A Gene Encoding Antigenic Peptides of Human Squamous Cell Carcinoma Recognized by Cytotoxic T Lymphocytes

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Summary

Except for melanomas, tumor antigens recognized by cytotoxic T lymphocytes (CTLs) are yet unidentified. We have identified a gene encoding antigenic peptides of human squamous cell carcinomas (SCCs) recognized by human histocompatibility leukocyte antigens (HLA)-A2601–restricted CTLs. This gene showed no similarity to known sequences, and encoded two (125- and 43-kilodalton [kD]) proteins. The 125-kD protein with the leucine zipper motif was expressed in the nucleus of the majority of proliferating cells tested, including normal and malignant cells. The 43-kD protein was expressed in the cytosol of most SCCs from various organs and half of lung adenocarcinomas, but was not expressed in other cancers nor in a panel of normal tissues. The three nonapeptides shared by the two proteins were recognized by the KE4 CTLs, and one of the peptides induced in vitro from peripheral blood mononuclear cells (PBMCs) the CTLs restricted to the autologous tumor cells. The 43-kD protein and this nonpeptide (KGSGKMKTE) may be useful for the specific immunotherapy of HLA-A2601+ epithelial cancer patients.

Many genes encoding tumor-rejection antigens recognized by CTLs were identified from cDNA of melanomas (1–6). Further, a large number of nonapeptides recognized by HLA class I–restricted CTLs cytotoxic to melanoma cells have been identified in the past five years (5–15). Some of them are under clinical trials as cancer vaccines, and major tumor regression in several HLA-A1–melanoma patients was reported in the vaccine therapy with the melanoma antigen (MAGE)-3 peptide (16). Therefore, these nonapeptides recognized by the CTLs could be a new tool for the specific immunotherapy of melanoma. However, no peptides are yet identified from human squamous cell carcinomas (SCCs)1 one of the major human cancers, except for a mutated CASP-8 (17). We previously reported the HLA-A2601–restricted and tumor-specific CTL line recognizing peptide antigen(s) expressed on SCCs (18). In this study, we have investigated a gene encoding tumor antigen recognized by this CTL line, and report a new gene encoding two novel proteins and three nonapeptides as the antigens recognized by the HLA-A2601–restricted CTLs.

Materials and Methods

Identification of 6A1-1D7 Genes. Expression-gene cloning methods developed by T. Boon and colleagues (4, 6) were used in this study to identify a gene coding tumor antigen recognized by the KE4 CTLs (18). In brief, messenger RNA (mRNA) of the KE4 tumor cells was converted to cDNA, ligated to SalI adapter, and inserted into the expression vector pSV-SPORT-1 (GIBCO BRL, Gaithersburg, MD). cDNA of HLA-A2601 or HLA-A0201 was obtained by reverse transcription PCR (RT-PCR), and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). cDNA of HLA-A2601 or HLA-A0201 was obtained by reverse transcription PCR (RT-PCR), and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). Both 200 ng of plasmid DNA pools or clones of the KE4 cDNA library and 200 ng of the HLA-A2601 cDNA were mixed with 1 μl of lipofectin in 70 μl of OPTI-MEM (GIBCO BRL, Gaithersburg, MD). cDNA of HLA-A2601 or HLA-A0201 was obtained by reverse transcription PCR (RT-PCR), and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). Both 200 ng of plasmid DNA pools or clones of the KE4 cDNA library and 200 ng of the HLA-A2601 cDNA were mixed with 1 μl of lipofectin in 70 μl of OPTI-MEM (GIBCO BRL) for 15 min. 30 μl of the mixture was then added to the VA13 (2 × 10⁵) cells and incubated for 5 h. 200 μl of the RPMI-1640 medium containing 20% FCS was then added and cultured for 2 d followed by adding the KE4 CTLs (10⁴ cells/well; reference 8). After a 18-h incubation, 100 μl of supernant was collected to measure IFN-γ by an ELISA kit (Otsuka Pharm. Co., Tokyo, Japan) in a duplicate assay. DNA sequencing was performed with dideoxynucleotide sequencing method using DNA Sequencing kit (Perkin-Elmer Corp., Foster, CA) and analyzed by ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer).
Northern Blot Analysis. Nylon membranes (Hybond-N+; Amersham, Buckinghamshire, UK) with UV-fixed total RNAs (5 μg/lane) extracted from the various cells or UV-fixed poly A+ RNA (2 μg/lane) from various tissues were prehybridized for 10 min and hybridized overnight at 65°C in the same solution (7% SDS, 1 mM EDTA, 0.5 M Na2HPO4, pH 7.2) containing 32P-labeled 6A1-1D7 probe. The membranes were washed three times at 65°C in a washing buffer (1% SDS, 40 mM Na2HPO4, pH 7.2), and then autoradiographed. The relative expression level of the squamous cell carcinoma antigen recognized by T cells 1 (SART-1) mRNA was calculated by the following formula: index = (SART-1 density of a sample/β-actin density of a sample) × (β-actin density of the KE4 tumor/SART-1 density of the KE4 tumor).

