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***Plasmodium yoelii* Sporozoites Infect CD36-Deficient Mice**

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Index Descriptors

Plasmodium; cell invasion; CD36; type I thrombospondin repeats

CD36 is an 88-kDA plasma membrane glycoprotein found on platelets, monocytes, and endothelial cells. It is a type B scavenger receptor and, as is typical for scavenger receptors, it has a broad binding specificity and participates in a wide range of biological processes (reviewed in Platt and Gordon 1998; Yamada *et al.* 1998; Silverstein and Febbraio 2000). Malariologists have long been interested in CD36 because of its central role in cytoadhesion of *Plasmodium falciparum*-infected erythrocytes (Barnwell *et al.* 1989; Oquendo *et al.* 1989; reviewed in Newbold *et al.* 1999). Many investigators have hypothesized that another stage of *Plasmodium*, the sporozoite, also interacts with CD36 (Rich *et al.* 1990; Asch *et al.* 1992; Maeno *et al.* 1994). This hypothesis is based on sequence identity between the circumsporozoite protein (CS; Dame *et al.* 1984) and the thrombospondin-related anonymous protein (TRAP; Robson *et al.* 1988), two abundant sporozoite proteins that function during attachment to and invasion of cells (reviewed in Chitnis *et al.* 1999), and the CD36-ligand, thrombospondin (TSP; Lawler and Hynes 1986).

An alignment of the orthologous motifs from these three proteins is shown in Table I. In TSP, this sequence is found within the type I thrombospondin repeats (TSR; Lawler and Hynes 1986). Although both CS and TRAP have sequences with some similarity to the entire TSR, the region of greatest identity is shown in Table I. In CS this sequence is called region II-plus (Sinnis *et al.* 1994) and, for simplicity, we will use this name when referring to this sequence in TRAP and TSP also. As shown in Table I, it contains an upstream conserved tryptophan residue, followed by the amino acids CSVTCG and a string of basic and hydrophobic residues.

The region II-plus motif is found in many proteins involved in cell–cell and cell–matrix interactions (Goundis and Reid 1988). In CS, TRAP, and TSP, it mediates binding to heparin and cell surface heparan sulfate proteoglycans (Prater *et al.* 1991; Frevert *et al.* 1993; Muller *et al.* 1993; Gantt *et al.* 1997). In TSP, this sequence also binds to CD36 (Catimel *et al.* 1992;

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Leung *et al.* 1992; Li *et al.* 1993; Pearce *et al.* 1995). Experiments with peptides have demonstrated that TSP binds to CD36 via the CSVTCG portion of this motif (Asch *et al.* 1992; Catimel *et al.* 1992; Dawson *et al.* 1997). In CS, peptide experiments have shown that the downstream basic residues are required for binding to heparan sulfate proteoglycans (Sinnis *et al.* 1994), and the function of the CSVTCG portion of the motif is not yet known (Gantt *et al.* 1997).

Sporozoites enter a vertebrate host during the bloodmeal of an infected anopheline mosquito. They are injected into the skin, far from their target organ, and must go through several cell barriers before they reach their destination, a hepatocyte (Sidjanski and Vanderberg 1997; Mota *et al.* 2001; Pradel and Frevert 2001). First they must cross an endothelial cell barrier to enter the blood circulation. From here they travel to the liver sinusoids where they then cross the continuous layer of sinusoidal cells and enter hepatocytes. There is evidence that sporozoite arrest in the liver is mediated by the interaction between CS and hepatic HSPGs (Pinzon-Ortiz *et al.* 2001 and reviewed in Sinnis and Sim 1997). After their arrest, the parasite must go through either Kupffer cells, the professional phagocytes of the liver sinusoids, or endothelial cells. CD36 is expressed on both Kupffer cells and sinusoidal endothelial cells (Volpes *et al.* 1990; Maeno *et al.* 1994). The sequence identity between the cell-adhesive regions of CS and TRAP and the CD36-binding region of TSP has led us and others to postulate that sporozoites may interact with CD36 as they traverse the sinusoids. The goal of this study was to investigate whether CD36 is required for sporozoite infectivity in the vertebrate host.

Initially we tested a monoclonal antibody against CD36, mAb 8A6 (Barnwell *et al.* 1989), in an *in vitro* sporozoite invasion assay with HepG2 cells, a hepatoma cell line that expresses CD36 (data not shown; Maeno *et al.* 1994) and supports sporozoite development *in vitro* (Hollingdale *et al.* 1983). It has been previously demonstrated that mAb 8A6 inhibits the interaction between CD36 and TSP (Pearce *et al.* 1994, 1995) and we hypothesized that it might also inhibit sporozoite invasion of HepG2 cells. As shown in Table II, the antibody had no effect on sporozoite invasion, suggesting that CD36 is not involved in this process. In this assay, however, sporozoites are added directly to a monolayer of target cells. While this is not a problem if one is looking for a receptor required for hepatocyte entry, it does not reflect conditions *in vivo*, where sporozoites are released at a distance from their target cell. It could be argued, therefore, that a negative result does not rule out the possibility that CD36 is used by sporozoites as they traverse the sinusoid. In fact studies show that in the liver, CD36 is found predominantly on endothelial cells and Kupffer cells and not on hepatocytes (Volpes *et al.* 1990). The localization of CD36 *in vivo*, therefore, is more consistent with a role in sporozoite migration to, and not invasion of, hepatocytes.

