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Role of zinc finger structure in nuclear localization of transcription factor Sp1

Tatsuo Ito [a], Makiko Azumano [a], Chisana Uwatoko [b], Kohji Itoh [a,*], Jun Kuwahara [a,b,*]

[a] Department of Medicinal Biotechnology, Institute for Medicinal Research, Graduate School of Pharmaceutical Sciences, The University of Tokushima, 1-78 Sho-machi, Tokushima 770-8505, Japan
[b] Faculty of Pharmaceutical Sciences, Doshisha Women's University, Kodo, Kyotanabe City, 610-0395, Japan

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ABSTRACT

Transcription factor Sp1 is localized in the nucleus and regulates gene expression. Our previous study demonstrated that the carboxyl terminal region of Sp1 containing 3-zinc finger region as DNA binding domain can also serve as nuclear localization signal (NLS). However, the nuclear transport mechanism of Sp1 has not been well understood. In this study, we performed a gene expression study on mutant Sp1 genes causing a set of amino acid substitutions in zinc finger domains to elucidate nuclear import activity. Nuclear localization of the GFP-fused mutant Sp1 proteins bearing concomitant substitutions in the first and third zinc fingers was highly inhibited. These mutant Sp1 proteins had also lost the binding ability as to the GC box sequence. The results suggest that the overall tertiary structure formed by the three zinc fingers is essential for nuclear localization of Sp1 as well as dispersed basic amino acids within the zinc fingers region.

In eukaryotic cells, the genetic information is stored in the nucleus, whereas protein synthesis occurs in the cytoplasm, and therefore the movement of a large amount of macromolecules between these two compartments is required [1]. This bidirectional traffic between the nucleus and the cytoplasm is routed through the nuclear pore complex (NPC) structure that penetrates and fuses with the double bilayer membrane of the nuclear envelope [2]. Small proteins of up to 40–60 kDa in size can pass through the NPC freely, but the nuclear transport of proteins of greater than 60 kDa, which are required to contain the nuclear localization signal (NLS) sequences, is restricted. A typical NLS first identified in the SV40 large T-antigen consists of a short stretch of basic amino acids (PKKKRKV) [3]. In general, NLSs are classified into two groups, i.e., monopartite and bipartite ones, according to the number of basic amino acid clusters [4,5]. The nuclear import of proteins containing these NLSs is mediated by a specific complex with transport factors such as importin α and β in an ATP-dependent manner [6–8]. In contrast, NLSs without basic amino acid clusters have been reported in recent years, although their structural features remain unclear [9–14].

Sp1 is a ubiquitous transcription factor isolated from HeLa cells, and activates many mammalian genes containing the GC box sequence [15–19]. The protein consists of three tandem repeats of C2H2 zinc finger motif at its carboxyl terminus and specifically binds the target DNA (terms "GC box" element). Sp1 must have NLSs to be imported into the nucleus via the NPC system due to its relatively large molecular mass (95 kDa/105 kDa). However, a typical NLS has not been found in the amino acid sequence of Sp1, and the nuclear transport mechanism is poorly understood. Previously, we found that the carboxyl terminal region (167 amino acids) containing the three C2H2 zinc fingers known as DNA binding domains were also responsible for nuclear localization of Sp1 [20,21].

In this study, we examined the contribution of zinc finger structure to nuclear import of Sp1 and found the importance of the overall tertiary structure formed by the three zinc fingers.

Materials and methods

Cell culture and transfection. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under a 5% CO_2 atmosphere. HeLa cells were seeded on 35-mm dishes at a density of 2.5×10^5 cells in DMEM containing 10% FBS and then incubated at 37 °C for 24 h. Transfection was performed by means of TransFast™ Reagent (Promega, Madison, WI), followed by incubation at 37 °C for 24 h.

