Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular & Biochemical Parasitology 144 (2005) 167–176
MOLECULAR & BIOCHEMICAL PARASITOLOGY

PfCG2, a *Plasmodium falciparum* protein peripherally associated with the parasitophorous vacuolar membrane, is expressed in the period of maximum hemoglobin uptake and digestion by trophozoites

Roland A. Cooper[a,1], Janni Papakrivos[b], Kristin D. Lane[c], Hisashi Fujioka[d], Klaus Lingelbach[b], Thomas E. Wellems[a,*]

[a] *Laboratory of Malaria and Vector Research, Twinbrook III, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-8132, USA*
[b] *Fachbereich Biologie/Zoologie, Philipps-Universität, Marburg 35032, Germany*
[c] *Department of Biological Sciences, Old Dominion University, Norfolk, VA, USA*
[d] *Institute of Pathology, Case Western Reserve University, Cleveland, OH, USA*

Received 23 February 2005; received in revised form 3 July 2005; accepted 14 July 2005
Available online 7 September 2005

Abstract

A *Plasmodium falciparum* gene closely linked to the chloroquine resistance locus encodes PfCG2, a predicted 320–330 kDa protein. In the parasitized erythrocyte, PfCG2 expression rises sharply in the trophozoite stage and is detected in electron-dense patches along the parasitophorous vacuolar membrane (PVM), in the cytoplasm and in the digestive vacuole (DV). Results of extraction and partitioning experiments show that PfCG2 is a peripheral membrane protein. Exposure of trophozoite-infected erythrocytes to trypsin-containing buffer after streptolysin O permeabilization indicates that PfCG2 is exposed to the erythrocyte cytosol at the outer face of the PVM. PfCG2 is highly susceptible to hydrolysis by aspartic and cysteine proteases and shows dose-dependent accumulation in the presence of protease inhibitors. These results suggest that PfCG2 is delivered from the outside face of the PVM to the DV, where it is broken down by parasite proteases. PfCG2 interacts with erythrocyte cytoplasm and may be associated with processes of hemoglobin uptake and digestion by erythrocytic-stage parasites.
Published by Elsevier B.V.

Keywords: Malaria; Endocytosis; Phagocytosis; Protein trafficking; Proteases

1. Introduction

During the pathogenic intraerythrocytic stage of infection by the human malaria parasite *Plasmodium falciparum*, much of the hemoglobin content of the host cell is digested by the growing parasite [1]. A generally accepted idea is that the parasite takes up hemoglobin via phagocytosis using specialized organelles known as cytostomes that result from the invagination of the parasite membrane and its apposed parasitophorous vacuolar membrane (PVM) [2]. In classical models of *Plasmodium* feeding, the deep pockets formed by the cytostomes capture cytosol of the host erythrocyte and subsequently pinch off as double membrane-delimited transport vesicles [2–4]. Fusion of these phagocytic transport vesicles with the parasite acidic digestive vacuole (DV) releases the hemoglobin and other molecules of the host erythrocyte cytosol for digestion by specialized aspartic-, cysteine- and metallo-proteases [5–7]. This process makes room in the host cell for the growing parasite and provides it with free amino acids utilized for protein synthesis. By the late trophozoite

Abbreviations: DV, digestive vacuole; IEM, immunoelectron microscopy; MAb, monoclonal antibody; PfALD, *Plasmodium falciparum* aldolase; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; PfEXP-1 or -2, *Plasmodium falciparum* exported protein 1 or 2; PfSERP, *Plasmodium falciparum* serine repeat protein; PVM, parasitophorous vacuolar membrane; RBC, red blood cell; SLO, streptolysin O

* Corresponding author. Tel.: +1 301 496 4021; fax: +1 301 402 2202.
E-mail address: twellems@niaid.nih.gov (T.E. Wellems).
[1] Current address: Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266, USA.

0166-6851/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.molbiopara.2005.07.009

stage, more than 60% of the host cell hemoglobin is digested [8]. Hemoglobin digestion also results in the release of free, soluble heme, a poisonous product that is detoxified by incorporation into microcrystals of hemozoin within the parasite DV [9].

The subject of the present report, PfCG2, was initially identified in a search for candidate genes from a 36-kb segment of *P. falciparum* chromosome 7 harboring the determinant of chloroquine resistance [10]. A direct involvement of PfCG2 in chloroquine resistance was ruled out, however, by subsequent results of PfCG2 allelic exchange experiments and the discovery of a nearby gene that encodes the chloroquine resistance transporter, PfCRT [11,12]. The physical proximity of the *pfcg2* and *pfcrt* genes helped explain the observed linkage between its polymorphisms and chloroquine resistance [12,13]. We remained intrigued, however, by the function of PfCG2, particularly as its expression profile is similar to that of PfCRT (unpublished results). Co-regulated expression of neighboring *Plasmodium* genes has been suggested in several cases to reflect their involvement in intersecting biologic processes [14], consistent with the reports of clustering of functionally related genes in the yeast and human genomes [15,16].

