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Spectroscopic characterization of the tumor antigen NY-REN-21 and identification of heterodimer formation with SCAND1

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Abstract

Human NY-REN-21 is a C_2H_2 type multi-finger protein, with a SCAN domain in the N-terminal region and a predicted coil central region. It represents a putative ortholog of mouse ZFP38, a transcriptional factor that recognizes a bipartite DNA motif and is unable to form homodimers. As shown in this work, NY-REN-21 contains a SCAN domain able to form homodimers and a central region that behaves as an intrinsically disordered protein. The SCAN domain is found in 71 human proteins and its ability to form homo- and heterodimers widens the number of genes that are regulated by this group of transcription factors. NY-REN-21 interaction with SCAND1 was identified using the yeast two-hybrid system and confirmed using recombinant proteins. SCAND1 is a truncated SCAN box protein, lacking the zinc finger region and the NY-REN-21/SCAND1 heterodimer is asymmetric concerning the DNA binding region. This result indicates that NY-REN-21 can function either as a homodimer or as a heterodimer with SCAND1.

Keywords: SCAN domain protein family; Intrinsically disordered protein; Zinc finger protein; Circular dichroism; Mass spectrometry; Yeast two-hybrid system

NY-REN-21 was identified in a SEREX screen (*ser*ological analysis of recombinant cDNA *ex*pression libraries) as an autologous antibody in patients with renal-cell carcinoma [1]. NY-REN-21 belongs to the multi-finger SCAN box protein family, containing the SCAN domain [2] in the N-terminal region and seven C_2H_2 type zinc fingers in the C-terminal region. NY-REN-21 also contains a 150 residue central region predicted to be intrinsically disordered under physiological conditions. Zinc finger transcriptional factors can exert either positive or negative regulation of gene targets [3]. Diversity in the modular multi-finger protein family is determined by the conserved domains found outside the zinc finger region. These domains include BTB/POZ [4], KRAB [5], and SCAN [2]. They usually control selective association of the transcription factors with other cellular factors essential for regulation, subcellular localization, DNA binding or transcription. The SCAN domain is restricted to vertebrates, acting as a mediator of both homo and heterodimerization [6–10]. It is an 82–88 amino acid highly conserved domain [7,8,10,11], forming a structurally independent module with 5 distinct α-helices in each monomer [12].

NY-REN-21 represents a putative ortholog of mouse ZFP38, also named CTfin51, Zipro1, and Ru49, sharing 82% amino acid identity. An important difference is observed in the N-terminal region where NY-REN-21 lacks 77 residues of the trans-activation region relative to ZFP38 (Fig. 1). ZFP38 functions as a transcriptional factor, containing three sequence repeats that show trans-activation activity [13]. It binds to a bipartite motif (5′AGTAC3′) showing higher affinity to motifs separated by 2–7 nucleotides [14]. Studies using transgenic mice

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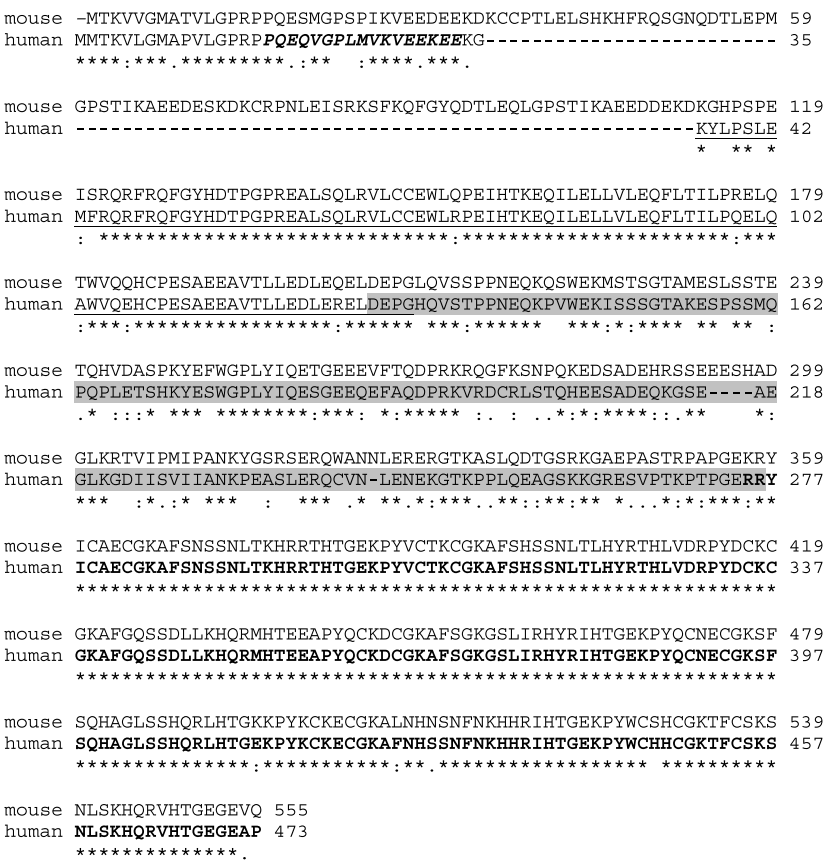

Fig. 1. Amino acid sequence alignment of the human NY-REN-21 and mouse ZFP38 proteins. The conserved domains are indicated for NY-REN-21 as follows: italic–bold, acidic region (residues 17–33); underlined, SCAN domain (residues 36–131); gray box, disordered central region (residues 128–276); bold, zinc finger region (residues 275–473).

revealed that ZFP38 functions in the proliferation of granule cell precursors in the developing cerebellum [15].

