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Single T Cell Receptor-Mediated Recognition of an Identical HIV-Derived Peptide Presented by Multiple HLA Class I Molecules

Takamasa Ueno, Hiroko Tomiyama, and Masafumi Takiguchi

A dual specific human CTL clone harboring one β and two inframer α transcripts of TCR was previously reported to recognize an HIV Pol-derived nonapeptide (IPLTEEAEL) endogenously presented by both syngeneic HLA-B*3501 and HLA-B*5101. In the current study, a retrovirus-mediated TCR transfer of individual α- and β-chains to TCR-negative hybridoma showed that Vα12.1 TCR in complex with Vβ5.6 were responsible for the peptide-specific response in the context of both HLA-B*3501 and HLA-B*5101, confirming single TCR-mediated dual specificity. The second TCR-α chain was not somehow expressed on the cell surface. Remarkably, the Vα12.1/Vβ5.6 TCR also recognized the same peptide presented by allogeneic HLA class I molecules that share the similar peptide-binding motifs, such as HLA-B*5301 and HLA-B*0702. The sensitivity of peptide recognition by the Vα12/Vβ5.6 TCR appeared to be comparable when the peptide was presented by syngeneic and allogeneic HLA class I molecules, with changes in T cell responsiveness caused largely by peptide-binding capacity. Moreover, the CTL clone bearing Vα12.1/Vβ5.6 TCR showed substantial cytolytic activity against the peptide-loaded cells expressing HLA-B*3501, HLA-B*5101, HLA-B*5301, or HLA-B*0702, providing further evidence that a single TCR complex can recognize the same peptide presented by a broad range of HLA class I molecules. A TCR with fine specificity for an HIV Ag but broad specificity to multiple HLA molecules may provide an advantage to the generation of allorestricted, peptide-specific T cells, and thus could be a potent candidate for immunotherapy against HIV infection. The Journal of Immunology, 2002, 169: 4961–4969.
similar peptide-binding motif (HLA-B7-like supertypes). Together, these findings indicate an additional level of cross-reactivity in T cells, demonstrating that the TCR complex interacts predominantly with the peptide Ag, with the HLA class I molecule behaving solely as a scaffolding component for the peptide rather than as a restriction element for the TCR.

Materials and Methods

Cloning of TCR-encoding genes and reconstruction of the TCR complex

Total RNA was prepared from CTL clones (∼1×10⁶ cells) using a total RNA isolation kit (Qiagen, Valencia, CA). cDNA clones encoding α and β TCR were obtained by reverse transcription using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA) with the isolated total RNA (∼50 ng) and a primer specific for Cα (5′-actgattgagcttcgtcgtgataca-3′) or Cβ (5′-ttgcttggtgagagctctgtcggt-3′), respectively. cDNA was then amplified by PCR using a high fidelity Pfu polymerase (Promega, Madison, WI) and was cloned into a plasmid. The DNA sequence of the α and β TCR-encoding genes was determined using an automatic DNA sequencer (LI-COR, Lincoln, NE). To obtain full-length TCR genes (i.e., containing not only the V and J regions, but also the C, transmembrane, and cytoplasmic domains), we used an overlapping PCR strategy using cDNA clones encoding α (pY1.4) and β (JUR-β-2) TCR chains from Jurkat cells (provided by Dr. T. W. Mak, University of Toronto, Toronto, Ontario, Canada) as templates.

Construction of retroviral vectors and gene transfer

Retrovirus-mediated gene transfer was used to reconstruct αβ TCR complexes on TG40/CD8 cells essentially as described by Kitamura et al. (20). Briefly, the genes of interest, i.e., full-length α and β TCR, were subcloned into the retroviral vector pMX, which was kindly provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan). The resulting constructs were first transfected into the ectotropic virus packaging cell line Platinum-E (also a gift from Dr. T. Kitamura) (21) using the transfection reagent Lipofectamine 2000 (Invitrogen, Groningen, The Netherlands). Two days later, culture supernatant containing recombinant virus was collected and then incubated with TG40/CD8 cells in the presence of 10 μg/ml polybrene for 6 h. The cells were cultured for an additional 2 days for analysis of TCR gene expression or selection of cells using appropriate antibiotics. Transduced TG40/CD8 cells showing bright staining by anti-mouse CD3e mAb were selected by fluorescence-activated cell sorting and used for further functional assays.

