JOURNAL OF PROTEOMICS 115 (2015) 157–176

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Plasmodium vivax trophozoite-stage proteomes

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ARTICLE INFO

Article history:
Received 22 July 2014
Accepted 21 December 2014
Available online 27 December 2014

Keywords:
Plasmodium vivax
Proteomics
Malaria
Trophozoite stage
Infected red blood cell
Protein oxidation/nitration

ABSTRACT

Plasmodium vivax is the causative infectious agent of 80–300 million annual cases of malaria. Many aspects of this parasite's biology remain unknown. To further elucidate the interaction of *P. vivax* with its *Saimiri boliviensis* host, we obtained detailed proteomes of infected red blood cells, representing the trophozoite-enriched stage of development. Data from two of three biological replicate proteomes, emphasized here, were analyzed using five search engines, which enhanced identifications and resulted in the most comprehensive *P. vivax* proteomes to date, with 1375 *P. vivax* and 3209 *S. boliviensis* identified proteins. Ribosome subunit proteins were noted for both *P. vivax* and *S. boliviensis*, consistent with *P. vivax*'s known reticulocyte host–cell specificity. A majority of the host and pathogen proteins identified belong to specific functional categories, and several parasite gene families, while 33% of the *P. vivax* proteins have no reported function. Hemoglobin was significantly oxidized in both proteomes, and additional protein oxidation and nitration was detected in one of the two proteomes. Detailed analyses of these post-translational modifications are presented. The proteins identified here significantly expand the known *P. vivax* proteome and complexity of available host protein functionality underlying the host–parasite interactive biology, and reveal unsuspected oxidative modifications that may impact protein function.

Biological significance
Plasmodium vivax malaria is a serious neglected disease, causing an estimated 80 to 300 million cases annually in 95 countries. Infection can result in significant morbidity and possible death. *P. vivax*, unlike the much better-studied *Plasmodium falciparum* species, cannot be grown in long-term culture, has a dormant form in the liver called the hypnozoite stage, has a reticulocyte host–cell preference in the blood, and creates caveolae vesicle

Abbreviations: 2D LC/MS/MS, two dimensional high performance liquid chromatography/tandem mass spectrometry; RBC, red blood cell; iRBC, infected red blood cell; CVC, caveolae vesicle complex; NHP, nonhuman primate; SCX, strong cation exchange; RP, reversed phase; CID, collision-induced dissociation; PSM, peptide-spectral match; emPAI, exponentially multiplied protein abundance index; NO, nitric oxide; ppm, parts per million; Xcorr, SEQUEST cross-correlation coefficient; Sp, SEQUEST preliminary score; z, charge; PEP, posterior error probability; HSP, heat shock protein; DOPA, 3,4-dihydroxyphenylalanine.

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http://dx.doi.org/10.1016/j.jprot.2014.12.010

complexes at the surface of the infected reticulocyte membranes. Studies of stage-specific *P. vivax* expressed proteomes have been limited in scope and focused mainly on pathogen proteins, thus limiting understanding of the biology of this pathogen and its host interactions. Here three *P. vivax* proteomes are reported from biological replicates based on purified trophozoite-infected reticulocytes from different *Saimiri boliviensis* infections (the main non-human primate experimental model for *P. vivax* biology and pathogenesis). An in-depth analysis of two of the proteomes using 2D LC/MS/MS and multiple search engines identified 1375 pathogen proteins and 3209 host proteins. Numerous functional categories of both host and pathogen proteins were identified, including several known *P. vivax* protein family members (e.g., PHIST, eTRAMP and VIR), and 33% of protein identifications were classified as hypothetical. Ribosome subunit proteins were noted for both *P. vivax* and *S. boliviensis*, consistent with this parasite species' known reticulocyte host–cell specificity. In two biological replicates analyzed for post-translational modifications, hemoglobin was extensively oxidized, and various other proteins were also oxidized or nitrated in one of the two replicates. The cause of such protein modification remains to be determined but could include oxidized heme and oxygen radicals released from the infected red blood cell's parasite-induced acidic digestive vacuoles. In any case, the data suggests the presence of distinct infection-specific conditions whereby both the pathogen and host infected red blood cell proteins may be subject to significant oxidative stress.

