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Identification of a Nonameric H-2K^k-Restricted CD8$^+$ Cytotoxic T Lymphocyte Epitope on the *Plasmodium falciparum* Circumsporozoite Protein

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Class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) against the circumsporozoite protein (CSP) protect mice against the rodent malaria parasite, *Plasmodium yoelii*, and vaccines designed to produce protective CTL against the *P. falciparum* CSP (PfCSP) are under development. Humans and B10.BR (H-2^k) mice have been shown to have CD8$^+$ CTL activity against a 23-amino-acid region of the PfCSP (residues 368 to 390 from the PfCSP 7G8 sequence) that is too long to bind directly to class I major histocompatibility complex molecules. To identify within this 23-amino-acid peptide a shorter peptide that binds to an H-2^k class I major histocompatibility molecule, a primarily CD8$^+$ (97.8%) T-cell line (PfCSP TCL.1) was produced by immunizing B10.BR mice with recombinant vaccinia virus expressing the PfCSP and stimulating in vitro spleen cells from these immunized mice with L cells transfected with the PfCSP gene (LPF cells). PfCSP TCL.1 lysed LPF cells and L cells pulsed with peptide PfCSP 7G8 368-390. When 15 overlapping nonamer peptides spanning the 368 to 390 sequence were tested, only one peptide, PfCSP 7G8 375-383 (Y E N D I E K K I), which includes an H-2K^k-binding motif, E at amino acid residue 2, and I at residue 9, sensitized targets for lysis by PfCSP TCL.1. Furthermore, a 10^3- to 10^4-fold lower concentration of the nonamer than that of the 23-amino-acid peptide was required to sensitize target cells for lysis by PfCSP TCL.1. Presentation by H-2K^k was demonstrated by using 3T3 fibroblast cells transfected with the murine H-2K^k or H-2D^k genes, and only the H-2K^k transfectants were lysed by PfCSP TCL.1 after incubation with peptide PfCSP 7G8 375-383. Binding to H-2K^k was confirmed by competitive inhibition of binding of labelled peptides to affinity-purified K^k molecules. Substitution of the anchor amino acid residue, E, at position 2 with A dramatically reduced binding to K^k and eliminated the capacity of the peptide to sensitize target cells for killing. Variation of non-anchor residues did not markedly reduce binding to K^k but in some cases eliminated the capacity of the peptide to sensitize targets for cytolysis by PfCSP TCL.1, presumably by eliminating T-cell receptor-binding sites. These data suggest that similar studies with human T cells will be required for optimal development of peptide-based vaccines designed to produce protective class I-restricted CD8$^+$ CTL against the PfCSP in humans.

A major focus of malaria vaccine development is the induction of CD8$^+$ cytotoxic T lymphocytes (CTL), which destroy infected hepatocytes (9, 10). This is because the sterile protective immunity induced by immunization of some strains of mice with radiation-attenuated sporozoites is eliminated by in vivo depletion of CD8$^+$ T cells (27, 38) and because adoptive transfer of CD8$^+$ CTL against the *Plasmodium berghei* (26) and *P. yoelii* (24, 37) circumsporozoite proteins (PbCSP and PyCSP, respectively) and against the *P. yoelii* sporozoite surface protein 2 (13) completely protect against sporozoite challenge.

The *P. falciparum* CSP (PfCSP) is a target for such malaria vaccine development. A 23-amino-acid peptide, PfCSP 7G8 368-390, has been demonstrated to include at least one CD8$^+$ CTL epitope in B10.BR mice (11, 14, 17, 34) and in humans (8, 16, 29), including one HLA-B35-restricted nonamer epitope (8). Peptides of 8 to 10 amino acids bind optimally to class I major histocompatibility complex (MHC) molecules for recognition of CD8$^+$ CTL, and the development of optimal malaria vaccines may depend on the identification of such short peptides. The current studies were designed to identify for the first time a nonamer peptide within PfCSP 368-390 that optimally sensitizes H-2^k target cells for cytolysis by CD8$^+$ CTL and to identify the H-2^k molecule to which the peptide binds.

MATERIALS AND METHODS

Mice. Female, 5- to 6-week-old B10.BR (H-2^k) mice (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. The experiments were conducted according to the guidelines for use of laboratory animals (19a).

Cell lines. L (H-2^k) and P815 (H-2^d) cell lines were purchased from the American Type Culture Collection (Rockville, Md.). L cells transfected with the PfCSP gene (LPF cells) (14) and 3T3 cells transfected with the genes for murine H-2K^k (NIH 3T3-K^k, G105-7.5 p4 [36]) or H-2D^k (NIH 3T3-D^k, G23-6.1 p4 [20]) have been described previously.