Up to now there are no experimental data available on NY-REN-21 structure and little is known about its function on transcriptional control. In this work, we present data on the spectroscopic characterization of NY-REN-21, confirming the helical structure of the SCAN domain and the intrinsically disordered nature of the central region. Unlike ZFP38, which apparently does not form homodimers [8], the SCAN domain of NY-REN-21 is able form homodimers. In addition, NY-REN-21 forms heterodimers with SCAND1, which is a SCAN family protein that lacks the zinc finger region [7]. Heterodimerization between different members of the SCAN protein family probably allows them to control expression of a larger number of genes and the identification of the NY-REN-21-SCAND1 interaction may help understand the mechanism of action of the multi-finger transcription factors.

Materials and methods

Construction of expression vectors. The NY-REN-21 cDNA (Accession No. NM_145914) was amplified by PCR from a human fetal brain library (Clontech HL4028AH) using primers ONZ111 and ONZ112 (oligonucleotides are listed in Table 1) and inserted into the *Nde*I and *Eco*RI sites of pET28a (Novagen) generating vector pET-NYREN21. pET-SCAN contains the SCAN domain (amino acids 36–131, Fig. 1) amplified using primers ONZ290 and ONZ291, and inserted into the *Bam*HI and *Eco*RI sites of pET-TEV (pET28a derivative containing a TEV protease site). pPROEX-SCAN was constructed by transferring the *Bam*HI–*Eco*RI fragment from pET-SCAN to pProEx-HTb (Invitrogen). pETGST-DCR contains the disordered central region (DCR, amino acids 128–276) amplified using primers ONZ323 and ONZ324, and inserted into the *Eco*RI and *Xho*I sites of pETGST-TEV (pET28a derivative containing GST followed by a TEV protease site). The zinc finger region (ZFR, amino acids 275–473) was amplified with primers ONZ321 and ONZ322, and cloned into the *Eco*RI and *Xho*I sites of pETGST-TEV and pProEX-HTa (Invitrogen), generating pETGST-ZFR and pPROEX-ZFR, respectively.

pGAD-C2 [16] (activation domain fusions) and pTL1 (DNA binding domain fusions) were used for yeast two-hybrid assays. pTL1 is a pBTM116 [17] derivative containing the *Escherichia coli* kanamycin marker. pTL1-SCAN and pGAD-SCAN contain the SCAN domain amplified using primers ONZ288 and ONZ289, and inserted into the *Bam*HI and *Eco*RI sites of pTL1 and pGAD-C2, respectively. pACT-SCAND1 contains the cDNA of SCAND1 isolated from the human fetal brain cDNA library. pET3-SCAND1 was constructed by inserting a *Bgl*II HA-SCAND1 fragment isolated from pACT-SCAND1 into the *Bam*HI site of pET3d (Novagen).

Protein expression and purification. Escherichia coli BL21(DE3)*slyD*$^{-}$ (a gift from Ryland Young, Texas A&M University) was used to express the recombinant proteins and the cultures were induced with 0.5 mM IPTG at an OD_{600} ~0.8. The induction time was 6 h at 20 °C for NY-REN-21; 4 h at 37 °C for the SCAN domain and GST-DCR; 4 h at both 25 °C and 37 °C for GST-ZFR and His-ZFR. Induction of GST-ZFR was also performed with 10 mM lactose for 16 h at 20 °C. Extracts were

Table 1
Oligonucleotides used in PCRs

Number	Sequence	Sites
ONZ111	5′TTACCATATGACCAAGGTACTAGGCATGGC 3′	*Nde*I
ONZ112	5′ TGAATTCTTACGGTGCTTCTCCCTCTCCAG 3′	*Eco*RI
ONZ288	5′ GTACGAATTCAAGTACCTTCCTAGCCTG 3′	*Eco*RI
ONZ289	5′ ACTGGATCCTCATCCTGGCTCATCCAGTTC 3′	*Bam*HI
ONZ290	5′ ACGGATCCCTAATGAAGTACCTTCCTAGCCTG 3′	*Bam*HI
ONZ291	5′GACTGAATTCTCATCCTGGCTCATCCAGTTC 3′	*Eco*RI
ONZ321	5′ CTTAGAATTCAGACGTTATATATGTGCTG 3′	*Eco*RI
ONZ322	5′ GTACCTCGAGTTACGGTGCTTCTCCCTCTC 3′	*Xho*I
ONZ323	5′ GACTGAATTCGATGAGCCAGGACACCAGG 3′	*Eco*RI
ONZ324	5′GATACTCGAGTTAACGTCTCTCTCCTGGGG 3′	*Xho*I

prepared in the appropriated buffers and cleared by centrifugation. Purification of histidine- and GST-tagged proteins was performed on standard affinity chromatography on metal-chelating and glutathione–Sepharose columns, respectively. NY-REN-21 extracts were prepared in buffer A (20 mM Hepes, pH 7.2, 100 mM NaCl, 20% glycerol (v/v), 5 mM β-mercapto-ethanol, 10 μM $ZnSO_4$, and 0.5 mM PMSF) and treated with DNAse I (2.5 μg/mL) for 40 min at room temperature prior to affinity chromatography. Affinity-purified NY-REN-21 was fractionated on a 1 mL heparin–Sepharose column equilibrated in buffer B (buffer A + 300 mM NaCl) and eluted with a 15-mL gradient of buffer C (buffer A + 1.5 M NaCl). SCAN domain extracts were prepared in buffer D (50 mM sodium phosphate, pH 7.0, 100 mM NaCl, and 5% glycerol) and purified using a nickel-chelating column. Following removal of the histidine-tag by TEV protease digestion, the domain was further purified using a 1 mL MonoQ column with buffer E as binding buffer (25 mM sodium phosphate, pH 7.0, 5% glycerol, 10 mM NaCl, and 1 mM DTT) and a gradient of buffer F (buffer E + 1 M NaCl) for elution. GST-DCR extracts were prepared in phosphate-buffered saline containing 5 mM β-mercapto-ethanol and 0.5 mM PMSF, and GST-DCR was affinity-purified using glutathione–Sepharose beads (GE Healthcare) according to the supplier's instructions. GST-DCR was cleaved using the TEV protease and GST removed by incubating the suspension extensively with glutathione–Sepharose beads. GST-ZFR affinity chromatography on glutathione–Sepharose beads was performed as described for GST-DCR.