Peptide binding to HLA class I molecules

Peptide-binding affinity for HLA class I molecules was assessed by an HLA stabilization assay as described (7, 22). In brief, a series of RMA-S transfectants expressing HLA-B*3501, HLA-B*5101, or HLA-B*5301 was cultured for 18 h at 26°C and then pulsed with various concentrations of peptide for 3 h at 37°C. Cells were then incubated at 37°C for 3 h and subsequently stained by an anti-HLA class I mAb, TP25.99, and FITC-conjugated IgG fraction of sheep anti-mouse Ig (SILENUS Labs, Victoria, Australia). The surface expression level of the HLA class I molecule was evaluated by a mean fluorescence intensity (MFI) determined by flow cytometric analysis (FACScalibur; BD Biosciences). The concentration of the peptide that yielded a half maximal binding level (BL50) was calculated by defining the surface expression level of HLA class I molecules on cells incubated continuously at 26°C for 24 h as maximal.

IL-2 assays for cellular activation

TCR recognition of peptide-HLA class I complexes was analyzed by cellular activation of TCR-transduced TG40/CD8 cells, measured by IL-2 production as previously described by Zumla et al. (17) with modifications. CIR cells or transfectants expressing HLA molecules (10⁴ cells/well) were preincubated in a 96-well microtiter plate for 30 min at 37°C in the absence or presence of various concentrations of peptide. TG40/CD8 cells (2×10⁴ cells/well) expressing a defined TCR complex were added to the culture medium containing RPMI 1640 and 10% FCS in a total volume of 200 μl. A portion of the culture supernatant (100 μl) was removed from each well after a 48-h incubation at 37°C, transferred to another culture plate, and frozen at −20°C. The amount of IL-2 produced by the TG40/CD8 cells was determined by analyzing the proliferative activity of the IL-2 indicator cell line CTLL-2. CTLL-2 cells (2×10⁴/well) were incubated with the prepared culture supernatants for 18 h at 37°C. [³H]Thymidine (Tdr; Amersham Pharmacia, Piscataway, NJ) was added to the cells and they were then incubated for an additional 6 h before being harvested onto glass fiber filters. After thorough washing of the filters with distilled water, the amount of [³H]Tdr incorporated by CTLL-2 cells that was retained on the filters was determined by a scintillation counter (MicroBeta TriLux; Wallac, Oy, Turku, Finland). The EC₅₀ value of the peptide was calculated as the concentration of peptide that exhibited a half-maximal activation of TCR-transduced TG40/CD8 cells with CD3e-mAb-mediated activation of the cells defined as maximal.

Cytotoxic assay

The cytotoxic activity of the CTL clones was determined by a standard ⁵¹Cr-release assay as previously described (7). When peptide-pulsed CIR cells were used as target cells, CIR cells or transfectants expressing HLA class I molecules were first incubated with 100 μCi Na₂⁵¹CrO₄ (Amersham Pharmacia) in saline for 1 h at 37°C, and then washed three times with a culture medium. The ⁵¹Cr-labeled CIR cells (2×10⁴ cells/well) were plated in a 96-well round-bottom microtiter plate in the absence or presence of the various concentrations of peptide. After 1 h, CTL clones (2×10⁴ cells/well) were added and the plate was incubated for an additional 4 h at 37°C. To determine maximum and spontaneous ⁵¹Cr release, 5% Triton X-100 or culture medium alone, respectively, was also added to different wells. A portion of the culture supernatant (100 μl) was then removed from each well and analyzed by a gamma counter. The percent specific lysis was calculated as previously described (7).

Results

Cloning of TCR-encoding genes and reconstruction of the TCR complex

Two CTL clones, designated 562 and 589, originally established from an HIV-infected patient (HLA-A24/A26, B35/B51, Cw3/−) following stimulation of peripheral lymphocytes with an HIV Pol-derived synthetic epitope peptide (HIV Pol4448-4456: H-IPLTTEAELOH) were previously found to exhibit dual specific recognition of the same
Following cloning and sequencing analysis of TCR-encoding genes, or infected with viruses expressing HIV Gag-Pol polyproteins (7). Following cloning and sequencing analysis of TCR-encoding genes, we previously reported that CTL clone 589 had two infame TCR-α transcripts (Vα10.1 and Vα12.1) and one TCR-β transcript (Vβ2.1). However, the CTL clone was not stained by anti-Vβ2.1 mAb (7).