1. Introduction

Plasmodium vivax malaria is a serious neglected disease with transmission in 95 countries [1] and an estimated 80 to 300 million yearly cases, extreme morbidity and the possibility of death [2,3]. Infection typically results in repeated episodes of paroxysms, with high fever and chills, and symptoms that include violent headaches, vomiting, diarrhea, and muscle aches. Clinical parameters can also include an enlarged spleen, thrombocytopenia and severe anemia, and disease ramifications can be a particular concern for pregnant women [4]. As a significant public health threat, a detailed examination of this parasite's biology and biochemistry is warranted for the development of possible vaccines, diagnostics and therapeutics that can reduce disease burden [1–3,5,6]. It is important for such studies to proceed in parallel with the most lethal and better studied species, *Plasmodium falciparum*. These two most predominant malaria-causing species are phylogenetically distant [7], and species-specific interventions will be important for today's global efforts to control, eliminate and ultimately eradicate malaria [8].

For each species of Plasmodium, the expressed proteome during the parasite's life cycle stages in the mosquito vector and its primate host would be expected to have stage-specific differences. This is also the case as the parasite develops in the blood over an approximate 48-hour period from the ring stage of development to a growing trophozoite and through its schizogonic multiplication phase. The trophozoite stage of development is critical for the parasite to undergo morphological changes, grow in size, and remodel the host red blood cell (RBC) to suit its development and release of new infectious merozoite forms into circulation. During this stage, the parasite is also consuming host hemoglobin from within the RBC and processing the toxic hematin byproduct into inert pigmented hematin crystals known as hemozoin [9].

Importantly, unlike *P. falciparum*, which invades RBCs of all ages, *P. vivax* specifically invades the young RBCs known as reticulocytes [10,11]. *P. vivax*, and a few other species including the human malaria species *Plasmodium ovale* [12] and the closely related simian malaria model species *Plasmodium cynomolgi* then begin to synthesize caveolae vesicle complexes (CVCs) [13]. These are elaborate structures that develop around the entire infected host cell membrane with the caveaole cup-like portion externalized and the vesicular and tubular structures internal within the host cell cytoplasm [12]. The CVCs have been observed from *P. cynomolgi* in 3-dimensions using electron tomography and by immuno-electron tomography showing the PHIST/CVC-8195 protein localized to the outer portions of CVC tubules [14,15]. Many other parasite-encoded infected RBC (iRBC) membrane proteins have been identified by SDS-PAGE analysis of purified *P. vivax* infected RBC membranes from *Saimiri boliviensis* monkey infections, with several others associated with the CVCs and other iRBC membrane structures [15] but these have remained uncharacterized. Critically, *P. vivax* and *P. cynomolgi* lack the knob-like morphology characteristic of *P. falciparum* iRBC surface structures, and which are known for expression of adhesive variant proteins that are associated with virulence [3,5,15]. Thus, *P. vivax* and *P. cynomolgi* iRBC biology is very different from *P. falciparum* (and other species) in many important respects that remain largely unexplored. These biological differences include the expression in *P. vivax* (and *P. cynomolgi* [16]) of members of a multigene family called vir, which encodes several hundred small presumptive variant antigen proteins with multiple predicted localizations [17–19]. This is in contrast to the ~60 member var gene family in *P. falciparum* and the related ~108 member SICAvar family in *Plasmodium knowlesi*, with each confirmed to encode large variant antigens that become positioned at the surface of the infected RBCs and undergo switching events in the course of an immune response [20,21].

Basic studies of *P. vivax* iRBCs are especially challenging because, unlike *P. falciparum* iRBCs, *P. vivax* iRBCs cannot be cultured continuously in vitro, requiring their isolation from