Sequence confirmation and characterization of the mouse Ssxa gene: Ssxa protein is cleaved and the N-terminal cleaved fragment translocates into the nucleus

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Received May 20, 2011; Accepted June 17, 2011

DOI: 10.3892/ijmm.2011.740

Abstract. This is the first demonstration of a stop codon in the sequence of mouse Ssxa and characterization of the biological behavior of Ssxa protein. Cancer testis antigen (CTA) is known as a target of immunotherapy against cancer, and Ssxa is one of the CTAs. Although a CTA would be useful to establish a mouse cancer vaccine model using endogenous antigen, the stop codon was not identified in the sequence of Ssxa cDNA that was previously reported. In this study, the gene sequence of Ssxa was different from the previous report in which several mouse CTAs were analyzed. Initially, we identified the correct cDNA sequence of mouse Ssxa by 3'-rapid amplification of cDNA ends and found a new exon containing the stop codon (Exon X). Ssxa mRNA expression was determined by reverse transcription-PCR (RT-PCR) in four mouse cancer cell lines and the testis but not in other normal organs. We found that the molecular weight of recombinant Ssxa protein is 12 kDa, and we generated an anti-Ssxa antibody which recognizes the C-terminus of Ssxa. Two vectors expressing fusion proteins (pSsxa-GFP and pGFP-Ssxa) were generated and fluorescence in the nucleus was observed only in the pGFP-Ssxa transfected cells. Therefore, we conclude that the N-terminal cleaved fragment of Ssxa, which has a KRAB domain (nuclear localization signal), translocates into the nucleus after cleavage of the C-terminus.

Introduction

Cancer testis antigens (CTAs) are promising candidates of antigen-specific cancer vaccines due to the fact that their normal expression is restricted to male germ cells in the testis, and they are not expressed in adult somatic tissues (1). Many clinical trials of cancer vaccination using CTAs have been carried out, but the effective immunological results in mouse models of cancer vaccine were not reflected in the clinical outcomes in human cancer patients. To improve the effects of cancer vaccines, it is necessary to understand the underlying anti-tumor immunological mechanism. However, exogenous antigens such as chicken ovalbumin, but not endogenous CTA are used in most mouse models of cancer vaccine.

Our final aim is to establish a mouse model of cancer peptide vaccine with endogenous CTA to investigate the cause of the disagreement between animal models and human clinical trials. Therefore, we initially analyzed several mouse cancer testis genes. Although the reported CTAs of humans are over 40, those of mice are only a few: Ssxa (2), Tsga10 (3), Mage-b (4) and OY-MS-4 (5). In the process, it was noticed that the gene sequence of Ssxa was different from the previous report (2). Therefore, the objectives of this study were to identify the correct cDNA sequence of Ssxa by 3'-rapid amplification of cDNA ends (3'-RACE) and to analyze the characteristics of Ssxa protein.

Materials and methods

Cells and mice. Four mouse cancer cell lines and one mouse fibroblast cell line: CT26 (BALB/c strain), LLC (C57/BL6 strain), B16 (C57/BL6 strain), EL4 (C57/BL6 strain) and NIH/3T3 (NIH Swiss strain) were purchased from ATCC. In addition, HEK293FT human kidney cells were purchased from Invitrogen (Carlsbad, CA). Male BALB/c mice, 8 weeks of age, were purchased from Japan Charles River Laboratory (Tokyo, Japan) and maintained under specific pathogen-free conditions. All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee using an approved protocol.

