

# Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum*

(malaria/cytoadherence/antigenic variation)

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**ABSTRACT** Parasite-derived proteins expressed on the surface of erythrocytes infected with *Plasmodium falciparum* are important virulence factors, since they mediate binding of infected cells to diverse receptors on vascular endothelium and are targets of a protective immune response. They are difficult to study because they undergo rapid clonal antigenic variation *in vitro*, which precludes the derivation of phenotypically homogeneous cultures. Here we have utilized sequence-specific proteases to dissect the role of defined antigenic variants in binding to particular receptors. By selection of protease-resistant subpopulations of parasites on defined receptors we (i) confirm the high rate of antigenic variation *in vitro*; (ii) demonstrate that a single infected erythrocyte can bind to intercellular adhesion molecule 1, CD36, and thrombospondin; (iii) show that binding to intercellular adhesion molecule 1 and CD36 are functions of the variant antigen; and (iv) suggest that binding to thrombospondin may be mediated by other components of the infected erythrocyte surface.

*Plasmodium falciparum* modifies the surface of its host erythrocyte to express ligands for endothelial cell receptors. This leads to sequestration of parasitized erythrocytes (PRBCs) in the microvasculature, and the available evidence suggests that organ dysfunction and severe pathology follow the accumulation of PRBCs at high density in particular organs (1, 2). Most field isolates can adhere via CD36 and thrombospondin (TSP), and a significant proportion can bind to intercellular adhesion molecule 1 (ICAM-1) (3–6). Binding to vascular cell adhesion molecule, E-selectin, and chondroitin 4-sulfate has also been reported (7, 8).

When ligands for endothelial cell receptors are expressed, parasite-specific antigens are also detected at the erythrocyte surface. In *P. falciparum* and a number of animal malaras, these antigens undergo clonal variation at a rate as high as 2% per generation (9–12); they are the target of host protective immune responses and are essential for the maintenance of chronic infection (10, 13, 14). Studies have suggested a single molecule, termed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expresses variant-specific epitopes and also mediates adhesion to endothelium (10–12, 15–17). The gene for this molecule has recently been cloned, but functional expression of the putative adhesive domains has not been reported (18, 19, 33), and so the evidence placing variant epitopes and endothelial ligands on a single molecule is to date indirect (12, 20–26).

Further progress in this area has been hampered by the high rate of variation, which means that even clonal cultures are extremely heterogeneous phenotypically. This heterogeneity combined with the existence of multiple endothelial receptors makes analysis of the system extremely complex.

To explore the relationship between particular variant antigenic types (VATs) and adhesion to particular receptors, we

decided to reexamine the protease sensitivity of the antigenic and adhesive phenotypes in a series of parasite variants. We reasoned that distinct VATs should be differentially sensitive to specific proteases because of their different primary sequences. We discovered that protease-resistant binding to CD36 and ICAM-1 occurred in a variant-specific fashion, whereas binding to TSP was a constant property of PRBCs. These observations, combined with surface-labeling data, strongly support the notion that variant antigens, PfEMP1 molecules, mediate PRBC adhesion to CD36 and ICAM-1 but suggest that an invariant molecule may mediate adhesion to TSP.

## MATERIALS AND METHODS

**Parasites.** The derivation and growth of A4 and its subclones have been described (12). Here we have used i+ and i- as superscripts to indicate ICAM-1 binding clones and ICAM-1 nonbinding clones, respectively. The subscripts <sub>tryp-ICAM</sub> and <sub>V8-CD36</sub> denote parasites treated with the relevant protease (trypsin or V8 protease) and then selected on the receptor stated. The VAT of a clone is referred to by the original nomenclature for that clone (e.g., A4VAT). All parasites that expressed the A4VAT could bind to ICAM-1 (e.g., C9<sup>i+</sup> and C28<sub>ICAM</sub>), but C clones that expressed alternative VATs bound to ICAM-1 at a significantly lower level (e.g., C18<sup>i-</sup>). The line IT6 had been derived elsewhere by selection of ItG-2F6 on purified ICAM-1 (23).

**Protease Digestion and Inhibition.** PRBCs were digested with V8 protease (10 units/ml; Boehringer Mannheim) at pH 7.8 for 5 min and inhibited by 2.5 mM diisopropyl fluorophosphate or digested with chymotrypsin, dispase, or L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin [1 mg (10 units)/ml; Sigma] at pH 7.2 for 5 min and inhibited by the addition of 0.1 mM TPCK, 0.5 mM EDTA, or soybean trypsin inhibitor (2 mg/ml), respectively. Treatment of cells with RPMI 1640 medium and the inhibitors in control experiments affected neither cytoadherence nor agglutination (data not shown).

**Sera and Agglutination Assays.** Serum pools were made from immune Gambian adults and healthy Europeans never exposed to malaria (25). Agglutination experiments were performed as described (12). Here 1000 PRBCs were counted in duplicate experiments. The number of agglutinates in defined size categories (3–5, 6–10, 11–20, >20 cells per agglutinate) was scored to show the size distribution of agglutinates in the sample.