In the current study, we first asked whether one or two TCR complexes are responsible for the dual recognition exhibited by CTL clone 589. The TCR-negative mouse T cell hybridoma cell line, TG40 (17), was transduced with human CD8α to give TG40/CD8 cells, which were then transduced with TCR genes. Cells transduced with Vα12.1 and Vβ2.1 TCR (TG40/CD8/TCRα12β2.1) were clearly stained by anti-CD3e, anti-Vα12, and Vβ2.1 mAbs (data not shown), indicating that the human-derived TCR was successfully reconstructed on the surface of the TG40/CD8 cells. This result is in accordance with a previous report demonstrating that αβ TCR was expressed on the surface of TG40 cells upon transduction with TCR-encoding genes isolated from Jurkat T cells (17). However, the observation that the cells were stained by anti-Vβ2.1 mAb appeared inconsistent with our previous finding that the parent CTL clone 589 was not stained by the same mAb (7). To clarify this issue, we reisolated TCR-encoding genes from CTL clone 589. This time, no TCR-α transcripts from the Vβ2.1 family were isolated, and instead a different TCR-β transcript classified in the Vβ5.6 family was isolated (Table I). CTL clone 562, isolated from the same patient as clone 589 (7), were also found to have the same TCR-β gene from the Vβ5.6 family, suggesting that the correct TCR-β chain recognizing the Pol448-456 peptide presented by B35 is from the Vβ5.6 family and not the Vβ2.1 family (see also Figs. 1 and 2). Therefore, we used the newly isolated Vβ5.6 gene for the remainder of this study. We again isolated two inframe α transcripts of Vα10.1 and Vα12.1 families in both CTL clones (Table I), as reported previously (7).

Expression of only one TCR complex bearing Vα12.1/Vβ5.6 on the cell surface

To address the issue of whether one or two TCR complexes is responsible for the dual recognition exhibited by CTL clone 589, TG40/CD8 cells were transduced with a retroviral vector expressing the Vβ5.6 TCR and puromycin-resistance genes. Puromycin-resistant cells were isolated and then transduced with retroviral vectors expressing either the Vα10.1 or Vα12.1 TCR coupled bi-cistronically with green fluorescent protein (GFP). Transduced cells were stained by anti-CD3e mAb and then analyzed by flow cytometry. As shown in Fig. 1A, TG40/CD8/Vβ5.6 cells transduced with Vα12.1 TCR were stained by anti-CD3e mAb, indicating that αβ TCR bearing Vα12.1/Vβ5.6 formed a complete TCR/CD3 complex on the transduced cells. In contrast, TG40/CD8/Vβ5.6 cells transduced with Vα10.1 TCR were not stained by CD3e mAb (Fig. 1A). Because these cells showed biocistronic expression of GFP, mRNA encoding Vα10.1 TCR should have been transcribed in GFP-positive cells. Moreover, TCR-α protein was observed in an immunoprecipitate using an anti-αα-Mab αF1 (Endogen) following detergent solubilization of GFP-positive cells (data not shown). This suggests that the Vα10.1 gene was transcribed and translated into protein in the transduced cells, but the TCR-α protein was somehow unable to form a heterodimer complex with the Vβ5.6 TCR protein and therefore was not present on the cell surface. It has been reported that particular combinations of murine αβ TCR are deficient in paring to form mature heterodimer complexes at the posttranslational stage (23), which may explain our observation. Taken together, these data suggest that only one TCR complex bearing Vα12.1/Vβ5.6 is functionally expressed on the surface of the parental CTL clones and, therefore, that this single Vα12.1/Vβ5.6 TCR complex is responsible for the dual specificity of the CTL clone for Pol448-456 peptide presented by both B35 and B51.

A single TCR complex bearing Vα12.1/Vβ5.6 can mediate both HLA-B35 and HLA-B51-restricted peptide recognition

To directly confirm that the Vα12.1/Vβ5.6 TCR complex has dual specificity, TG40/CD8 cells were transduced with a biocistronic retroviral vector containing Vα12.1 and Vβ5.6 or Vβ2.1 genes, and then cells showing bright staining with anti-CD3e mAb were sorted by flow cytometry.

We first tested the ability of the transduced TG40/CD8 cells to bind to the PE-labeled B35 tetramer (Fig. 1B). Cells expressing Vα12.1/Vβ5.6 or Vα12.1/Vβ2.1 TCR were similarly stained with anti-CD3e mAb (Fig. 1B), indicating that they expressed a comparable level of TCR/CD3 complex on their surface. In contrast, cells expressing the Vα12.1/Vβ5.6 TCR were stained by the B35 tetramer, whereas the cells expressing the Vα12.1/Vβ2.1 TCR were not stained (Fig. 1B). This indicates that only the Vα12.1/Vβ5.6 TCR can specifically interact with the Pol448-456 peptide.

