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Entamoeba histolytica rhomboid protease 1 has a role in migration and motility as validated by two independent genetic approaches

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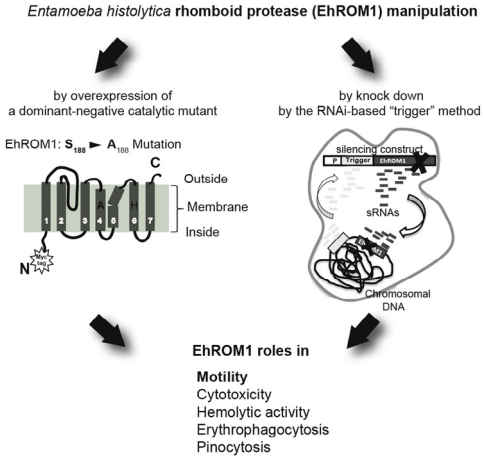
HIGHLIGHTS

- Catalytic mutant and gene downregulation of *Entamoeba* rhomboid protease 1.
- ROM1 has roles in numerous aspects of parasite biology, including virulence.
- New roles in parasite migration and motility noted for ROM1.
- ROM1 role in cell migration mimics roles in mammalian and apicomplexan systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Rhomboid proteins represent a recently discovered family of intramembrane proteases present in a broad range of organisms and with increasing links to human diseases. The enteric parasite *Entamoeba histolytica* has evolved multiple mechanisms to adapt to the human host environment and establish infection. Our recent studies identified EhROM1 as a functional *E. histolytica* rhomboid protease with roles in adhesion to and phagocytosis of host cells. Since those studies were performed in a non-virulent strain, roles in parasite virulence could not be assessed. We focused this study on the comparison and validation of two genetic manipulation techniques: overexpression of a dominant-negative catalytic mutant of EhROM1 and knock down of EhROM1 using a RNAi-based silencing approach followed by functional studies of phenotypic analyses in virulent parasites. Both the EhROM1 catalytic mutant and parasites with EhROM1 downregulation were reduced in cytotoxicity, hemolytic activity, and directional and non-directional transwell migration. Importantly, the role for EhROM1 in cell migration mimics similar roles for rhomboid proteases from mammalian and apicomplexan systems. However, the EhROM1 catalytic mutant and EhROM1 downregulation parasites had different phenotypes for erythrophagocytosis, while complement resistance was not affected in either strain. In summary, in this study we genetically manipulated *E. histolytica* rhomboid protease EhROM1 by two different approaches and identified similarly attenuated phenotypes by both approaches, suggesting a novel role for EhROM1 in amebic motility.

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1. Introduction

Intramembrane proteolysis mediated by rhomboid proteases was initially described in *Drosophila* (Lemberg et al., 2005; Urban and Wolfe, 2005; Urban et al., 2001). Rhomboid proteases belong to a family of seven transmembrane domain serine proteases harboring serine and histidine as a catalytic dyad (Lemberg et al., 2005; Urban et al., 2001), which catalyze the release of membrane-bound substrates. Rhomboid proteases (ROMs) are conserved among all kingdoms of life (Lee et al., 2001; Urban et al., 2001) and are known to control a wide range of biologically and medically important processes ranging from insulin resistance and type 2 diabetes by the mitochondrial rhomboid protease PSARL (Walder et al., 2005), mitochondrial dysfunction and Parkinson's disease (Greene, 2012; Meissner et al., 2011), mitochondrial adaptation to stress (Greene, 2012; Sanjuan Szklarz and Scorrano, 2012), shedding thrombomodulin and roles in wound healing by RHBDL2 (Cheng et al., 2011), and ER-associated degradation and extraction of misfolded membrane proteins by the endoplasmic reticulum resident rhomboid protease RHBDL4 (Greenblatt et al., 2012).

Several parasitic species such as *Trichomonas vaginalis*, *Naegleria gruberi*, *Giardia lamblia, Leishmania major, Cryptosporidium parvum*, *Babesia bovis*, *Theileria* spp., and *Trypanosoma* species are known to accommodate multiple (often redundant) rhomboid genes within their genomes. (Santos et al., 2012). However, systematic studies to characterize the roles of these proteases have only been performed extensively in *Toxoplasma gondii*, *Plasmodium* spp. and *Entamoeba histolytica* (Baxt et al., 2008, 2010; Brossier et al., 2005, 2008; Buguliskis et al., 2010; Dowse et al., 2008), where these proteases are found to have multiple important roles. *T. gondii* contains six rhomboid proteases, one in the mitochondria, and five others that are expressed at different life cycle stages and localized in different cellular compartments. Roles of *T. gondii* proteases range from intracellular growth, maintenance of micronemal adhesin gradient (thus assuring directional gliding), apical attachment, and efficient host cell invasion (Brossier et al., 2005, 2008; Buguliskis et al., 2010; Dowse et al., 2008; Santos et al., 2011). Human and rodent malaria parasites, *P. falciparum* and *P. bergehi*, contain eight rhomboid genes with redundant and multiple roles in parasite development in the asexual blood stage (ROM4, 6, 7 and 8) and in the mosquito or liver stage (ROM1, 3, 9 and 10). Moreover, ROM3 has a vital function in sporogony (Lin et al., 2013), ROM1 is important for proper formation of the *Plasmodium parasitophorous* vacuole (Vera et al., 2011), whereas ROM4 is involved in shedding of various surface adhesins. Shedding of the EBA175 adhesin is an essential event for sialic acid dependent invasion of red blood cells by the merozoite stage (Brossier et al., 2005, 2008; Dowse et al., 2008; O'Donnell et al., 2006) and removing extracellular adhesive domains of a transmembrane protein TRAP from sporozoite surface is essential for gliding motility and infectivity (Ejigiri et al., 2012).