The relative antigenic phenotype of a culture was determined by the mixed agglutination assay (25). The rates of agglutination of each pair of PRBCs tested were equalized in

Abbreviations: ICAM-1, intercellular adhesion molecule 1; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; PRBC, parasitized erythrocyte; TSP, thrombospondin; VAT, variant antigenic type.

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preliminary experiments. One hundred agglutinates of 5–10 cells were counted in at least two separate experiments for each pair of samples tested.

**Adhesion Assays and Selection of Parasites for Adherent Phenotype.** Adhesion of PRBCs to purified proteins was measured as described using triplicate spots of CD36 (12.5 ng/ml), ICAM-1 (25  $\mu$ g/ml), and TSP (40  $\mu$ g/ml) in plastic dishes (12). PRBCs were used at 1–2% hematocrit and 2–10% parasitemia in duplicate experiments. Results are expressed as the number of PRBCs bound per mm<sup>2</sup> of surface area and normalized to 1.0% hematocrit and 1.0% parasitemia.

Reversal of adhesion by human sera was investigated using a modified version of a published method (21). Briefly PRBCs were resuspended in identical dishes and rocked in a hybridization machine (Genetic Research Instrumentation) for 15 min before unbound cells were removed. Immune or control serum (1:8 in binding medium) was then added, and the dishes were rocked for a further 15 min. The nonadherent cells were removed by four gentle washes in binding medium, and bound cells were counted as above.

For selection of parasites, purified trophozoites (>90% parasitemia) were added to plastic dishes coated with the above concentration of receptor as described (12). The unbound parasites were gently washed off with binding medium, and the adherent parasites were removed and cultured as above.

**Measurement of Stage Specificity of Surface Phenotypes.** Large numbers (>10<sup>10</sup>) of PRBCs from A4<sup>+</sup> were synchronized using sorbitol lysis and Plasmagel flotation in four successive cycles of growth (12). In the last two cycles, sorbitol was applied exactly 1 hr after the first appearance of bursting schizonts to give 1-hr synchrony. At specific times, samples were tested for agglutination or receptor binding as described (12), but each assay was abbreviated to a 15-min incubation.

**Identification of Radioiodinated Proteins at the PRBC Surface.** Cultures were sorbitol-synchronized and immediately treated with Plasmagel to remove mature trophozoites and debris. Trophozoites were enriched 24 hr later using Plasmagel. Control, uninfected erythrocytes were treated identically. Purified trophozoites were returned to culture for 1 hr to allow recovery.

PRBCs (2.0–4.0  $\times$  10<sup>8</sup>) were washed twice in RPMI medium, washed twice in phosphate-buffered saline (PBS), and labeled by a modification of the lactoperoxidase/<sup>125</sup>I method (15). Cells were equilibrated in 0.5 ml of PBS containing 0.1  $\mu$ M potassium iodide for 2 min; then 1.2 units of lactoperoxidase (Sigma) and 0.5 mCi (1 Ci = 37 GBq) of Na<sup>125</sup>I (Amersham) were added. Six microliters of freshly prepared 0.03% (vol/vol) hydrogen peroxide was added initially, and the contents were mixed vigorously to initiate the reaction. Three microliters of 0.03% hydrogen peroxide was added each minute for 5 min with thorough mixing. The reaction was quenched by PBS/50  $\mu$ M sodium thiosulfate after 6 min, and the suspension was transferred to new tubes. The cells were washed twice in PBS/10 mM potassium iodide and extracted with detergent after protease or control treatment (15). Sam-

ples were precleared with protein A-Sepharose and immunoprecipitated using standard procedures.

## RESULTS

**Effect of Proteases on Agglutination.** A number of proteases were screened for their effect on the agglutination of PRBCs from a single clone (A4<sup>+</sup>). Chymotrypsin, dispase, and trypsin all dramatically reduced the number of PRBCs in agglutinates. With V8 protease, agglutinates still formed after treatment, and their mean size was increased. This effect was observed at high concentrations (40 units/ml) of V8 protease and after long digestion times (30 min), which indicated that a limit digestion had occurred (data not shown).

The effect of the same proteases on agglutination of a number of subclones of A4<sup>+</sup> was then investigated. Two observations provided a basis for further phenotypic examination. First, PRBCs from C9<sup>+</sup> (an identical VAT to A4<sup>+</sup>) and C18<sup>-</sup> (a different VAT from A4<sup>+</sup>) showed control and enhanced levels of agglutination, respectively, after treatment with V8 protease (Table 1). The size of the resistant agglutinates was increased in both parasites (data not shown). Agglutination of all other parasites tested was completely abolished by the same treatment. Second, agglutination of A4<sup>+</sup> and C9<sup>+</sup> was partially resistant to trypsin, whereas agglutination of all other parasites was completely sensitive (Table 1). Chymotrypsin and dispase completely abolished agglutination of all PRBCs tested (data not shown).

