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Cryptic CTL Epitope on a Murine Sarcoma Meth A Generated by Exon Extension as a Novel Mechanism

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Using the recently developed ELISPOT cloning methodology, we obtained cDNA clone S35 coding for the Ag epitope recognized by a murine sarcoma Meth A-specific CTL clone AT-1. Analysis of truncated S35 constructs and overlapping peptides revealed that the peptide epitope was LGAEAI(R)FL. AT-1 CTL lysed peptide-pulsed CMS8 cells at a nanomolar concentration, and the peptide strongly stimulated IFN-γ production in AT-1 CTL. Sequence homology indicated that the S35 was derived from a mouse homologue of human retinoic acid-regulated nuclear matrix-associated protein (ramp). The ramp gene consisted of 15 exons. The majority of the ramp mRNA was the transcript normally spliced between exons 14 and 15, but a minor population of mRNA with an extended exon 14 was also present in Meth A cells. The epitope was derived from the newly created open reading frame, which resulted from extension of exon 14 after splicing of the adjacent intronic sequence. *The Journal of Immunology, 2003, 170: 4862–4868.

Since the first Ag recognized by T cells was discovered by Boon and colleagues (1), a number of human tumor Ags have been identified (2, 3). A smaller number of murine tumor Ags recognized by T cells have also been identified (2, 4–11). The murine tumor Ags defined to date were derived from differentiation Ags, e.g., TRP-2 (11); genetically altered Ags, e.g., pRL1(6), extracellular signal-regulated kinase 2 (7); and viral Ags (8, 9). Most human tumor Ags fall into these categories, suggesting the relevance of murine tumor models for exploring human cancer Ags (12). Murine tumor models for which the tumor Ags have been molecularly identified are extremely useful in helping to establish the basis of immunotherapy (13, 14).

Antigenic peptide epitopes recognized by T cells were generally thought to be derived from proper full-length protein molecules by degradation either in the cytosol for presentation on MHC class I or in the endosomes for presentation on MHC class II. However, recently, CTL peptide epitopes derived from polypeptides produced by unusual events that occurred during transcription, splicing, or translation have been identified in tumors and in viral infections (15). For instance, epitopes derived from frameshifts (16–18), downstream AUG initiation for translation (16), improper splicing of introns (19–21), and reverse strand transcription (22) have been identified.

Meth A, CMS4, CMS5a, CMS5j, CMS8, CMS9, and CMS13 are methylcholanthrene-induced sarcomas in BALB/c mice (24, 25). Parental Meth A, Meth A (p), is resistant to CTL lysis. Meth A (sv) is a CTL lysis-sensitive variant line that occurred during in vivo passages of Meth A (p) provided by H. Shiku (Mie University School of Medicine, Mie, Japan). RL male 1 is a radiation-induced leukemia (26), and RVA, RVC, and RVD are radiation leukemia virus-induced leukemias in BALB/c mice (6). P815 is a methylcholanthrene-induced mastocytoma in a DBA/2 mouse (27). T1.1.1 and T4.8.3 are derivatives of L cells (H-2d) transfected with the H-2Ld and H-2Dd gene, respectively (28).

Materials and Methods

Tumors and cell lines

Meth A, CMS4, CMS5a, CMS5j, CMS8, CMS9, and CMS13 are methylcholanthrene-induced sarcomas in BALB/c mice (24, 25). Parental Meth A, Meth A (p), is resistant to CTL lysis. Meth A (sv) is a CTL lysis-sensitive variant line that occurred during in vivo passages of Meth A (p) provided by H. Shiku (Mie University School of Medicine, Mie, Japan). RL male 1 is a radiation-induced leukemia (26), and RVA, RVC, and RVD are radiation leukemia virus-induced leukemias in BALB/c mice (6). P815 is a methylcholanthrene-induced mastocytoma in a DBA/2 mouse (27). T1.1.1 and T4.8.3 are derivatives of L cells (H-2d) transfected with the H-2Ld and H-2Dd gene, respectively (28).