Table I. TCR-encoding genes reisolated from CTL clones 562 and 589

<table>
<thead>
<tr>
<th>V Gene</th>
<th>J Segment</th>
<th>CDR3 Sequence</th>
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<tbody>
<tr>
<td>Vα10.1</td>
<td>TRAV27*01</td>
<td>TRAJ56*01 CAGATNSKLTFGKG</td>
</tr>
<tr>
<td>Vα12.1</td>
<td>TRAV9*01</td>
<td>TRAJ53*01 CALSHNOSGSNLYLTFGKG</td>
</tr>
<tr>
<td>Vβ5.6</td>
<td>TRBV5*01</td>
<td>TRBJ2-5*01 CASSFRGGKTQYPFG</td>
</tr>
</tbody>
</table>

* Alignment of V and J regions of the TCR genes were analyzed by the International ImMunoGeneTics database (http://imgt.cines.fr:8104). Complementarity-determining region 3 (CDR3) sequences for α and β TCR are shown.
B35 complex. We could not investigate the dual specificity of the Vα12.1/Vβ5.6 TCR for Pol448–456 peptide/B51 complexes using tetramers as, despite many attempts to prepare a B51 tetramer in complex with the Pol448–456 peptide, we have not yet succeeded in re- folding the Pol448–456 peptide/B51 complex under cell-free conditions. Considering that the Pol448–456 peptide has previously been shown to be endogenously present on B51 molecules (7), distinct mechanisms might play a role in the exogenous and endogenous peptide-assembling pathway of B51 molecules (24).

We next tested the ability of the TCR-expressing TG40/CD8 cells to recognize the Pol448–456 peptide presented by B35 and B51 as assessed by a cellular activation assay (Fig. 2A). Both TG40/CD8/TCRα12β2.1 and TG40/CD8/TCRα12β5.6 cells were comparably activated by anti-CD3ε mAb (Fig. 2A), confirming a functional TCR/CD3-mediated signaling system in these cells. The transduced TG40/CD8 cells were then incubated in the presence or absence of 1 μM Pol448–456 peptide either with C1R cells or with a series of transfectants expressing HLA-A*0201 (A2), B35, and B51. TG40/CD8/TCRα12β5.6 cells exhibited substantial activation when cocultured with C1R-B3501 and C1R-B5101 in the presence of the peptide, but not when cocultured with C1R-A0201 in the presence of the peptide or with any of the cells in the absence of the peptide (Fig. 2A). In contrast, neither TG40/CD8 nor TG40/CD8/TCRα12β2.1 cells appeared to be activated in response to any of the C1R cells tested, even in the presence of 1 μM Pol448–456 peptide (Fig. 2A). These data clearly indicate that a single TCR complex bearing Vα12.1/Vβ5.6 has dual specificity recognizing Pol448–456 peptide presented by both B35 and B51. However, it should be noted that TG40/CD8/TCRα12β5.6 cells did show a small response to peptide-pulsed C1R and C1R-A0201 cells, although these responses were significantly lower compared with their response to peptide-pulsed C1R-B3501 and C1R-B5101 cells (Fig. 2A). This response could be due to the low level of endogenous HLA-B*3503 expressed on C1R cells (25)(see also Fig. 4B), which could bind Pol448–456 peptide and present it to the T cells.

To determine whether TG40/CD8/TCRα12β5.6 cells have a different sensitivity to Pol448–456 peptide presented by B35 or B51, TG40/CD8/TCRα12β5.6 cells were stimulated by the C1R transfectants in the presence of various concentrations of Pol448–456 peptide. As shown in Fig. 2B, the response of TG40/CD8/TCRα12β5.6 cells to both C1R-B3501 and C1R-B5101 cells was proportional to peptide concentration, with EC50 values of 2.6 and 140 nM for B35 and B51, respectively.

