Mouse Homologue of the Human SART3 Gene Encoding Tumor-rejection Antigen

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We recently isolated a human SART3 (hSART3) gene encoding a tumor-rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes (CTLs). The hSART3 was also found to exist as an RNA-binding nuclear protein of unknown biological function. In this study, we cloned and analyzed the homologous mouse SART3 (mSART3) gene in order to understand better the function of hSART3, and to aid in establishing animal models of specific immunotherapy. The cloned 3586-bp cDNA encoded a 962-amino acid polypeptide with high homology to hSART3 (80% or 86% identity at the nucleotide or protein level, respectively). Nonapeptides recognized by the HLA-A2402-restricted CTLs and all of the RNA-binding motifs were conserved between hSART3 and mSART3. The mSART3 mRNA was ubiquitously expressed in normal tissues, with low level expression in the liver, heart, and skeletal muscle. It was widely expressed in various organs from as early as day 7 of gestation. mSART3 was mapped to chromosome 5, a syntenic region for human chromosome 12q23-24, and its genomic DNA extended over 28-kb and consisted of 19 exons. This information should be important for studies of the biological functions of the SART3 protein and for the establishment of animal models of specific cancer immunotherapy.

Key words: SART3 — Tumor-rejection antigen — RNA-binding protein — HLA-A2402 — Cytotoxic T lymphocytes

One of the most significant advances in the field of tumor immunology has been the recent identification of genes encoding tumor-rejection antigens that are recognized by HLA-class-I-restricted and tumor-specific cytotoxic T lymphocytes (CTLs).1, 2) The potential application of these findings to the development of cancer vaccines has brought nearer the prospect of specific cancer immunotherapy. Indeed, several peptides encoded by these genes are now under clinical trial as cancer vaccines, and major tumor regression has been observed in some melanoma patients.3, 4) We have recently isolated a human SART3 (hSART3) gene encoding a tumor-rejection antigen from cDNA of a human esophageal cancer cell line.5) Two SART3-derived peptides, at positions 109–118 and 315–323, that are recognized by HLA-A2402-restricted CTLs, are able to induce HLA-A2402-restricted and tumor-specific CTLs from peripheral blood mononuclear cells (PBMCs) of HLA-A24 positive (HLA-A24+) cancer patients. hSART3 encodes a Mr 140 000 protein expressed in both the cytosol of the majority of proliferating cells, including non-tumorous cell lines, and the nucleus of the majority of cancer cells. However, the SART3 protein was undetectable in normal tissues except for the testis and fetal liver, regardless of its ubiquitous expression at the mRNA level.5) There are several motifs in the sequence of the SART3 protein: nuclear localization signals3) around positions 612–615 and 641–647, a ribonucleoprotein-1 (RNP-1) motif,7) one of the well-characterized RNA-binding motifs, at positions 746–753 and 841–848, and a tyrosine phosphorylation motif8) at positions 309–316. The involvement of the SART3 protein in tyrosine phosphorylation (data not shown) and the RNA-binding capacity of the SART3 protein9) have both been demonstrated. These results suggest that the hSART3 gene plays an important role in cellular proliferation. However, its biological function remains unknown. To understand better the biological function of hSART3 and to aid in establishing animal models of cancer immunotherapy, we have cloned a mouse homologue (mSART3) of the hSART3 gene, and here report the characterization, expression pattern, chromosomal localization, and genomic organization of this homologue.

MATERIALS AND METHODS

Homology searches and cloning of the mSART3 gene

The hSART3 cDNA sequence was used as a query in BLAST2 homology searches against mouse Expressed sequence tag (EST) databases. An EST clone AA674246 was obtained from Genome Systems (St. Louis, MO). A mouse brain cDNA library (TaKaRa, Otsu) was screened.
Northern blot analysis

Total RNAs were isolated using the acid guanidium thiocyanate-phenol-chloroform method. The RNA samples (5 or 10 μg/lane) were electrophoresed on 1% agarose gels in the presence of formaldehyde, transferred to a nylon membrane (Hybond-N+; Amersham, Buckinghamshire, UK), and probed with 32P-labeled probes. The membrane was hybridized overnight at 65°C in a hybridization buffer (7% sodium dodecyl sulfate (SDS), 1 M Na2HPO4, pH 7.2), and then autoradiographed. A 1.1-kbp fragment of a mouse embryo multiple tissues northern blot filtered was purchased from Clontech (Palo Alto, CA) and used as a probe. A 1.1-kbp fragment of AA674246 was radiolabeled with a Multiprime DNA labelling system (Amersham Pharmacia Biotech) and used as a probe. A 1.1-kbp fragment of the EST clone AA674246 showed the highest homology, and was therefore used as a probe to clone the mouse homologue of the hSART3 gene. A 3586-bp cDNA clone was obtained from a mouse brain cDNA library by means of a combination of cDNA library screening with probe hybridization and 5'-RACE. The sequence flanking the presumed start site (5'-AGATGGCC-GCGCAAGATGGCGACGAC-GG-3' and 5'-CGTACTTGGCGATTTCCCCCTCTG-3', and the other was 5'-GAGCTTCGGACGACCATC-3' and 5'-ACAAACTCAATGGGGGAGAC-3'). The isolated fragments were subcloned into a vector pCR-XL-TOPO (Invitrogen, Carlsbad, CA), and obtained clones were subsequently sequenced with gene-specific primers. The exon-intron junctions were determined by comparing the sequence with the mouse cDNA sequence using GeneWorks software (IntelliGenetics, Mountain View, CA). Introns were further amplified with appropriate primers and their sizes were estimated.