Peptides. The peptides used in this study were based on the PfCSP 7G8 sequence and other variant sequences. They were synthesized as described previously (12) by the tea bag method with the exception of peptides PfCSP 374-379 and 379-390, which were synthesized by the F-moc, t-Bu strategy for solid-phase peptide synthesis as described previously (19). Purity was assessed by reverse-phase high-performance liquid chromatography. Peptides were dissolved in distilled water.

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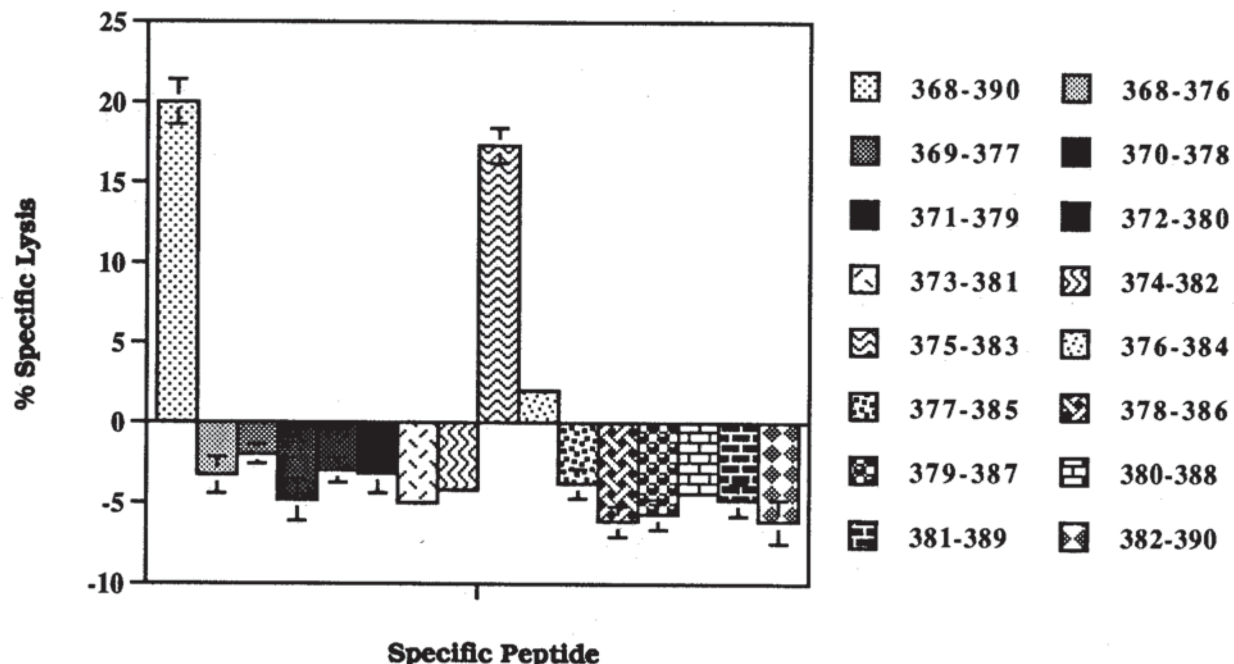

FIG. 1. Recognition of nonamer peptides by PfCSP TCL.1. Target cells were produced by incubation of L cells overnight with the 23-amino-acid peptide PfCSP 7G8 368-390 or each of 15 nonamers derived from peptide PfCSP 7G8 368-390 (Table 1) in the presence of 100 μCi of ^{51}Cr. The effector cells (PfCSP TCL.1) were added to the target cells at an effector-to-target ratio of 25:1, and the percent specific lysis was assessed. Symbols are identified from left to right.

Immunization. Ten B10.BR mice were immunized intravenously with a single dose of 10^7 PFU of recombinant vaccinia virus expressing PfCSP (V71) (14, 17).