TEV protease digestion and limited proteolysis assays. TEV protease digestions were performed in buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, and 1 mM DTT for 16 h at 25 °C (TEV protease/fusion protein ratio 1:20). For limited proteolysis, purified DCR and SCAN domain were subjected to digestion with trypsin (1%, w/w) at room temperature in buffer containing 50 mM sodium phosphate, pH 7.2, 100 mM NaCl, 5% glycerol, and 0.5 mM DTT. Samples were collected at various times and the reactions were stopped by adding Tricine–SDS–PAGE sample buffer containing 1 mM PMSF and heated at 95 °C for 5 min. The proteolysis products were analyzed on Tricine–SDS–PAGE gels.

Circular dichroism and fluorescence spectroscopy. Circular dichroism (CD) spectra were acquired on a Jasco-810 spectropolarimeter at 20 °C using a 50 nm/min scanning rate and a spectral bandwidth of 0.5 nm. Ellipticity is reported as the mean residual ellipticity [θ] (deg cm^2 $dmol^{-1}$). CD spectra of NY-REN-21 were acquired at 82 μM using a 0.01 mm path length cell over the range of 190–260 nm in buffer containing 20 mM Hepes, pH 7.2, 900 mM NaCl, 20% glycerol (v/v), 5 mM β-mercapto-ethanol, 10 μM $ZnSO_4$, and 0.5 mM PMSF. EDTA-treated samples were incubated in the same buffer with 2 mM EDTA for 30 min prior spectrum acquisition. The CD spectra of DCR and the SCAN domain at 10 μM in 10 mM sodium phosphate buffer, pH 7.2, 20 mM NaCl, and 1 mM DTT were acquired using a 0.1 mm path length cell over the range of 190–260 nm. The SCAN domain was subjected to thermal unfolding at 5 μM and 10 μM from 10 °C to 95 °C. Ellipticity at 222 nm was measured using 0.5 °C intervals and spectra were collected at 5 °C intervals over the λ range of 190–260 nm. Midpoint transition temperatures were calculated as the center of the Gaussian fit of the first derivative of the denaturation curves. Refolding assays started at 95 °C and the temperature lowered to 20 °C. DCR intrinsic fluorescence was measured at 10 μM in 10 mM sodium phosphate buffer, pH 7.2, 20 mM NaCl, and 1 mM DTT using an ISSK2 fluorimeter. Excitation was at 280 nm and emission at 340 nm was recorded from 300 to 400 nm.

Mass spectrometry. The SCAN domain (6 mg/mL) in 100 mM ammonium bicarbonate buffer, pH 8.0, was analyzed by direct infusion electrospray ionization on a Q-Tof Ultima API mass spectrometer (Waters/Micromass) with a nanoflow interface. The instrument settings were 3 kV for the spray voltage, 100 V for the cone voltage, cone gas at 30 L/h, and source temperature of 100 °C. The final spectra were processed using the MaxEnt I program (Waters/Micromass). For mass spectrometry analysis, the recombinant DCR was dialyzed against 100 mM ammonium bicarbonate buffer, pH 8.0, containing 5 mM β-mercapto-ethanol, dried, and suspended in water/acetonitrile (1:1, v/v) containing 0.1% TFA. Data were acquired and analyzed as described for the SCAN domain.

Yeast two-hybrid assays. The yeast two-hybrid system was used to test homodimerization of the NY-REN-21 SCAN domain. The assay was performed in strain L40 [18] co-transformed with vectors pTL1-SCAN and pGAD-SCAN. The test and control strains were incubated in YNB medium supplemented with adenine and either histidine or 15 mM 3-amino-triazole (3-AT). Filter assays to test β-galactosidase expression were performed as described previously [18]. Strain L40 transformed with pBTM-Nip7 and pACT-Nop8 was used as a positive control [19]. The combinations pBTM-Nop8/pGAD-SCAN [19], pTL1/pGAD-SCAN, and pTL1-SCAN/pGADC2 co-transformed into L40 served as negative controls. A large-scale screen was performed to identify the NY-REN-21 SCAN domain-interacting partners. For this purpose, strain L40 containing pTL1-SCAN was transformed with a human fetal brain cDNA library fused to the activation domain of pACT2 (Clontech). Positive clones were selected on YNB medium supplemented with adenine and 7 mM 3-AT, followed by β-galactosidase filter assays [18].

Protein interaction assays. The proteins were co-expressed in *E. coli* BL21(DE3)*slyD*$^-$ using the combinations pET3-SCAND1/pET-NYREN21, pET3-SCAND1/pET-SCAN, and pET3-SCAND1/pET-Nip7 (pET-Nip7, unpublished results). Extracts were prepared in affinity buffer (100 mM sodium phosphate, pH 7.2, 100 mM NaCl, 5% glycerol, and 0.5 mM PMSF) and incubated with 25 μL Ni–NTA agarose beads (Qiagen) for 45 min at 4 °C. Subsequently, the agarose beads were washed with 5 mL of wash buffer (affinity buffer + 200 mM NaCl) and the bound proteins eluted in 50 μL of wash buffer containing 200 mM imidazole. The bound proteins were analyzed by SDS–PAGE and SCAND1 was identified by immunoblotting using an anti-HA antiserum (Santa Cruz Biotechnology).

