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Identification of a novel nuclear speckle-type protein, SPOP

Yasuo Nagai[a,b], Tatsuya Kojima[a], Yoshinao Muro[c], Takahisa Hachiya[d], Yuji Nishizawa[b], Takashi Wakabayashi[b], Masatoshi Hagiwara[a,*]

[a]*Department of Endocrinology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113, Japan*
[b]*Department of Anatomy, Nagoya University School of Medicine, Nagoya 466, Japan*
[c]*Department of Dermatology, Nagoya University School of Medicine, Nagoya 466, Japan*
[d]*Medical and Biological Laboratories Co., Ltd (MBL), Ina 396, Japan*

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Abstract A novel antigen recognized by serum from a scleroderma patient was identified by expression cloning from the HeLa cell cDNA library. The cloned cDNA encoded a 374-amino acid protein with a relative molecular mass of 47 000 and a predicted amino acid sequence 62.7% identical to the hypothetical protein of *Caenorhabditis elegans*, T16H12.5. The deduced amino acid sequence had a typical POZ domain and an unidentified region conserved during evolution. No zinc finger or RNA recognition motifs were found in this clone. The 2 kbp mRNA encoding the novel clone SPOP (speckle-type POZ protein) was found to be expressed in all human tissues examined. HA-tagged SPOP, transfected and overexpressed in COS7 cells, exhibited a discrete speckled pattern in the nuclei and was co-localized with the splicing factor, snRNP B′/B. Deletion analysis revealed that both the POZ domain and the evolutionarily conserved region at the amino-terminus are required for the nuclear speckled accumulation of SPOP.

Key words: Autoantibody; cDNA cloning; Nuclear protein; POZ domain

1. Introduction

Human autoantibodies have proven to be useful reagents for identifying novel nuclear antigens. Several autoantibodies are known to recognize proteins which are highly conserved in evolution and are involved in essential intracellular functions such as DNA replication, transcription, and splicing of pre-mRNA [1]. In a previous study, we isolated cDNA clones encoding transcription factor ZF5 [2], which is present in speckle structures, and a novel MAP kinase kinase (MKK6) [3] from a HeLa cDNA library using autoantibodies. In the present study, a novel human cDNA was isolated by this immunoscreening approach with serum from a scleroderma patient and designated SPOP (speckle-type POZ protein).

2. Materials and methods

2.1. cDNA cloning and sequence analysis of SPOP

Patient K.M. had a scleroderma disease and her antiserum (#232) reacted with nuclear proteins of HEp-2 cells and showed a typical coarse speckled pattern. To identify the nuclear antigen, the #232 serum was used for immunoscreening of 5×10^5 recombinants from a HeLa Uni-ZAP XR cDNA library (Stratagene) as previously described [3]. pBluescript SK$^-$ plasmid clones containing cDNA inserts were isolated by in vivo excision as recommended by the manufacturer (Stratagene). The cDNA clones were sequenced on both strands by primer walking using Dye Deoxy terminators (Applied Biosystems) and an ABI 373A DNA sequencer (Applied Biosystems). We designated one clone containing the full length coding region pBS6111.

2.2. Northern blot analysis

A human multiple tissue blot containing approximately 2 μg of poly(A)$^+$ RNA per lane from eight different human tissues (Clontech) was hybridized with the ^{32}P-labeled 1.5 kbp cDNA probe in Express-Hyb solution (Clontech) according to the manufacturer's instructions.

2.3. Plasmid constructs

HA-tagged SPOP with the entire protein coding region (wild-type) was constructed by the polymerase chain reaction (PCR). A 24-base synthetic single-stranded DNA PCR primer, 5′-A (GTCGACATGT-CAAGGGTTCCAAGT), and a 3′-A primer (GCGGCCGCAC-GACTCACTATAGGGC) were used to prime a PCR with pBS6111 as the template DNA. The amplified fragment was digested with *Sal*I and *Not*I and ligated into the linearized pME-HA vector [3], resulting in the pME-HA-SPOP plasmid. Deletion mutants of SPOP were also generated by PCR. For HA-SPOP (Δ71–374) and HA-SPOP (Δ192–374), PCR fragments were amplified with the 5′-A primer as the antisense primer and 3′-B (GCGGCCGCTCGCAAACACCATTT-CA) or 3′-C (GCGGCCGCTCCTAACTCATCTGCCAG). HA-SPOP (Δ1–185) was also constructed as described above with 5′-B (GTCGACCTGGCAGATGAGTTAGGAG) as the sense primer.