Cloning of the SART-1 Gene. We tentatively named this gene encoding a tumor antigen recognized by the KE4 CTLs as the SART-1 gene. The SART-1 clone was obtained from both PBMCs (SuperScriptII† Human Leukocyte cDNA Library in pCMV-SPORT; Gibco BRL) and KE4 cDNA libraries by the standard colony hybridization method with the 32P-labeled 6A1-1D7 cDNA as a probe. Sequence data of the SART-1 are available from EMBL/GenBank/DDBJ under accession number AB006198. The difference of the sequence at nucleotide (nt) position 812 of the SART-1 between PBMCs and KE4 was further analyzed by treatment of the PCR products with an SceI restriction enzyme. Amplification was performed for 30 cycles (1 min at 94°C, 2 min at 56°C, and 2 min at 72°C) with the primers of 5'-CGTGAATTCTCACCGTGCTCCAGCC-3' (forward primer) and 5'-TGGGAATTCGATGAGGATCCCGAGC-3' (reverse primer).

Preparation of Glutathione S-transferase Fusion Proteins and Rabbit Abs. For SART-1-1-668 (GST, glutathione S-transferase) protein, the full length of SART-1 was digested with EcoRI and NcoI at the multiple cloning site of pCMV-SPORT, and then ligated into the pGEX-4T-2 vector (Pharmacia Biotech AB, Uppsala, Sweden). For SART-1-1-668/GST protein, the SART-1 cDNA fragment (nt 1,663-2,449) was amplified by PCR using a forward primer 5'-GCTCGGAATTCACGTGCCACTATGGG-3' and a sr-1 primer. Amplified product was digested with EcoRI and NotI, and ligated to the pGEX-5X-3 (Pharmacia Biotech AB). For SART-1-1-668/GST protein, the SART-1 cDNA fragment (nt 1,663-2,866) was amplified by PCR using a sf-1 primer and a reverse primer 5'-CGTGAATTCTCACCGTGCTCCAGCC-3'. Amplified product was digested with EcoRI and NotI, and ligated to the pGEX-5X-3 (Pharmacia Biotech AB). For SART-1-1-668/GST protein, the SART-1 cDNA fragment (1,781-2,449) was amplified by PCR using a forward primer 5'-GAGAATTCCATGGACTTTGAACGGGATG-3' and a sr-1 primer. Amplified product was digested with EcoRI and NcoI, and ligated to the pGEX-5X-3. Polynuclease anti-SART-1-1-668/GST, anti-SART-1-1-668/GST, anti-SART-1-1-668/GST, and anti-SART-1-1-668/GST Abs were prepared by immunization of rabbits with purified SART-1-1-668/GST, SART-1-1-668/GST, SART-1-1-668/GST, and SART-1-1-668/GST proteins, respectively, by the methods previously reported (19).

Preparation of SART-1-1-tag Fusion Protein in Expression Vector Constructs. For preparation of the SART-1-1-668/myc, the SART-1 of positions 29-2,449 was amplified by PCR using a forward primer 5'-GCTCGGAATTCACGTGCCACTATGGG-3' and a reverse primer 5'-AGGGAATTCCTCGGTGATGTTGGTGTTC-3' (sr-2). Amplified product was digested with EcoRI, and ligated to pcDNA3.1/Myc-His vector (Invitrogen). The gene encoding a tag peptide was ligated to the 2,438 position before the stop codon of the third frame, which was used as the SART-1-1000/myc. Similarly, the SART-1 fragment of positions 1,663-2,449 or 1,782-2,449 was amplified by PCR using a sf-1 and a sr-2 primer or a sf-2 primer and a sr-2 primer, and the amplified product was used for preparation of the SART-1-1-668/myc or SART-1-1-668/myc respectively.

Western Blot Analysis. The samples were lysed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.2 mM PM SF (Sigma Chemical Co., St. Louis, MO), and 0.03 one trypsin inhibitor unit (TIU)/ml aprotinin, sonicated, and centrifuged at 14,000 rpm for 20 min, and the supernatant was used as the cytosol fraction. Then, the pellet was lysed with a buffer consisting of 7.2 M urea, 1.6% Triton X-100, 0.8% dithiothreitol, and 2% lithium dodecyl sulfate, and centrifuged, and the supernatant was used as the nuclear fraction. The lysate was separated by SDS-PAGE. The proteins in acrylamide gel were blotted to Hybond™-polyvinylidene difluoride (PVDF) membrane (Amersham) and were incubated with appropriate Abs for 4 h at room temperature. The other methods of Western blot analysis were previously described (19).

KE4 CTL, Its Sublines, and CTL Assay. HLA-A2601-restricted and SCC-specific CTL line (KE4 CTL) established from an esophageal cancer patient (16) was used in this study as effector cells for identification of the peptide antigens encoded by the SART-1 gene. KE4 CTL sublines were established from the parental KE4 CTL line by the limiting dilution culture as reported (20). In brief, 1 cell/well (round bottomed 96-well microculture plate) of the parental KE4 CTL line was incubated with the culture medium alone for up to 30 d. The sublines were tested for their cytotoxicity to the KE4 (A2402/2601, KE3 (A2402/A0201), and V13 fibroblast cell lines in a 6-h 51Cr-release assay as reported (18) at an E/T ratio of 5:1, and the 51Cr release assay was used in this study.

