Identification and Characterization of Multiple HLA-A24-Restricted HIV-1 CTL Epitopes: Strong Epitopes Are Derived from V Regions of HIV-1[1]

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HIV-1-specific CTL has a crucial role in the elimination of the virus. However, a restricted number of common HLA class I alleles has been studied for their presentation of HIV-1 CTL epitopes. We have attempted to identify HIV-1 CTL epitopes presented by HLA-A*2402 using reverse immunogenetics. Fifty-three HLA-A*2402-binding HIV-1 peptides were used to induce specific CTL in PBL of four HIV-1-infected individuals carrying HLA-A24. Twelve peptides were strongly suggested to be HLA-A*2402-restricted HIV-1 CTL epitopes because these peptides induced the specific CTL that killed both the target cells pulsed with the specific peptides and those infected with the vaccinia HIV-1 recombinant virus in at least one HIV-1-infected individual. Of these epitopes, 11 were confirmed by the generation of the specific CTL clones. Six were the Env epitopes while three, one, and one were derived from Gag, Pol, and Nef proteins, respectively. Analysis of 12 HIV-1-infected individuals using these peptides showed that 5 derived from the Env protein and one from the Nef protein were strong epitopes. These strong epitopes were derived from the diverse region of HIV-1 while weak epitopes were conserved in the HIV-1 clade B strain. Analysis of CTL recognition of mutations in these strong epitopes suggested that the mutations in the Env epitopes may critically influence CTL recognition in vivo. *The Journal of Immunology,* **1997, 159: 6242–6252.**

HIV-1-specific CTL can be detected early after HIV infection (1, 2) and in individuals repeatedly exposed to HIV-1 yet remaining uninfected (3, 4). Strong HIV-1-specific CTL activity is detected in long-term nonprogressors whereas rapid progressors have low HIV-1-specific CTL activity (5). These findings suggest that the infection of HIV-1 is protected by the specific CTL and the progression to AIDS can be controlled by the maintenance of its activity. In most HIV-1 infected individuals, high HIV-1-specific CTL activity is detected in an early clinical course, but the CTL activity falls within a few years (5). There are several hypotheses for the mechanism of the failure of elimination of HIV-1. Nowak et al. (6) proposed an "antigenic diversity threshold" theory that CTL response to variable HIV-1 epitopes will cause an ever-increasing antigenic diversity of the virus population, which will create weak epitopes, so that the immune system in HIV-1-infected individuals fails to eliminate the virus. In contrast, the recent study of Wolinsky et al. (7) showed that an increase in genetic diversity of HIV-1 was found in long-term nonprogressors whereas conserved HIV-1 populations were observed in rapid progressors, implying that an increasing antigenic diversity may stimulate the immune responses. TCR antagonism is another explanation for the failure of elimination of HIV-1. Recent studies showed a case of TCR antagonism for HIV-1-specific, HLA-B8-restricted CTL (8, 9), although it is unknown how much TCR antagonism contributes to the failure of immunologic elimination of HIV-1.

The identification of epitopes recognized by HIV-specific CTL is expected to analyze the CTL response in the course of HIV-1 infection and to be useful for the development of vaccines for the treatment and prevention of HIV infection. Most T cell epitopes of virus proteins have been mapped by the joint use of CTL lines, clones, recombinant vaccinia viruses, and overlapping synthetic peptides (10–15). Difficulties in the generation of human CTL clones and the complexity of the mapping procedure yielded incomplete knowledge of HIV-1 CTL epitopes. We recently identified multiple HIV-1 CTL epitopes presented by HLA-B*3501 molecules (16) using reverse immunogenetics, which is a strategy to identify CTL epitopes from a panel of peptides matched to an HLA class I-binding peptide motif (17). In the present study, we have employed this strategy to identify HIV-1 CTL epitopes presented by HLA-A*2402, which is the most popular HLA class I allele in Japan (18) and a common allele worldwide (19). The epitopes were confirmed by generating the specific CTL clones and were further characterized. The analysis of HLA-A24-restricted HIV-1 CTL in HIV-1-infected individuals carrying HLA-A24 showed that they have strong HLA-A24-restricted CTL for multiple epitopes derived from the diverse region of HIV-1.

Materials and Methods

Cells

C1R cells expressing HLA-A*2402 (C1R-A*2402) and RMA-S cells expressing HLA-A*2402 (RMA-S-A*2402) were previously generated (20,

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21). C1R and RMA-S cells were cultured in RPMI 1640 medium containing 10% FCS. C1R-A*2402 and RMA-S-A*2402 cells were cultured in RPMI 1640 medium containing 10% FCS and 0.15 mg/ml hygromycin B. EBV-transformed cell line Tm (HLA-A11/A24, B52/B52, Cw7/Cw*1202) were previously generated (22) and were maintained in RPMI 1640 medium supplemented with 10% FCS.

Synthetic peptides

Peptides were prepared utilizing an automated multiple peptide synthesizer (Shimadzu model PSSM-8, Shimadzu Co., Kyoto, Japan), with F-moc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry.

Peptide-binding assay

Binding of the HIV-1 peptides to the HLA-A*2402 molecule was examined by the HLA-A*2402 stabilization assays, using RMA-S-A*2402 cells as described previously (21). RMA-S-A*2402 cells were cultured for 16 h at 26°C and then were pulsed with peptides at concentrations of 10^{-3} to 10^{-9} M for 1 h at 26°C. After incubation for 3 h at 37°C, peptide-pulsed cells were stained by MB40.5 mAb anti-HLA class I and the fluorescent-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity was measured with FACScan (Becton Dickinson, Mountain View, CA).

*Evaluation of HLA-A*2402-binding peptides*

Peptides at a concentration of 10^{-4} M giving more than 25% of the mean fluorescence intensity (MFI)[3] of RMA-S-A*2402 cells were evaluated as HLA-A*2402-binding peptides. The MFI of RMA-S-A*2402 cells was obtained by subtracting the MFI value of RMA-S-A*2402 cells incubated at 37°C for 3 h stained with MB40.5 mAb from the MFI value of RMA-S-A*2402 cells cultured at 26°C stained with MB40.5 mAb. The peptide concentration that yields the half-maximal levels of the MFI (the BL_{50} value) was calculated.

Induction of peptide-specific CTL in PBLs from HIV-1-infected individuals carrying HLA-A24

Peptide-specific CTL was induced from PBLs of four HIV-1-seropositive individuals carrying HLA-A24. A total of 1×10^6 PBLs were cultured with HLA-A*2402-binding peptides at a concentration of 10^{-6} M in culture medium (RPMI 1640 medium, 10% FCS, and 200 U/ml human IL-2). Then these cells were stimulated with 1×10^6 of irradiated autologous PHA blast prepulsed with the corresponding peptide at 10^{-5} M every week. After the fourth stimulation, peptide-specific CTL activity was tested by CTL assay.

Generation of CTL clones

Peptide-specific CTL clones were generated from established HIV-1-specific bulk CTL culture by seeding at 0.8 cells/well in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (5×10^5 irradiated allogeneic PBLs from a healthy donor and 1×10^6 irradiated C1R-A*2402 cells prepulsed with the corresponding peptide at 10^{-6} M in RPMI 1640 supplemented with 10% FCS and 200 U/ml human rIL-2). Wells positive for growth after 2 wk were transferred into 48-well plates together with 1 ml of the cloning mixture. They were tested for CTL activity by 4-h ^{51}Cr release assay. Positive clones were maintained in RPMI 1640 medium containing 200 U/ml human rIL-2 and were stimulated weekly with peptide-pulsed C1R-A*2402 cells.

CTL assay

Cytotoxicity was measured in standard ^{51}Cr release assay as previously described (16). Target cells (5×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline, and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (5×10^3/well) were added into a 96-well round-bottom microtiter plate (Nunc) with the indicated amount of the corresponding peptide. After 1-h incubation, effector cells were added and the mixtures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the cpm in the supernatant in the wells containing only target cells (cpm spn). The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. The specific lysis = (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the counts per minute in the supernatant in wells containing both target and effector cells.

