Sequence Analysis of Genes Encoding Rodent Homologues of the Human Tumor-rejection Antigen SART-1

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H uman SART-1 (hSART-1) gene encodes a 125 kD protein with a leucine-zipper motif expressed in the nucleus of all proliferating cells, and a 43 kD protein expressed in the cytosol of most epithelial cancers. In this study, two rodent genes (rSART-1 and mSART-1) homologous to hSART-1 were cloned from cDNA libraries of murine brain and a rat tumor cell line, respectively. mSART-1 and rSART-1 were highly homologous to hSART-1 with 86% and 84% identity at the nucleotide level, and 95% and 91% at the protein level, respectively. The leucine zipper domain and two basic amino acid portions that bind DNA, as well as peptide sequences recognized by human cytotoxic T lymphocytes (CTLs), were all conserved in these rodent genes. Nuclear protein homologous to the 125 kD hSART-1 protein, but not to the 43 kD cytosol SART-1 protein, was detectable with specific antibody in the nuclear fractions of rodent tumor cell lines, and normal rodent fetal liver and testis. These rodent genes should be a novel tool for studies on the biological roles of the SART-1 gene, and also in the construction of animal models of specific immunotherapy using SART-1 gene products.

Key words: Tumor rejection antigen — Cytotoxic T lymphocyte — Rodent genes

A number of antigens recognized by HLA-class-I-restricted and tumor-specific cytotoxic T lymphocytes (CTLs) have recently been isolated, raising the hope that they might be used to develop cancer vaccines for specific immunotherapy. Indeed, several peptides encoded by these genes are under clinical trial as cancer vaccines, and major tumor regression has been seen in melanoma patients. We have recently identified a SART-1 gene encoding tumor antigens recognized by the CTLs from cDNA of human esophageal cancer. The SART-1 was suggested to be a bicistronic gene encoding two (125 kD and 43 kD) proteins. The 125 kD protein is expressed in the nucleus of proliferating cells, including normal and malignant cells, but not in non-proliferating cells, or in any normal tissues other than testis and fetal liver. In contrast, the 43 kD protein is expressed in the cytosol of head and neck, esophageal and lung squamous cell carcinomas (SCC) and lung adenocarcinomas, but not in leukemia or melanomas, or in any normal tissues or cell lines other than fetal liver and testis. The human bicistronic LAP gene has been shown to be involved in regulation of hepatocyte proliferation. These results suggest that the SART-1 gene is involved in cellular proliferation, although the mechanisms of this involvement are unknown. In this study, rodent genes homologous to hSART-1 were cloned with the aim of better understanding the biological roles of the SART-1 gene and also to provide animal models for specific immunotherapy with SART-1 gene products.

MATERIALS AND METHODS

Cloning of rodent SART-1 genes A murine cDNA library was obtained from “SuperScript” Murine Brain cDNA Library in pCMV-SPORT 2 ( Gibco BRL, Gaithersburg, MD) and the rat cDNA library was prepared according to the manufacturer’s instructions ( Gibco BRL). In brief, mRNA of the SCC-131 rat tumor cells was converted to cDNA, ligated to αII adapter, and inserted into the expression vector pSV-SPORT-1 ( Gibco BRL). The murine and rat SART-1 homologue clones were obtained from the murine and rat cDNA plasmid libraries, respectively, by the colony hybridization method using 32P-labeled 641-1D7, a truncated human SART-1 cDNA, as a probe. Briefly, the cDNA library was plated out at approximately 100,000 colonies per screen onto nitrocellulose filters ( NEN Research Products, Boston, MA) on agar plates, and cultured for 10 h. Replicate daughter filters were prepared and colonies were

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Fig. 1. Comparison of (A) nucleotide sequences between murine, rat, and human SART-1 cDNA and (B) the predicted amino acid sequences. The murine and rat SART-1 genes were cloned from murine and rat cDNA libraries, respectively. They consisted of 2,418 bp encoding a protein with 806 aa.
lysed. After prehybridization for 3 h at 42°C in 50% (by vol.) formamide, hybridization was performed by adding to the prehybridization solution the denatured labeled 6A1–1D7 cDNA probe together with 10 µg/ml denatured salmon sperm DNA. The hybridization was carried out overnight at 42°C. The filter was washed twice at room temperature with 2× salt sodium citrate (SSC) and 0.2× SSC supplemented with 0.1% sodium dodecyl sulfate (SDS), followed by autoradiography at −80°C for approximately 5 h using BIOMAX (Kodak, Rochester, NY). Putative positive colonies were subjected to a second round of screening to facilitate the isolation of colonies. The largest clone of 2,513 or 2,532 base pairs was purified from the murine or rat cDNA library, respectively. DNA sequencing was performed using the dideoxynucleotide sequencing method with a DNA Sequencer Kit (Perkin Elmer, Applied Biosystems Division, Foster, CA) with an ABI “PRISM” 377 DNA Sequencer.

**Expression of SART-1 at the mRNA and protein levels**

Samples used for the study were fetal rat (Wistar) livers from two prenatal rats in a 19-day pregnant rat, and adult rat testis, liver, spleen, and kidneys. Mouse cell lines, Colon 26 (colon cancer), 3LL (lung cancer), MN134 (hepatoma), Meth A (fibrosarcoma), and B16 (melanoma), and rat cell lines, SCC-131 and SCC-158 (external auditory meatus squamous cell carcinoma) were donated by the Japanese Cancer Research Resources Bank (JCRB, Tokyo), and KNRK (Kirsten sarcoma virus transformed normal kidney) was donated by American Type Culture Collection (ATCC, Rockville, MD). Tissues were sonicated for 60 to 90 s with an Astron ultrasonic processor (Heat Systems, Farmingdale, NY) before isolation of RNA. The SART-1 mRNA expression in these tissues and cells was investigated by the reverse transcriptase-polymerase chain reaction (RT-PCR) method using specific primers (SART-1f 700: 5′-CCAAGTTACTGGAGA-TGG-3′ and SART-1r1045: 5′-TTGGACAGGATAGAGGAGG-3′). There was no risk of false positives due to small amounts of DNA contaminating the RNA preparation, since the primers corresponded to sequences located in different exons. Amplification was performed for 35 cycles of 1 min at 94°C, 2 min at 56°C and 2 min at 72°C. The detection of β-actin mRNA and the methods of the western blot analysis to detect the 125 kD hSART-1800 protein and the 43 kD hSART-1259 antigens were previously described.9)

**RESULTS**

The nucleotide sequences of mSART-1 and rSART-1 are shown in Fig. 1A. Both are highly homologous to hSART-1, with 86% and 84% identity at the nucleotide level, respectively. The open-reading frames (ORF) of both mSART-1 and rSART-1 were 2,418 bp in length and
encoded a protein of 806 amino acids (aa). The predicted aa sequences are shown in Fig. 1B. The mSART-1 sequence contains one small insertion at nt positions 1,388 to 1,405, resulting in an encoded protein of 806 aa, which is 6 aa (ALEDEE) longer than the human hSART-1800 (hSART-1800) protein. Similarly, rSART-1 contains an insertion at nt positions 1,415 to 1,432, resulting in an encoded protein of 806 aa. hSART-1800 showed 95% and 91% homology with mSART-1806 and rSART-1806, respectively. Both mSART-1 and rSART-1 encode a leucine zipper motif at around nt positions 1,119 to 1,198 (corresponding peptide, RELEEIRTKLRLQAQSLNTVG-PRLAS) and at around nt positions 1,139 to 1,222 (corresponding peptide, RELEEIRTLRLQAQSLTVG-PRLAS), respectively. Two basic aa-rich portions at aa positions 31 to 42 (RHREHKKHKHRS) and 400 to 414 (KKTKRRVKKIRKEK) of the hSART-1 were completely conserved in both mSART-1 and rSART-1.

The rodent SART-1 genes were expressed at the mRNA level in all samples tested (8 normal rat tissues, 2 fetal liver, 5 mouse cell lines, and 3 rat cell lines). Representative results are shown in Fig. 2A.

We then investigated whether the rabbit anti-hSART-1800 or anti-hSART-1259 polyclonal antibody (Ab) reacted to the rodent proteins corresponding to the hSART-1800 or hSART-1259 protein using western blot analysis. Anti-hSART-1800 Ab recognized the approximately 127 kD band of mSART-1806 or rSART-1806 in all the murine and rat tumor cell lines tested (SCC-131, SCC-158, B16, 3LL, MH134, Meth-A, KNRK, Colon 26) and in normal rat fetal liver and testis (Fig. 2B). The molecular weight was a little larger than that of hSART-1800. In contrast, no band reactive to anti-hSART-1259 Ab was seen (Fig. 2B).

DISCUSSION

The nucleotide sequences of both mSART-1 and rSART-1, as well as the predicted aa sequences, were all highly homologous to those of hSART-1. There were no significant differences among the human and rodent proteins in terms of hydrophobicity pattern analyses, such as Kyte-Doolittle hydropathy.12) Both the mSART-1 and rSART-1 genes encode a leucine zipper motif that is highly homologous to that of hSART-1 (at nt positions 1,119 to 1,202).10) This leucine zipper motif is known to form a homo- or hetero-dimer that can bind DNA and modulate the transcription of target genes. The basic aa domain that is capable of binding to DNA is often associated with this motif.10) Two basic aa-rich portions of hSART-1 were completely conserved in both mSART-1 and rSART-1. This high homology between rodent and human indicates that the SART-1 gene might play an important role at the M-phase with respect to the regulation of cellular proliferation, over a wide range of species.

The nucleotide sequences of hSART-1 encoding antigenic peptides of human cancer cells were also well conserved in both mSART-1 and rSART-1. These antigenic peptides encoded by hSART-1 are SART-1258-345 (KLDEE-ALLK) and SART-1365-370 (VLSGSQSKM) recognized by the HLA-A26-restricted CTL,9) and the SART-1906-910 peptide (EYRGFTQDF) recognized by the HLA-A24-restricted CTL (Kikuchi et al., unpublished results). All three peptides are shared among the hSART-1258, hSART-1906, mSART-1906, and rSART-1906. The anchor residues of mouse class I, H-2Kd, were already reported as tyrosine (Y) or phenylalanine (F) at the 2nd position and isoleucine (I), leucine (L), or valine (V) at the 9th position of 9mer antigenic peptides.10) Six different peptides with H-2Kd binding motifs were found in both mouse and rat SART-1 (aa positions 240–248, 265–273, 389–397, 398–406, 565–573, and 626–634). Our recent data have shown that one of them has the ability to induce MHC (major histocompatibility complex)-restricted and peptide-specific CTL in Balb/c mice (H-2Kd) (Yamaguchi et al., unpublished data). These results suggest that rodent SART-1 genes might be a novel tool for developing animal models of specific immunotherapy with the SART-1 gene product.

The SART-1 mRNA was ubiquitously expressed in all the rodent normal tissues and tumor cell lines tested. This is in agreement with the results from the northern blot analysis of human SART-1 mRNA published previously.10) We next investigated the expression of the rodent proteins corresponding to the hSART-1800 or hSART-1259 protein by western blot analysis with the rabbit anti-hSART-1800 or anti-hSART-1259 polyclonal Ab. Anti-hSART-1800 Ab recognized the 127 kD band of mSART-1806 or rSART-1806 in all the murine and rat tumor cell lines, and in normal rat fetal liver and testis. The molecular weights of these proteins were a little larger than that of hSART-1800. In contrast, no band reactive to anti-hSART-1259 Ab was seen. There are two possible explanations for this failure. First, this Ab might not have recognized the rodent protein corresponding to hSART-1259, since at least 4 out of 259 aa were different (at the aa positions 526, 542, 540, and 617 of the human peptide sequence). Secondly, the failure might be due to the fact that the start ATG at position 1,663–1,665 responsible for the hSART-1259 protein was not found in either mSART-1 or rSART-1. Alternatively, rodent SART-1 genes might not be bicistronic, since a Shine-Dalgarno (S-D) like sequence observed in hSART-1 (AGG GGG at positions 1,681–1,686) was not found in either mSART-1 (AGG GGA at positions 1,695–
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REFERENCES

1) van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A. and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science, 254, 1643–1647 (1991).
2) Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., Miki, T. and Rosenberg, S. A. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. USA, 91, 3515–3519 (1994).
3) Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T. and Rosenberg, S. A. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc. Natl. Acad. Sci. USA, 91, 6458–6462 (1994).
4) Brichard, V., Van Pel, A., Wölfel, T., Wölfel, C., De Plaen, E., Lethé, B., Coulie, P. and Boon, T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med., 178, 489–495 (1993).
5) Robbins, P. F., El-Gamil, M., Li, Y. F., Kawakami, Y., Loftus, D., Appella, E. and Rosenberg, S. A. A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J. Exp. Med., 183, 1185–1192 (1996).
6) Guiloux, Y., Lucas, S., Brichard, V. G., Van Pel, A., Vitret, C., De Plaen, E., Brasseur, F., Lethé, B., Jotereau, F. and Boon, T. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanoma is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. J. Exp. Med., 183, 1173–1183 (1996).
7) Rosenberg, S. A., Yang, J. C., Schwartzzenbruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H. and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., 4, 321–327 (1998).
8) Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., 4, 328–332 (1998).
9) Shichijo, S., Nakao, M., Imai, Y., Takasu, H., Kawamoto, M., Niya, F., Yang, D. Y., Yamana, H. and Itoh, K. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J. Exp. Med., 187, 277–288 (1998).
10) Nakao, M., Yamana, H., Imai, Y., Toh, Y., Toh, U., Kimura, A., Yanoma, S., Kakegawa, T. and Itoh, K. HLA-A2601-restricted CTLs recognize a peptide antigen expressed on squamous cell carcinoma. Cancer Res., 55, 4248–4252 (1995).
11) Descombes, P. and Schibler, U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell, 67, 569–579 (1991).
12) Kyte, J. and Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol., 157, 105–132 (1982).
13) Ron, D. and Habener, J. F. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev., 4, 439–453 (1990).
14) Rammensee, H.-G., Friede, T. and Stevanovic, S. MHC ligands and peptide motifs: first listing. Immunogenetics, 41, 178–228 (1995).
15) Larsen, B., Peden, J., Marsu, S., Brady, K., Maldonado, R., Wills, N. M., Fayet, O., Atkins, J. F. and Gesteland, R. F. Upstream stimulators for recoding. Biochem. Cell Biol., 73, 1123–1129 (1995).
16) Sachs, A. B., Sarnow, P. and Hentze, M. W. Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell, 89, 831–838 (1997).