Constructions of D deletion Mutants. The pcDNA3/6A1-1D7 plasmid, a derivative of the pcDNA3 vector containing a 990-bp DNA fragment of the 6A1-1D7 gene corresponding to the nucleotide positions 1,517-2,506 of the SART-1 gene and a CMV promoter for directing transcription, was digested with NcoI for preparation of deletion mutants. The linear 6A1-1D7/myc DNA digested with NcoI and NotI was ligated to the pGEX-5X-3 (Pharmacia Biotech AB). For SART-1-1-668/GST protein, the SART-1 cDNA fragment (nt 1,663-2,866) was amplified by PCR using a sf-1 primer and a reverse primer 5'-CGTGAATTCTCACCGTGCTCCAGCC-3'. Amplified product was digested with EcoRI and NotI, ligated to the pGEX-5X-3, and the recloned DNA was amplified by PCR using a forward primer 5'-GAGAATTCCATGGACTTTGAACGGGATG-3' and a sr-1 primer. Amplitied product was digested with EcoRI and NcoI, and ligated to the pGEX-5X-3. Polynuclease anti-SART-1-1-668/GST, anti-SART-1-1-668/GST, anti-SART-1-1-668/GST, and anti-SART-1-1-668/GST Abs were prepared by immunization of rabbits with purified SART-1-1-668/GST, SART-1-1-668/GST, SART-1-1-668/GST, and SART-1-1-668/GST proteins, respectively, by the methods previously reported (19).

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Western Blot Analysis. The samples were lysed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.2 mM PM SF (Sigma Chemical Co., St. Louis, MO), and 0.03 one trypsin inhibitor unit (TIU)/ml aprotinin, sonicated, and centrifuged at 14,000 rpm for 20 min, and the supernatant was used as the cytosol fraction. Then, the pellet was lysed with a buffer consisting of 7.2 M urea, 1.6% Triton X-100, 0.8% dithiothreitol, and 2% lithium dodecyl sulfate, and centrifuged, and the supernatant was used as the nuclear fraction. The lysate was separated by SDS-PAGE. The proteins in acrylamide gel were blotted to Hybond™-polyvinylidene difluoride (PVDF) membrane (Amersham) and were incubated with appropriate Abs for 4 h at room temperature. The other methods of Western blot analysis were previously described (19).
Peptides and AA Says. In this manuscript, amino acid (aa) positions were named based on the sequence of the predicted SART-1 protein because all the synthesized peptides were located in the region shared by both the SART-1 and SART-2 proteins that was translated in the third frame. A series of 22 different 10-mer, according to the predicted aa sequences corresponding to a part of deduced SART-1 protein (aa positions 730–800: SHR–FGKGSQKTKERMKQKDLDEALEKKMSDSTDPLGTVAILQEGKQAQKPTYIVLSGSGKSMNANTITIK), were prepared. Each peptide is a 10 mer that shares the same aa with the following peptide at positions 4–10. Six different nonapeptides were also prepared in which each of the three 10 mer (SART-1, 736–745, SART-1, 748–757, SART-1, 784–793) was deleted at position 1 or 10. Further, each aa of the three nonapeptides was extended by glycine (G) when it was not glycine or by threonine (T) when it was glycine to determine aa residues critical for binding to HLA-A2601 and CTL-mediated recognition. These peptides were purchased from Biologica (Nagoya, Japan). The purity was >70% in most of the peptides, and >95% in those used for induction of CTLs. For detection of antigenic peptides, the HLA-A2601 or A0201 cDNA (as a control) was transfected to the VA13 (2 × 104) cells and incubated for 5 h. Then, 200 μl of the RPMI-1640 medium containing 20% FCS was added and cultured for 2 d, followed by adding the peptides at the concentration of 10 μM in most experiments, or 10 nM to 50 μM in certain experiments. 2 h later, the supernatant was removed and the KE4 CTLs (106 cells/well) were added, incubated for 18 h, and 100 μl of supernatant was collected to measure IFN-γ by an ELISA kit in a duplicate assay.

Induction of CTL by Nonapeptides. PBMCs (2 × 106) from a KE4 patient that had been cryopreserved in a nitrogen tank were thawed in the morning of experiments, and were incubated with a nonapeptide (10 μM) in a well of a 24-well plate containing 2 ml of the culture medium. PBMCs from HLA-A2601–healthy volunteers were also used. At days 7 and 14 of culture, cells were collected, washed, and stimulated with antigen presenting cells consisting of the irradiated autologous PBMCs that had been pre-incubated with the same nonapeptide (10 μM) for 24 h followed by washing with PBS. The ratio of the responder to stimulator cell was 10:1. Cells were harvested at day 21 of the culture, and most of them were tested for their CTL activity in a 6-h 51Cr-release assay. Some of them were used for preparation of the CTL sublines by incubation of 10 cells/well (round bottomed 96-well microculture plate) with the culture medium in the presence of irradiated allogenic PBMCs as feeder cells. These cells from the macroculture were tested for their activity at 10 d of culture to produce IFN-γ by an ELISA. Several sublines from well-proliferating wells were further expanded in the culture medium alone in wells of a 24-well plate, and were tested for their cytotoxicity to the KE4 and KE3 tumor cells at an E/T ratio of 5:1 in a 6-h 51Cr-release assay at 15 d of the culture.

Results

Identification of the 6A1-1D7 Gene. The total of 106 cDNA clones from cDNA library of the KE4 tumor cells were tested for their ability to stimulate IFN-γ production by the KE4 CTLs after cotransfection with the HLA-A2601 into the VA13 human fibroblast cells. This method allows identification of genes encoding tumor-rejection antigens (1–6). After repeated experiments for the several candidate clones, one clone (6A1-1D7) was confirmed to encode a tumor antigen recognized by the KE4 CTLs when cotransfected with HLA-A2601 (Fig. 1 A). The sequence of this cDNA clone proved to be 990 bp long. Expression of this gene was investigated by Northern blot analysis. A band of ~2.6 kb was observed in all the normal tissues and tumor cell lines tested (Fig. 2). The relative level of mRNA expression was within the range of 2.3 ± 2.9 in all the samples except for testis (the expression level: 7.5) and pancreas (17.4) (Fig. 2). These results suggest that this gene was ubiquitously expressed at the mRNA level with higher expression in testis and pancreas, and that the 990-bp-long cDNA was incomplete.