Treatment with zinc-chelating agent TSQ. N-(6-methoxy-8-quinoly)-4-toluenesulfonamide (TSQ) (DOJINDO Laboratories, Kumamoto, Japan) was first dissolved in ethanol, then diluted with

Abbreviations: NLS, nuclear localization signal; GFP, green fluorescent protein; TSQ, toluene sulfonamide quinoline; EMSA, electrophoretic mobility shift assays; CD, circular dichroism

* Corresponding authors. Fax: +81 88 633 7290 (K. Itoh); fax. +81 774 65 8648 (J. Kuwahara).
E-mail addresses: kitoh@ph.tokushima-u.ac.jp (K. Itoh), jkuwahar@dwc.doshisha.ac.jp (J. Kuwahara).

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140 mM sodium acetate (pH 10). HeLa cells were cultured in DMEM containing 10% FBS, 5% CO_2, 5 μM TSQ, and 1 mM CaEDTA for 24 h. Six hours after transfection, TSQ (5 μM) and CaEDTA (1 mM) were added again to the medium. Fixed cells were observed by confocal laser scanning fluorescence microscopy. Fluorescence derived from formation of Zn(II)–TSQ complex was observed under the condition (Ex: 367 nm, Em: 495 nm).

Visualization. After 24 h incubation at 37 °C, the cells were further incubated at 30 °C for 4 h in order to generate a fluorophore in EGFP. The cells were washed twice with phosphate buffered saline (PBS), fixed with a 2% (w/v) paraformaldehyde/PBS for 30 min on ice and washed with PBS, then mounted with 50% (v/v) glycerol/PBS containing 1% (w/v) n-propyl gallate. The fixed cells were visualized with a confocal laser scanning microscopy (Leica TCS4 D NT, Heiderberg, Germany) (FITC; AeX = 488 and Aem = 630 nm), fluorescence microscopy IX-70 (Olympus Co., Tokyo, Japan), or Axioskop (Zeiss, Oberkohen, Germany). Fluorescence derived from EGFP was observed under the condition (Ex: 488 nm, Em: 507 nm).

Determination of Sp1 localization. The GFP intensity was detected by fluorescence microscopy. Multiple fields were examined to count about 100 positive cells, and subcellular localizations of GFP-Sp1 fusion proteins were classified as nuclear (N), and both nuclear and cytoplasmic (N&C) for each HeLa cell. The percentages showed the ratio of number of cells showing (N&C) against that of whole cells expressing GFP-Sp1. The number of cells showing (N) and (N&C) were derived from three independent experiments, each sampling about 100 different cells. The I value indicates the ratio of the Sp1 mutants improperly and incompletely located to the nucleus and namely reflects the extent of nuclear localization inhibition of Sp1 mutants.

$$I\ [\%] = \{(N\&C) + (C)\}/\{(N\&C) + (C) + (N)\} \times 100$$

Data are the means ± S.E. for three independent experiments.

Plasmid construction. The expression vectors for constructing GFP-fused Sp1 mutants bearing amino acid substitutions in zinc fingers were prepared by site-directed mutagenesis and two-step PCR. The zinc finger-coding regions were amplified by PCR using a plasmid containing the full-length of Sp1 cDNA as a template, and the amplified product was subcloned into pEGFP-C3 (Clontech) to construct a plasmid vector encoding GFP-fused Sp1 and GFP-fused SV40 NLS. The coding region of the Sp1 zinc finger domains was excised by digestion with restriction enzymes and subcloned into an *Escherichia coli* expression vector, pAR3039 (Novagen, Madison, WI).

Expression and purification of recombinant Sp1 zinc finger proteins. The plasmid vectors, pAR3039/Sp1 zinc fingers and their site-directed mutants were introduced into *E. coli* BL21 (DE3). Cells were grown at 37 °C in LB (Luria broth) medium containing 50 μg/mL ampicillin. Protein expression was induced by the addition of 1 mM IPTG (isopropyl-1-thio-β-D-galactoside). Cells were resuspended in 8 M urea buffer (50 mM Tris–HCl, 50 mM NaCl, 8 M urea, 1.4 mM 2-mercaptoethanol, pH 8.0) and then lysed by gentle sonication. After centrifugation at 10,000*g* for 30 min, the insoluble pellet was resuspended in 8 M urea buffer, followed by standing for 1.5 h at room temperature. After centrifugation, the supernatant was purified by cation exchange chromatography, and then the purified protein was dialyzed and refolded in 10 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl and 5 μM $Zn(II)Cl_2$.