Generation of PfCSP TLC.1. Four weeks after immunization with V71, mice were euthanized and spleen cells were isolated. Spleen cells (5×10^6 per well) were added to 24-well culture plates (Costar, Cambridge, Mass.) in 2-ml complete T-cell medium, a 1:1 mixture of RPMI 1640 (GIBCO, Grand Island, N.Y.) and Eagle Hanks amino acid (GIBCO) medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μm 2-mercaptoethanol (GIBCO). Spleen cells were stimulated in vitro for 6 days with mitomycin (100 μg/ml)-treated LPF cells (3×10^5 per well). Six days later, stimulated cells were passed through a lymphocyte separation medium (Organon Teknika Corporation, Durham, N.C.), and 3×10^5 T cells were stimulated with LPF cells (3×10^5) and irradiated (3,300 rads) syngeneic spleen cells in a 24-well plate in the presence of 5% rat concanavalin A supernatant (Collaborative Research Inc, Bedford, Mass.). After four cycles of stimulation, the T-cell line designated PfCSP TCL.1 was tested for cytolytic activity.

Flow cytometry analysis. Samples containing 10^6 PfCSP TCL.1 cells were incubated with fluorescein isothiocyanate (FITC)-labelled RM4-5 (anti-CD4 monoclonal antibody) or FITC-labelled 53-5.8 (anti-CD8 monoclonal antibody) (PharMinogen, San Diego, Calif.) diluted in Hanks balanced salt solution (HBSS) supplemented with 1% fetal calf serum and 0.1% sodium azide for 30 min at 4°C and then washed three times. Stained cells were analyzed on an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, Fla.). Ten thousand cells per sample were analyzed. Dead cells were excluded by propidium iodide gating.

In vitro production of IFN-γ and IL-2 by PfCSP TCL.1. PfCSP TCL.1 was cultured in the presence of LPF cells as described above. Supernatants were collected at 24 and 72 h and stored at −70°C. Gamma interferon (IFN-γ) was measured in 72-h supernatants by two-site capture enzyme-linked immunosorbent assay using immobilized monoclonal antibody HB170 (anti-IFN-γ), rabbit polyclonal anti-mouse IFN-γ, and peroxidase-conjugated donkey anti-rabbit immunoglobulin (Jackson Immuno Research Laboratories, Inc., West Grove, Pa.) (2). Interleukin 2 (IL-2) was measured in 24-h supernatants by a proliferation assay with CTLL (a mouse CTL line; ATCC TIB-214) as described previously (28). The quantity of IL-2 was estimated from a standard curve generated by incubating CTLL cells with known quantities of recombinant IL-2 and measuring proliferation (28).

CTL assay. CTL assays were performed as described previously (17). Briefly, target cells were pulsed overnight with 100 μCi of ^{51}Cr (Dupont New England Nuclear, Boston, Mass.) and individual peptides as described in the figure legends. In some experiments, the target cells were treated with 5 nM recombinant IFN-γ (Genzyme, Cambridge, Mass.) to enhance peptide presentation by the target cells (11). Target cells were then washed three times, and 5×10^3 cells were plated in a 96-well U-bottom plate (Costar). Effector cells were washed and added to the wells containing target cells. After 6 h of incubation at 37°C in 5% CO_2, supernatants were collected by using the Skatron (Sterling, Va.) SCS system, and the amount of ^{51}Cr released in the supernatants was determined by using a gamma counter (Clinnigamma; Pharmacia LKB Nuclear, Inc., Gaithersburg, Md.). The mean (± standard error) percentage of specific lysis in triplicate wells was calculated as previously described (17).

Peptide-MHC class I-binding assay. The biochemical peptide-K^k-binding assay used affinity-purified K^k molecules and was conducted as previously described (21, 32). Briefly, affinity-purified K^k molecules in detergent solution were incubated with ^{125}I-labelled influenza nucleoprotein peptide 50-57 (SDYEG RLI) and increasing concentrations of unlabelled PfCSP peptides. After equilibrium had been reached (48 h, 18°C), the free and bound labelled peptides were separated by spin column gel filtration. The concentration of PfCSP peptides needed to inhibit nucleoprotein peptide 50-57 binding by 50% was determined and related to the concentration of unlabelled nucleoprotein peptide 50-57 needed to block labelled peptide binding by 50% in the same experiments. The lower the concentration of peptide required, the greater the relative affinity of the peptide.

RESULTS AND DISCUSSION

Characterization of PfCSP-specific CTL line. After 4 weeks of in vitro stimulation, PfCSP TCL.1 was shown to lyse L cells pulsed with peptide PfCSP 7G8 368-390 (Fig. 1) and LPF cells (Fig. 2). The cytolytic activity was MHC restricted since PfCSP TCL.1 did not lyse mismatched P815 cells (H-2^d) pulsed with peptide PfCSP 7G8 368-390 (data not shown). Microfluorometic analysis of PfCSP TCL.1 showed a 97.8% homogeneous population of $CD8^+$, $CD4^-$ cells (data not shown).