Table I. *Binding of 8-mer to 11-mer peptides carrying two anchor residues of HLA-A*2402 binding self-peptides to HLA-A*2402 molecules*

Peptides	Position	Sequence	BL_{50} (M)
High binders			
SF2-Env219-8	Env 219–226	HYCTPAGF	2.1×10^{-6}
SF2-Env385-8	Env 385–392	FYCNTTQL	3.3×10^{-6}
SF2-Pol493-9	Pol 493–501	TYQIYQEPF	9.5×10^{-7}
SF2-Pol496-9	Pol 496–504	IYQEPFKNL	2.9×10^{-6}
SF2-Env310-9	Env 310–318	IYIGPGRAF	3.8×10^{-6}
SF2-Env385-9	Env 385–393	FYCNTTQLF	1.1×10^{-6}
SF2-Env584-9	Env 584–592	RYLRDQQLL	2.4×10^{-6}
SF2-Env636-9	Env 636–644	NYTNTIYTL	1.1×10^{-6}
SF2-Env679-9	Env 679–687	WYIKIFIMI	4.0×10^{-6}
SF2-Nef138-10	Nef 138–147	RYPLTFGWCF	3.1×10^{-7}
SF2-Env219-10	Env 219–228	HYCTPAGFAI	7.3×10^{-6}
SF2-Env431-10	Env 431–440	MYAPPIGGQI	1.8×10^{-6}
SF2-Env636-10	Env 636–645	NYTNTIYTLL	7.8×10^{-6}
SF2-Gag485-11	Gag 485–495	LYPLTSLRSLF	8.5×10^{-6}
SF2-Env793-11	Env 793–803	KYWWSLLQYWI	5.4×10^{-6}
Medium binders			
SF2-Nef130-8	Nef 130–137	NYTPGPGI	5.1×10^{-5}
SF2-Gag85-8	Gag 85–92	LYCVHQRI	8.1×10^{-5}
SF2-Gag133-8	Gag 133–140	NYPIVQNL	1.6×10^{-5}
SF2-Gag263-8	Gag 263–270	IYKRWIIL	1.5×10^{-5}
SF2-Pol335-8	Pol 335–342	IYQYMDDL	3.2×10^{-5}
SF2-Env190-8	Env 190–197	NYTNYRLI	2.0×10^{-5}
SF2-Pol114-9	Pol 114–122	QYDQIPVEI	1.0×10^{-5}
SF2-Pol210-9	Pol 210–218	PYNTPMFAI	6.2×10^{-5}
SF2-Pol342-9	Pol 342–350	LYVGSDLEI	7.9×10^{-5}
SF2-Pol472-9	Pol 472–480	VYYDPSKDL	5.6×10^{-5}
SF2-Env481-9	Env 481–489	LYKYKVIKI	6.3×10^{-5}
SF2-Env766-9	Env 766–774	SYRRLRDLL	6.6×10^{-5}
SF2-Gag263-10	Gag 263–272	IYKRWIILGL	2.3×10^{-5}
SF2-Gag278-10	Gag 278–287	MYSPTSILDI	3.1×10^{-5}
SF2-Gag442-10	Gag 442–451	SYKGRPGNFL	5.2×10^{-5}
SF2-Gag485-10	Gag 485–494	LYPLTSLRSL	2.9×10^{-5}
SF2-Pol281-10	Pol 281–290	KYTAFTIPSI	5.0×10^{-5}
SF2-Pol425-10	Pol 425–434	IYAGIKVKQL	2.2×10^{-5}
SF2-Pol940-10	Pol 940–949	VYYRDNKDPL	6.3×10^{-5}
SF2-Env483-10	Env 483–492	KYKVIKIEPL	4.0×10^{-5}
SF2-Nef84-11	Nef 84–94	TYKAALDISHF	2.5×10^{-5}
SF2-Pol269-11	Pol 269–279	AYFSVPLDKDF	2.7×10^{-5}
SF2-Env9-11	Env 9–19	NYQHLWRWGTL	2.5×10^{-5}
SF2-Env219-11	Env 219–229	HYCTPAGFAIL	2.3×10^{-5}
SF2-Env584-11	Env 584–594	RYLRDQQLLGI	2.0×10^{-5}
SF2-Env710-11	Env 710–720	GYSPLSFQTRL	3.5×10^{-5}
SF2-Env766-11	Env 766–776	SYRRLRDLLLI	1.8×10^{-5}
Low binders			
SF2-Nef118-8	Nef 118–125	IYHTQGYF	5.2×10^{-4}
SF2-Gag78-8	Gag 78–85	LYNTVATL	2.7×10^{-4}
SF2-Gag278-8	Gag 278-285	MYSPTSIL	7.1×10^{-4}
SF2-Pol559-8	Pol 559–566	EYWQATWI	2.0×10^{-4}
SF2-Pol797-8	Pol 797–804	GYIEAEVI	3.1×10^{-4}
SF2-Env584-8	Env 584–591	RYLRDQQL	3.8×10^{-4}
SF2-Env835-8	Env 835–842	AYRAILHI	1.2×10^{-4}
SF2-Pol581-9	Pol 581–589	WYQLEKEPI	1.3×10^{-4}
SF2-Pol941-9	Pol 941–949	YYRDNKDPL	2.5×10^{-4}
SF2-Gag297-10	Gag 297–306	DYVDRFYKTL	7.4×10^{-4}
SF2-Env766-10	Env 766–775	SYRRLRDLLL	1.6×10^{-4}
No binders			
SF2-Nef84-8	Nef 84–91	TYKAALDI	$>10^{-3}$
SF2-Pol473-8	Pol 473–480	YYDPSKDL	$>10^{-3}$
SF2-Pol908-8	Pol 908–915	GYSAGERI	$>10^{-3}$
SF2-Env766-8	Env 766–773	SYRRLRDL	$>10^{-3}$
SF2-Gag442-9	Gag 442–450	SYKGRPGNF	$>10^{-3}$
SF2-Pol908-11	Pol 908–918	GYSAGERIVDI	$>10^{-3}$

[3] Abbreviations used in this paper: MFI, mean fluorescence intensity; spn, spontaneous.

Table II. *Induction of HLA-A24-restricted, HIV-1 peptide-specific CTL in PBL of four HIV-1-infected, HLA-A24-positive individuals by four in vitro stimulations with HLA-A*2402-binding HIV-1 peptides*

Peptides	Position	Sequence	YY		YK		KM		SG	
			C1R-A*2402	C1R	C1R-A*2402	C1R	C1R-A*2402	C1R	C1R-A*2402	C1R
High binders										
SF2-Env310-9	Env 310–318	IYIGPGRAF	−2[a]	−4[a]	−2	8	**19**[b]	−4	**17**	3
SF2-Env385-9	Env 385–393	FYCNTTQLF	−6	1	−2	0	0	5	**61**	19
SF2-Env584-9	Env 584–592	RYLRDQQLL	**28**	10	**89**	46	**45**	34	12	5
SF2-Env636-9	Env 636–644	NYTNTIYTL	7	0	**42**	9	3	12	−1	4
SF2-Env679-9	Env 679–687	WYIKIFIMI	12	−1	13	NT[c]	10	4	**22**[d]	7[b]
SF2-Nef138-10	Nef 138–147	RYPLTFGWCF	**37**	19	**15**[e]	0[e]	**26**[e]	−1[e]	**73**	8
SF2-Gag485-11	Gag 485–495	LYPLTSLRSLF	0	−1	1	2	−1	2	**52**	0
Medium binders										
SF2-Gag133-8	Gag 133–140	NYPIVQNL	2	12	**51**	0	−3	2	3	4
SF2-Gag263-8	Gag 263–270	IYKRWIIL	3	10	**21**	22	3	4	14	6
SF2-Pol342-9	Pol 342–350	LYVGSDLEI	**30**	1	2	−7	9	−3	3	−7
SF2-Env766-9	Env 766–774	SYRRLRDLL	2	−1	2	−13	−0	0	**16**	−7
SF2-Gag263-10	Gag 263–272	IYKRWIILGL	**41**	−1	−12	10	−8	−1	2	7
SF2-Pol281-10	Pol 281–290	KYTAFTIPSI	**50**	41	**50**	28	−4	−1	12	10
SF2-Pol940-10	Pol 940–949	VYYRDNKDPL	3	3	−1	0	3	5	**40**	12
SF2-Env483-10	Env 483–492	KYKVIKIEPL	**40**	10	3	0	**20**	−1	**23**	−3
SF2-Nef84-11	Nef 84–94	TYKAALDISHF	3	0	4	0	1	−3	**28**	NT
SF2-Env584-11	Env 584–594	RYLRDQQLLGI	**76**	47	**50**	28	**30**	9	14	−12
Low binders										
SF-Nef118-8	Nef 118–125	IYHTQGYF	**18**	20	3	4	7	1	11	NT
SF2-Gag78-8	Gag 78–85	LYNTVATL	**40**	29	8	4	−7	6	12	−1
SF2-Pol797-8	Pol 797–804	GYIEAEVI	4	1	**17**	−10	6	2	9	4
SF2-Env584-8	Env 584–591	RYLRDQQL	**58**	10	**23**	9	12	2	−5	NT
SF2-Env835-8	Env 835–842	AYRAILHI	4	2	**15**	4	4	5	−2	−1
SF2-Gag297-10	Gag 297–306	DYVDRFYKTL	10	1	**23**	−1	2	0	**45**	41

