International Journal for Parasitology 40 (2010) 1257–1268

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Plasmodium CDP-DAG synthase: An atypical gene with an essential N-terminal extension

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ARTICLE INFO

Article history:
Received 15 January 2010
Received in revised form 10 March 2010
Accepted 10 March 2010

Keywords:
Malaria
Plasmodium falciparum
Plasmodium knowlesi
Phospholipid
CDP-DAG synthase

ABSTRACT

Cytidine diphosphate diacylglycerol synthase (CDS) diverts phosphatidic acid towards the biosynthesis of CDP-DAG, an obligatory liponucleotide intermediate in anionic phospholipid biosynthesis. The 78 kDa predicted *Plasmodium falciparum* CDS (PfCDS) is recovered as a 50 kDa conserved C-terminal cytidylyltransferase domain (C-PfCDS) and a 28 kDa fragment that corresponds to the unusually long hydrophilic asparagine-rich N-terminal extension (N-PfCDS). Here, we show that the two fragments of PfCDS are the processed forms of the 78 kDa pro-form that is encoded from a single transcript with no alternate translation start site for C-PfCDS. PfCDS, which shares 54% sequence identity with *Plasmodium knowlesi* CDS (PkCDS), could substitute for PkCDS in *P. knowlesi*. Experiments to disrupt either the full-length or the N-terminal extension of PkCDS indicate that not only the C-terminal cytidylyltransferase domain but also the N-terminal extension is essential to *Plasmodium* spp. PkCDS and PfCDS introduced in *P. knowlesi* were processed in the parasite, suggesting a conserved parasite-dependent mechanism. The N-PfCDS appears to be a peripheral membrane protein and is trafficked outside the parasite to the parasitophorous vacuole. Although the function of this unusual N-PfCDS remains enigmatic, the study here highlights features of this essential gene and its biological importance during the intra-erythrocytic cycle of the parasite.

1. Introduction

Malaria is caused by infection with *Plasmodium* parasites and leads to nearly 1 million deaths annually (Greenwood et al., 2008; WHO, 2008). All of the clinical symptoms of the disease are produced during the asexual erythrocytic stages of the parasite life cycle. One of the drastic changes occurring upon infection is an unusually high, sixfold increase in the content of essential phospholipids (PLs) in *Plasmodium*-infected erythrocytes (Holz, 1977; Vial et al., 1990). Since PL salvage from the host plasma is not significant and the mature host erythrocyte lacks biosynthetic pathways, this drastic increase in PLs and the prodigious proliferative capacity of malaria parasites relies on its own PL biosynthetic machinery (Holz, 1977; Sherman, 1984; Vial et al., 2003; Vial and Mamoun, 2005). *Plasmodium* PL biosynthesis, as in other eukaryotic cells, involves the cytidine diphosphate diacylglycerol (CDP-DAG)-dependent pathways for phosphotidylinositol (PI), phosphatidylserine (PS) and phosphatidylglycerol (PG), and on the Kennedy pathways for the de novo synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Vial et al., 1982, 2003). In addition, a plant-like serine decarboxylase phosphoethanolamine N-methyltransferase pathway generates PC and PE (Elabbadi et al., 1997; Pessi et al., 2005).

CDP-DAG, an obligatory liponucleotide intermediate in PL biosynthesis is synthesised by CDP-DAG synthase (CDS) from phosphatidic acid (PA) and cytidine triphosphate (CTP) (Carter, 1968). In prokaryotes, CDP-DAG is the common precursor for the biosynthesis of all PLs, essentially the anionic PG and PS which are subsequently converted to cardiolipin and PE, respectively (Raetz and Kennedy, 1973). In eukaryotic cells, CDS diverts the common precursor PA towards the CDP-DAG-dependent pathways at the expense of the Kennedy pathways. The CDS-mediated synthesis of CDP-DAG is essential and its utilization is tightly regulated. The low level of CDP-DAG compared with PA (Thompson and MacDonald, 1975, 1976) appears to be the crucial factor in determining the differential synthesis of downstream PLs. The fundamental role of CDS in PL metabolism has been shown in *Escherichia coli* (Ganong et al., 1980; Ganong and Raetz, 1982) and *Saccharomyces cerevisiae* (Shen and Dowhan, 1997). Together with this, CDS has also been shown to be crucial in PI-mediated signal transduction cascades (Wu et al., 1995a; Saito et al., 1997).