Antibodies

Anti-L3T4 (CD4) mAb, a rat Ab of the IgG2b Ig class, produced by hybridoma GK1.5 (29), was provided by F. Ficht (University of Chicago, Chicago, IL), and anti-Lyt-2.2 (CD8) mAb (30), a mouse Ab of the IgG2a class, produced by hybridoma 19/178, was provided by U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-H-2Kd and anti-H-2Dd are mouse Abs produced by hybridomas HB159 and HB102, respectively. Anti-H-2Ld mAb is a mouse IgG2a Ab produced by hybridoma 30-5-7 (31). Anti-IFN-γ mAb, a rat Ab of the IgG1 class, produced by hybridoma R4-6A2, was obtained from American Type Culture Collection (Manassas, VA) (32). Polyclonal rabbit anti-IFN-γ serum was

"3 Abbreviations used in this paper: ramp, retinoic acid-regulated nuclear matrix-associated protein; EST, expressed sequence tag; MMC, mitomycin C; ORF, open reading frame; SEREX, serological analysis of Ags by recombinant expression cloning."
produced by immunization with murine rIFN-γ (23). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Southern Biotechnology (Birmingham, AL).

Peptide synthesis

Peptides were synthesized by standard solid-phase methods using F-moc chemistry in a peptide synthesizer model AMS422; ABIMED Analyzer Technik, Langenfeld, Germany). Cleavage of the peptide from the resin and removal of side chain protecting groups were conducted using 95% trifluoroacetic acid.

Deletion constructs

Deleted S35 cDNA was amplified by PCR using pCAGGS-S35 plasmid as the template. PCR amplification was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min. The PCR fragments were cloned into the expression vector pCI-neo (Promega, Madison, WI).

Site-directed mutagenesis

Site-directed mutagenesis was done using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primers S35-S683-SITEMU (5'-CTTACGCTTCTTCTGATCACCTT-3') and S35-AS717-SIT EM (5'—CTGGGAGATTTAGTGGAATCTGGAGAAAGAAA-3') were used to generate the mutations (A to C) at nt 699 and (G to A) at nt 701. The mutants were sequenced to confirm the orientation and nucleotide sequence.

cDNA library construction

mRNA was isolated from Meth A (sv) cells using a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). First-strand cDNA was synthesized using random hexamer primer (Life Technologies, Rockville, MD). The cDNA was inserted into the EcoRI sites of expression vector pCAGGS (provided by A. Shibuya, University of Tsukuba, Tsukuba, Japan), according to the manufacturer’s instructions (SuperScript Plasmid Choice System; Life Technologies). The recombinant plasmids were electroporated into DH10B Escherichia coli bacteria (Life Technologies). The cDNA library was divided and stocked as pools containing ~10,000 bacterial colonies.

RT-PCR

mRNA was purified using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia). mRNA was reverse transcribed into single-strand cDNA using Moloney murine leukemia reverse transcriptase and oligo(dT)18 as a primer (Amersham Pharmacia), and cDNAs were tested for integrity by amplification of β-actin transcripts in a 30-cycle reaction. RT-PCR was performed by 30 amplification cycles, and the products were analyzed by agarose gel electrophoresis.

Transfection

Recombinant plasmids were purified by a Wizard Plus Series 9600 DNA purification kit (Promega, Madison, WI), and cDNAs were digested with EcoRI and XhoI and transfected into 1 × 10^6 or 1 × 10^7 cells in 24- or 96-well plates, respectively, with plasmid pd3027 containing the polyoma T Ag (33) using lipofectamine (Life Technologies [Birmingham, AL]). The cDNA library was divided and stocked as pools containing ~10,000 bacterial colonies.

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DNA sequencing and homology search

The DNA sequence was determined using a BigDye terminator cycle sequencing ready reaction kit (PerkinElmer Applied Biosystems, Foster City, CA) and a DNA sequencer ABI PRISM (PerkinElmer Applied Biosystems). The computer search for sequence homology was performed by the BLAST program on GenBank database.