[a] Relative percentage of specific lysis equals percentage of specific lysis of C1R-A*2402 cells (C1R cells) pulsed with the peptides minus percentage of specific lysis of C1R-A*2402 cells (C1R cells) pulsed without peptides. Specific lysis of the target cells was examined using an E:T ratio of 20:1.
[b] Bold shows positive responses (more than 15% relative percentage of specific lysis of C1R-A*2402 cells).
[c] NT, not tested.
[d] Data after the second stimulation.
[e] Data after the third stimulation.

CTL assay for the target cells infected with recombinant vaccinia virus

The recombinant vaccinia virus containing *gag/pol*, *nef*, or *env* gene of HIV-1 SF2 were generated as described previously (16). Target cells (C1R and C1R-A*2402 cells) were cultured with 10 plaque-forming units of recombinant or wild-type vaccinia virus per target cell overnight. These infected cells (5×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline, and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (5×10^3/well) were added with effector cells, and the mixtures were incubated for 4 h (for target cells infected with the vaccinia recombinant containing *gag/pol* and *env*) or 6 h (for target cells infected with the vaccinia recombinant containing *nef*) at 37°C. The activities of the CTL clone for target cells infected with recombinant vaccinia virus expressing corresponding proteins (Gag and Pol, Nef or Env) were tested at an E:T ratio of 2:1.

Results

*Binding of the HIV-1 peptides carrying the anchor residues to HLA-A*2402 molecules*

From 8-mer to 11-mer peptides carrying the HLA-A*2402-binding peptide motif (Tyr at position 2 and Phe, Leu, or Ile at the C terminus) (23) were searched out from the amino acid sequence of Gag, Pol, Nef, and Env proteins in HIV-1 SF2 strain and 59 peptides (21 of 8-mer peptides, 18 of 9-mer peptides, 17 of 10-mer peptides, and 12 of 11-mer peptides) were synthesized. Binding affinities of these peptides were tested by HLA-A*2402 stabilization assay using RMA-S-A*2402 cells. Among these 59 peptides, 6 peptides (four 8-mer peptides, one 9-mer peptide, and one 11-mer peptide) did not bind to HLA-A*2402 molecules while 53 peptides bound to HLA-A*2402 molecules (Table I). According to the BL_{50}, the binding peptides were classified into three classes: 15 high binders (two 8-mer, seven 9-mer, four 10-mer, and two 11-mer), 27 medium binders (six 8-mer, six 9-mer, eight 10-mer, seven 11-mer), and 11 low binders (seven 8-mer, two 9-mer, and two 10-mer). They contained 4 Nef, 11 Gag, 15 Pol, and 23 Env peptides. These 53 HLA-A*2402-binding HIV peptides were used to induce the specific CTL in PBL of HIV-1-infected individuals carrying HLA-A24.

*Induction of the specific CTL from PBL of HIV-1-infected individuals by HLA-A*2402-binding HIV-1 peptides*

The specific CTL was induced from four HIV-1-seropositive individuals carrying HLA-A24, YY (HLA-A24/-, -B51/59, -Cw1/-); YK (HLA-A24/-, -B46/52, -Cw1/-); KM (HLA-A24/26, -B44/61, -Cw3/-); and SG (HLA-A24/26, -B35/51, -Cw3/-) by stimulating their PBLs with HLA-A*2402-binding HIV-1 peptides. These bulk CTLs were restimulated with PHA-induced autologous cells pulsed with the peptide at 1-wk intervals. After the fourth stimulation, cytotoxic activity of each bulk CTL was tested against C1R and C1R-A*2402 cells pulsed with the corresponding peptide. A total of 23 of the 53 peptides induced the specific CTL (more than 15 relative percentage of specific lysis; percentage of specific lysis of C1R-A*2402 pulsed with the peptide minus percentage of specific lysis of C1R-A*2402 pulsed without the peptide) from at least one of the four individuals (Table II).

To clarify whether these peptides are processed and presented by HLA-A*2402 molecules, the cytolytic activity of these peptide-specific bulk CTL was evaluated using C1R and C1R-A*2402

Table III. *Killing activity of the CTL induced by the HIV-1 peptide for the target cells infected with the HIV-1-recombinant vaccinia virus*

Peptides	Position	Sequence	YY		YK		KM		SG	
			C1R-A*2402	C1R	C1R-A*2402	C1R	C1R-A*2402	C1R	C1R-A*2402	C1R
High binders										
SF2-Env310-9	Env 310–318	IYIGPGRAF					9[a]	7	**15**[b]	8
SF2-Env385-9	Env 385–393	FYCNTTQLF							**13**	11
SF2-Env584-9	Env 584–592	RYLRDQQLL	**13**[c]	1[c]	**63**	0	**28**	2	**20**	7
SF2-Env636-9	Env 636–644	NYTNTIYTL			−5	6				
SF2-Env679-9	Env 679–687	WYIKIFIMI			7				**11**[c]	16[c]
SF2-Nef138-10	Nef 138–147	RYPLTFGWCF	**30**	0	7	−3	**15**[d]	6[d]	**23**[d]	−3[d]
SF2-Gag485-11	Gag 485–495	LYPLTSLRSLF							−7	−2
Medium binders										
SF2-Gag133-8	Gag 133–140	NYPIVQNL			**24**	−10				
SF2-Gag263-8	Gag 263–270	IYKRWIIL			**12**	1				
SF2-Pol342-9	Pol 342–350	LYVGSDLEI	3	3						
SF2-Env766-9	Env 766–774	SYRRLRDLL							**22**	−6
SF2-Gag263-10	Gag 263–272	IYKRWIILGL	**22**	−9						
SF2-Pol281-10	Pol 281–290	KYTAFTIPSI	3		−5					
SF2-Pol940-10	Pol 940–949	VYYRDNKDPL							−1	−42
SF2-Env483-10	Env 483–492	KYKVIKIEPL	3	−2					9	
SF2-Nef84-11	Nef 84–94	TYKAALDISHF							8	
SF2-Env584-11	Env 584–594	RYLRDQQLLGI	**72**	−4	**57**	−9	**29**		**16**	−4
Low binders										
SF2-Nef118-8	Nef 118–125	IYHTQGYF	3	−9						
SF2-Gag78-8	Gag 78–85	LYNTVATL	3							
SF2-Pol797-8	Pol 797–804	GYIEAEVI			**12**					
SF2-Env584-8	Env 584–591	RYLRDQQL	**29**	5	**22**	4				
SF2-Env835-8	Env 835–842	AYRAILHI			−1	−1				
SF2-Gag297-10	Gag 297–306	DYVDRFYKTL			8	22			−11	−4

[a] Relative percentage of specific lysis equals percentage of specific lysis of C1R-A*2402 cells (C1R cells) infected with the HIV-1-recombinant vaccinia virus minus percentage of specific lysis of C1R-A*2402 cells (C1R cells) infected with wild-type vaccinia virus. Specific lysis of the target cells was examined using an E:T ratio of 20:1.

[b] Bold shows positive responses (more than 10% relative percentage of specific lysis of C1R-A*2402 cells).

[c] Data after the second stimulation.

