Identification of a Unique Antigen Peptide pRL1 on BALB/c RLo1 Leukemia Recognized by Cytotoxic T Lymphocytes and Its Relation to the Akt Oncogene

By Akiko Uenaka,* Toshiro Ono,† Toshifumi Akisawa,‡ Hisashi Wada,† Tatsui Yasuda,§ and Eiichi Nakayama†

From the *Department of Tumor Immunology, The Center for Adult Diseases, Osaka 537; the †Department of Parasitology and Immunology; and the ‡Institute of Cellular and Molecular Biology, Okayama University Medical School, Okayama 700; and the §Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsumin University, Osaka 573-01, Japan

Summary

BALB/c radiation leukemia RLo1 is an immunogenic tumor. We established bulk and cloned cytotoxic T lymphocyte (CTL) lines from regressor (BALB/c × C57BL/6)F1 (CB6F1) spleen cells that recognized RLo1 specifically. We then obtained antigen peptide recognized by CTL from RLo1 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 RLo1 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 RLo1 is a highly immunogenic tumor. In BALB/c mice hybridized with certain mouse strains, inocula of RLo1 cells initially grew, formed a tumor, and then regressed. The different immune responsiveness to RLo1 rejection antigen of the various mouse strains used for producing F1 hybrids have been ascribed to a gene located in the H-2K region (11). Spleen cells from regressor (BALB/c × C57BL/6)F1 (CB6F1)1 mice generate CD8+ CTL after in vitro stimulation (12). CTL have been shown to recognize RLo1, 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 RLo1. Adoptive transfer of CTL to BALB/c nu/nu mice protected the recipient mice from subsequent challenge with RLo1 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 RLo1 rejection response is mediated by CD8+ CTL. In this study, we identified a unique rejection antigen peptide, pRL1, that is recognized on RLo1 by CTL derived from semisynthetic CB6F1 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 CB6F1 mice were purchased from Japan SLC Co. (Shizuoka, Japan). Breeding pairs of

1 Abbreviations used in this paper: CB6F1, (BALB/c × C57BL/6)F1; r, recombinant.
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.** RLO1, RLO4, and RL08 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 DVA/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-2d) transfected with the H-2Ld and H-2Dd gene, respectively (19), and were provided by Dr. N. Shidara (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. Kabo (National Jewish Center, Denver, CO). Anti-CD3e 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-2Kd and anti-H-2Dd 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-2-2d mAb is a mouse IgG2a antibody produced by a hybridoma 30-5/7 (23). Anti-IAa mAb is a mouse IgG2b antibody produced by hybridoma MK-D6 (24). Anti-IEKd 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 fast atom bombardment mass spectrometry (model 9020-DF; Applied Biosystems) in 0.1% TFA with an acid elution from Affinity-purified H-2Ld.

**Acid Elution from Affinity-purified H-2Ld.** 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-Ld 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 × 10⁸) were cultured with Con A at a concentration of 5 μg/ml for 3 d.

**Establishment and Maintenance of CTL Clones.** CBF6, spleen cells (5 × 10⁶) from regorressor mice were cultured with 5 × 10⁶ irradiated (100 Gy) RL01 cells in tissue culture flasks (model 25100, Corning Glass Co., Corning, NY) for 5 d at 37°C under 5% CO₂ 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 × 10⁶) were maintained as a bulk CTL line by weekly restimulation with 10⁴ irradiated (100 Gy) RL01 stimulator cells and 5 × 10⁶ irradiated (20 Gy) CBF6, splenic feeder cells in the presence of human recombinant IL-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 10⁴ irradiated RL01 cells and 10⁴ irradiated CBF6, splenic feeder cells in the presence of IL-2 in 96-well culture plates (model 25860, Corning Glass Co.). After 10–14 d, the cytotoxicity of clonally
Results

Generation of Bulk and Cloned CTL Lines Specific for RLcY1. Inoculation of 10⁶ RLcY1 cells into the back of CB6F₁ 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) RLcY1 cells, cytotoxicity against the RLcY1 cells was generated in 5 d (12). The culture was maintained as a bulk CTL line by weekly repeated stimulation with 10⁶ irradiated (100 Gy) RLcY1 cells and 5 × 10⁶ irradiated (20 Gy) CB6F₁ 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 10⁶ irradiated RLcY1 cells and 10⁶ irradiated CB6F₁ 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 RLcY1 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 RLcY1, but not RLcY4, RLQ8, BALB-RVA, C, D, P815, or normal splenic Con A blasts from BALB/c, C57BL/6, C3H, and (BALB/c × B6)F₁ mice. The results of a typical experiment are shown in Fig. 1A.