RESULTS

Isolation of mSART3 cDNA

Several mouse cDNA clones containing nucleotide sequences with high similarity to the hSART3 cDNA were found in the EST database. The EST clone AA674246 showed the highest homology, and was therefore used as a probe to clone the mouse homologue of the hSART3 gene. A 3586-bp cDNA clone was obtained from a mouse brain cDNA library by means of a combination of cDNA library screening with probe hybridization and 5'-RACE. The sequence flanking the presumed start site (5'-AGATGGCC-GCGCAAGATGGCGACGAC-GG-3' and 5'-CGTACTTGGCGATTTCCCCCTCTG-3', and the other was 5'-GAGCTTCGGACGACCATC-3' and 5'-ACAAACTCAATGGGGGAGAC-3'). The isolated fragments were subcloned into a vector pCR-XL-TOPO (Invitrogen, Carlsbad, CA), and obtained clones were subsequently sequenced with gene-specific primers. The exon-intron junctions were determined by comparing the sequence with the mSART3 cDNA sequence using GeneWorks software (IntelliGenetics, Mountain View, CA). Introns were further amplified with appropriate primers and their sizes were estimated.

Chromosomal localization of mSART3

Chromosomal localization of mSART3 was determined by PCR analysis of a mouse/hamster radiation hybrid panel (Research Genetics, SW Huntsville, AL). This panel of 100 radiation hybrid clones of the whole mouse genome was created by fusing irradiated mouse embryo primary cells (129aa) with hamster recipient cells (A23). The 3'-untranslated region primers (5'-AATGGGAGACTTTGTCATC-3' and 5'-AACTACAA-TCTAATGGGGGAGAC-3'), which generate a 320-bp DNA fragment, were used under the following conditions: 94°C for 2 min, 40 cycles at (94°C for 30 s, 56°C for 30 s and 72°C for 30 s), and 72°C for 7 min. The occurrences of PCR products in each clone were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. The mapping results were analyzed on the radiation hybrid mapping server of the mouse genome at the Whitehead Institute/MIT Center (http://www.genome.wi.mit.edu/cgi-bin/mouseRh/rhmapper/auto/rhmapper.cgi).

Isolation of genomic DNA

The mSART3 genomic DNA fragments were obtained by PCR from BALB/c mouse genomic DNA. Oligonucleotide primer pairs used for the PCR were designed from the mSART3 cDNA sequence. One primer pair was 5'-CCGCAAGATGGCGACGAC-GG-3' and 5'-CGTACTTGGCGATTTCCCCCTCTG-3', and the other was 5'-GAGCTTCGGACGACCATC-3' and 5'-ACAAACTCAATGGGGGAGAC-3'. The isolated fragments were subcloned into a vector pCR-XL-TOPO (Invitrogen, Carlsbad, CA), and obtained clones were subsequently sequenced with gene-specific primers. The exon-intron junctions were determined by comparing the sequence with the mSART3 cDNA sequence using GeneWorks software (IntelliGenetics, Mountain View, CA). Introns were further amplified with appropriate primers and their sizes were estimated.
Mouse Homologue of the Human SART3 Gene

Fig. 1. Deduced amino acid sequence of the mSART3 gene and comparison with those of homologues. (A) Alignment with the hSART3 protein. Identical amino acids are indicated by shading. Two hSART3-derived peptides (CTL epitopes) recognized by HLA-A2402-restricted CTLs are underlined. The following putative motif sites are also underlined: nuclear localization signal (NLS), RNP-1, and tyrosine phosphorylation motif (P-tyr). (B) Multiple alignment with the C-terminal regions of putative SART3 proteins of humans, mice, C. elegans, A. thaliana, and D. melanogaster. Highly conserved regions are double-underlined. RNP-1 motif sites are also indicated. Amino acid positions are shown on the right.
**mSART3 mRNA expression**  Expression of the *mSART3* mRNA in various tissues of adult mice was analyzed by northern blot analysis. *mSART3* mRNA was ubiquitously expressed in adult mouse tissues, and a single species of message (approximate size, 3.6 kb) was observed (Fig. 2A). Relative expression levels of the mRNA ranged between 0.1 and 1.1, when the expression level in the thymus was considered to be 1.0. mRNA expression was high in the testis (1.0), thymus (1.0), spleen (1.1), and lung (1.0), but low in the liver (0.2), heart (0.2), and skeletal muscle (0.1). Expression of the *mSART3* mRNA was further analyzed at various developmental stages. It was expressed from the early prenatal stages—as early as day 7 of gestation (E7)—and increased thereafter (Fig. 2B). The expression of *mSART3* mRNA was detected in all the organs tested at different embryonic stages (gestational days 14, 16, 18; E14, E16, E18) and at postnatal day 1 (P1). The level of expression of *mSART3* was relatively

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constant during developmental stages in the brain, lung and spleen, whereas it slightly declined in the thymus, and was greatly reduced in the liver (Fig. 2C).

Chromosomal localization of mSART3 Chromosomal location of the mSART3 gene was determined by radiation hybrid mapping. The mSART3 was located on chromosome 5, 14.3 cR distal from the D5Mit317 marker gene (Fig. 3). This position is syntenic to the human chromosome 12q23-24, to which the hSART3 has been mapped (Accession No. D63879/SGC31638).

Genomic Structure of mSART3 Genomic DNA of mSART3 gene was amplified by PCR, and a total of approximately 28 kb of genomic DNA was partially sequenced to determine the intron-exon structure of the mSART3 gene. As shown in Fig. 3, the mSART3 mRNA was a multiply spliced transcript consisting of at least 19 exons and 18 introns within the sequenced region. The exon-intron structure and boundary sequences of mSART3 are presented in Table I.

**DISCUSSION**

The mSART3 gene was highly homologous to hSART3 at both the nucleotide and protein levels. Peptide sequences capable of inducing HLA-A24-restricted CTLs, RNP-1 motifs, nuclear localization signals, and a tyrosine

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**Table I. Genomic Structure and Boundary Sequences of mSART3**

| Exon | Size (bp) | Position in cDNA | Intron size (kb) | Splice acceptor *a* | Splice donor *a* |
|------|-----------|------------------|------------------|---------------------|-----------------|
| 1    | 322       | 8–329            | ~6.9 kb          | —                   | CAGgttgccgga    |
| 2    | 127       | 330–456          | ~1.1 kb          | tgctcttagCTG        | AAGtaagtagcc    |
| 3    | 105       | 457–561          | ~1.5 kb          | tgctcttagAGC        | TCAGtaggaccc    |
| 4    | 185       | 562–746          | ~1.7 kb          | gtctcttagGTC        | CGGgtagccccaa   |
| 5    | 52        | 747–798          | 957 bp           | gattccagCTG         | ACGtaagagccca   |
| 6    | 125       | 799–923          | ~2.5 kb          | ttctcttagAGA        | CTGtaggaggcc    |
| 7    | 156       | 924–1079         | 998 bp           | gtctcttagCTG        | CTAgtaagataaa   |
| 8    | 139       | 1080–1218        | 527 bp           | ttgctcttagGAT       | CTGtaggacccag   |
| 9    | 108       | 1219–1326        | ~1.1 kb          | gcattcctagCCA       | AGGtagcaagttg   |
| 10   | 78        | 1327–1404        | 118 bp           | ttctcttagACT        | AGGtagaagccg    |
| 11   | 59        | 1405–1463        | 901 bp           | tgtctcttagGTA       | GAGtagccggtt    |
| 12   | 110       | 1464–1573        | ~2.2 kb          | gtctcttagGCT        | ACGtaggagggaa   |
| 13   | 113       | 1574–1686        | ~0.7 kb          | tgtctcttagGCC        | AAGtaggagggcc   |
| 14   | 77        | 1687–1763        | ~1.1 kb          | tctctcttagGGA       | AAGtagacactg    |
| 15   | 169       | 1764–1932        | 592 bp           | cattccttagGCC       | AAGtagccgctg    |
| 16   | 452       | 1933–2384        | 380 bp           | gtctcttagAGC         | AAGtagctctttt   |
| 17   | 153       | 2385–2537        | 313 bp           | gtctcttagAGG         | AAGtagcttggg    |
| 18   | 191       | 2538–2728        | ~1.1 kb          | gtctcttagGCG        | CGGtagaaagtg    |
| 19   | 835       | 2729–3563        | —                | ctctcttagCCG         | —               |

*a* Exon sequences are shown in uppercase letters, intron sequences are shown in lowercase letters, and invariant ag (splice acceptor) and gt (splice donor) nucleotides are shown in boldface type.

*b* Approximate size based on agarose gel electrophoretic mobility of intron-spanning PCR products relative to standards.
phosphorylation motif were all conserved in both the hSART3 and mSART3 (Fig. 1A). In addition to mSART3, putative SART3 homologues were found in C. elegans, A. thaliana, and D. melanogaster. These genes also have an RNP-1 motif in the COOH-terminal (Fig. 1B). Physiological roles of RNP motif proteins have been discerned from the consequences of loss of expression or mutations in the RNP proteins that result in developmental disorders in humans and other organisms. A deletion mutant of 4f-rnp, a putative SART3 homologue of Drosophila, has been reported to have a lethal effect. These facts suggest that the SART3 protein plays critical roles in the development and maintenance of various organs, although its biological functions remain to be clarified.

RNP motif proteins, such as La proteins, are common targets in autoimmune disease, especially in patients with systemic lupus erythematosus. Many autoantigens seem to be components of subcellular nuclear particles involved in important cell functions, such as DNA replication, DNA transcription, RNA processing, and cell division. Autoantibodies are often detectable in cancer patients, although their molecular bases have not been well clarified. We previously identified the SAT1 gene as encoding a tumor-rejection antigen recognized by CTLs. The same gene was also identified the hSART3 gene as encoding a tumor-rejection antigen, while Gu et al. reported it as an RNA-binding nuclear protein. These results suggest that the SART3 antigen is a possible self antigen eliciting autoantibodies. The molecular mechanisms involved in this phenomenon should be elucidated.

mSART3 was mapped to mouse chromosome 5, 14.3 cR distal from the D5Mit317 marker gene, which is a region syntenic to the human chromosome, 12q23-24, that includes the hSART3 gene. Some of the mouse genes around this region have already been mapped, and each human counterpart was localized to 12q23-24. The human chromosome 12q23-24 is one of the best-characterized regions of the human genome. Several genes mapped on this region are involved in inherited diseases, including many kinds of metabolic diseases, Brachydactyly type C, Noonan syndrome, and spinal muscular atrophy. In addition, this region is known to be rearranged in a variety of cancers (e.g., chronic lymphoproliferative disorders, non-Hodgkin’s lymphomas, germ cell tumors, and astrocytomases). No phenotype or disease loci associated with this region has yet been reported on the mouse chromosome.

In this study, we isolated the mSART3 genomic DNA and determined the intron-exon boundaries of the mSART3 gene. Two hSART3 genomic DNA clones (Accession Nos. AY395743 and B83376), which contained sequences around intron-exon junctions, were obtained from the database. These boundaries in the hSART3 gene were found at positions equivalent to those observed in the mSART3 gene. All mSART3 introns belonged to the predominant vertebrate splicing GT-AG class of introns. Both the mSART3 cDNA and its genomic clone should be novel models for improving our understanding of the molecular basis of these diseases.

The expression of mSART3 at the mRNA level was ubiquitous in normal tissues with high-level expression in the spleen, thymus, lymph node, lung, testis, and brain, and low levels in the liver, heart, and skeletal muscle. The hSART3 mRNA was also ubiquitously expressed in normal tissues, whereas the expression was low in the thymus and PBMCs. The meaning of the difference of expression in the lymphoid organs between mouse and human SART3 gene is unclear. The mRNA expression of both the mSART3 and hSART3 in the testis was high. A similar expression pattern, i.e., ubiquitous with high-level expression in the testis, was previously reported for SART1. Although these genes are ubiquitously expressed in normal and malignant cells or tissues at the mRNA levels, protein expression levels in the normal and malignant cells were very different. Expression of the SART3 protein as well as SART1 43-kD protein was limited in the malignant tumor cells or well proliferating cells, and undetectable in the normal adult tissues except for the testis.

Two peptides, hSART3109–118 and hSART3315–323, encoded by the hSART3 gene were recognized by HLA-A2402-restricted CTLs and were able to induce HLA-A2402-restricted and tumor-specific CTLs in PBMCs of cancer patients. Thus, SART3 and its peptides at positions 109–118 (hSART3109–118) and 315–323 (hSART3315–323) are appropriate molecules for use in specific immunotherapy for HLA-A24+ patients with various histological types of cancer. Because of the expression of SART3 proteins in the normal testis, the testis is a possible target organ of collateral effects of the specific immunotherapy. It should be noted that no severe collateral effect in the testis has been reported in the clinical trials of melanoma antigen (MAGE) specific immunotherapy against melanoma patients, although the MAGE gene family proteins are similarly expressed in the normal testis.

The anchor motifs on the antigenic peptides to bind mouse class I major histocompatibility complex, H-2Kd, are similar to those for the human HLA-A2402, and the sequence of the hSART3315–323 peptide is conserved in mSART3. Moreover, fourteen different peptides with H-2Kd binding motifs were also found in mSART3. These results suggest that mSART3 and its peptides should be useful as novel tools for developing animal models of specific cancer immunotherapy.
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