The Vα12.1/Vβ5.6 TCR recognizes the same peptide presented by multiple HLA class I molecules

A group of HLA class I alleles has been shown to share peptide-binding motifs and have the ability to bind a series of identical peptides (14). Among them, HLA-B7-like supertypes are defined
as HLA-B alleles with a preference for peptides with proline at position 2 (P2) and hydrophobic/aromatic residues at the C terminus (11, 13). These alleles include HLA-B*0702 (B7), B35, B51, HLA-B*5301 (B53), and HLA-B*7801 (B78). Although there are many polymorphic amino acid residues among different HLA class I alleles (Fig. 3), it may be possible that the Pol448–456 peptide, which has Pro at P2 and a hydrophobic residue at the C terminus, binds to all these HLA molecules, and is recognized by the same TCR. To test this possibility, TG40/CD8/TCR\textsubscript{12}/H9251\textsubscript{12}/H9252\textsubscript{5.6} cells were incubated separately with C1R cells expressing different HLA class I molecules in the presence or absence of various concentrations of Pol448–456 peptide, and then their activation was analyzed (Fig. 4A). The HLA expression levels were all shown to be comparable among the C1R transfectants, as assessed by staining with w6/32 mAb (Fig. 4B), which has a broad range of specificity to various HLA class I alleles and recognizes a conformational epitope of the HLA class I complex including \beta_2M and the polymorphic H chain (26). Remarkably, as shown in Fig. 4A, TG40/CD8/TCR\textsubscript{12}/B5\textsubscript{7} and B53 (EC\textsubscript{50} values of 690 and 270 nM, respectively), and moderate release in response to peptide presented by HLA-B*5201 (B52) (EC\textsubscript{50} of 1.4 \mu M). In contrast, the cells showed only a low level of IL-2 release when the peptide was presented by A2 and B78 (EC\textsubscript{50} > 10 \mu M). Given that the \textsubscript{12}/B5\textsubscript{6} TCR was isolated from peripheral T cells of an individual who carries B35 and B51 alleles but not B7 or B53, it is of significant interest that this TCR showed peptide-specific recognition restricted by allogeneic B7 and B53.

Apparent differences in T cell sensitivity for peptide presented by allogeneic and syngeneic HLA class I molecules is caused by different peptide-binding activities of these molecules

We next examined whether the apparent decreased sensitivity of TG40/CD8/TCR\textsubscript{12}/B5\textsubscript{6} cells to Pol448–456 peptide presented by
allogeneic HLA molecules is caused by decreased binding affinity of these molecules to the peptide. Using mutant peptides with different C-terminal anchor residues and hence with different binding activities to HLA class I molecules, we compared T cell responsiveness (EC50 value as determined by a cellular activation assay) and peptide-binding activity (BL50 value as determined by a HLA stabilization assay using RMA-S cells), and sought to analyze changes in TCR-peptide-HLA interactions (EC50/BL50 ratio) when the peptide was presented by self (B35) and nonself (B53) HLA class I molecules.

As revealed by HLA stabilization assays using an RMA-S transfected expressing B35, Pol448-456 peptide bound to self B35 molecules, with a BL50 value of 1.2 μM (Fig. 5A, Table II). Substitution of Leu to Ile at the C terminus (Pol448-456-9I) resulted in a ~35-fold reduced binding activity to B35, whereas substitution of Leu to Phe (Pol448-456-9F) showed a comparable binding activity to B35 (Fig. 5A, Table II). In cellular activation assays, TG40/CD8/TCRαβ256 cells responded comparably to Pol448-456 and Pol448-456-9F peptides, but showed ~38-fold reduced sensitivity to the Pol448-456-9I peptide (Fig. 5B). Consequently, virtually the same EC50/BL50 values were obtained for the peptides tested (Table II), indicating that the Vα12/Vβ5.6 TCR can tolerate changes of amino acid side chains at the C terminus of peptides presented by B35.

B35 and B53 share an identical α2 region and have five amino acid differences in the α1 region (Fig. 3). X-ray structural analyses have shown that three (residues 77, 80, and 81) of these five amino acids form part of the F pocket and therefore contribute in part to the preference of these HLA molecules for particular peptide C-terminal anchor residues (27, 28). Indeed, an HLA stabilization assay showed that Pol448-456 peptide bound to nonself B53 with a BL50 value of 68 μM (Fig. 5C, Table II), a ~50-fold decrease in binding activity compared with that for B35. Both Pol448-456-9I and Pol448-456-9F peptides showed 1.9- and 3.2-fold increased binding activity to B53, respectively (Fig. 5C), highlighting the differences between B35 and B53 in their binding peptide preference. In cellular activation assays, TG40/CD8/TCRαβ256 cells responded with 2.4- and 3.4-fold increased sensitivity to the Pol448-456-9I and Pol448-456-9F peptides presented by B53, respectively, compared with Pol448-456 peptide (Fig. 5D, Table II). Thus, similarly to peptides presented by B35, virtually the same EC50/BL50 values were obtained among the peptides tested (Table II), indicating that the Vα12/Vβ5.6 TCR can tolerate changes in amino acid side chains at the C terminus of peptides presented by B53.