Reverse transcription-PCR (RT-PCR). Total cellular RNA was isolated from each cell line or normal tissues (brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis) of 8-week-old male BALB/c mice using the TRIzol reagent (Invitrogen). Each 20 μl cDNA synthesis reaction mixture contained 1 μg

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Key words: cancer testis antigens, tumor immunology, cancer vaccine

total-RNA, 4 μl First Strand buffer (Invitrogen), 10 mmol/l of each deoxynucleotide triphosphate, 1 μl (200 units) SuperScript II (Invitrogen), 2 μl of 0.1 mol/l DTT (Invitrogen), and 1 μl Oligo(dt) (Invitrogen). The reverse transcription reaction was carried out for 50 min at 42˚C and inactivated by heating at 70˚C for 15 min. The PCR reaction mixture contained 1 μl reverse transcription reaction products, 0.1 μl Taq DNA polymerase (Promega, Madison, WI), 4 μl reaction buffer (Promega), 160 mmol/l of each deoxynucleotide, and 20 pmol of each of the 3' and 5' primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH sense, 5'-AGTATGA TGACATCAAGAAGG-3'; antisense, 5'-ATGGTATTCAAG AGAGTAGGG-3') and Ssxa (Exon 2 sense, 5'-GGTCTCATT CAGTGAACCTCGGAA-3'; Exon 5 antisense, 5'-GATTTC TTCATATATCACAGGCTTCTTC-3'; Exon X antisense, 5'-TCACCCACGGGATTCTTCCT-3'). The conditions for GAPDH PCR were 94˚C for 60 sec, 58˚C for 60 sec, 72˚C for 60 sec and the conditions for Ssxa PCR were 94˚C for 60 sec, 52.6˚C for 60 sec, 72˚C for 60 sec. Each cDNA was amplified for 40 cycles. All PCR products were electrophoresed on a 2.0% agarose gel and visualized by ethidium bromide staining.

3'-Rapid amplification of cDNA ends (3'-RACE). The used primers were as follows [adapter plus oligo(dt), 5'-GGCCACG CGTCGACTAGTTTTTTTTTTTTTTTT-3'; adapter primer, 5'-GGCCACGCGTCGACTAG-3'; gene specific primer, 5'-GG TCTCATTCAGTGAACCTCGGAA-3'; nested gene specific primer, 5'-CTTCGAGGATATCTCTAAAT-3'. The conditions for PCR and for nested PCR were 94˚C for 60 sec, 58˚C for 60 sec, 72˚C for 60 sec. Each cDNA was amplified for 40 cycles.

Recombinant Ssxa protein. Recombinant Ssxa protein was produced using the GST (glutathione S-transferase) gene fusion system. The Ssxa cDNA was originally cloned from testis cDNA of 8-week-old male BALB/c mice and inserted into pGEX-6P-2 (GE Healthcare UK Ltd., Little Chalfont, UK). JM109 competent cells (Takara Bio, Inc., Shiga, Japan) were transformed with pGEX-6P-2-Ssxa and were incubated in LB culture media with ampicillin (100 μg/ml) by shaker flask at 37˚C for 16 h. To induce the GST-Ssxa fusion protein, 1.0 mM IPTG was added followed by 5 h of incubation. The bacterial bodies were collected by centrifugation and the pellet was dissolved in lysis buffer. After freeze (-80˚C)/thaw (30˚C) cycles were repeated three times, the supernatant was collected by centrifugation and applied into GSTrap FF 1 ml columns (GE Healthcare UK, Ltd.). After washing the column 2 times, PreScission protease (GE Healthcare UK Ltd.) was injected and incubated at 4˚C for 16 h. Finally, PBS was injected into the column and Ssxa protein was collected in microtubes.

Generation of rabbit polyclonal antibodies for Ssxa. Rabbit polyclonal antibodies were generated by injecting 200 μg keyhole limpet hemocyanin (KLH)-conjugated peptide of the Ssxa protein into a rabbit subcutaneously once a week for a total of 6 times. One peptide sequence of Ssxa [C1 (76-89): N-VTKSVLSDSDEVSS-C] was selected based on immunogenicity from the website (http://immunax.dfci.harvard.edu/Tools/antigenic.pl) and the other peptide sequence [C2 (94-101): N-DKRKNPVV-C] was selected from the novel position we identified in this report.