2.4. Cell culture and immunofluorescence

Monkey kidney COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS on glass coverslips in 24-well dishes and transfected with 2 μg aliquots of plasmid DNA in DEAE-dextran. After 36–48 h, cells were fixed with 3.5% paraformaldehyde in PBS for 5 min and then permeabilized in 0.1% Triton X-100/PBS solution. Anti-HA tag staining was performed by incubation for 1 h at room temperature with 12CA5 anti-HA monoclonal antibody 1:1000 diluted in PBS with 1% BSA. Cells were then washed twice with PBS and incubated for 1 h at room temperature with 1:50 fluorescein-conjugated anti-mouse IgG antibody. Intracellular localization of antigens was analyzed by MRC 1024 confocal microscopy (Bio-Rad).

2.5. Western blotting and immunoprecipitation

COS7 cells were transfected with 10 μg plasmid DNA per 6-well dish as described above, harvested 48 h thereafter by scraping into ice-cold NET-2 (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 0.05% NP-40, and 1 mM PMSF) and disrupted by sonication. Total cell extracts were precleared with protein G-Sepharose beads (Pharmacia) for 60 min at 4°C.

Immunoprecipitation was performed with protein G-Sepharose antibodies in NET-2 for 60 min at 4°C followed by washing four times with NET-2. Samples were separated on 15% SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Hybond C, Amersham). After blocking with 10% dried milk powder in TBS-T (20 mM Tris-Cl, 137 mM NaCl, and 0.1% Tween-20) for 60 min and incubation with 12CA5 monoclonal antibody for another 60 min at room temperature, the membranes were washed and incubated with the secondary antibody (goat anti-mouse IgG, HRP conjugated [Amersham]) for 60 min at room temperature. Detection was performed by ECL (Amersham) with X-ray films exposed for 1–15 min.

*Corresponding author. Fax: (81) (3) 5803-5836.
E-mail: m.hagiwara.end@mri.tmd.ac.jp

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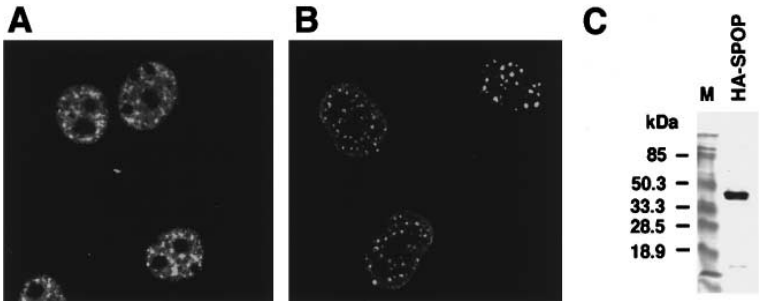

Fig. 1. Subcellular location of SPOP. A: Immunofluorescence staining of COS7 cells with autoimmune serum #232 taken from a scleroderma patient. B: Localization of HA-tagged SPOP recombinant protein. COS7 cells were transfected with HA-tagged SPOP expression vector, incubated with 12CA5 anti-HA antibody and then observed under the confocal microscope. C: Immunoblotting of HA-tagged SPOP expressed in COS7 cells. The total cell extract from the COS7 cells transiently expressing HA-SPOP was resolved by 10% SDS-PAGE and processed for immunoblotting with 12CA5 antibody.