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0020-7519/$36.00
doi:10.1016/j.ijpara.2010.03.006

CDS is conserved from prokaryotes to eukaryotes (Fig. 1) (Sparrow and Raetz, 1985; Wissing et al., 1992; Kopka et al., 1997; Monaco and Feldman, 1997). *E. coli* has a single copy of *cds* encoding a small protein of 27 kDa (Sparrow and Raetz, 1985) with eight hydrophobic domains. In higher eukaryotes such as *Drosophila melanogaster* (Wu et al., 1995a) and mammals, several isoforms originating from different genes or alternate splicing (Volta et al., 1999; Inglis-Broadgate et al., 2005) are present in the endoplasmic reticulum (ER) and in the mitochondria (Kleppinger-Sparace and Moore, 1985; Shen et al., 1996). Eukaryotic CDS proteins are much larger than prokaryotic CDS proteins with an extended N-terminal end and long loops connecting the hydrophobic domains (Heacock and Agranoff, 1997; Kopka et al., 1997; Saito et al., 1997).

Remarkably, CDS proteins of *Plasmodium* spp. and *Toxoplasma gondii*, both belonging to the phylum Apicomplexa, possess an unusual very long N-terminal extension compared with other eukaryotic CDS proteins (Fig. 1). The N-terminal extensions vary in length and have no significant sequence similarity to any known protein. Only *Plasmodium falciparum* CDS (PfCDS) has been characterised (Martin et al., 2000). Surprisingly, the 667 amino acids long 78 kDa predicted protein was not recovered in blood stage parasites. Instead two fragments of 28 and 50 kDa corresponding to the N-terminal extension (N-PfCDS) and the conserved C-terminal cytidylyltransferase domain (C-PfCDS), respectively, were detected. These fragments could be the products of spliced variants or generated by proteolytic processing. When expressed in COS (CV-1 (simian) in Origin, and carrying the SV40 genetic material) cells, cytidylyltransferase enzymatic activity was confirmed for the C-PfCDS (Martin et al., 2000). Here, we show that the non-cytidylyltransferase N-PfCDS is essential for the intra-erythrocytic stages of the parasite and results from the processing of the 78 kDa pro-form PfCDS. We have also gained insight into the biological and biochemical properties of N-PfCDS.

2. Materials and methods

2.1. Reagents

RPMI 1640, albumax I, TRIZOL, DNase1 and Superscript RNA polymerase were purchased from Invitrogen. Plasmion was from Laboratoire Fresenius Kabi, France. The pArl1 vector and attB vectors were kind gifts from Dr. Michael Lanzer (University Medical School, Heidelberg, Germany) (Przyborski et al., 2005) and Dr. David Fidock (Columbia University, New York, USA) (Nkrumah et al., 2006), respectively. WR99210, an inhibitor of dihydrofolate reductase, was a gift from D. Jacobus Pharmaceuticals, Princeton, New Jersey, USA. Rabbit polyclonal antisera N26 and I28 were raised earlier in our laboratory (Martin et al., 2000). Saponin, Blasticidin, Hoechst 33342 and mouse monoclonal antibodies against human Band3 and FLAG® peptide (DYKDDDDK) were purchased from Sigma. Paraformaldehyde (PAF) and glutaraldehyde were from EMS Sciences, USA. The anti-GFP antibody, cocktail of complete protease inhibitors was from Roche, France. Mouse monoclonal anti-serine repeat antigen (SERA) antibody and rabbit anti-exported protein 1 (EXP1) antiserum were gifts from Dr. Jean-Francois Dubremetz (University of Montpellier 2, France) (Debrabant et al., 1992), and Professor Klaus Lingelbach (Philipps University of Marburg, Germany) (Gunther et al., 1991), respectively. The anti-Bip antibody (MRA-19) was obtained from MR4. Alexa conjugated secondary antibodies were purchased from Molecular Probes, France, and Vectashield with DAPI from Vector Laboratories (USA). Streptolysin O (SLO) was from ELITech, France. Phospholipids

