A Cyclophilin B Gene Encodes Antigenic Epitopes Recognized by HLA-A24-Restricted and Tumor-Specific CTLs

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A Cyclophilin B Gene Encodes Antigenic Epitopes Recognized by HLA-A24-Restricted and Tumor-Specific CTLs

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We have studied Ags recognized by HLA class I-restricted CTLs established from tumor site to better understand the molecular basis of tumor immunology. HLA-A24-restricted and tumor-specific CTLs established from T cells infiltrating into lung adenocarcinoma recognized the two antigenic peptides encoded by a cyclophilin B gene, a family of genes for cyclophilins involved in T cell activation. These two cyclophilin B peptides at positions 84–92 and 91–99 induced HLA-A24-restricted CTL activity against tumor cells in PBMCs of leukemia patients, but not in epithelial cancer patients or in healthy donors. In contrast, the modified peptides at position 2 from phenylalanine to tyrosine, which had more than 10 times higher binding affinities to HLA-A24 molecules, could induce HLA-A24-restricted CTL activity against tumor cells in PBMCs from leukemia patients, epithelial cancer patients, or healthy donors. PHA-activated normal T cells were resistant to lysis by the CTL line or by these peptide-induced CTLs. These results indicate that a cyclophilin B gene encodes antigenic epitopes recognized by CTLs at the tumor site, although T cells in peripheral blood (except for those from leukemia patients) are immunologically tolerant to the cyclophilin B. These peptides might be applicable for use in specific immunotherapy of leukemia patients or that of epithelial cancer patients. The Journal of Immunology, 1999, 163: 4994–5004.

Many genes encoding tumor Ags recognized by CTLs were recently identified from melanoma cDNA (1–8). Tumor Ags from the other tumors recognized by CTLs were also characterized, including HER-2/neu (9, 10), prostate-specific Ag (11, 12), mucin (13), CEA (14), and SART1 (15). These Ags are for the most part not truly tumor-specific Ags; rather, they are self Ags expressed in normal cells and tissues. T cells infiltrating into tumor sites (2, 3, 16) or into eye in patients with Vogt-Koyanagi-Harada disease (17) recognize these melanoma Ags and lyse both melanoma cells and normal melanocytes. Administration of the wild gp100 peptide failed to induce CTLs in most cases of melanoma patients (18), suggesting that T cells in the circulation of melanoma patients are also generally tolerant to the gp100, regardless of the in vitro results of CTL induction by this peptide (19). These results suggest that T cells in the circulation are usually immunologically tolerant to peptides of self Ags, whereas those at tumor site or at an immuno-privileged site recognized them. In contrast to the wild-type, the modified gp100 peptide was more efficient for induction of CTLs in vitro (20), and was able to induce CTLs in vivo; the subsequent administration of IL-2 resulted in tumor regression in melanoma patients (18). The HLA-A24 allele is found in ~60% of the Japanese population (in a majority, of 95%, the genotype is A*2402, 20% of Caucasians, and 12% of Africans (21). Therefore, we have studied Ags recognized by HLA-A24-restricted and tumor-specific CTLs to better understand the molecular basis of tumor immunology, reporting that a cyclophilin B gene encoded antigenic epitopes recognized by the CTLs.

Materials and Methods

Generation of the CTL line

The HLA-A24-restricted and tumor-specific CTL (GK-CTL) line was established from tumor-infiltrating lymphocytes (TILs) of a patient with lung adenocarcinoma (HLA-A*0206/A*2402, B39/B52, Cw7/) by incubation of TILs with IL-2 alone for more than 60 days using previously reported methods (15). Briefly, the patient’s TILs were incubated with the culture medium (45% RPMI 1640 medium, 45% AIM-V medium; Life Technologies, Walkersville, MA), 10% FCS (Equitech Bio, Ingram, TX) with 100 U/ml of IL-2 (Shionogi Pharmaceutical, Osaka, Japan). These IL-2-activated TILs were tested for CTL activity every 7 days to various cancer and normal cells by a 6-h 51Cr-release assay and an IFN-γ assay with an ELISA (limit of sensitivity: 10 pg/ml) as reported elsewhere (15, 22). The HLA class I genotypes of these tumor cells were described previously (15, 22, 23). GK-CTL sublines were established from the parental CTL line by incubation of cells at 1, 2, or 4 cells/well of 96-well U-bottom microculture plates with the culture medium and IL-2 (100 U/ml) in the presence of irradiated (30 Gy) HLA-A24+ allogenic PBMCs as feeder cells, as reported (23). The surface phenotype of the CTL line and sublines was investigated by an immunofluorescence assay with FITC-conjugated anti-CD3, -CD4, or -CD8 mAb (22). For inhibition of CTL activity, 100 µg/ml of anti-class I (W6/32, IgG2a) or anti-CD8 (IgG2a), anti-class II (H-DR-1, IgG2a), anti-CD4 (IgG1, anti-CD4 (IgG1) mAb were used as reported previously (22). Anti-CD14 mAb (IgG2a) or -CD13 (IgG1) was served as an isotype-matched control mAb. A two-tailed Student t test was employed for the statistical analysis in this study.