Results and discussion

Expression and CD analysis of NY-REN-21

NY-REN-21 is a modular protein with a SCAN domain at the N-terminal region, a predicted disordered central region (DCR), and seven C_2H_2-type zinc fingers (ZFR) at the C-terminal region (Fig. 1). The central and the zinc finger regions confer high instability to the protein. Nevertheless,

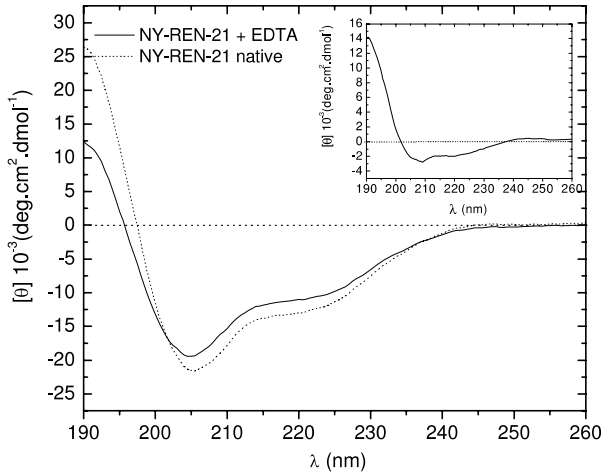

Fig. 2. Circular dichroism analysis of NY-REN-21. The dotted line represents the untreated protein and the solid line the sample treated with 2.0 mM EDTA for 30 min prior to spectrum acquisition. Inset: difference spectrum between NY-REN-21 native and NY-REN-21 treated with EDTA.

high levels of NY-REN-21 were obtained by inducing *E. coli* cells at 20 °C during 6 h. The recombinant protein was purified by affinity followed by a chromatography on heparin and was relatively stable in the heparin elution buffer containing 20 mM Hepes, pH 7.2, ~0.9 M NaCl, 20% glycerol (v/v), 5 mM β-mercapto-ethanol, 10 μM $ZnSO_4$, and 0.5 mM PMSF. Dialysis against lower salt buffers led NY-REN-21 to aggregate and precipitate (data not shown). The CD spectrum of NY-REN-21 revealed a partially unfolded protein with a deeper minimum at 205 nm that must represent the contribution of the central coil region and possibly of partially unfolded zinc finger domains (Fig. 2). The C_2H_2 zinc finger is a structurally independent domain showing a typical β–β–α compact fold in which the zinc atom is bound by two cysteine and two histidine residues [20]. The zinc finger is prone to oxidation being highly unstable particularly when not bound to the cognate DNA [21]. In addition, chelating of the zinc ion by EDTA causes loss of the α-helix content of zinc finger proteins [22]. As expected, the CD spectrum of NY-REN-21 treated with 2 mM EDTA showed a moderate reduction of signal in the λ 200–230 nm range and a sharper reduction in the 190–200 nm range (Fig. 2). This loss of structure upon EDTA treatment indicates that the zinc finger region was at least partially folded. In order to analyze the contribution of each NY-REN-21 region to the final structure of the protein and to obtain data about their respective functions, we constructed truncated proteins and their characterization is described in the following sections.

Analysis of the disordered central region and the zinc finger region

Sequence analysis algorithms [23,24] suggested that the NY-REN-21 region comprising amino acids 128–276 falls into the group of intrinsically disordered proteins. GST-DCR showed an aberrant electrophoretic mobility, migrating with a molecular weight on SDS–PAGE gels similar to GST-ZFR, which is 50 amino acids longer (Figs. 1 and 3A). Mass spectrometry was used to determine whether the correct DCR was produced. Its predicted molecular mass is 16967.5 Da and the mass spectrum of DCR cleaved from GST by TEV protease revealed a product of 16965.25 Da, which is quite close to the predicted molecular mass, and two additional products of 17041.25 and 17119.25 Da (Fig. 3B). The additional products correspond, respectively, to DCR containing one and two β-mercapto-ethanol molecules bound to its cysteine residues. Since GST-DCR was purified in the presence of β-mercapto-ethanol, we concluded that DCR was correctly expressed in *E. coli*. Its aberrant electrophoretic mobility may be due to a higher resistance of the unfolded protein against the polyacrylamide gel. This type of aberrant mobility has been described for *Saccharomyces cerevisiae* Gir2, which is an intrinsically disordered protein [25].

The techniques most employed to demonstrate the intrinsically unstructured nature of proteins are proteolysis, CD, and fluorescence spectroscopy, although there has been an increasing use of nuclear magnetic resonance and small angle X-ray scattering [26,27]. In order to determine whether DCR was a natively unstructured protein, we used a proteolysis assay and CD and fluorescence spectroscopy. DCR was fully digested following 1 h of incubation with trypsin, whereas most of the SCAN domain, used as a control, remained undigested (Figs. 3C and D). This result was an important indication that DCR is unstructured. CD and fluorescence analyses supported this finding. The CD spectrum of DCR showed a minimum at 199 nm which is typical of disordered structure (Fig. 4A). DCR contains two tryptophan residues and its fluorescence emission spectrum shows a maximum near 355 nm (Fig. 4B) which is consistent with the fluorescence emission of free or water-exposed tryptophan residues of unfolded protein chains. The actual function of the DCR of NY-REN-21 is not known but it is speculated that the flexible region of these modular proteins is important for transcriptional complex assembly [28] or can act as a spacer, regulating the distance between adjacent domains [29].