Electrophoretic mobility shift assays (EMSA). EMSA was performed using a double stranded oligonucleotide containing the GC box consensus sequence: 5′-GGGGCGGGGC-3′ [22]. The single stranded oligonucleotide was 5′-end-labeled using [γ-^{32}P]-ATP and T4 polynucleotide kinase and subsequently annealed with its complementary strand. The EMSA reactions were carried out in a total volume of 20 μL, containing 0.1 pg of ^{32}P-labeled probe, ~100 ng of recombinant protein, 10 mM Tris–HCl buffer (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM 2-mercaptoethanol, 0.1 mg/mL BSA and 25 ng/μL poly (dI–dC). Samples were incubated at 30 °C for 30 min. The resulting protein/DNA complexes were electrophoretically analyzed on 5% native polyacrylamide gels in Tris-borate buffer (pH 8.0) for 3 h at 70 V and 4 °C. The gels were dried and then subjected to autoradiography. Binding of the recombinant Sp1 and the zinc finger mutants to GC box oligonucleotides was evaluated from bound-form ratio and radioactivity.

Circular dichroism measurements. CD spectra, from 190 to 250 nm, were recorded in 10 mM Tris–HCl buffer (pH 8.0) at room temperature using a Jasco J-600 spectropolarimeter (Tokyo, Japan).

Results and discussion

Effect of TSQ, a Zn (II) chelating ligand, on nuclear localization of Sp1 zinc fingers

In order to examine the involvement of the steric structure of Zn (II)-bound zinc fingers in the nuclear localization of Sp1, the zinc-specific fluorescent probe TSQ [23] was used. We transiently expressed fusion proteins composed of green fluorescent protein and Sp1 zinc finger domains (GFP-zf) in HeLa cells pre-treated with 5 μM TSQ, and then the subcellular localization of GFP-zf was examined by fluorescence microscopy. In the absence of TSQ, GFP-zf was exclusively localized to the nucleus (Fig. 1A), indicating that the Sp1 zinc finger domains functioned as the NLS. However, in the presence of TSQ, the fluorescence of GFP was observed in both the nucleus and cytoplasm, and the nuclear localization of GFP-zf was partially, but significantly inhibited (Fig. 1A). In contrast, the GFP fusion protein with the SV40 large T-antigen NLS was localized to only the nucleus in the absence and presence of

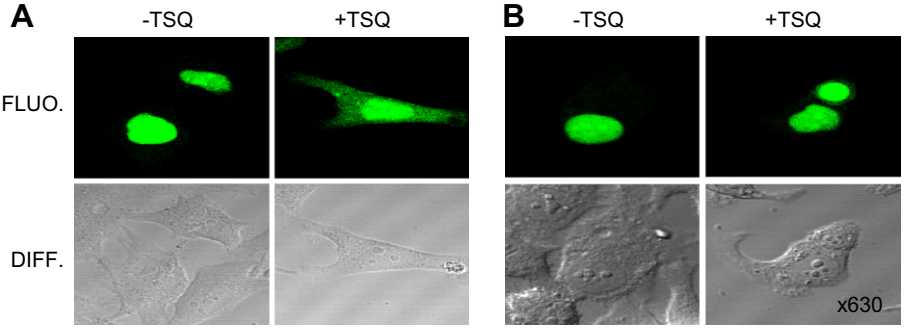

Fig. 1. Effect of TSQ on subcellular localization of GFP-zf. Each expression vector was transfected into HeLa cells pre-treated with or without 5 μM TSQ. One hour after transfection, the cells were cultured in the presence and absence of 5 μM TSQ for 23 h, and the subcellular localization of GFP-fused proteins were examined at the end of the culture. Intracellular distribution of (A) GFP-zf and (B) GFP-fuzed SV40 NLS. FLUO and DIFF indicate confocal laser scanning microscopic and differential interference microscopic images, respectively.