Identification of the cyclophilin B gene

Methods for identification of a gene coding for tumor Ag recognized by CTLs were reported previously (15). In brief, poly(A)+ RNA of the
performed with dideoxynucleotide sequencing method using a DNA sequencing kit (Perkin Elmer, Foster, CA), and the sequence was analyzed by the ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

**Northern blot analysis**

mRNA expression of a cyclophilin B (Cyp-B) gene on various tumor cells and tissues was investigated by Northern blot analysis with a 32P-labeled Cyp-B probe according to previously reported methods (15). β-actin probe was used as a control. The relative expression level of the mRNA was calculated by the following formula: index = (Cyp-B density of a sample/β-actin density of a sample) × (β-actin density of unstimulated PBMCs/β-actin density of unstimulated PBMCs) (15). Expression of the HLA class I or HLA-A24 Ags on these cells was studied by staining of the cells with anti-class I (w6/32) mAb recognizing a polymorphic region of HLA class I molecule or anti-HLA-A24 mAb recognizing a polymorphic region of HLA-A24 molecule (One Lamda, Canoga Park, CA), and the expression was measured by FACSscan analysis (Becton Dickinson, San Jose, CA) as reported previously (23).

**Detection of TCR repertoire**

TCR Vβ chain mRNA expression was investigated with the RT-PCR method described previously (24). Briefly, total RNA of the GK-CTL line and its sublines was converted to cDNA. cDNA was individually mixed with 10 μl of lipofectin in 70 μl of OPTI-MEM (Life Technologies) for 15 min. Thirty microliters of the mixture was then added to the COS-7 (2 × 107) cells and incubated for 5 h, and 200 μl of the RPMI 1640 medium containing 10% FCS was added and cultured for 2 days, followed by incubation with CTLs (10⁴ cells/well). After an 18-h incubation, 100 μl of supernatant was collected to measure IFN-γ production by an ELISA in a duplicate assay as reported (15). This concentration (100 ng/well) of HLA-A*2402 cDNA was chosen based on the fact that the expression level of HLA-A24 Ags on the surface of COS-7 cells transfected with 50, 100, 200, or 400 ng/well of HLA-A*2402 cDNA was 35, 42, 40, or 20%, respectively. The average cell IFN-γ production was calculated by the following formula:

\[
\text{IFN}-\gamma\text{ production (pg/ml)} = \frac{\text{index}}{\text{cell number} \times \text{assay volume (ml)}}
\]

where index = (Cyp-B density of a sample/β-actin density of a sample) × (β-actin density of a sample/β-actin density of unstimulated PBMCs).

**Peptides and evaluation of HLA-A*2402 binding**

Eleven different peptide sequences capable of binding to HLA-A24 among the 208 amino acids of Cyp-B (Cyp-B77-85, Cyp-B93-99, Cyp-B101-107, Cyp-B110-122, Cyp-B124-132, Cyp-B135-141, Cyp-B144-151, Cyp-B152-161, and Cyp-B169-184) were synthesized based on a previous report regarding the HLA-A24-binding motif (26). HIV-derived peptide (RYPLTFGWCF) capable of binding to HLA-A*2402 Gag was used as a negative control (27). Cyp-B110-122 peptide was also provided for a case in which the methionine of the upstream was used as the start codon (28, 29). The modified peptides of Cyp-B84-92 and Cyp-B91-99 at position...
2 from phenylalanine to tyrosine (Cyp-B84–92F-Y and Cyp-B91–99F-Y, respectively) were synthesized because these modified peptides are expected to have a stronger binding motif to HLA-A*2402 molecules (26). These peptides were kindly provided by Dr. Kanou (Sumitomo Pharmaceutical, Osaka), and their purity was 85–95%.

Estimated scores of half time of dissociation of each Cyp-B peptide for HLA-A24 molecule were calculated based on HLA peptide motif search results with computer analysis (30) as follows; Cyp-B17, 9.3; Cyp-B23, 9.8; Cyp-B29, 6.0; Cyp-B34, 28.0; Cyp-B34, 2F-Y, 280.0; Cyp-B81, 15.0; Cyp-B92, 2F-Y, 150.0; Cyp-B122, 6.6; Cyp-B92, 2F-Y, 79.2; Cyp-B122, 30.0; Cyp-B152, 3.6; and Cyp-B152, 16.5, 3.5. Among them, the four peptides (Cyp-B44–49, Cyp-B44–492F-Y, Cyp-B91–99, and Cyp-B91–992F-Y) capable of inducing CTL activity in PBMCs were further evaluated for their binding activity to HLA-A24 molecules using RMA-S-A*2402 cells, as reported previously (31). In brief, brief at a concentration of 10^{-7} M, with more than 25% of the mean fluorescence intensity (MFI) of RMA-S-A*2402 cells cultured at 26°C, were evaluated as binding peptides. Relative MFI of RMA-S-A*2402 cells was obtained by subtracting the MFI value of peptide-unloaded RMA-S-A*2402 cells stained with TP25.99 mAb from that of peptide-loaded cells. The half-maximal binding level (BL50), which is the peptide concentration yielding the half-maximal MFI, was calculated. Binding peptides were classified according to the BL50 into three categories: high binder (BL50 < 10^{-5} M), medium binder (10^{-5} M < BL50 < 10^{-4} M), and low binder (BL50 > 10^{-4} M).

