Identification of a Unique Antigen Peptide pRL1 on BALB/c RLσ1 Leukemia Recognized by Cytotoxic T Lymphocytes and Its Relation to the *Akt* Oncogene

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Summary

BALB/c radiation leukemia RLσ1 is an immunogenic tumor. We established bulk and cloned cytotoxic T lymphocyte (CTL) lines from regressor (BALB/c × C57BL/6)F_1 ($CB6F_1$) spleen cells that recognized RLσ1 specifically. We then obtained antigen peptide recognized by CTL from RLσ1 by acid extraction. Analysis of the acid extract by reversed-phase high performance liquid chromatography (HPLC) on a semipreparative C18 column revealed that fractions eluted in 23 min (peak a) and 26 min (peak b) showed sensitization activity on the P815 target for specific CTL. On further purification of these fractions by HPLC and direct sequencing by Edman degradation, we identified the CTL-recognizing RLσ1 peptide pRL1a (IPGLPLSL) in peak a and its possible precursor peptide pRL1b (SIIPGLPLSL) in peak b. Sequence homology indicated that these peptides were derived from the 5′ untranslated region of c-*akt* oncogene.

Tumor rejection antigens were first found on methylcholanthrene-induced fibrosarcomas in mice (1–5). Immunization of syngeneic mice with a tumor was shown to render these mice resistant to successive challenge by the same tumor. A characteristic of tumor rejection antigens is extremely high polymorphism. In fact, even two tumors in the same animal possess different rejection antigens (6). These individually distinct antigens were later found on a variety of tumors of many other histological types in different species.

Studies on adoptive transfer showed that the tumor rejection response is mediated by T cells (7, 8); $CD8^+$ T cells are predominantly responsible for rejection, whereas $CD4^+$ T cells are involved to various extents depending on the tumor. It is generally accepted that the effector cells are mainly $CD8^+$ CTL and that $CD4^+$ T cells help $CD8^+$ CTL precursors to differentiate into effector cells (9, 10). In cases not involving $CD4^+$ T cells, $CD8^+$ helper cells participate in CTL induction (10).

BALB/c radiation-induced leukemia RLσ1 is a highly immunogenic tumor. In BALB/c mice hybridized with certain mouse strains, inocula of RLσ1 cells initially grew, formed a tumor, and then regressed. The different immune responsivenesses to RLσ1 rejection antigen of the various mouse strains used for producing F_1 hybrids have been ascribed to a gene located in the *H-2K* region (11). Spleen cells from regressor (BALB/c × C57BL/6)F_1 ($CB6F_1$)[1] mice generate $CD8^+$ CTL after in vitro stimulation (12). CTL have been shown to recognize RLσ1, but not other RL series of leukemias, radiation–leukemia virus–induced leukemias, fibrosarcomas, or blasts from normal lymphoid cells. These findings suggest that there is a unique (individually distinct) antigen on RLσ1. Adoptive transfer of CTL to BALB/c *nu/nu* mice protected the recipient mice from subsequent challenge with RLσ1 cells (13). In vivo depletion of $CD8^+$ T cells abrogated the rejection, whereas depletion of $CD4^+$ T cells had little effect (14, 15). These findings suggest that the RLσ1 rejection response is mediated by $CD8^+$ CTL. In this study, we identified a unique rejection antigen peptide, pRL1, that is recognized on RLσ1 by CTL derived from semisyngeneic $CB6F_1$ mice. Sequence homology analysis revealed that pRL1 is derived from the 5′ untranslated region of c-*akt* oncogene (16).

Materials and Methods

Mice. BALB/c, C57BL/6 (B6), and $CB6F_1$ mice were purchased from Japan SLC Co. (Shizuoka, Japan). Breeding pairs of

[1] *Abbreviations used in this paper:* $CB6F_1$, (BALB/c × C57BL/6)F_1; r, recombinant.

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BALB.B mice were provided by Dr. H. Fujiwara (Osaka University Medical School, Osaka, Japan). These mice were bred in our animal center.

Tumors and Cell Lines. RL♂1, RL♂4, and RL♀8 are radiation-induced leukemias in BALB/c mice (12). RVA, RVC, and RVD are leukemias induced by injection of radiation leukemia virus into neonatal BALB/c mice (17). P815 is a methylcholanthrene-induced mastocytoma in a DBA/2 mouse (18). These tumors were maintained in the strain of origin. T1.1.1 and T4.8.3 are derivatives of L cells (H-2^k) transfected with the H-$2L^d$ and H-$2D^d$ gene, respectively (19), and were provided by Dr. N. Shinohara (Mitsubishi Kasei Institute of Life Science, Machida, Japan).