To determine if PfCSP TCL.1 secreted IFN-γ or IL-2 in response to stimulation with LPF cells, culture supernatants were collected at 24 and 72 h after initiation of stimulation. Supernatants collected at 72 h contained 1,427 U of IFN-γ per ml, compared with 8.51 U of IFN-γ per ml in supernatants from cultures of PfCSP TCL.1 that had not been exposed to LPF cells. As in other systems (37), supernatants collected 24 h after initiation of stimulation of PfCSP TCL.1 with LPF cells did not contain detectable amounts of IL-2.

Nonamer from PfCSP recognized by PfCSP TCL.1. Class

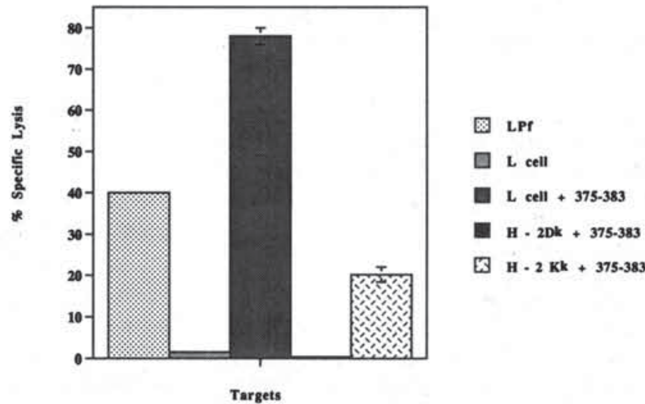

FIG. 2. Lysis of H-2K^k-transfected targets by PfCSP TCL.1. 3T3 cells transfected with murine H-2 K^k (G105-7.5) and H-2D^k (G23-6.1) genes and untransfected L cells were sensitized overnight in the presence of peptide PfCSP 7G8 375-383 at 0.4 μM and ^{51}Cr. LPF cells were labeled overnight with ^{51}Cr only. These target cells were then used in a CTL assay at a 40:1 effector-to-target ratio.

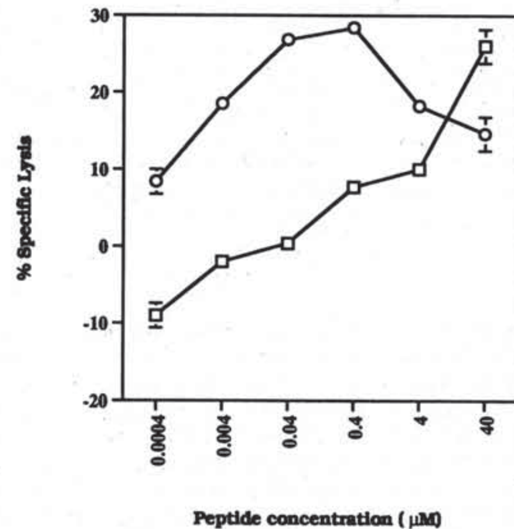

FIG. 3. Comparison of lysis by PfCSP TCL.1 of L cells incubated with a 23- or 9-amino-acid peptide. Target cells (L cells) were incubated overnight with various concentrations of the 23-amino-acid peptide, PfCSP 7G8 368-390 (□), or the 9-amino acid-peptide, PfCSP 7G8 375-383 (○), and ^{51}Cr and studied in a CTL assay with PfCSP TCL.1 effector cells at an effector-to-target ratio of 25:1.

I-restricted CTL epitopes are generally 8 to 10 amino acids in length. To map the peptide responsible for cytolytic activity within PfCSP 368-390, several truncated peptides and overlapping peptides were studied. Peptide PfCSP 374-390 (D Y E N D I E K K I C K M E K C S) sensitized target cells for lysis by PfCSP TCL.1, but peptide PfCSP 379-390 (I E K K I C K M E K C S) did not (data not shown). These data indicated that from one to five amino acids from residues 374 to 378 were included within the minimal epitope. This was confirmed by studying 15 overlapping nonamers from the PfCSP 7G8 368-390 sequence (Table 1). In three different experiments, only peptide PfCSP 375-383 (Y E N D I E K K I) was recognized by PfCSP TCL.1 (Fig. 1). When peptide PfCSP 375-383 was compared with peptide PfCSP 368-390 for sensitizing target cells, a nonamer concentration 10^3- to 10^4-fold lower than that of the longer peptide sensitized targets for similar specific lysis (Fig. 3).