CTL induction by peptide

PBMCs were incubated with 10 μM of a peptide in one well of a 24-well plate. At days 7 and 14 of culture, the cells were retransformed with the irradiated (30 Gy) autologous PBMCs as APCs that had been preincubated with the same peptide (10 μM) for 2 h at an effector cell to APC ratio of 4 to 1. Effector cells were harvested at day 21 of the culture and tested for both their surface phenotypes and activity to produce IFN-γ in response to various target cells by an ELISA at different E:T cell ratios. For a 14Cr-release assay, the cryopreserved PBMCs from day 21 of culture were thawed and cultured in wells of 96-well U-bottom microculture plates in the absence of any peptide but in the presence of 100 U/ml of IL-2 and irradiated autologous PBMCs (2 × 10^6 cells/well) as feeder cells. HLA-A2402-restricted autologous PBMCs from healthy donors were used as feeder cells in some leukemia patients. Proliferating cells were transferred to wells of the 24-well plate at days 10–14 after reculture in the absence of feeder cells, and were further expanded in culture with IL-2 alone. These CTLs were retested for their surface phenotypes and CTL activity by an IFN-γ assay at around day 25 of culture, and then these cells were tested for their cytotoxicity by a 6-h 14Cr-release assay at different E:T ratios at around day 28–35. CTL sublines were established from the parental CTL line by incubation of cells at 1, 10, or 100 cells/well of 96-well U-bottom microculture plates with the culture medium and IL-2 (100 U/ml) in the presence of autologous PBMCs as feeder cells as reported (15, 23).

CTL precursor frequency analysis

The detailed methods of CTL precursor frequency analysis were described elsewhere (23). In this study, the Cyp-B44–492 peptide-stimulated PBMCs from a leukemia patient were plated at 1, 2, 5, 10, 20, 40, and 80 cells per well of 96-well microculture plates, and were cultured with the cloning medium (25% RPMI 1640 medium, 55% AIM-V medium, 20% FCS, and 100 U/ml of PHA, and 0.1 mM MEM nonessential amino acids solution) in the presence of irradiated allogeneic PBMCs of three healthy volunteers as feeder cells. PBMCs cultured without peptide were served as a negative control. Cells from each well were tested at 9 to 15 days after peptide stimulation for IFN-γ production in response to target cells. Wells were considered positive if they contained effector cells producing much higher levels (>100 pg/ml) of IFN-γ in response to the HLA-A*2402 MT-2 and Bec-2 cells as compared with the IFN-γ levels in response to the HLA-A*2402 RPMI8402 and Ban-B1 cells, respectively. Data were analyzed by the minimum X² method with the 95% confidence intervals, and the CTL precursor frequency was calculated by Taswell’s method (32).

**Results**

**Establishment of the HLA-A24-restricted and tumor-specific CTL**

The cells of the GK-CTL (CD3+CD4+CD8+ line, derived from TILs of lung adenocarcinoma, produced significant levels of IFN-γ by recognition of the majority of HLA-A*2402 epithelial cancer cells and the other cancer cells, leukemia cells, and virus-transformed cells, but did not react to any of the HLA-A*2402 target cells. This CTL line failed to respond to several HLA-A*2402+ epithelial cancer cells.
epithelial cancer cells. Representative results are shown in Table I. This CTL line showed significant levels of cytotoxicity against HLA-A*2402+ 11-18 lung adenocarcinoma cells, HT1376 bladder tumor cells, KOPT-K1 T cell lymphoma cells, and virus-transformed B cells (SSB and Bec-2), but not against any of the HLA-A*2402+ target cells or COS-7 cells (Fig. 1A). This CTL line, however, did not lyse HLA-A24+ PHA-activated normal T cells (PHA-blaster cells) from the PBMCs of healthy donors, whereas lymphokine-activated killer (LAK) cells showed HLA class I-nonrestricted cytotoxicity to all the target cells tested (Fig. 1B). CTL-mediated IFN-γ production by recognition of tumor cells was inhibited by 100 μg/ml of anti-class I (W6/32) or anti-CD8, but not by anti-class II (H-DR-1), anti-CD4, or isotype-matched irrelevant anti-CD13 or anti-CD14 mAb (Fig. 1C). These results indicate that the GK-CTL line showed HLA-A24-restricted cytotoxicity against tumor cells and virus-transformed cells, but not against normal cells. Because of its wide spectrum of reactivity, this CTL line may recognize widely shared Ags on HLA-A*2402 molecules.