The EC50/BL50 values obtained when the same peptide (either Pol448-456, Pol448-456-9I, or Pol448-456-9F) was presented by B35 or B53 were also all comparable (~2-fold differences; Table II). This indicates that the apparent decreased sensitivity of TG40/CD8/TCRαβ256 cells to Pol448-456 peptide presented by allogeneic HLA molecules is largely caused by the decreased binding activity of these molecules for the peptide and that the Vα12/Vβ5.6 TCR binds to the peptide-HLA complex with a comparable affinity whether the peptide is presented by B35 or B53.

It should be noted that, although TG40/CD8/TCRαβ256 cells recognized the Pol448-456 peptide presented by B51 but not by B78 (Fig. 4A), in the course of this HLA stabilization assays we observed poor binding of Pol448-456 peptide to both B51 and B78 (BL50 >1 mM). Because the ability of Pol448-456 peptide to be endogenously presented by B51 has previously been shown in a peptide-elution study using C1R-B5101 cells infected with recombinant vaccinia virus expressing the HIV Pol protein (7), the reason for not being able to detect binding of Pol448-456 peptide to B51 in the HLA stabilization assay is unclear. However, it has previously been documented that peptide binding to B51 is generally low (29) and that endogenous assembling of B51 for Ag presentation is slow (24) compared with B35. This could explain why Pol448-456 peptide binding to HLA-B51 could not be detected in our HLA stabilization assay.

A parental CTL clone killed peptide-pulsed target cells in the context of multiple HLA molecules

To further confirm that the Vα12.1/Vβ5.6 TCR has multiple specificity and can recognize a single peptide presented by a broad range of B7-like HLA supertype molecules, we tested the ability of parental CTL clone 589 to kill peptide-loaded target cells expressing different HLA molecules. Although the CTL clone has two inframe TCR-α transcripts, our data showed that only one (Vα12.1) is functionally expressed on their cell surfaces and is thus the determinant for Ag specificity in the CTL clone. In support of this, staining of the clone with anti-Vα12.1 mAb as well as Pol448-456/B35 tetramer showed virtually no negative subsets in the cell populations (data not shown).

As shown in Fig. 6, the CTL clone showed specific lysis activity against peptide-loaded C1R cells expressing B35, B51, B53, or Vβ5.6 TCR.

Table II. Summary of peptide-binding activity and T cell responsiveness in the context of self and nonself HLA class I molecules

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>HLA-B*3501</th>
<th>HLA-B*5301</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BL50 (M)</td>
<td>EC50 (M)</td>
</tr>
<tr>
<td>Pol448-456</td>
<td>IPLTEEAEL</td>
<td>1.2 × 10⁻⁶</td>
<td>2.6 × 10⁻⁶</td>
</tr>
<tr>
<td>Pol448-456-9F</td>
<td>----F</td>
<td>1.0 × 10⁻⁴</td>
<td>2.1 × 10⁻⁶</td>
</tr>
<tr>
<td>Pol448-456-9I</td>
<td>----I</td>
<td>4.3 × 10⁻⁵</td>
<td>1.0 × 10⁻⁷</td>
</tr>
</tbody>
</table>

* The BL50 and EC50 values were determined from the data shown in Fig. 5, A and C, and B and D, respectively. Dashes denote identical amino acids.
B7. The CTL clone was most sensitive to target cells expressing B35 (Fig. 6), consistent with the finding that recognition of Pol448–456 peptide by TG40/CD8/α12β5.6 cells was 50-fold more potent when the peptide was presented by B35 compared with when it was presented by B51 (Fig. 2B).