H-2K^k-restricted recognition of peptide PfCSP 375-383 by PfCSP TCL.1. Peptide 375-383, YENDIEKKI, conforms to an H-2K^k-binding motif: a glutamic acid or aspartic acid residue at the 2nd position and an isoleucine residue at the 8th or 9th position of a 8- or 9-amino-acid-long peptide (1, 6, 22, 23, 33) (Table 2). To confirm the H-2K^k restriction of this peptide, we assessed the capacity of PfCSP TCL.1 to lyse peptide-pulsed BALB/c 3T3 fibroblasts (H-2^d) transfected with the gene encoding H-2K^k or H-2D^k. In three different experiments, PfCSP TCL.1 lysed only the H-2K^k targets (Fig. 2).

Effect of variation in peptide sequence on binding to H-2K^k and on cytolytic activity of PfCSP TCL.1. The binding of a peptide to class I MHC is generally regulated by amino acids at positions 2 and the C′ terminus of 8- to 10-amino-acid-long

TABLE 1. Relative binding of peptides from PfCSP to MHC class I K^k and ability to label target cells for lysis by PfCSP TCL.1

Parasite strain	Amino acid residue		Relative binding (μM)[a]	% Specific lysis[b]	
	Position	Sequence		20 μM	0.4 μM
7G8	368–390	K P K D E L D Y E N D I E K K I C K M E K C S	>1,000	20.0	
7G8	368–376	K P K D E L D Y E	>1,000	−3.5	
7G8	369–377	P K D E L D Y E N	>1,000	−2.4	
7G8	370–377	K D E L D Y E N D	>1,000	−5.0	
7G8	371–379	D E L D Y E N D I	0.2	−2.3	−0.1
7G8	372–380	E L D Y E N D I E	>1,000	−4.7	
7G8	373–381	L D Y E N D I E K	>1,000	−4.7	
7G8	374–382	D Y E N D I E K K	>1,000	−4.8	
7G8	375–383	Y E N D I E K K I	0.1	17.3	33.3
7G8	376–384	E N D I E K K I C	300	3.5	
7G8	377–385	N D I E K K I C K	>1,000	−3.8	
7G8	378–386	D I E K K I C K M	>1,000	−6.1	
7G8	379–387	I E K K I C K M E	>1,000	−5.8	
7G8	380–388	E K K I C K M E K	>1,000	−4.6	
7G8	381–389	K K I C K M E K C	>1,000	−4.9	
7G8	382–390	K I C K M E K C S	>1,000	−6.1	
3D7	375–383	Y A N D I E K K I	9.4	1.18	1.2
GAM5	375–383	Y A D D I E K K I	22.0	0.1	0.1
PNG3	375–383	C E S D I E K K I	0.2	11.8	12.0

[a] The lower the concentration of peptide, the higher the relative binding of the peptide to K^k.
[b] Targets were labelled with a 20 or 0.4 μM concentration of the peptides in two different experiments.

TABLE 2. Known MHC class I H-2K^{k}-restricted CTL epitopes

Sequence	Protein	Reference
Y E N D I E K K I	PfCSP	This study
S D Y E G R L I	Influenza A nucleoprotein	1
S E F L L E K R I	Simian virus 40	6, 23
F E S T G N L I	Influenza A hemagglutinin	6, 33
F E A N G N L I	Influenza A hemagglutinin	6
I E G G W T G M I	Influenza A hemagglutinin	6