Identification of a gene

A total of 10^5 clones from the cDNA library of the HT1376 tumor cells, which induced the highest IFN-γ production by the GK-CTLs, were tested for their ability to stimulate IFN-γ production by the GK-CTLs after cotransfection with the HLA-A*2402 cDNA into COS-7 cells. This method allows identification of genes encoding tumor-rejection Ags (15). After the first and second screening, one clone (6A1-4F2) was found to confer recognition by the GK-CTLs (Fig. 2). Namely, the CTLs produced significant amounts of IFN-γ by the third screening (Fig. 2). Cyp-B, a family of cyclophilins involved in T cell activation (34, 35), is reported to be ubiquitously expressed in normal cells and tissues, with a higher expression in lymphoblastoid cells (28, 29, 33). We have investigated mRNA expression of Cyp-B by Northern blots analysis, and a part of the results is shown in Fig. 3. Cyp-B mRNA was ubiquitously expressed in tumor cells, PHA-blaster cells, and unstimulated PBMCs, with the highest expression in HT1376 tumor cells from which Cyp-B gene was cloned.

HLA-A24 expression and the ability to stimulate IFN-γ production

Levels of IFN-γ production by CTLs largely differed among the HLA-A*2402 tumor cells, as shown in Table I. Therefore, we
investigated the correlation between levels of HLA-A24 expression on the surface and the activity to stimulate IFN-\(\gamma\) production by the CTLs in the HLA-A*2402 homozygous tumor cells (Fig. 3). PBMCs of an HLA-A24 homozygous donor were served as a control. Both HLA-A24 expression and the ability to stimulate IFN-\(\gamma\) production were highest or higher in HT1376 or SKG-I tumor cells, respectively. In contrast, HLA-24 expression was very low in MKN-45 and LC99A tumor cells, and was undetectable in LK79 cells. None of them stimulated IFN-\(\gamma\) production by CTLs.

The Cyp-B mRNA was detectable in all these tumor cells. These results suggest that the level of HLA-A24 expression, but not that of Cyp-B expression, correlates with the ability to stimulate IFN-\(\gamma\) production.

Identification of Cyp-B peptide recognized by GK-CTLs

Twelve different Cyp-B-derived peptides were synthesized, loaded onto the HLA-A*2402-transfected COS-7 cells at a concentration of 10 \(\mu\)M, and were tested for their ability to induce IFN-\(\gamma\) production by GK-CTLs (Fig. 4A). Among these peptides, three peptides (Cyp-B77-85, Cyp-B84-92, and Cyp-B91-99) stimulated the significant levels of IFN-\(\gamma\) production by CTLs. Among these peptides, two peptides (Cyp-B84-92 and Cyp-B91-99) stimulated IFN-\(\gamma\) production in a dose-dependent fashion (Fig. 4B). The significant level of IFN-\(\gamma\) production was induced by Cyp-B91-99 peptide as low as 0.01 \(\mu\)M or by Cyp-B84-92 peptide as low as 10 \(\mu\)M. In contrast, a dose dependency was not obtained in the remaining

**FIGURE 4.** Determination of Cyp-B peptides recognized by the GK-CTLs. A, Each of the 12 different Cyp-B peptides or an HIV-derived peptide (a negative control) was loaded at a concentration of 10 \(\mu\)M to COS-7 cells that had been transfected with HLA-A*2402 cDNA 2 days before. Two hours later, the CTLs were added at an E:T ratio of 20 and incubated for 18 h, followed by harvesting of supernatant for measurement of IFN-\(\gamma\) by the ELISA in triplicate assays. The background of IFN-\(\gamma\) production by the CTL in response to the HLA-A*2402-transfected COS-7 cells alone (50 pg/ml) was subtracted from the figure. B, Cyp-B77-85 (●), Cyp-B84-92 (○), or an HIV-derived peptide (□) (a negative control) was loaded at various doses to COS-7 cells that had been transfected with HLA-A*2402 2 days before, and 2 h later the CTLs were added at an E:T ratio of 20. The background of IFN-\(\gamma\) production by the CTLs in response to the HLA-A*2402-transfected COS-7 cells alone (50 pg/ml) was subtracted from the figure.
GK-CTL subline 2-40

No pep.  
Cyp B84-92  
Cyp B91-99  
HIV pep.  

IFN-γ production (pg/ml)

* p<0.05

GK-CTL subline 2-3

No pep.  
Cyp B84-92  
Cyp B91-99  
HIV pep.  

IFN-γ production (pg/ml)

Peptide specificity of the CTL sublines. GK-CTL sublines were established from the parental CTL line by incubation of cells at 2 cells/well of 96-well U-bottom microculture plates with the culture medium and IL-2 (100 U/ml) in the presence of HLA-A24\(^*\) allogenic PBMCs as feeder cells as reported (23). Eighty different sublines were tested for their reactivity to CIR-A\(^*\)2402 cells pulsed with Cyp-B84-92, Cyp-B91-99, or HIV-derived peptide (a negative control) at an E:T ratio of 5 to 1 in the triplicate assays. Four or three sublines showed the reactivity to Cyp-B84-92 or Cyp-B91-99 peptide, respectively. Representative results of each subline are shown in this figure. Some other sublines failed to respond to any peptides tested, while the remaining sublines showed LAK activity (data not shown).

