A GBP 130 Derived Peptide from *Plasmodium falciparum* Binds to Human Erythrocytes and Inhibits Merozoite Invasion *in Vitro*

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The malarial GBP 130 protein binds weakly to intact human erythrocytes; the binding sites seem to be located in the repeat region and this region's antibodies block the merozoite invasion. A peptide from this region (residues from 701 to 720) which binds to human erythrocytes was identified. This peptide named 2220 did not bind to sialic acid; the binding site on human erythrocyte was affected by treatment with trypsin but not by chymotrypsin. The peptide was able to inhibit Plasmodium falciparum *merozoite invasion of erythrocytes. The residues* F_{701}, K_{703}, L_{705}, T_{706}, E_{713} *(FYKILTNTDPNDEVERDNAD) were found to be critical for peptide binding to erythrocytes.*

Key words: *Plasmodium falciparum* - GBP 130 - binding assay

Interactions between *Plasmodium falciparum* merozoite and erythrocyte proteins are essential for the parasite's survival. Inhibition of these interactions is one of the main strategies for malaria control. Merozoite proteins involved in erythrocyte recognition, binding and invasion are specific targets for the development of effective weapons against malaria (Camus & Hadley 1985, Kochan et al. 1986, Perkins & Rocco 1988).

One of these erythrocyte binding proteins is the glycophorin binding protein (GBP 130) (Perkins 1984), which contains 11 highly conserved 50-residue-long repeats and a charged 225-residue-long N-terminal region. GBP 130 binds weakly intact human erythrocytes and this binding is not significantly modified by erythrocyte neuraminidase treatment (Perkins 1988). GBP 130 binds to glycophorin coupled to an acrylamide matrix (Perkins 1984). Although the specificity of its binding has been questioned (Van Scharavendijk et al. 1987), the binding sites seem to be located in the repeat region, since this region alone is able to bind to glycophorin (Kochan et al. 1986). Antibodies raised against a recombinant fusion protein containing 4.5 of the 50-amino acid repetitive units from GBP 130 inhibit *in vitro* merozoite invasion into erythrocytes (Kochan et al. 1986). Furthermore, immunization of splenectomized Saimiri monkeys with parasite proteins induces a protective immune response against *P. falciparum* (Dubois et al. 1984), in which the main antigen is a 96 kDa thermostable protein that was first described in 1984 as GBP 130 (Bonnefoy et al. 1988, 1994). GBP 130 is also recognized by antibodies involved in the formation of "Immune Clusters of Merozoites" when the *P. falciparum* parasites are cultured in the presence of immune sera from *Aotus* monkeys (Lyon et al. 1986, 1989, Chulay et al. 1987). On the contrary, a three repeat (GBP3R) recombinant protein was not recognized by these antibodies; also immunization of *Aotus* monkeys with this protein provided no protection whatsoever (Aronson et al. 1991).

In order to identify GBP 130 sequences relevant to its binding to human erythrocytes, sequential non-overlapping 20 mer peptides, spanning the entire sequence (Kochan et al. 1986), were synthesized and tested in erythrocyte binding assay. A peptide (named 2220) with a high binding activity to erythrocytes was identified. This peptide has a 150 nM affinity constant and is located in the GBP 130 repeat region. The binding activity of this peptide is not dependent on erythrocyte-sialic acid. The binding of this peptide to trypsin treated erythrocyte is affected, but not by chymotrypsin. This peptide also inhibited merozoite invasion of erythrocytes.

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MATERIALS AND METHODS

Peptide synthesis - Peptides were synthesized by the solid phase method (Merrifield 1963, Houghten 1985) with 100 mg p-methyl benzyhydrylamine resin (substitution 0.74 meq g^{-1}). Standard N α t-Boc protected amino acids were employed (Bachem). Peptides were cleaved by the Low-High HF technique (Tam et al. 1983), purified by RP-HPLC and freeze-dried.

Amino acid analysis was performed on all peptides and amino acid sequence on those having high binding activity. In order to allow radiolabeling of peptides which did not contain a Tyr residue, the underlined residue was replaced with Tyr (Fig. 1); replacements are based on volumetric and charge analysis.

