of the gut, and the role of EhGPCR-1 in this process is a current line of research in our laboratory.

In summary, this study demonstrates the utility of a heterologous yeast expression system in the characterization of EhGPCR-1, a GPCR putatively used in phagocytosis by pathogenic *E. histolytica*. EhGPCR-1 is activated by bacterial LPS, suggesting that this GPCR may be used to initiate phagocytosis upon the recognition of bacterial prey. Functional studies supported the role of LPS in engulfment of *E. coli* by *E. histolytica*. EhGPCR-1 is the first GPCR to be characterized in *E. histolytica*, and it may represent an important chemotherapeutic target in this pathogen. While this may be the only GPCR expressed in *E. histolytica* despite the unexplained presence of nonexpressed GPCR-encoding genes, other protozoan GPCRs may represent innovative drug targets, and their roles in regulating protozoan physiology merit further investigation.

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