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Peptide specificity of the CTL sublines and TCR repertoire

GK-CTL sublines were established from the parental line by incubation at 1, 2, or 4 cells/well, and their peptide specificity were tested. Among 80 different sublines tested, four sublines recognized CIR-A\(^*\)2402 cells pulsed with Cyp-B84-92, but not either Cyp-B91-99 or HIV-derived peptide as a negative control, whereas the other three sublines recognized CIR-A\(^*\)2402 cells pulsed with Cyp-B91-99, but not the others. Representative results of each subline are shown in Fig. 5. The remaining sublines failed to respond to any peptides tested. Then, the TCR V\(\beta\) repertoire of these two sublines shown in Fig. 5 along with the parental GK-CTL lines were analyzed by the RT-PCR. TCR V\(\beta\) usage in the parental line, 2-40 and 2-3 sublines were polyclonal with the preferential uses of V\(\beta\)-1 and V\(\beta\)-15 in the parental line, V\(\beta\)-1 and V\(\beta\)-16 in 2-40 subline, and V\(\beta\)-1 and V\(\beta\)-16 in the 2-3 line.

Induction of CTL activity by peptides

Cyp-B84-92, Cyp-B84-92F-Y, Cyp-B91-99 and Cyp-B91-99F-Y peptides were tested for their respective ability to stimulate IFN-γ production in PBMCs of HLA-A24\(^*\) leukemia patients. HIV-derived peptide was used as a negative control in certain experiments. PBMCs from all five HLA-A24\(^*\) patients (two patients with acute lymphoblastic leukemia [ALL], two with acute myeloid leukemia, and one with Hodgkin’s lymphoma) produced significant levels of IFN-γ in response to the HLA-A*2402\(^*\) MT-2 and Bec-2 cells when stimulated three times in vitro by either Cyp-B84-92 or Cyp-B91-99. The modified peptides induced CTL activity with levels of activity lower than induced by their wild-types. Representative results of one ALL patient are shown in Table II. PBMCs from these five patients were comprised of less than 10% leukemia cells at the time of harvesting, IFN-γ production by the peptide-induced CTLs in response to HLA-A*2402\(^*\) tumor cells was inhibited by anti-CD8, but not by anti-CD4 mAb (data not shown).

These four peptides were then tested for their ability to induce CTL activity in PBMCs from epithelial cancer patients. The PBMCs of four patients (one with stomach cancer, two with lung adenocarcinoma, and one with laryngeal squamous cell carcinoma) of the six HLA-A24\(^*\) patients produced the significant levels of IFN-γ in response to the HLA-A*2402\(^*\) target cells when stimulated by Cyp-B84-92F-Y or Cyp-B91-99F-Y, but not when stimulated by the wild peptides. Representative results of a stomach cancer patient are shown in Table II.

Similarly, these peptides were tested with PBMCs of healthy donors. PBMCs from five of six HLA-A24\(^*\) healthy donors showed HLA-A24-restricted IFN-γ production when stimulated by the modified peptides, but not when stimulated by any of the wild peptides. Representative results are shown in Table II. In PBMCs from the remaining healthy donor, the Cyp-B91-99 induced higher levels of HLA-A24-restricted IFN-γ production. The Cyp-B91-99F-Y induced lower but significant levels of CTL activity (data not shown).

Twenty-five to 40% of CD3\(^+\)CD4\(^-\)CD8\(^+\) cells were observed in the population of the PBMCs showing the HLA-A24-restricted CTL activity at day 21 by analysis of their surface phenotypes (data not shown). The remaining cells were mostly CD3\(^+\)CD4\(^-\)CD8\(^-\). To confirm CTL activity by a 6-h 51Cr-release assay, these PBMCs at 21 days were further incubated for an additional 23–28 days in the absence of any peptides but in the presence of IL-2 and feeder cells (irradiated autologous PBMCs for most cases, HLA-A24\(^*\) allogenic PBMCs for some leukemia patients). CTL activity of these cells was redetermined by an IFN-γ assay before their use in a 6-h 51Cr-release assay. When stimulated with Cyp-B84-92 (Fig. 6a) or Cyp-B84-92F-Y (Fig. 6b), PBMCs of a HLA-A24\(^*\) leukemia patient lysed the HLA-A*2402\(^*\) tumor cells (BALL-1 cells from B cell type ALL, or 11-18 lung adenocarcinoma cells) and the virus-transformed cells (MT-2 or Bec-2 cells), but did not lyse either HLA-A24\(^*\) PHA-activated T cells from a healthy donor or any HLA-A24\(^*\) target cells (RPMI8402, HL-60 cells of monocye type from a patient with acute promyelocytic leukemia, or COS-7 cells). When stimulated with the Cyp-B84-92F-Y, PBMCs of a HLA-A24\(^*\) stomach cancer patient also lysed the
HLA-A*2402^1^ KOPT-K1 and the MT-2 cells, but did not lyse any HLA-A24^2^ target cells (Fig. 6c). Similarly, when stimulated with the Cyp-B_84–92 F-Y (Fig. 6d) or the Cyp-B_91–99 F-Y (Fig. 6e), PBMCs of HLA-A24^1^ healthy donors lysed the HLA-A*2402^1^ tumor cells (KOPT-K1, MT-2, and 11–18 tumor cells), but did not lyse any HLA-A24^2^ PHA-activated T cells from healthy donors, including the autologous T cells, or any HLA-A24^2^ target cells. Forty to 50% of CD3^1^ CD4^2^ CD8^1^ cells were observed in the population of PBMCs showing the HLA-A24-restricted CTL activity at the time of 51 Cr-release assay. The remaining cells were mostly CD3^1^ CD4^1^ CD8^2^.