3. Results

Immunostaining of COS7 cells with the antiserum (#232) from the scleroderma patient resulted in a rough speckled pattern in nuclei (Fig. 1A). As this serum #232 did not react with known spliceosomal antigens such as Sm, SS-A, and SS-B (data not shown), we started an immunoscreening of a human HeLa cell cDNA library to identify the antigen. Approximately 5×10^5 phage plaques were screened, and five positive clones were isolated. One clone, designated clone 6111, carried a 1.6-kbp insert containing an open reading frame which encodes a protein of 374 amino acids. The initiation codon was found 33 bp downstream of the in-frame stop codon and no polyadenylation signal was observed. The HA-tagged recombinant protein expressed in COS7 cells had a relative molecular mass of 47 000 (Fig. 1C) and showed a discrete speckled pattern in the nuclei (Fig. 1B).

A BLAST search of GenBank suggested that the cDNA encodes a novel protein which has the POZ (poxvirus and zinc finger) domain [4], but no other functional domains such as the zinc finger or the RNA recognition motif (Fig. 2B). The protein, designated speckle-type POZ protein (SPOP), is 62.7% identical to a hypothetical protein, T16H12.5, in *Caenorhabditis elegans* which is also a member of the POZ family (Fig. 2A). In addition to the POZ domain, we found that the amino-terminus of SPOP (amino acids 17–151) is highly conserved between SPOP and the T16H12.5 protein (91% identity), and named the domain the NS (N-terminus of SPOP) domain (Fig. 2B). Northern blot analysis of poly(A)$^+$ RNA from human tissues revealed the ubiquitous presence of a 2.0 kbp long SPOP mRNA in all tissues examined (Fig. 3).

To examine whether the intranuclear localization of SPOP is identical to that of spliceosomes, co-localization between SPOP and snRNP B′/B was analyzed. The nuclear speckles of SPOP recognized by anti-HA monoclonal antibody in the transfected COS7 cells were also stained with an affinity-purified autoimmune antiserum to snRNP B′/B, although diffuse background staining was present in this case (Fig. 4). As SPOP lacks any of the consensus sequences observed in many spliceosomal proteins such as the arginine-serine (RS) domain and the RNA recognition motifs (RRM), it may be deduced that it associates with other spliceosomal proteins which have signals required for nuclear speckled localization. In an attempt to identify the partner for SPOP in spliceosomes, we carried out co-immunoprecipitation studies of HA-tagged SPOP transfected in COS7 cells with several autoimmune sera under low-salt conditions preserving protein-protein interactions. However, no positive band was obtained from precipitates with anti-snRNP B′/B or anti-snRNP-70 antisera (data not shown).

To identify the domain required for intranuclear speckled localization of SPOP, several deletion mutants of SPOP were constructed and expressed in COS7 cells. The structures of the mutant SPOP proteins are shown in Fig. 5A. Immunofluorescence studies were performed with anti-HA antibody with

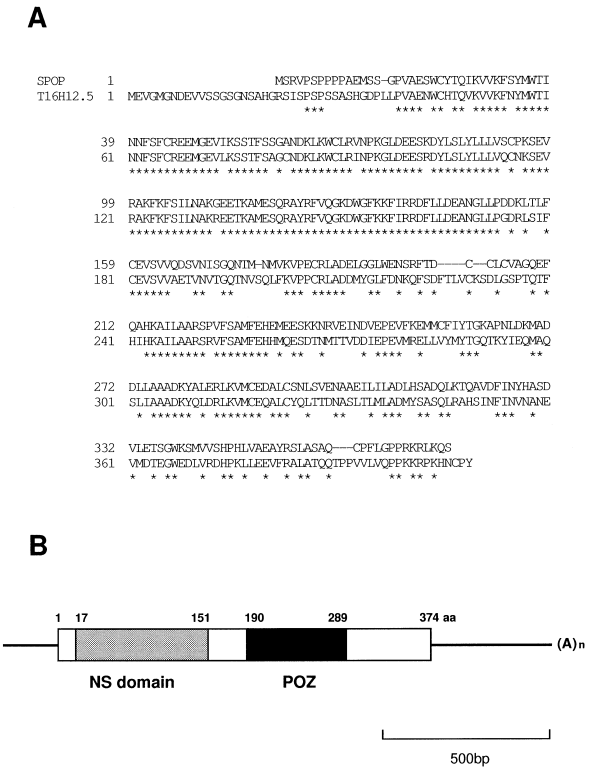

Fig. 2. Structure of human SPOP. A: The amino acid sequence alignment of SPOP and T16H12.5. The positions of identical amino acids in these two proteins are indicated by asterisks. Gaps (-) were introduced to optimize the alignment. B: Schematic representation of the SPOP cDNA is shown with the coding region indicated by the outlined box. The N-terminus of SPOP (NS) domain with the N-terminal side of *C. elegans* hypothetical protein T16H12.5, and the POZ domain (POZ) are shown by dotted and solid boxes, respectively. The numbers above the boxes indicate the positions of amino acids.