Radiolabeling - Peptides were labeled with ^{125}I by the chloramine T method (Urquiza et al. 1996); 3.2 µl $Na^{125}I$ (17.2 mCi μg^{-1}) was oxidized with 28 µg chloramine T and added to 5 µg of peptide. The reaction was stopped by addition of 14 µg sodium bisulfite in isotonic PBS (pH 7.4). The radiolabeled peptide was purified by passage through a sephadex G-10 column.

Screening assay - Increasing concentrations of radiolabeled peptide (16-100 nM) were added to a human erythrocyte suspension ($3*10^8$ cells), in the absence (total binding) or presence (non-specific binding) of unlabeled peptide (1.25 µM), and incubated for 1 h at room temperature (Urquiza et al. 1996). After incubation, cells were washed five times with isotonic PBS. The specific binding was calculated as the difference between total and non-specific binding. For each peptide, the slope of the specific binding curve (specific bound CPM/ total added CPM) was calculated (Fig. 1). Based on previous studies, peptides with slope values greater than or equal to 2%, were considered as being high specific binding activity (HSBA) (Urquiza et al. 1996).

The binding assay with erythrocytes from other species (*Aotus* monkeys, rabbits, horses, goats, and chickens) was performed under the same experimental conditions. For saturation assays, $3*10^7$ cells were incubated in 140 µl and radiolabeled peptide concentration ranged from 20 to 400 nM.

Treatment of erythrocytes with neuraminidase - Erythrocytes (20% hematocrit, in buffer containing 5 mM sodium acetate, 140 mM NaCl, 10 mM $CaCl_2$, and 0.1 mM PMSF, pH 5.1) were treated with 250 µU neuraminidase (ICN 9001-67-6) per ml of packed cells at 37°C for 1 h. Finally the cells were washed with TBS (Camus & Hadley 1985) and used in binding assay.

Treatment of erythrocytes with trypsin and chymotrypsin - Erythrocytes [20% hematocrit in TBS buffer (5 mM Tris-HCl, 140 mM NaCl, pH 7.4)] were treated with trypsin (Sigma T-1005) or chymotrypsin (Sigma C-4129) at a final concentration of 100 µg ml^{-1} at 25°C for 1 h. They were then washed with TBS buffer containing 0.1 mM EDTA and 0.1 mM PMSF (Camus & Hadley 1985). These erythrocytes were used in binding assay with ^{125}I-labeled peptide 2220.

Competition binding assay - In order to identify the residues involved in peptide binding to erythrocytes, a competition binding assay was carried out with peptide 2220 glycine analogues (Fig. 4). Human erythrocytes ($1*10^8$) in 100 µl were incubated with 20 nM of ^{125}I-labeled peptide 2220 and with 0, 100 and 800 nM of each non-radiolabeled analogue peptide for 1 h at room temperature. The erythrocytes were then washed five times with 25 volume of PBS. The assay was done in triplicate. It was considered that a critical amino acid replacement had taken place in any peptide analogue which was not able to inhibit at least 50% of the ^{125}I-labeled 2220 peptide binding.

Invasion and development inhibition assays - The peptides were tested for their ability to inhibit erythrocyte invasion by merozoites. For this purpose *in vitro P. falciparum* FCB-2 strain cultures were used. A synchronized culture, in schizont stage (Trager & Jensen 1976), was seeded in 96-well plates with non-infected erythrocytes and with the peptide (200 µM final concentration, a final hematocrit of 1.5% and a final parasitemia of 1.2%). After 18 h, the culture supernatant was removed and complete media was added with 1.33 µCi ml^{-1} of ^3H-hypoxanthine. Plates were then incubated for 30 h and harvested in fiberglas filters. To determine the development inhibition capacity, a simultaneous assay was performed under similar conditions, but peptides were added at the ring-stage after synchronization. Schizonts were harvested after 30 h of development inhibition test incubation. Two criteria were used to assess invasion and development: (1) the amount of newly formed rings was determined by measuring the incorporation of ^3H-hypoxanthine (metabolic labeling of rings); (2) alternatively, the number of newly formed rings was determined by microscopic examination in Giemsa stained thin smears. Routinely, 10,000 erythrocytes were examined and the percentage was calculated.