[d] Data after the third stimulation.

cells infected with HIV-1 recombinant vaccinia virus expressing HIV-1 Gag/Pol, Nef, or Env proteins as target cells (Table III). Eleven peptides (SF2-Env636-9, SF2-Gag485-11, SF2-Pol342-9, SF2-Pol281-10, SF2-Pol940-10, SF2-Env483-10, SF2-Nef84-11, SF2-Nef118-8, SF2-Gag78-8, SF2-Env835-8, and SF2-Gag297-10) were excluded from the candidates for the epitopes because the CTL induced with these peptides did not kill C1R-A*2402 cells infected with the recombinant vaccinia virus expressing the corresponding HIV-1-SF2 proteins. The CTL induced with other peptides lysed C1R-A*2402 cells infected with these recombinant vaccinia virus (more than 10% relative specific lysis; the percentage of specific lysis of C1R-A*2402 infected with the vaccinia recombinant virus minus the percentage of specific lysis of C1R-A*2402 infected with wild-type vaccinia virus). Thus, it was demonstrated that these 12 peptides are processed and presented by HLA-A*2402 molecule. On the other hand, these peptides failed to induce the specific CTL in 4-wk cultures of PBL (four stimulations with the peptide) from four HIV-1-seronegative, HLA-A24-positive individuals (data not shown). This results excluded the possibility that the specific CTL was induced by in vitro multiple stimulations with the peptides.

*Confirmation and characterization of HLA-A*2402-restricted HIV-1 CTL epitopes by the specific CTL clones*

To confirm the HIV-1 epitopes presented by HLA-A*2402 molecules, we attempted to generate the specific CTL clones from the CTL lines that specifically killed C1R-A*2402 cells infected with recombinant vaccinia virus expressing HIV-1 proteins. No clone was obtained from the CTL line specific for the peptide SF2-Env310-9, although at least two clones were obtained from the CTL lines for the other 11 peptides. All the CTL clones were capable of killing C1R-A*2402 cells pulsed with the corresponding peptides in a peptide concentration-dependent fashion (Table IV, and Fig. 1). The concentration of the peptides at which the half-maximum lysis occurred was 0.1 to 1000 nM varying among each clone specific for the epitopes. These clones also showed the specific cytolytic activity against C1R-A*2402 cells infected with the recombinant vaccinia virus expressing the full length of Gag/Pol, Nef, or Env protein, but not C1R cells or C1R-A*2402 cells infected with wild-type vaccinia virus (Table IV). Thus, these results confirmed that these 11 peptides are HLA-A*2402-restricted HIV-1 CTL epitopes. CTL clones specific for four epitopes showed much lower specific cytolytic activity against the target cells infected with the vaccinia recombinant than that against the target cells pulsed with the peptides (Table IV), suggesting that these naturally processed peptides are poorly recognized by the CTL clones. This might be explained by the small amounts of HLA-A*2402-peptide complex expressed on the surface of the vaccinia-recombinant infected cells.

CTL clones specific for two Gag-derived epitopes, SF2-Gag133-8 and SF2-Gag263-8, were generated from the individual YK while those specific for SF2-Gag263-10 were established from the individual YY. SF2-Gag263-8 (IYKRWIIL) is included in the 10-mer epitope SF2-Gag263-10 (IYKRWIILGL). In spite of the fact that binding affinities (BL_{50}) of the two peptides were similar (1.5×10^{-5} M for SF2-Gag263-8 and 2.3×10^{-5} M for SF2-Gag263-10), the concentrations of the 8-mer peptide required to

Table IV. *Recognition of the HLA-A*2402-restricted CTL clones for processed HIV-1 epitopes*

Epitopes	Clone No.	C1R				C1R-A*2402			
		Peptide(−)	Peptide(+)[a]	Wild-type vaccinia	Recombinant vaccinia	Peptide(−)	Peptide(+)[a]	Wild-type vaccinia	Recombinant vaccinia
SF2-Gag133-8	3	1[b]	13	−1	3	4	63	1	13
	7	0	19	−1	4	−2	72	22	60
SF2-Gag263-8	6	1	4	1	8	3	41	4	17
	16	1	2	2	11	1	43	5	15
SF2-Gag263-10	3	2	4	3	2	3	55	1	32
	17	1	3	3	1	2	64	5	32
SF2-Pol797-8	25	1	27	3	2	4	62	4	15
	40	7	30	10	6	10	67	10	22
SF2-Nef138-10	8	9	20	8	7	5	75	9	20
	18	7	24	8	8	2	82	7	22
SF2-Env385-9	2	−4	7	1	0	−1	61	2	49
	7	−3	5	−1	0	0	52	4	43
SF2-Env584-8	11	3	8	3	0	0	79	1	65
	15	2	7	2	−1	1	86	−1	66
SF2-Env584-9	24	3	18	6	6	1	72	4	66
	32	−1	13	4	2	−2	56	1	60
SF2-Env584-11	1	0	22	2	1	0	69	1	63
	2	1	26	0	0	−1	73	1	65
SF2-Env679-9	3	0	20	0	3	2	60	2	54
	22	7	22	4	2	3	75	3	68
SF2-Env766-9	4	−1	1	4	0	2	19	1	60
	7	0	3	2	0	2	18	1	59

[a] The target cells were loaded with the peptide at 1 μM.
[b] Percentage of specific lysis.

attain half-maximum cytotoxicity of the specific CTL clone (SF2-Gag263-8-6) were approximately 20 times higher than that for the 10-mer epitope-specific CTL clone (SF2-Gag263-10-3) (Fig. 2). An 8-mer peptide-specific CTL clone, SF2-Gag263-8-6, killed C1R-A*2402 cells pulsed with the 8-mer peptide, but not C1R-A*2402 cells pulsed with the 10-mer peptide. In contrast, the 10-mer peptide-specific CTL clone, SF2-Gag263-10-17, killed C1R-A*2402 cells pulsed with both the 8-mer and the 10-mer peptides. Taken together, these results suggest that these peptides are presented as distinct epitopes.

CTL clones specific for the epitope derived from Pol protein (SF2-Pol797-8) were established from the individual YK. These CTL clones specific for SF2-Pol797-8 peptide not only killed C1R-A*2402 cells pulsed with this peptide but also weak C1R cells pulsed with the peptide (Table IV, Fig. 1). However, the minimum peptide concentration recognized by these CTL clones was about 100 times higher for C1R cells than for the target cells. Because HLA-A*2402 and HLA-Cw4 have a similar peptide motif (23, 24), it is speculated that these CTL clones cross-recognize the peptide presented by HLA-Cw4 that was expressed on C1R cells. However, since the CTL clones cannot kill the C1R cells infected with the recombinant vaccinia virus (Table IV), it is thought that the peptide is not presented by HLA-Cw4.

CTL clones specific for the SF2-Nef138-10 peptide (RY PLTEGWCF) were generated from the individual YY. The clones killed both the C1R-A*2402 target cells pulsed with the specific peptide and those infected with the HIV-1 Nef recombinant vaccinia virus. This 10-mer epitope includes the 8-mer sequence RY PLTFGW. Our recent study showed that Trp at the C terminus is also an anchor for HLA-A*2402 molecules (21). Therefore, it remains a possibility that the 8-mer peptide is an HLA-A*2402-restricted epitope rather than the 10-mer peptide. The CTL clones specific for the SF2-Nef-138-10 failed to kill the C1R-A*2402 cells pulsed with the 8-mer peptide (Fig. 3). These results indicate that the 10-mer Nef peptide but not the 8-mer peptide was presented as epitope by HLA-A*2402 molecules.

CTL clones specific for six Env-derived epitopes were generated from three individuals: CTL clones specific for SF2-Env385-9, SF2-Env679-9, and SF2-Env-766-9 from the individual SG, CTL clones specific for SF2-Env584-8 and SF2-Env584-11 from the individual YY, and CTL clones specific for SF2-Env584-9 from the individual YK. Two CTL clones specific for SF2-Env766-9 showed relatively low killing activity against C1R-A*2402 cells pulsed with the specific peptide (18%), but considerably high activity against the Env-expressing, recombinant vaccinia virus-infected C1R-A*2402 cells (about 60% specific lysis, Table IV). These findings imply the possibility that naturally processed peptide has another sequence. The amino acid sequence of HIV-1-SF2 strain around SF2-Env766-9 is LFSYRRLRDLLLI (amino acids 764–776). Phe at position 2 is also anchor peptide for HLA-A*2402 molecule in addition to Tyr (21). Therefore, we examined whether the CTL clones specific for SF2-Env766-9 recognize SYRRLRDL, SYRRLRDLLL, SYRRLRDLLLI, LFSYR RLRDL, and LFSYRRLRDLL. Both SF2-Env766-9 and SF2-Env766-7 clones failed to kill the target cells pulsed with these peptides except the epitope peptide SF2-Env766-9 (data not shown). These results confirmed that SF2-Env766-9 is HLA-A*2402-restricted CTL epitope.

