Development and characterization of polyclonal antibodies against the linker region of the telomere-binding protein TRF2

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Background: TRF2 (telomeric repeat binding factor 2) is an essential component of the telomere-binding protein complex shelterin. TRF2 induces the formation of a special structure of telomeric DNA and counteracts activation of DNA damage-response pathways telomeres. TRF2 has a poorly characterized linker region (udTRF2) between its homodimerization and DNA-binding domains. Some lines of evidence have shown that this region could be involved in TRF2 interaction with nuclear lamina.

Results: In this study, the fragment of the TERF2 gene encoding udTRF2 domain of telomere-binding protein TRF2 was produced by PCR and cloned into the pET32a vector. The resulting plasmid pET32a-udTRF2 was used for the expression of the recombinant udTRF2 in E. coli RosettaBlue (DE3). The protein was isolated and purified using ammonium sulfate precipitation followed by ion-exchange chromatography. The purified recombinant protein udTRF2 was injected into guinea pigs to generate polyclonal antibodies. The ability of anti-udTRF2 antibodies to bind endogenous TRF2 in human skin fibroblasts was tested by western blotting and immunofluorescent staining.

Conclusions: In this study, the recombinant protein udTRF2 and antibodies to it were generated. Both protein and antibodies will provide a useful tool for investigation of the functions of the udTRF2 domain and its role in the interaction between TRF2 and nuclear lamina.

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1. Introduction

Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. They protect chromosomes from degradation and prevent activation of reparative systems of the cell, which can recognize chromosome ends as double-stranded breaks. Telomeres' role in cancerogenesis and aging has been the object of numerous studies. Mammalian telomeres consist of TTAGGG repeats. The G-rich strand of telomere contains a single-stranded overhang on its 3'-end. This overhang invades the double-stranded telomeric DNA, base pairing with the C-strand and displacing the G-strand. The strand invasion results in the formation of a large duplex lariat structure named the t-loop. Telomeric DNA is bound with a specific protein complex called shelterin, which is composed of the following six subunits: TRF1, TRF2, POT1, TIN2, TPP1 and Rap1 [1].

Telomere-binding protein TRF2 binds directly to the telomeric DNA. TRF2 is required for the t-loop formation and protects telomeres from non-homologous end joining (NHEJ) and ATM-kinase pathway [2]. TRF2 contains a DNA-binding Myb/SANT domain, homodimerization domain (TRF homology domain (TRFH)), a basic N-terminal domain (B domain or GAR domain) and a poorly characterized linker region, which is named udTRF2 in this study (Fig. 1a). Computer analysis of TRF2 sequence has shown that this domain has a limited similarity to several sequences of rod domains of intermediate filaments, including nuclear lamins [3]. Lamins are the components of the nuclear envelope, so the domain of TRF2 with unknown functions is likely to be responsible for the interaction of telomeres with the nuclear envelope. Investigation of this interaction may shed light on the processes of aging because a mutation in the LMNA gene encoding lamin A leads to Hutchinson–Gilford progeria syndrome (HGPS), which is characterized by premature aging, abnormalities of nuclear membrane and telomeres shortening [4,5].

In the current work, the recombinant protein corresponding to udTRF2 was obtained, and polyclonal antibodies against it were raised in guinea pigs.
2. Materials and methods

2.1. Plasmid construction

The fragment of the TERF2 gene (GenBank accession no. NM_005652.4) encoding the udTRF2 domain of telomere-binding protein TRF2 was produced by PCR. The plasmid vector pCDNA3hTRF2Full containing the full-length human TERF2 gene was used as a matrix for PCR. The plasmid was obtained from Dr. Eric Gilson (Department of Medical Genetics, CHU Nice, France). The following primers were used in PCR: TRF2udNDE (gccatatgtctgagtccgctgcctcaa) and TRF2udHind (gcaagcttttattttgttatattggttgtactgtct). The forward primer, TRF2udNDE, contained a restriction site for NdeI restriction endonuclease and start-codon ATG. The reverse primer, TRF2udHind, contained a restriction site for HindIII restriction endonuclease and stop-codon TAA. The PCR-product was cloned into the cloning vector pTZ57R/T (ThermoScientific). The plasmid was named pTZ57R-udTRF2. The cloned fragment was excised from the plasmid using restriction endonuclease FauNDI, which is an isoschizomer of NdeI, and HindIII and ligated into the pET32a vector, which was digested with the same enzymes. The resulting plasmid was named pET32a-udTRF2 and its sequence was verified by sequencing on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The plasmid was transformed into the expression strain E. coli RosettaBlue (DE3) (Novagen).

2.2. Protein expression and purification

The transformed bacteria E. coli RosettaBlue (DE3)-pET32a-udTRF2 were cultivated on the LB medium supplemented with 50 mg/l ampicillin at 37°C until the optical density of the culture at 500 nm reached 0.5. Expression was induced by adding 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) (Fermentas), and cells were cultured during 2.5 h at 37°C. The control culture was cultivated at the same conditions without IPTG. The resulting plasmid was cloned into the cloning vector pTZ57R/T (ThermoScientific). The plasmid was named pTZ57R-udTRF2. The cloned fragment was excised from the plasmid using restriction endonuclease FauNDI, which is an isoschizomer of NdeI, and HindIII and ligated into the pET32a vector, which was digested with the same enzymes. The resulting plasmid was named pET32a-udTRF2 and its sequence was verified by sequencing on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The plasmid was transformed into the expression strain E. coli RosettaBlue (DE3) (Novagen).

