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Two alleles of the 175-kilodalton *Plasmodium falciparum* erythrocyte binding antigen

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EBA-175, erythrocyte binding antigen 175, is a 175-kDa antigen of *Plasmodium falciparum* which has been shown to be involved in the recognition of erythrocytes by merozoites and may be involved in the process of erythrocyte invasion. Invasion of erythrocytes by Camp strain merozoites is inhibited by pre-treatment of red blood cells by EBA-175 from the heterologous strain, FCR-3. The sequence of the Camp strain has been published and we report here the sequence of the FCR-3 strain. The sequences are nearly identical except for a 423-bp segment in the FCR-3 strain, F-segment, that is not found in the Camp strain and a 342-bp segment, C-segment, present in the Camp strain but not in the FCR-3 strain. The locations of these two segments are different in Camp and FCR-3 EBA-175 genes and there is little DNA or amino acid sequence homology between them. The essentially dimorphic alleles, F-segment and C-segment, are conserved in all isolates examined to date. Evidence of genetic cross-over between the FCR-3 and the Camp EBA-175 genes was not observed in the analysis of a limited number of wild isolates. The continued study of the biological relevance of these sequence divergences in EBA-175 may further elucidate the sequence of events resulting in merozoite invasion of erythrocytes.

Key words: *Plasmodium falciparum*; Erythrocyte binding antigen; Allele

Introduction

Invasion of erythrocytes by *Plasmodium falciparum* merozoites is a multi-step process involving numerous ligands and receptors [1–8]. One merozoite protein involved in initial erythrocyte binding is a 175-kDa antigen,

EBA-175 [9,10]. This microneme associated protein is released from rupturing schizont-infected erythrocytes [11] and preferentially binds the Neu5Ac(α2,3)-Gal-determinant on the O-linked tetrasaccharide of glycophorin A [12–14].

EBA-175 binding and *P. falciparum* invasion are correlated. For example, EBA-175 binds human, *Aotus* monkey [9] and mouse erythrocytes [15] and *P. falciparum* merozoites invade these cells. However, EBA-175 binds erythrocytes with modified glycophorin less efficiently [16], and these erythrocytes have reduced invasion efficiencies [8].

The gene encoding the Camp strain EBA-175 protein has been cloned and sequenced [10]. Contained in the carboxy-terminus of the 1435 amino acid (aa) Camp EBA-175 is a 42-

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Abbreviations: EBA-175, 175-kDa erythrocyte binding antigen; PCR, polymerase chain reaction; aa, amino acid.

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession number L07755.

aa peptide, named EBA-peptide 4. Antibodies against this region inhibit the binding of Camp EBA-175 to erythrocytes and inhibit homologous parasite invasion of erythrocytes in vitro [10]. The receptor-ligand interactions between erythrocytes and merozoites may be strain dependent. Merozoites from *P. falciparum* FCR-3 or Camp strains show decreased invasion of erythrocytes previously treated with *P. falciparum* EBA-175 from the heterologous strain [9]. Because of the observation that FCR-3 strain EBA-175 inhibits Camp strain merozoite invasion and an observed restriction fragment length polymorphism of the EBA-175 gene between FCR-3 and Camp DNA (B.K.L. Sim, unpublished observation), the cloning and sequencing of the FCR-3 strain EBA-175 was undertaken.

Materials and Methods

Gene isolation. Genomic DNA was isolated from cloned FCR-3 strain parasites [17], digested with *Xba*I and fractionated on a sucrose gradient. Samples of the gradient fractions were probed with a nick-translated 566-bp fragment from Camp DNA (bp 527–1093). Positive fractions were pooled, purified over an Elutip-D column, (Scheicher and Schuell) ligated into Bluescript SK+ (Stratagene Cloning Systems) and transformed into DH5 α cells (Gibco-BRL). Transformed colonies were screened with a ³²P-end-labeled oligonucleotide (bp 4295–4321) from the carboxy-terminus of Camp EBA-175. One colony containing a 3988-bp fragment was grown and sequenced in both directions (Sequenase; U.S. Biochemical Corp.). In addition, sucrose gradient fractions of the FCR-3 DNA, were screened with a nick-translated 423-bp fragment which included the EBA-175 start codon of the Camp strain. Positive fractions were used as templates in a polymerase chain reaction (PCR) using 50 pmol of a sense primer (bp 1–18 of the Camp EBA-175 sequence) and an antisense primer (bp 527–552 of the Camp EBA-175 sequence). This fragment was cloned and multiple clones