Antibodies. Anti-L3T4 (CD4) mAb, a rat antibody of the IgG2b immunoglobulin class, produced by hybridoma GK1.5 (20), was provided by Dr. F. Fitch (University of Chicago, Chicago, IL). Anti-Lyt-2.2 (CD8) mAb, a mouse antibody of the IgG2a class, produced by hybridoma 19/178 was provided by Dr. U. Hämmerling (Memorial Sloan-Kettering Cancer Center, New York). Anti-TCR-β mAb, a hamster antibody of the IgG class, produced by hybridoma H57-597 (21) was provided by Dr. R. Kubo (National Jewish Center, Denver, CO). Anti-CD3ϵ mAb, a hamster antibody of the IgG class, produced by hybridoma 145-2C11 (22), was provided by Dr. J. A. Bluestone (University of Chicago). Anti-H-$2K^d$ and anti-H-$2D^d$ mAbs are mouse antibodies of the IgG2a class produced by hybridomas KD40 and DD98, respectively, that were established by hybridization of P3U1 myeloma and spleen cells from BALB.B mice that had been immunized with BALB/c lymphoid cells. Anti-H-$2L^d$ mAb is a mouse IgG2a antibody produced by a hybridoma 30-5-7 (23). Anti-IA^d mAb is a mouse IgG2b antibody produced by hybridoma MK-D6 (24). Anti-$IE^{k,d}$ mAb is a mouse IgG2b antibody produced by hybridoma ISCR3 (25). These mAbs were provided by Dr. N. Shinohara.

Peptide Synthesis and Purification. Peptides were synthesized by standard solid-phase methods using Fmoc chemistry in a peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA). Cleavage of the peptide from the resin and removal of the side chain protecting groups were carried out using 95% trifluoroacetic acid (TFA). The peptides were purified to >98% homogeneity by reversed-phase HPLC on a preparative C8 column (10 × 100 mm, 20 μm particle size; Applied Biosystems) in 0.1% TFA with an acetonitrile gradient. The purity of peptides was assayed in an analytical C18 column (4.6 × 150 mm, 5 μm particle size; Chemco, Osaka, Japan). The integrity of the purified peptides was confirmed by fast atom bombardment mass spectrometry (model 9020-DF; Shimazu, Kyoto, Japan).

Sequence Analysis. Sequencing was performed by NH_2-terminal Edman degradation using a protein sequencer (model 477A; Applied Biosystems, Inc.) coupled to a phenyl thiohydantoin analyzer (model 120A; Applied Biosystems, Inc.). Amino acid sequence homology was investigated using the National Biomedical Research Foundation (NBRF) database using GENETYX-MAC (Genetic Bio Database Software, Software Development Co., Tokyo, Japan).

Acid Extraction of Whole Cells and HPLC Analysis. Samples of 2.5 × 10^{11} RL♂1 ascites cells from 250 BALB/c mice were homogenized by 10 strokes in 0.1% TFA with a Dounce homogenizer and sonicated (Sonifier W-185; Branson Sonic Power, Danbury, CT) for 3 min. The homogenates were then stirred at pH 2.0 in 0.1% TFA for 30 min. The supernatants obtained by centrifugation at 100,000 *g* for 30 min were filtered with a molecular cut-off membrane (m.w. 5000, PLCC; Millipore Corp., Bedford, MA). These procedures were done at 4°C. The filtrates obtained from 10 mice were lyophilized, resolved in 0.1% TFA, and analyzed by reversed-phase HPLC (model 625L; Waters/Millipore, Milford, MA) on a semipreparative C18 column (ODP, 10 × 250 mm, 10 μm particles; Asahipak, Kawasaki, Japan). Solvent A was 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. The gradient for chromatography was 20–60% B over 40 min. The fractions (2.0 ml) collected at 1 min intervals were assayed for sensitization activity on P815 cells for anti-RL♂1 CTL (see below). Reversed-phase HPLC on a semipreparative C18 (ODP) column was repeated 25 times. Active fractions were pooled and subjected to reversed-phase HPLC on an analytical C2/C18 column (SuperPac™ Pep-S, 4 × 250 mm, 5 μm particles; Pharmacia LKB, Uppsala, Sweden) at neutral pH. For peak a obtained in the first round of HPLC analysis on the C18 (ODP) column (see Fig. 2 in the Results section), solvent A was 25% acetonitrile, and solvent B was 40% acetonitrile, with a gradient of 0–100% B over 30 min. For peak b obtained on the C18 (ODP) column, solvent A was water, and solvent B was 50% acetonitrile, with a gradient of 0–100% B over 30 min followed by isocratic elution with 100% B for 10 min. Fractions (1.0 ml) collected at 1 min intervals were assayed for sensitization activity as described above. Active fractions were pooled and that of peak a but not peak b was subjected to gel-filtration (Protein-Pak™ 60 column, 7.8 × 300 mm, 10 μm particles; Waters Millipore) and then reversed-phase HPLC on a C2/C18 column under acidic conditions. For peak a, solvent A and B were 25 and 40% acetonitrile, respectively, containing 0.1% TFA. The gradient was 0–100% B over 60 min. For peak b, solvent A was 0.1% TFA, and solvent B was 50% acetonitrile, containing 0.1% TFA. The gradient was 0–100% B for 30 min followed by isocratic elution with 100% B over 10 min. Fractions (250 and 100 μl) were collected at intervals of 0.25 and 0.1 min, respectively, and tested for sensitizing activity.

