Gene 550 (2014) 245–252

Contents lists available at ScienceDirect

Gene

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

Molecular characterization of calreticulin from *Anopheles stephensi* midgut cells and functional assay of the recombinant calreticulin with *Plasmodium berghei* ookinetes

Nahid Borhani Dizaji [a,b], Hamid Reza Basseri [a], Saied Reza Naddaf [b], Mansour Heidari [c,d,*]

[a] Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran
[c] Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran
[d] Stem Cell Preparation Unit, Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 27 October 2013
Received in revised form 2 June 2014
Accepted 19 August 2014
Available online 20 August 2014

Keywords:
Calreticulin
Anopheles stephensi
Plasmodium berghei
Molecular analysis
Protein characterization
Interaction assay
Localization
TBV

ABSTRACT

Transmission blocking vaccines (TBVs) that target the antigens on the midgut epithelium of *Anopheles* mosquitoes are among the promising tools for the elimination of the malaria parasite. Characterization and analysis of effective antigens is the first step to design TBVs. Calreticulin (CRT), a lectin-like protein, from *Anopheles albimanus* midgut, has shown antigenic features, suggesting a promising and novel TBV target. CRT is a highly conserved protein with similar features in vertebrates and invertebrates including anopheline. We cloned the full-length *crt* gene from malaria vector, *Anopheles stephensi* (AsCrt) and explored the interaction of recombinant AsCrt protein, expressed in a prokaryotic system (pGEX-6p-1), with surface proteins of *Plasmodium berghei* ookinetes by immunofluorescence assay. The cellular localization of AsCrt was determined using the baculovirus expression system. Sequence analysis of the whole cDNA of AsCrt revealed that AsCrt contains an ORF of 1221 bp. The amino acid sequence of AsCrt protein obtained in this study showed 64% homology with similar protein in human. The AsCrt shares the most common features of CRTs from other species. This gene encodes a 406 amino-acid protein with a molecular mass of 46 kDa, which contains a predicted 16 amino-acid signal peptides, conserved cysteine residues, a proline-rich region, and highly acidic C-terminal domain with endoplasmic reticulum retrieval sequence HDEL. The production of GST-AsCrt recombinant protein was confirmed by Western blot analysis using an antibody against the GST protein. The FITC-labeled GST-AsCrt exhibited a significant interaction with *P. berghei* ookinete surface proteins. Purified recombinant GST-AsCrt, labeled with FITC, displayed specific binding to the surface of *P. berghei* ookinetes in comparison with control. Moreover, the expression of AsCrt in baculovirus expression system indicated that AsCrt was localized on the surface of Sf9 cells. Our results suggest that AsCrt could be utilized as a potential target for future studies in TBV area for malaria control.

1. Introduction

Malaria remains a major health problem in Africa, Asia, and South America, with about 3.3 billion people at risk in 2011 (WHO, 2011). For decades, approaches that target *Anopheles* vectors and malaria parasites by insecticides and anti-plasmodia combinations were the most commonly used tools for malaria control. Malaria used to be an endemic parasitic disease in many parts of Iran and had been widely prevalent for a long time. Before starting any anti-malarial campaign in Iran about 60% of population was living in malaria endemic areas. In hyper-endemic areas, approximately 30 to 40% of the total mortality was due to malaria. With application of preventive and control measures from late 1940s (Edrissian, 2006), the numbers of case patients have dramatically declined and the disease disappeared from many areas. The latest official records reported 3000 case patients in a population of 74 million in year 2010. Malaria transmission is now confined to the southeastern part of Iran including Sistan and Baluchistan, Hormozgan, and Kerman Provinces. This area is bordered with Afghanistan and Pakistan, where both species of *Plasmodium falciparum* and *Plasmodium vivax* are prevalent and an influx of many patients in Iran is annually reported (Raeisi et al., 2009; WHO, 2010). Today, efforts are being made to develop effective vaccines to reduce disease transmission by interrupting the parasite development in *Anopheles* mosquito's midgut. These transmission blocking vaccines (TBVs) target the sexual stage of the parasite and

Abbreviations: TBVs, Transmission blocking vaccines; CRT, Calreticulin; As, *Anopheles stephensi*; CPB, Carboxypeptidase B; P-domain, Proline rich region; CRD, Carbohydrate recognition domain; GST, Glutathione S-transferase; ORF, Open reading frame; CSF1, CRT signature family 1; CSF2, CRT signature family 2; ESC, Embryonic stem cells; BAE, Bovine aortic endothelial.