were sequenced in both directions in order to insure that there were no PCR incorporation errors.

Patient samples. In order to investigate the extent of heterogeneity within EBA-175 in natural isolates, patient samples were obtained from Africa and South East Asia. Cloned cultured isolates were also analyzed. All samples were collected and analyzed for the presence of F-segment and C-segment according to previously described methods [18]. Ten patient samples were also amplified using a sense oligonucleotide, KK-30 (bp 2275–2295), and an antisense oligonucleotide, Sub 2 (bp 3539–3555), from homologous regions flanking the two allelic regions in the FCR-3 and Camp sequences. The amplified products were probed with F-segment, C-segment or both as previously described [18].

Results

The FCR-3 EBA-175 gene contains 1462 aa, which is 27 aa larger than the Camp strain gene (1435 aa). The majority of the polymorphism between the FCR-3 and the CAMP strain EBA-175 genes comes from a 423-bp (141 aa) segment, named the F-segment allelic region, which is not present in the Camp strain DNA and a 342-bp (114 aa) sequence, named the C-segment allelic region, which is not observed in the FCR-3 strain DNA. The F-segment begins at aa 777 of the FCR-3 strain and the C-segment begins at aa 868 of the Camp strain. The amino acid sequence of the F-segment and the C-segment are given in Fig. 1. Outside these two regions, there are 7 single base changes, 6 of which result in aa changes from the Camp gene (FCR-3 aa: 478-K, 481-K, 584-Q, 664-R, 932-K, 1058-V; Camp aa: 478-N, 481-I, 584-K, 664-S, 791-E, 1031-E).

The PCR analysis of cultured isolates, using primers and probes specific for C-segment and F-segment [18] showed that one of 8 clones (12.5%) is positive for the F-segment allelic region and 7 of 8 (87.5%) are positive for the C-segment allelic region. However, of the 26

F	768	TEIAHRTETRTDERKNQEPANKDLKNPQQSVGENGTKDLLQEDLGGSRSSEDEVTEQFGVNHGIPKGEDQTLGKSDAIPNIGEPETGISTTEESRHE
C	768	TEIAHRTET-----
F	864	EGHNKQALSTSVDEPELSDTLQLHEDTKENDKLPLESSTITSPTESGSSDTEETPSISEGPKGNEQKKRDDDLSKISVSPENSRPETDAKDTSNL
C	777	-----PSISEGPKGNEQKKRDDDLSKISVSPENSRPETDAKDTSNL
F	960	LKLKGDVDISMPKAVIGSSPNDNINVTEQGDNISGVNSKPLSDDVRPDK-----
C	819	LKLKGDVDISMPKAVIGSSPNDNINVTEQGDNISGVNSKPLSDDVRPDKKELEDQNSDESVRTQDTANTEDVIRKENADKDEDEKGADEERHSTSE
F	1009	-----NHEEV
C	916	EETVVNHISKSPSINNGDDSGSGSATVSESSSNTGLSIDDDRNGDTFSLSSPEEKMLTDNEGNSLNHEEV

Fig. 1. Amino acid sequence of the FCR-3 strain (F) and the Camp strain (C) beginning at aa 768. At FCR-3 aa 777 there is a 141-aa sequence, F-segment, that is not present in the Camp strain. After this region the two sequences are homologous until aa 1009 of the FCR-3 strain where the Camp strain diverges with a 114-aa sequence, C-segment. After this divergence the two sequences are nearly identical until the stop codon. (—) represents areas not present in the heterologous strain.