RESULTS

Human erythrocytes binding peptides - In the GBP-130 peptide screening binding assay, according to Urquiza et al. (1996), three patterns were found: high specific binding peptides, non-specific binding peptides and low or non-binding peptides. Only one high specific binding peptide was found, and named 2220 (Fig. 1). This peptide is located in the GBP-130 repeat sequence, for which binding

activity has already been reported (Kochan et al. 1986). Some other peptides (2204, 2208, 2212, 2217), with very similar sequences to peptide 2220 (R**DN** is substituted by R**RN**), show lower binding activities than peptide 2220. Peptides which only share the **N**-terminal (2201, 2206, 2209, 2214, 2222) or the C-terminal (2202, 2210, 2215, 2218, 2223) sequence with peptide 2220 showed no specific binding to erythrocytes.

In order to investigate whether the peptide labeling, which introduces an iodine atom into a tyrosine residue, affected its interaction with human erythrocytes, an additional assay was carried out with peptide 2220 labeled with non-radioactive NaI. Fig. 2 shows that both iodinated and non-iodinated peptides identically displaced the radiolabeled ligand. Therefore, iodinated tyrosine can be excluded as significantly altering the erythrocyte-peptide interaction.

A saturation binding assay was performed for peptide 2220 (Fig. 3). The Hill plot, obtained by transformation of experimental data, is shown in the inset. Using saturation and Hill analysis, a 150 nM dissociation constant and a Hill coefficient of 2 were calculated and each cell was determined to have 8,000 binding sites.

Peptide Number	Sequence	Residues	Binding activity 1.0 2.0
2189	M R L S K V S D I K S T G V S N Y K N F	1-20	
2190	N S K N S S K Y S L M E V S K K N E K K	21-40	
2191	N S L G A F H S K K I L L I F G I I Y V	41-60	
2192	V L L N A Y I C G D K Y E K A V D Y G F	61-80	
2193	R E S R Y L A E G E D T C A R K E K T T	81-100	
2194	Y R K S K Q K T S T R T V A T Q T K K D	101-120	
2195	E E N K S V V T E E Q K V E S D Y E K Q	121-140	
2196	K R T K K V V K K Q I N Y G D T E N Q K	141-160	
2197	E G K N V K K V Y K K E K K K E E S G K	161-180	
2198	P E E N K H A N E A S K K K E P K A Y K	181-200	
2199	V S Q K P S T S T R S N N E V K Y R A A	201-220	
2200	S N Q E T L T S A D P E G Q I M R E Y A	221-240	
2205	D N K E D L T S A D P E G Q I M R E Y A	321-340	
2213	N K E D L T S A D P E G Q I M R E Y A A	521-540	
2221	N K E D L T S A D P E G Q I M R E Y A A	721-740	
2203	P E G Q I M R E Y A S D P E Y R K H L E	281-300	
2207	P E G Q I M R E Y A A D P E Y R K H L E	381-400	
2211	E G K I M R E Y A A D P E Y R K H L E I	481-500,581-600	
2219	E G Q I M R E Y A S D P E Y R K H L E I	681-700	
2201	A D P E Y R K H L E I F Y K I L T N T D	241-260	
2206	A D P E Y R K H L E V F H K I L T N T D	341-360	
2209	A D P E Y R K H L E V F H K I L T N T D	441-460	
2214	D P E Y R K H L E I F Y K I L T N T D P	541-560	
2222	D P E Y R K H L E I F H K I L T N T D P	741-760	
2204	I F Y K I L T N T D P N D D V E R R N A	301-320	
2208	Y F H K I L T N T D P N D E V E R R N A	401-420	
2212	F H K Y L T N T D P N D E V E R R N A D	501-520	
2217	Y H K I L T N T D P N D E V E R R N A D	601-620	
2220	F Y K I L T N T D P N D E V E R D N A D	701-720	
2202	P N D E V E R R N A D N K E D Y T S A D	261-280	
2210	P N D E V E R R N A D N K E Y T S S D V	461-480	
2215	N D E V E R R N A D N K E E Y T S S D P	561-580	
2218	N D E V E R R N A D N K E D Y T S A D P	661-680	
2223	N D E V E R Q N A D N Q E A Y	761-774	

Fig. 1: GBP 130 synthetic peptides' specific binding activity to human erythrocytes. Amino acid sequences are given in the left column. Binding activity: is the slope value of specific binding curve. Underlined amino acids were substituted with Tyr to allow radiolabeling. Each one of the black bars represents the slope of the specific binding curve, which is named high specific binding activity (HSBA). Peptides with HSBA ≥ 2% were considered as high specific binding peptides to erythrocytes, since these peptides recognize more than 200 specific binding sites per cell at low concentrations of radiolabeled peptide (200 nM).