Identification of the SART-1 Gene. A 2,506-bp-long gene was independently cloned from the cDNA libraries of KE4 tumor and PBMCs of healthy donors using the 6A1-1D7 as a probe (Fig. 3). The (nt) sequences of these clones were identical with the expectation of the position 812 (cytosine in the KE4 versus thymine in PBMCs). This would be due to a genetic polymorphism, but not due to a point mutation, since the samples in which the nt position 812 was cytosine were the KE4 CTLs, a B cell line from the KE4 patient (Bec-1), fetal liver, COS cells, and 16 of 22 solid tumor cell lines tested, whereas it was thymine in testis, VA13 cells, and the other six tumor cell lines. An aa translated from these codons in the third frame is identical (ACC, ACU = threonine). KE4 CTLs also recognized VA13 cells cotransfected with these new genes and HLA-A2601 (Fig. 1 B). Both clones contained the 6A1-1D7 at positions 1–812. This 2,506-bp-long gene showed no similarity to known sequences, and was tentatively named as the SART-1 gene. Although the SART-1 mRNA was ubiquitously expressed, the KE4 CTLs did not recognize...
We then intended to investigate the SART-1 protein expression in various cells and tissues by Western blot analysis with anti-SART-1 1,600/GST and -SART-1 6A1-1D7 Abs, since both the SART-1 and 6A1-1D7 encoded a tumor antigen recognized by the KE4 CTls. The first AUG codon resided at positions 39–41 of the SART-1 in the first frame with suitable context (CCACU AU GG; Fig. 3) for initiation of protein synthesis (21, 22). The SART-1 thus contains an open reading frame (ORF) of 2,400 bp encoding a protein of 800 aa residues (SART-1 800). In contrast, the first AUG codon of the 6A1-1D7 exists at positions 1,663–1,665 with unfavorable content (GGAGG-AU GA) in the first frame. Between this first AUG and the stop UGA (1,864–1,866) codon of the 6A1-1D7, there is one Shine-Dalgarno (S-D) sequence (AGGGAG, 1,771–1,775), and the other S-D-like sequence (AGGGG, 1,681–1,685), and the other S-D sequence (AGGAGG, 1,771–1,775) that are known to induce frame shifting in prokaryotic mRNAs (23, 24). A protein of 259 aa (SART-1 259) could be translated if any of these S-D sequences induces -1 frame shifting and change the stop codon from the positions 1,864–1,866 of the first frame to the positions 2,439–2,441 of the third frame. If not, a peptide of 67 aa could be translated in the first frame.

Expression of the SART-1 800 Protein. An Ab to the SART-1 1,600/GST recognized a 125-kD band of SART-1 1,600 protein after cleavage of GST with thrombin (data not shown), and recognized a 125-kD band in the nuclear fraction of PBMs activated with 10 μg/ml of PHA (PHA blasts), KE4 tumor, and Bec-1, but not unstimulated PBMs (Fig. 4 A). No protein in the cytosol was recognized by this Ab in any samples tested. The 125-kD band was also expressed in the nucleus of the majority of tumor tissues, tumor cell lines, and normal cell lines tested, but was not expressed in normal tissues except for testis and fetal liver. The summary is shown in Table 1. When the SART-1 of positions 29–2,449 (SART-1 1,600) was transfected to VA13, intensities of the 125-kD band in both the nuclear and cytosol fractions increased (Fig. 4 A). Furthermore, this Ab and anti-myc monoclonal Ab recognized a 132-kD band of the VA13 cells transfected with the SART-1 of positions 29–2,449 in conjunction with pcDNA3.1/Myc-His vector (SART-1 1,600/myc) (Fig. 4 A). The different migration of these bands (125 and 132 kD) will be due to a tag peptide (theoretically ~5 kD). These results suggest that the 125 kD of the SART-1 1,600 protein was expressed in the nucleus of proliferating cells including normal and malignant cells, but not in nonproliferating cells, nor any normal tissues except for testis and fetal liver.

Expression of the SART-1 259 Protein. An Ab to the SART-1 6A1-1D7/GST recognized a 43-kD band of the recombinant SART-1 6A1-1D7 protein after cleavage of GST with factor Xa (Fig. 4 B). Therefore, the SART-1 259 could be translated by the mechanism of -1 frame shifting in the prokaryotic mRNA, and be recognized by anti-SART-1 6A1-1D7/GST Ab. This Ab also recognized a 43-kD protein in the cytosol of KE4 and TE9 esophageal SCC cell lines, fresh esophageal SCCs, and lung SCCs and adenocarcinomas, but not PBMs (Fig. 4 B). No protein in the nucleus.