IFN-γ ELISA

CTL were stimulated with targets for 18 h, and IFN-γ in the culture supernatant was assayed by the sandwich ELISA method. Anti-IFN-γ mAb (R4-6A2) was used as the capture Ab, and polyclonal rabbit anti-IFN-γ Ab was used as the detection Ab. HRP-conjugated anti-rabbit IgG Ab (MBL, Nagoya, Japan) and o-phenylenediamine dichlorohydride were used as the indicators.

CTL clones

Spleen cells (5 × 10^7) from Meth A (sv)-rejected (BALB/c × C57BL/6)F1 mice were stimulated with 5 × 10^6 mitomycin C (MMC)-treated Meth A (sv) cells in a culture flask (353014; BD Biosciences, Franklin Lakes, NJ) for 6 days 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, 100 μg/ml of streptomycin, and 50 μM 2-ME. The cells were restimulated with MMC-treated Meth A (sv) in the presence of 20 U of rIL-2 (Takeda Pharmaceutical Industries, Osaka, Japan). After the second in vitro stimulation, 13 CTL clones specific for Meth A were established by limiting dilution. Recognition of 11 clones was Dd restricted, and that of 2 clones was K^d restricted. One of the Dd-restricted CTL clones designated as AT-1 was used in this study.

Cytotoxicity assay

Tumor cells were labeled by incubating 2 × 10^6 cells with 2 MBq of Na_2^111In (New England Nuclear, Boston, MA) in 0.3 ml of medium for 90 min at 37°C under 5% CO_2 in air. The cells were washed and used as targets. For peptide sensitization, the labeled target cells were incubated with peptides for 30 min at room temperature. In direct assays, 10^5 labeled target cells (100 μl) were incubated with effector cells (100 μl). In Ab-blocking assays, serially diluted mAb (100 μl) was added to the mixture of effector cells and 10^5 labeled target cells (100 μl). After incubation for 4 h at 37°C under 5% CO_2 in air, the supernatants were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: (a - b)/c - b × 100, in which 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 the target cells with 1% Nonidet P-40.

ELISPOT assay

Nitrocellulose disk membranes (14 mm diameter) and 96-well nitrocellulose membrane-based plates (Millipore S4510; Millipore, Bedford, MA) were coated with anti-IFN-γ mAb (R4-6A2) in 0.05 M bicarbonate buffer (pH 9.6) at 4°C overnight. After washing, membranes were blocked with RPMI 1640 containing 10% FCS and 50 μM 2-ME for 1 h at 37°C. CTL were incubated with stimulator cells on the coated membrane in 24- or 96-well plates for 18 h at 37°C under 5% CO_2 in air. After culture, the membranes were thoroughly washed with distilled water and incubated with polyclonal rabbit anti-IFN-γ Ab for 1.5 h at 37°C. The IFN-γ spots were developed by alkaline phosphatase-conjugated anti-rabbit IgG Ab and a substrate kit (Bio-Rad, Hercules, CA) and counted with a dissecting microscope.

Results

Cytotoxicity and IFN-γ ELISPOT by CTL clone AT-1

The CTL clone AT-1 was established from Meth A (sv)-rejected (BALB/c × C57BL/6)F1 spleen cells by repetitive in vitro stimulation with MMC-treated Meth A (sv) cells. As shown in Fig. 1B, AT-1 specifically recognized parental Meth A, Meth A (p), and the Meth A (sv) variant sensitive for CTL lysis, in IFN-γ ELISPOT assays. Meth A (sv), but not Meth A (p), was lysed in a standard 4-h 51Cr release assay (Fig. 1A). Failure of Meth A (p) lysis by CTL appeared to be due to intrinsic resistance to lysis because lysis by anti-allogeneic H-2d CTL was also not observed (34) and data not shown. Meth A Ag specificity of the AT-1 CTL clone was confirmed because none of BALB/c methylcholanthrene sarcomas, CMS45, CMS55a, CMS55j, CMS8, CMS9, and CMS13; BALB/c radiation leukemia RL male 1; or DBA2 mastocytoma P815 targets was recognized. As shown in Fig. 1, C and D, AT-1 cytotoxicity and IFN-γ ELISPOTs for Meth A (sv) were CD8 dependent and restricted by H-2D^d because they were blocked by anti-CD8 mAb and anti-H-2D^d mAb, while no blocking was observed with anti-CD4 mAb, anti-H-2K^d mAb, or anti-H-2L^d mAb.

