Mass spectrometric identification of an HLA-A*0201 epitope from Plasmodium falciparum MSP-1

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Abstract

Cytotoxic T lymphocytes (CTL) directed against Plasmodium falciparum-derived antigens were shown to play an important role for the protection against malaria. Although several CTL epitopes have been identified from P. falciparum sporozoite-derived antigens, none has been described for the merozoite form. Since the merozoite surface protein (MSP)-1 is a known target of the immune response, we focused on this protein to identify HLA-A*0201-associated epitopes. Using our mass spectrometry-based method [the 'predict–calibrate–detect' (PCD) approach], we were able to identify an MSP-1-derived epitope in the peptide mixture naturally associated with HLA-A*0201 molecules purified from an MSP-1-expressing cell line. CTLs against this epitope were generated from HLA-A*0201 monochain transgenic mice (HHD). They specifically killed MSP-1-expressing HLA-A2-positive target cells. Thus, we describe here the first MHC class I epitope from the merozoite form of P. falciparum. This epitope can be used as a tool for the immunomonitoring of natural or vaccine-induced CTL immune responses against malaria and could eventually be proposed as a component of an anti-malaria peptide-based vaccine.

Introduction

Plasmodium falciparum is the most pathogenic parasite causative of malaria. Yearly, 300–400 millions clinical cases of P. falciparum infections are reported among which 2–3 millions are deadly (1). Moreover, an increase of the incidence of the disease has been observed due to the emergence of drug-resistant parasites and of insecticide-resistant mosquitoes (2). For these reasons, a protective and cost-effective vaccine against P. falciparum is needed [review by Ballou et al. (3)]. Individuals are exposed to P. falciparum in its sporozoite form that is delivered by anopheles mosquitoes. It infects hepatocytes which release the merozoite form. Then, merozoites infect circulating red blood cells and induce the life-threatening malaria symptoms. Penetration in red blood cells is mediated by several proteins located at the parasite’s surface. Mostly, merozoite surface protein (MSP)-1, MSP-2, MSP-3 and apical membrane antigen 1 are known to participate in this process. Antibodies to any of these proteins can block infection but MSP-1 seems to be the main target of natural (4, 5) and vaccine-induced (6–11) immunity to P. falciparum. MSP-1 is a 190-kDa precursor protein expressed at the surface of the pathogen during the late stages of erythrocytic schizogony. After schizont rupture and release of the merozoite into the plasma, MSP-1 undergoes two subsequent processing that raise four fragments p83, p30, p38 and p42 (from N-term to C-term) (12, 13). The full-length MSP-1 as well as processing fragments expressed in Escherichia coli are evaluated as vaccines (3). While induced MSP-1-specific antibodies are easily monitored by ELISA or western blotting, cell-mediated immunity has been scarcely assessed. Since T cells are instrumental in a natural or vaccine-induced immunity against P. falciparum, there is a need to develop tools that allow the monitoring of the CD8 response against this pathogen in natural infections and vaccine-induced immune responses. Indeed, the monitoring of total anti-MSP-1 IgG was shown to be an inexact correlate of immunity (14). Moreover, Egan et al. (7) and Kumar et al. (8) showed that generation of MSP-1 processing blocking antibodies in vaccinated Aotus vociferans monkeys is not a good prognosis for protection against P. falciparum. Thus, to date, rechallenge with attenuate pathogen remains the only reliable method to monitor immune response against P. falciparum MSP-1-based vaccines, raising obvious ethical questions. The precise characterization of the immunogenic sequences from MSP-1 involved in the cytotoxic immune response, i.e. the MHC class I epitopes, is critical for the in vitro monitoring of the natural or therapeutically induced anti-P. falciparum cytotoxic

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T-lymphocyte (CTL) response in patients or volunteers using the tetramer technology, ELISPOT assays, intracellular cytokine staining or cytotoxicity assays as well as for the development of peptide-based vaccines. In this article, we report the identification by mass spectrometry (MS) (15) of the first HLA class I epitope derived from the \textit{P. falciparum} MSP-1 antigen.

**Methods**

**Tumor cell lines**

EL4S3-Rob/HHD cells are mouse β2m-deficient EL4 cells expressing the HHD molecule (16). HeLa A2 p83/30 cl.3 was kindly provided by Prof. H. Bujard. They express HLA-A2 and, in the presence of doxycyclin, the MSP-1 p80/30 protein from a codon-optimized synthetic gene (17, 18). All cells were kept in RPMI enriched with 10% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin (Life Technologies) supplemented at 1 mg ml\(^{-1}\) G418 (PAA Labor, Linz, Austria) and 0.3 mg ml\(^{-1}\) Hygromycin B (Roche, Mannheim, Germany) for HeLa A2 p83/30 cl.3 transfectants.

**Epitope prediction**

Prediction of potential HLA-A\(^*\)0201 ligands was carried out as previously described by Rammensee et al. (19). Briefly, proteins were screened against a matrix pattern which evaluates every amino acid within nonamer or decamer peptides fitting to the HLA-A\(^*\)0201 motif. Anchor residues are valued 10, other residues 0–10, reflecting amino acid preferences for certain positions within the peptide. The theoretical maximum score for a candidate peptide is 36; scores for abundant natural ligands are typically between 32 and 34. Such motif predictions are available using the database SYFPEITHI on our web page at http://www.uni-tuebingen.de/uni/kxi.

**Peptides**

Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Foster City, CA, USA) following the 9-fluorenylmethyloxycarbonyl/tert. butyl (Fmoc/tBu) strategy. After removal from the resin by treatment with trifluoroacetic acid/phenol/ethanedithiol/thioanisole/water (90/3.75/1.25/2.5/2.5 by volume) for 1 or 3 h (arginine-containing peptides), peptides were precipitated from methyl-tert. butyl ether, washed once with methyl-tert. butyl ether and twice with diethyl ether and re-suspended in water prior to lyophilization. Synthesis products were analyzed by HPLC (System Gold, Beckman Instruments, Fullerton, CA, USA) and matrix-assisted laser desorption/ionization time of flight (TOF) MS (G2025A, Hewlett-Packard, Palo Alto, CA, USA). Peptides of <80% purity were purified by preparative HPLC.

**Peptide-binding assay**

T2 cells were used for peptide-binding studies. A 1 mM peptide stock solution in PBS 10% dimethylsulfoxide was made and cells were incubated with the peptide at a final concentration of 100 μM in RPMI overnight at 37°C. HLA surface expression was monitored on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) after staining of the cells with the primary antibody BB7.2 FITC labeled (DPC Biermann, Bad Nauheim, Germany). Results in Table 1 show the ratio between the mean fluorescence of the stained cells incubated with 100 μM of peptide and the mean fluorescence of the stained cells incubated without peptide. This ratio should be >1.2 in order to conclude that the peptide can significantly bind to HLA-A2. As control, the dominant HLA-A2 epitope from influenza matrix M1 protein was used.

**Isolation of HLA-A2-bound peptides**

HLA-bound peptides were isolated from HeLa A2 p83/30 cl.3 cells according to a slightly modified standard protocol.
was 20–50 nl min \(^{-1}\) from Proxeon Biosystems (Odense, Denmark). The needle Gold-coated glass capillary nanoflow needles were obtained by interpretation of fragment spectra using com-
mal 16 S. In MS/MS experiments, sequence information was with argon atoms. Fragmentation was completed after maxi-
pendent acquisition at the given retention time by collision
online nanocapillary HPLC–MS/MS experiments, fragmenta-
tion that the system was free of any residual peptide. For
formed prior to any subsequent HPLC–MS run in order to en-
HPLC-coupled MS
Predicted MSP-1 epitopes in the form of synthetic peptides and natural HLA class I ligands in HeLa A2 p83/30 cl.3 were analyzed by a reversed-phase Ultimate HPLC system (Dionex, Amsterdam, The Netherlands), coupled to a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrome-
ter (Q-TOF, Micromass, Manchester, UK) equipped with a micro-electrospray ionization (ESI) source. Samples were loaded onto a C\(_{18}\) pre-column for concentration and desalting. After loading, the pre-column was placed in line for separation by a fused silica microcapillary column (75 \(\mu\)m i.d. \(\times\) 250 mm) packed with 5 \(\mu\)m C\(_{18}\) reversed-phase material (Dionex). Sol-
vent A was 4 mM ammonium acetate/water. Solvent B was 2 mM ammonium acetate in 80% acetonitrile/water. Both sol-
vents were adjusted to pH 3.0 with formic acid. A binary gra-
dient of 15–50% B within 120 min was performed, applying a flow rate of 200 \(\mu\)l min \(^{-1}\) reduced to \(~300\) nl min \(^{-1}\) by the
Ultimate split-system. A gold-coated glass capillary (PicoTip, New Objective, Cambridge, MA, USA) was used for sample introduction into the micro-ESI source. A blank run was per-
formed prior to any subsequent HPLC–MS run in order to en-
sure that the system was free of any residual peptide. For
online nanocapillary HPLC–MS/MS experiments, fragmenta-
tion of the parent ion was achieved by automated data de-
dependent acquisition at the given retention time by collision
with argon atoms. Fragmentation was completed after maxi-
mal 16 s. In MS/MS experiments, sequence information was obtained by interpretation of fragment spectra using com-
puter-assisted database searching tools.

Nanoflow ESI–MS
Gold-coated glass capillary nanoflow needles were obtained from Proxeon Biosystems (Odense, Denmark). The needle was filled with 3 \(\mu\)l of sample diluted in 50% methanol/water/ 1% formic acid. A stable spray was observed applying a needle voltage of 800–1200 V, a backpressure of 2 psi and a source temperature of 40\(^{\circ}\)C. The estimated flow rate was 20–50 nl min \(^{-1}\). For nanoflow ESI–MS/MS experiments, fragmentation was achieved by collision with argon atoms. Q1 was set to the mass of interest \(\pm 0.5\) Da and an opti-
mized collision energy applied. The integration time for the TOF analyser was 1 s with an interscan delay of 0.1 s.

Generation of mouse CTLs and cytotoxicity assays
The research using mice has complied with all relevant federal guidelines and institutional policies. HHD mice (16) were injected s.c. twice at 2 weeks interval into the flank with 10 \(\mu\)g of synthetic KLKEFIPKV peptide mixed with 10 \(\mu\)g of oligonu-
cleotide CpG DNA 1826 (22) (Curevac GmbH, Tübingen, Germany) and 90 \(\mu\)l CFA (Sigma, Taufkirchen, Germany). Ten days after the last injection, spleens were removed and splic-
nocytes were put in culture in 10 ml alpha-MEM medium sup-
plemented with 10% FCS (HyClone, Logan, UT, USA), 2 mM l-glutamine, 1% penicillin–streptomycin (Life Technologies), 50 \(\mu\)M \(\beta\)-mercaptoethanol, 10 mM HEPES (Sigma) and 2% of the supernatant from \(\alpha\)-methyl-mannoside-inactivated Con A-stimulated rat spleen. Peptide was added at a final concentra-
tion of 1 \(\mu\)M. Four days later, 30 units of recombinant IL-2 (Prolfkirkin, CHIRON) were added. At day 6, cytotoxicity of the cultures was tested on 5000 unloaded or peptide-loaded EL453-Rob/HHD (16) in an 8-h \(^{51}\)Cr release assay. Specific lysis was calculated as follows: (experimental release – sponta-
aneous release)/(total release – spontaneous release) \times 100. CTL cultures exhibiting detectable peptide specific cytotoxicity were re-stimulated weekly using 20 millions of peptide-
loaded (1 \(\mu\)M peptide for 1 h) fresh irradiated (28 Gy) HHD-II splenocytes. Long-term cultures were used for cytolytic assays on 5000 \(^{51}\)Cr-labeled target cells eventually in the presence of a 50-fold excess of non-labeled EL453-Rob/HHD cells loaded or not with the synthetic KLKEFIPKV peptide.

Results and discussion
Epitope prediction
Potential HLA-A*0201-binding peptides were predicted from the MSP-1 p83/30 protein sequence using our prediction algo-
rithm available at the web page http:\\www.uni-tuebingen.de/ uni/xxi (19). A total of 16 peptides predicted to be the best binders (score >26) were synthesized and tested for HLA-
A*0201 binding by a cell surface MHC class I stabilization assay. The results are presented in Table 1. As positive con-
trol, we used the dominant epitope from Influenza Virus Matrix 1 protein (peptides 58–66). One peptide from MSP-1 (KLKE-
FIPKV) was found to be a very strong binder to HLA-A2. Four
peptides were defined as intermediate binders (KLLDKINEI, SMDQKLLEV, YLIDGYEEI and VLKKEVIDKL) inducing a slight but detectable accumulation of HLA-A2 at the surface of T2 cells (mean fluorescence ratio >1.2). The other 11 peptides showed very weak or undetectable binding capacities. Over-
all, the prediction was fitting with the experimental results: the best binders were peptides with the highest scores.

Detection of a predicted MSP-1 p83/30 epitope by an HPLC–MS system
HeLa-A2 MSP-1 p83/30 cl.3 cells were grown in roller bottles and a cell pellet of 28 ml was produced. It was used for
HLA-A2 purification and peptide extraction as previously de-
dscribed (23). The peptide mixture eluted from HLA-A2 was analyzed on the HPLC–MS system. The resulting HPLC–MS profiles of the sample peptide pool was compared with the
HPLC–MS profile obtained by analyzing synthetic standard
peptides (the self-ligand p68,68–176 YLPLPAIYHI selected as internal control) and synthetic MSP-1 predicted epitopes which were recorded on the same analytical system under identical conditions independently. Co-elution of peptides from the natural sample with molecular masses correspond-
ing to the internal standard (the self-ligand p68,68–176 YLPL-
PAIYHI) confirmed the quality of the peptides eluted from the purified HLA-A2 molecules. A natural ligand with the re-
tention time and mass of the predicted MSP-1 p83/30 pep-
tide MSP-1, 674–692 (KLKEFIPKV) was also detected (Fig. 1). The amino acid sequence of this peptide was confirmed in

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a second experiment by online nanocapillary HPLC–MS/MS, shown in Fig. 1(C). A nanoflow ESI–MS/MS experiment of the synthetic MSP-1 674–682 recorded under identical conditions as in the nanocapillary HPLC–MS/MS experiment confirmed further the identity of the peptide (data not shown). The self-peptide p68 168–176 was present at \( 77 \) pmol per \( 10^{10} \) cells as estimated by comparison of signal intensities of eluted and synthetic peptide. Assuming a loss of peptides during HLA extraction of 50%, this corresponds to \( 9400 \) copies per cell. MSP-1 674–682 was present at \( 3.4 \) pmol per \( 10^{10} \) cells, corresponding to 412 copies per cell. Thus, the predicted HLA-A2 epitope from MSP-1 that showed the highest experimental binding capacity (Table 1) was the peptide that is processed from the endogenously expressed MSP-1 protein and was detectable on a molecular level by MS.

**Generation of anti-MSP-1674-682 HLA-A*0201 restricted mouse CTLs**

HHD-II mice are deficient for H-2 class I molecules and express a chimeric monochain HLA-A*0201 molecule able to educate a diverse repertoire of CD8+ cells (16). These mice received s.c. injections of a synthetic peptide corresponding to the MSP-1674-682 epitope emulsified in CFA adjuvant.
(Sigma) together with 10 μg of oligonucleotide CpG 1826 DNA (22). Ten days after the boost, splenocytes of immunized mice were stimulated in vitro with the synthetic MSP-1674–682 peptide. After 6 days of culture, specific cytoxicity of the CTL line was tested on EL4S3-Rob/HHD target cells (mB2-microglobulin-deficient EL4 cells expressing the HHD molecule) loaded or not with the MSP-1674–682 peptide (data not shown). T-cell lines showing specific killing of peptide-loaded cells were re-stimulated weekly using irradiated, peptide-loaded fresh splenocytes from HHD-II mice. The CTL lines were then used in cytotoxic assays. The target cells were 51Cr-labeled EL4S3-Rob/HHD cells and HeLa A2 p83/30 cells induced with doxycyclin to express the MSP-1 p83/30 protein. The target cells were pre-incubated or not with the synthetic MSP-1674–682 peptide. Figure 2(A) presents the results obtained with two independent T-cell lines (made from two different HHD mice). The CTL lines specifically killed EL4S3-Rob/HHD loaded with the MSP-1674–682 peptide. They also killed doxycyclin-treated HeLa A2 p83/30 target cells whether or not the cells were loaded with the MSP-1674–682 peptide. To confirm that the killing of HeLa-A2 MSP-1 p83/30 by the CTL lines was due to the recognition of the MSP-1674–682 peptide in the context of HLA-A*0201, a cold target inhibition was performed. A 50-fold excess of non-labeled EL4S3-Rob/HHD cells loaded or not with the KLKEFIPKV peptide was added to the 51Cr-labeled, doxycyclin-induced HeLa-A2 MSP-1 p83/30 target cells. As shown in Fig. 2(B), EL4S3-Rob/HHD cells loaded with the peptide but not unloaded EL4S3-Rob/HHD cells could inhibit the killing of doxycyclin-induced HeLa-A2 MSP-1 p83/30 target cells. These experiments showed that the KLKEFIPKV peptide identified by MS is presented by HLA-A*0201 molecules at the surface of cells expressing the MSP-1 p83/30 protein.

Thus, using a combination of epitope prediction and HPLC–MS, the predict-calibrate-detect method (15, 23), an HLA-A*0201-ligand derived from the malaria pathogen P. falciparum antigen MSP-1 was identified. Since mice cannot be infected by P. falciparum, we could not test in vivo whether the natural infection results in the priming of T cells against the KLKEFIPKV epitope. However, since cross-priming is a robust process (24), we anticipate that either directly (presentation by infected cells) or indirectly (presentation by antigen presenting cell that took up dying infected cells) the KLKEFIPKV peptide will be presented by HLA-A*0201 at the cell surface during infection by P. falciparum. Further studies using T lymphocytes from patients vaccinated against P. falciparum or infected by this pathogen are needed in order to confirm that in the human population, the KLKEFIPKV epitope identified through our work is recognized by human cytotoxic T cells. Those studies would finalize the validation of this epitope as a new tool for the monitoring of natural or therapeutically induced cellular immunity against P. falciparum and as candidate for an anti-malaria peptide-based vaccines designed to induce the detection and destruction of cells such as hepatocytes containing and producing the pathogenic merozoite form of P. falciparum.

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**Abbreviations**
- CTL: cytotoxic T lymphocytes
- ESI: electrospray ionization
- MS: mass spectrometry
- MSP: merozoite surface protein
- TOF: time of flight
- PCD: predict-calibrate-defect
References