**Effect of Proteases on Adhesion to Individual Receptors.** The effect of V8 protease and trypsin on binding to purified CD36, ICAM-1, and TSP was then examined. PRBCs from A4<sup>+</sup> and C9<sup>+</sup> bound to CD36 at  $\approx$ 20% of control levels after digestion with V8 protease, whereas PRBCs from C18<sup>-</sup> bound to CD36 at control levels after digestion (Table 1). V8 protease completely ablated binding to CD36 in all other parasites. Hence, the effect of V8 protease on binding to CD36 correlated with its effect on agglutination because resistant phenotypes were observed only in PRBCs from A4<sup>+</sup>, C9<sup>+</sup>, and C18<sup>-</sup>. Trypsin abolished binding to CD36 in all cases.

Only a subset of parasite clones bound to ICAM-1 and this property was universally sensitive to V8 protease (Table 1). Trypsin digestion caused either a slight reduction in ICAM-1 binding of PRBCs from A4<sup>+</sup> and C9<sup>+</sup> or completely destroyed adhesion if PRBCs from C28<sub>ICAM</sub> were examined.

In contrast, the effects of V8 protease and trypsin on binding to TSP were independent of the line used and showed partial resistance in all cases (Table 1). The mean levels of resistant binding were 86.8%  $\pm$  10.7% for V8 protease and 21.8%  $\pm$  3.5% for trypsin.

Thus the effect of specific proteases on the binding of PRBCs to CD36 and ICAM-1 was variant specific, but their effect on adhesion to TSP was independent of antigenic type. Protease-resistant adhesion in some variants could have two possible explanations. The modification of the whole surface by proteases could alter the receptor–ligand interaction

Table 1. Effect of V8 protease and trypsin on agglutination and receptor binding of various parasite clones

Parasite clone	V8 protease				Trypsin			
	Agglut.	CD36	ICAM-1	TSP	Agglut.	CD36	ICAM-1	TSP
A4	102 $\pm$ 6	23.0 $\pm$ 6.3	2.3 $\pm$ 1.1	89.3 $\pm$ 14.1	25 $\pm$ 3.2	0.1 $\pm$ 0.5	70.4 $\pm$ 8.2	25.2 $\pm$ 4.1
C9	95.4 $\pm$ 8.8	23.1 $\pm$ 7	2.5 $\pm$ 0.5	80.8 $\pm$ 18.5	10.2 $\pm$ 1.3	0.4 $\pm$ 1	87.5 $\pm$ 16	23.1 $\pm$ 5
C18	171 $\pm$ 18.8	99 $\pm$ 5.2	ND	91.4 $\pm$ 18.5	0 $\pm$ 0.7	0.2 $\pm$ 1.2	ND	21.5 $\pm$ 3.2
C24	0.3 $\pm$ 1.2	1 $\pm$ 0.8	ND	106 $\pm$ 10.3	0 $\pm$ 0.8	0.2 $\pm$ 0.4	ND	19.8 $\pm$ 4.4
C28	0.20 $\pm$ 0.9	2 $\pm$ 0.9	ND	75 $\pm$ 20.9	0 $\pm$ 0.9	0.3 $\pm$ 1.7	ND	26 $\pm$ 7.6
C28 <sub>ICAM</sub>	0 $\pm$ 0.8	2.4 $\pm$ 1.7	1 $\pm$ 0.7	ND	2 $\pm$ 1.7	0.5 $\pm$ 1	5.2 $\pm$ 2.7	ND

Binding was measured to CD36, ICAM-1, and TSP as described in *Materials and Methods*, and agglutination was measured in immune serum at a 1:8 dilution in RPMI. Results are the mean percentage  $\pm$  SEM of undigested control binding to each receptor from at least two separate experiments. ND, not done. Agglutination (Agglut.) is expressed as the percentage of a mock protease-treated control culture.

through nonspecific mechanisms such as surface charge. Alternatively, the phenotypic heterogeneity of the clones caused by rapid antigenic variation may have resulted in the presence of protease-resistant, antigenically variant subpopulations within the cultures. These hypotheses were tested by ligand-specific selection of the protease-resistant cells.

**Analysis of PRBCs Selected on Purified Receptors After Protease Digestion.** A4<sup>i+</sup> was selected on CD36 after digestion of mature stages with V8 protease. The resulting population (A4<sub>V8-CD36</sub>) was grown for three cycles and bound strongly to CD36 and TSP, but not to ICAM-1, and formed agglutinates in immune serum, but not in control serum (Table 2). These properties were resistant to V8 protease but were completely sensitive to trypsin.

The mixed agglutination assay was used to characterize the antigenic relationship of A4<sub>V8-CD36</sub> to its parent, A4<sup>i+</sup>, and to C18<sup>i-</sup>. A4<sub>V8-CD36</sub> showed 12% mixed agglutination with A4<sup>i+</sup> and 72% with C18<sup>i-</sup> (data not shown). Therefore, it appeared that parasites of the A4<sub>V8-CD36</sub> (and hence C18<sup>i-</sup>) phenotype represented the V8 protease-resistant subpopulation within A4<sup>i+</sup>. To confirm that this population had not arisen by chance during growth after selection, PRBCs were used in the mixed agglutination assay immediately after protease treatment. V8 protease-digested PRBCs from both A4<sub>V8-CD36</sub> and C18<sup>i-</sup> (as well as A4<sup>i+</sup>) expressed the C18VAT, which was therefore unchanged by digestion with this protease (data not shown). Thus, V8 protease-resistant agglutination and adhesion to CD36 was restricted to a single VAT in this clone family.