In contrast, PBMCs that failed to show HLA-A24-restricted IFN-γ production at day 21 did not proliferate well in most cases. LAK cell activity was observed in the remaining few cases in which PBMCs proliferated well (data not shown).

HLA-A24^1^ PBMCs failed to respond to an HIV-derived peptide taken as a negative control (data not shown). HLA-A24^2^ PBMCs from any of healthy donors (n = 5) or patients (n = 7, three with hematological malignancy and four with solid cancer) did not exhibit HLA-A24-restricted CTL activity when stimulated with any of the four Cyp-B peptides tested. Either LAK cell activity or no cytotoxic activity was observed in these PBMCs (data not shown).

**Peptide-specificity and CTL precursor frequency**

To confirm the peptide specificility, the sublines were established from the PBMCs from a healthy donor that had been stimulated with Cyp-B_84–92 F-Y peptide. Among the four sublines tested, one subline 100-1 responded to CIR-A*2402 cells pulsed with Cyp-B_91–99 F-Y peptide used for stimulation, but not pulsed with Cyp-B_84–92 F-Y (Fig. 7). This CTL-mediated IFN-γ production was inhibited by 100 μg/ml of anti-class I (W6/32) or anti-CD8, but not by anti-class II (H-DR-1), anti-CD4, or isotype-matched irrelevant mAb (Fig. 7).

CTL precursor frequency was determined in PBMCs of a patient with ALL after the third stimulation in vitro to better understand the ability of Cyp-B peptide to induce CTL activity. PBMCs cultured without peptide were served as a negative control (Fig. 7).

### Table II. Induction of HLA-A24-restricted CTL activity in PBMCs by Cyp-B-derived peptides^a^

<table>
<thead>
<tr>
<th>Donors (Disease)</th>
<th>HLA-A</th>
<th>Peptide</th>
<th>IFN-γ Production (pg/ml) by Recognition of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia patient 1 (B cell-ALL)</td>
<td>A24/A2</td>
<td>Cyp-B_84–92</td>
<td>456</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_84–92 F-Y</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_91–99</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_91–92 F-Y</td>
<td>0</td>
</tr>
<tr>
<td>Stomach cancer patient 1</td>
<td>A24/26</td>
<td>Cyp-B_84–92</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_84–92 F-Y</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_91–99</td>
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<tr>
<td></td>
<td></td>
<td>Cyp-B_91–92 F-Y</td>
<td>292</td>
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<td>HD 1</td>
<td>A24/A31</td>
<td>Cyp-B_91–92 F-Y</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyp-B_84–92</td>
<td>203</td>
</tr>
</tbody>
</table>

^a^ PBMCs from HLA-A24^1^ patients (ALL and stomach cancer) or HLA-A24^+^ healthy donors were stimulated in vitro with 10 μM of the peptide shown above. After the third stimulation, PBMCs were tested for their ability to produce IFN-γ by recognition of various target cells at an E:T ratio of 2. Values represent the mean of IFN-γ production of the triplicate determinants. Background of IFN-γ production by the PBMCs alone (50–200 pg/ml) was subtracted from the values shown in the table.

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**FIGURE 6. Induction of CTLs by Cyp-B peptides.** The peptide-stimulated PBMCs were tested for their cytotoxicity at different E:T ratios in a 6-h 51 Cr-release assay against HLA-A*2402^+^ tumor cells (BEC-2, MT-2, KOPT-K1, BALL-1, or 11–18 cells), PHA-activated T cells from three healthy donors (HLA-A24^+, A24^−^, A33, or A2/A30), and HLA-A*2402^+^ target cells (RMBl8402, HL-60, or COS-7 cells). a and b, CTL activity of PBMCs from an HLA-A24^−^ leukemia patient (ALL) stimulated with Cyp-B_84–92 and Cyp-B_84–92 F-Y, respectively. c, CTL activity of PBMCs from an HLA-A24^+^ patient with stomach cancer stimulated with Cyp-B_84–92 F-Y. d and e, CTL activity of PBMCs from HLA-A24^+^ healthy donors stimulated with the Cyp-B_84–92 F-Y and the Cyp-B_91–92 F-Y, respectively.
control. The frequency was found to be 1/1368 or undetectable (1/30,000) in the stimulated PBMCs or those cultured without peptide, respectively (Fig. 8).

**Evaluation of binding activity**

Binding affinities of the four peptides were analyzed. HBV-derived peptide (WFHISCLTF) capable of binding to HLA-A2402 and HIV-derived peptide (VPVKLKPGM) capable of binding to HLA-B35 were served as positive and negative controls, respectively (31). All the four peptides bound to HLA-A2402 molecules with different affinities (Fig. 9). Cyp-B_{84-92}F-Y and Cyp-B_{91-99}F-Y were evaluated as high binders, Cyp-B_{91-99} as a median binder, and Cyp-B_{84-92} as a low binder.