TSQ (Fig. 1B), indicating that TSQ had no effect on the general nuclear import-export system for the protein containing the NLS. This inhibitory effect of TSQ was specific for the Sp1 zinc finger fused to GFP. These results suggest that not only the amino acid sequence of the Sp1 zinc finger but also its conformation should be important for regulation of the nuclear localization of Sp1.

The subcellular localization of Sp1 zinc finger mutants

To more directly study the correlation between the structure of zinc fingers and the nuclear localization of Sp1, we generated a series of Sp1 zinc finger mutants by site-directed mutagenesis (Fig. 2A), where the second Cys and/or the first His residues in two pairs of well conserved Cys and His amino acids which are responsible for zinc binding in each C2H2 zinc finger were systematically changed to Ala residues. These mutations were expected to lead to defect in zinc-coordination. Group I mutants (GFP-zf1C, -zf2C and -zf3C) have a single Cys-to-Ala substitution in a single zinc finger among the three zinc fingers of Sp1. Group II mutants (GFP-zf12C, -zf23C and -zf13C) have also a single Cys-to-Ala substitution in double zinc fingers among the three. Group III mutants (GFP-zf1CH, -zf2CH and -zf3CH) have double Cys-to-Ala and His-to-Ala substitutions in a single zinc finger among the three. Group IV mutants (GFP-zf12CH, -zf23CH and -zf13CH) have also double Cys-to-Ala and His-to-Ala substitutions in double zinc fingers among the three. Group V mutant (GFP-zf123CH) has double Cys-to-Ala and His-to-Ala substitutions in all of the three zinc fingers of Sp1.

As shown in Fig. 2B, Group I mutants (GFP-zf1C, -zf2C and -zf3C) were seen only in the nucleus like GFP-zf, and their nuclear localization was not inhibited at all (data not shown). On the other hand, the other mutants were observed in both the nucleus and cytoplasm. Quantitative analysis of the nuclear localization revealed that the nuclear localization of two Group IV mutants (GFP-zf13C and -zf13CH) and the Group V (GFP-zf123CH) one containing substitutions in the first and third zinc fingers were remarkably inhibited compared to that of GFP-zf (Fig. 2C). These results suggest the following two structural possibilities. First, the local structures of the first and third finger domains among the three zinc fingers of Sp1 could be important for its zinc finger-mediated nuclear localization. Second, the entire structure formed by all three zinc fingers may be critical because it was greatly altered by mutations in two zinc fingers at both termini.

In previous work, we have demonstrated that removal of zinc ion from the three zinc fingers of Sp1 and collapse of the β–β–α structure by stepwise modification of the coordinating Cys residues with a mercury reagent, *p*-(hydroxymercuri)benzenesulfonic acid (PMPS), associated with each other and quite synergistically