Similar experiments were carried out with A4<sup>i+</sup> and C9<sup>i+</sup>, which exhibited trypsin-resistant binding to ICAM-1. The results obtained with adhesion to CD36 suggested that the most likely explanation for this observation was the presence of an antigenically distinct subpopulation within these lines. To investigate this hypothesis, PRBCs from A4<sup>i+</sup> were selected twice on purified ICAM-1 after trypsin digestion. This line (designated A4<sub>tryp-ICAM</sub>) formed trypsin-resistant agglutinates in immune serum, whereas V8 protease treatment abolished agglutination. Undigested PRBCs from A4<sub>tryp-ICAM</sub> bound to CD36, ICAM-1, and TSP. After digestion with trypsin, ICAM-1 binding was increased 4- to 5-fold, binding to CD36 was abolished, and, as for all other parasites, binding to TSP was reduced (Fig. 1). The mixed agglutination test showed that A4<sub>tryp-ICAM</sub> was antigenically distinct from A4<sup>i+</sup> and that trypsinization produced a novel antigenic phenotype in both cultures (data not shown).

To investigate the apparent independence of TSP binding from other surface properties, PRBCs from IT6 (23) were selected on TSP after digestion with either trypsin (IT6<sub>tryp-TSP</sub>) or V8 protease (IT6<sub>V8-TSP</sub>). After selection and further growth, the antigenic phenotype and protease sensitivity of adhesion of these cells were indistinguishable from the unselected parental line [20.5% ± 6.8% of control TSP binding after trypsin digestion (*n* = 2) and 73.5% ± 12.4% after V8 protease (*n* = 2)]. To show that no changes had occurred during growth after selection, PRBCs from C24<sup>i-</sup> were selected directly on TSP, and the antigenic phenotype of the resulting population

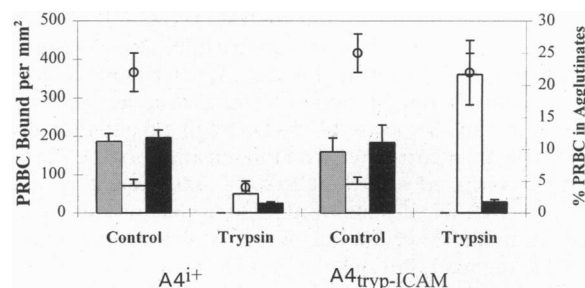


FIG. 1. Effect of trypsin on binding and agglutination. Cells were treated with RPMI medium or trypsin and divided for analysis in a spot binding assay, a standard agglutination assay, and a mixed agglutination assay. Results are the mean values ± SEM from at least three experiments. ○, Agglutination; shaded bars, CD36; open bars, ICAM-1; solid bars, TSP.

(C24<sub>TSP</sub>) was investigated upon removal from the TSP-coated dish. The mixed agglutination test demonstrated that C24<sub>TSP</sub> and C24<sup>i-</sup> were antigenically indistinguishable. Furthermore, PRBCs from C24<sub>TSP</sub> and mock-selected PRBCs from C24<sup>i-</sup> bound at similar levels to CD36 and TSP (data not shown).

These data suggested that binding to TSP was a constant property of all PRBCs and that the partial effects of proteases could best be explained by their effects on the RBC glycocalyx (for instance by changing surface charge or protein topography) rather than their effects on parasite-derived ligands. To test this hypothesis, parasites were treated with proteases at ring stage (before they had become adherent) and returned to culture until they became adherent trophozoites. Binding of these cells to TSP before and after protease digestion was compared to binding of cells that had been mock treated earlier in the cell cycle. All PRBCs exhibited the same adhesion characteristics observed previously (data not shown). Thus the partial protease resistance of the adherence to TSP was a result of parasite-induced changes.

**Surface Molecules.** The only differences between the surface-labeled patterns of proteins in different VATs occurred in molecules that fulfilled the characteristics of PfEMP1. PRBCs expressing the A4VAT expressed one major and several minor members of the PfEMP1 family between 225 and 290 kDa, whereas PRBCs from C18<sup>i-</sup> expressed a single major 225-kDa PfEMP1; a 240-kDa equivalent was identified in PRBCs from C28<sup>i-</sup> (Fig. 2A). Surface labeling of A4<sub>V8-CD36</sub> showed a band with an identical molecular mass to that of C18<sup>i-</sup>, thus confirming the conclusion from phenotypic observations that these two parasites were indistinguishable (Fig. 2A).