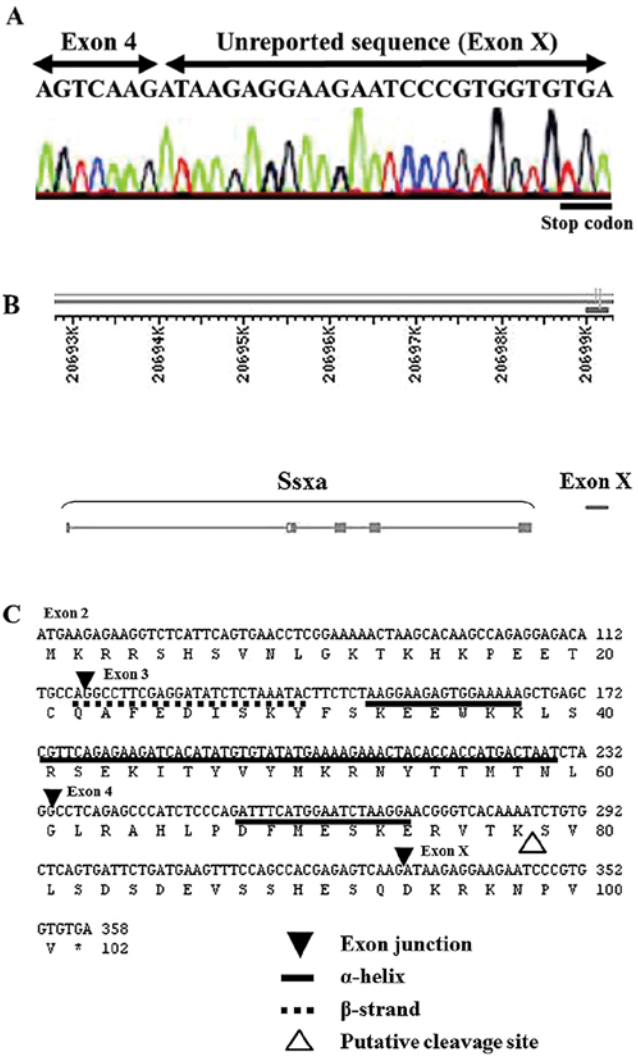

Figure 1. (A) DNA sequencing analysis for Ssxa cDNA (3' end of Exon 4 and 26 bases of new sequence were shown). The unknown sequence (Exon X) was found downstream of Exon 4. Exon X contains the stop codon. (B) Basic local alignment search tool (BLAST) search for Exon X. Exon X is located 662 base pairs downstream from the Exon 5 ends. (C) Nucleotide and amino acid sequences of the Ssxa gene. Exon junctions are marked by closed arrowheads. α-helices are shown by the solid lines, and the β-strand is indicated by the dashed line. The putative cleavage site is shown by an open arrowhead. Nucleotide and amino acid numbers are listed on the right.

Transfection. GFP (green fluorescent protein), IRES (internal ribosome entry site)-GFP, Ssxa-IRES-GFP, GFP-Ssxa and Ssxa-GFP (fusion proteins of Ssxa and GFP) were cloned into vector pcDNA3.1 (+) (Invitrogen). As a transfectant, HEK293FT cells were used with Lipofectamine LTX (Invitrogen) according to the instructions of the manufacturer.

Western blot analysis. Briefly, samples were resolved using 10% SDS-PAGE gels and were then transferred to a nitrocellulose membrane (Amersham, Aylesbury, UK) for Western blot analysis. GFP Monoclonal Antibody Living Colors (Takara Bio, Inc.) or rabbit polyclonal antibodies for Ssxa were used as primary antibodies. Peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as the secondary antibody. Immunoreactivity