Ld Restriction for Specific CTL To Recognize RLcY1 Antigen. The H-2 restriction molecule for specific CTL to recognize RLcY1 antigen was determined by antibody blocking experiments. The cytotoxicities of the bulk and six cloned CTL lines were all blocked by anti-Ld mAb (30-5-7), but not by anti-Kd mAb (KD40), anti-IAd mAb (MK-D6), anti-IEd mAb (ISCR3), or anti-Dd mAb (DD98). The results of a typical experiment are shown in Fig. 1B.

Fractionation of Acid Extract from Whole RLcY1 Cells by Reversed-phase HPLC. A sample of 2.5 × 10¹¹ RLcY1 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 ¹¹⁵Cr-labeled P815 target and cytotoxicity by RLcY1 CTL was determined. As shown in Fig. 2A, 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. 2B.

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-Ld mAb, but not anti-Kd mAb, anti-IAd mAb, anti-IEd mAb, or anti-Dd mAb. Results were essentially similar with the bulk and all six cloned CTL lines (data not shown).

Competitive Inhibition of RLcY1 Cytotoxicity by P815 Cells Sensitized with Active Fractions. P815 cells sensitized with active fractions were used as inhibitors of RLcY1 cytotoxicity in com-

Figure 1. Direct cytotoxicity (A) and antibody blocking (B) of anti-RLcY1 CTL in 4-h ¹¹⁵Cr-release assay. The effector/target cell ratios in B were 10 with bulk RLcY1 CTL and 5 with clone 33. The bulk and all six cloned CTL lines tested gave essentially similar results. NMS, normal mouse serum.
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 51Cr-release assay (A). Samples of 5 µl of each fraction collected in a volume of 2 ml/min were added to 51Cr-labeled P815 target. The effector cells used were the bulk RLcY1 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 RLcY1 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 Lα Molecules from RLcY1. A sample of \(4.0 \times 10^8\) RLcY1 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-Lα 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).

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...
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'. Fractions 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 (Ld-transfected L cell line) cells was observed at concentrations as low as 1-100 nM with the bulk and cloned RLo'I CTL lines. The sensitization activity of 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 264-283 of v-Akt (16, 26) (Table 1). Decamer peptide pRL1b (SIIPGLPLSL) differs from Akt in having an NH2-terminal Ser residue in place of the Ile residue at position 269 of v-Akt.

| Peptide   | Amino Acid Sequence |
|-----------|---------------------|
| pRL1a     | IPGLPLSL            |
| pRL1b     | SIIPGLPLSL          |
| Akt       | REETLIIIPGLPLSGLATDT |

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) 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 PLA, 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 PLA 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 Ld-specific 2C CTL. 2C CTL was established by immunization of BALB.B (H-2b) mice with H-2b 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 RLcYl that is recognized by specific CTL and showed that it is derived from the akt oncogene.

An acid extract of RLcYl 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 RLcYl specific CTL lines. Antibody blocking indicated that recognition of the P815 target sensitized with either the peak a or b fraction by RLcYl CTL was restricted to Ld like that of the RLcYl target (data not shown). Competitive inhibition assays showed that the RLcYl 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 unlabelled RLcYl. These findings suggest that the peptides in the peak a and b fractions bind to the Ld molecule and create a common antigenic epitope that is dominantly recognized by anti-RLcYl 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 Ld-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 peptides present in FCS. There are several reports of generation of optimal sized peptides from larger fragments by specific extracellular peptides 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-2Dd (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 NH2-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-4b-positive cells, H-4-positive H2Kk-restricted CTL B21W9 recognized two HPLC fractions (38). But in peptide mixtures eluted from purified Kk molecules of the same cells, CTL recognize only one. In our study, using an acid extract from affinity-purified Ld molecules of RLcYl, 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 Ld molecules on the RLcYl cell surface and that it is generated from the pRL1b (SIIPGLPLSL) peptide by removal of Ser and Ile from the NH2 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 Ld molecule is much lower than that of pRL1a and, therefore, resulted in poor recovery of the peptide on elution from affinity-purified Ld 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 × 1011 cells was ~40 pmol, the number of pRL1 peptides on a single RLO1 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 an 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 NH2 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 IIIPLGLPLSL also showed sensitization activity, Ser at the NH2 terminus of pRL1b is not responsible for sensitivity to cleavage by dipeptidase.

In addition to the RLO1 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.

We thank Dr. Lloyd J. Old for his continuous encouragement and invaluable advice; and Drs. Toshitada Takahashi, Teizo Fujita, Kenji Shimizu, Yuichi Obata, and Hitoshi Akedo for valuable suggestions and discussion. We also thank Ms. Junko Mizuochi for preparation of the manuscript.

This work was supported in part by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Education, Science, and Culture.

Address correspondence to Dr. Eiichi Nakayama, Department of Parasitology and Immunology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, Japan.