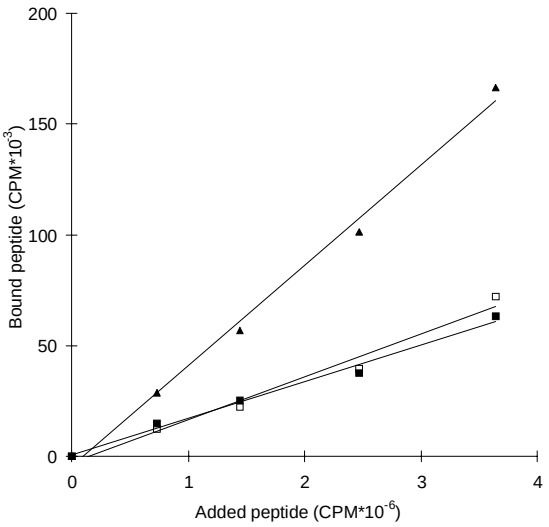

Fig. 2: peptide ^{125}I-2220 binding to human erythrocyte. Total binding: binding to erythrocyte in absence of competition (▲). Non specific-binding: binding in presence of non-labeled peptide (□). Non specific binding in presence of peptide iodinate with non-radioactive NaI (■).

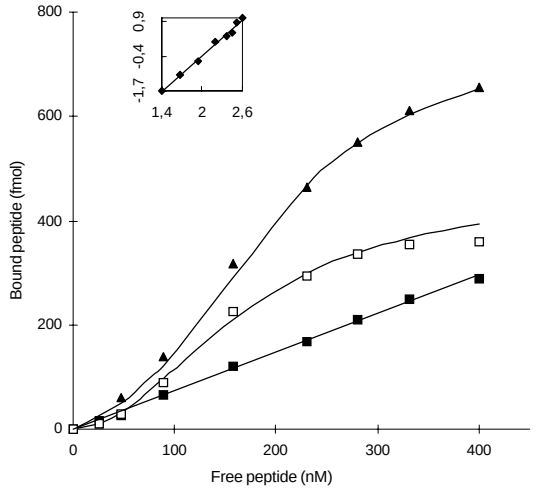

Fig. 3: saturation curve for peptide 2220. The graph shows the total (▲) binding, non-specific (■) binding and specific (□) binding of the radiolabeled peptide to human erythrocytes. In the Hill plot (inset graph) the axis are: the abscissa is log F, and the ordinate is log (B/Bmax-B). B: fmol bound peptide; Bmax: maximum fmol bound peptide; F: nM free peptide

Peptide 2220 and interaction with enzyme treated erythrocytes - In order to determine whether the peptide 2220 interacts with sialic acid or sialoglycoproteins, binding assays were performed with enzyme treated erythrocytes. Table I shows the percentages of specific binding compared to peptide binding of normal human erythrocytes. Human erythrocytes treated with neuraminidase showed a 373% increase in specific binding for peptide 2220. However, human erythrocytes treated with trypsin showed markedly lower affinity for peptide 2220, with a reduction to 35%. It was also noted that, when human erythrocytes were treated with chymotrypsin, there was no effect on peptide 2220 specific binding.

Peptide 2220 bound mainly to human erythrocytes - Peptide 2220 was assayed with erythrocytes of different species with the aim of determining if the binding is species specific (Table I). Peptide 2220 possesses a higher specific binding to human erythrocytes than to erythrocytes from other species. *Aotus* and rabbit erythrocytes showed specific binding values of 59% and 44% respectively, compared to human erythrocytes. No binding was observed to goat, horse or chicken erythrocytes.