**FIGURE 7.** Peptide specificity of the peptide-induced CTLs. CTL sublines were established from PBMCs from a healthy donor that had been stimulated with Cyp-B_{91-99}F-Y peptide. These PBMCs were thawed and cultured in the absence of peptide, but in the presence of IL-2 and irradiated autologous PBMCs (1 x 10^6 cell/well) as feeder cells at 100 cells/well of a 96-well U-bottom microculture plate for 25 days. The cells from the four wells containing well-proliferating cells were tested at an E:T ratio of 2 to 1 in the triplicate assays for their reactivity to CIR-A*2402 cells pulsed with Cyp-B_{84-92}F-Y or Cyp-B_{91-99}F-Y in the presence or absence of 100 μg/ml of mAb shown in the figure. Among them, one subline 100-1 recognized CIR-A*2402 cells pulsed with Cyp-B_{91-99}F-Y peptide used for stimulation, but not Cyp-B_{84-92}F-Y as shown in the figure. The other three sublines showed LAK activity (data not shown).

**FIGURE 8.** CTL precursor frequency. The CTL precursor frequency was determined in PBMCs of a patient with ALL after the third stimulation in vitro with Cyp-B_{84-92}. PBMCs cultured without peptide were served as a negative control. Detailed methods are shown in Materials and Methods. The frequency was found to be 1/1368 or undetectable (<1/30,000) in the stimulated PBMCs or those cultured without peptide, respectively.

**FIGURE 9.** Binding of Cyp-B-derived peptides to HLA-A*2402 molecules. The four peptides (Cyp-B_{84-92}, Cyp-B_{81-92}F-Y, Cyp-B_{91-99}, and Cyp-B_{91-99}F-Y) were evaluated for their binding activity to HLA-A24 molecules using RMA-S-A*2402 cells as reported previously (31). Detailed methods are shown in Materials and Methods. Experiments were repeated three times with consistent results, and data of one experiment are shown in the figure.
Discussion

This study has reported that a Cyp-B gene encodes antigenic epitopes recognized by the HLA-A24-restricted and tumor-specific CTLs. Recent studies show that immunologic cancer Ags are not truly foreign or nonself, but rather are self Ags (1–4, 7, 9–15). Cyp-B is a self Ag playing an important role in protein folding with peptidyl-prolyl isomerase activity (28, 34, 35). Cyp-B, in conjunction with calcium-modulating cyclophilin B-binding protein, transmits a signal from TCRs to the calcineurin, which in turn is involved in regulation of intracellular calcium for lymphocyte activation (36). The region of Cyp-B from positions 85 to 100, which includes the peptides (Cyp-B84–92 and Cyp-B91–99) capable of inducing CTLs, is important due to its biological activity of signal transmission that induces lymphocyte activation (35–37). This region is highly conserved in other members of cyclophilins (35–37). Further, this region contains the sequence critical for binding to cyclophilin A (38, 39), an immunosuppressive agent widely used in organ transplantation. Therefore, this region will be crucial for its biological function. However, it is presently unclear why this region contains epitopes recognized by CTLs. This domain might be ubiquitinated after transmission of a signal to the calcineurin, and thus be processed and loaded to the groove of HLA-class I molecules.

Among 12 peptides tested, relative affinities of the Cyp-B84–92 and Cyp-B91–99 were modest and lower than the several other Cyp-B peptides by HLA peptide motif search results. These results are consistent with those from the others (40). Peptides with intermediate affinity, but not with high affinity, seem to be recognized by HLA-A2-restricted melanoma-specific CTLs (40).

Cyp-B mRNA was ubiquitously expressed in tumor cells with the various levels of expression. Capability of tumor cells to stimulate IFN-γ production by the CTLs correlated with the levels of HLA-A24 molecules, but not with Cyp-B mRNA level. Therefore, HLA-class I expression on tumor cells could be a major limiting factor in CTL-mediated recognition of Cyp-B peptide.

TCR Vβ usage of the GK-CTL line and its sublines tested were polyclonal with the preferential use of several Vβ-chains. These results are consistent with our previous investigation determined by the methods of DNA sequencing of TCR Vα and Vβ usage of CTL clones established from TILs (25). These results along with the other studies of sublines suggest that the GK-CTL line consists of a polyclonal population of CTLs reactive to HLA-A24+ tumor cells, and a few of them recognize the Cyp-B-derived peptides.

The wild-type Cyp-B peptides failed to induce HLA-A24-restricted CTL activity against tumor cells in PBMCs in most cases tested except for those from leukemia patients. These results suggest that T cells in peripheral blood, except for those from the peripheral blood of leukemia patients, are immunologically tolerant to the Cyp-B. T cells in peripheral blood of healthy donors also seem to be usually immunologically tolerant to other self Ags (16–20, 41). Administration of the gp100 peptide failed to induce CTLs in most cases of melanoma patients (18), suggesting that T cells in the circulation of melanoma patients are also generally tolerant to self Ags, regardless of the fact that CTLs reacting to these Ags were detectable in vitro by repeated stimulation with a relevant peptide (16–20, 41). CTLs reacting to MAGE-3 are also not detectable in the PBMC of melanoma patients, even after in vivo administration (42). Cumulatively, these results suggest that T cells in the circulation are immunologically tolerant to self Ags recognized by CTLs that are located at the tumor site or at an immuno-privileged site.