NY-REN-21 is predicted to contain seven C_2H_2 zinc fingers (ZFR) in the C-terminal region (amino acids 275–473). The histidine-tagged ZFR was totally insoluble but the GST-ZFR could be purified from *E. coli* extracts, although its characterization was troublesome (Fig. 3A). A huge amount of DNA co-purified with ZFR even using the strategy that was successful to obtain DNA-free NY-REN-21 (data not shown). DNA affects protein quantitation and TEV protease cleavage was unsuccessful due to the quick degradation of the ZFR moiety of the fusion protein. Further characterization of the ZFR was not possible.

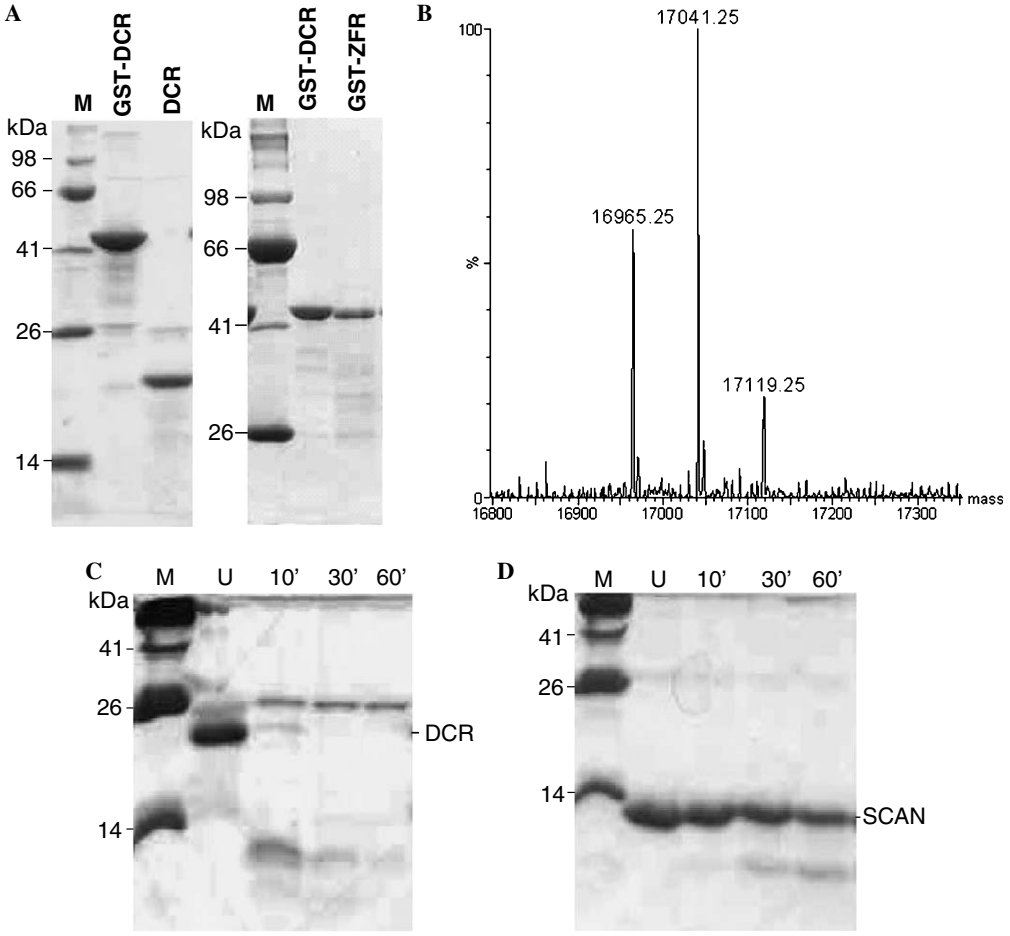

Fig. 3. Purification and analysis of the disordered central region (DCR). (A) Coomassie-stained SDS–PAGE gel showing purified DCR (left panel) and comparison of the electrophoretic mobility of GST-DCR and GST-ZFR (right panel). GST-DCR and GST-ZFR: respective purified fusion proteins; DCR: purified disordered central region following cleavage by TEV protease. (B) Mass spectrometry analysis of DCR. The deconvoluted spectra revealed products of 16965.25 Da corresponding to DCR (predicted molecular mass 16967.5 Da) and two additional products (17041.25 and 17119.25 Da) corresponding to DCR containing one and two β-mercapto-ethanol molecules, respectively. (C,D) Coomassie-stained SDS–PAGE gels showing trypsin proteolysis of DCR (C) and the SCAN domain (D), which was used as a control. The incubation time is indicated above each lane. U, Untreated protein. M, Molecular weight standards.