TABLE I

Binding of 2220 peptide to enzyme treated human erythrocyte and non-human erythrocytes

Erythrocytes	Specific binding (%)
Humans	
non-treated	100 ± 8
neuraminidase	373 ± 31
trypsin	35 ± 4
chymotrypsin	95 ± 9
Aotus	59 ± 6
Rabbits	44 ± 7
Goats	0 ± 4
Horses	0 ± 2
Chickens	8 ± 4

Critical residues for peptide-erythrocyte interaction - The importance of each residue in the binding of peptide 2220 to erythrocytes was determined by analyzing the ability of glycine analogs to inhibit the binding of the unmodified peptide (competition binding assay, Fig. 4). Residues F_{701}, K_{703}, L_{705}, T_{706} and E_{713} were critical, since the replacement of any of these amino acids dramatically reduced their binding affinity (Fig. 4).

Effect on in vitro peptide 2220 P. falciparum cultures - To determine whether the peptide 2220 is biologically relevant, *in vitro* invasion and development inhibition assays were performed. Both assays were performed in triplicate and six peptides were used as controls: peptides 2189 and 2197 (medium specific-binding); peptides 2204 and 2208 (low specific-binding); and peptides 2214 and 2215 (non-binding). As shown in Table II, the invasion process is inhibited only by peptide 2220 (91% inhibition). The slides of this assay, staining with

TABLE II

Inhibition of parasite invasion and development to erythrocyte by GBP 130 peptides

Binding activity	Peptides (200 µM)	Inhibition of invasion				Inhibition of development			
		^{3}H-hypoxanthine		Giemsa		^{3}H-hypoxanthine		Giemsa	
		%	SD	%	SD	%	SD	%	SD
High	2220	91	±7	86	±9	7	±17	2	±5
Medium	2189	0	±4	0	±6	59	±9	67	±8
	2197	0	±5	2	±4	0	±1	1	±1
Low	2204	0	±1	2	±2	0	±2	2	±3
	2208	0	±2	0	±1	5	±3	0	±2
No-binding	2214	0	±3	4	±4	0	±1	1	±2
	2215	0	±9	0	±3	0	±4	0	±1
Control	Chloroquine	98	±17	100	±11	99	±4	100	±10

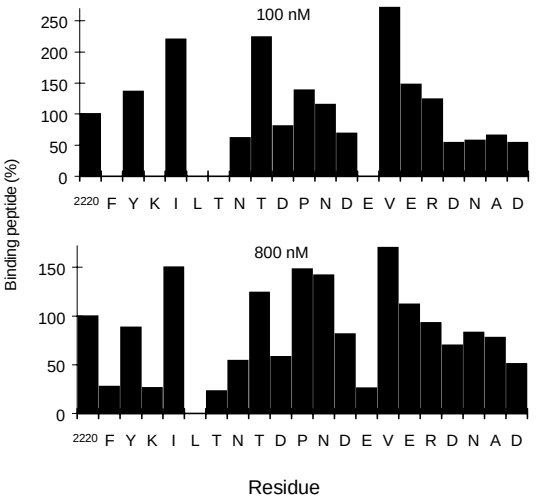

Fig. 4: specific binding to erythrocytes of peptide 2220 in competition assays with glycine analog peptides. The height of the black bars is proportional to the binding inhibition of the original radiolabeled peptide (20 nM) by the original or peptide unlabeled analogues (100 and 800 nM). The letter at the bottom represents the amino acid changed by glycine.

Giemsa show that the parasites in the slide of peptide 2220 has the same morphology of the control, but the number of parasited erythrocytes was diminished. None of the control peptides showed any effect on *P. falciparum* parasite invasion. One control peptide (2189) markedly affected the development of the parasite (59%), but did not inhibit invasion. This suggests that it may have a toxic effect on the intracellular parasite, but is not capable of interacting with erythrocyte proteins to block the invasion process.

DISCUSSION

Merozoite surface proteins play an important role in erythrocyte invasion, since they determine the first contact between erythrocyte and merozoite. Among these, GBP 130 has been implicated as being an erythrocyte binding protein in merozoites (Perkins 1984, 1988). Peptide 2220 was identified as being a part of the GBP 130 sequence which binds specifically to erythrocytes. This peptide belongs to the repeat region which binds to human erythrocytes; antibodies against this region inhibited *in vitro* erythrocyte invasion by merozoites (Kochan et al. 1986). Peptides 2204, 2208, 2212, 2217 and 2220 have a very similar sequence, including the residues critical for binding to erythrocytes. However, the 2220 peptide has the highest binding affinity. The change of R for D in position 717 increases the affinity of peptide 2220 to erythrocytes.