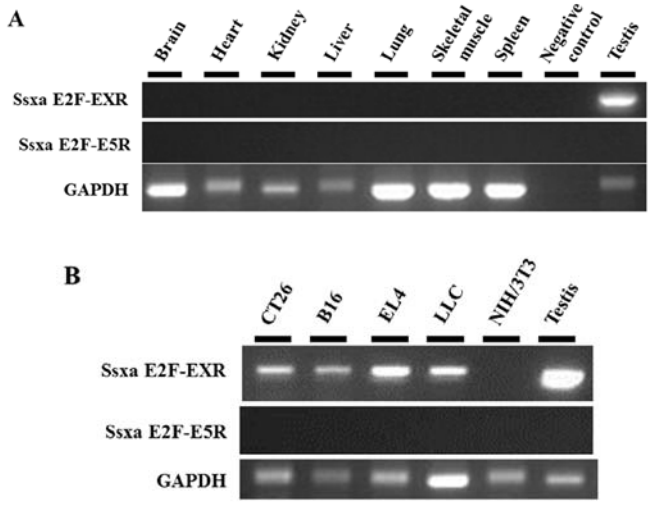

Figure 2. (A) RT-PCR analysis for Ssxa expression. mRNA expression of Ssxa was not found in normal tissues except the testis. (B) Four mouse tumor cell lines expressed Ssxa mRNA.

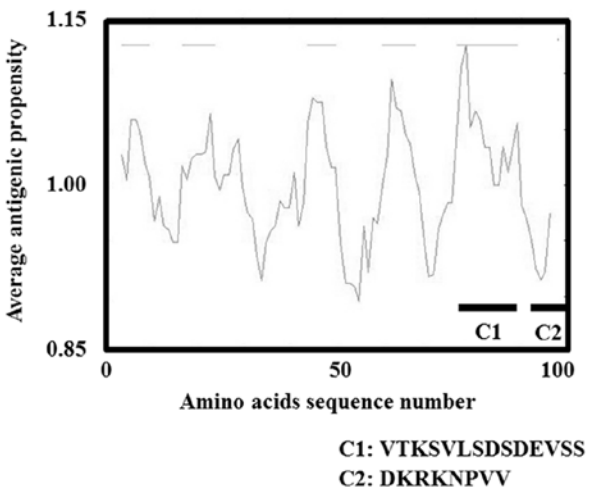

Figure 3. Prediction of the antigenic peptides in the Ssxa protein. One peptide sequence of Ssxa [C1 (76-89): N-VTKSVLSDSDEVSS-C] was selected based on immunogenicity from the website (http://immunax.dfci.harvard.edu/Tools/antigenic.pl). The other peptide sequence [C2 (94-101): N-DKRKNPVV-C] was selected from the novel position identified in this report.

was detected by an enhanced chemiluminescence detection system (Amersham).

Immunohistochemistry. Immunohistochemical reactions were carried out using the universal immunoenzyme polymer method. Each slide was deparaffinized in xylene, rehydrated through a graded series of ethanol/water, and treated in a pressure cooker for 10 min. Endogenous peroxidase activity was blocked by a 15-min incubation with hydrogen peroxide. After 3 washes in PBS that contained 1% Tween-20, the sections were incubated in 10% normal goat serum (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) for 30 min. Samples then were incubated overnight with mSSXA-C2 antibody (1:100 dilution), GFP Monoclonal Antibody Living Colors (1:400 dilution), or negative control rabbit polyclonal antibody (1:100 dilution) at 4˚C. After 3 additional washes, the sections were incubated with Histofine Simple Stain MAX-PO (Multi; Nichirei) or Histofine Simple Stain Mouse MAX-PO (M, R or Rat; Nichirei) for 30 min at room temperature. Reaction products were observed by incubation for approximately 5 min with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei) followed by washing in distilled water. Sections were counterstained in hematoxylin for 30 sec and then were mounted in Permount (Micro Slides; Muto-Grass, Tokyo, Japan).

Results

New Ssxa cDNA sequence. To confirm the full-length Ssxa cDNA sequence, a 3'-RACE experiment was performed with mouse testis mRNA. DNA sequencing analysis revealed that the Ssxa Exon 5 dose not exist, and a new exon which has another sequence (Exon X) was identified (Fig. 1A) (GenBank accession no. AB618486). Exon X was found to be located 662 base pairs downstream from the Exon 5 ends (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 1B). The present sequence has a conventional translational terminal codon, and Ssxa protein consists of 101 amino acids (Fig. 1A) (1C; GenBank protein ID BAJ83776.1).