wild isolates analyzed, 10 isolates (38%) produced a single amplified DNA fragment corresponding to the F-segment allelic regions, 3 isolates (12%) produced a fragment corresponding to the C-segment allelic region, and 13 isolates (50%) contained 2 DNA fragments corresponding to the F-segment and C-segment allelic regions indicating a mixed infec-

tion (Fig. 2). Ten mixed infection isolates were analyzed with oligonucleotides KK-30 and Sub 2 which flank the two allelic forms. This would have produced a 1623-bp fragment if both forms had been present or a 860-bp fragment if neither allelic form had been present. None of the isolates produced a fragment of either length (data not shown).

Fig. 2. Southern blot analysis of cloned (A and B) and wild (C and D) isolates of *P. falciparum*. (A) and (C) were probed with an FCR-3 specific oligonucleotide (16); (B) and (D) with a Camp specific oligonucleotide (DH1) [18]. (A and B) Lane 1, 3D7; lane 2, Camp; lane 3, 7G8; lane 4, Thai Tn; lane 5, Honduras; lane 6, IMTM; lane 7, Brazil; lane 8, KD7; lane 9, negative control. (C and D) Patient isolates: lanes 1,3,4,6,7 and 8 contain mixed infections; lane 2 is positive for a Camp-like allele of EBA-175; lane 5 is positive for an FCR-3-like allele of EBA-175; lane 9 is a negative control.

Discussion

The EBA-175 gene is one of a few genes whose protein product has been correlated with a biological function, namely erythrocyte invasion. Before the gene was cloned, Camus and Hadley noticed a difference in the ability of FCR-3 and Camp merozoites to invade erythrocytes that had been pre-treated with purified EBA-175 from the heterologous strain [9]. This observation prompted a search for differences between the two proteins. Once the gene for the Camp strain was isolated and sequenced, the investigation of the FCR-3 gene was undertaken to identify regions of the gene which could account for the functional differences observed.

The genes from the FCR-3 and the Camp clones are highly conserved and outside the two major regions of divergence, F-segment and C-segment, there are only 7 single base changes which are widely spaced and most likely arose from unrelated genetic mutations occurring during gene replication. However, the two blocks of aa constituting the F-segment and C-segment regions are major elements which appear to have moved into or

out of the gene as discrete units.

Interestingly, the F-segment region DNA is flanked by a 7-bp repeat, GAAACTC. This 7-bp sequence occurs once in the Camp EBA-175 gene, exactly at the position that the F-segment region would be inserted if a genetic crossover had occurred. If this was to occur, and the resulting gene product was not detrimental to the parasite, one would expect to find an isolate that contained both F-segment and C-segment regions. Analysis of a limited number of cultured and wild isolates failed to detect any EBA-175 gene containing both F-segment and C-segment within a single EBA molecule. It is possible that the presence of both F-segment and C-segment together might disrupt the conformation of a functional domain responsible for erythrocyte binding. That domain may reside near the previously described peptide 4 region [10], which may be important for recognition of sialic acid residues on the erythrocyte.

It is also possible that the regions F-segment and C-segment themselves have a function involved in erythrocyte attachment by merozoites. The two sequences have a 56-aa overlap between the end of F-segment and the beginning of C-segment, beginning at aa 869 of the FCR-3 strain. Experiments to examine the function of the F-segment and the C-segment have shown that when these two regions are synthesized in vitro they bind to erythrocytes in a sialic acid independent manner (Kain, manuscript submitted). Together with the data that the peptide 4 region is putatively involved in the sialic acid dependent binding of EBA-175 and that F-segment or C-segment may participate in a second binding domain within the same protein, the molecular basis of merozoite invasion becomes even more interesting. The conservation of function in these two allelic forms emphasizes the importance of the EBA-175 molecule to parasite survival and therefore the importance of EBA-175 as a malarial vaccine candidate.

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