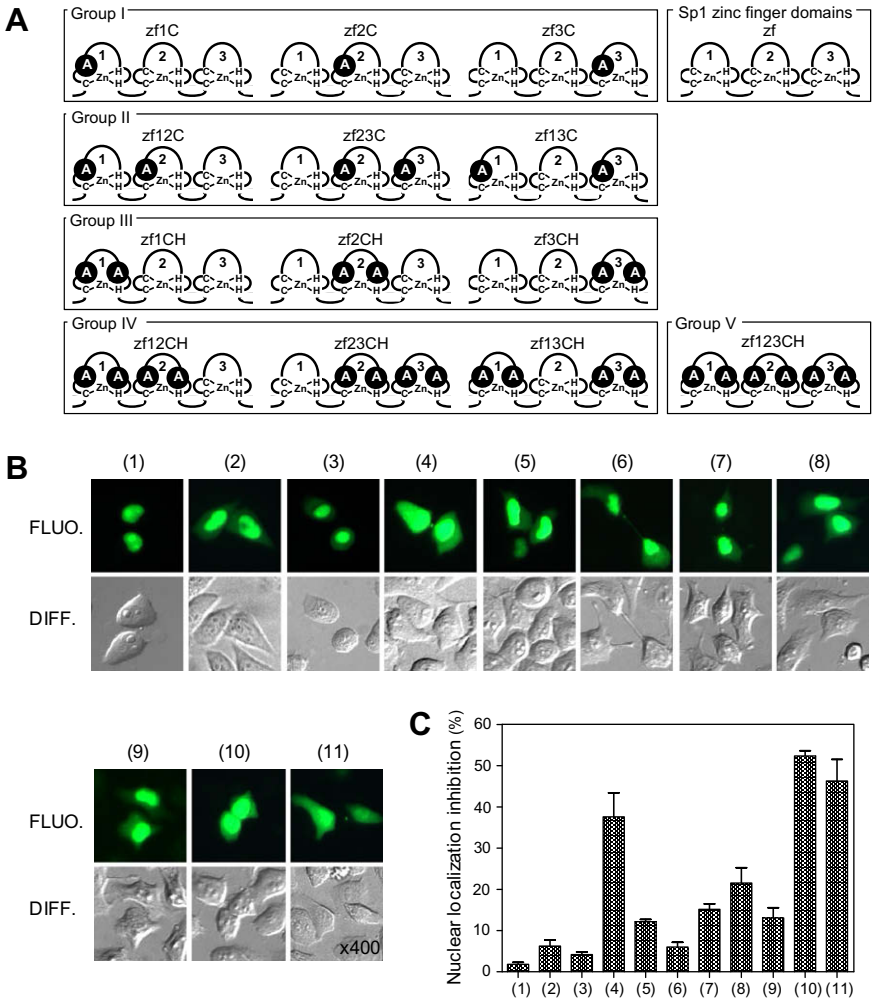

Fig. 2. (A) Schematic representation of Sp1 zinc finger mutants. The indicated schematics are the zinc finger domains in GFP-fused Sp1 zinc finger mutants. The three zinc fingers are sequentially numbered. Amino acids substituted with alanine are shown in black closed circles. A, alanine; C, cysteine; H, histidine. (B) Intracellular distribution of GFP-fused Sp1 zinc finger mutants. FLUO and DIFF indicate fluorescent microscopic and differential interference microscopic images, respectively. (C) Nuclear localization inhibition (%) of GFP-fused Sp1 zinc finger mutants. The average values were determined in three independent experiments. The numbers represent as follows: (1) zf, (2) zf12C, (3) zf23C, (4) zf13C, (5) zf1CH, (6) zf2CH, (7) zf3CH, (8) zf12CH, (9) zf23CH, (10) zf13CH, and (11) zf123CH, respectively.

occurred [24]. In other words, structure of individual finger domain could be stabilized by finger–finger interaction among the three. Anisotropic and slower overall tumbling for the first three zinc fingers of TFIIIA in heteronuclear NMR relaxation experiment has indicated the presence of interaction among the three-finger domains [25].

Next, we compared nuclear localization of GFP-zf123CH with that of GFP-zf in the presence of TSQ. As shown in Fig. 3, nuclear import of GFP-zf in HeLa cells was inhibited when the cells were pre-treated with TSQ. On the other hand, nuclear localization of GFP-zf123CH was markedly suppressed even under the condition without TSQ, and further suppression on addition of TSQ could not be observed. It is suggested that the little conformational change in the zinc fingers should be caused by TSQ since the entire structure of the expressed GFP-zf123CH had already changed.