The secondary structure of Ssxa protein was predicted by Phyre server ver0.2 (http://www.sbg.bio.ic.ac.uk/~phyre/)(6), and it was inferred that the putative cleavage site is between Lys78 and Ser79 by using the general PC (proprotein convertase) cleavage site predictor (ProP 1.0 server; http://www.cbs.dtu.dk/services/ProP/) (7) (Fig. 1C).

mRNA expression of Ssxa in mouse normal tissues and tumors. According to the above result, RT-PCR with Exon 2 sense primer and Exon X antisense primer reveled that mRNA expression of Ssxa was not found in normal mouse tissues except for the testis (Fig. 2A). Ssxa mRNA expression was found in four mouse tumor cell lines (CT26, LLC, B16 and EL4), but not in the NIH/3T3 cells derived from normal mouse fibroblasts (Fig. 2B upper gel). Based on the sequence from the previous report (2), RT-PCR with Exon 2 sense primer and Exon 5 antisense primer did not detect mRNA expression of Ssxa even in the testis (Fig. 2 middle gel).

Generation of rabbit polyclonal antibodies for Ssxa. According to the sequence data of Ssxa, two polypeptides (C1, C2) were synthesized to generate antibodies. Immunogenicity of these peptides is shown in Fig. 3. The C1 peptide sequence was selected in regards to immunogenicity and the C2 peptide was selected from Exon X identified in this report. Two rabbit polyclonal antibodies for Ssxa, mSSXA-C1 and mSSXA-C2, were generated using the C1 and C2 peptides.

Construction of the Ssxa expression vector and detection of the Ssxa protein. To investigate Ssxa expression and the biological behavior, several expression vectors were constructed (Fig. 4A). Moreover, recombinant Ssxa protein was purified using *E. coli* and silver stain analysis revealed that the molecular weight of the whole recombinant Ssxa protein is 12 kDa (data not shown).

Western blotting using the mSSXA-C2 antibody detected the Ssxa protein much more efficiently than use of the mSSXA-C1 antibody (Fig. 4B). Although the band of the