* Corresponding author at: Stem Cell Preparation Unit, Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran.
E-mail address: mheidari@sina.tums.ac.ir (M. Heidari).

http://dx.doi.org/10.1016/j.gene.2014.08.036
0378-1119/

prevent the spread of the disease through the community; these vaccines can be an additional tool for the elimination of the malaria parasite in malaria endemic areas. TBVs are designed based on either the antigens expressed on sexual stages of the malaria parasite or *Anopheles* midgut proteins that interact with sexual stages and assist the motile form (ookinete) to traverse the *Anopheles* midgut cells. In the latter form, identification and characterization of molecules from the midgut epithelium of *Anopheles* mosquitoes that interact with antigens on gametes, zygotes or ookinetes are the primary steps to design such vaccines.

Several studies have reported that glycan-proteins and proteins on the microvilli of the midgut of *Anopheles* mosquitoes are involved in the invasion of ookinetes to midgut epithelial cells and in the transformation of ookinetes into oocysts (Wilkins and Billingsley, 2010). The majority of studies are based on direct analysis of molecules that are capable of attaching to *Plasmodium* proteins. Some of *Anopheles* midgut molecules include laminin γ1 (Vlachou et al., 2001), collagen type IV (Adini and Warburg, 1999), integrin (Mahairaki et al., 2005b), chondroitin sulfate proteoglycans (Dinglasan et al., 2007), non-peptide molecules such as sugars (Ramasamy et al., 1997; Zieler et al., 2000), annexinB11 (Mahairaki et al., 2005a), membrane alanyl-amino-peptidase (APN) (Vogel, 2010), carboxypeptidase B (CPB) (Lavazec et al., 2007), and calreticulin (CRT) (Rodríguez et al., 2007). In *Anopheles gambiae crt* gene is located on chromosome 2R (Holt et al., 2002) and encodes a 406 amino-acid sequence residue (46 kDa). In silico analysis revealed major features of CRT protein family in AsCrt, including an N-terminal signal peptide, and two highly conserved CRT family signature motifs. Three highly conserved cysteine sites suggested disulfide band formation sites. Two out of three cysteine residues in the N-terminal region of CRT make a disulfide bridge, which shapes the protein structure by folding. Also, four histidine residues, which bind to Zn^{+2} are supposed to be involved in the conformation of CRT, and consequently in the functional activity of AsCrt. The position of histidine residues is conserved in the CRTs of different organisms ranging from higher plants to humans (Michalak et al., 1992).The AsCrt amino-acid composition also represented a central P-domain of about 70 residues with three repeats of an acidic 17 amino-acid motifs and two sets of triplicate conserved domains. In proline rich region (P-domain), two sets of triplicated motif characteristics of CRT family were detected. The well conserved tryptophan residues in the first set of triplicated motif (DWD segment) and in the second one (GXW segment) were located in carbohydrate recognition domain (CRD), which might be involved in the formation of the extended hairpin fold, as shown in the rat calreticulin by others (Martin et al., 2006).

The P-domain of CRTs might possess potential sites for post-translational modification (Michalak et al., 1999). No potential N-glycosylation site was predicted in AsCrt. In some well-studied mammalian CRTs, glycosylated sites show variable patterns. The distinct patterns of glycosylation in CRTs from different species and even various tissues suggest that their biological functions could be mediated through their specific post-translational modifications (Decca et al., 2007). Depending on the organism, the CRTs may have one or more potential N-linked glycosylation sites (Jethmalani et al., 1994; Khanna et al., 1987; Matsuoka et al., 1994; Michalak et al., 1999; Peter et al., 1992), e. g., the CRTs in bovine and mouse have two potential N-glycosylation sites at Asn162 and Asn327 positions, while Asn327 doesn't appear to be glycosylated in bovine CRT (Matsuoka et al., 1994). Glycosylation of CRTs is more common in plants than in animals (Michalak et al., 1999).