The genome sequences of different *Plasmodium* species contain clear, single-copy homologues of PfCG2 with extensive conserved regions (Supplemental figure). These conserved regions, which may contain motifs with important functional roles, are interrupted in PfCG2 by highly polymorphic regions including a poly-asparagine tract and four domains of tandem repeats [10]. In addition, PfCG2 sequences from different *P. falciparum* lines show frequent polymorphisms in the number and arrangement of these repeats and in individual amino acids at positions elsewhere in the protein [10,17–19]. PfCG2 (320–330 kDa) thus resembles some other large-molecular weight *P. falciparum* proteins that have polymorphic regions of low complexity, hydrophilic character and domains of simple tandem repeats [20]. Individual functions of these *P. falciparum* proteins have been generally difficult to establish.

PfCG2 is found in electron-dense patches at the periphery of the parasitophorous vacuolar space, in the cytoplasm and in the DV of *P. falciparum* trophozoites and schizonts [10,21]. Here we report further on the localization and erythrocyte-stage expression profile of this protein and describe evidence that PfCG2 is transiently exposed at the outer face of the PVM that surrounds the growing parasite in the erythrocyte. PfCG2 is internalized to the parasite DV, where it is appears to be quickly broken down by parasite proteases. We consider the implications of these findings, particularly as they might relate to endocytosis or the pathway of hemoglobin ingestion by malaria parasites.

2. Materials and methods

2.1. Parasite culture

Parasites were grown in O-positive human red blood cells (RBC) using RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 0.5% Albumax (Invitrogen), 0.25% sodium bicarbonate and 0.01 mg ml^{-1} gentamicin under an atmosphere of 90% nitrogen, 5% oxygen and 5% carbon dioxide. For stage-specific experiments, the parasites were maintained in synchrony by treatment with 10% sorbitol solution [22]. Parasites were of the FCB strain [10] unless otherwise noted.

2.2. Localization of PfCG2 by immunoelectron microscopy (IEM)

Anti-PfCG2 monoclonal antibody (MAb) B4D12 was purified from ascites fluid by passage over protein G-linked Sepharose (Amersham Biosciences Inc., Piscataway, NJ, USA) as recommended by the manufacturer. Samples of the *P. falciparum* strain Dd2 at various developmental stages in erythrocytes were fixed and processed for IEM as described [10], except that 5-nm gold-conjugated anti-mouse IgG was used (Amersham Biosciences Inc.). Images were made using a Zeiss CEM902 electron microscope (Oberkochen, Germany).

2.3. SDS-PAGE and immunoblotting

Parasite samples were solubilized in SDS-PAGE sample buffer (100 mM Tris, 6.5 mM EDTA, 20% glycerol (v/v), 2% SDS (w/v), 2% 2-mercaptoethanol (v/v), 0.02% bromphenol blue (w/v), pH 6.8) and then heated to 95 °C for 5 min. Samples for analysis of PfCG2 were separated by 5 or 4–12% gradient SDS-PAGE (Invitrogen, San Diego, CA, USA). Proteins were transferred to nitrocellulose or PVDF membranes and incubated with MAb B4D12 at 1:1000 dilution for 1 h at room temperature, or overnight at 5 °C, followed by incubation with HRP conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:25,000 dilution. Signals were detected using enhanced chemiluminescent reagents (Pierce, Rockford, IL, USA). In some cases, an alkaline phosphatase-conjugated secondary antibody was used and signals were detected with the Promega ProtoBlot system (Promega Corporation, Madison, WI, USA). Membranes were also probed with antibodies specific to the parasite proteins PfSERP, PfALD, PfCRT, PfEXP-1 and PfEXP-2 [12,23].

2.4. High pH extraction

Synchronous parasite cultures consisting of trophozoite or schizont stages, representing 10^8–10^9 parasites, were washed twice by centrifugation with PBS (10 mM potassium phosphate, 138 mM NaCl, 2.7 mM KCl; pH 7.4) at 5 °C. Parasites were isolated by treating cells with 0.01% saponin in PBS for 1 min. The parasite pellet was washed twice again with PBS at 5 °C. Samples were taken at the time of the final wash and treated as follows. A sample serving as a control was placed directly in SDS-PAGE sample buffer, heated at 95 °C for 5 min and stored at −70 °C until analysis by SDS-PAGE and immunoblotting. Sets of duplicate samples were resuspended in 100 μl of 100 mM Tris buffer, pH 7.5, or in 100 mM carbonate (Na_2CO_3) buffer, pH 11, and one sample from each set was incubated immediately on wet ice for 30 min. The other sample from each set was snap-frozen and thawed before incubating on ice. All samples were centrifuged at 105,000 × *g* for 30 min. Pellets and supernatants were separated by SDS-PAGE, blotted to PVDF or nitrocellu-