Figure 2. Expression of the SART-1. 21 tumor cell lines (KE4, KE3, KUMa-1, NSC-4, QG6, SQ-1, A549, MKN-28, Colo201, SW 620, KIN-1, KYN-1, M 56, N 73, NALM-1; reference 18), PBMs, Bec-1, COS, or 16 tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas on human multiple tissue Northern blot, and spleen, thymus, prostate, testis, uterus, small intestine, colon, and peripheral blood leukocyte on human multiple tissue Northern blot IV; Clontech Lab., Inc., Palo Alto, CA) were provided for Northern blot analysis with the 6A1-1D7 as a probe. Some of the results are shown in the figure.

Figure 3. Nucleotide sequence of the SART-1. The cloned cDNA (6A1-1D7) was initially provided for the nucleotide sequencing. The sequence of 6A1-1D7 is 990 bp long (positions 1,517–2,506, underlined by the dotted line) in the first frame are used for protein synthesis. One S-D and each of the two different S-D-like sequences are marked by the dot on the top. The 2,506-bp-long SART-1 was then cloned from DNA libraries of the KE4 tumor and human PBMs. The SART-1 has an ORF 2,400 bp encoding 800 aa when the first AUG codon (39–41, underlined by the bold line) and stop codon (2,439–2,441, underlined by the dotted line) are used for protein synthesis in the third frame. There was only one nt difference at the position 812 (marked by G) between the SART-1 of KE4 tumor and human PBMs. These sequence data are available from the EMBL/GenBank/DDBJ under accession number A8006198.
The 43-kD protein was expressed in the cytosol of all the head and neck SCC tissues tested, 60% of esophageal SCCs, and half of the lung SCCs and lung adenocarcinomas, but not observed in leukemia, melanomas, nor any normal tissues, normal cell lines, or normal cells except for fetal liver and testis (Table 1). These results suggest that the

Table 1. Expression of the SART-11800 and SART-1259 Proteins in Normal and Cancer Cells and Tissues

|                  | SART-11800 (nucleus)* | SART-1259 (cytosol)† |
|------------------|-----------------------|----------------------|
|                  | Cell lines | Tissues | Cell lines | Tissues |
| Normal           |            |         |            |         |
| PBMC             | 0/5        | —       | 0/5        | —       |
| PHA blast        | 2/2        | —       | 0/2        | —       |
| Fibroblast       | 2/2        | —       | 0/2        | —       |
| Fetal liver      | —          | 1/1     | —          | 1/1     |
| Newborn liver    | —          | 0/1     | —          | 0/1     |
| Liver            | —          | 0/1     | —          | 0/1     |
| Placenta         | —          | 0/1     | —          | 0/2     |
| Esophagus        | —          | 0/2     | —          | 0/4     |
| Pancreas         | —          | 0/1     | —          | 0/1     |
| Cancer           |            |         |            |         |
| Head and neck SCC| 2/2        | 2/2     | 3/5        | 7/7     (100%) |
| Esophageal SCC   | 5/5        | 3/5     | 4/6        | 18/30   (60%) |
| Lung cancer      |            |         |            |         |
| Adenocarcinoma   | 3/3        | 7/7     | 3/6        | 16/35   (47%) |
| SCC              | 2/2        | 3/4     | 3/3        | 8/17    (47%) |
| Leukemia         | 4/4        | 4/4     | 0/16       | 0/10    (0%) |
| Melanoma         | 1/1        | —       | 0/2        | 0/10    (0%) |

* Expression of the SART-11800 protein in the nucleus of various normal and cancer cells and tissues was investigated by Western blot analysis with anti-SART-11800 Ab.
† Expression of the SART-1259 protein in the cytosol of various normal and cancer cells and tissues was investigated by Western blot analysis with anti-SART-1259 Ab.
§ Number of positive per total samples tested are shown.

was detected by this Ab in any samples tested (data not shown). The 43-kD protein was expressed in the cytosol of all the head and neck SCC tissues tested, 60% of esophageal SCCs, and half of the lung SCCs and lung adenocarcinomas, but not observed in leukemia, melanomas, nor any normal tissues, normal cell lines, or normal cells except for fetal liver and testis (Table 1). These results suggest that the

from various organs were also studied. (A) Expression of the SART-11800 protein was investigated by Western blot analysis with anti-SART-11800 Ab. Anti-myc monoclonal Ab (Invitrogen) was also used for analysis of VA13 transfected with the SART-11800/myc. (B) Expression of the SART-1259 was investigated with anti-SART-1259 Ab. In the left gel, 70-, 43-, and 27-kD bands corresponded to the SART-1259/myc, SART-1259, and GST, respectively. The data of the cytosol fraction were shown. No bands were detected by this Ab in the nucleus of any samples tested (data not shown). (C) Expression of the SART-1259 was investigated with anti-SART-1259 Ab. The data of the cytosol fraction were shown. (D) Anti-myc and anti-SART-1259 Abs were used for analysis of COS cells transfected with the SART-1259/myc or SART-1259. The total lysate was used for experiments.
SART-1259 protein was translated by the mechanism of −1 frame shifting using an internal ribosomal entry site in human mRNAs primarily from SCCs and adenocarcinomas, and was recognized by this Ab.