All PfEMP1 molecules in this clone family were sensitive to trypsin and V8 protease because the major high molecular mass band(s) disappeared completely after digestion. However, a single protein of 175 kDa with the characteristics of PfEMP1 was observed after V8 digestion for the V8 protease-resistant parasites C18<sup>i-</sup> and A4<sub>V8-CD36</sub>. A similar band was present at low levels in parasites of the A4VAT (e.g., C9) but

Table 2. Effect of trypsin and V8 protease on agglutination and binding of A4<sub>V8-CD36</sub>

Treatment	Agglutination,* % PRBC in agglutinates		Binding,† cells per mm <sup>2</sup>		
	Immune serum	Control serum	CD36	TSP	ICAM-1
None (control)	12.4 ± 4.2	0.2 ± 0.2	364.6 ± 55.6	189.7 ± 25.4	10.0 ± 2.4
Trypsin	0	0	0	32.1 ± 6.8	0
V8 protease	16.7 ± 3.6	0	361.7 ± 25.8	174.7 ± 16.4	0.5 ± 0.2

Mature stage cells were removed from culture and treated with RPMI (control) or protease as described in *Materials and Methods*. After inhibition of enzymes, the PRBCs were divided into aliquots for use in agglutination assays or spot binding assays.

\*Results are mean values ± SEM from duplicate determinations in two separate experiments.

†Binding data are mean values ± SEM from triplicate determinations in duplicate dishes in two experiments and were corrected to 1.0% parasitemia and 1.0% hematocrit.

was absent from the other parasites tested (Fig. 2A). A 50-kDa fragment was observed in digestion supernatants after V8 proteolysis of the same lines (Fig. 2B). When labeled cells were digested with a serial dilution of V8 protease, the appearance of the membrane-associated 175-kDa fragment and the soluble 50-kDa fragment correlated with the sensitivity of the 225-kDa PfEMP1 associated with the C18VAT (data not shown). The PfEMP1s from all variants were immunoprecipitated by antibodies in immune serum, but not by control serum, and the 175-kDa fragment, but not the 50-kDa supernatant polypeptide, was immunoprecipitated from C18<sup>i-</sup> by the same serum (data not shown).

Band 3 was surface labeled by <sup>125</sup>I and migrated as a characteristic trypsin-resistant smear of ≈85 kDa in all PRBCs. It was sensitive to V8 protease, and a surface-labeled smear of ≈65 kDa was immunoprecipitated by monoclonal antibodies to band 3 in all digested PRBCs (data not shown).

Binding to ICAM-1 was generally associated with higher molecular mass forms of the variant antigen: C28<sup>i-</sup> expressed a band of 240 kDa, but after selection on ICAM-1 to produce