Discussion

The experiments presented in this study provide functional evidence that a single TCR complex has the ability to recognize a broad range of self and nonself HLA class I molecules while retaining fine specificity for a foreign antigenic peptide. The cross-reactivity of the TCR to the same peptide presented by multiple HLA class I molecules including B35, B51, B53, and B7 was confirmed using two different T cell systems and two different assay systems. In the first system, the Vα12.1/Vβ5.6 TCR was reconstructed on the surface of TG40/CD8 cells (a TCR-negative mouse T cell hybridoma cell line expressing human CD8α) and Ag recognition by the TCR was assessed by analyzing IL-2 release by the TCR-expressing TG40/CD8 cells. In the second system, the parental CTL clone was confirmed to exclusively express Vα12.1/Vβ5.6 TCR on its surface and Ag recognition by the TCR was assessed by determining the cytolytic activity of the CTL clone. In addition, modulation of peptide-binding affinity to different HLA class I molecules by introduction of mutant C-terminal anchor residues in the Pol448–456 peptide revealed that peptide recognition by Vα12.1/Vβ5.6 TCR was comparable when the peptide was presented by different HLA class I molecules. A TCR with fine specificity for an HIV Ag but broad specificity to multiple HLA molecules may provide an advantage to the generation of alloreactive, peptide-specific T cells, and thus could be a potent candidate for immunotherapy against HIV infection based on TCR gene transfer technology (30–32).

As many as 30% of human T cells have been reported to coexpress two different TCR-α chains on their cell surface (9). These T cells may be involved in some forms of unconventional recognition, such as allostereactivity and autoimmunity (9). Alternatively, dual receptor T cells may provide a benefit to the immune system by extending the immune repertoire for foreign Ags (33). Dual specificity of these T cells may be via the two different αβ TCR complexes each recognizing a different peptide-HLA complex. Indeed, in our previous study (7), a dual-specific CTL clone recognizing Pol448–456 peptide presented by B35 and B51 was found to have one β and two inframer α TCR transcripts. However, using retroviral gene transfer of the two αβ TCR complexes into TCR-negative TG40/CD8 cells, we showed that the dual specificity was not mediated through the two different αβ TCR complexes. Instead, a single αβ TCR complex bearing Vα12.1/Vβ5.6 exhibited multiple specificities, recognizing Pol448–456 peptide presented by multiple HLA class I molecules.

It is of note that the parental CTL clone showed a >10-fold higher sensitivity to Pol448–456 peptide presented by B35 compared with TG40/CD8 cells transduced with the Vα12.1/Vβ5.6 TCR. The latter recognized Pol448–456 peptide at concentrations down to 1 nM, but the parental CTL clone exhibited significant cytotoxic activity even at a peptide concentration of 0.1 nM. It is conceivable that certain costimulatory molecules present on the surface of the CTL clone, but not on the mouse-derived TG40/CD8 cells, may enhance ligand recognition by the CTL clone, or that CTL clones have some intrinsic property that allowed them to achieve low-density ligand recognition. In fact, T cells have been shown to increase their affinity/avidity for an epitope following Ag stimulation through changes in their membrane organization and a redistribution of signaling molecules (reviewed in Ref. 34). We thus propose that the system used in this study involving reconstruction of the TCR complex on the surface of TG40/CD8 has a marked advantage in investigation of ligand recognition by TCRs because the effect(s) of costimulatory molecules or membrane architectures can be excluded.

Five of the HLA class I alleles analyzed in this study, B35, B51, B52, B53, and B78, are members of a serologically cross-reacting group, whereas the sixth, B7, is a member of a different serological group. However, all except B52 preferentially bind peptides with (Pro at P2) in their binding peptides, with B52 preferentially binding peptides with Gln at P2 (14). There are only two amino acid differences between B51 and B52 alleles: Asn63 and Phe67 of B51 are replaced by Glu63 and Ser67 in B52 (19). Because structural analysis of the B51 molecule has shown that the Phe67 residue forms a part of the B pocket (35), the Phe to Ser substitution in B52 most likely accounts for its different peptide-binding preference. It is thus likely that the observed 10-fold reduced recognition by Vα12.1/Vβ5.6 TCR-bearing T cells of Pol448–456 peptide presented by B52 compared with peptide presented by B51 is due to reduced binding of Pol448–456 peptide to the B52 molecule.

Allelic difference in HLA-B*3501 and HLA-B*5301 stems five amino acid residues found in the α1 region associated with HLA-Bw4/Bw6 epitopes. Structural studies of B35 and B53 have shown that three of the five polymorphic residues form the part of F pocket and thereby attribute in part to the preference of these HLA molecules for particular C-terminal anchor residues in binding peptides (27, 28). By precise comparison of the data for T cell responsiveness and peptide-binding capacities, we show in this study that the Vα12.1/Vβ5.6 TCR interacted comparably with the Pol448–456 peptide presented by both self B35 and nonself B53. The tolerance of the Vα12.1/Vβ5.6 TCR for polymorphic differences between different HLA class I molecules could be due to this TCR making relatively strong contacts with other regions of the antigenic surface of the peptide-HLA complex, such as charged interactions through the three Glu residues located at P5, P6, and P8 of the Pol448–456 peptide. It is also likely that this TCR recognizes the peptide and shared structural features of several HLA-B molecules, as reflected in their common propensity to bind the same peptide, because crystal structures of several peptide/MHC class I complexes have consistently shown that 70–80% of the peptide surface is buried in the binding cleft of MHC, leaving only 20–30% of the peptide surface exposed for interaction with solvent or TCR (36).

