Molecular & Biochemical Parasitology 193 (2014) 33–44

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Crystal structure and putative substrate identification for the *Entamoeba histolytica* low molecular weight tyrosine phosphatase

Alicia S. Linford[a,**], Nona M. Jiang[b], Thomas E. Edwards[c,d], Nicholas E. Sherman[e], Wesley C. Van Voorhis[c,f], Lance J. Stewart[c,d], Peter J. Myler[c,g,h], Bart L. Staker[c,d], William A. Petri Jr.[b,i,j,*]

[a] Department of Biochemistry and Molecular Genetics, University of Virginia Health System, Charlottesville, VA 22908, USA
[b] Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, VA 22908, USA
[c] Seattle Structural Genomics Center for Infectious Disease (SSGCID), USA
[d] Emerald Bio, Bainbridge Island, WA 98110, USA
[e] Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA 22908, USA
[f] Department of Medicine, University of Washington, Seattle, WA 98195, USA
[g] Seattle Biomedical Research Institute, Seattle, WA 98109, USA
[h] Departments of Global Health and Medical Education & Biomedical Informatics, University of Washington, Seattle, WA 98195, USA
[i] Department of Medicine, University of Virginia, Charlottesville, VA 22908, USA
[j] Department of Pathology, University of Virginia, Charlottesville, VA 22908, USA

ARTICLE INFO

Article history:
Received 24 August 2013
Received in revised form 12 January 2014
Accepted 22 January 2014
Available online 15 February 2014

Keywords:
Entamoeba histolytica
Low molecular weight protein tyrosine phosphatase (LMW-PTP)
LMW-PTP crystal structure
Substrate-trapping
LMW-PTP putative substrate identification

ABSTRACT

Entamoeba histolytica is a eukaryotic intestinal parasite of humans, and is endemic in developing countries. We have characterized the *E. histolytica* putative low molecular weight protein tyrosine phosphatase (LMW-PTP). The structure for this amebic tyrosine phosphatase was solved, showing the ligand-induced conformational changes necessary for binding of substrate. In amebae, it was expressed at low but detectable levels as detected by immunoprecipitation followed by immunoblotting. A mutant LMW-PTP protein in which the catalytic cysteine in the active site was replaced with a serine lacked phosphatase activity, and was used to identify a number of trapped putative substrate proteins via mass spectrometry analysis. Seven of these putative substrate protein genes were cloned with an epitope tag and overexpressed in amebae. Five of these seven putative substrate proteins were demonstrated to interact specifically with the mutant LMW-PTP. This is the first biochemical study of a small tyrosine phosphatase in Entamoeba, and sets the stage for understanding its role in amebic biology and pathogenesis.

1. Introduction

The unicellular protozoal human parasite *Entamoeba histolytica* has two stages in its life cycle: infective cysts and motile trophozoites [1]. *E. histolytica* infection can result in amebic colitis and liver abscesses; an estimated 50 million symptomatic clinical cases of amebiasis occur every year worldwide, resulting in 100,000 deaths [1,2]. *E. histolytica* cysts are spread to human hosts via the fecal-oral route via contaminated food or water, and infection with this organism is endemic in many parts of the developing world [2]. Outbreaks in developed countries have occurred when drinking water has become contaminated with human fecal matter such as in the city of Tbilisi in the Republic of Georgia in 1998 [3], and in Chicago in 1933 during the World's Fair [4].

Phosphorylation and dephosphorylation of protein tyrosine residues play important roles in regulating cellular processes [5]. Low molecular weight protein tyrosine phosphatases (LMW-PTPs) are found in most organisms including Archaea, bacteria, and eukaryotes [6]. In general, an organism has one or two LMW-PTP genes: *E. histolytica* has two, the commensal species *Entamoeba dispar* and the reptile parasite *Entamoeba invadens* each have one, as does the green alga *Chlamydomonas reinhardtii* and the plants *Arabidopsis thaliana* and *Oryza sativa* [7]. The black cottonwood tree *Populus trichocarpa* has two [7], as does Drosophila [8]. All mammals, including humans [6], have a single gene yielding two active isoforms [9]. Mammalian LMW-PTPs have been observed to be overexpressed in certain tumors, and thus are considered oncogenes [10].