Entamoeba histolytica is an intestinal parasitic protozoan that causes colitis and liver abscess. *E. histolytica* is estimated to cause about 40,000–100,000 deaths annually worldwide (Stauffer and Ravdin, 2003; Wertheim et al., 2012) and is ranked third as a cause of death among parasites (Haque et al., 2003). *E. histolytica* possesses several factors that influence tissue invasion including the surface *N*-acetyl D-galactosamine-inhibitable lectin (Gal/GalNAc lectin) (Petri et al., 1987; Ravdin et al., 1985; Saffer and Petri, 1991), cysteine proteases (Bracha et al., 2006; Moncada et al., 2006; Tillack et al., 2006), pore-forming amoeba pores (Baxt et al., 2010; Bracha et al., 2003; Bujanover et al., 2003) and saposin-like proteins (Winkelmann et al., 2006). Additionally, phagocytosis (Labruyere and Guillen, 2006) and motility are prominent pathological features of invasive amoebiasis (Griffin, 1972). Motility of *E. histolytica* pathogenicity is linked to its ability to destroy colonic epithelium and travel to extra-intestinal sites of infection and parasites with reduced motility are less virulent (Guillen, 1996; Labruyere and Guillen, 2006; Voigt et al., 1999).

The genome of *E. histolytica* contains four genes annotated as rhomboid proteases, with only EhROM1 described to date as an active rhomboid protease (Baxt et al., 2008, 2010). Our previous data imply that EhROM1 functions as the sheddase for the amebic Gal/GalNAc lectin on the juxtamembrane site of the host cell (Baxt et al., 2008). EhROM1 knockdown within the genetic background of the *E. histolytica* G3 strain revealed roles for EhROM1 in parasite adhesion and phagocytosis of host cells (Baxt et al., 2010). However, since the *E. histolytica* G3 strain is inherently avirulent, studies on cytotoxicity could not be performed (Bracha et al., 2003; Bujanover et al., 2003).

In this work, our main purpose was to verify two independent approaches for genetic manipulation of ROM1 within a virulent parasite strain to allow studies of virulence-associated phenotypes. We overexpressed a catalytic site mutant of EhROM1 (to function in a dominant-negative manner) and achieved RNAi-based downregulation of ROM1 in a virulent *E. histolytica* strain. We demonstrate that both approaches designed to interfere with EhROM1 activity resulted in parasites with remarkably attenuated transwell migration, significantly reduced cytotoxicity, and defects in pinocytosis. In addition, our study highlights parasite migration as a novel functional property of amoebic rhomboid proteases. Thus, EhROM1 has multi-factorial roles in *E. histolytica* pathogenesis as both motility and migration are prominent pathological features of invasive amoebiasis (Griffin, 1972).

2. Materials and methods

2.1. Plasmid construction

The full-length coding region of EhROM1 (EHI_197460) was amplified by PCR from *E. histolytica* HM-1:IMSS genomic DNA using the following primers: TCCCCCGGGCATTCTCCACCACATAACAATATA (forward primer), GCCGCTCGAGTTAATTGCATTTTCCAACATTGAGTA (reverse primer) containing SmaI and XhoI restriction sites, and cloned into a Topo TA pCR® 2.1 vector (Invitrogen, USA). In order to generate the EhROM1 catalytic mutant, the serine at the amino acid position 188 was substituted with alanine using the following primer: GCAACGAACCTGCAGCTCC. Site directed mutagenesis was performed to insert the desired mutations into the DNA motif using the Quikchange site directed mutagenesis protocol (Stratagene). Both, wild type and mutated version of EhROM1 were sub cloned into the pKT-3M vector (Saito-Nakano et al., 2004) at the SmaI and XhoI (New England Bio Labs Inc., USA) restriction sites resulting in an N-terminal triple Myc tag fusion. Correct gene insertion was confirmed by sequencing. EhROM1 silencing was achieved by generation of antisense small RNAs by the "trigger" method described by Morf et al. (2013). In brief, the first 132 bp of the EHI_197520 coding sequence to which large numbers of antisense small RNAs map is designated as a "trigger"-sequence. This trigger was fused with the full-length open reading frame of the EhROM1 gene and resulted in significant downregulation of EhROM1 gene expression.

2.2. Generation of stable parasite transfectants

Entamoeba histolytica HM-1:IMSS trophozoites were transfected with each construct ("trigger" T-EhROM1-s; "catalytic mutant" EhROM1-SA; or "EhROM1 overexpression" EhROM1-OX) as previously described (Baxt et al., 2010; Saito-Nakano et al., 2004). Briefly, mid-log parasites were seeded into 25 mm Petri dishes, sealed with parafilm and allowed to grow at 37 °C for 24 h. Transfection mixture containing 15–20 μg of plasmid DNA and 20 μl of SuperFect (Qiagen, USA) in a total volume of 200 μl of M199 medium (Gibco, USA) was incubated for 10 min at room temperature (RT). Plated amoebae were