The gp41-derived epitopes, SF2-584-8, -9, and -11 overlap one another. However, the peptide concentration required to reach half-maximum cytolysis of peptide-pulsed target cells by the specific CTL clones was approximately 50 nM for the 8-mer, 0.5 nM for the 9-mer, and 5 nM for the 11-mer peptide (Fig. 1). The differences in the cytolytic activities of these clones reflects the differences between the binding affinities of each peptide to HLA-A*2402 molecules (Table I). The CTL clones generated from the CTL line induced with each of the three peptides were capable of lysing C1R-A*2402 cells pulsed with these three peptides regardless of the peptide length (Fig. 4). Therefore, it is not clear that these three peptides were independently processed and presented by HLA-A*2402 molecules.

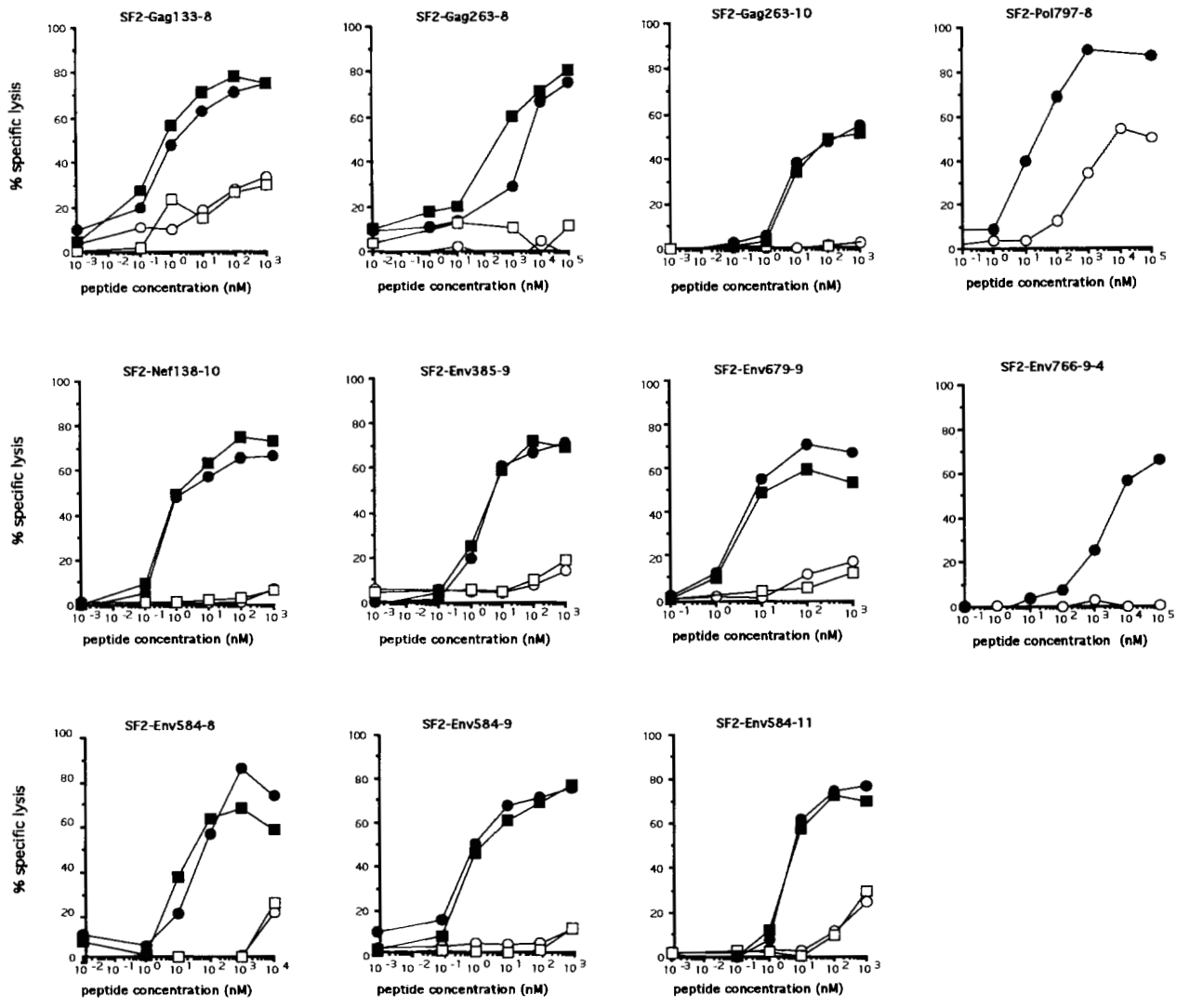

FIGURE 1. Recognition of HIV-1 epitope peptides by HLA-A*2402-restricted CTL clones. Cytotoxicity of each CTL clone for target cells pulsed with titrated peptides was tested at an E:T ratio of 2:1. C1R-A*2402 cells (closed symbols) and C1R cells (open symbols) were used as target cells. Except the clones (SF2-Pol797-8-25 and SF2-Env766-9-4) specific for SF2-Pol797-8 and SF2-Env766-9, two CTL clones for each epitope were used as follows: SF2-Gag133-8-7, SF2-Gag263-8-6, SF2-Gag263-10-3, SF2-Nef138-10-8, SF2-Env385-9-2, SF2-Env-679-9-3, SF2-Env584-8-11, SF2-Env585-9-24, and SF2-Env584-11-1 CTL clones (●○); SF2-Gag133-8-15, SF2-Gag263-8-16, SF2-Gag263-10-17, SF2-Nef138-10-18, SF2-Env385-9-7, SF2-Env-679-9-18, SF2-Env584-8-15, SF2-Env585-9-32, and SF2-Env584-11-2 CTL clones (■□).

CTL activity for the 11 epitopes in HIV-1-infected individuals carrying HLA-A24

To investigate whether these epitopes were common and strong among HIV-1-infected individuals carrying HLA-A24, PBL from 12 HIV-1-seropositive individuals carrying HLA-A24 was stimulated with each of the 12 epitopes including SF2-Env310-9. CTL activity against Tm-EBV-transformed cells expressing HLA-A24 pulsed with the corresponding peptide was measured 1 wk after the induction with each peptide. As shown in Table V, relatively low cytolytic activity against SF2-Gag133-8 was observed in the bulk CTL of only 1 individual, MYZ. Bulk cultures from all the 12 individuals did not develop CTL activity against either SF2-Gag263-8 or -10. Similarly, no CTL activity was observed against SF2-Pol797-8 in the bulk cultures from these individuals. These results show that four epitopes from Gag and Pol proteins are weak epitopes. In contrast, SF2-Nef138-10-stimulated bulk cultures from seven individuals showed specific CTL activity (Table V), indicating that this is a strong epitope.