peptides (4, 18, 35). It has been proposed that the binding affinity depends on hydrogen bonding of two charged termini of a peptide and conserved class I residues located at either end of the peptide-binding cleft (5, 15, 31). After peptide binding to class I MHC, the peptide-MHC complex must be recognized by the T-cell receptor. Amino acid residues not buried in the grooves of the MHC molecule are thought to be responsible for interaction with the T-cell receptor. For example, peptide 371-379 (D E L D Y E N D I) also has a glutamic acid at position 2 and an isoleucine at position 9, but cells incubated with this peptide are not lysed by PfCSP TCL.1 (Fig. 1; Table 1). This may be due to low-affinity binding to the MHC because other amino acid residues are required for optimal binding or, more likely, because the amino acids required for recognition by the PfCSP TCL.1 T-cell receptor are not present in the 371-379 peptide. To further assess the importance of specific amino acid residues in binding and cytolytic activity, we synthesized the three known variants of peptide PfCSP 7G8 375-383, (Y E N D I E K K I) (Table 1) and assessed the capacity of these three peptides and the 15 overlapping nonamers from 368 to 390 to bind to H-2K^{k}. In parallel, we assessed the capacity of the variant sequences to sensitize targets for killing by PfCSP TCL.1. Of the 15 overlapping nonamers, only peptide 371-379 and peptide 375-383 bound to H-2K^{k} (Table 1). These data clearly indicate that the inability of peptide 371-379 to sensitize target cells for lysis by the T-cell line is due to inadequate recognition by the T-cell receptor, not to inadequate binding to H-2K^{k}. The three known variant sequences of peptide 375-383 are Y A N D I E K K I (NF54/3D7), Y A D D I E K K I (GAM5), and C E S D I E K K I (PNG3) (3, 30, 10). Only the PNG3 (C E S D I E K K I) peptide, which retains the H-2K^{k}-binding motif (E at position 2 and I at position 9), shows binding to H-2K^{k} similar to that of the original 375-383 peptide (Table 1). Although the other two peptides bound to H-2K^{k} with greater relative affinity than did any of the other nonamers, they had 100- to 220-fold lower levels of relative binding than did the 7G8 peptide (Table 1). Peptides with D at position 2 and I at position 8 are also known to bind to H-2K^{k} (1) (Table 2). In fact, the standard peptide used in our binding assays (Table 1) is S D YE G R L I from influenza nucleoprotein (1) (Table 2), a peptide that was shown to have approximately 10-fold higher levels of relative binding to H-2K^{k} (10 nM) than the 7G8 peptide in the same experiment. Interestingly, the Y A D D I E K K I (GAM5) peptide has a D in position 3 and an I in position 9, a spacing similar to the position 2 and 8 spacing of the influenza peptide, yet the level of binding is 2,000-fold lower than that of the influenza peptide and 2-fold lower than the binding level of the Y A N D I E K K I peptide. These findings suggest that placement of the first anchor amino acid residue in position 2 as opposed to position 3 may be critical for its capacity to bind.

Having established the relative binding of the variant peptides, we assessed their capacity to sensitize target cells for lysis. Substituting an alanine for the glutamic acid in position 2 in the NF54/3D7 peptide reduced binding by 100-fold and eliminated cytolytic activity. Peptide GAM5, Y A D D I E K K I, a peptide that varies from the 7G8 sequence at positions 2 and 3, did not sensitize cells for lysis. In contrast, the PNG3 peptide, which has binding characteristics similar to those of the 7G8 peptide, was able to sensitize target cells for lysis, albeit at a lower level of specific lysis than the 7G8 peptide (Table 1). Substitutions in positions 1 and 3 had only a minimal effect on binding and lysis, indicating that the T-cell receptor recognition is more dependent on other amino acid residues.

These data clearly identify for the first time a K^{k}-restricted 9-amino-acid peptide epitope on PfCSP and demonstrate that amino acid changes at positions responsible for binding to the class I MHC molecule or the T-cell receptor can eliminate the capacity of peptides to be recognized by a T-cell line. Such findings underscore the importance of conducting similar studies in humans to identify target peptides for vaccine development from the PfCSP, the *P. falciparum* sporozoite surface protein 2 (25, 39), *P. falciparum* liver-stage antigen-1 (7, 8), and other preerythrocytic proteins. Furthermore, these data clearly demonstrate that if such peptide vaccines are to be effective in a general population of diverse HLA background and against polymorphic *P. falciparum* parasites, the peptide vaccines will have to include multiple variant sequences that bind to multiple class I HLA molecules.

In studies of CTL in humans immunized with radiation-attenuated sporozoites, we previously reported that peptide PfCSP 7G8 368-390 includes a CTL epitope(s) recognized by three of the four individuals studied (16). A nonamer that binds to HLA-B35 has been identified in this sequence (PfCSP 7G8 368-376) and shown to be recognized by CTL from HLA B35-positive humans naturally exposed to malaria in The Gambia (8). Only two of the three responders that we identified in the first study and none of the four responders that we identified in a study in Kenya (29) were HLA B35 positive. Thus, there must be other CTL epitopes that bind to other HLA molecules included within this 23-amino-acid peptide. Likewise, although we have mapped a nonamer epitope recognized by the H-2K^{k}-restricted CTL line, PfCSP TCL.1, this does not exclude the possibility that there are other H-2^{k}-restricted epitopes within this sequence. For example, since peptide PfCSP 7G8 371-379 binds so well to K^{k}, it is likely that immunization with this peptide, with a recombinant protein including the peptide, or with a native sporozoite protein would induce CTL against this peptide.

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