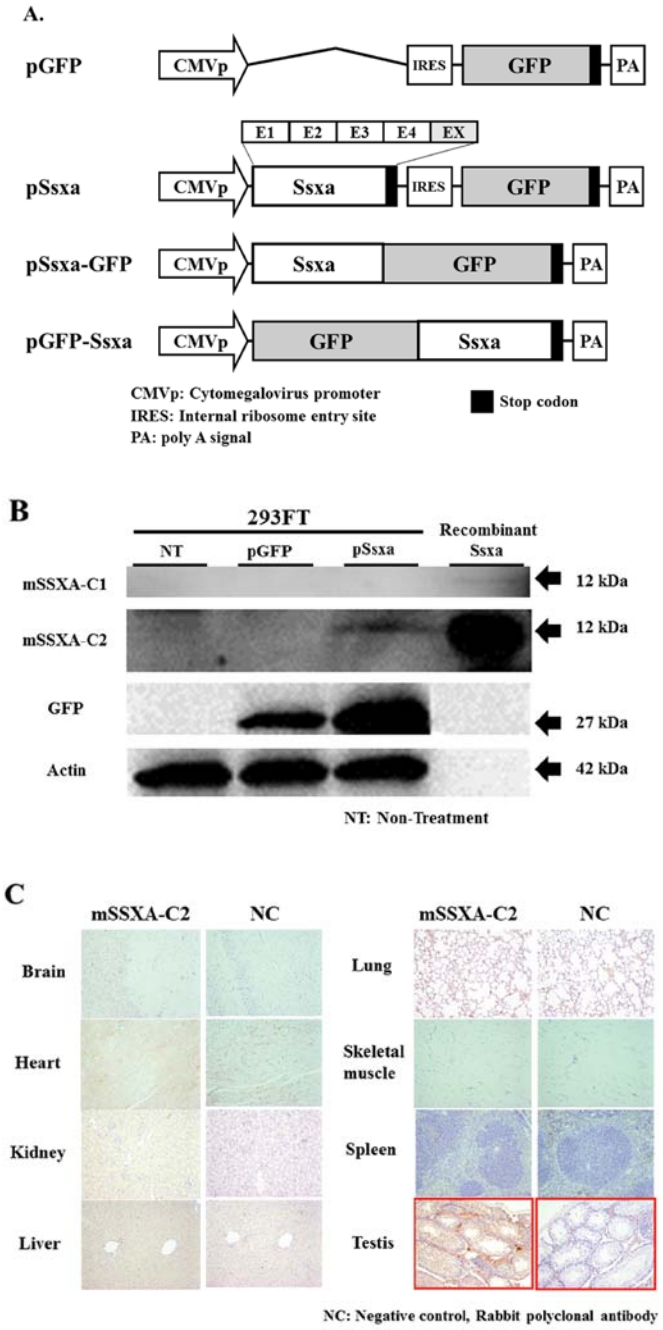

Figure 4. (A) Construction of vectors expressing GFP (pGFP), Ssxa and GFP (pSsxa), Ssxa-GFP fusion protein (pSsxa-GFP), and GFP-Ssxa fusion protein (pGFP-Ssxa). (B) Western blotting with the rabbit polyclonal antibody for peptides of Ssxa (mSSXA-C1 or mSSXA-C2). The mSSXA-C2 antibody detected the Ssxa protein much more efficiently than the mSSXA-C1 antibody. (C) Immunohistochemistry with the mSSXA-C2 antibody for the mouse normal tissues. Expression of Ssxa protein was observed only in the testis.

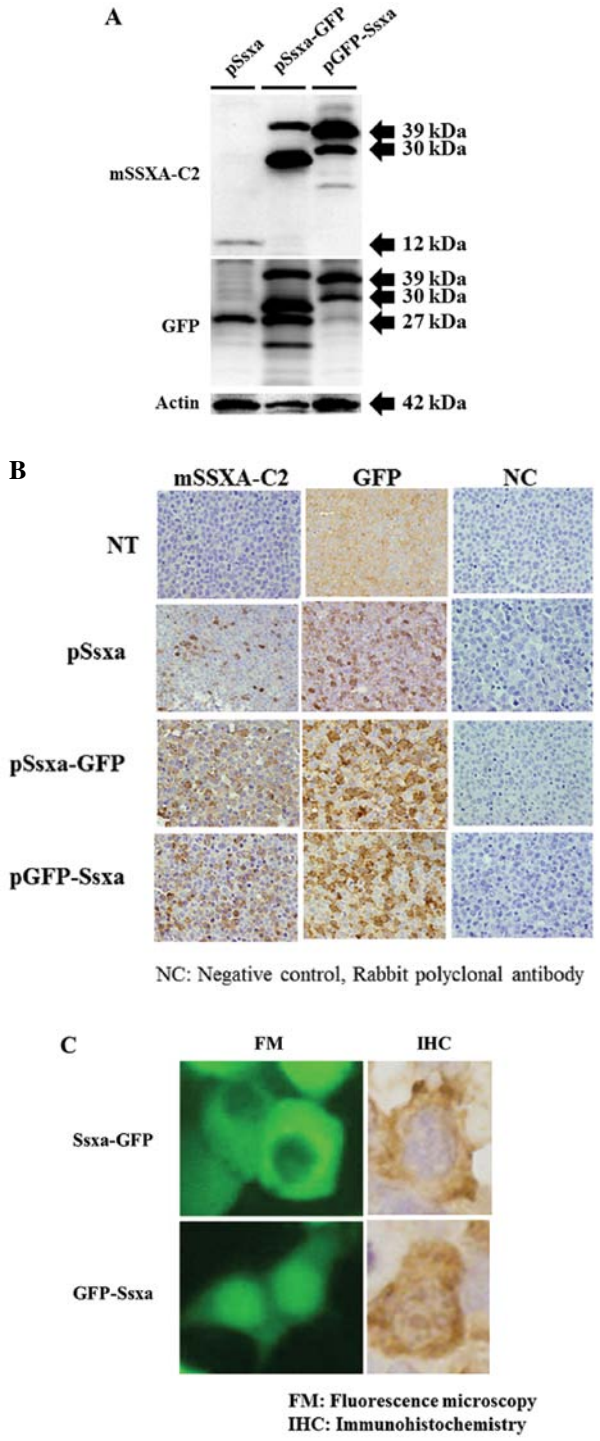

Figure 5. (A) Western blot analysis for the fusion proteins of Ssxa and GFP. mSSXA-C2 antibody was used for the primary antibody. Ssxa is cleaved *in vivo*; the second band in the lane of HEK293FT transfectant with pSsxa-GFP was the resolvent of the first band. Measuring the intensity of the bands, 74.1% of Ssxa was cleaved *in vivo*. (B,C) Intracellular distribution of the Ssxa-GFP and GFP-Ssxa fusion proteins. The HEK293FT cells were transfected with the pSsxa-GFP or the pGFP-Ssxa and observed by (B) immunohistochemistry (primary antibody: GFP Monoclonal Antibody Living Colors) and the (C) fluorescence microscopy. When applying extreme attention reviewing the specimen induced by the GFP-Ssxa fusion protein, nuclear staining was observed. The N-terminal cleavage part of Ssxa protein translocates to the nucleus.

recombinant Ssxa was thick, that of Ssxa protein derived from HEK293FT cell transfected pSsxa was very thin. Expression of Ssxa protein detected by immunohistochemistry with the mSSXA-C2 antibody was observed only in the testis (Fig. 4C).

Analyses of fusion proteins of Ssxa and GFP. For easy analysis, the vectors expressing the fusion proteins of Ssxa and GFP, Ssxa-GFP and GFP-Ssxa, were generated (Fig. 4A). Western blotting using mSSXA-C2 antibody as the primary antibody showed the band around 39 kDa, which is theoretically the correct size of the fusion protein of Ssxa and GFP (Fig. 5A top blot). However, other thick bands were detected around 30 kDa

in the lane of the HEK293FT transfectant with the pGFP-Ssxa. These lower bands were detected also by the anti-GFP antibody (Fig. 5A middle blot). In the lane of the transfectant with pSsxa, the mSSXA-C2 antibody detected only one band of Ssxa at the size of 12 kDa, and the anti-GFP antibody detected only one band of GFP at the size of 27 kDa.

Immunohistochemistry revealed that Ssxa and the fusion proteins were expressed in the cytoplasm (Fig. 5B). Likewise, there was the Ssxa-GFP fusion protein in the cytoplasm observed by fluorescence microscopy (Fig. 5C). However, the GFP-Ssxa fusion protein seemed to be mostly present in the nucleus than in the cytoplasm observed by fluorescence microscopy. When applying extreme attention reviewing the specimen induced by the GFP-Ssxa fusion protein staining of the nuclei is also observed in the specimen for which the GFP monoclonal antibody was used as the primary antibody (Fig. 5C right lower panel).

Discussion

The human SSX gene was first identified through its involvement in the characteristic t(X; 18) translocation in synovial sarcoma (8). It has been reported that the human SSX functions as a transcriptional repressor (9), and the human SYT-SSX1 protein plays a critical role in the tumorigenesis of synovial sarcomas through increased transcriptional activity (10). Serological analysis of tumor cDNA expression libraries (SEREX) showed that SSX2 was recognized by antibodies from melanoma and breast cancer patients (11,12). The human SSX gene contains a KRAB domain in their N-terminal sequence and SSX-RD (repressor domain) at the C-terminal. The KRAB domain is a highly charged 75 amino acid motif that defines a large subfamily of Cys2His2-type (Krüppel-type) zinc finger DNA binding proteins, which act as potent transcriptional repressors (13-15). The KRAB domain binds to nuclear import proteins and is required for efficient nuclear localization (16).