To investigate this possibility, we developed rabbit Abs against GST fusion protein with a peptide of 67 aa in the first frame (SART-11-2,675/GST) and a protein of 219 aa in the third frame (SART-1219/GST), since each of the two proteins is necessary for construction of the SART-1259. Both anti-SART-11-2,675/GST and anti-SART-1219/GST Abs recognized a 43-kD band in the cytosol of the KE-4, but not of PBMCs or PHA blasts (Fig. 4 C). These Abs also recognized a 43-kD protein of the other SCCs and lung adenocarcinomas, and the pattern of the reactivity was almost identical to that of anti-SART-16A1-1D7/GST Ab shown in Table 1. The results suggest that this 43-kD protein consists of both a peptide of 67 aa in the first frame and a protein of 219 aa in the third frame. Furthermore, we prepared the plasmid construct in which the part of the SART-1 at nt positions 1,663–1,665 with the mechanism of codon A31 I/Myc-His vector (SART-16A1-1D7/myc and SART-1219/myc, respectively). When the SART-16A1-1D7/myc was transfected into COS cells, two bands (48 and 43 kD) were detected with both anti-myc monoclonal and anti–SART-1219/GST Abs, whereas only a 43-kD band was detected in COS cells transfected with the SART-1219/myc (Fig. 4 D). The 48-kD protein might consist of 43 kD of SART-1259 plus 5 kD of a tag peptide that would be initiated by the AUG codon at positions 1,663–1,665 with the mechanism of −1 frame shifting. On the other hand, the 43-kD protein might consist of the 38-kD protein of the SART-1219 plus 5 kD of a tag peptide that would be initiated by the AUG codon at positions 1,782–1,784 in the third frame.

Identification of Regions Containing Antigenic Peptides for CTLs. To identify antigenic peptides encoded by the SART-1 gene, we investigated the capability of deletion mutants of both the 6A1-1D7 and SART-1 genes to stimulate IFN-γ production by the KE4 CTLs, since both genes encoded tumor antigens recognized by the KE-4 CTL as shown in Fig. 1. Higher levels of IFN-γ production were observed in the 6A1-1D7 (full length), 6A1-1D7a, and 6A1-1D7b when cotransfected with HLA-A2601 and -A0201, and 2 d later these cells were tested for their ability to stimulate IFN-γ production by the KE4 CTLs. The background of IFN-γ production by the KE4 CTLs in response to VA13 cells transfected with both each mutant and HLA-A2601 (~50 pg/ml) was subtracted in the figure.

Figure 5. Identification of regions containing antigenic peptides for CTL. Deletion mutants of 6A1-1D7 gene (6A1-1D7, 6A1-1D7a, 6A1-1D7b, 6A1-1D7, and 6A1-1D7b) and the full length of 6A1-1D7 in A, or mutants of SART-1 gene (SART-1-1-2,506, SART-1-1-2,355, SART-1-1-2,252, SART-1-1-2,251, and the others) and the full length of SART-1 in B were cotransfected into VA13 cells (2 × 10^9) with HLA-A2601 or -A0201, and 2 d later these cells were tested for their ability to stimulate IFN-γ production by the KE4 CTLs. The background of IFN-γ production by the KE4 CTLs in response to VA13 cells transfected with both each mutant and HLA-A2601 (~50 pg/ml) was subtracted in the figure.

To confirm the presence of a peptide-specific CTL, 80 KE4 CTL sublines were tested for their reactivity to each of the three 10 mer (SART-1-1-2,506, SART-1-1-2,355, and SART-1-1-2,252). 4, 5, or 6 of 80 of the KE4 CTL sublines showed the SART-1-1-2,506, SART-1-1-2,355, or SART-1-1-2,252 peptide-specific reactivity, respectively. The representative results are shown in Fig. 7.

In the SART-1-1-2,485 peptide, the ability to stimulate IFN-γ production was observed at 50 nM with the maximal level at 3 μM (Fig. 8). This ability was observed as low as 10 nM with the maximal level at 0.78 μM in the cases of both the SART-1-1-2,485 and SART-1-1-2,485 peptides.
Figure 6. Determination of peptide antigens. A series of 22 SART-1 oligopeptides (10 mer; 10 μM) in A or 6 different nonapeptides (10 μM) from three 10 mer (SART-1 1736-745, SART-1 1748-757, and SART-1 1784-793) with deletion of one aa at position 1 or 10 in B were loaded for 2 h to the VA13 cells (2 x 10⁴) transfected with HLA-A2601 or -A0201. For IFN-γ production, the KE4 CTLs (10⁴) were added, incubated for 18 h, and the culture supernatant was collected for measurement of IFN-γ by the ELISA in duplicate assays. The background of IFN-γ production by the KE4 CTLs in response to each peptide loaded to the VA13 cells transfected with HLA-A0201 (~50 pg/ml) was subtracted in the figure. In a ⁵¹Cr-release assay, these VA13 cells were labeled with Na₂ ⁵¹CrO₄ for 1 h followed by adding the KE4 CTLs (5 x 10⁴). 6 h later, the supernatant was harvested for measurement of the radioactivity in triplicate assays as reported (18). The background of percent lysis by the KE4 CTLs of the VA13 cells that were transfected with HLA-A0201 and loaded by each peptide was <5%.