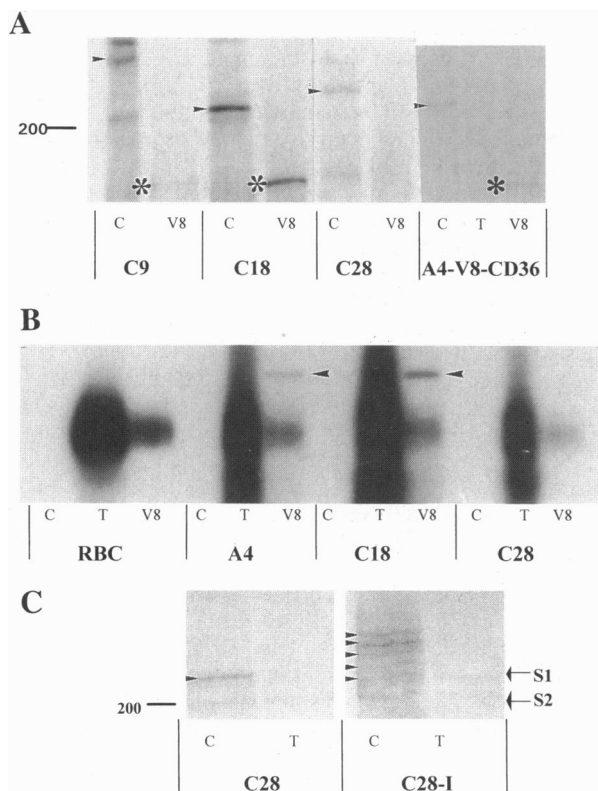


FIG. 2. (A) Protease-sensitive <sup>125</sup>I-labeled proteins. PRBCs were labeled by the lactoperoxidase technique and treated with either RPMI medium (C), trypsin (T), or V8 protease (V8) before separation on an SDS/5% polyacrylamide gel. Triton-insoluble extracts from equal numbers of PRBCs are shown from C9<sup>i+</sup>, C18<sup>i-</sup>, C28<sup>i-</sup>, and A4<sub>V8-CD36</sub>. PfEMP1 molecules are designated with arrowheads, and a 175-kDa protein produced after V8 protease digestion is denoted by an asterisk. A molecular size marker (200 kDa) is shown on the left. (B) <sup>125</sup>I-labeled proteins released after proteolysis. Supernatants from radioiodinated cells were separated on an SDS/10% polyacrylamide gel. Samples were from equivalent numbers of uninfected erythrocytes (RBC) and PRBCs from A4<sup>i+</sup>, C18<sup>i-</sup>, and C28<sup>i-</sup>. The 50-kDa bands in A4<sup>i+</sup> and C18<sup>i-</sup> are indicated by arrowheads. (C) Changes in PfEMP1 expression after ICAM-1 selection. Equal numbers of radio-labeled PRBCs from C28<sup>i-</sup> (C28) and C28<sub>ICAM</sub> (C28-I) were treated with either RPMI (C) or trypsin (T). Triton-insoluble pellets were prepared and separated on a 5% polyacrylamide gel. PfEMP1 molecules are indicated by arrowheads, and the spectrin doublet (S1, S2) is due to internal labeling of a small number of cells. The 200-kDa size marker is shown on the left.

C28<sub>ICAM</sub>, multiple higher molecular mass bands were evident (Fig. 2C). A4<sub>tryp-ICAM</sub>, selected from A4<sup>i+</sup> on ICAM-1 after trypsin digestion, showed two protease-sensitive proteins with the characteristics of PfEMP1. The 270-kDa band was present at a higher level in A4<sub>tryp-ICAM</sub> than in A4<sup>i+</sup>, and the 190-kDa PfEMP1 was unique for A4<sub>tryp-ICAM</sub>.

Unlike the V8 protease-resistant C18VAT, no large labeled protease-resistant fragment was uniquely associated with A4<sub>tryp-ICAM</sub> after trypsin treatment. PRBCs were also labeled after trypsinization to identify the trypsin-resistant receptor for ICAM-1, but no such high molecular mass proteins were seen.

**Linkage of Surface Phenotypes.** The relationship between adherence to specific ligands was studied further by measuring their time of appearance in the cell cycle and their sensitivity to immune serum.

PRBC binding to CD36 and ICAM-1 occurred simultaneously with the onset of agglutination 16 hr postinvasion (Fig. 3A). However, binding to TSP was observed consistently 14 hr postinvasion when the same PRBCs did not bind to other ligands or from agglutinates.

PRBC adhesion to CD36 and ICAM-1 was partially reversed by immune serum, but little or no effect was seen with the same serum on binding to TSP (Fig. 3B). These observations were consistent with the findings that the protease-resistant adhesion to CD36 and ICAM-1 was reversed (92.7% ± 2.3% reversal; n = 4) in A4<sup>i+</sup>, C18<sup>i-</sup>, and A4<sub>tryp-ICAM</sub>, but the resistant adhesion to TSP in the same parasites was unaffected by immune serum (8.1% ± 7.8% reversal; n = 4). The antibody reversal of binding to ICAM-1 in trypsinized PRBCs from A4<sup>i+</sup> and A4<sub>tryp-ICAM</sub> (88.9% ± 0.5% reversal; n = 2) was greater than that of undigested controls (29.4% ± 9.4% reversal; n = 6) and may be due to the greater spatial accessibility after digestion.

## DISCUSSION

Analysis of the surface phenotypes of malaria-infected erythrocytes is complicated by the rapid appearance of antigenic

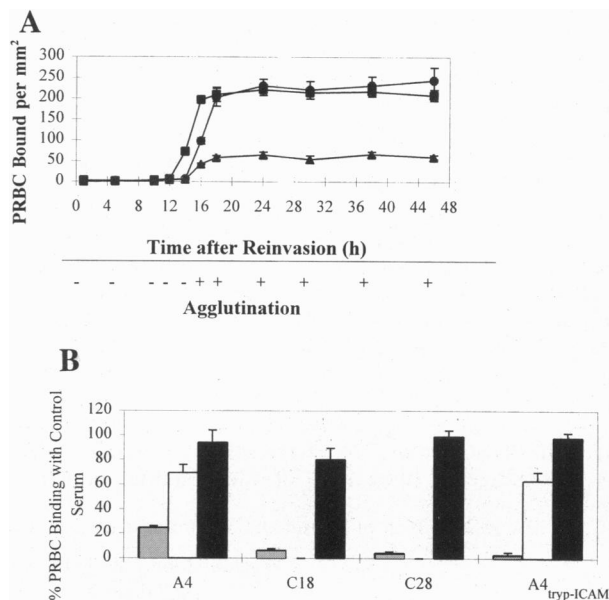


FIG. 3. (A) Staging of onset of agglutination and receptor-specific adhesion of PRBCs. Synchronized cultures of A4<sup>i+</sup> were used in shortened agglutination and spot binding assays at different times during the cell cycle. Binding data are mean numbers of cells bound per mm<sup>2</sup> ± SEM from two separate experiments; agglutination in immune serum was scored as - or + as described in *Materials and Methods*. ●, CD36; ■, TSP; ▲, ICAM-1. (B) Effect of immune serum on binding to CD36, ICAM-1, and TSP. All comparisons are between the relative effects of control serum and immune serum. Data are the mean percentages of binding ± SEM of control serum from three separate experiments. Shaded bars, CD36; open bars, ICAM-1; solid bars, TSP.

and adhesive heterogeneity, even in clonal populations (12, 26). To circumvent this problem, we have used sequence-specific proteases to dissect the role of particular VATs in receptor-specific adhesion.