Relationship of DNA binding activity with the structure of Sp1 zinc finger mutants

As described above, it was suggested that the suppression of nuclear localization by transient depletion of free zinc ion in HeLa cell by TSQ or by substitutions of the zinc-coordinating amino acids was possibly caused by the altered secondary structure of zinc finger domains. In order to examine these possibilities, we analyzed and compared DNA binding and secondary structure of the zinc finger domains of the wild-type (zf) with those of the mutant (zf13CH). The wild-type zf specifically bound to GC box element, which is the target DNA of Sp1, in a concentration-dependent manner, while the zf13CH mutant failed to bind to the DNA (Fig. 4B). Circular dichroism (CD) spectroscopy is often used to detect secondary structure of protein. The CD spectrum of wild-type zf showed that the zf purified from insoluble extract can be properly refolded by dialysis with zinc ion and can form a β–β–α structure. In contrast, significant change of CD spectra between zf and zf13CH was observed (Fig. 4C). Generally, removal of zinc from C2H2 zinc finger can easily destroy the β–β–α structure, especially α helical structure, which is characterized by signals at 208 nm and at 222 nm [24]. Ala substitutions of two zinc-coordinating ligands in the zf13CH may cause the incorrect secondary structure of wild-type zf in addition to loss of zinc atom leading to incomplete nuclear import as well as loss of DNA binding.

Our findings that the overall tertiary structure formed by the three zinc fingers should be essential for nuclear localization of Sp1 are consistent with the results of related 3-zinc finger proteins, NGFI-A [26] and EKLF/KLF-1[27]. In contrast, Pandya and Townes have pointed out the possibility that the tertiary structure of the C2H2 zinc fingers of EKLF/KLF-1 is not necessary for its nuclear localization because the HA-(H295A,H323A,H353A) is localized exclusively to the nucleus, whereas the mutant zinc seriously impair its function of gene activation [28]. The mutant has single Ala substitution with the first zinc-chelating His residue in all of three zinc fingers.

It seems that the zinc finger structure of the mutant, HA-(H295A,H323A,H353A), might not be ruined but form a distinct secondary structure from that of the wild-type. In our study, a single Ala substitution of the second Cys residue in a single zinc finger did not interfere its nuclear import at all. A single Ala substitution at that position might hardly disrupt its finger structure, in contrast to chemical modification of Cys residue with a reagent containing a mercury atom, *p*-(hydroxymercuri)benzenesulfonic acid (PMPS) [24]. A single Ala substitution with one of the four zinc-chelating amino acids of a zinc finger might not necessarily disrupt its folding structure but induce an altered secondary structure dependent on its mutated position. Therefore, these distinctive structures might be responsible for the different results on nuclear localization and DNA binding in the zinc finger proteins described above. Pandya and Townes also have clearly demonstrated the basic amino acids distributed over the three zinc fingers of EKLF/KLF-1 are critical for its nuclear localization [28]. These basic amino acids are also conserved in Sp1.