Acid Elution from Affinity-purified H-$2L^d$. Cells were lysed in a solution (lysis buffer) of 0.5% NP-40, 10 mM Tris-HCl (pH 7.5), 0.2 mM p-amidinophenyl (pA)-PMSF, 5 mM EDTA, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. The supernatant obtained by centrifugation at 100,000 *g* for 30 min was diluted to 0.1% NP-40 and loaded onto anti-L^d mAb (30-5-7)–coupled Affigel-hydrazide (Bio-Rad Laboratories, Inc., Richmond, CA). The column was washed extensively with 60 vol of a solution of 10 mM Tris-HCl (pH 7.5), 0.2 mM pA-PMSF, 5 mM EDTA, and 150 mM NaCl, and then bound material was eluted with 0.1% TFA. The eluate was filtered on a PLCC membrane with a cut off of m.w. 5,000 (PLCC 5,000). The filtrate was lyophilized and dissolved in 0.1% TFA for reversed-phase HPLC.

Generation of Con A Blasts. Spleen cells (2 × 10^7) were cultured with Con A at a concentration of 5 μg/ml for 3 d.

Establishment and Maintenance of CTL Clones. $CB6F_1$ spleen cells (5 × 10^7) from regressor mice were cultured with 5 × 10^6 irradiated (100 Gy) RL♂1 cells in tissue culture flasks (model 25100, Corning Glass Co., Corning, NY) for 5 d at 37°C under 5% CO_2 in air. The culture medium was RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM 2-mercaptoethanol. The harvested cells (5 × 10^4) were maintained as a bulk CTL line by weekly restimulation with 10^5 irradiated (100 Gy) RL♂1 stimulator cells and 5 × 10^6 irradiated (20 Gy) $CB6F_1$ splenic feeder cells in the presence of human recombinant IL-2 (rIL-2) (Takeda Chemical Industries, Osaka, Japan) at a concentration of 5 ng/ml in 24-well culture plates (76-033-05; Flow Laboratories, McLean, VA). Similar cultures from different mice were used for cloning. Cells were diluted to 3–0.3 cells/well and stimulated with 10^4 irradiated RL♂1 cells and 10^6 irradiated $CB6F_1$ splenic feeder cells in the presence of rIL-2 in 96-well culture plates (model 25860, Corning Glass Co.). After 10–14 d, the cytotoxicity of clonally

growing cells was tested. Six clones were chosen and maintained by weekly stimulation as described above.

Cell-mediated Cytotoxicity Assay. Tumor cells and Con A blasts were labeled by incubating 2×10^6 cells with 2 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in 0.3 ml of medium for 1 h at 37°C under 5% CO_2 in air. The cells were then washed and used as target cells. In direct assays, 10^4 labeled target cells (100 μl) were incubated with the effector cell suspension (100 μl). In antibody blocking assays, serially diluted mAb (100 μl) was added to the culture of effector cells (50 μl) and labeled target cells (50 μl). In competitive inhibition assays, unlabeled inhibitor cells (50 μl) with or without sensitization by the peptide-containing HPLC fraction were added at different ratios to 10^4 ^{51}Cr-labeled RLσ1 target cells (100 μl), and then the effector cells (50 μl) were added. In sensitization assays, 5 –10 μl of HPLC fractions or peptide solution in 100 μl of medium were added to 10^4 ^{51}Cr-labeled target cells (50 μl) and incubated for 60 min at room temperature before adding effector cells (50 μl). After incubation for 4 h at 37°C under 5% CO_2 in air, the supernatants (100 μl) were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: $[(a-b)/(c-b)] \times 100$, where a is the radioactivity in the supernatant of target cells mixed with effector cells, b is that in the supernatant of target cells incubated alone, and c is that in the supernatant after lysis of target cells with 1% NP-40.

Results

Generation of Bulk and Cloned CTL Lines Specific for RLσ1. Inoculation of 10^6 RLσ1 cells into the back of $CB6F_1$ mice results in formation of a tumor and then its regression (11). When spleen cells obtained from mice after tumor regression were cultured with irradiated (100 Gy) RLσ1 cells, cytotoxicity against the RLσ1 cells was generated in 5 d (12). The culture was maintained as a bulk CTL line by weekly repeated stimulation with 10^5 irradiated (100 Gy) RLσ1 cells and 5×10^6 irradiated (20 Gy) $CB6F_1$ splenic feeder cells in the presence of rIL-2 in 24-well culture plates. A culture from different mice was used for cloning. The cells were diluted to 3–0.3 cells/well and stimulated with 10^4 irradiated RLσ1 cells and 10^6 irradiated $CB6F_1$ splenic feeder cells in the presence of rIL-2 in 96-well culture plates. After culture for 10–14 d, the cytotoxicity of clonally growing cells was tested using half the culture. The other half was maintained as a clone by repeated stimulation as described above. We established six cloned CTL lines: clone 12, 14, 31, 33, 44, and 103.

The cytotoxicities of all the CTL lines against RLσ1 were eliminated by anti-Thy-1.2 mAb and anti-Lyt-2.2 (CD8) mAb, but not anti-L3T4 (CD4) mAb and complement, and were blocked by anti-CD3ε mAb (125-2C11), anti-TCR-β mAb (H57-597), and anti-Lyt-2.2 (CD8) mAb, but not anti-TCR-δ mAb (3A10) or anti-L3T4 (CD4) mAb in the absence of exogenously added complement.

The direct cytotoxicities of the bulk and the six cloned CTL lines were essentially similar. All the CTL lines showed cytotoxicity against RLσ1, but not RLσ4, RL♀8, BALB-RVA, C, D, P815, or normal splenic Con A blasts from BALB/c, C57BL/6, C3H, and $(BALB/c \times B6)F_1$ mice. The results of a typical experiment are shown in Fig. 1 *A*.