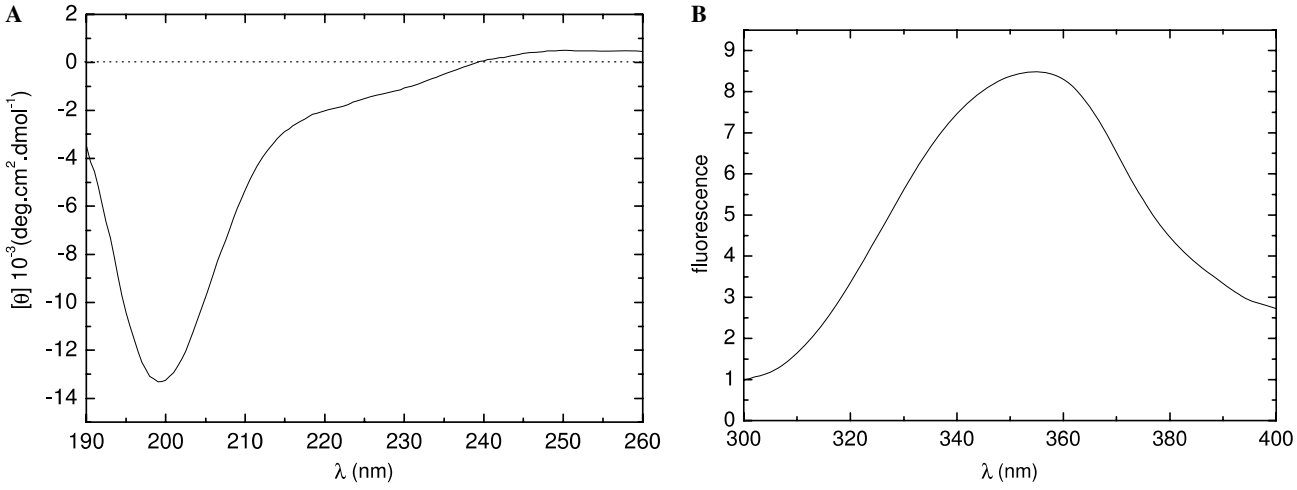

Fig. 4. Spectroscopic analysis of the disordered central region (DCR). (A) Circular dichroism spectrum of DCR showing a minimum at 199 nm. (B) Fluorescence analysis of DCR. The excitation and emission wavelengths used were 280 and 340 nm, respectively. The maximum fluorescence emission at 355 nm shows that the tryptophans are exposed to the solvent.

Secondary structure and oligomerization analysis of the SCAN domain

The SCAN domain showed a typical α-helical CD spectrum and could be partially refolded following thermal denaturation (Fig. 5A). The SCAN domain of ZNF174 was reported to show a concentration-dependent midpoint transition temperature as a consequence of the dimeric structure [10]. An unfolding behavior consistent with a dimer was also observed for the NY-REN-21 SCAN domain, which showed a midpoint transition temperature of 60 °C at 5 μM and 62.8 °C at 10 μM (Fig. 5B). Oligomerization of SCAN domains has been widely documented, forming either homo or heterodimers with other SCAN-containing proteins [6–10]. In the mammalian two-hybrid system, mouse ZFP38 did not form homodimers and interacted only with SRE-ZBP [8]. NY-REN-21 lacks 77 amino acids of the acidic region as compared to ZFP38 (Fig. 1) but this difference should not interfere with the oligomerization mediated by the SCAN domain. In order to determine whether the SCAN domain of NY-REN-21 was able to form homodimers, experiments were performed using the yeast two-hybrid system and mass spectrometry. The yeast two-hybrid assay was performed in strain L40 carrying the SCAN domain (amino acids 36–131) fused both to the *lexA* DNA binding domain and to the *GAL4* activation domain. This strain contains *HIS3* and *lacZ* as two-hybrid reporters, both of which were expressed at high levels, indicating that the SCAN domain formed homodimers with high affinity (Fig. 6). Parallel controls were performed with L40 transformed with vectors pBTM-Nop8/pGAD-SCAN, pTL1/pGAD-SCAN, and pTL-SCAN/pGAD-C2, which did not activate the two-hybrid markers (Fig. 6). Electrospray ionization analysis of the native SCAN domain in ammonium bicarbonate buffer identified a monomer as a minor product (14896 Da) and a dimer as a major product (29793 Da, Fig. 6C). The SCAN domain contains a cysteine residue and its oxidation could form a dimer linked by a disulfide bond. To exclude this possibility, a new analysis was performed under denaturing conditions in buffer containing 50% acetonitrile, 0.1% formic acid, without reducing the cysteine residues. The molecular mass obtained (14896 Da) corresponded to the monomer (Fig. 6D), indicating that the dimer was formed by specific, non-covalent interactions. Size-exclusion chromatography also showed a retention time consistent with the size of a dimer (data not shown). It is quite intriguing that no homodimer was described for mouse ZFP38 [8] since the two proteins share 97% amino acid similarity in the SCAN domain (Fig. 1).

