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Production of Murine Leukemia RL♂1 Rejection Antigen Peptide pRL1a by Proteolysis of Natural Precursor pRL1b

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In this study, we demonstrated that NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) (SIIPGLPLSL) are not involved in the recognition by RL♂1-specific cytotoxic T lymphocyte. The sensitization activity observed with pRL1b (SI-pRL1a) was not greater than that of peptides substituted with irrelevant amino acids at these positions. In serum-free medium, pRL1a retained sensitization activity, but pRL1b (SI-pRL1a) did not. Furthermore, addition of bestatin to serum-containing medium blocked sensitization by pRL1b (SI-pRL1a). On the other hand, the addition of captopril enhanced it, probably by inhibiting the degradation of pRL1a by ACE. pRL1a-D peptide with D-Ile in place of the L-Ile residue of pRL1a (IPGLPLSL) showed sensitization, but SI-pRL1a-2,3D peptide, which has D-Iles in place of the L-Ile residues of pRL1b (SI-pRL1a), and which was not cleaved between the two D-Iles, did not. The findings suggest that pRL1a is the antigenic peptide bound to L^d molecules and pRL1b (SI-pRL1a) peptide is its natural precursor, which generates pRL1a via proteolysis.

Key words: BALB/c leukemia RL♂1 — Cytotoxic T lymphocyte — Tumor rejection antigen peptide — Natural precursor peptide — Protease

Cytotoxic T lymphocytes (CTL) have been shown to play a significant role in eradication of certain human and mouse tumors.[1)] CTL exert their effect by recognizing antigen peptides in association with MHC molecules on the tumor cell surface.[2)] We recently identified the tumor rejection antigen peptide pRL1 recognized by CTL on BALB/c radiation leukemia RL♂1 by acid extraction, purification by high-performance liquid chromatography (HPLC) and direct sequencing.[3)] Sequence homology analysis revealed that pRL1 peptides were derived from the 5′ untranslated region of the c-*akt* proto-oncogene.[4, 5)] Insertion of MuLV LTR and six nucleotides of unknown origin results in enhanced production of an altered AKT protein and creation of tumor rejection antigen peptides derived from the normally untranslated region of the gene.[6)]

Octapeptide pRL1a (IPGLPLSL) was eluted from both affinity-purified L^d molecules and whole cell extract from RL♂1, and the decapeptide pRL1b (SI-pRL1a) (SIIPGLPLSL) was eluted predominantly from whole cell extract.[3)] Both pRL1a and pRL1b (SI-pRL1a) showed the sensitization activity on P815 target cells for lysis by bulk CTL and six cloned CTL lines obtained from (BALB/c×C57BL/6)F_1 (CB6F_1) mice that had rejected RL♂1 in serum-containing medium, although the sensitization activity of pRL1b (SI-pRL1a) is about 10-fold less than that of pRL1a. In this study, we investigated the proteolysis of pRL1b (SI-pRL1a) *in vitro* by proteases in fetal calf serum (FCS) and demonstrated that the octapeptide pRL1a is the antigenic peptide bound to H-2L^d molecules and the decapeptide pRL1b (SI-pRL1a) is its natural precursor.

MATERIALS AND METHODS

Cell line P815 is a methylcholanthrene-induced mastocytoma line obtained from a DBA/2 mouse.[7)]

CTL clones We used a bulk CTL line and CTL clone 44, which were established from spleen cells of (BALB/c×C57BL/6)F_1 (CB6F_1) mice in which RL♂1 tumor had regressed following stimulation with mitomycin C (MMC)-treated RL♂1 cells.[3)] The cells were maintained by weekly stimulation with MMC (25 μg/ml)-treated CB6F_1 splenic feeder cells (5×10^6) and MMC (50 μg/ml)-treated RL♂1 cells (1×10^5) in the presence of human recombinant IL-2 (Takeda Chemical Industries, Osaka) at a concentration of 5 ng/ml in 24-well tissue culture plates. Culture medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 50 μ*M* 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin.

Protease inhibitors Captopril and bestatin were purchased from Sigma Chemical Co. (St. Louis, MO). They were dissolved in PBS at a concentration of 10 m*M* and stored at −20°C until use.