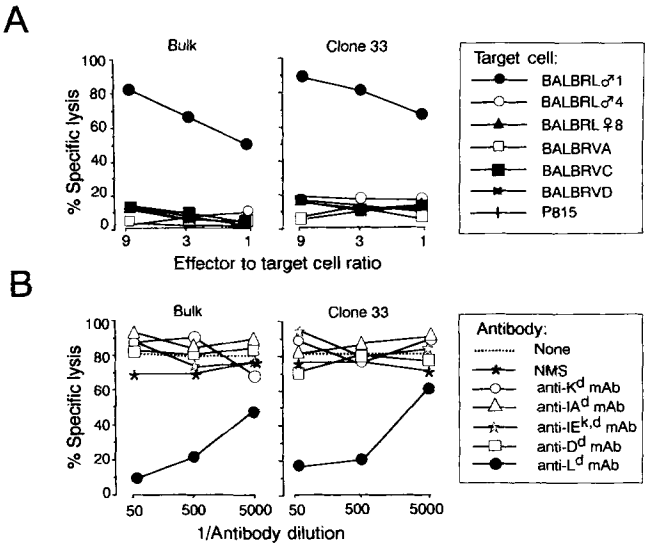

Figure 1. Direct cytotoxicity (*A*) and antibody blocking (*B*) of anti-RLσ1 CTL in 4-h ^{51}Cr-release assay. The effector/target cell ratios in *B* were 10 with bulk RLσ1 CTL and 5 with clone 33. The bulk and all six cloned CTL lines tested gave essentially similar results. NMS, normal mouse serum.

L^d Restriction for Specific CTL To Recognize RLσ1 Antigen. The H-2 restriction molecule for specific CTL to recognize RLσ1 antigen was determined by antibody blocking experiments. The cytotoxicities of the bulk and six cloned CTL lines were all blocked by anti-L^d mAb (30-5-7), but not by anti-K^d mAb (KD40), anti-IA^d mAb (MK-D6), anti-$IE^{k,d}$ mAb (ISCR3), or anti-D^d mAb (DD98). The results of a typical experiment are shown in Fig. 1 *B*.

Fractionation of Acid Extract from Whole RLσ1 Cells by Reversed-phase HPLC. A sample of 2.5×10^{11} RLσ1 ascites cells from 250 BALB/c mice was homogenized with 0.1% TFA. The homogenate was centrifuged at 100,000 *g* for 30 min, and materials of <5,000 daltons were obtained by filtering the supernatant on a molecular cut-off membrane and were separated by reversed-phase HPLC on a semipreparative C18 (ODP) column at an elution rate of 2 ml/min with an increasing concentration of acetonitrile. Each HPLC fraction (5 μl) was incubated with ^{51}Cr-labeled P815 target and cytotoxicity by RLσ1 CTL was determined. As shown in Fig. 2 *A*, sensitization activity was observed in two fractions, peaks a and b, with elution times of 23 and 26 min. The sensitization activities of the peaks a and b fractions were observed with the bulk and all six cloned CTL lines. The results of a typical experiment are shown in Fig. 2 *B*.

Antibody blocking of sensitization of P815 with the active fractions was investigated. In these experiments, an active fraction and antibody were present in the culture throughout the assay. Sensitization was blocked by addition of anti-L^d mAb, but not anti-K^d mAb, anti-IA^d mAb, anti-$IE^{k,d}$ mAb, or anti-D^d mAb. Results were essentially similar with the bulk and all six cloned CTL lines (data not shown).

Competitive Inhibition of RLσ1 Cytotoxicity by P815 Cells Sensitized with Active Fractions. P815 cells sensitized with active fractions were used as inhibitors of RLσ1 cytotoxicity in com-

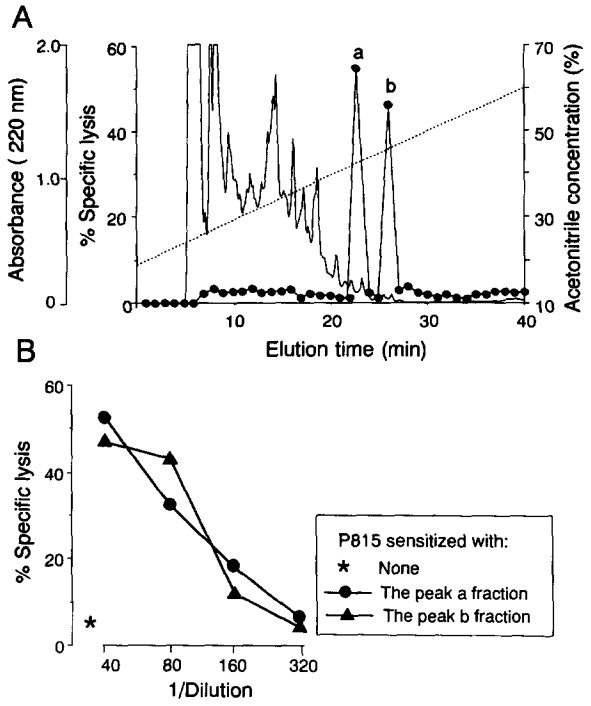

Figure 2. Reversed-phase HPLC on a semipreparative C18 (ODP) column with a 1%/min acetonitrile gradient and sensitization activity of each fraction in 4-h ^{51}Cr-release assay (*A*). Samples of 5 μl of each fraction collected in a volume of 2 ml/min were added to ^{51}Cr-labeled P815 target. The effector cells used were the bulk RLσ1 CTL. Active fractions eluted in 23 min (peak a) and 26 min (peak b) were serially diluted and tested for sensitization activity (*B*). Essentially similar sensitization activity was observed with the bulk and all six cloned CTL lines tested.

petitive inhibition assays. P815 cells sensitized with either the peaks a or b fraction inhibited the RLσ1 cytotoxicities of the bulk and the six cloned CTL lines. The results of a typical experiment are shown in Fig. 3.