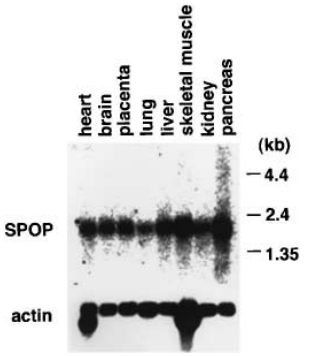

Fig. 3. Northern blot analysis of human tissues with the SPOP cDNA probe (upper panel). The blot was subsequently stripped and rehybridized with β-actin control probe (lower panel).

COS7 cells, 48 h after transfection of the mutant vectors. In contrast to the nuclear speckled localization of full-length SPOP (Fig. 5B), SPOP (Δ71–374) produced a speckled appearance throughout the entire cytoplasm without nuclear staining (Fig. 5C). SPOP (Δ192–374) showed a diffuse nuclear pattern suggesting the presence of a nuclear localization signal (NLS) in amino acids 71–191 of SPOP, though no known NLS consensus was found to be included. The N-terminal deletion of SPOP (Δ1–185) resulted in a minute speckled localization in the nucleus which was distinct from the more discrete speckled pattern observed on the wild-type SPOP (Fig. 5D). Thus at least two distinct protein-protein interaction domains of SPOP may determine the intracellular localization of the protein. The C-terminal peptide of SPOP retained immunoreactivity recognized by antiserum #232, while the N-terminal peptides described above were not immunoprecipitated by this serum, indicating that the epitope of SPOP for #232 serum is located in the C-terminal half of the protein.

4. Discussion

We report here the isolation of a cDNA encoding SPOP, a novel nuclear antigen recognized by serum from a scleroderma patient. Immunoprecipitation analysis of deletion mutants indicated that the carboxy-terminal half of SPOP contains the epitope for the antibodies used for the cDNA screening. The protein was found to be expressed in all human tissues examined and bears high similarity at the amino acid level to a hypothetical protein (T16H12.5) in chromosome III of

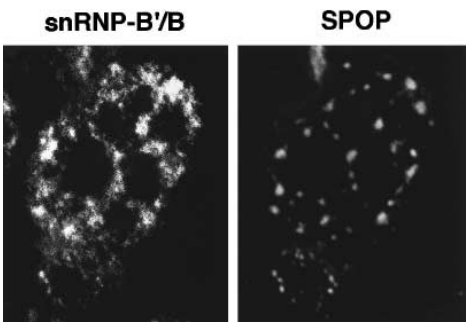

Fig. 4. Co-localization of SPOP with a spliceosomal protein, snRNP B'/B. COS7 cells transfected with HA-tagged SPOP expression vector were stained with anti-Sm human antibodies affinity-purified from the SLE patient serum with recombinant B'/B protein (A) and with anti-HA monoclonal antibody (B).

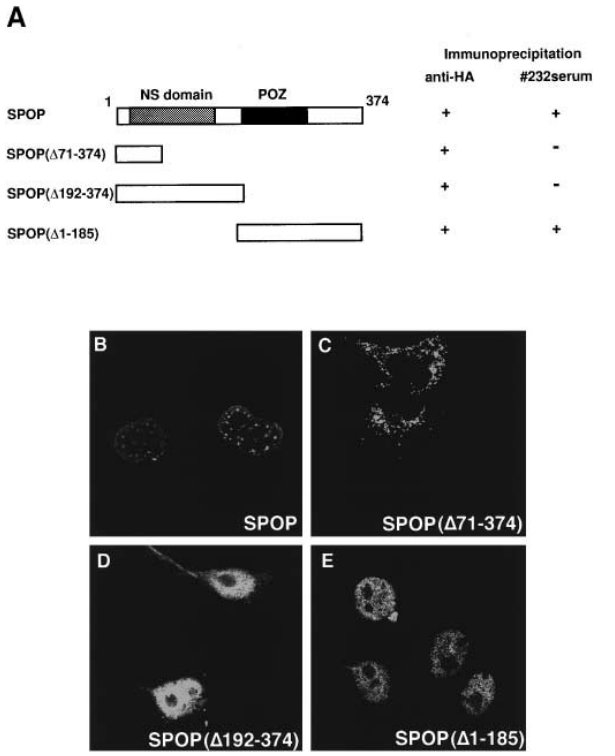

Fig. 5. Schematic illustration of the SPOP deletion mutant forms and results for their subcellular localization. A: Schematic representation of wild-type and mutant SPOPs along with results of immunoprecipitation experiments using the anti-HA antibody and the autoimmune serum (#232) performed as described above. B: Differential localization of SPOP variants in COS7 cells transfected with the indicated SPOP deletion mutants. All cells are stained with the 12CA5 antibody.

C. elegans, with 63% identity and 82% similarity [5]. No obvious function of *C. elegans* T16H12.5 protein has yet been reported.

The POZ (for poxvirus and zinc finger) or BTB (for Broad Complex, tramtrack and bric à brac) [6] is a conserved and hydrophobic domain, composed of approximately 120 amino acids, and present generally at the NH_2-terminal end of numerous proteins. POZ proteins have been divided into three classes [7]. The first comprises examples interacting with DNA and most of these are zinc finger proteins such as Ttk, GAGA and ZF5, except for the *Drosophila* E(var)3–93D product, stretches of whose charged amino acids may bind to DNA. We recently identified a human homologue of ZF5 as an autoimmune antigen [2]. The second class of POZ proteins includes a *C. elegans* protein, the *Drosophila kelch* gene product, and viral proteins from three distinct poxvirus genera. All these share an array of six imperfect repeats, each approximately 50 amino acids long, forming an actin-binding domain. The third class comprises proteins containing a POZ domain in the absence of any other known DNA- or protein-binding motifs. Although several members of this subfamily have been identified by genomic sequence analysis in poxviruses, *C. elegans* and man, the functions of these genes are unknown. Our present data indicate that SPOP should be classified in this group as the human homologue of *C. elegans* T16H12.5.

Conserved residues, the majority of which are hydrophobic, are thought to mediate protein-protein interactions. The POZ domain is predicted to be folded to form four α-helices, sep-

arated by β-sheet structures [4]. In the present study, HA-tagged SPOP, transfected and expressed in COS7 cells, exhibited a discrete speckled pattern of nuclear distribution, co-localizing with a splicing factor, snRNP B′/B. As SPOP has no RRM, it is conceivable that it may be anchored to the spliceosome by protein-protein interactions with some spliceosomal factor(s) through its POZ domain located within amino acids 190–289. Thus, a deletion mutant lacking the POZ domain was found to be diffusely localized throughout the nucleus. In addition to the POZ domain, the NS domain is surprisingly conserved between SPOP and T16H12.5 protein in *C. elegans* (91% identity). Deletion of this region also resulted in the loss of speckled localization. These data indicate that either the amino- or the carboxy-terminal half is sufficient for nuclear localization of SPOP, but that both of them are required for aggregation at spliceosomal sites. The staining pattern of nuclear speckles in COS7 cells with the patient serum was not identical to that of HA-tagged SPOP, suggesting the existence of other major antigen(s) in the autoimmune serum. Actually four other distinct clones were isolated which were all immunopositive with the serum but did not overlap with any part of SPOP (data not shown).

Recent findings have suggested that *C. elegans* gene mel-26, which is closely related to T16H12.5, acts as a post-meiotic negative regulator of mel-1, an oocyte meiosis-specific gene [8]. The phenotype of T16H12.5 loss-of-function *C. elegans* mutants should now be analyzed to provide clues to the physiological function of SPOP.

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