* Corresponding author at: Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, VA 22908, USA. Tel.: +1 434 924 5621.
** Corresponding author.
Tel.: +1 434 243 2726.
E-mail addresses: asl2c@virginia.edu (A.S. Linford), wap3g@virginia.edu (W.A. Petri Jr.).

0166-6851/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molbiopara.2014.01.003

The active site, or P-loop, of LMW-PTPs has the conserved sequence CLGNICR, conforming to the general PTP sequence CX_5R [5,11]. The cysteine residue performs the nucleophilic attack on the phosphorus atom of the substrate phosphate group, producing a covalent phosphoenzyme intermediate [12,13]. Mutating the active site cysteine to a serine or alanine creates an enzyme lacking detectable catalytic activity [13]. Cysteine to serine (Cys to Ser) mutants bind substrates and substrate analogs with the same affinity as the wild-type PTP [12]. These mutants are used to isolate and identify PTP substrates by "substrate trapping" either in vivo or in vitro, since they bind substrate but do not carry out the catalytic reaction.

E. histolytica has 20 genes annotated as PTPs or putative PTPs [14,15], far fewer than the 107 PTPs that the human genome contains [7,16]. The two *E. histolytica* LMW-PTP proteins (GenBank: XP_656359, coded by GenBank: XM_651267, and GenBank: XP_653357, coded by GenBank: XM_648265), are identical except for a single conservative residue change at position 85 in the protein sequence: XP_656359 has an alanine and XP_653357 a valine. Both genes are expressed in cultured trophozoites, clinical isolates, and cysts [17,18]. XM_651267, the gene encoding XP_656359, was cloned and expressed for this study, as was its Cys to Ser substrate-trapping mutant form. This LMW-PTP had never been studied before and determination of its structure could be a starting point for designing drugs targeting it. In mammalian cells, LMW-PTPs play roles in controlling cell proliferation, motility, and adhesion through dephosphorylation of such substrates as growth factor receptors and cytoskeleton-associated proteins [11,16,19–21]. Identifying *E. histolytica* LMW-PTP putative substrates by use of a substrate-trapping Cys to Ser mutant LMW-PTP is a start to elucidating cellular pathways regulated by the action of this LMW-PTP.

2. Materials and methods

2.1. Alignment of LMW-PTP protein sequences

The wild-type *E. histolytica* LMW-PTP protein sequence (GenBank: XP_656359) was input into BLAST [22] to identify, select, and align LMW-PTP sequences from other representative species; LALIGN [23] was also used for alignments. The one exception was the *E. invadens* LMW-PTP, where the ameba database AmoebaDB [14] hosted by EuPathDB [24] was used for selection and alignment. Phylogenetic trees for the relative genetic distances between the LMW-PTPs were created with or without an outgroup using the phylogeny.fr web site [25] or mirror site [26], which was used in its "One-Click" mode, utilizing MUSCLE for alignment, PhyML for phylogeny, and TreeDyn for tree rendering [25,26]. Gblocks (which can be used to eliminate divergent regions and poorly aligned positions after alignment is performed) was not used in this analysis. PTP1B was included as the outgroup for one tree. The sequence for human PTP1B was input into BLAST [22] to obtain the sequence for the *E. histolytica* PTP1B homologue, which in turn was input into BLAST [22] to identify sequences for PTP1B homologues in the same organisms for which the LMW-PTPs had been selected for comparison.