The specific cytotoxicity was observed in bulk cultures stimulated with SF2-Env766-9, SF2-Env679-9, and SF2-Env385-9 in two, three, and three individuals, respectively (Table V). The specific cytotoxicity for SF2-Env766-9 in two individuals was weak while that for the other two Env epitopes was strong. These findings suggest that the former is a weak epitope while the latter epitopes are strong. However, since CTL clones specific for Env766-9 showed high killing activity for the target cells infected with the vaccinia recombinant virus but low killing activity for the target cells pulsed with the peptides at 1 μM (Table IV and Fig. 1), it still remains a possibility that this epitope is effectively processed in HIV-1-infected target cells and is recognized as a strong epitope by T cells. For the overlapping epitopes, SF2-Env584-8, -9, and -11, three types of induction were observed (Table V): bulk culture from the individuals YY and KM showed cytotoxic activity for all the three epitopes, those from the individuals YK, SK, and AK had cytolytic activity for SF2-Env584-8 and -11, but not for -9, and those from the individual SG, OCH, MYZ, and ON revealed killing activity for SF2-Env594-9, and −11, but not

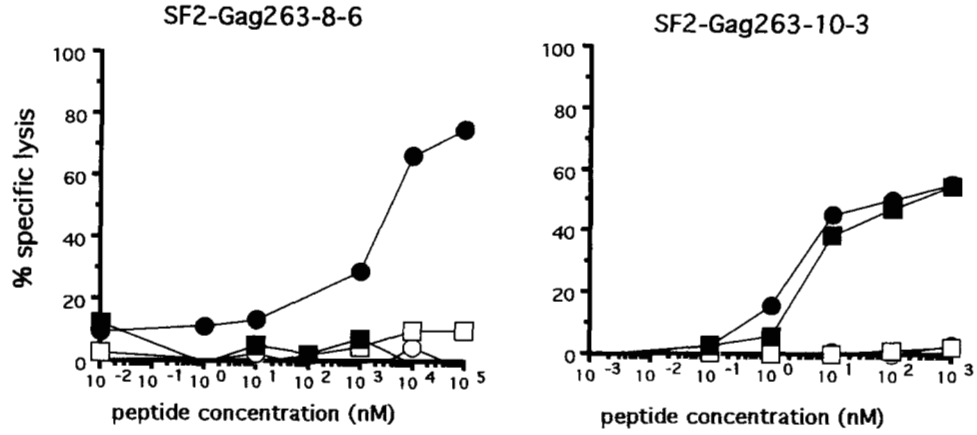

FIGURE 2. Cross-recognition of SF2-Gag263-8 and SF2-Gag263-10 epitopes by the specific CTL clones. Cytotoxicity of the CTL clones SF2-Gag263-8-6 and SF2-Gag263-10-3 was tested for C1R-A*2402 cells pulsed with SF2-Gag263-8 (●), or SF2-Gag263-10 (■) and C1R cells pulsed with SF2-Gag263-10 (○) or SF2-Gag263-10 (□) at an E:T ratio of 2:1.

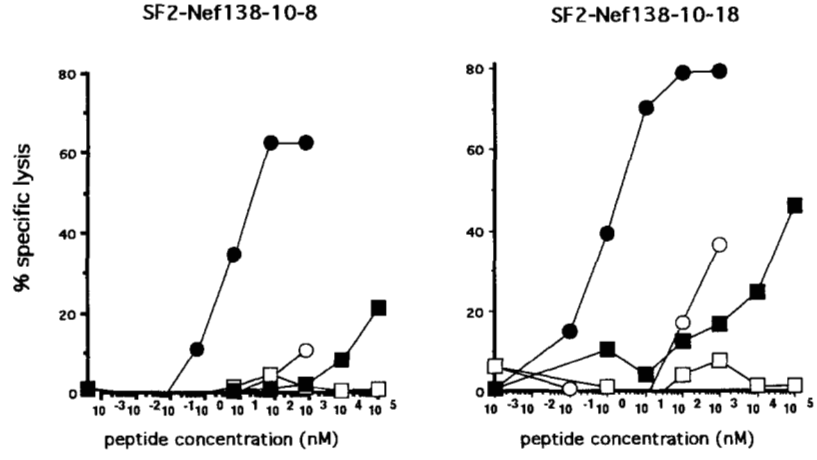

FIGURE 3. Recognition of SF2-Nef138-8 peptide by SF2-Nef138-10-specific CTL clones. Cytotoxicity of the CTL clones SF2-Nef138-10-8 and SF2-Nef138-10-18 was tested for C1R-A*2402 cells pulsed with SF2-Nef138-10 (●) or SF2-Nef138-8 (■) and C1R cells pulsed with SF2-Nef138-10 (○) or SF2-Nef138-8 (□) at an E:T ratio of 2:1.

for −8. A low cytolytic activity was observed for only the 9-mer epitope in the cultures from the individual SHG, and no CTL activity was gained from the individual ICH and AZ. These results suggest that these three epitopes may be independently presented by HLA-A*2402 molecules. The specific cytotoxicity for SF2-Env310-9 was weakly observed in only 1 of the 12 HIV-1-infected individuals although the CTL clone for this peptide was not generated. This finding suggests that SF2-Env310-9 may be a weak epitope even if this is a CTL epitope presented by HLA-A24.

We induced PBL of three HIV-1-seronegative individuals carrying HLA-A24 by these peptides to exclude the possibility of nonspecific CTL induction by these peptides. No CTL activity for the target cells pulsed with each peptide was observed in the bulk culture cells 1 wk after the induction of PBL with the peptides (data not shown), showing that the specific CTL was not induced in PBL of the HIV-1-seronegative individuals. Taken together, these results demonstrated that the five Env-derived epitopes and the Nef epitope are effectively recognized by T cells in HIV-1-infected individuals carrying HLA-A*2402.

*The effect of mutations in strong CTL epitopes on HLA-A*2402-restricted CTL recognition*

In order to clarify the effect of mutations in the HLA-A*2402-restricted CTL epitopes on CTL recognition, we investigated whether the mutations affect the recognition of the CTL clones specific for seven strong epitopes derived from Env and Nef proteins. For this purpose, we selected the mutations that were observed in the clade B isolated in North America.

All five mutations of SF2-Env385-9 reduced the recognition of the CTL clone (Fig. 5*A*). Especially the clone failed to recognize two mutants, SF2-Env385-9-5S7K and SF2-Env385-9-7K, although the effect of these mutations on peptide binding to HLA-A*2402 is minimum, indicating that the mutation of Lys for Gln at position 7 is critical for TCR recognition.

The same mutations in three epitopes, SF2-Env584-8, SF2-Env584-9, and SF2-Env584-11, showed a different effect on the recognition of the CTL clones (Fig. 5, *B*, *C*, and *D*). The mutations of Phe for Tyr at position 2 and Lys for Arg at positon 4 critically affected the recognition of SF2-Env584-8-15 clone, but the effect of these mutations on the recognition of SF2-Env584-9-32 and SF2-Env584-11-1 clones was minimum. These findings imply that the 8-mer peptides may be separately presented from the 9-mer and the 11-mer peptides by HLA-A*2402 molecules. Two mutations of Gly and Gln for Arg at position 4 critically affected the recognition of all three CTL clones. These mutations at position 4 did not reduce the binding to HLA-A*2402 molecules. These results together indicate that the mutations at position 4 influences TCR recognition.

Three mutations (2S, 4R, and 4R8I) of SF2-Env679-3 affected the recognition of CTL clones (Fig. 5*E*). This is thought to result

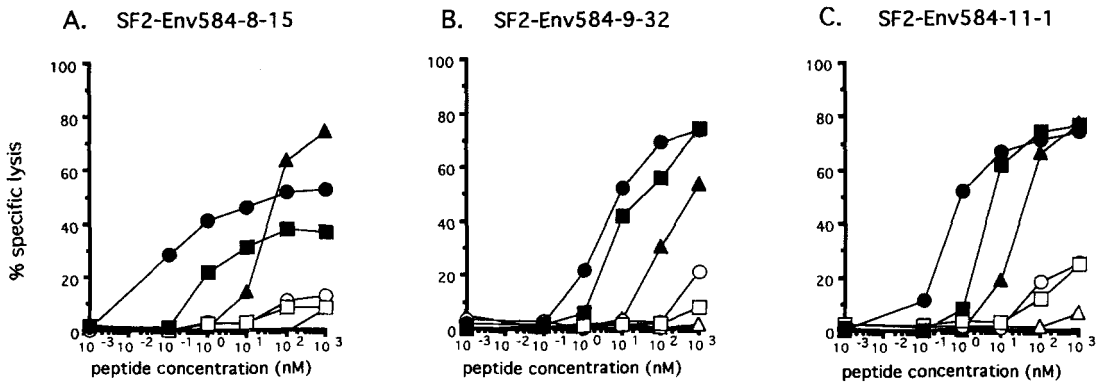

FIGURE 4. Cross-recognition of SF2-Env584-8, SF2-Env584-9, and SF2-Env584-11 epitopes by the specific CTL clones. Cytotoxicity of the CTL clones SF2-Env584-8-15 (*A*), SF2-Env584-9-32 (*B*), and SF2-Env584-11-1 (*C*) was tested for C1R-A*2402 pulsed with SF2-Env584-8 (▲), SF2-Env584-9 (●), or SF2-Env584-11 (■), and C1R cells pulsed with SF2-Env584-8 (△), SF2-Env584-9 (○), SF2-Env584-11 (□) at an E:T ratio of 2:1.