Cloning of Meth A Ag recognized by AT-1 CTL from Meth A (sv) cDNA library by ELISPOT assays

For cloning the gene that coded for the Ag recognized by AT-1 CTL, a cDNA library from Meth A (sv) mRNA was prepared. Approximately 4 × 10^8 cDNA clones were screened by ELISPOT assay, according to the previously described method (23). In the first round of screening, 40 cDNA pools, each containing 10,000 bacterial cDNA clones, were prepared. Three micrograms of DNA
from each pool and polyoma T were cotransfected into $1 \times 10^5$ CMS5a cells. After incubation for 24 h, the transfectants were collected and transferred onto anti-IFN-γ mAb-coated nitrocellulose membranes in 24-well culture plates, and 50,000 AT-1 CTL were added. As shown in Fig. 2, A and B, 18 spots were observed in pool number 9 after culture for 18 h. For the second round of screening, bacteria in pool number 9 in the first screening were diluted into 40 pools, each containing 1,000 bacterial cDNA clones. Transfection and assay were performed as above. For the third round of screening, bacteria in positive pool number 18 in the second screening were diluted into 40 pools, each containing 100 bacterial cDNA clones. Transfection and assay were performed as above. In the fourth and fifth rounds of screening, 100 ng DNA and polyoma T were cotransfected into $1 \times 10^5$ CMS5a cells in 96-well plates. After the incubation, the cells were transfected onto anti-IFN-γ mAb-coated nitrocellulose membrane-based 96-well plates, and 5,000 AT-1 CTL were added. ELISPOTs were then detected. Positive pool number 39 in the third screening and positive pool number 4 in the fourth screening were thus sequentially examined. Finally, the cDNA clone S35 was obtained in the fifth screening. AT-1 was stimulated with S35-transfected CMS5a, CMS8, and CMS13, or H-2Dd-transfected L cells screening. AT-1 was stimulated with S35-transfected CMS5a, examined. Finally, the cDNA clone S35 was obtained in the positive pool number 4 in the fourth screening were thus sequentially detected. Positive pool number 39 in the third screening and positive pool number 18 in the second screening were diluted into 40 pools, each containing 1,000 bacterial cDNA clones. Transfection and assay were performed as above. For the third round of screening, bacteria in positive pool number 18 in the second screening were diluted into 40 pools, each containing 100 bacterial cDNA clones. Transfection and assay were performed as above. In the fourth and fifth rounds of screening, 100 ng DNA and polyoma T were cotransfected into $1 \times 10^5$ CMS5a cells in 96-well plates. After the incubation, the cells were transfected onto anti-IFN-γ mAb-coated nitrocellulose membrane-based 96-well plates, and 5,000 AT-1 CTL were added. ELISPOTs were then detected. Positive pool number 39 in the third screening and positive pool number 4 in the fourth screening were thus sequentially examined. Finally, the cDNA clone S35 was obtained in the fifth screening. AT-1 was stimulated with S35-transfected CMS5a, CMS8, and CMS13, or H-2Dd-, but not H-2Ld-transfected L cells (data not shown).

Identification of the peptide epitope recognized by AT-1 CTL in S35

The cDNA clone S35 was 937 bp long (Fig. 3). To identify the peptide epitope recognized by AT-1 CTL, truncated S35 was prepared and transfected into CMS8 cells. IFN-γ production from AT-1 after stimulation with truncated S35-transfected CMS8 cells was assayed by ELISA after culture for 18 h. As shown in Fig. 4, experiment I, 3' truncated S35, nt 1–799 and 1–700, but not nt 1–665 or 1–350, stimulated AT-1 for IFN-γ production. Therefore, we synthesized 9-mer overlapping peptides covering the region A spanning nt 665–700 and tested for stimulation of AT-1 IFN-γ production by peptide-pulsed CMS8 cells. However, no stimulation of AT-1 IFN-γ production was observed with any of the synthetic peptides. We then prepared 5' truncated S35 nt 48–700 and nt 443–700 and tested their stimulatory activity (Fig. 4, Expt. II). No stimulation was observed with either mutant. As shown in Fig. 5, A and B, we synthesized 9-mer overlapping peptides covering the region B containing nt 1–665, nt 799 and 1–700, but not H-2Ld-transfected L cells (data not shown).