There might be another type of self Ag to which CTLs are easily detectable in PBMCs of healthy donors by in vitro stimulation with a relevant peptide. These self Ags would include tyrosinase (43), prostate-specific Ag (PSA) (12), and SART1 (23). Although mechanisms involved in this reactivity are not clear at the present time, one explanation might be based on antigenic mimicry. In particular, the SART1 peptide at positions 690–698 is highly homologous to a peptide of the membrane protein of Saccharomyces cerevisiae, a well characterized nonpathogenic fungus (23).

The modified Cyp-B peptides induced HLA-A24-restricted CTL activity against tumor cells in PBMCs from leukemia patients, epithelial cancer patients, and healthy donors. The modified gp100 peptide, which is more efficient for induction of CTLs in vitro, was able to induce CTLs in vivo, and the subsequent administration of IL-2 resulted in tumor regression in some melanoma patients (18). Therefore, the Cyp-B84–92F-Y and Cyp-B91–99F-Y peptides would be applicable for use in specific immunotherapy of HLA-A24+ cancer patients. We are now studying whether Cyp-B-derived peptides capable of binding to HLA-A2 molecules can be recognized by HLA-A2-restricted CTLs established from TILs of an HLA-A*0207+ colon cancer patient.

Binding activity of Cyp-B91–99 to HLA-A24 molecules was >10 times higher than that of Cyp-B84–92, which is consistent with the results of the experiments of dose dependency. Cyp-B84–92F-Y and Cyp-B91–99F-Y peptides were >10,000 and >10 times higher than Cyp-B84–92 and Cyp-B91–99 peptides, respectively. Although the binding activity of Cyp-B91–99F-Y was >10 times higher than that of Cyp-B84–92F-Y, large differences between the two modified peptides with regard to ability to induce CTLs were not observed in this study.

Although Cyp-B mRNA is ubiquitously expressed in normal cells, with a higher expression in lymphoblastic cell lines (28, 29, 33), neither the GK-CTL nor the CTLs induced by Cyp-B peptide lysed PHA-activated normal T cells. In contrast to PHA-activated T cells, the cells of melanocyte cell lines were susceptible to lysis by the MART-1 peptide-induced CTLs (17). This discrepancy might be partly due to the fact that the cells from melanocyte cell lines used in the study are well adapted for in vitro culture, whereas PHA-activated T cells are not. EBV-transformed B cells that are also adapted for in vitro culture were susceptible to lysis by both the GK-CTL line and the CTLs induced by the Cyp-B peptides. Several molecules in the activated T cells, including a family of serpins are involved in their resistance for the CTL-mediated lysis (44, 45). Another possibility can be attributed to the different posttranslational modification of the epitopes between normal and cancer cells. Some of the previously identified CTL epitopes have posttranslational modifications, and the modifications have had a significant impact on the ability of the CTLs to recognize those peptides (46, 47). Further studies, including determination of molecules involved in the resistance to lysis, are needed to clarify this issue.

This study used autologous PBMCs as peptide-pulsed APCs in all the cases to avoid allogenic stimulation. Autologous PBMCs were also used as feeder cells for expansion of the peptide-stimulated PBMCs in most cases. However, in few cases of leukemia patients, allogenic but HLA-A24+ PBMCs from healthy donors were used because of limiting number of available cells. Therefore, we investigated the possible negative influence of the allogenic stimulation. The Cyp-B91–99F-Y-stimulated PBMCs from a melanoma patient were lyzed by the GK-CTL line and its sublines tested were well adapted for in vitro culture, whereas PHA-activated T cells are not. EBV-transformed B cells that are also adapted for in vitro culture were susceptible to lysis by both the GK-CTL line and the CTLs induced by the Cyp-B peptides. However, in the case of allogenic stimulation, the levels of cytotoxicity were significantly higher in the former CTLs, which also failed to lyse any of the three PHA-blast cells (two with
In contrast, the CTLs expanded in the presence of allogeneic feeder cells showed the cytotoxicity to one (HLA-A24) of the three PHA-blast cells (data not shown). These results suggest that the autologous feeder cells are better if available, and the allogeneic HLA-A24+ feeder cells could be alternatively useful with attention to the allogeneic influence.

In summary, this study showed that the wild-type Cyp-B peptides could induce HLA-A24-restricted CTL activity in PBMCs be alternatively useful with attention to the allogenic influence. These results suggest that the autologous feeder cells are better if available, and the allogenic HLA-A24+ peptide could be applicable for use in specific immunotherapy of a relatively large number of leukemia patients, while the modified peptides could be applicable for leukemia patients and epithelial cancer patients.

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References