Figure 7. CTL sublines recognizing each nonapeptide. 80 KE4 CTL sublines were tested for their ability to produce IFN-γ by recognition of each of the three 10 mer (SART-1 1736-745, SART-1 1748-757, and SART-1 1784-793) that was loaded at 10 μM on the VA13 cells for 2 h transfected with HLA-A2601 or -A0201. Detailed methods are shown in the legend for Fig. 6. Four, five, or six of 80 of the KE4 CTL sublines reacted to the SART-1 1736-745, SART-1 1748-757, or SART-1 1784-793, respectively. The representative results from the peptide-specific CTL sublines (N o. 48 and 53: the SART-1 1736-745-specific CTLs; N o. 3 and 60: the SART-1 1748-757-specific CTLs; and N o. 13 and 36: the SART-1 1784-793-specific CTLs) are shown in the figure. The other CTL sublines were mostly not reactive to any of the 10 mer, and only a few sublines were reactive to two of the three 10 mer (data not shown). None of the sublines were reactive to all the three peptides.
Determination of aa Required for CTL-mediated Recognition. Each aa of the three nonapeptides (SART-1 736-744, SART-1 749-757, and SART-1 785-793) was substituted by glycine (G) when it was not glycine or by threonine when it was glycine. These peptides, along with the parental nonapeptides, were tested for their ability to stimulate IFN-γ production by the parental KE4 CTLs. The representative results are shown in Fig. 9. The ability of the SART-1 736-744 disappeared or extremely decreased when glutamic acid (E) or threonine (T) at position 9 (9E-G in Fig. 9) or 8 (8T-G in Fig. 9) was substituted, respectively. It also decreased when glycine or methionine (M) at position 4 or 6 was substituted, whereas it slightly increased when glycine or serine (S) at position 2 or 3 was substituted to threonine or glycine, respectively. The ability of SART-1 749-757 disappeared when leucine (L) or alanine (A) at position 2 or 6 was substituted. It also decreased when glutamic acid or leucine at position 5 or 8 was substituted. In a case of the SART-1 785-793, the ability disappeared when glycine or serine at position 4 or 8 was substituted. It decreased when leucine or serine at position 2 or 5 was substituted, whereas slightly increased when serine at position 3 was substituted.

Induction of CTLs by the Nonapeptides. The three nonapeptides (SART-1 736-744, SART-1 749-757, and SART-1 785-793) were tested for their ability to induce the CTLs against the autologous tumor cells from PBMCs of a KE4 patient. PBMCs stimulated with the SART-1 736-744 and their subline No. 1 showed higher levels of the KE4 autologous tumor cell lysis than those of the KE3 allogenic tumor cell lysis (Table 2). In contrast, PBMCs cultured with IL-2 alone or stimulated with SART-1 749-757 or SART-1 785-793 equally lysed both the tumor cells. The cells from the total of 144 microcultures (48 microcultures from PBMCs alone, 48 stimulated with the SART-1 736-744, and 48 with the
Table 2. Cytotoxicity in PBMCs Stimulated with the T Three N onapeptides

| Effector cells       | Percent specific lysis (E/T ratio of 5:1) | KE3  | KE4  |
|----------------------|------------------------------------------|------|------|
| PBMC alone           | Bulk culture                             | 11.50| 12.10|
| PBMC stimulated      | Bulk culture                             | 15.80| 28.50|
| with SART-T-1736-744 | Subline N.o. 1                           | 0.00 | 12.00|
| PBMC stimulated      | Bulk culture                             | 26.10| 27.50|
| with SART-T-1259-757 | Subline N.o. 1                           | 0.00 | 0.00 |
| PBMC stimulated      | Bulk culture                             | 12.00| 13.00|
| with SART-T-1785-793 |                                         |      |      |

*S The three nonapeptides (SART-T-1736-744, SART-T-1124-757, and SART-T-1785-793) were tested for their ability to induce CTLs against the KE4 tumor cells from PBMCs of a KE4 patient. After the stimulation, PBMCs or the sublines were tested for their cytotoxicity against the autologous KE4 and allogenic KE3 tumor cells at an E/T ratio of 5:1 in triplicate determinants in a 6-h 3H Cr-release assay. The mean values of percent specific lysis are shown in the table.

SART-T-1736-744 were independently tested for their activity to produce IFN-γ by recognition of the KE3, KE4 tumor, and VA13 cells. The cells from 10 of 48 of the microcultures from the PBMCs stimulated with the SART-T-1736-744 produced higher levels of IFN-γ by recognition of the KE4, but not the other cells. The representative result of one microculture showing positive IFN-γ production is shown in Fig. 10. In contrast, the cells from none of 48 of the microcultures besides one from either PBMCs alone or PBMCs stimulated with the SART-T-1259-757 produced IFN-γ by recognition of the KE4 cells. The representative result of one microculture (subline 11 from PBMCs alone or subline 14 from PBMCs with the SART-T-1249-757) showing negative IFN-γ production is shown in the figure.

Discussion

The results of this study suggest that SART-T-1 gene is a bicistronic gene encoding two proteins, SART-T-1259 (125 kD) in the nucleus and SART-T-1259 (43 kD) in the cytosol. Most eukaryotic mRNAs have a single ORF and a single functional initiation site, which is usually the AUG codon that lies closest to the 5' end (21, 22). However, there are some viral mRNAs that break this rule; two proteins are translated from either the same or a different ORF (25–27). Several human genes are also suggested to be bicistronic. Liver-enriched transcriptional-activator protein (LAP) mRNA was found to be translated into two proteins, LAP and the liver-enriched transcriptional-inhibitory protein (LIP) (28, 29). The LIP contains the DNA-binding and dimerization domains, but is devoid of the transcription-activation domain. LAP and LIP seem to exhibit antagonistic activities. Another example is the glycoprotein (gp) 75 encoding two different polypeptides, gp75 recognized by sera from cancer patients and a peptide with 24 aa recognized by CTLs (30). However, the mechanism of posttranscriptional regulation in human mRNAs is scarcely understood at the present time. Therefore, SART-T-1 shall be a novel tool to explore the mechanism.