^{51}Cr-release assay P815 cells were labeled with 1.85 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1 h at 37°C under 5% CO_2 in air and used as targets. In sensitization assays, HPLC fraction or peptide solution (5–10 μl) was added to 5×10^3 labeled target cells (100

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Table I. Designation and Amino Acid Sequences of Peptides

Designation	Amino acid sequence									
pRL1a			I	P	G	L	P	L	S	L
pRL1b (SI-pRL1a)	S	I	I	P	G	L	P	L	S	L
pRL1b (SI-pRL1a) (SL-)	S	I	I	P	G	L	P	L		
pRL1a-D			I[a)]	P	G	L	P	L	S	L
SI-pRL1a-2,3D	S	I[a)]	I[a)]	P	G	L	P	L	S	L
II-pRL1a	I	I	I	P	G	L	P	L	S	L
KK-pRL1a	K	K	I	P	G	L	P	L	S	L

a) D-Ile substituted for L-Ile.

μl) before addition of effector cells (100 μl) in 10% FCS-containing or serum-free RPMI 1640 medium. In the sensitization assay under a serum-free condition, P815 cells which had been maintained in 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN) medium were used. For protease inhibition, labeled target cells were preincubated with peptides in FCS-containing RPMI 1640 medium containing 1×10^{-6} *M* captopril or bestatin for 1 h at 37°C under 5% CO_2 in air before addition of effector cells. 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 with a gamma counter. The percentage of specific lysis was calculated as 100× (experimental release−spontaneous release)/(maximum release−spontaneous release).

Peptide synthesis The peptides used in this study are shown in Table I. D-Ile was purchased from Watanabe Chem. Co. (Hiroshima). Peptides were synthesized by standard solid-phase methods using Fmoc chemistry in an Applied Biosystems 430A peptide synthesizer (Foster City, CA). The peptide was cleaved from the resin and the side chain protecting groups were removed using 95% trifluoroacetic acid (TFA). The peptides were purified to >98% homogeneity by reversed-phase HPLC on a Brownlee Aquapore C8 preparative column (RP300, Applied Biosystems) in 0.1% TFA with an acetonitrile gradient. Peptide purity was assayed with a Vydac C18 analytical column (218TP104, Vydac, Hesperia, CA). The integrity of the purified peptides was further confirmed by fast bombardment mass spectrometry (Model 9020-DF, Shimadzu, Kyoto).

Purification of degraded peptides Peptide (2.5×10^{-4} *M*), in 10% FCS-containing PBS was incubated at 37°C, with or without 1×10^{-6} *M* protease inhibitors. After incubation, the peptide solution was filtered with a molecular cut-off membrane (m.w. 10,000, Centricut, Kurabo Co., Osaka). The filtrate was then analyzed by reversed-phase HPLC on a Pharmacia SMART System (μRPC C2/C18 SC 2.1/10, LKB, Uppsala, Sweden). Solvent A was 0.1% TFA and solvent B was 80% acetonitrile containing 0.1% TFA, with a gradient of 30% B to 55% B over 25 min, followed by isocratic elution with 55% B for 3 min and 55% B to 0% B over 5 min, at a flow rate of 100 μl/min, with a fraction size of 50 μl. Each fraction was collected using the automatic peak fractionation function of the SMART System, and used for sensitization assay and sequencing.

Peptide sequencing Direct sequencing was performed by amino-terminal Edman degradation using an Applied Biosystems 477A protein sequencer coupled to a 120A PTH analyzer (Applied Biosystems).

RESULTS

No involvement of NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) for the epitope recognized by CB6F$_1$ anti-RL♂1 CTL pRL1a and pRL1b (SI-pRL1a) peptides that were recognized by CB6F$_1$ anti-RL♂1 CTL have been identified on RL♂1 cells by acid extraction from RL♂1 whole cells, purification by reversed-phase HPLC, and direct sequencing. As shown in Fig. 1A, sensitization activity of pRL1b (SI-pRL1a) for the P815 target is about 10-fold less than that of pRL1a in ^{51}Cr-release assay using 10% FCS-containing RPMI 1640 medium. To investigate the involvement of NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) in the epitope recognized by anti-RL♂1 CTL, the sensitization activity of pRL1b (SI-pRL1a) was compared with that of II-pRL1a and KK-pRL1a substituted with irrelevant amino acids. No greater sensitization activity was observed with pRL1b (SI-pRL1a) in comparison with those peptides. The results were essentially similar with bulk and cloned CTL lines and suggested that there is no involvement of NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) in the epitope.