Received for publication 17 May 1994 and in revised form 18 July 1994.

References

1. Gross, L. 1943. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res. 3:326.
2. Foley, E.J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. Cancer Res. 13:835.
3. Prehn, R.T., and J.M. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. J. Natl. Cancer Inst. 18:769.
4. Klein, G., H.O. Sjögren, E. Klein, and K.E. Hellström. 1960. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res. 20:1561.
5. Old, I.J., E.A. Boyse, D.A. Clarke, and E.A. Carswell. 1962. Antigenic properties of chemically induced tumors. Ann. NY Acad. Sci. 101:80.
6. Globerson, A., and M. Feldman. 1964. Antigenic specificity of benzo(a)pyrene-induced sarcomas. J. Natl. Cancer Inst. 32:1229.
7. Greenberg, P.D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. 49:281.
8. Melief, C.J.M. 1992. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. Adv. Cancer Res. 58:143.
9. Rosenberg, A.S., T. Mizuochi, S.O. Sharrow, and A. Singer.
10. Singer, A., T.i. Munitz, H. Golding, A.S. Rosenberg, and T. Mizuochi. 1987. Recognition requirements for the activation, differentiation, and function of T-helper cells specific for class I MHC alloantigens. *Immunol. Rev.* 98:143.

11. Sato, H., E.A. Boyse, T. Aoki, C. Iritani, and L.J. Old. 1973. Leukemia-associated transplantation antigens related to murine leukemia virus. The X.1 system: immune response controlled by a locus linked to H-2. *J. Exp. Med.* 138:593.

12. Nakayama, E., H. Shiku, T. Takahashi, H.F. Oettgen, and L.J. Old. 1979. Definition of a unique cell surface antigen of mouse leukemia RLcrl by cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 76:3486.

13. Keyaki, A., K. Kuribayashi, S. Sakaguchi, T. Masuda, J. Yamashita, H. Handa, and E. Nakayama. 1985. Effector mechanisms of syngeneic anti-tumour responses in mice. II. Cytoxic T lymphocytes mediate neutralization and rejection of radiation-induced leukaemia RLcrl in the nude mouse system. *Immunology.* 56:141.

14. Nakayama, E., and A. Uenaka. 1985. Effect of in vivo administration of Lyt antibodies. Lyt phenotype of T cells in lymphoid tissues and blocking of tumor rejection. *J. Exp. Med.* 161:345.

15. Udono, H., M. Mino, H. Shiku, and E. Nakayama. 1989. The roles of CD8+ and CD4+ cells in tumor rejection. *Jpn. J. Cancer Res.* 80:649.

16. Bellacosa, A., J.R. Testa, S.P. Staal, and P.N. Tsichlis. 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science (Wash. DC).* 254:274.

17. Stockert, E., A.B. DeLeo, P.V. O'Donnell, Y. Obata, and L.J. Old. 1979. G(ASKL2): a new cell surface antigen of the mouse related to the dualtropic mink cell focus-inducing class of murine leukemia virus detected by naturally occurring antibody. *J. Exp. Med.* 149:200.

18. Dunn, T.B., and M. Potter. 1957. A transplantable mast-cell neoplasm in the mouse. *J. Natl. Cancer Inst.* 18:587.

19. Margulies, D.H., G.A. Evans, K. Ozato, R.D. Camerini-Otero, K. Tanaka, E. Appella, and J.G. Seidman. 1983. Expression of H-2Dα and H-2Lα mouse major histocompatibility antigen genes in L cells after DNA-mediated gene transfer. *J. Immunol.* 130:463.

20. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quintans, M.R. Loken, M. Pierres, and F.W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* 131:2445.

21. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine αβ T cell receptors. *J. Immunol.* 142:2736.

22. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.

23. Ozato, K., T.H. Hansen, and D.H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2Lα antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473.

24. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.

25. Watanabe, M., T. Suzuki, M. Taniguchi, and N. Shinohara. 1983. Monoclonal anti-la murine alloantibodies crossreactive with the la-homologues of other mammalian species including humans. *Transplantation (Baltimore).* 36:712.

26. Bellacosa, A., T.F. Franke, M.E. Gonzalez-Portal, K. Datta, T. Taguchi, J. Gardner, J.Q. Cheng, J.R. Testa, and P.N. Tsichlis. 1993. Structure, expression, and chromosomal mapping of c-akt: relationship to v-akt and its implications. *Oncomine.* 8:745.

27. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Ann. Rev. Immunol.* 7:601.

28. Van der Bruggen, P., and B. Van den Eynde. 1992. Molecular definition of tumor antigens recognized by T lymphocytes. *Curr. Opin. Immunol.* 4:608.