It is of note that SART-T-1 encodes a leucine zipper motif around nt positions 1,125-1,202 in the third frame (corresponding peptide: R E L E E I R A K L R L O A Q S L S T V G - P R L A S). The leucine zipper motif is known to form homo- or heterodimers that can bind DNA and modulate transcription of many genes (31, 32). Indeed, the SART-T-1 gene product bound to DNA (our unpublished results). Although its biological functions are currently unknown, the SART-T-1259 protein might be involved in regulation of gene transcription, because it was localized in the nucleus of proliferating cells, possessed a leucine zipper motif, and bound to DNA. In contrast, the SART-T-1259 protein without leucine zipper motif expressed in the cytosol of SCCs and adenocarcinomas might inhibit the activity of the SART-T-1259. If this is the case, these proteins might be involved in regulation of proliferation of epithelial cells and their malignant transformation.

The region of antigenic peptides encoded by the 6A1-1D7 and SART-T-1 genes was 62 aa from the COOH terminus shared by the SART-T-1259 and SART-T-1200. Therefore, both proteins could be used for antigen processing to present the antigenic peptides on the groove of the HLA-A2601 molecule, although the SART-T-1259 protein, but not the SART-T-1200, is expected to be used as a major source of the antigenic peptides recognized by the KE4 CTL because of its preferential expression in the cytosol of tumor cells.
The three 10 mer and their nonapeptides in the region of SART-1 protein at positions of 730–800 were identified by an IFN-γ assay as antigenic peptides recognized by the HLA-A2601-restricted KE4 CTLs. Because of the presence of CTLs sublines reacting to each of the three 10 mer among the 80 sublines tested, the parental KE4 CTL line would consist of the mixtures of these peptide-specific CTL clones. The other sublines were either not reactive to any of the 10 mer or reactive to two of the three 10 mer. Among these 10 mer, SART-1736-745, and also SART-1749-757 to some extent, but not SART-1738-749, had the activity in a 51Cr-release assay. SART-1736-744, but not the others, possessed the ability to induce CTLs in PBMCs against the autologous tumor cells. Although the molecular basis for this discrepancy is presently unclear, SART-1736-744 might be naturally expressed on the HLA-A2601 allele of the KE4 tumor cells.

Our results suggest that threonine and glutamic acid at positions 8 and 9 of SART-1736-744 (KGSKMKTE), leucine and alanine at positions 2 and 6 of SART-1736-745 (KLDEEALLK), and glycine and serine at positions 4 and 8 of SART-1738-749 (VLSGSGKSM) are critical for the recognition of each peptide by the parental KE4 CTLs. In addition, glycine and methionine at positions 4 and 6 of SART-1737-746, glutamic acid and leucine at positions 5 and 8 of SART-1739-747, and leucine and serine at positions 2 and 5 of SART-1735-749 are important for the recognition. The binding motif for HLA-A2601 has not been determined as far as we know, and the KE4 CTL did not react to HLA-A2601 + SCC and thus seemed to be HLA-A2601 restricted. The P pocket residues of these two subtypes are different (33), and therefore a binding motif at position 9 for them may be different from each other. Subsequently, it is difficult to compare our results of aa residues at position 9 to others, showing that valine or a hydrophobic residue at position 9 is important for binding to HLA-A2601 (34, 35). With regard to the position 2, threonine, leucine, or valine was reported as the motif for binding to HLA-A26. Our results indicated that leucine of both SART-1736-745 and SART-1738-749 was required for binding to HLA-A2601 allele, and substitution of glycine to threonine at position 2 of the SART-1736-744 rather increased its activity to induce IFN-γ production by the parental KE4 CTLs.

From the results of the experiments of dose-dependent reactions, both SART-1739-757 and SART-1738-753 seemed to have higher affinity for binding to the groove of HLA-A2601 molecule than that of SART-1736-744. Modified gp100 nonapeptides are reported to be more potent for induction from PBMCs of HLA-A26–restricted CTLs cytotoxic to melanoma cells (36). Therefore, a modified SART-1736-744 peptide at position 2 from glycine to threonine, or probably to leucine or valine, may increase affinity of the binding to HLA-A2601, which in turn increases the ability to induce CTLs restricted to HLA-A2601 + SCCs. This issue needs to be tested for development of better cancer vaccines.

The SART-1736-746 peptide, but not the others, induced from the patient’s PBMCs the CTLs restricted to the autologous tumor cells. CTL precursors in the patient’s PBMCs increased by 10-fold after three rounds of stimulation with the peptide in vitro. This peptide failed to induce CTLs in PBMCs from any of three HLA-A2601 + healthy donors tested under the conditions used in this study (data not shown). The SART-1739 protein was expressed in the cytosol of the majority of SCCs tested and half of lung adenocarcinomas. Because of its preferential expression in the cytosol of SCCs and adenocarcinomas, the SART-1739 protein, but not the SART-1700, could be a major source of antigenic peptides recognized by CTLs. The HLA-A26 allele is found in ~22% of Japanese, 17% of Caucasians, and 14% of Africans (37). The A2601 subtype is found most frequently among the A26 subtypes (38). Therefore, the SART-1739 protein along with the SART-1736-744 peptide could be useful for specific immunotherapy of relatively large numbers of HLA-A2601 patients with SCCs or adenocarcinomas as a cancer vaccine and also an antigen in vitro to induce CTLs for adoptive cellular therapy.

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