Anopheles albimanus midgut CRT has been shown on the apical surface of midgut and also shows a specific adhesion property to the surface protein Pvs25 of *Plasmodium vivax* ookinetes (Rodríguez et al., 2007). Orthologues of AsCrt have been found in *An. gambiae* DRMs (detergent resistant membranes) (Parish et al., 2011). DRMs are enriched in lipid rafts and associated proteins and provide molecular protein–protein and protein–glycan interactions between host cell and pathogen (Riethmuller, J et al. 2006). CRT is involved in many host–parasite interactions (Jaubert et al., 2002), e.g., CRT on the surface of *Pieris rapae* hemocytes mediates the phagocytosis (Asgari and Schmidt, 2003) and in wax moth *Galleria mellonella* larvae this protein is involved in early stage of encapsulation (Choi et al., 2002). CRTs have been also reported to be involved in the regulation of cell adhesion in vertebrates (Williamson et al., 1993), cell trafficking and signal transduction of the cells (Orr et al., 2003) such as the regulation of intracellular Ca^{2+} homeostasis and Ca^{2+}-dependent signal pathways (Gelebart et al., 2005).

The present study aimed to identify and characterize the nucleic acid and amino-acid sequences of CRT from *Anopheles stephensi*, the major malaria vector in Iran. We also explored the interaction between recombinant calreticulin (CRT) of *An. stephensi* and surface proteins of *Plasmodium berghei* ookinetes in order to show the binding feature of this protein to their antigens on the parasite surface. This protein might be considered as a potential vector antigen in fundamental studies and a candidate for the development of a TBV.

2. Materials and Methods

2.1. Mosquito Preparation

An. stephensi mosquitoes were obtained from the insectary of Public Health Faculty of Tehran University of Medical Science. All mosquitoes were maintained at 28 ± 2 °C and 70 ± 10% relative humidity with 12:12 hr light and dark photoperiods adjusted by fluorescent lighting. The adult mosquitoes were fed with 5% sucrose solution and the females on guinea pig blood.

2.2. Nucleic Acid Isolation and Identification of Full-length AsCrt Gene Sequence

An. stephensi mosquito was homogenized in sterile PBS 1×. Then, genomic DNA was isolated using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's protocol. In order to isolate RNA, the midgut of mosquitoes was dissected in sterile PBS 1× under a binocular. Ten midguts were pooled and RNA extraction was performed using Ambion kit (RNAqueous®-Micro Kit) according to the manufacturer's instructions. The cDNA was synthesized by the Oligodt and MMLV enzyme (Fermentas, Burlington, ON, Canada) and its integrity was checked by the amplification of 118 bp housekeeping S7 gene using the primers S7F: 5′-ATGGCTATGGTGTTCGGTTC-3′ and S7R: 5′-TGATGATCGGCCTTCTTGTTG-3′. A partial 1059 bp sequence of the *crt* gene was amplified by RT-PCR assay using the primers CRTF: 5′-GATGCCGATGCCGACAAAG-3′ and CRTR: 5′-TTACAGCTCA TCGTGWCCCTC-3′ designed corresponding to the conserved sequence of *crt* genes in *An. albimanus* (accession number: DQ206710.1) and *An. gambiae* (accession number: AF457551.1). Amplification was performed in a Corbett thermal cycler (model: CG1-96, AUS) programmed for 3 min at 95 °C for an initial denaturation step, followed by 30 cycles of 30 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C.

Transcription start site of AsCrt was identified by the 5′ RACE PCR technique using the rapid amplification of cDNA ends kit (Gibco/BRL, Rockville, MD) according to the manufacturer's instructions. Single strand cDNA was synthesized using a gene-specific antisense primer 5CRTR: 5′-TCCTTGTTGATCAGATGGTTC-3′. The cDNA was utilized for the amplification of 5′-flanking region of AsCrt gene via two rounds of PCR using gene specific reverse primers, CRTR1: 5′-CGTGCAGATCCTTC TGGTC-3′ in the first round and CRTR2: 5′-ATGTTCTGCTCGTGCTTCAC-3′ in the second round, and an adaptor supplied in the kit as forward in both rounds. The PCR amplification program included an initial denaturation step for 3 min at 95 °C, 30 s at 95 °C, 45 s at 64 °C with a 1 °C decrease in two cycles down to 55 °C, and then for 14 cycles at 55 °C, 1 min at 72 °C for extension, and finally 10 min at 72 °C.

PCR products of RACEs were cloned into T/A vector (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions