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Identification and Characterization of HIV-1 Epitopes Presented by HLA-A*2603: Comparison Between HIV-1 Epitopes Presented by A*2601 and A*2603

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ABSTRACT: Human leukocyte antigen (HLA)-A*26 is one of the alleles associated with a slow progression to AIDS. Identification and characterization of HIV-1–specific epitopes presented by this allele are necessary for studies on the immunopathogenesis of AIDS and vaccine development in Asia, where three HLA-A*26 subtypes are frequently found. In the present study, we sought to identify HLA-A*2603–restricted HIV-1 epitopes by using reverse immunogenetics and to compare them with HLA-A*2601–restricted ones recently identified. We found that 31 of 110 HIV-1 peptides bound to HLA-A*2603 and that only two peptides (Gag169-177 and Env63-72) induced specific $CD8^+$T cells by stimulating peripheral blood mononuclear leukocytes from HIV-1–infected individuals carrying HLA-A*2603. The specific cytotoxic T lymphocyte clones killed HIV-1 recombinant vaccinia-infected cells, indicating that these two peptides were naturally occurring peptides presented by HLA-A*2603. Gag169-177–specific $CD8^+$T cells were frequently detected in both HLA-A*$2601^+$and -A*2603^+individuals with chronic HIV-1 infection, whereas Env63-72–specific ones were frequently detected only in the HLA-A*2603^+individuals. Gag169-177 peptide bound equally to both HLA-A*26 antigens, whereas Env63-72 peptide bound to A*2603 much more strongly than to A*2601. These findings suggest that the relative affinity of these peptides for the HLA-A*26 subtypes determines whether these peptides are recognized as epitopes in HIV-1-infected individuals carrying these alleles. *Human Immunology 66, 1155–1166 (2005).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: HIV-1; CTL; HLA-A*2603; epitope; reverse immunogenetics

ABBREVIATIONS

CTL	cytotoxic T lymphocyte
FCS	fetal calf serum
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
IFN-γ	interferon-γ
mAb	monoclonal antibody
PBMC	peripheral blood mononuclear leukocyte
r-HIV-1-vaccinia	HIV-1 recombinant vaccinia virus

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) and neutralizing antibodies have a crucial role in the clearance of virus in the infected host. Studies on influenza virus and cytomegalovirus have shown that CTLs play an important role in antivirus immune systems [1–3]. Similarly, HIV-1–specific CTLs also contribute to the response in both acute and chronic phases of HIV-1 infection [4–6]. Several studies have provided direct evidence for high levels of HIV-1–specific CTLs in HIV-1–infected individuals in whom HIV-1 replication is controlled [7, 8], suggesting that HIV-1–specific CTLs may control HIV-1 replication. However, because HIV-1–specific CTLs fail to completely eradicate HIV-1 from infected individuals, HIV-1 may escape from the host immune system. There

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are several proposed mechanisms that would allow HIV-1–infected cells to escape from being killed by HIV-1–specific $CD8^+$T cells [9–14]. A mutation within the viral epitopes recognized by CTLs is one of these mechanisms [9, 10]. Therefore, identification and characterization of such HIV-1 epitopes are necessary for studies on the immunopathogenesis of AIDS. In addition, because HIV-1–specific CTLs are expected to suppress HIV-1 replication *in vivo*, information about these epitopes is also necessary for studies aimed at developing HIV-1 vaccines and immunotherapy to induce HIV-1–specific CTLs, either of which might be expected to prevent HIV-1 infection and the progression to AIDS.

We previously devised a strategy to determine HIV-1 epitopes by testing whether HIV-1–specific CTLs are induced in peripheral blood mononuclear leukocytes (PBMCs) from HIV-1–seropositive individuals by stimulating the cells with HLA class I–binding HIV-1 peptides [15]. Subsequent studies employing this strategy, which is called reverse immunogenetics, identified a large number of HIV-1 epitopes presented by HLA-A*1101, HLA-A*2402, HLA-A*2601, HLA-A*3303, HLA-B*3501, and HLA-B*5101 [16–23], indicating that this strategy is very useful to identify HIV-1 CTL epitopes.

HLA-A*26 is one of the common alleles in Asian countries. This allele is one of the HLA alleles associated with a slow progression to AIDS [24]. Therefore, the identification and characterization of HIV-1 epitopes presented by HLA-A*26 are required for analysis of CTL responses and escape mutations in progressors and slow progressors carrying HLA-A*26. Three HLA-A*26 subtypes—HLA-A*2601, -A*2602, and -A*2603—have been identified and shown to have allele gene frequencies of 8.1%, 2.3%, and 2.4%, respectively, in Japan [25, 26]. HIV-1 clade B and clade B/C are predominantly found in Japanese and Chinese HIV-1–infected individuals, respectively. Therefore, identification of HIV-1 clade B epitopes is required for studies on AIDS pathogenesis and vaccine development in Japan and China. We recently reported 4 HLA-A*2601–restricted HIV-1 epitopes [20]. In the present study, we identified HIV-1 epitopes presented by HLA-A*2603 by using the reverse immunogenetics technique. Additionally, we compared the identified epitopes with the HLA-A*2601-restricted epitopes recently identified.

MATERIALS AND METHODS

Cells

C1R and TAP-defective cells of mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2603 (C1R-A*2603) were generated by transfecting the C1R cells with the HLA-A*2603 gene [27]. RMA-S transfectants expressing HLA-A*2603 (RMA-S-A*2603) were previously generated [28]. C1R-A*2603 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin, and RMA-S-A* 2603 in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Synthetic Peptides

Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2603 binding motifs. Peptides were prepared by using an automated multiple peptide synthesizer, with the Fmoc (9-Huorenylmethyloxycarbonyl) strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% purity were used in the present study.

HLA-Stabilization Assay

RMA-S-A*2603 cells express empty HLA-A*2603 on their cell surface when they are cultured at 26 °C. The surface expression of empty HLA-A*2603 rapidly decreases after RMA-S-A*2603 cells are incubated at 37 °C, whereas HLA-A*2603 molecules are stably expressed on the surface of the cells at 37 °C if they bind peptides. Binding of HIV-1–derived peptides to HLA-A*2603 was measured as previously described by using RMA-S-A*2603 cells [28]. Briefly, RMA-S-A*2603 cells were cultured for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class $I\alpha_3$domain-specific monoclonal antibody (mAb) TP25.99 [29] and the FITC-conjugated immunoglobulin G fraction of sheep anti-mouse immunoglobulin (Silenius Laboratories, Hawthorn, Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACSCalibur (BD Bioscience, San Jose, CA). HLA-A*2603–binding peptides were defined as those which at a concentration of 10^{-3}M caused a >25 % increase in MFI compared with the MFI of control RMA-S-A*2603 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL_{50} value. Binding peptides were classified into three categories according to the BL_{50}: high binding ($BL_{50} < 10^{-5}$), medium binding ($10^{-5} \leq BL_{50} < 10^{-4}$), and low binding ($BL_{50} \geq 10^{-4}$).

Patients

Blood samples were collected with informed consent from four chronically HIV-1 clade B–infected patients with HLA-A*2603 (KI-001, KI-010, KI-093, and KI-177) at the AIDS Clinical Center, International Medical Center of Japan. The laboratory data of these patients are

as follows: KI-001 (viral load, 8.3×10^3, CD4 count, 223), KI-010 (viral load, undetectable, CD4 count, 525), KI-093 (viral load, 7.1×10^3, CD4 count, 373), and KI-177 (viral load, 5.1×10^5, CD4 count, 684). KI-117 had not been treated with anti-retroviral therapy, whereas the other three patients had.

Detection of Interferon-γ–Producing $CD8^+$T Cells After Stimulation of PBMCs With Peptide-Pulsed C1R-A*2603 Cells

After C1R-A*2603 cells had been incubated for 60 minutes with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These C1R cells (8×10^4 per well) and peptide-pulsed cultured PBMC cells (2×10^4 per well) were added to a 96-well round-bottomed plate and then incubated at 37 °C for 6 h after the addition of Brefeldin A (10 μg/ml). Next, the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4°C for 20 minutes, and then permeabilized at 4 °C for 10 minutes with phosphate-buffered saline supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and then stained with anti-interferon [IFN]-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in phosphate-buffered saline containing 2% paraformaldehyde, and then the percentage of $CD8^+$cells positive for intracellular IFN-γ was calculated from data on 5000 cells obtained by flow cytometry.

Detection of IFN-γ–producing $CD8^+$T Cells After Stimulation With C1R-A*2603 Cells Infected With Recombinant HIV-1 Vaccinia

C1R-A*2603 cells were infected for 1 h at 37 °C with 10 plaque-forming units (per target cell) of recombinant vaccinia virus expressing the desired HIV proteins (Gag and Pol, Nef, Env from SF2), which had been previously generated [30], or of wild-type vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells in a 96-well round-bottomed plate at 37 °C for 6 h after Brefeldin A (10 μg/ml) had been added. The ability of the effector cells to produce IFN-γ was tested at an E: S ratio of 1:4. The cells were then stained with anti-CD8 mAb, fixed with 4% paraformaldehyde at 4 °C for 20 min, and permeabilized at 4 °C for 10 min with the permeabilizing buffer. Next, they were resuspended in permeabilizing buffer and then stained with anti-IFN-γ mAb. The cells were finally resuspended in phosphate-buffered saline containing 2% paraformaldehyde, and then the percentage of $CD8^+$cells positive for intracellular IFN-γ was calculated from data obtained by flow cytometry.

Generation of CTL Clones

Gag169-177–specific CTL clones and Env63-72–specific CTL clones were generated from the HIV-1-specific bulk-cultured T cells by limiting dilution in U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from the healthy donors and 1×10^5irradiated C1R-A*2603 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human rIL-2).

CTL Assay for Target Cells Loaded With Peptides

Cytotoxicity was measured by the standard ^{51}Cr release assay. Target cells (2×10^5) were incubated for 60 min with 150 μCi $Na_2{}^{51}CrO_4$in saline, and washed 3 times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) with the desired amount of the corresponding peptides and incubated for 1 hour at 37 °C. Effector cells at an effector-to-target (E:T) ratio of 2:1 were then added, and the mixtures were incubated for 4 hours at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% TritonX-100. The percent specific lysis was calculated by using the following formula: percentage specific lysis = $100 \times$ (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells.

CTL Assay for Target Cells Infected With HIV-1 Recombinant Vaccinia Virus

C1R-A*2603 cells were infected with 10 plaque-forming units per target cell of HIV-1 recombinant vaccinia virus expressing the desired proteins (Gag and Pol, Nef, Env derived from SF2), or wild-type vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and were then incubated with 150 μCi $Na_2{}^{51}CrO_4$in saline. After incubation for 60 minutes, the infected cells were washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to each well of a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) with effector cells at an effecter-to-target ratio of 2:1. The mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous

TABLE 1 List of HLA-A*2603-binding HIV-1 peptides

	Sequence	Position	BL50
High binding	EVIPMFSAL	Gag 169-177	6.7×10^{-7}
	EVIPLTEEA	Pol 446-454	1.4×10^{-6}
	EVHNVWATHA	Env 63-72	1.9×10^{-6}
Medium binding	ELYPLTSLRSL	Gag 484-494	1.3×10^{-5}
	ETINEEAAEW	Gag 205-214	3.3×10^{-5}
	ETPGIRYQY	Pol 293-301	5.9×10^{-5}
	EVFRPGGGDM	Env 464-473	6.2×10^{-5}
Low binding	ELKKIIGQV	Pol 872-880	1.1×10^{-4}
	EVAQRAYRA	Env 830-838	5.5×10^{-4}
	ELVSQIIEQLI	Pol 671-681	6.3×10^{-4}
	EVHNVWATHAC	Env 63-73	10^{-4}–10^{-3}
	DIVIYQYMDDL	Pol 1332-342	10^{-4}–10^{-3}
	EVNIVTDSQYA	Pol 647-657	10^{-4}–10^{-3}
	ETKLGKAGYV	Pol 1603-613	10^{-4}–10^{-3}
	EVYYDPSKDL	Pol 1471-480	10^{-4}–10^{-3}
	EILGHRGWEA	Env 782-791	10^{-4}–10^{-3}
	EVVIRSDNF	Env 272-280	10^{-4}–10^{-3}
	DIVIYQYM	Pol 332-339	10^{-4}–10^{-3}
	EVNIVTDSQY	Pol 647-656	10^{-4}–10^{-3}
	EVQLGIPHPA	Pol 244-253	10^{-4}–10^{-3}
	ELRSLYNTV	Gag 74-82	10^{-4}–10^{-3}
	ELYPLTSLRS	Gag 484-493	10^{-4}–10^{-3}
	EICGHKAIGTV	Pol 121-131	$>10^{-3}$
	EIKGEIKNCSF	Env 147-157	$>10^{-3}$
	ETWEAWWMEY	Pol 551-560	$>10^{-3}$
	EILGHRGWEAL	Env 782-792	$>10^{-3}$
	EVYYDPSKDLV	Pol 471-481	$>10^{-3}$
	EVVLGNVTENF	Env 82-92	$>10^{-3}$
	ETVPVKLKPGM	Pol 161-171	$>10^{-3}$
	DLLLIAARTV	Env 786-795	$>10^{-3}$
	ELVSQIIEQL	Pol 671-680	$>10^{-3}$

^{51}Cr release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% TritonX-100. The percent specific lysis was calculated by the following formula: percentage specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effecter cells.

RESULTS

Identification of HLA-A*2603-Binding HIV-1 Peptides

HLA-A*2603-binding peptides have 2 anchor residues, Val, Thr, Ile, Leu or Phe at position 2 [P2] and Tyr, Phe, Met or Leu at the C-terminus [27]. A previous study demonstrated that acidic amino acids (Asp and Glu) and a broad range of amino acids with the exception of positively charge amino acids function as an anchor at position 1 and the C terminus, respectively [28]. Therefore, to identify HLA-A*2603–binding HIV-1 peptides, 8-mer to 11-mer sequences containing the anchor residues Asp or Glu at position 1; Val, Thr, Ile, Leu or Phe at position 2; and all amino acids except positively charged amino acids at the C-terminus were selected from the sequence of Gag, Pol, Nef, and Env proteins in the HIV-1 SF2 strain; and then 110 peptides matched to these sequences were synthesized. The binding affinity of these synthesized peptides for the HLA-A*2603 molecule was tested by using an HLA-stabilization assay. Thirty-one of 110 peptides bound to HLA-A*2603 (Table 1). On the basis of their binding ability (BL50), the binding peptides were classified into 3 classes—*i.e.*, high (BL50 $< 10^{-5}$), medium ($10^{-5} \leq BL_{50} < 10^{-4}$), and low affinity ($BL_{50} \geq 10^{-4}$). Of the 31 HLA-A*2603-binding peptides, 3, 4, and 24 peptides were high-, medium-, and low-affinity peptides, respectively (Table 1). One 8-mer, seven 9-mers, twelve 10-mers, and eleven 11-mer peptides bound to HLA-A*2603.

Further analysis demonstrated that the ability of peptides carrying Glu at P1 to bind HLA-A*2603 was much stronger than that of those carrying Asp at P1 (Table 2), indicating that HLA-A*2603 favored Glu at P1. Similarly, the results showed that HLA-A*2603 favored Val

TABLE 2 Effect of residues at P1, P2 on the binding of peptides to HLA-A*2603

Amino acid at position 1 (NBP*1/NTP*2)		Amino acid at position 2 (NBP*1/NTP*2)			
E	D	V	T	L	I
28/72 (38.9%)	3/38 (7.9%)	13/24 (54.2%)	5/22 (33.7%)	7/37 (18.9%)	6/27 (22.2%)

*1 Number of binding peptides.
*2 Number of total peptides tested.

and Thr more than Leu and Ile at P2 (Table 2). These results support those of a previous study [20].

Induction of HIV-1 Peptide-Specific CD8$^+$T Cells From PBMCs of HLA-A*2603$^+$HIV-1–Infected Individuals

PBMCs from four chronically HIV-1–infected individuals with HLA-A*2603 (KI-001, KI-010, KI-093, and KI-177) were cultured for 10–16 days after they had been stimulated with cocktails of HLA-A*2603-binding peptides (cocktail 1: Gag169-177, Pol446-454, Env63-72, Gag484-494, and Gag205-214; cocktail 2: Pol293-301, Env464-473, Pol121-131, Env147-157, Pol551-560, and Env63-73; cocktail 3: Pol332-342, Pol872-880, Pol647-657, Env830-838, Pol603-613, and Pol471-480; cocktail 4: Env782-792, Env782-791, Env272-280, Pol471-481, Env82-92, Pol332-339 and Pol647-656; and cocktail 5: Pol161-171, Pol671-681, Env786-795, Pol244-253, Pol671-680, Gag74-82, and Gag484-493). Peptides with similar binding affinity were pooled in the same cocktails to reduce the effect of peptide competition on binding to HLA-A*2603. Additionally, they were restimulated with phytohaemagglutinin (PHA)-induced autologous cells pulsed with cocktail peptides and then were cultured for 5–7 days. The cultured cells were subsequently tested for IFN-γ production by CD8$^+$T cells after the cultured cells had been stimulated with peptide cocktail-pulsed C1R-A*2603 cells. The cocktail 1 and cocktail 2 induced the specific CD8$^+$T cells among PBMCs from four HIV-1 infected individuals and one individual, respectively. In contrast, cocktail 3, 4, or 5 failed to induce specific CD8$^+$T cells among PBMCs from any of the four individuals (data not shown).

To determine which peptides in these cocktails induced the specific CD8$^+$T cells, we stimulated the cultured cells from these four individuals with C1R-A*2603 cells pulsed with each peptide in cocktails 1 and 2. Only Gag169-177 and Env63-72 peptides induced the specific CD8$^+$T cells (Figure 1). Gag169-177–specific CD8$^+$T cells were detected as a high percentage of the cultured cells from three individuals (KI-001, KI-010, and KI-093), whereas Env63-72–specific ones were detected among those from only one individual (KI-177, Table 3). These results were confirmed in a different experiment using PBMC from KI-093, KI-010, and KI-177 (data not shown).

Identification of naturally occurring HIV-1 epitopes presented by HLA-A*2603

To clarify whether Gag169-177 and Env63-72 are naturally occurring peptides, we investigated the ability of these peptide-specific CD8$^+$T cells to produce IFN-γ after stimulation of them with C1R-A*2603 cells infected with recombinant HIV vaccinia virus. IFN-γ–producing cells were weakly induced in the cultures containing the Gag169-177–specific CD8$^+$T cells after stimulation with r-HIV vaccinia-infected cells, whereas they were not detected in those stimulated with wild-type vaccinia-infected cells (Figure 2). On the other hand, IFN-γ–producing cells were strongly induced in the cultures containing the Env63-72–specific CD8$^+$T cells after stimulation with r-HIV vaccinia-infected cells

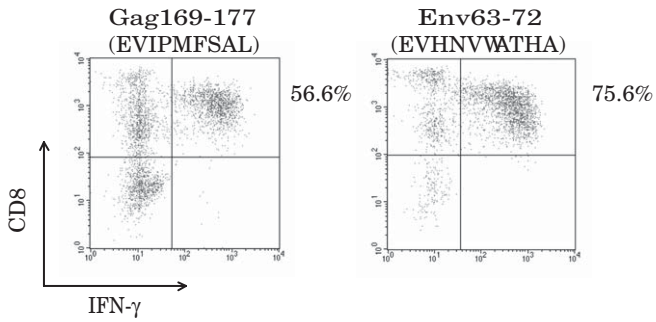

FIGURE 1 Induction of HIV-1–specific CD8$^+$T cell from PBMCs of HIV-1–infected individuals carrying HLA-A*2603. Peripheral blood mononuclear leukocytes from 2 HIV-1–infected HLA-A*2603$^+$individuals (KI-093 and KI-177) were stimulated with the cocktail of HLA-A*2603–binding peptides, and then were cultured for 11–14 days. The cultured cells were stimulated with C1R-A*2603 cells pulsed with individual peptides in the cocktails of HLA-A*2603–binding peptides. Interferon (IFN)-γ–producing CD8$^+$T cells were measured by using flow cytometry. Two peptides (Gag169-177 and Env63-72) induced specific CD8$^+$T cells. The percentage of IFN-γ–producing CD8$^+$T cells is presented to the right of each plot.

TABLE 3 Induction of peptide-specific $CD8^+$ T cells in cultured cells stimulated with each peptide

Sequence	Position	% of IFN-γ-producing cells in $CD8^+$ T cells			
		KI-001	KI-010	KI-093	KI-177
EVIPMFSAL	Gag 169-177	37.6	21.3	56.7	2.5
EVHNVWATHA	Env 63-72	1.8	2.8	0.5	75.6

(Figure 2). The difference in IFN-γ production between these two CTLs stimulated with r-HIV vaccinia-infected cells might be explained by lower expression of the Gag169-177 epitope on r-HIV vaccinia-infected cells though this possibility remains unproved. These results indicate that Gag169-177 and Env63-72 are naturally occurring HIV-1 epitope peptides.

Killing of Epitope Peptide-Pulsed and HIV-1-Recombinant Vaccinia–Infected Cells by Specific CTL Clones

Because IFN-γ–producing cells were weakly induced in the cultures containing the Gag169-177–specific $CD8^+$ T cells after stimulation with r-HIV vaccinia-infected cells, it remained unclear whether Gag169-177 is truly a naturally occurring HIV-1 epitope. Therefore, we first established Gag169-177– and Env63-72–specific CTL clones for further study. Two Gag169-177–specific (Gag169-A*2603-14 and Gag169-A*2603-23) and two Env63-72-specific (Env63-A*2603-1 and Env63-A*2603-10) CTL clones were established from patients KI-010 and KI-177, respectively. These clones effectively killed epitope peptide-pulsed C1R-A*2603 cells, but failed to kill the cells that had not been pulsed with the peptides (Figure 3A). These CTL clones effectively killed C1R-A*2603 cells infected with r-HIV-1 vaccinia virus but not the cells infected with the wild-type vac-

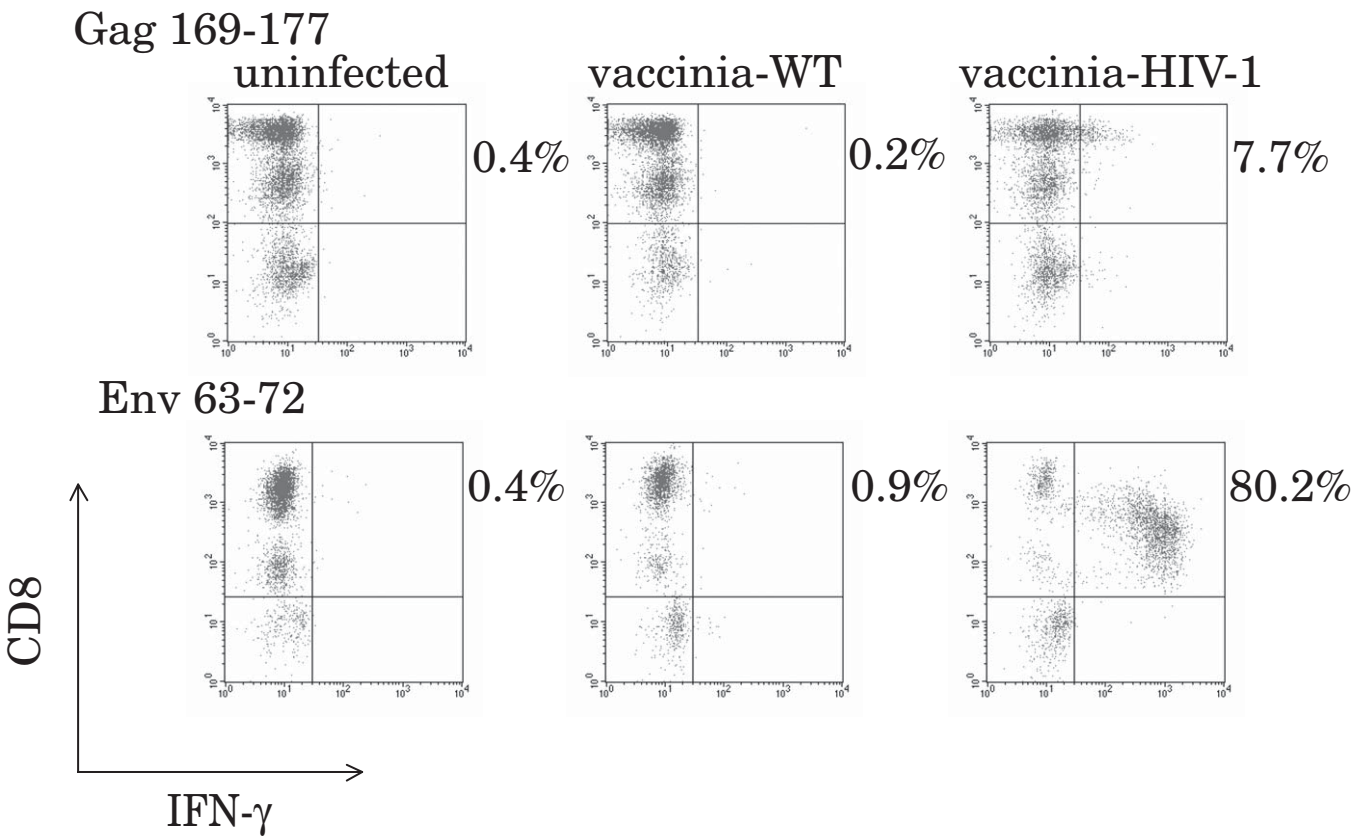

FIGURE 2 Recognition of HLA-A*2603–restricted HIV-1 epitopes on r-HIV-1 vaccinia–infected cells by Gag166-177 and Env 63-72–specific $CD8^+$T cells. Cultures containing Gag169-177 or Env63-72–specific $CD8^+$T cells were examined for interferon (IFN)-γ production after having been stimulated with C1R-A*2603 cells infected with wild-type vaccinia (vaccinia-WT), with r-HIV-1 vaccinia (vaccinia-HIV-1), or left uninfected (uninfected). IFN-γ–producing $CD8^+$T cells were measured by using flow cytometry. The percentage of IFN-γ–producing $CD8^+$T cells is presented at the right of each plot.

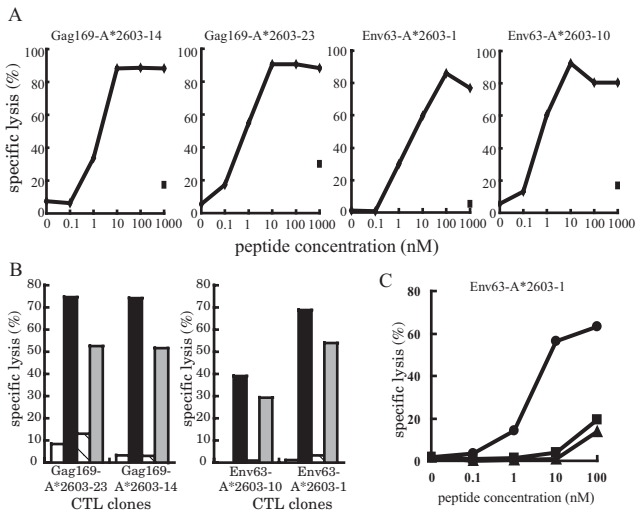

FIGURE 3 Killing of epitope peptide-pulsed or r-HIV-1 vaccinia–infected cells by Gag169-177 and Env63-72–specific CTL clones. (A) The cytotoxic activities of HLA-A*2603–restricted cytotoxic T lymphocyte (CTL) clones toward C1R-A*2603 cells prepulsed with 0.1–1000 nM epitope peptides (◆) and C1R cells prepulsed with 1000 nM epitope peptides (■) were measured at an effecter-to-target ratio (E:T) of 2:1. (B) The activities of HLA-A*2603–restricted CTL clones toward C1R-A*2603 cells infected with r-HIV-1 vaccinia (shaded bar), wild-type vaccinia (cross-hatched bar), or left uninfected (opened bar) or prepulsed with 1 μM epitope peptide (filled bar) were tested at an E:T ratio of 2:1. C. The cytotoxic activity of an Env63-72–specific CTL clone, Env-63-A*2603-1, toward C1R-A*2603 cells prepulsed with Env63-72 or two shorter peptides were measured at an E:T of 2:1. C1R-A*2603 cells prepulsed with 0.1–1000 nM Env63-72 (●), Env63-71 (■), or Env63-70 (▲) peptide were used as target cells.

cinia virus (Figure 3B). These results confirmed both of these peptides to be naturally occurring ones presented by HLA-A*2603.

The newly identified Env63-72 epitope carries Ala at its C-terminus. It remains possible that Env63-72 is not a minimal epitope, because Ala at the C-terminus has not been reported to be anchor for HLA-A*2603 [27]. To clarify that Env63-72 is minimal epitope, we investigated the binding of two shorter peptides, EVHNVWATH and EVHNVWAT, and the activity of Env63-72–specific CTL clone to kill target cells prepulsed with these peptides. The binding ability of these shorter peptides was more than 100-fold lower than that of the Env63-72 peptide (data not shown). The Env63-72–specific CTL clone weakly killed the target cells prepulsed with these shorter peptides at 100 nM, but failed to kill those with the peptides at 1 or 10 nM, whereas this clone effectively killed the target cells prepulsed with the 10-mer peptide (Figure 3C). These results indicate that the Env63-72 10-mer peptide was indeed the minimal epitope.

Comparison of Ability of the Epitope Peptides to Induce Specific CTLs Between HLA-A*2603– and HLA-A*2601–Restricted Epitopes

We recently identified 4 HLA-A*2601-restricted HIV-1–specific epitopes [20]. The frequency of specific CTLs and the peptide-binding ability were compared between HLA-A*2601 and HLA-A*2603 (Table 4). Gag169-177-specific $CD8^+$T cells were detected in five of seven HIV-1–infected HLA-A*2601 individuals and in all four HIV-1–infected HLA-A*2603 individuals, suggesting that Gag169-177 was an immunodominant epitope in HIV-1–infected individuals carrying either HLA-A*26 subtype. Pol604-612-specific $CD8^+$T cells were predominantly detected in HLA-A*2601 individuals, whereas this epitope peptide failed to bind to HLA-A*2603, suggesting that T cells were found in three of the four HIV-1–infected HLA-A*2603 individuals but in none of the HLA-A*2601 ones. Therefore Env63-72 may be recognized as an immunodominant epitope only in HLA-A*2603 individuals. This peptide could not be presented by HLA-A*2603. Two other epitope (Pol647-656 and Env464-473)-specific $CD8^+$T cells were detected in only one of seven HIV-1–infected HLA-A*2601 individuals, but in none of the four HIV-1–infected HLA-A*2603 individuals, thus indicating them not to be immunodominant epitopes. Env63-72–specific $CD8^+$T cells were found in three of the four HIV-1–infected HLA-A*2603 individuals but in none of the HLA-A*2601 ones. Therefore Env63-72 may be recognized as an immunodominant epitope only in HLA-A*2603 individuals.

TABLE 4 Comparison of binding affinity of HLA-A*26 epitope peptides between HLA-A*2601 and A*2603

	Binding affinity (BL50)			Epitopes	
	A*2601	A*2603	A*2603/A*2601	A*2601	A*2603
Gag169-177	1.1×10^{-6}	6.7×10^{-7}	0.61	5/7	4/4
Env63-72	10^{-4}–10^{-3}	1.9×10^{-6}	0.002–0.02	0/7	3/4
Pol604-612	2.5×10^{-5}	No binding	>40	4/7	Not tested
Pol647-656	4.6×10^{-5}	10^{-4}–10^{-3}	2.17–21.7	1/7	0/4
Env464-473	1.7×10^{-6}	6.2×10^{-5}	36.47	1/7	0/4

TABLE 5 Variation of HLA-A*2603-restricted epitopes in clades A–E

Epitope	Sequence	Clade A	Clade B	Clade C	Clade D	Clade E
Gag169-177	EVIPMFSAL	10/18	83/101	2/118	7/9	16/17
	------T--	4/18	9/101	109/118	2/9	
	------A--		3/101			
	-I-------	1/18				
	----V----	1/18				
	G--------	1/18				
	K--------		2/101			
	-G-------		1/101			
	------P--					1/17
	-I----A--		1/101			
	-M----A--		1/101			
	------TT-		1/101			
	-I----T--			4/118		
	-E----T--			1/118		
	----I-T--			1/118		
	----V-T--	1/18				
	-I--I-T--			1/118		
Env63-72	EVHNVWATHA	21/64	118/176	106/122	1/49	25/35
	-A--------	6/64	24/176	3/122	3/49	2/35
	-K--------	18/64	11/176		1/49	
	----I-----	1/64	4/176	6/122	3/49	3/35
	-M--------	9/64	3/176	2/122		1/35
	-A--I-----	1/64	2/176	1/122	34/49	1/35
	G---------		2/176			
	----M-----		1/176			
	-----S----	1/64	1/176			
	-K--I-----	1/64	1/176		2/49	
	-K---S----	2/64				
	-------I--		1/176			
	-I--------		3/176			
	--------Y-		1/176			
	-------K--		1/176			
	--------Q-		1/176			
	-R--------	1/64	1/176			
	--Y-I-----					
	-Q--------	1/64				
	-Q-I------	1/64				
	-A---S----	1/64				
	-G--------			1/122		
	----A-----			1/122		
	----T-----			1/122		
	------G-F-			1/122		
	GA--I-----				2/49	
	-A--I--S--				2/49	
	-A-DI-----				1/49	
	---------V		1/176			1/35
	------T---					1/35
	-MRSI-----					1/35

Recognition of Specific CTL Clones for the Variant Epitopes

We searched HIV-1 sequences of HIV-1 clades A–E, which are the major subtypes in the world (HIV sequence database, Los Alamos, NM), to identify variants of these epitopes. The sequences of Gag169-177 (EVIPMFSAL) and Env63-72 (EVHNVWATHA) were found in 83 of 101 HIV-1 clade B isolates (82.2%) and in 118 of 176 HIV-1 clade B isolates (67.0%), respectively (Table 5). We investigated the binding ability of three variants of Gag169-177 that were found at relatively high frequency in HIV-1 clade B isolates. The binding ability of two of these Gag167-177 variants, Gag 169-177-7T and Gag169-177-7A, to HLA-A*2603 was almost identical to that of Gag167-177, whereas Gag 169-177-1K failed to bind to HLA-A*2603 (Figure

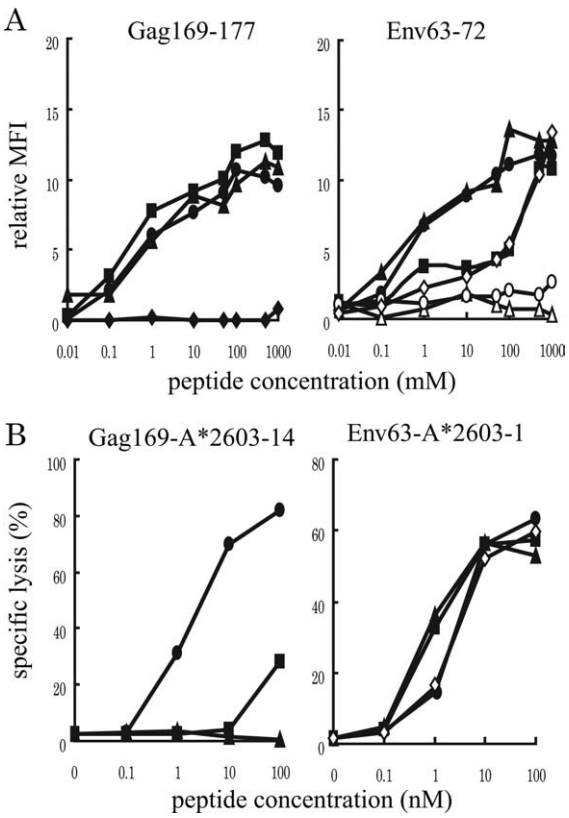

FIGURE 4 Binding of variant epitope peptides to HLA-A*2603 and the recognition of these peptides by specific CTL clones. (A) Binding affinities of natural HIV-1 clade B variant peptides to HLA-A*2603. (Left) The binding of Gag169-177 (●) peptide and its mutants -7T (■), -7A (▲) and -1K (◆) to HLA-A*2603. (Right) The binding affinities of Env63-72 (●) and its variants -2A (■), -2K (△), -5I (▲), -2M (○), -2I (◇) for HLA-A*2603. (B) Recognition of natural HIV-1 clade B variant peptides by HLA-A*2603–restricted CTL clones. (Left) The cytotoxic activities of the Gag169-177–specific cytotoxic T lymphocyte (CTL) clone toward C1R-A*2603 cells prepulsed with Gag169-177 (●) and its variants Gag169-177-7T (■), or -7A (▲). (Right) The cytotoxic activities of Env63-72–specific CTL clone toward C1R-A*2603 cells prepulsed with Env63-72 (●) or its variants Env63-72 -2A (■), -5I (▲), or -2I (◇).

4A). These results strongly suggest that the Gag167-177–specific CTL clone could not recognize Gag 169-177-1K peptide. Therefore, we investigated the ability of the Gag167-177–specific CTL clone to kill C1R-A*2603 cells prepulsed with Gag 169-177-7T or Gag169-177-7A peptide. The Gag 167-A*2603-14 clone hardly killed the target cells prepulsed with these peptides (Figure 4B). These results indicate that these 3 mutations are critical for the binding to HLA-A*2603 or the recognition of TCR on the specific CTLs.

We also investigated the binding ability of five variants of Env63-72 that were found at relatively high frequency in HIV-1 clade B isolates. The binding ability of the Env-63-72-5I variant peptide was almost identical to that of Env63-72 peptide, whereas that of Env-63-72-2I and Env-63-72-2A variant peptides was decreased. Env-63-72-2M and Env-63-72-2K variant peptides failed to bind to HLA-A*2603 (Figure 4A). Therefore, we investigated whether the specific CTL clone would recognize the former three variant peptides. The Env 63-A*2603-1 CTL clone effectively killed C1R-A*2603 cells prepulsed with Env-63-72-5I, Env-63-72-2I, or Env-63-72-2A variant peptide (Figure 4B). These results suggest that the two variants Env-63-72-2M and Env-63-72-2K may be escape mutants.

DISCUSSION

A previous study that analyzed the sequences of self-peptides eluted from HLA-A*2603 molecules identified the anchor residues of HLA-A*2603-binding peptides (P2: Val, Thr, Ile, Phe, or Leu; C terminus: Phe, Tyr, Met, or Leu) [27]. A subsequent study using an HLA-A*2603 stabilization assay confirmed the anchor residues at P2 and the C-terminus by using mutated peptides at P2 and the C-terminus, and further revealed by using 38 peptides that Glu/Asp and non-polar amino acids are preferred at P1 and the C-terminus, respectively [28]. In the present study using 110 8- to 11-mer peptides, we confirmed these anchor residues at P1, P2, and the C-terminus. The ability of peptides carrying Glu at P1 to bind HLA-A*2603 was much stronger than that of those carrying Asp at P1, indicating that HLA-A*2603 favored Glu at P1. Similarly, the results showed that HLA-A*2603 favored Val and Thr more than Leu and Ile at P2 (Table 2). Similar results were observed for the binding of the peptides to HLA-A*2601 [20].

The Env63-72 epitope carries Ala at its C-terminus. A previous study of peptide elution from HLA-A*2603 showed that peptides eluted from this allele carry Tyr, Phe, Met or Leu, but not Ala at the C-terminus [27], implying the possibility that Env63-72 10-mer peptide is not minimal epitope. However, the results on binding of these shorter peptide to HLA-A*2603 and CTL recognition of the peptides showed that this 10-mer peptide was in fact the minimal epitope. A previous study demonstrated that a peptide carrying Ala at the C-terminus effectively bound to HLA-A*2603 [28]. The present study also revealed that six peptides carrying Ala at their C-terminus bound to HLA-A*2603. Thus, peptides carrying Ala at the C-terminus can effectively bind to HLA-A*2603, and it is likely that peptides carrying C-terminal Ala can be presented as T-cell epitopes. In fact, 22 HIV-1 CTL epitopes carrying Ala at the C-terminus have been reported (Los Alamos National Laboratory HIV Molecular Immunology Data Base: http://www.hiv.lanl.gov/content/immunology/index.html).

Gag169-177–specific $CD8^+$T cells were induced in chronically HIV-1–infected individuals with either HLA-A*2603 or HLA-A*2601, whereas Env63-72–specific and Pol604-612–specific ones were induced only in HLA-A*2603 and HLA-A*2601, respectively. The ability of the Gag169-177 peptide to bind HLA-A*2603 was nearly the same as that to bind HLA-A*2601. The binding ability of Env63-72 peptide toward HLA-A*2603 was much higher than that toward HLA-A*2601, but that of Pol604-612 toward HLA-A*2603 was much weaker than that toward HLA-A*2601 (Table 4). These findings suggest that the relative binding ability of these peptides to these HLA-A*26 subtypes may determine whether these peptides are recognized as epitopes in HIV-1–infected individuals carrying these alleles. These alleles differ at residues 74, 76, and 77 on the α_1 helix (A*2601: D74, A76, and N77; A*2603: H74, V76, and D77) [31, 32]. This indicates that HLA-A*2603 molecules carry a negatively charged F-pocket, which is determined by D77. The difference in F-pocket between these alleles may have an effect on the binding of both Env63-72 and Pol604-612 peptides. Thus, the subtle differences between HLA class I subtypes influenced the binding affinity of HIV-1 peptides and the recognition of specific T cells. These findings suggest a restricted advantage of HIV-1 vaccine development using an HLA peptide-binding super-motif for HIV-1 vaccine development.

The sequence of the Gag169-177 epitope was relatively conserved in clades B, D, and E (Table 5), implying that this peptide may be recognized as epitopes in the HIV-1–infected individuals infected with clade D or E virus. Indeed, a previous study revealed that HLA-A*1101–restricted clade B CTL epitopes, whose sequences were conserved between clades B and E, were recognized as epitopes by CTLs in the clade E–infected individuals [33]. Several variants were found at positions 1, 2, and 7 in clade B (Table 5). The substitution to Lys at position 1 of this epitope critically affected the binding to HLA-A*2603. The substitutions to Thr and Ala at position 7 of the epitope affected T-cell recognition but did not affect the binding to HLA-A*2603, indicating that these substitutions affected the recognition of TCR. Thus, these variants, which are found at a relatively high frequency in clade B, critically affect the recognition by Gag169-177–specific CTLs. Interestingly, the Gag169-177 variant carrying Thr at position 7 (Gag169-177-7T) is the consensus sequence in clade C. A previous study showed that HLA-A*1101–restricted HIV-1 clade B mutant epitopes, which were predominantly found in clade E, were frequently recognized as CTL epitopes in clade E–infected individuals [33], implying the possibility that Gag169-177-7T is recognized as an epitope in the clade C–infected individuals. Forty-five of 176 clade B isolates (25.6%) carried a mutation at position 2 of the Env63-72 epitope (Table 5). The common variants are the substitution of Val by Ala, Lys, Met, or Ile at position 2. Of these variants, the mutations to Lys and Met critically affected the binding to HLA-A*2603. These variants may thus be escape mutants.

Interestingly, Gag169-177 includes the HLA-Cw*0102-restricted 8-mer epitopes (VIPMFSAL) [34]. HLA-Cw*0102 is the most common HLA-C allele in Japan [26]. Therefore, induction of both HLA-A*2601/2603–restricted CTLs and HLA-Cw*0102–restricted ones would be expected in donors carrying both alleles. Therefore, we speculated that this epitope would be useful for the development of an HIV-1 vaccine for the Japanese population. The HLA-B*57-restricted immunodominant epitope KF11, which is KAFSPEVIPMF [35], overlaps with Gag169-177. Thus, the region including these epitopes might be used in an HIV-1 vaccine.

HLA-A*26 is one of the HLA alleles associated with a slow progression to AIDS [24]. Because this allele is found in approximately 20 % of Japanese and Asian populations [25, 26], the identification of HLA-A*26–restricted HIV-1 epitopes is important for studies on the immunopathogenesis of AIDS and vaccine development in Asian countries. In the present study, we identified 2 HLA-A*2603-restricted HIV-1 epitopes. Gag169-177 was presented by both HLA-A*2601 and -A*2603, whereas only HLA-A*2603 presented Env63-72. Gag 169-177 is a relatively conserved epitope, and the specific CTLs were frequently detected in chronically HIV-1–infected individuals with either HLA-A*2601 or HLA-A*2603. We therefore speculate that Gag169-177–specific CTLs play an important role in the suppression of HIV-1 replication in slow progressors. If Gag169-177–specific CTLs have a strong ability to suppress HIV-1 replication, the vaccine that can induce Gag169-177 epitope would be expected to be useful in the areas where HLA-A26 is frequently found. Further analysis of these epitopes is expected to be beneficial to studies on the immunopathogenesis of AIDS and vaccine development.

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