The interaction between peptide 2220 and erythrocytes is charge-independent, since peptides 2202 and 2205, which have a similar charge, did not bind specifically to erythrocytes. Consequently, the amino acid sequence is the determinant factor in the binding. Peptide 2220, iodinated with non-radioactive NaI, displaced the radiolabeled ligand identically to displacement produced by the non-labeled peptide (Fig. 2), showing that the modification caused by iodination of the peptide does not affect this peptide's interaction with erythrocytes. A 150 nM affinity constant shows that the interaction between peptide 2220 and erythrocytes is very strong. This interaction could be mediated by salt-bridges, since peptide 2220 has a high proportion of charged amino acids (8/20) and the E_{713} amino acid is critical to binding. A Hill coefficient of 2 suggests positive cooperativity.

Peptide 2220 shows a high increase in specific binding to neuraminidase treated erythrocytes (Table I), indicating that the interaction does not involve sialic acid molecules. This agrees with previous observations, which demonstrate that GBP 130 receptors are not dependent on sialic acid (Perkins 1988). The binding of peptide 2220 to erythrocytes was affected by the treatment with trypsin and not by chymotrypsin. As trypsin cleaves the external proteic part of glycophorin A and C (Pasvol 1984), it is possible that glycophorin A, C or other related molecules act as a receptor for this peptide. However, as the binding was not completely abrogated by trypsin treatment, other proteins (such as glycophorin B) could be involved in the binding. The membrane receptor of peptide 2220 has not been identified, although it is known that its binding activity is sensitive to trypsin treatment.

Binding competition assays with peptide 2220 glycine analogues show that some amino acids (FYKILTNTDPNDEVERDNAD) were critical in the binding; probably these residues are not only involved in direct binding interactions (salt-bridges and hydrophobic), but could also be essential for the induction of the tridimensional structure required for binding. In some cases the replacement of 2220 residues by glycine led to peptides with higher binding affinity (I_{704}, P_{710}, N_{711}, V_{714}). Probably these residues' side chains hinder optimal 2220 peptide-receptor interactions. Substitution of two different threonine residues (T_{706} and T_{708}) has a very different effect on peptide binding (Fig. 4). This further emphasizes that peptide 2220 binding is highly dependent on its primary structure, and not only on a particular amino acid composition.

The binding assays with peptide 2220 and erythrocytes from different species (Table I) showed that this peptide binds to human erythrocytes with higher affinity than to erythrocytes from other species. However, it also interacts with *Aotus* monkeys' erythrocytes, albeit to a lesser extent. This might explain why both species are susceptible to *P. falciparum* infection. Peptide 2220 also binds significantly to rabbit erythrocytes. It has been demonstrated that some proteins involved in merozoite invasion are able to bind to rabbit erythrocytes (Orlandi et al. 1990), but *P. falciparum* merozoites do not invade these erythrocytes (Breuer et al. 1983). Probably the binding domain of the peptide receptor on the erythrocyte is different in the studied species, which will change the affinity constant of this interaction.

Two assays were carried out in order to test the peptide effect on *in vitro* cultures of *P. falciparum*. In one assay, peptide was added before merozoites were released at the schizont stage. In this assay, peptide 2220 inhibited invasion by 91%. The peptide may affect merozoite release or erythrocyte invasion. However, we did not see delay in parasite growth or death parasites. This could be indicating that peptide 2220 may compete for the receptor sites on the erythrocyte and block the merozoite-invasion. In another assay, when peptide was added after invasion, ring stage peptide 2220 did not affect parasite development. However, the difference in membrane permeability between ring stage and schizont stage restricts the interpretation of this results. The peptide concentration needed for invasion-inhibition was higher than the affinity constant value; this may be due to higher GBP 130 affinity than peptide 2220, or because the effective concentration is diminished by proteolytic degradation. Interestingly, peptide 2220 belongs to a repeat region which binds to glycophorin and elicits antibodies able to block merozoite invasion of erythrocytes (Kochan et al. 1986).

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