Requirement of serum proteases for sensitization of P815 targets with pRL1b (SI-pRL1a) To investigate the requirement of serum proteases in FCS added to the culture medium for sensitization of P815 targets with pRL1b (SI-pRL1a), serum-free medium (1% Nutri-

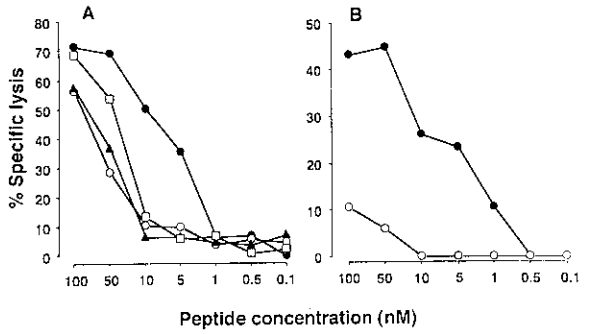

Fig. 1. Sensitization activity of peptides in 10% FCS-containing (A) and serum-free (B) RPMI 1640 medium. Peptides were pRL1a(●), pRL1b (SI-pRL1a) (○), II-pRL1a (▲) and KK-pRL1a (□). Peptide sensitization was done by incubating ^{51}Cr-labeled P815 target cells with various concentrations of peptides for 1 h at room temperature. Cytotoxicity was determined by 4-h ^{51}Cr-release assay using RL♂1-specific CTL clone 44. Effector-to-target cell ratios were 3 in A and 8 in B.

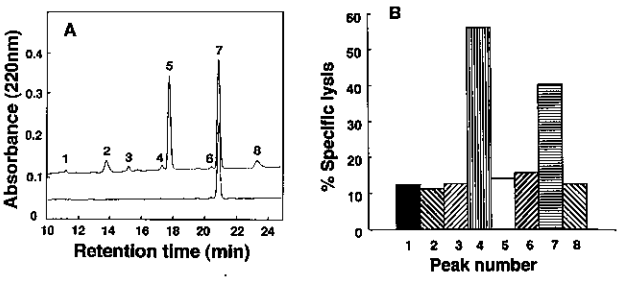

Fig. 2. Reversed-phase HPLC analysis (A) and sensitization activity (B) of degraded products of pRL1b (SI-pRL1a) peptide generated by serum proteases. In A, pRL1b (SI-pRL1a) peptide was incubated for 30 min at 37°C in 10% FCS-containing PBS and serum-free PBS (lower line). Peptide concentration in PBS was 250 μg/ml. After incubation the peptides were collected by filtration through a molecular cut-off membrane (m.w. 10,000). In B, ^{51}Cr-labeled P815 target cells were incubated with 5 μl of each peak fraction for 1 h at room temperature. Cytotoxicity was determined by 4-h ^{51}Cr-release assay using a bulk CTL line. The effector-to-target cell ratio was 6.

doma) was used for ^{51}Cr-release assay. Whereas half-maximal sensitization of pRL1a peptide for P815 target cells was unchanged (5 n*M*), the sensitization activity of pRL1b (SI-pRL1a) was markedly decreased (Fig. 1B).

Generation of pRL1a by proteolysis of pRL1b (SI-pRL1a) in FCS-containing medium Proteolysis by serum proteases of pRL1b (SI-pRL1a) peptide incubated for 30 min at 37°C in 10% FCS-containing PBS was analyzed by reversed-phase HPLC on a SMART System. Amino acid sequences of peptides in peak fractions were determined by Edman degradation. As shown in Fig. 2A, peak 7 corresponded to pRL1b (SI-pRL1a). The major product was peak 5, an octapeptide pRL1b (SI-pRL1a) (SL-) (SIIPGLPL) lacking the two COOH-terminal amino acid residues. Other products and their amino acid sequences were peak 1, II; peak 2, SIIPGLP and IPGL; peak 3, SIIPGLPLS; peak 4, IPGLPLSL (pRL1a). No peptide was found in peaks 6 and 8. Sensitization activities of peak fractions on P815 targets for lysis by anti-RL♂1 CTL were studied (Fig. 2B). Significant sensitization was observed with only peak 4 (pRL1a) and peak 7 (pRL1b (SI-pRL1a)). The results were essentially similar with bulk and cloned CTL lines.