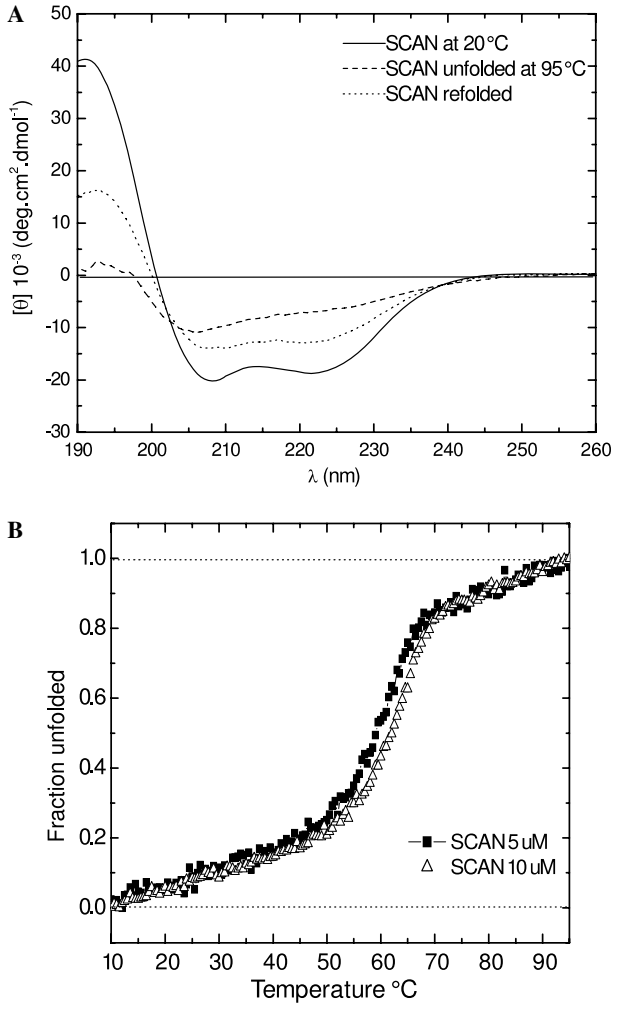

Fig. 5. Analysis of the recombinant SCAN domain. (A) CD spectra of the SCAN domain (10 μM). Solid line, spectrum acquired at 20 °C; dashed line, spectrum acquired at 95 °C; dotted line, spectrum of refolded SCAN domain following thermal denaturation at 95 °C and refolding to 20 °C. (B) Thermal unfolding analysis of the SCAN domain. The molar ellipticity at 222 nm was determined at 5 μM (■) and 10 μM (Δ) in the range from 10 °C to 95 °C and plotted as a ratio of folded versus unfolded protein.

Identification of the SCAN domain/NY-REN-21 interaction with SCAND1

The ability of this particular SCAN domain to interact with other SCAN family proteins was investigated by taking advantage of the yeast two-hybrid system. Five positive clones containing the cDNA encoding SCAND1 (also named SDP1 and RAZ1) were isolated from a human fetal brain library. The assay for the *HIS3* reporter was performed in medium containing high 3-AT concentration (up to 30 mM) which is a strong indication of positive interaction (Fig. 7A). Positive interaction was also observed for the *lacZ* reporter gene (Fig. 7B). Direct interaction of NY-REN-21 with SCAND1 was assayed using recombinant proteins. HA-tagged SCAND1 was co-expressed in *E. coli* with histidine-tagged NY-REN-21 or with histidine-tagged SCAN domain. A parallel control assay was carried out with HA-tagged SCAND1 co-expressed with histidine-tagged Nip7p, an unrelated protein. The histidine-tagged proteins were affinity-purified on nickel columns and SCAND1 co-purified with both full-length NY-REN-21 and the SCAN domains of NY-REN-21 (Fig. 7C), confirming the heterodimer formation between these two proteins.

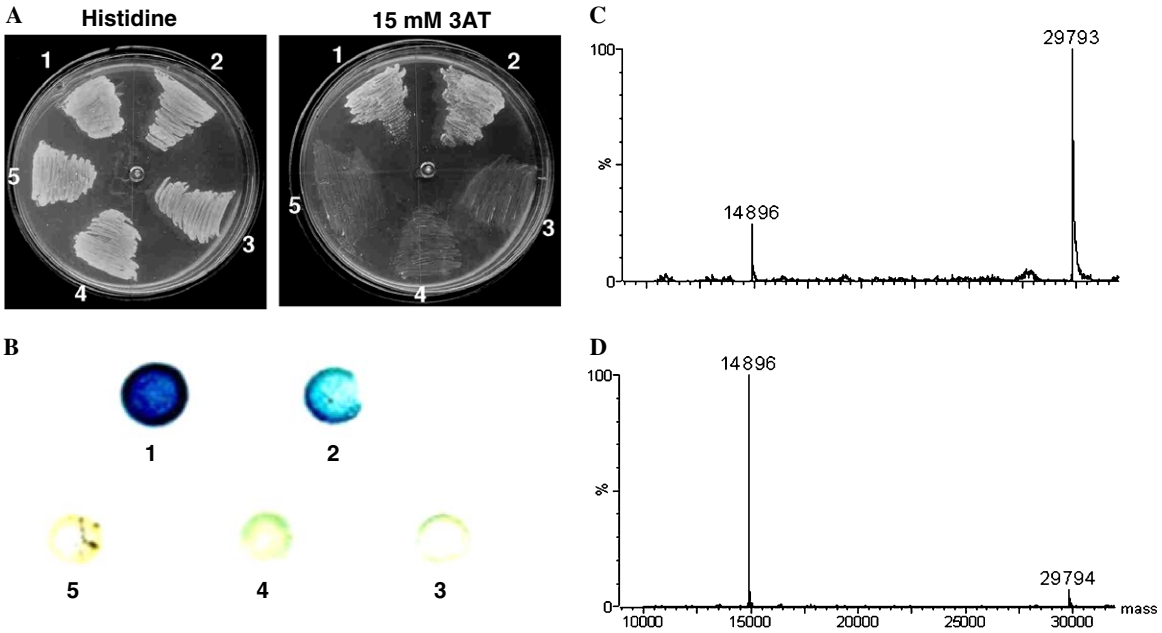

Fig. 6. SCAN domain dimerization analysis. (A,B) Yeast two-hybrid interaction assay on synthetic minimal medium (containing either histidine or 15 mM of 3-AT) and β-galactosidase filter assay, respectively. 1, Test strain containing the SCAN domain fused both to the DNA binding domain of *lexA* (DB-SCAN) and to the *GAL4* activation domain (AD-SCAN); 2, positive control (DB-Nip7/AD-Nop8); 3, 4, and 5 indicate the combinations DB-SCAN/AD, DB/AD-SCAN, and DB-Nop8/AD-SCAN used as negative controls. DB, DNA binding domain. AD, Activation domain. (C,D) Mass spectra of the SCAN domain in ammonium bicarbonate buffer and in ammonium bicarbonate buffer containing 50% acetonitrile, 0.1% formic acid, respectively.