FIGURE 1. Direct cytotoxicity (A) and IFN-γ ELISPOT (B) of AT-1 CTL against various target cells. Blocking of cytotoxicity (C) and IFN-γ ELISPOT (D) of AT-1 CTL by various Abs. Cytotoxicity was measured by a standard 4-h 51Cr release assay. The number of target cells in A and C was 10,000. Effector to Meth A (sv) target cell ratio in C was 10. The number of AT-1 cells used in the ELISPOT assays in B and D was 10,000. The effector to stimulator (B) and Meth A (sv) (D) cell ratio was 4. Ab dilution in D was 1/100.

FIGURE 2. ELISPOT cloning of the gene coding for the Ag recognized by AT-1 CTL from a Meth A (sv) cDNA expression library. In A, the first, second, and third screenings for 10,000, 1,000, and 100 bacterial colonies per pool, respectively, were done by large-scale ELISPOT assays in 24-well culture plates, and the fourth and fifth screenings for 10 bacterial colonies and single clones, respectively, were done by small-scale assays in 96-well culture plates. Arrows and the figures beside them indicate positive pools examined and the number of ELISPOTs, respectively, B, ELISPOTs developed on the membranes from the first and the fifth screenings.
Analysis of cDNA that coded for the Ag recognized by AT-1 CTL

According to a GenBank BLAST search, nt 700–811 of S35 showed 86% homology to a region of the human ramp gene, suggesting that S35 is a part of a mouse homologue of ramp (35). The region of nt 1–699 showed no homology with any sequence. To obtain full-length cDNA, RT-PCR was conducted using human ramp nt 124–145 as the 5′ primer and S35 nt 923–937 as the 3′ primer with Meth A (p) cDNA. A 2.5-kb PCR product was obtained and cloned. The nucleotide sequence and the deduced amino acid sequence of the cloned products showed 85% homology, respectively, to human ramp. Subsequently, a longer clone of 3805 bp, spanning exons 1–15 corresponding to those in human ramp, was obtained. A number of mouse expressed sequence tags (ESTs) with homology to this sequence were identified. In ESTs, selective deletion spanning nt 2207–3568 was observed, suggesting that this region was an intronic sequence (Fig. 6). The nucleotide sequence in the above 2.5-kb PCR product was the same as that in the 3805-bp product, except for lacking the intronic sequence. Sequence homology indicated that this intron was between exons 14 and 15. Therefore, the 3805-bp PCR product appeared to be derived from an immature mRNA. The splicing donor site GT at nt 71 and 72 of S35 corresponded to nt 2939 and 2940, respectively. In contrast, the splicing acceptor site AG at nt 699 and 700

FIGURE 3. The genomic nucleotide sequence of extended exon 14, the altered intron, the exon 15, and putative amino acid sequence in the Meth A ramp. The nucleotide region included in clone S35 is indicated by the arrows showing the boundaries. The 3′ end of exon 14 in normal ramp was indicated by the vertical dotted line. The splicing donor (S35 nt 71) and acceptor (S35 nt 700) sites were indicated by arrowheads. Exon 15 starts at S35 nt 701. The putative amino acid sequences derived from extended exons 14 and 15 in the Meth A ramp were underlined. The amino acids sequence for CTL epitope was shown in bold.
in S35 corresponded to nt 3567 and 3568, respectively. RT-PCR analysis showed that most of the ramp mRNA underwent normal splicing between exons 14 and 15 in either Meth A (p) or Meth A (sv). However, an RT-PCR product that lacked S35 nt 71–700 was also obtained using nt 2017–2041 at 3′ end of exon 14 as the sense primer, and a combined sequence of nt 3582–3569 (S35 nt 714–701) at 5′ end of exon 15 and nt 2938–2927 (S35 nt 70–59) as the antisense primer. These results indicated that ramp mRNA with extended exon 14, as shown in Fig. 7, was expressed in Meth A.