2.2. Cloning of substrate-trapping mutant LMW-PTP

The substrate-trapping mutant Cys to Ser LMW-PTP gene was cloned using *E. histolytica* genomic DNA as a template. PCR was used for site-directed mutagenesis to alter the codon at residue number 7 in the XM_651267 sequence from TGT to AGT in the LMW-PTP active site, using the forward primer *Bam*H1TyrPhosFmut (5′-ATG GGA TCC ATG AAG TTG TTG TTT GTA AGT TTA GGC AAC ATT TGT CGA TCT CCT-3′) and the reverse primer *Sal*ITyrPhosR (5′-GGC GTC GAC TTA ATT AAT AAG TTT TCC TTC TTC TAG TTT AAT GAT TTA ATT CTC ACA AGC ATC-3′). PCR products were digested with *Bam* H1 and *Sal* I and cloned into plasmid pBluescript II KS(+) (Agilent Technologies, Santa Clara, CA, USA). Mutant LMW-PTP protein was recombinantly expressed and purified at the Seattle Structural Genomics Center for Infectious Disease (SSGCID) as previously described [27,28].

2.3. Recombinant protein crystallization and X-ray diffraction

Recombinant wild-type LMW-PTP protein was expressed and purified as previously described [27,28]. Crystallization (26–31 mg/ml) and X-ray diffraction conditions were as follows for the wild-type LMW-PTP structures: 3ILY (apo structure): Molecular Dimensions ProPlex Screen condition A9, 0.1 M MES pH 6.0, 20% PEG MME 2000, 0.2 M NaCl, 20% glycerol as cryo-protectant; 3IDO (substrate analog HEPES bound): Molecular Dimensions ProPlex condition F1, 0.1 M HEPES pH 7.0, 20% PEG 8000, 25% glycerol as cryo-protectant; 3JS5 (substrate analog HEPES bound; higher resolution than 3IDO) Emerald Bio PACT screen condition E6, 20% PEG 3350, 0.2 M sodium formate; 3JVI (product analog sulfate bound) Emerald Bio Wizard II screen condition 41, 2.0 M ammonium sulfate, 0.1 M Tris–HCl pH 7.0, 0.2 M lithium sulfate. The expression, crystallization, and X-ray diffraction work was performed by the SSGCID protein crystallization team. The crystallographic X-ray data and refinement statistics are shown in Supplemental Table 1.

2.4. Comparison of LMW-PTP solved structures

To identify and compare LMW-PTPs with solved structures similar to the *E. histolytica* LMW-PTP, the structure **PDB: 3JS5 (substrate mimic HEPES bound) was input into both the RSCB protein database [29] and NCBI's VAST+ database [30] in order to find structural homologues. VAST+ was then used to compare the three-dimensional structures of selected LMW-PTP homologues to the *E. histolytica* LMW-PTP using geometric criteria.

2.5. Testing recombinant wild-type and mutant LMW-PTPs for phosphatase activity

Phosphatase activity was tested with the SensoLyte *p*NPP Colorimetric Protein Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA) using the supplied Assay Buffer in 100 μl volumes in a 96-well plate. 10–200 ng wild-type LMW-PTP was tested, and absorbance was measured at 405 nm (A_{405}). HALT™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was used to test inhibition in one set of samples.

2.6. Culture of trophozoites

E. histolytica strain HM1:IMSS trophozoites were grown axenically in TYI-S-33 (trypticase-yeast extract-iron-serum) medium supplemented with 1X Diamond's vitamins (SAFC Biosciences, Lenexa, KS, USA), 15% heat-inactivated bovine serum (Gemini Bio-Products, West Sacramento, CA), 100 U penicillin/ml and 100 mg streptomycin sulfate/ml (Gibco/Life Technologies, Grand Island, NY, USA), at 37 °C in T-25 tissue culture flasks [31].

2.7. Transfection of trophozoites

HM1:IMSS strain *E. histolytica* trophozoites were transfected as previously described using the lipofection technique [32], but with the following changes: 30 μg plasmid DNA (>1 μg/μl solution, or a dried pellet) was suspended in supplemented M199 medium in 200 μl volume in 2 ml sterile microcentrifuge tubes, and 30 μl