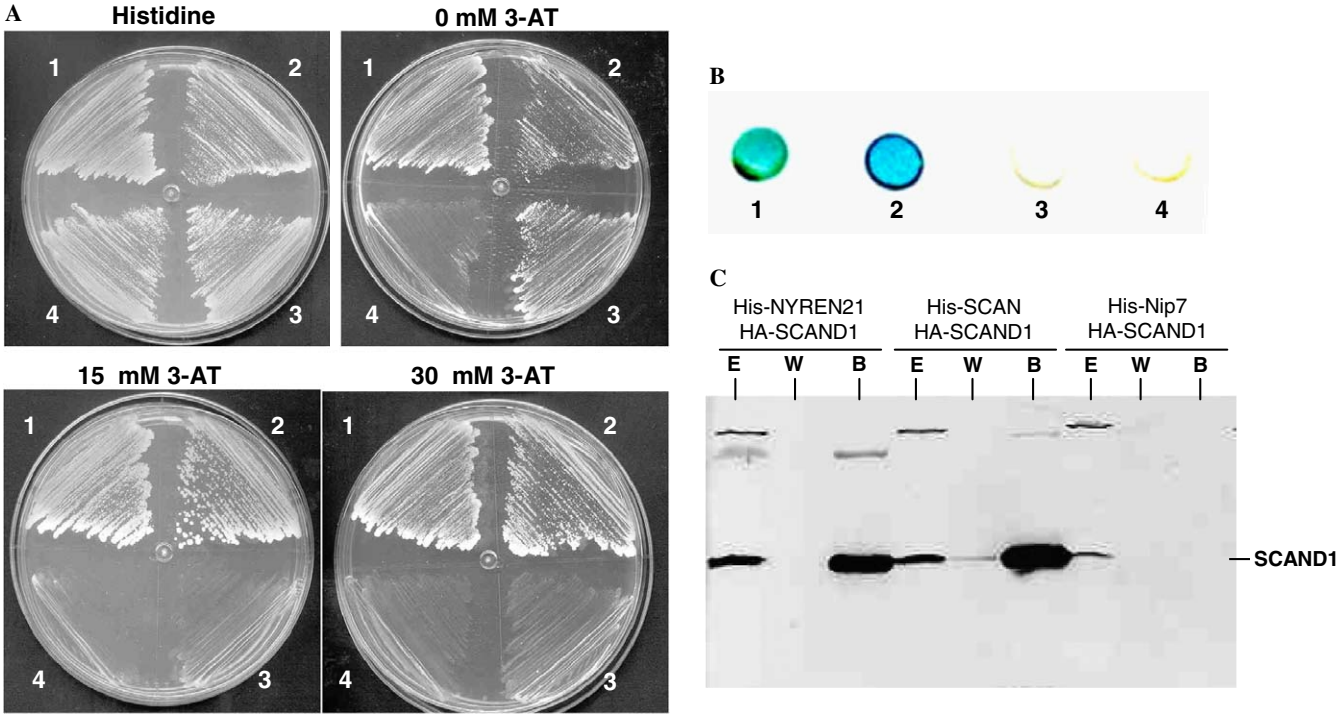

Fig. 7. Interaction between SCAN/NY-REN-21 and SCAND1. (A,B) Yeast two-hybrid interaction assay on synthetic minimal medium (containing histidine 0, 15 or 30 mM of 3-AT) and β-galactosidase filter assay, respectively. 1, Test strain containing the SCAN domain of NY-REN-21 fused to the DNA binding domain of *lexA* (DB-SCAN) and SCAND1 fused to the *GAL4* activation domain (AD-SCAND1); 2, positive control strain containing DB-SCAN/AD-SCAN; 3 and 4, negative control strains containing the combinations DB/AD-SCAND1 and DB-Nop8/AD-SCAN, respectively. (C) "Pull-down" assay of His-NY-REN-21/HA-SCAND1, His-SCAN/HA-SCAND1 and His-Nip7p/HA-SCAND1 (negative control). The histidine-tagged proteins were purified by affinity chromatography and the interacting HA-tagged SCAND1 (indicated) was detected by immunoblotting with an anti-HA antibody. Samples from the extract (E), the wash fraction (W) and from the resin-bound fraction eluted with imidazole (B) were fractionated by SDS–PAGE followed by immunoblot analysis.

Human SCAND1 and its mouse ortholog PGC-2 [30] are particular members of the SCAN domain protein family because they lack the zinc finger region. As a consequence, the heterodimer formed by NY-REN-21/SCAND1 is asymmetric concerning the DNA binding region since it contains just one zinc finger region and this asymmetric structure may have important implications on the control of gene expression by NY-REN-21. SCAND1 is expressed in a diverse number of tissues with higher levels detected in prostate, kidney, thyroid, liver, and thymus [9,31]. SCAND1 may play an important role in the mechanism of transcription regulation. In addition to NY-REN-21, SCAND1 interacts with the transcription factors MZF1B [7], ZNF202, ZNF191 [9], and with the nuclear receptors PPARγ2, α e δ e ERα [30]. Its interaction with PPARγ2 increased the trans-activation activity of PPARγ2 in transiently transfected cells [30]. Interestingly, SCAND1 and PGC-2 fused to the *GAL4* activation domain resulted in proteins unable to activate or repress reporter genes in transiently transfected HeLa cells. However, a truncated mutant containing the N-terminal region of SCAND1 and lacking the SCAN domain showed trans-activation activity suggesting that the SCAN domain hinders the trans-activation function of SCAND1 [30]. In conclusion, we have presented data showing that the SCAN domain of NY-REN-21 is able to form homodimers and asymmetric heterodimers with SCAND1. There are few reports in the literature on NY-REN-21 function and identifying the interactions among the SCAN family members is essential for the understanding of the regulatory mechanism used by this group of transcription factors to control gene expression.

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