Two mouse homologs of the human SSX were identified, Ssxa and Ssxb (2). Only one Ssxa member has been found, whereas Ssxb contains at least 12 closely related members. To confirm the full-length Ssxa cDNA sequence, a 3'-RACE experiment was performed with the testis mRNA of an 8-week-old BALB/c mouse which attained sexual maturity. DNA sequencing analysis revealed that Ssxa Exon 5 did not exist, and a new Exon (Exon X) was identified. Although this cloned sequence was different from the previously reported sequence, the repetition of the previous experiment could not be performed because the sequences of the primers were not reported (2). Moreover, RT-PCR with Exon 2 sense primer and Exon X antisense primer revealed restricted mRNA expression of Ssxa in the testis and four mouse tumor cell lines. The present sequence of Ssxa actually exists and seems to be correct as a CTA. The feature in the sequence newly discovered in the present study is that the stop codon was detected. The open reading frame of Ssxa was not confirmed in the previous report because the stop codon was not identified. Therefore, there were difficulties performing the experiment using the cDNA of Ssxa.

To analyze the tissue distribution and behavior of Ssxa protein, two rabbit polyclonal antibodies for Ssxa were generated. To predict the antigenic peptides of Ssxa, the website was used and the predictions were based on a table that reflected the occurrence of amino acid residues in experimentally known segmental epitopes (Kolaskar and Tongaonkar antigenicity) (17). Western blotting revealed that the mSSXA-C2 antibody detected the Ssxa protein much more efficiently than the mSSXA-C1 antibody. The C2 peptide is suitable for the generation of rabbit polyclonal antibody for Ssxa.

Interestingly, two thick bands were shown in both respectively GFP-Ssxa and Ssxa-GFP lanes by Western blotting of the fusion protein of GFP and Ssxa. The lanes correspond to the existence of two bands of the fusion protein if Ssxa is cleaved in the putative cleavage site. Moreover, in the lane of pSsxa-GFP, the lower band was thicker than the higher one. The density of the lower band was 74.1% of the total density. The data suggest that most of Ssxa is immediately cleaved *in vivo*, and this is in agreement with the data that the thick band was not detected in the pSsxa transfectant. Based on the putative cleavage site we came to a conclusion that the molecular weight of the C-terminal cleavage part is 2.5 kDa, which is compatible with the results of Western blotting. On the other hand, in the lane of pGFP-Ssxa, the higher band is thicker. The lower band in the lane of pGFP-Ssxa does not correspong to the cleaved fusion protein, but to the shorter GFP fused Ssxa protein because the cDNA of GFP has other start codons at 234 and 264 base pairs downstream of the first start codon.

Although it has been reported that human SSX protein exists in the nucleus (18), it was unknown that mouse Ssxa translocates into the nucleus. Immunohistochemistry using mSSXA-C2 antibody, which recognizes the C-terminus of Ssxa, showed cytoplasmic staining in pSsxa transfected HEK293FT cells. However, observation of pGFP-Ssxa transfected HEK293FT cell by fluorescence microscopy showed increased GFP fluorescence in the nucleus than in the cytoplasm. These data suggest that the Ssxa N-terminal cleaved fragment translocates into the nucleus after cleavage. The Ssxa N-terminal cleaved fragment contains the KRAB domain, which is a nuclear localization signal, and may function as a transcriptional regulator.

Here, we have shown a new sequence of Ssxa cDNA and have characterized the biological behavior of Ssxa protein. However, several problems still remain. If an antibody for the N-terminal cleaved fragment of Ssxa is generated, it will become possible to obtain more objective evidence because immunohistochemistry using the mSSXA-C2 antibody, which recognizes the peptides expressed from Exon X, could not detect Ssxa expression in tumor cell lines that express endogenous Ssxa.

We are currently attempting to establish the pure cancer peptide vaccine mouse model with this Ssxa, reveal the antitumor immunological mechanism and explore the procedure to enhance the immunological reaction in an effective manner.

This is the first report showing the complete protein coding sequence for Ssxa. Moreover, mouse Ssxa is cleaved in the vicinity of the C-terminus, and the N-terminus fragment translocates into the nucleus.

Acknowledgements

We thank Hiraku Shida and Naomi Saito for their technical support in immunohistochemical analysis. Sequence data from this article have been deposited with the GenBank accession no. AB618486 and the protein ID BAJ83776.1.

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