Fractionation of the Acid Eluate of Affinity-purified L^d Molecules from RLσ1. A sample of 4.0 × 10^{10} RLσ1 ascites cells from 40 BALB/c mice was suspended in lysis buffer containing 0.5% NP-40 and incubated for 30 min at 4°C with shaking. The cell lysate obtained by ultracentrifugation was loaded onto an anti-L^d mAb-binding affinity column and the eluate in 0.1% TFA was collected. Materials of <5,000 daltons were obtained by filtering the eluate on a molecular cut-off membrane and were separated by reversed-phase HPLC on a semipreparative C18 column as described above. Each fraction (20 μl) was tested for sensitization activity. Fraction 23, but not fraction 26, showed sensitization activity (Fig. 4).

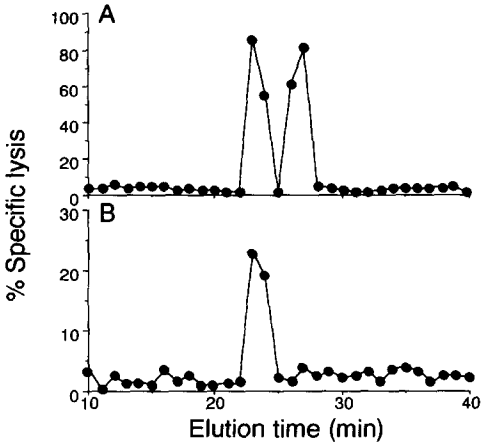

Figure 4. Sensitization activity of fractions separated by reversed-phase HPLC on a semipreparative C18 (ODP) column of an acid extract from whole cells (*A*) and an acid eluate from affinity-purified L^d molecules (*B*).

Further Purification of CTL-recognizing Peptides. For purification of active peptides, fractions in peaks a and b separated on a semipreparative C18 (ODP) column were further separated by reversed-phase HPLC on an analytical C2/C18 column at neutral pH and collected in volumes of 1 ml/min. As shown in Fig. 5, the active peptide in peak a was eluted

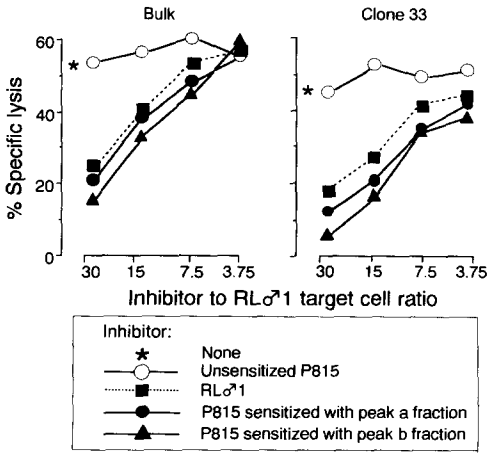

Figure 3. Competitive inhibition assays. Unlabeled inhibitor cells (50 μl) were serially diluted and added to 10^4 ^{51}Cr-labeled RLσ1 target cells (100 μl). Effector cells were then added. Cytotoxicity was determined by 4-h ^{51}Cr-release assay. The effector/target cell ratio was 5 with bulk CTL and 3 with clone 33.

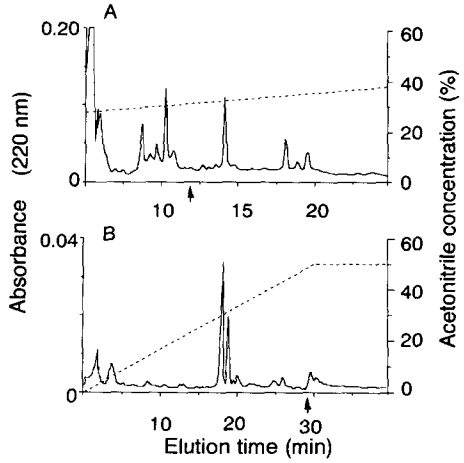

Figure 5. Reversed-phase HPLC fractions of peak a (*A*) and b (*B*) on the semipreparative C18 (ODP) column shown in Fig. 2 *A* were further separated by reversed-phase HPLC on an analytical C2/C18 column with an acetonitrile gradient of 0.5 (*A*) or 1.66% (*B*) per min at neutral pH. *Arrows* indicate active fractions.

in fraction 12, and the active peptide in peak b was eluted in fraction 30. Fraction 12 derived from peak a at a neutral pH was purified by repeated HPLC (seven times) and was subjected to gel filtration. The active fraction was then separated by reversed-phase HPLC in acidic conditions on an analytical C2/C18 column in fractions of 250 μl/15 s and each fraction was tested for sensitization activity (Fig. 6 *A*). Fractions with peak sensitization activity were denoted as a'. Fraction 30 from peak b in neutral pH conditions was purified by repeated HPLC (six times) and was separated by reversed-phase HPLC in acidic conditions on an analytical C2/C18 column and collected in fractions of 100 μl/6 s and each fraction was similarly tested for sensitization activity (Fig. 6 *B*). The fractions with peak sensitization activity were denoted as b'.