29. Van den Eynde, B., B. Lethé, A. Van Pel, E. De Plaen, and T. Boon. 1991. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J. Exp. Med.* 173:1373.

30. Lethé, B., B. Van den Eynde, A. Van Pel, G. Corrardin, and T. Boon. 1992. Mouse tumor rejection antigens P815A and P815B: two epitopes carried by a single peptide. *Eur. J. Immunol.* 22:2283.

31. Van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science (Wash. DC).* 254:1643.

32. Traversari, C., P. Van der Bruggen, I.F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costese, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453.

33. Gaugler, B., B. Van den Eynde, P. Van der Bruggen, P. Romeo, J.J. Gaforivo, E. De Plaen, B. Lethé, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 179:921.

34. Brichard, V., A. Van Pel, T. Wölfel, C. Wölfel, E. De Plaen, B. Lethé, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 178:489.

35. Wölfel, T., A. Van Pel, V. Brichard, J. Schneider, B. Seliger, K.-H. Meyer zum Büschenfelde, and T. Boon. 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur. J. Immunol.* 24:759.

36. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kβ molecule. *Nature (Lond.)*. 348:213.

37. Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.)*. 348:252.

38. Falk, K., O. Rötzschke, and H.-G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.)*. 348:248.

39. Udaka, K., T.J. Tsomides, and H.N. Eisen. 1992. A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell.* 69:989.

40. Udaka, K., T.J. Tsomides, P. Walden, N. Fukusen, and H.N. Eisen. 1992. A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8+ T-cell clone. *Proc. Natl. Acad. Sci. USA.* 90:11272.
41. Henderson, K.A., A.L. Cox, K. Sakaguchi, E. Appella, J. Shabanowitz, D.F. Hunt, and V.H. Engelhard. 1993. Direct identification of an endogenous peptide recognized by multiple HLA-A2.1-specific cytotoxic T cells. Proc. Natl. Acad. Sci. USA. 90:10275.
42. Cox, A.L., J. Skipper, Y. Chen, R.A. Henderson, T.L. Darrow, J. Shabanowitz, V.H. Engelhard, D.F. Hunt, and C.L. Slingluff, Jr. 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science (Wash. DC). 264:716.
43. Mandelboim, O., G. Berke, M. Fridkin, M. Feldman, M. Eisenstein, and L. Eisenbach. 1994. CTL induction by a tumor-associated antigen octapeptide derived from a murine lung carcinoma. Nature (Lond.). 369:67.
44. Sherman, L.A., T.A. Burke, and J.A. Biggs. 1992. Extracellular processing of peptide antigens that bind class I major histocompatibility molecules. J. Exp. Med. 175:1221.
45. Kozlowski, S., M. Corr, T. Takeshita, L.F. Boyd, C.D. Pendleton, R.N. Germain, J.A. Berzofsky, and D.H. Margulies. 1992. Serum angiotensin-1 converting enzyme activity processes a human immunodeficiency virus 1 gp160 peptide for presentation by major histocompatibility complex I molecules. J. Exp. Med. 175:1417.
46. Joyce, S., P. Tabaczewski, R.H. Angeletti, S.G. Nathenson, and I. Stroynowski. 1994. A nonpolymorphic major histocompatibility complex Ib molecule binds a large array of diverse self-peptides. J. Exp. Med. 179:579.
47. Christinck, E.R., M.A. Luscher, B.H. Barber, and D.B. Williams. 1991. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. Nature (Lond.). 352:67.
48. Bishop, J.M. 1991. Molecular themes in oncogenesis. Cell. 64:235.
49. Skipper, J., and H.J. Stauss. 1993. Identification of two cytotoxic T lymphocyte-recognized epitopes in the Ras protein. J. Exp. Med. 177:1493.
50. Yanuck, M., D.P. Carbone, C.D. Pendleton, T. Tsukui, S.F. Winter, J.D. Minna, and J.A. Berzofsky. 1993. A mutant p53 tumor suppressor protein is a target for peptide-induced CD8+ cytotoxic T-cells. Cancer Res. 53:3257.
51. Peace, D.J., J.W. Smith, W. Chen, S.-G. You, W.L. Cosand, J. Blake, and M.A. Cheever. 1994. Lysis of ras oncogene-transformed cells by specific cytotoxic T lymphocytes elicited by primary in vitro immunization with mutated ras peptide. J. Exp. Med. 179:473.
52. Ahmed, N.N., T.F. Franke, A. Bellacosa, K. Datta, M.E. Gonzalez-Portal, T. Taguchi, J.R. Testa, and P.N. Tschilis. 1993. The proteins encoded by c-akt and v-akt differ in post-translational modification, subcellular localization and onco-genic potential. Oncogene. 8:1957.