The newly created ORF from nt 12722 of S35 contained the epitope sequence.

The ramp mRNA expression was examined by RT-PCR. The normally spliced ramp mRNA was overexpressed, but the exon 14 extension occurred only rarely in Meth A. The ramp mRNA expression was observed strongly in testis and weakly in adrenal gland, esophagus, and ovary in normal tissues. Its overexpression was observed in all tumor cell lines examined, including three other methylcholanthrene sarcomas, CMS5a, CMS8, and CMS13; a radiation leukemia RL male 1; three radiation leukemia virus-induced leukemias, RVA, RVC, and RVD; and a mastocytoma P815. No expression of exon 14-extended variant ramp was observed in those tumors.

**Discussion**

In this study, we demonstrated that the Ag peptide recognized by the Meth A-specific CTL clone AT-1 was derived from a mouse homologue of the human ramp gene. The peptide epitope recognized by AT-1 CTL was generated from an ORF that resulted from extension of exon 14. Three-prime truncated S35 nt 1–700, but not nt 1–665, stimulated AT-1 CTL IFN-γ production. This appeared to be caused by loss of the splicing acceptor site AG at nt 699 and 700 of S35. Disruption of the site by the introduction of point mutations resulted in a loss of stimulatory activity. These findings indicated that splicing of the intronic sequence between nt 71 and 72 as a typical splicing donor site with the adjacent sequence and nt 699 and 700 as an acceptor site was necessary for transcription of the nucleotides containing nt 1–70. Thus, S35 appeared to be

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**FIGURE 4.** Localization of the nucleotide sequence in S35 coding for the antigenic peptide recognized by AT-1 CTL. The deleted fragments were amplified by PCR using the full-length S35 cDNA as template and were cloned into pCI-neo vector with EcoRI and SalI sites. IFN-γ production by AT-1 CTL was measured by ELISA after culture with CMS8 transfected with the mutants. Mutations at nt 699 (A to C) and nt 701 (G to A) in experiment III were introduced by site-directed mutagenesis kits.

**FIGURE 5.** Identification of an antigenic peptide recognized by AT-1 CTL. A, Reading frames in region B of S35 in Fig. 4. B, Serial 9-mer overlapping peptides synthesized for reading frame 3 in region B. AT-1 CTL (10,000) were cultured with CMS8 targets (10,000) in presence of synthetic peptides (1.0 μM) for 18 h, and IFN-γ in the supernatant was measured by ELISA. C, CMS8 cells were 51Cr labeled and pulsed with p8 peptide (S35 peptide, LGAEAFRL) at various concentrations. AT-1 CTL to target cell ratio was 10. Cytotoxicity was measured by a 4-h 51Cr release assay.

**FIGURE 6.** The EST database analysis. Top horizontal line represents nucleotide number of 3805-bp PCR product of Meth A ramp. *+, Indicates location of the CTL epitope in S35. The intron normally spliced between exons 14 and 15 corresponds to nt 2207–3568. Arrows represent selected EST sequences in the databank with sequence homology.
Peptide strongly stimulated AT-1 CTL IFN-γ production. Analysis of Ags by recombinant expression cloning (SEREX) analysis. Matsutake and Srivastava (40) then showed that the ribosomal protein L11 was the dominant Ag in the humoral immune response against Meth A by serological analysis. L11 showed a normal configuration of exons and introns by nucleotide sequencing. However, we could also obtain clones spliced between S35 nt 71 and 700. We concluded that ramp mRNA with extended exon 14 constituted only a minor population in Meth A cells.

The nonamer LGAEAIIFRL was the antigenic peptide, as described above. The peptides QLGAEAIIFRL, LGAEAIIFRLV, or QLGAEAIIFR1L showed less efficient stimulation of AT-1 CTL when pulsed on CMS8 cells. The peptide LGAEAIIFRL had alanine instead of proline as a typical motif amino acid at position 3 for the H-2D^d-binding peptide (37). However, AT-1 CTL lysed peptide-pulsed CMS8 cells at nanomolar concentrations, and the peptide strongly stimulated AT-1 CTL IFN-γ production. Analysis of the acid eluate from Meth A (p) and Meth A (sv) cells showed that a peptide with the same elution time as the synthetic nonamer peptide was present in both cell extracts. A preliminary study suggested the presence of a precursor peptide for the nonamer epitope peptide. The relationship between those peptides is now under investigation.