Amino Acid Sequencing by Edman Degradation. The amino acid sequences of peptides in active fractions of peaks a' and b' were analyzed by automated Edman degradation. The octamer peptide IPGLPLSL was identified in the fraction of peak a', and the decamer peptide SIIPGLPLSL was identified in the fraction of peak b'.

Characterization of Synthetic Peptides. Peptides IPGLPLSL and SIIPGLPLSL were synthesized and their sensitization activities were investigated. As shown in Fig. 7 *A*, sensitization on P815 and T1.1.1 (L^d-transfected L cell line) cells was observed at concentrations as low as 1–100 nM with the bulk and cloned RLO'1 CTL lines. The sensitization activity of

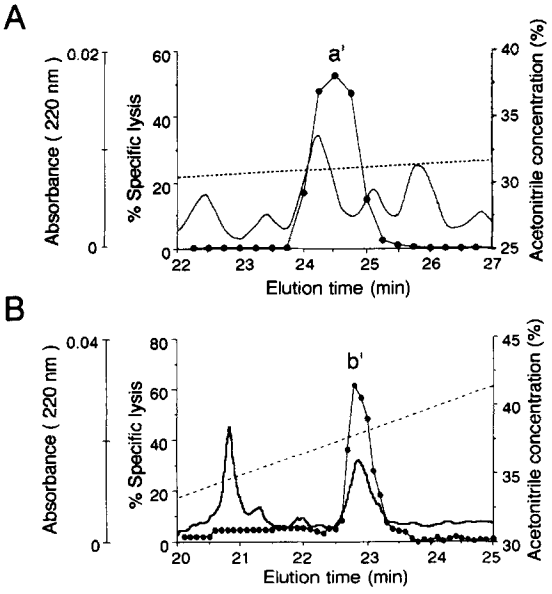

Figure 6. Fraction 12 shown in Fig. 5 *A* derived from peak a in Fig. 2 *A* was separated by reversed-phase HPLC on an analytical C2/C18 column in 0.1% TFA with an acetonitrile gradient of 0.25%/min and fractions in a volume of 250 μl/15 s were collected (*A*). Fraction 30 in Fig. 5 *B* derived from peak b in Fig. 2 *A* was separated by reversed-phase HPLC on an analytical C2/C18 column in 0.1% TFA with an acetonitrile gradient of 1.66%/min and fractions of 100 μl per 6 s were collected (*B*). The materials in fractions that showed maximal sensitization (a' and b') as well as adjacent fractions were sequenced by automated Edman degradation.

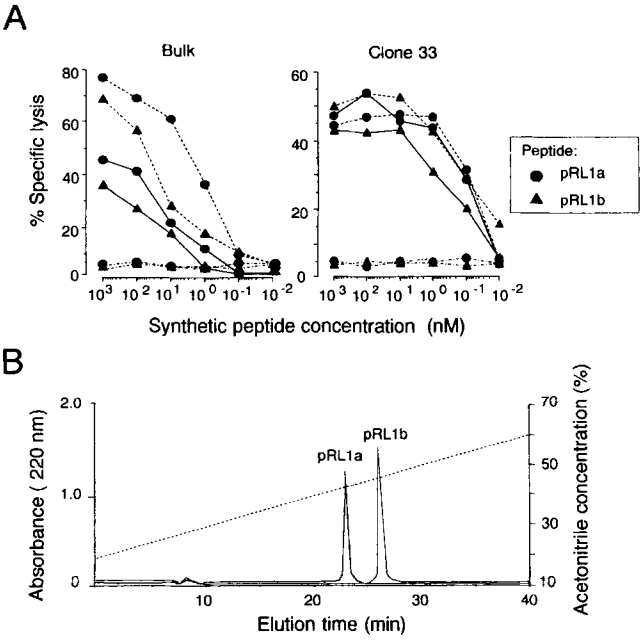

Figure 7. Sensitization (*A*) and reversed-phase HPLC analysis (*B*) of synthetic peptides pRL1. (*A*) Cytotoxicity by RLO'1 CTL of T1.1.1 (L^d-transfected L cell line) (·····), T4.8.3 (- - -) (D^d-transfected L cell line), and P815 (——) target cells sensitized with synthetic pRL1 peptides examined by 4-h ^{51}Cr-release assay. The amino acid sequences of pRL1a and b are IPGLPLSL and SIIPGLPLSL, respectively. Target cells were preincubated with the peptides for 1 h at room temperature before addition of the effector cells. Effector/target cell ratios were 5 for the bulk CTL, and 3 for clone 33. (*B*) Synthetic pRL1a and b subjected independently to reversed-phase HPLC on a semipreparative C18 (ODP) column as shown in Fig. 2. The elution times of pRL1a and pRL1b were 23 min and 26 min, respectively.