Effects of protease inhibitors on proteolysis of pRL1b (SI-pRL1a) and on the sensitization activity for P815 Effects of the angiotensin-converting enzyme (ACE) inhibitor captopril and the aminopeptidase inhibitor bestatin added to FCS-containing PBS on the proteolysis of pRL1b (SI-pRL1a) peptide were investigated by reversed-phase HPLC. As shown in Fig. 3, addition of captopril blocked the generation of pRL1b (SI-pRL1a) (SL-) (SIIPGLPL), which lacks the Ser and Leu residues at the COOH terminus of pRL1b (SI-pRL1a). On the other hand, addition of bestatin reduced the generation of pRL1a. The results suggested involvement of ACE in the production of pRL1b (SI-pRL1a) (SL-) and of aminodipeptidase in the production of pRL1a. In ^{51}Cr-release assay, the addition of captopril to pRL1b (SI-pRL1a) enhanced its sensitization activity, probably by inhibiting the degradation of pRL1a by ACE. On the other hand, addition of bestatin reduced it (Fig. 3D).

Effect of substitution of D-Ile for L-Ile of pRL1a and pRL1b (SI-pRL1a) peptides on sensitization activity for P815 targets We synthesized pRL1a-D peptide in which NH_2-terminal D-Ile was substituted for L-Ile of pRL1a and SI-pRL1a-2,3D in which D-Iles were substituted for L-Iles of pRL1b (SI-pRL1a) at positions 2 and 3. The sensitization activities of these peptides were investigated. No cleavage between the two Ile residues in SI-pRL1a-2,3D was observed in FCS-containing medium (data not shown). As shown in Fig. 4, pRL1a-D showed moderate sensitization activity, but SI-pRL1a-2,3D showed none.

DISCUSSION

We have demonstrated that NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) are not involved in recognition by RL♂1-specific CTL. The sensitization activity observed with pRL1b (SI-pRL1a) was not greater than those of peptides substituted with irrelevant amino acids at these positions. In serum-free medium, pRL1a retained its sensitization activity for target cells, while

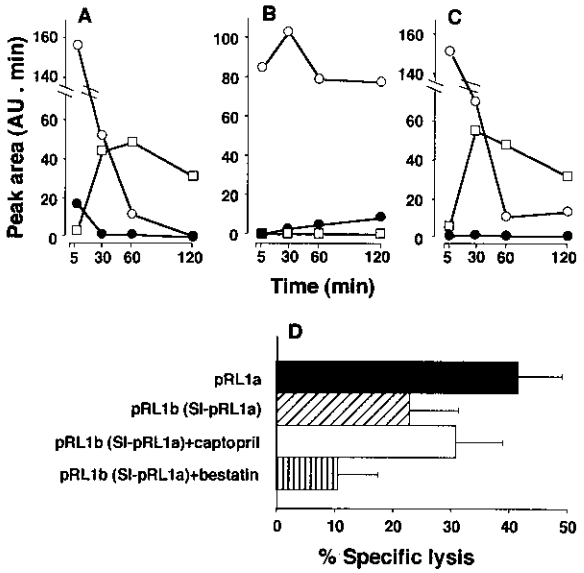

Fig. 3. Effect of protease inhibitors on degradation of pRL1b (SI-pRL1a) peptide (A–C) and on sensitization activity (D). pRL1b (SI-pRL1a) peptide was incubated in 10% FCS-containing PBS in the absence (A), or the presence of 1×10^{-6} *M* captopril (B) and bestatin (C) for an indicated time at 37°C and then analyzed by reversed-phase HPLC as described in Fig. 2. The amount of pRL1b (SI-pRL1a) (○), pRL1b (SI-pRL1a) (SL-) (□) or pRL1a (●) peptide was calculated from the chromatographic peak area. In D, ^{51}Cr-labeled P815 target cells were incubated with 5×10^{-8} *M* pRL1a and pRL1b (SI-pRL1a) in 10% FCS-containing RPMI 1640 medium, in the presence or absence of 1×10^{-6} *M* captopril or bestatin for 1 h at room temperature. The effector cells were then added. The cytotoxicity was determined by 4-h ^{51}Cr-release assay using CTL clone 44. Results are shown as the mean±SD of triplicate cultures. The effector-to-target cell ratio was 3.