A very high level of cross-reactivity is an intrinsic and necessary characteristic of ligand recognition of the TCR (37). For example, the murine 2C TCR recognizes the octapeptide LSPFFPD with syngeneic Kβ and also with allogeneic Lβ (38, 39), and this TCR also recognizes another octapeptide EQYKFYSV with Kβ and allogeneic Kb (1, 40). Cross-reactivity of TCR has often been explained by the molecular mimicry model, proposing that different peptide/MHC complexes may form antigenic surfaces which are similar in shape, charge, or both (41, 42). In contrast, Zhao et al. (43) have reported in their structural study that rather than simple molecular mimicry, unpredictable arrays of common and differential contacts on the different peptide/MHC complexes are used for their recognition by the xeno-reactive murine TCR AHIII12.2. In addition, a recent detailed mutational study focusing on 15 TCR contact sites on the HLA-A2 molecule recognized by an A6 TCR specific for the Tax peptide presented by HLA-A2 has shown that only three amino acids (Arg56, Lys66, and Ala69) located on the α1 helix affect T cell recognition (44). However, in contrast, a study of mouse 2C TCR binding to the QL9 peptide presented by class I MHC H2-Ld estimated that 37% of the binding energy results from recognition of peptide, with 63% resulting from recognition of the MHC (45). Considering that different types
of interactions might be expected within different TCR/pptide/ MHC complexes, mutational analysis and crystallographic structural analysis of the Vol2.1/Vβ5.6 TCR and the Pol448/αβ/ TCR in complex with self and nonself HLA class I molecules could provide further insights into the molecular details of how a single TCR recognizes different HLA class I molecules while retaining its peptide specificity.

It should also be noted that, in repeated experiments, TG40/ CD8/TCRαβ5.6 cells recognized the Pol448/αβ peptide presented by B7 but not by B78. The failure to detect binding of Pol448/αβ peptide to B78 as assessed by an HLA stabilization assay appeared to directly correlate with a lack of responsiveness by TG40/CD8/TCRαβ5.6 cells to peptide-loaded C1R cells expressing B78. Consistent with these observations, the preferred binding peptide motif of B78 (Pro, Ala, or Gly at P2 and hydrophobic amino acid residues at P6) (46), does not fully match the Pol448/αβ peptide sequence (which has Gln at P6), whereas the preferred binding peptide motif of B7 (Pro or Leu or Phe at P9) (14) does match the Pol448/αβ peptide sequence. In addition, B78 and B51 have identical α2 helices and B78 and B35 differ by only one amino acid in the α1 helix at residue 74 (Asp and Tyr in B78 and B35, respectively), whereas B7 and B35 differ by 20 amino acids including the difference at residue 74 (Asp in B7 as well). It may be possible that the Tyr74 residue found in B35, B51, B52, and B53 plays an important role in binding to Pol448/αβ peptide and that the change to Asp74 is responsible for the loss of this binding in B78. However, because B7 also has an Asp74 resi-idue, the other amino acid changes might contribute to compensating for the decreased peptide binding, TCR binding, or both. It is obvious that staining the CTL clones or the TCR-expressing TG40/CD8 cells using an HLA tetramer with B7, B51, or B53 could provide additional arguments on the ligand recognition of this cross-reactive TCR. However, we have not yet succeeded in refolding the Pol448/αβ peptide with these HLA molecules despite many attempts, probably because the HLA complex with a low-binding peptide is relatively unstable during the course of the refolding step.

In summary, by focusing on various HLA class I alleles that share similar peptide-binding motifs, we demonstrated that a single αβ TCR complex (Vol2.1/Vβ5.6) has the ability to specifically recognize a foreign peptide presented by multiple self (B35 and B51) and nonself (B53 and B7) HLA class I molecules. Consid-ering that this TCR was isolated from an HIV-infected patient (HLA-A24/26, B35/51, Cw3/−), T cells bearing this TCR should become the primary targets of T cell-mediated immune defense in individu-als with HIV infection.

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