SIIPGLPLSL was slightly less than that of IPGLPLSL. Analysis by reversed-phase HPLC on a semipreparative C18 column revealed that the peptide IPGLPLSL was eluted in 23 min, and the peptide SIIPGLPLSL was eluted in 26 min (Fig. 7 *B*). Peptides IPGLPLSL and SIIPGLPLSL were denoted as pRL1a and b, respectively.

Amino Acid Sequence Homology. Amino acid sequence homology was investigated. Octamer peptide pRL1a (IPGLPLSL) was identical to amino acid residues 271–278 of the product of v-Akt (16, 26) (Table 1). Decamer peptide pRL1b (SIIPGLPLSL) differs from Akt in having an NH_2-terminal Ser residue in place of the Ile residue at position 269 of v-Akt.

Table 1. *Amino Acid Sequences of pRL1 and Akt*

pRL1a				I	P	G	L	P	L	S	L
pRL1b			S	I	I	P	G	L	P	L	S	L
Akt	R	E	E	T	L	I	I	I	P	G	L	P	L	S	L	G	A	T	D	T

The amino acid sequence of Akt corresponds to residues 264–283 of v-Akt in which the nucleotide sequence is in 5' untranslated region of c-*akt* next to the ATG codon (26).

Therefore, we synthesized IIIPGLPLSL and investigated its sensitization activity. Results showed that its sensitization activity was as high as that of pRL1b (SIIPGLPLSL) (data not shown).

Discussion

$CD8^+$ CTL recognize a peptide fragment in association with a MHC class I molecule (27). The amino acid sequences of naturally occurring peptides recognized by CTL have been determined by two alternative approaches. One is determination of amino acid sequences based on information on the nucleotide sequences that are responsible for susceptibility of target cells to lysis by CTL. Boon's group identified several genes (28) identified several genes encoding tumor rejection antigens in mice and humans. Mouse mastocytoma P815 expresses several distinct antigens that are recognized by syngeneic (DBA/2) CTL. By transfecting a cosmid library derived from P815 cells into a variant that did not express P815A and P815B antigens and by packaging directly the DNA of a transfectant with λphage extracts, they isolated gene *P1A*, which directs the expression of both antigens (29). One variant that had lost only antigen A displayed a point mutation. Synthetic peptides that corresponded to the normal sequence located in the region of this point mutation sensitized target cells (30). The *P1A* gene in this cell line is not mutated, but the gene is not expressed normally. They extended their analyses to human tumors and identified the *MAGE* gene family in a malignant melanoma. Nonapeptides encoded by *MAGE-1* and *-3* genes are recognized in association with HLA-A1 (31–33). Furthermore, they found that two nonapeptides encoded by the tyrosinase gene are recognized in association with HLA-A2 on tumors by autologous CTL (34, 35).

The other approach for determination of the amino acid sequences of naturally occurring peptides recognized by CTL involves extraction of the peptides from MHC molecules, and their purification and direct sequencing. Peptides that sensitize targets to lysis by specific CTL can be extracted with acid from cells expressing appropriate class I molecules (36–38), and attempts have been made to sequence these peptides. There are several reports on determinations of their amino acid sequences. Udaka et al. identified an octapeptide p2Ca (39) and a 16-mer peptide p2Cb (40) that includes the entire p2Ca from BALB/c spleen cells that are recognized by allogeneic L^d-specific 2C CTL. 2C CTL was established by immunization of BALB.B ($H\text{-}2^b$) mice with $H\text{-}2^d$ cells. p2Ca and b peptides are derived from mouse 2-oxoglutarate dehydrogenase. Henderson et al. (41) reported a nonamer peptide 1049 recognized by xenogeneic HLA-A2.1-specific murine CTL by mass spectrometry. Recently, Cox et al. (42) demonstrated that peptide 946 associated with the HLA-A2.1 molecule on melanomas recognized by CTL from different patients by mass spectrometry. Mandelboim et al. (43) reported an octapeptide on murine Lewis lung carcinoma (3LL) recognized by specific CTL. This peptide originates from mutated connexin 37. In this study, we identified a pRL1 peptide on BALB/c radiation-induced leukemia RLσ1 that is recognized by specific CTL and showed that it is derived from the *akt* oncogene.

An acid extract of RLσ1 cells was fractionated by reversed-phase HPLC on a semipreparative C18 column. Fractions eluted in 23 min (peak a) and 26 min (peak b) showed sensitization activity on P815 cells for the bulk and all six cloned RLσ1 specific CTL lines. Antibody blocking indicated that recognition of the P815 target sensitized with either the peak a or b fraction by RLσ1 CTL was restricted to L^d like that of the RLσ1 target (data not shown). Competitive inhibition assays showed that the RLσ1 cytotoxicities of the bulk and cloned CTL lines were inhibited by P815 target cells sensitized with either the peak a or b fraction as efficiently as by unlabeled RLσ1. These findings suggest that the peptides in the peak a and b fractions bind to the L^d molecule and create a common antigenic epitope that is dominantly recognized by anti-RLσ1 CTL. Amino acid sequence analysis by Edman degradation revealed an octamer peptide pRL1a (IPGLPLSL) in the peak a fraction and a decamer peptide pRL1b (SIIPGLPLSL) in the peak b fraction. The sensitization activities of these peptides on P815 and the L^d-transfected L cell line T1.1.1 were observed at concentrations as low as 1–100 nM with the bulk and all six cloned CTL lines tested. In a 4-h cytotoxicity assay, sensitization activity of the pRL1a peptide was observed in medium with or without serum, whereas that of the pRL1b peptide was diminished in medium without serum (data not shown). These findings suggest conversion of pRL1b to pRL1a by peptidases present in FCS. There are several reports of generation of optimal sized peptides from larger fragments by specific extracellular peptidases in cells sensitized with exogenous peptides to class I–restricted cytotoxic T cells. Exogenously added 11-mer peptide 147–158/R^- of the influenza virus nucleoprotein (form altered at residues 147–158, in which the Arg at position 156 is deleted) was presented resulting from removal of the COOH-terminal Thr and Gly by the angiotensin-converting enzyme (ACE) present in FCS (44). Cleavage by ACE was also observed with the HIV-1 gp160-derived 15-mer peptide p18 presented by $H\text{-}2D^d$ (45). Furthermore, recently, it has been shown that the sensitization activity of the naturally occurring 16-mer peptide p2Cb (see above) appears to be due to cleavage of eight NH_2-terminal residues in medium containing serum during the course of cytotoxicity assay (40).

There is a report that in a whole cell extract of minor H antigen $H\text{-}4^b$–positive cells, H-4–specific K^b-restricted CTL B21W9 recognized two HPLC fractions (38). But in peptide mixtures eluted from purified K^b molecules of the same cells, CTL recognize only one. In our study, using an acid extract from affinity-purified L^d molecules of RLσ1, the reversed-phase HPLC fraction eluted in 23 min but not that eluted in 26 min from a semipreparative C18 column had sensitization activity. This finding suggests that the octamer peptide pRL1a (IPGLPLSL) is the CTL-recognizing peptide that is presented by L^d molecules on the RLσ1 cell surface and that it is generated from the pRL1b (SIIPGPLPSL) peptide by removal of Ser and Ile from the NH_2 terminus. Thus, pRL1b could be an intermediate in the processing

pathway from intracellular protein to the octamer bound to class I molecules. Udaka et al. (40) showed that incubation of p2Cb with purified proteasomes resulted in a new peak of material with sensitizing activity that had the same retention time as octamer peptide p2Ca, which was concluded previously to be the active peptide recognized by 2C cells. The findings suggest that p2Cb is a precursor of p2Ca, the final product in the processing pathway. An alternative possibility is that the binding affinity of pRL1b to the L^d molecule is much lower than that of pRL1a and, therefore, resulted in poor recovery of the peptide on elution from affinity-purified L^d molecules.

It is usually difficult to determine the sequence of a CTL-recognizing peptide directly because of limitation in the amount of peptide available. A sample of 5–10 nmol of starting material would be required for several rounds of purification for obtaining a single peak that can be assumed to contain one or two peptides (46), although only 150–200 target peptides presented on a class I molecule seem to be required for target cell lysis when recognized by CTL (47). In the present study, in three sequential purification procedures by HPLC, the final yield was ~5%. Since the yield from 2.5×10^{11} cells was ~40 pmol, the number of pRL1 peptides on a single RLσ1 cell was calculated to be ~2,000.

Oncogenes are often mutated or overexpressed in malignant cells (48). C57BL/10 mice immunized with vaccinia virus expressing either the mutated or nonmutated *ras* gene were found to generate CTL specific for the peptides used for immunization (49). BALB/c mice immunized with spleen cells pulsed with mutated p53 peptide generated specific CTL (50). Furthermore, CTL were generated in B6 spleen cells by primary in vitro stimulation with mutated ras peptide (51). The present finding that the *akt* oncogene codes for pRL1 supports the findings that the oncogene product could be the epitope of a tumor rejection antigen that can be recognized by CTL. The v-*akt* gene was found in a defective clone of the AKT8 virus, which was an acute transforming retrovirus isolated from a AKT T cell lymphoma, and was generated by the in frame fusion of *gag* and c-*akt*. The protein encoded by v-*akt* is a tripartite gag-X-c-akt fusion protein of which X is a 21-amino acid peptide derived primarily from the 5′ untranslated region of the gene (26). Acquisition of transforming properties by v-*akt* appeared to be due to the addition of the *gag-X* sequences at the NH_2 terminus (52). Myristylation and membrane association of v-Akt are likely to be critical for the oncogenic properties. C-*akt* mRNA is expressed in all tissues tested (26). The pRL1 peptides shown in the present study correspond to the sequence in the 5′ untranslated region of c-*akt*. pRL1a is derived from residues 271–278 and pRL1b from residues 269–278 of v-Akt. The amino acid residue Ile at position 269 in v-Akt is replaced by Ser in pRL1b. Since the decamer peptide IIIPGLPLSL also showed sensitization activity, Ser at the NH_2 terminus of pRL1b is not responsible for sensitivity to cleavage by dipeptidase.

In addition to the RLσ1 unique antigen demonstrated in this study, a number of unique antigens recognized by specific CTL have been identified in other leukemias. Whether the unique antigen peptides on these leukemias are also derived from the *akt* oncogene or from other genes remains to be seen.

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