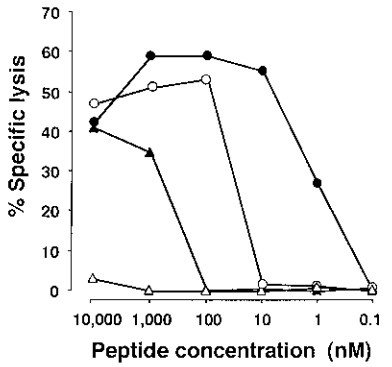

Fig. 4. Sensitization activity of peptides substituted with D-Ile in place of L-Ile of pRL1a and pRL1b (SI-pRL1a) on P815 target cells. Peptides were pRL1a (●), pRL1a-D (▲), pRL1b (SI-pRL1a) (○) and SI-pRL1a-2, 3D (△) (see Table I). Sensitization is described in the legend to Fig. 1. Cytotoxicity was determined by 4-h ^{51}Cr-release assay using CTL clone 44. The effector-to-target cell ratio was 3.

pRL1b (SI-pRL1a) did not. Analysis of proteolysis of pRL1b (SI-pRL1a) peptide by proteases in fetal calf serum showed that the peptide was degraded into five major fragment peptides. Among these peptides, only IPGLPLSL (pRL1a) showed sensitization activity on the target cells. Addition of bestatin to serum-containing medium blocked sensitization by pRL1b (SI-pRL1a). On the other hand, the addition of captopril enhanced it, probably by inhibiting the degradation of pRL1a by ACE. Furthermore, whereas pRL1a-D peptide substituted with D-Ile in place of the L-Ile residue of pRL1a (IPGLPLSL) showed sensitization activity, this was not the case for SI-pRL1a-2,3D peptide, which is substituted with D-Iles in place of the L-Ile residues of pRL1b (SI-pRL1a) (SIIPGLPLSL) at the second and third positions, and which is resistant to cleavage between the two D-Iles (data not shown). The findings suggest that cleavage of NH_2-terminal Ser and Ile residues of pRL1b (SI-pRL1a) by proteases in serum-containing medium resulted in the generation of antigenic peptide pRL1a. We previously demonstrated that the octapeptide pRL1a was eluted from both affinity-purified L^d molecules and whole cell extract from RL♂1 cells. On the other hand, the decapeptide pRL1b (SI-pRL1a) was eluted predominantly from whole cell extract.[3] Taken together, the findings suggest that pRL1a is the final product which is presented on L^d molecules and pRL1b (SI-pRL1a) peptide is the natural precursor of pRL1a.

It has been shown that some peptides which optimally sensitize target cells are produced *in vitro* by cleavage of longer peptides by the serum protease ACE.[8–11] ACE was also shown to affect the intracellular processing of a class I antigen peptide.[12] These findings indicate the presence of intermediate products during antigen processing and the involvement of proteases in the production of the antigenic peptides. Previously, Udaka *et al.*[13] identified an octapeptide p2Ca and a 16-mer peptide p2Cb (that includes the entire p2Ca) from BALB/c spleen cells, which are recognized by allogeneic L^d-specific 2C CTL, and suggested that p2Cb might be a natural precursor of p2Ca.[14] Thus, pRL1b (SI-pRL1a) has been shown to be another natural precursor peptide that is further cleaved to generate MHC-binding peptides.

Endogenous protein antigens are degraded into peptides by the proteasome in the cytoplasm, and then the peptides are translocated to the endoplasmic reticulum through major histocompatibility complex (MHC)-encoded peptide transporters, TAP1 and TAP2. TAP shows a size selectivity, efficiently translocating peptides of 9–13

amino acids.[15–17] The peptides are further trimmed at their NH_2 terminus to a size of 8–10 amino acids in length in the endoplasmic reticulum so as to fit the MHC class I peptide binding groove.[18, 19] Then the peptides are presented on the cell surface.[20–22] pRL1 peptide has been shown to be derived from the 5′ untranslated region of c-*akt* gene by insertion of MuLV LTR and 6 nucleotides of unknown origin, in which the Met codon is present.[6] Localization of pRL1b (SI-pRL1a) peptide in the cytoplasmic compartment was not determined in the present study. However, sonication of RL♂1 cells in TFA caused extensive damage to the cell walls. Thus, it is likely that the intermediate pRL1b (SI-pRL1a) peptide in the cytoplasmic compartments leaked out during the preparation and was purified by HPLC in the sensitization assay of ^{51}Cr-labeled target cells.

MHC class I binding peptides have allele-specific motifs. L^d binding peptides commonly possess Pro at position 2 which interacts with the B pocket, and an aliphatic residue at the COOH-terminal position which interacts with the F pocket of the peptide binding cleft of L^d molecules.[23] The amino acid sequence of pRL1a is consistent with the known L^d motif, and direct binding of pRL1b (SI-pRL1a) peptide to L^d molecules is unlikely.

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