There have been many reports describing abnormally generated CTL epitopes. Those include epitopes derived from the untranslated region sequence by gene insertion (5), frameshift (16, 17) sometimes within the epitope sequence (18), the remaining intronic sequence (19–21), and reverse strand (22). Furthermore, posttranslational alteration can also be recognized by the CTL (38). The finding that the cryptic antigenic CTL epitope generation was not a rare event suggests the presence of many more CTL epitopes than expected based on the normal transcription of the gene and its translation to protein, followed by Ag processing in the cytoplasm. Exon extension with the requirement of splicing for the epitope expression shown in this study is a novel mechanism for generating cryptic CTL Ags.

In this study, we also demonstrated the usefulness of ELISPOT cloning for identification of an Ag recognized by T cells, which we have described recently (23), using a model tumor Ag system (6). The method of cDNA expression cloning of the gene coding for Ags recognized by T cells was improved by incorporating the IFN-γ ELISPOT assay instead of ELISA for assaying cytokines in the culture supernatant to detect T cell response. Using the ELISPOT assay for the detection of T cell response, we could screen >10,000 cDNA clones in a pool on a 14-mm membrane in a 24-well plate. This made screening of a 2 × 10^5 cDNA library possible in a single plate. In the conventional method, one would use 96-well plates for transfection of each cDNA pool containing 50–100 clones of the library and for assaying the secreted cytokines. It would usually take several months to complete such screening of a cDNA library. ELISPOT cloning greatly shortened the screening period, and therefore overcame possible ambiguities in the results due to background fluctuation.

Meth A was established as a transplantable tumor line in 1963, and thereafter has been used by many investigators for analysis of tumor Ag and tumor immune response because of its rather strong immunogenicity. BALB/c mice immunized with the tumor showed resistance to tumor growth, and the immunity was mediated predominantly by CD8 T cells with help from CD4 T cells. Ono et al. (39) showed that the ribosomal protein L11 was the dominant Ag in the humoral immune response against Meth A by serological analysis. Matsutake and Srivastava (40) then showed that CD4 T cells generated in stimulation with Meth A recognized L11 that was mutated. However, analysis of the Meth A Ag recognized by CD8 T cells has been hampered because Meth A was resistant to CTL. Even allogeneic CTL (B6 anti-BALB/c CTL) did not lyse Meth A cells in a 3^1Cr release assay. This could be due to the lack or the presence of the cell surface molecules on Meth A, which are the ligands to killer cell-activating receptors (e.g., NKG2D) (41, 42) or killer cell-inhibitory receptors (43), respectively, on Meth A CTL.

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The present study showed that using a variant cell line Meth A (sv), the ramp-derived peptide epitope was recognized by April 28, 2007. The method of cDNA expression cloning of the gene coding for Ags was published recently (23), using a model tumor Ag system (6). The method of cDNA expression cloning of the gene coding for Ags recognized by T cells was improved by incorporating the IFN-γ ELISPOT assay instead of ELISA for assaying cytokines in the culture supernatant to detect T cell response. Using the ELISPOT assay for the detection of T cell response, we could screen >10,000 cDNA clones in a pool on a 14-mm membrane in a 24-well plate. This made screening of a 2 × 10^5 cDNA library possible in a single plate. In the conventional method, one would use 96-well plates for transfection of each cDNA pool containing 50–100 clones of the library and for assaying the secreted cytokines. It would usually take several months to complete such screening of a cDNA library. ELISPOT cloning greatly shortened the screening period, and therefore overcame possible ambiguities in the results due to background fluctuation.

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The present study showed that using a variant cell line Meth A (sv), the ramp-derived peptide epitope was recognized by April 28, 2007.

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