We have obtained strong evidence that the variant antigen, PfEMP1, mediates adhesion to two endothelial cell surface receptors, CD36 and ICAM-1. We argue that the binding sites for CD36 and ICAM-1 are carried on the same molecule, which expresses variant antigenic epitopes, for three reasons. First, the effect of protease treatment on CD36 and ICAM-1 binding in clonally derived parasites was a variant-specific phenomenon. Second, selection of parasites on the basis of protease-resistant receptor binding resulted in enrichment of particular antigenic types bearing PfEMP1 molecules with unique biochemical properties. Third, both phenomena were activated simultaneously in the cell cycle and were at least partially reversible by the action of immune serum. Binding to TSP on the other hand was an invariant property of all the clones examined, was equally affected by a variety of proteases in a range of antigenic variants, appeared earlier in the cell cycle, and was not reversed by immune serum.

Molecular information on the relationship between PfEMP1, adhesion, and antigenicity was obtained by analyzing the effect of the same proteases on surface-labeled PRBCs expressing a range of VATs. Antigenic polymorphisms were associated with size polymorphisms of PfEMP1, and each VAT correlated with a PfEMP1 of unique molecular mass. The high rate of clonal antigenic variation was biochemically confirmed by the observations that multiple proteins with the operational characteristics of PfEMP1 were present within cloned parasites and that it was possible in some circumstances to select out minor subpopulations of parasites bearing particular PfEMP1 molecules. Under these latter circumstances, enrichment by selection for protease-resistant adhesion to CD36 or ICAM-1 gave rise to PfEMP1 molecules of characteristic size and properties.

The presence of the C18VAT was predictive of V8 protease-resistant agglutination and adhesion to CD36: V8 protease treatment of this clone released a soluble PfEMP1 fragment of 50 kDa showing that the binding site for CD36 must lie proximal to the cleavage site in these parasites.

Two lines of evidence provide strong support that PfEMP1 also contains domains that mediate adhesion to ICAM-1. First, trypsin-resistant binding to this receptor was associated with a particular antigenic phenotype ( $A4_{\text{tryp-ICAM}}$ ). Second, selection on the basis of adhesion alone (of trypsinized PRBC to ICAM-1) selected for this antigenic type. At the molecular level,  $A4_{\text{tryp-ICAM}}$  expressed two members of the PfEMP1 family, which were both sensitive to trypsin and V8 protease. The protease-resistant phenotype was thus not correlated with a protease-resistant molecule. We conclude that the  $^{125}\text{I}$ -labeled tyrosine residues had been cleaved from PfEMP1 by trypsin but that the ICAM-1 binding domain remained intact and unlabeled at the cell surface. Treatment with trypsin revealed a novel antigenic phenotype but ablated adhesion to CD36. Taken together, these data imply that PfEMP1 must contain separate domains that mediate ligand-specific adhesion and can have antigenically distinct epitopes.

Trypsin-resistant adhesion of PRBCs to human umbilical vein endothelial cells and C32 melanoma cells has previously been associated with the expression of a trypsin-insensitive PfEMP1 (27, 28). The identity of the host cell molecules that mediated the resistant adhesion was not defined in these studies because trypsin abolished the ability of the selected parasites to bind to all known purified endothelial cell surface receptors, including ICAM-1.

It is also noteworthy that, in general, the clones that bound to ICAM-1 expressed larger forms of PfEMP1 than non-ICAM-1 binding equivalents. This is consistent with the necessity for a larger molecule or an extra domain in PfEMP1 to

enable parasites to bind to ICAM-1. These findings agree closely with recent work demonstrating the importance of high molecular mass forms of PfEMP1 in PRBC adhesion to human umbilical vein endothelial cells (17).

There was no relationship between adhesion to TSP, antigenic phenotype, and PfEMP1 expression. Binding to TSP was a constant feature of all PRBCs tested and was reduced by proteases to similar levels in all parasites. Selection of protease-resistant adhesion to TSP had no effect on the antigenic type of the culture and did not produce a line with enhanced resistance to further protease treatment. Subpopulations of variant parasites cannot, therefore, account for these observations. Treatment of PRBCs with the same proteases at ring stage did not alter the protease sensitivity of TSP binding expressed later in the cell cycle. The partial protease resistance of this phenotype is thus a property conferred by parasite-directed modification of the surface. Furthermore, immune serum had no effect on adhesion to TSP, even in protease-digested cells. Finally, binding to TSP in a highly synchronized culture was activated 2 hr before agglutination and adhesion to ICAM-1 and CD36.