Table V. *The CTL activity for the HLA-A24-restricted, HIV-1 epitopes in the cultured cells after the first in vitro stimulation with the peptides*

Epitopes	HIV-1-Infected Individuals Carrying HLA-A24												No. of Individuals Who Have Specific CTL Activity
	YY	YK	KM	SG	ICH	OCH	SK	AK	MYZ	AZ	ON	SHG	
SF2-Gag133-8	1[a]	1	3	−4	−1	−9	−2	−0	**15**[b]	3	3	−8	1
SF2-Gag263-8	3	−5	−5	3	2	−3	6	−1	2	4	7	−2	0
SF2-Gag263-10	−2	1	2	5	−5	−10	7	1	1	−5	7	0	0
SF2-Pol797-8	2	0	8	−1	1	0	8	−1	6	7	0	−8	0
SF2-Nef138-10	**54**	**18**	**49**	**33**	6	**11**	**14**	3	−4	7	**55**	8	7
SF2-Env310-9	2	2	−14	−9	3	5	**19**	−5	1	0	2	−3	1
SF2-Env385-9	−9	6	−4	**45**	4	−1	**20**	−6	**37**	−8	6	6	3
SF2-Env584-8	**50**	**23**	**41**	−3	−5	1	**22**	**26**	7	2	−18	0	5
SF2-Env584-9	33	9	**62**	**10**	3	**26**	4	5	**53**	−0	**62**	**10**	7
SF2-Env584-11	**22**	**16**	**60**	**11**	2	**27**	**13**	**14**	**22**	−6	**48**	3	9
SF2-Env679-9	1	**32**	9	**15**	0	−5	3	5	−2	−6	12	**58**	3
SF2-Env766-9	−12	−6	−9	0	−6	**11**	−1	6	1	**11**	7	2	2

[a] Relative percentage of specific lysis equals percentage of specific lysis of EBV-transformed cell line Tm loaded with the peptide (1 μM) minus percentage of specific lysis of Tm pulsed without the peptide.

[b] Specific lysis of the target cells was examined using an E:T ratio of 50:1. Bold shows positive responses (more than 10% relative percentage of specific lysis of Tm).

from reduction of peptide binding or failure of TCR recognition. The mutation of Ser for Tyr at position 2 reduced the binding of the peptide to HLA-A*2402 molecules while it is assumed that two other mutations at positions 4 and 8 affect TCR recognition because these mutations did not reduce peptide binding to HLA-A*2402 molecules. Two mutations (3H, 3H4H) reduced the recognition of SF2-Env766-9-4 CTL clone while two other mutations (3H6T and 3H9I) critically affected the recognition of the CTL clone (Fig. 5*F*). These mutations did not reduce the binding of the peptide to HLA-A*2402 molecules. Taken together, the results indicate that these mutations affect TCR recognition.

On the other hand, the effect of the mutations in SF2-Nef-138-10 on the recognition of CTL clone was minimum (Fig. 5*G*), implying that this epitope can be recognized by CTL in most HLA-A24-positive, HIV-1-seropositive individuals. In contrast, 17 of 31 mutations in 6 Env epitopes critically affected recognition of the CTL clones. Thus, these findings suggest that mutations in Env epitopes influence CTL recognition in vivo.

Discussion

We recently showed that a strategy called reverse immunogenetics is very useful for the identification of HLA-B*3501-restricted HIV-1 CTL epitopes (16). In the present study, we used this strategy to identify HLA-A*2402-restricted HIV-1 CTL epitopes and showed that at least 11 were HLA-A24-restricted CTL epitopes in addition to 1 HLA-A*2402-restricted HIV-1 epitope corresponding to 1 (SF2-Env584-9) of these 11 epitopes, which was shown in a previous study (14). Thus, we showed the second case that reverse immunogenetics is very useful to identify HIV-1 CTL epitopes.

The sequence Env584-594 of HIV-1 SF2 provided three candidates (8-mer, 9-mer, and 11-mer) for HLA-A*2402-restricted epitopes. Although CTL clones established by induction of each peptide were not able to discriminate against each other, CTL activity in PBL of the 10 HIV-1-infected individuals induced by these peptides showed three different patterns for these peptides. Moreover, the recognition of two mutants by the CTL clone specific for the 8-mer was different from that by the CTL clones specific for the 9-mer and 11-mer. These observations suggest that these three epitopes are independently processed and presented by HLA-A*2402 molecules. Since CTL activity for at least one of these epitopes was induced in 10 of the 12 HIV-1-infected individuals, they are considered as strong epitopes. SF2-Env310-9 induced the specific CTL in only 1 out of 12 HIV-1-infected individuals after the first stimulation and in two out of four individuals after the fourth stimulation. However, we failed to generate the CTL clones specific for SF2-Env310-9, although CTL cloning was attempted three times.