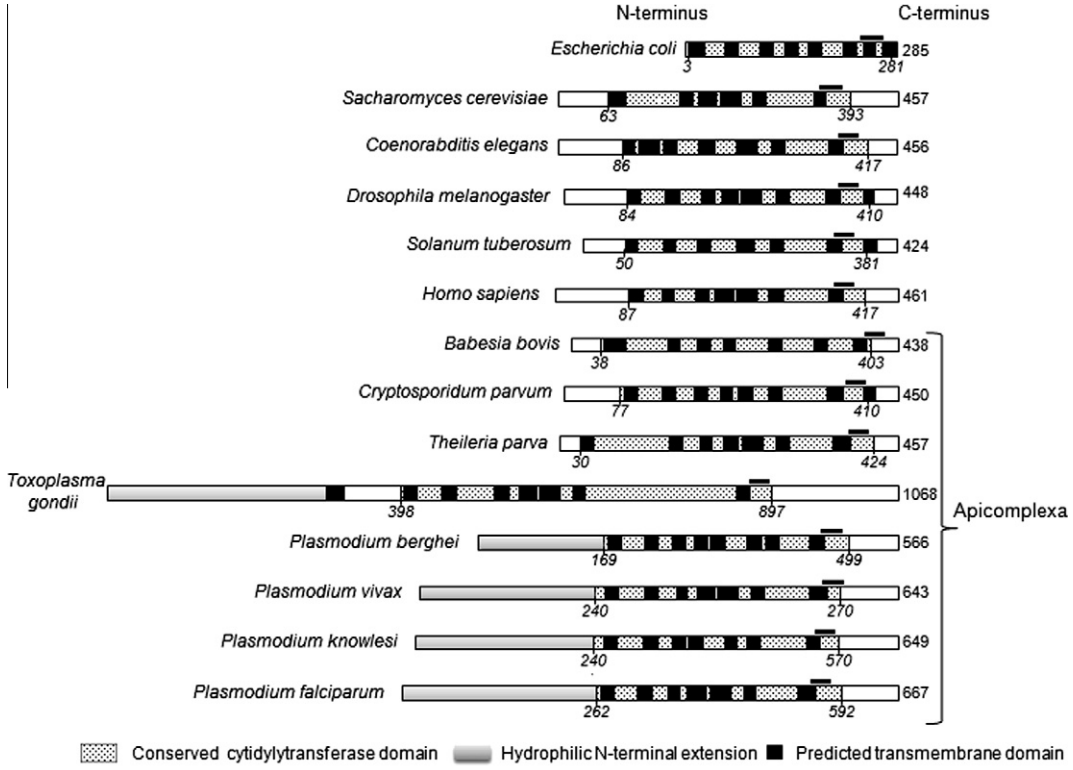

Fig. 1. Schematic drawing showing cytidine diphoshate diacylglycerol synthase (CDS) in different organisms. In all organisms CDS protein contains a cytidylyltransferase domain (dotted box) which in turn consists of several predicted hydrophobic segments (black box) and a phosphatidate cytidylyltransferase signature motif (black line). In addition to the conserved cytidylyltransferase domain, CDS from *Toxoplasma gondii* and *Plasmodium* spp. contain an N-terminal extension (grey box). The numbers at the end of each protein indicate its length in amino acids. The cytidylyltransferase domain prediction was done using the Simple Modular Architecture Research Tool algorithm.