Which molecule(s) then are responsible for adhesion to TSP? Three candidates exist: a constant region of PfEMP1 situated close to the membrane, a novel invariant parasite-encoded protein, or a modified host protein. Against the first possibility is the fact that the time of onset in the cell cycle of PfEMP1-mediated antigenic changes is later than the appearance of TSP binding. However, if the affinity of this interaction is higher, then such a result could be obtained. A novel parasite protein could be responsible, but no such molecule has yet been identified. We therefore favor the explanation that parasite-induced modifications to a host surface protein occur, which reveal cryptic regions that contain TSP-binding domains. It has already been shown that erythrocytes of the SS genotype can interact with TSP *in vitro* (29). We suggest that band 3 may mediate these effects. This molecule aggregates during erythrocytes senescence, revealing novel epitopes that are involved in the removal of aging cells from the circulation (27). Others have shown that band 3 undergoes further changes during intraerythrocytic parasite growth, which are related to novel adhesive properties (30, 31). The authors deduced that CD36 was the receptor involved, since peptides from band 3 could inhibit parasite adhesion to CD36-transfected target cells. They have, however, used intact cells rather than purified protein in their binding assays. Since CD36 may act as a receptor for TSP (32), a contribution of TSP to adhesion in whole cell assays cannot be discounted.

Our data support the notion that a single PRBC can bind to at least three receptors. This can be deduced from the experiments with  $A4_{\text{tryp-ICAM}}$ , which bound to ICAM-1 but not CD36 after trypsin treatment. Trypsinized PRBCs from this line selected on ICAM-1 could bind to ICAM-1, CD36, and TSP in the next cycle of growth. Since trypsin-resistant ICAM-1 binding was unique to  $A4_{\text{tryp-ICAM}}$  within this clone tree, the ability to bind to three receptors must be present on a single cell. Another laboratory has also concluded that a single PRBC can bind to more than one receptor (28). These parasites were selected on whole cells, and one of the receptors involved was unidentified. If  $A4_{\text{tryp-ICAM}}$  was to be selected after trypsinization on a cell bearing surface TSP, then in addition to specific selection for ICAM-1, binding a general selection for all phenotypes present in the culture would occur because of the invariant nature and partial protease resistance of binding to TSP. It is therefore imperative to use purified proteins for these experiments.

Using the above data, we propose a model to explain the adhesive and antigenic properties of *P. falciparum*-infected erythrocytes based on the domain-like structure of PfEMP1 (18, 19, 33) and an as yet undefined surface molecule(s). Fig. 4 shows the proposed order of functional domains and pro-

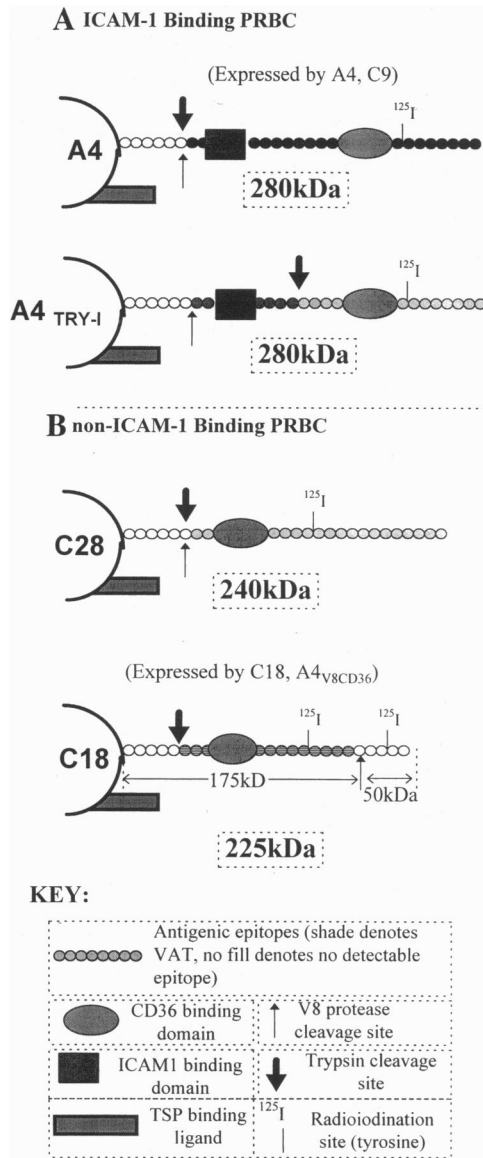


FIG. 4. Domain model of PfEMP1 in particular VATs within the IT clone tree. Proposed organization of the major VAT expressed by each clone. Antigenic epitopes are recognized by antibodies in immune serum in agglutination assays and cytoadherence reversal experiments. A single site for each protease is depicted for simplicity (not drawn to scale).

tease cleavage sites in the PfEMP1 molecule in different variant types. In these variants, the CD36 binding activity always lies distal to the ICAM-1 binding activity (if present). It is also clear that for A4<sub>TRY-I</sub>-ICAM multiple antigenic epitopes exist, which would elicit an antibody response in exposed individuals but which remain cryptic in intact cells.

Full sequence from a number of different PfEMP1 variants together with heterologous expression of the adhesive domains will, however, be required to establish properly the relationship between structure and function.

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