To investigate the role of CD36 *in vivo*, we tested sporozoite infectivity in CD36-deficient mice. These mice were generated by homologous recombination in 129 Sv embryonic stem cells as previously described (Febbraio *et al.* 1999, 2000). No CD36 protein is present in these mice in any tissue. In addition, they have been backcrossed four times to C57Bl/6j to achieve a background homozygosity of greater than 90%. Control mice were C57Bl/6j of the same sex and age, raised in the same facility. Mice were injected with *Plasmodium yoelii* sporozoites, and 40 h later their livers were harvested, total RNA was extracted, and a

competitive RT-PCR assay was used to quantify *Plasmodium* rRNA. In this assay, a known concentration of competitor template is added to each PCR so that simultaneous amplification of parasite and competitor templates occurs. The amplification products of the competitor and parasite DNA differ in size and can be separated by gel electrophoresis and analyzed by densitometry. Results of this experiment are shown in Fig. 1. The PCRs in Figs. 1A and 1B were performed with 1 and 5 pg of competitor, respectively. When 5 pg of competitor was used the intensities of the competitor and target bands were equivalent and there were no significant differences between wild-type and CD36 null mice. Densitometry analysis of these results is plotted in Fig. 1C.

It is important to note that only sporozoites which invade and undergo many cycles of replication are detected in this assay since the small amount of ribosomal RNA present in the injected sporozoites is below the sensitivity of the assay (Briones *et al.* 1996; P. Sinnis, unpublished results). Since we saw no difference between the experimental and the control groups, our data indicate that sporozoite localization to invasion of and development in hepatocytes occur with equal efficiency in both groups.

In conclusion, CD36 is not required for infection of hepatocytes by *P. yoelii* sporozoites. Although our data suggest that CD36 is not utilized by sporozoites during their journey to the hepatocyte, we have not excluded the possibility that CD36 is part of a redundant receptor system and, when absent, the sporozoite uses other molecules to enter or traverse cells.

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FIG. 1.

Plasmodium sporozoites are infective in CD36-deficient mice. CD36 null mice and controls were injected intravenously with 4×10^4 *Plasmodium yoelii* sporozoites and 40 h later their livers were harvested and total liver RNA was isolated using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). A competitive RT-PCR assay was used to measure parasite rRNA in the livers (Briones *et al.* 1996). RTs were performed with 1 µg of total RNA and random hexamers (PE Biosystems, Branchburg, NJ). PCRs of this cDNA were performed using primers that recognize *P. yoelii*-specific sequences within the 18S rRNA, in

the presence of a competitor template as previously described (Briones *et al.* 1996). The reactions were run on an agarose gel, stained with ethidium bromide, photographed, and analyzed by densitometry. Shown are PCR products of reactions using parasite rRNA primers and (A) 1 pg or (B) 5 pg of parasite rRNA competitor. The competitor is 333 bp and the target DNA is 393 bp. Molecular size markers (M); bp: 1000, 750, 500, 300, 150, and 50. (C) The photograph in B was scanned and analyzed by densitometry using the Gel plotting macro of the program NIH IMAGE (<http://rsb.info.nih.gov/nih-image>). For each amplification reaction a target/competitor ratio was calculated and this ratio was used to determine the amount of parasite RNA per microgram of liver RNA. The mean for each group of mice is plotted with error bars showing the range of values calculated. This experiment was repeated with seven mice per group with identical results.

TABLE I

Alignment of Region II-plus Motifs from TSP, CS, and TRAP

Protein	Sequence ^a
Thrombospondin	P <u>W</u> SSCSVTCGDG <i>VITRIR</i>
<i>P. falciparum</i> CS Protein	E <u>W</u> SPCSVTCGNGI <i>QVRIK</i>
<i>P. yoelii</i> CS Protein	E <u>W</u> SQCSVTCGSG <i>VRVRKR</i>
<i>P. falciparum</i> TRAP	E <u>W</u> SPCSVTCGKGTR <i>SRKR</i>

^aThe conserved tryptophan is underlined, CSVTCG is shown in boldface, and the downstream basic and hydrophobic residues are in italics. The amino acid sequences derive from the following sources: TSP (Lawler and Hynes 1986), *P. falciparum* CS (Dame *et al.* 1984), *P. yoelii* CS (Lal *et al.* 1987), and *P. falciparum* TRAP (Robson *et al.* 1988).

TABLE II

Monoclonal Antibody to CD36 Does Not Inhibit *Plasmodium* Sporozoite Invasion of HepG2 Cells

Expt. ^a	Treatment	Number of EEFs ^b
1	Control	249 ± 4
	MAb 8A6	238 ± 13
2	Control	714 ± 53
	Mab 8A6	620 ± 22

^aExperiments 1 and 2 were performed with HepG2 cells (ATCC HB8065; Rockville, MD), plated in DMEM with 10% fetal calf serum in labtek chamber slides, and allowed to grow overnight. *Plasmodium berghei* sporozoites in medium with or without 100 µg/ml mAb 8A6 were added to HepG2 cells. After 2 h at 37°C, unattached sporozoites were removed, fresh medium was added, and 2 days later cells were fixed and exoerythrocytic forms (EEFs) were visualized with mAb 2E6 (directed against the liver stage of *P. berghei*) and goat anti-mouse Ig conjugated to horseradish peroxidase.

^bNumber of EEFs per 20 fields. Each point was plated in triplicate and shown are the means with standard deviations.