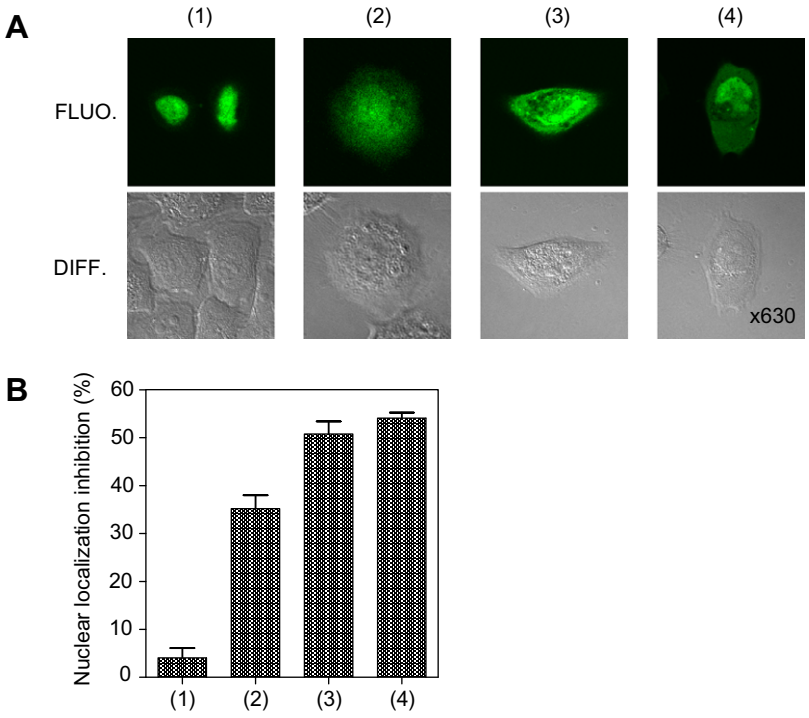

Fig. 3. Effect of TSQ on subcellular localization of GFP-fused Sp1 zinc finger mutant. (A) Intracellular distribution of GFP-fused Sp1 zinc finger mutant. FLUO and DIFF indicate confocal laser scanning microscopic and differential interference microscopic images, respectively. (B) Nuclear localization inhibition (%) of GFP-fused Sp1 zinc finger mutant. The average values were determined in three independent experiments. The numbers represent as follows: (1) zf, (2) zf pre-treated with 5 μM TSQ, (3) zf123CH, and (4) zf123CH pre-treated with 5 μM TSQ, respectively.

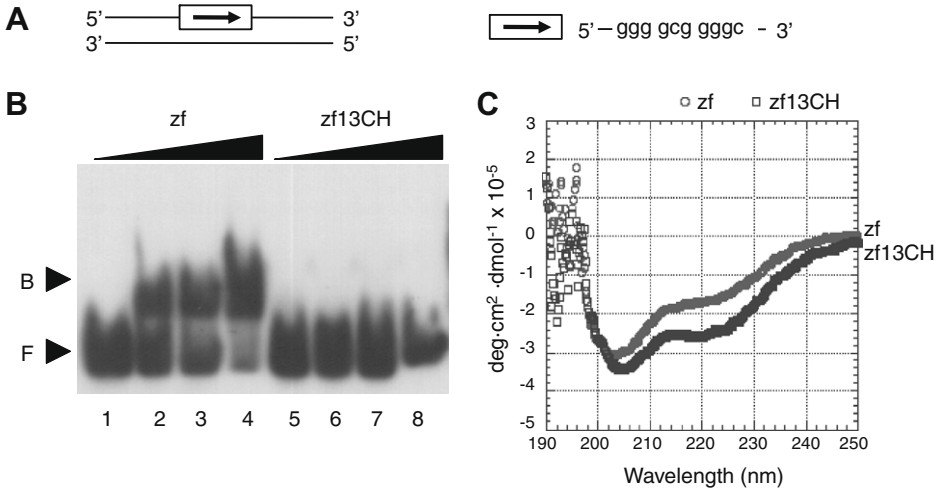

Fig. 4. Structural and functional analysis of zinc finger domains of Sp1 and its mutants. (A) Sequence of the GC box DNA probe. (B) EMSA with zf and zf13CH. Lanes 1–4, 0, 330, 660, and 1650 nM zf, and lanes 5–8, 0, 330, 660, and 1650 nM zf13CH, respectively. F and B show free DNA probe and protein–DNA complex species, respectively. (C) CD spectra of zf (o) and zf13CH (□).

Finally, the overall tertiary structure formed by the three zinc fingers should be important for nuclear localization of Sp1 as well as basic amino acids distributed within the zinc fingers region.

Conclusion

In summary, we elucidated that the Sp1 zinc finger known as the DNA binding domain plays a role as a NLS, and the properly folded secondary structure of the zinc finger is required for this function. The results we presented here will be useful for studies on the nuclear localization mechanism of Sp1.

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