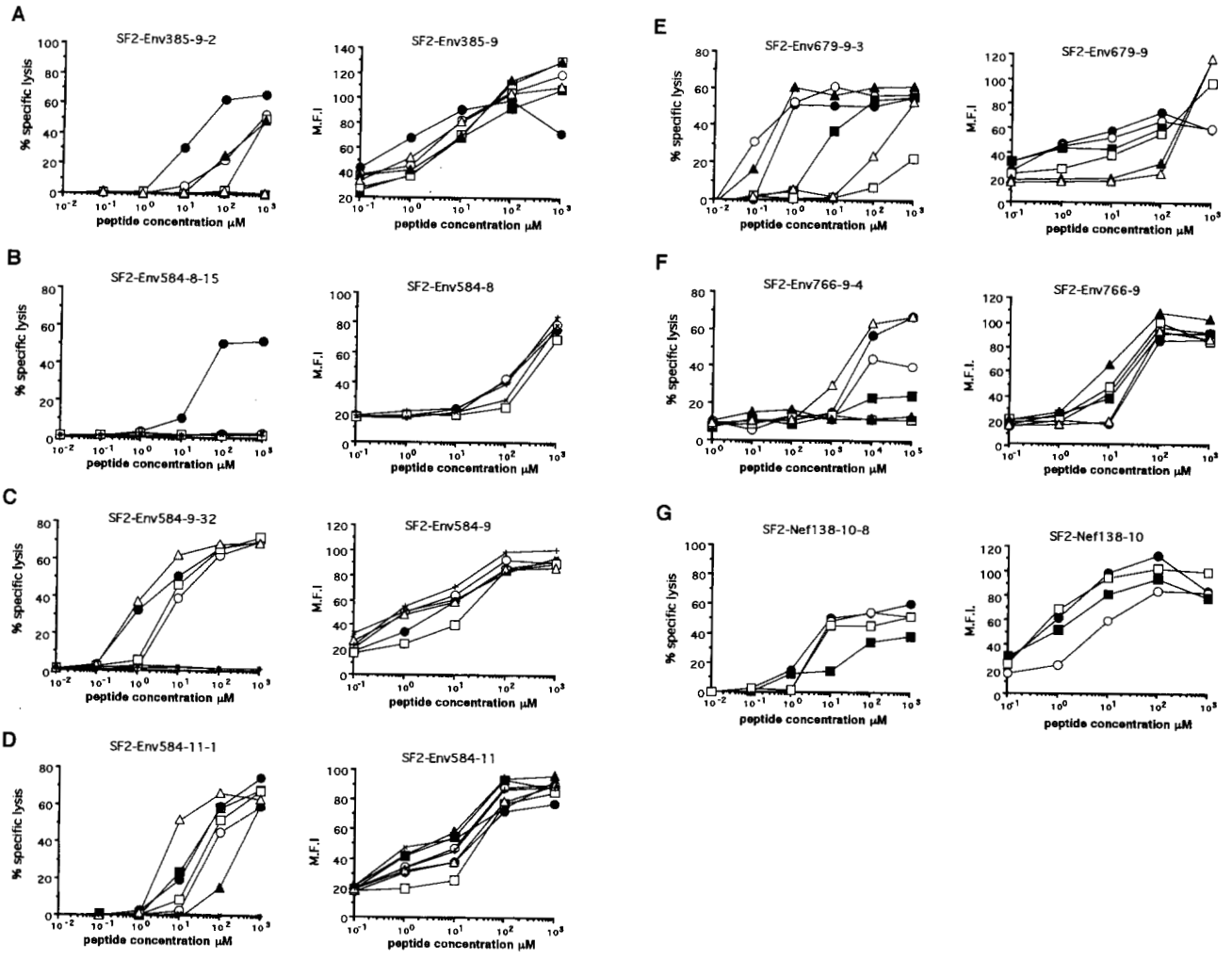

FIGURE 5. Recognition of the HIV-1 peptide-specific, HLA-A24-restricted CTL clones for the mutant epitopes and binding of these mutant peptides to HLA-A*2402 molecules. Recognition of seven CTL clones (*A*, SF2-Env385-9-2; *B*, SF2-Env584-8-15; *C*, SF2-Env584-9-32; *D*, SF2-Env584-11-1; *E*, SF2-Env679-9-3; *F*, SF2-Env766-9-4; *G*, SF2-Nef138-10-8, *left figures*) for the mutant sequences of the presumably immunodominant epitopes were investigated. Specific lysis of each CTL clone for C1R-A*2402 cells pulsed with the peptide at concentrations of 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M was tested at an E:T ratio of 2:1. Binding of the original peptide and that of the mutant peptides to HLA-A*2402 molecules was compared (*right figures*). Each symbol represents as follows; original peptides (●); *A*, 5S (○), 6S (❑), 5S7K (■), 6A (▲), 7K (△); *B*, *C*, and *D*, 4K (○), 10E (■), 2F (❑), 4K10R (▲), 4K9M (△), 4Q (+), 4G (×); *E*, 5L (○), 4R (■), 4R8I (❑), 9V (▲), 2S (△); *F*, 3H (○), 3H4H (■), 3H6T (❑), 3H9I (▲), 3Y (△), and *G*, 10Y (○), 2F (■), 5C (❑).

Therefore, we evaluated that this is not a CTL epitope or a very weak epitope. Since SF2-Env310-9 is located in the V3 loop and very diverse, it is necessary to determine the sequence of Env310-318 in the viruses from each individual and then to induce the specific CTL with the corresponding peptide in PBL for each individual. Of the other three Env epitopes, SF2-Env385-9 and SF2-Env679-9 were considered as strong epitopes because strong CTL activity specific for these epitopes was observed in 3 out of 12 HIV-1-infected individuals. SF2-Env766-9 may be another strong epitope although the CTL specific for the target cells pulsed with the peptide were weakly induced in only two individuals, because the CTL clones specific for this epitope killed the target cells infected with the vaccinia recombinant virus much more effectively than those pulsed with the peptide.

The HLA-A*2402-restricted HIV-1 epitopes consist of four high binders, five medium binders, and two low binders. Moreover, four of the six strong CTL epitopes were high binders while the other two (SF2-Env584-11 and SF2-Env584-8) were epitopes that overlapped with the high binder and strong epitope, SF2-Env584-9. These findings strongly suggested that immunogenicity of HLA-A*2402-binding peptides is dependent on their affinity to HLA-A*2402 molecules. However, since this association was not observed in the HLA-B*3501-restricted HIV-1 CTL epitopes (16, 25), it may be specific in the HLA-A*2402-restricted epitopes. This association in other HLA class I alleles will be clarified when many CTL epitopes presented by these HLA class I molecules are identified.

HLA-A*2402-restricted strong epitopes (the Nef and Env epitopes) are derived from the diverse region. In contrast, the weak epitopes (the Gag and Pol epitopes) are conserved in HIV-1 clade B strain (Table VI). This association was not observed in the HLA-B35-restricted HIV-1 CTL epitopes (25). Besides, it is not clear in HLA-A2-restricted, apparently immunodominant HIV-1 epitopes; Pol 346,347,348-354 (26), Pol 476-484 (27), and Env120-128 (28) are derived from the conserved region, while Gag77-85 (29) and Env814,815-823,824 (28) are derived from the diverse region. These observations imply that some mutations in HIV-1 CTL

Table VI. *Mutations of HLA-A*2402-restricted CTL epitopes in HIV-1 clade B strain*

Epitopes	No. of Isolates Carrying Mutant Epitopes per Total Number of Isolates	No. of Mutations
Strong epitopes		
SF2-Nef138-10	27/64	10
SF2-Env385-9	84/10 (73*/104)	32
SF2-Env584-8	48/71 (27*/71)	7
SF2-Env584-9	51/71 (30*/71)	10
SF2-Env584-11	54/71 (33*/71)	13
SF2-Env679-9	18/71	8
Possible strong epitope		
SF2-Env766-9	15/68	7
Weak epitopes		
SF2-Gag-133-8	13/32	3
SF2-Gag263-8	2/32	1
SF2-Gag263-10	2/32	1
SF2-Pol797-8	0/21	0

[a] The number of isolates carrying mutant epitopes for consensus sequences (Theoretical Biology and Biophysics/Los Alamos National Laboratory, Los Alamos, NM). The consensus sequence reflects residues conserved 50% or better in the sequences of the clade B.

epitopes may stimulate CTL responses restricted by particular HLA class I molecules.

Recent studies of Wolinsky et al. (7) revealed that a vigorous HIV-1-specific CTL response is associated with a slow rather than a rapid rate of development of AIDS. They also showed that a high frequency of amino acid substitutions of HIV-1 was observed in slow and nonprogressors while rapid progressors possess HIV-1 carrying a low frequency of the substitutions. However, only one HLA-B7-restricted Env epitope of HIV-1 from non- and slow progressors expressing HLA-B7 was compared with one HLA-Cw4-restricted Env epitope of HIV-1 from rapid progressors expressing HLA-Cw4. Therefore, the conclusion that the increase of antigenic diversity of HIV-1 stimulates the CTL responses that prevent the progression of AIDS should be reevaluated by the detailed analysis of diversity of CTL epitopes presented by many HLA class I molecules between non- or slow progressors and rapid progressors or in the clinical course of the disease in each individual. Especially, analysis of the association among mutations on the epitopes in isolated viruses from HIV-1-infected individuals carrying HLA-A24, the CTL responses for these epitopes and the progress of the disease may be important to clarify this idea because HLA-A*2402 molecules present the epitopes in rather diverse regions that are strongly recognized by T cells.

HLA-A2 is the most investigated HLA class I allele in the study of HIV-1 CTL epitopes. So far, 13 HLA-A2-restricted CTL epitopes have been suggested (27–37) although only 8 were identified as epitopes by the CTL killing of target cells infected with the vaccinia recombinant virus or elution of the specific peptides from infected target cells (26–29, 31, 34, 35). Our studies also showed that HLA-B35 molecules can present at least 9 CTL epitopes (16). The present study, moreover, supported that HLA class I molecules can present multiple HIV-1 CTL epitopes. Although not all of these epitopes are immunodominant, at least 6 of 12 HLA-A24-restricted epitopes and 5 of 9 HLA-B35-restricted epitopes were presented as strong epitopes. Analysis of CTL recognition for multiple immunodominant epitopes and their mutations are required to evaluate precisely the immunity of HIV-1-infected individuals and to clarify the mechanism of immune escape of HIV-1.

In the present study, we identified multiple HIV-1 CTL epitopes presented by HLA-A24. Since HLA-A24 is the most popular allele in Japanese (70.4%) and very frequently observed in other populations (18, 19), these HLA-A24-restricted HIV-1 CTL epitopes are strong candidates for the vaccine of HIV-1 for not only Japanese but also other populations. The combination of this allele and TAP2.1 or TAP2.3 is associated with shorter time to AIDS onset (38). The molecular mechanism of HLA-A24 molecules in rapid progression of AIDS is unknown. Analysis of CTL recognition of the mutations in seven strong epitopes showed that approximately 50% of mutations in the Env epitopes affect the recognition of CTL clones. This implies that HIV-1 carrying these mutations can escape the immunologic surveillance via CTL. Further study of CTL recognition for these HLA-A*2402-restricted HIV-1 CTL epitopes in HIV-1-infected individuals will clarify the role of CTL in HIV-1 infection. The analysis of mutations in HLA-A*2402-restricted HIV-1 epitopes and CTL responses to these mutant epitopes in the clinical course of HLA-A24-positive, HIV-1-seropositive individuals are now under investigation.

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