2.3. Polyclonal antibodies production

Two four-month-old male guinea pigs weighing 400–500 g (purchased from Rappolovo nursery, Leningrad region, Russia) were used for immunization. All animal procedures were performed according to the EU Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes. Pre-immunization sera were taken one week before immunization and tested by western blot. The animals were immunized with 20 μg of udTRF2 mixed with the Freund adjuvant three times with 10-day intervals. Injections were performed subcutaneously (first injection) and intramuscularly (second and third injections). The blood samples were taken 10 d after the last injection. The immune antiserum was obtained by centrifugation at 10,000g for 10 min.
2.4. Western blotting

Lysates of induced and control bacterial cultures and human skin fibroblast lysate were separated by 12% SDS-PAGE and transferred to the PVDF membrane (Millipore, Hertfordshire, UK) by Electroblot (BioRad Lab Ltd., Hertfordshire, UK) with the current density 1 mA × sm⁻² for 1 h in an electrophoresis buffer containing 10% ethanol. After transfer, the membrane was incubated in a blocking solution (5% skimmed milk, 0.05% Tween 20, 1 × PBS). The first antibodies were anti-TRF2 (ab4182, Abcam, dilution 1:500), preimmune serum (dilution 1:500) and immune serum anti-udTRF2 (dilutions 1:500, 1:1000, 1:2000). The blots were incubated with antibodies diluted in 1 × PBS for 1 h RT, washed with PBS 3 × 10 min and incubated in the second antibodies (anti-guinea pig or anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody produced in goat (Sigma), dilution 1:10,000). The blots were detected using BCIP-NBT in an alkaline-phosphatase buffer (50 mM Tris–HCl (pH 9.5), 5 mM MgCl₂, 100 mM NaCl) for around 30 min.

2.5. Immunocytochemistry

Human skin fibroblasts were obtained from the Russian Cell Culture Collection (Institute of Cytology RAS, St. Petersburg). The cells were cultivated on glass coverslips in Petri dishes in DMEM supplemented with glutamine, glucose, 10% fetal calf serum and antibiotics. Cells were washed with PBS and fixed in 2% paraformaldehyde with 0.1% Triton X-100 for 20 min at 4°C. After washing with PBS, cells were incubated in the blocking solution (3% BSA in 1× PBS) for 1 h and in antibodies solution (anti-udTRF2 and anti-TRF2 (Abcam), dilutions 1:100). The second antibodies were Alexa-488 goat anti-guinea pig IgG antibody (Molecular Probes). The preparations were mounted in Antifade Gold with DAPI (Molecular Probes) and analyzed with an LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss) equipped with argon (488 nm) and helium–neon (543 nm) laser sets.

3. Results

3.1. Production and purification of recombinant udTRF2

Fragments of the TERF2 gene encoding the linker region of telomeric protein TRF2 (udTRF2-domain) were amplified by PCR. This fragment encodes amino-acid residues from 245 to 445 situated between the homodimerization domain (TRFH) and C-end DNA-binding domain (Myb-domain) (Fig. 1a). Initially, we digested the PCR fragment by restrictases and tried to insert the fragment directly into the expression vector, but we failed to obtain the desired clones. Therefore, the PCR fragment was cloned into the cloning vector pTZ57R/T and then subcloned into the expression vector pET32a. This vector contains tags (two His-tags, Trx-tag and S-tag) which may interfere with the peptide of interest during the production of polyclonal antibodies. To eliminate nucleotide sequences encoding these tags, we used restriction endonuclease FauND I, which has two restriction sites in pET32a flanking the tag sequences. The resulting plasmid pET32a-udTRF2 (Fig. 1b) was transformed into E. coli RosettaBlue (DE3) competent cells.

Protein expression was carried out as described in Materials and methods. After 2.5 h of cultivation, the optical density OD₅₀₀ of the induced culture was half that of the control culture (0.75 vs. 1.5), which meant good expression level. SDS-PAGE analysis of bacterial lysates revealed a major protein band in the induced culture that was not present in the control culture.

The apparent molecular weight of the protein determined by SDS-PAGE was found to be 25 kDa (Fig. 2), which approximately corresponds to the calculated molecular weight of udTRF2. To investigate whether the recombinant protein is expressed in the soluble or insoluble state, the supernatant and the pellet obtained after lysis were analyzed by SDS-PAGE. The analysis showed that the protein is mostly expressed in a soluble form and only a small part of it accumulates in inclusion bodies (Fig. 2).

The recombinant protein udTRF2 was purified from the soluble fraction of bacterial lysate using ammonium sulfate precipitation followed by ion-exchange chromatography (Fig. 3). The purity of the recombinant protein determined by gel analysis in ImageJ was about 95%.

3.2. Generation and characterization of antibodies

The purified recombinant protein udTRF2 was injected into guinea pigs to produce polyclonal antibodies. The immune sera were tested by western blotting as described in Materials and methods. Commercial rabbit polyclonal antibodies against TRF2 (ab4082, Abcam) were used as a control. These antibodies recognize an
Amino-acid sequence within TRF2 that partly overlaps udTRF2. Anti-udTRF2 antiserum appeared to reveal two proteins with apparent molecular weights of 25 kDa and 30 kDa in the induced bacterial culture lysate and one protein of 30 kDa in the control bacterial lysate. Commercial antibodies revealed one 25-kDa protein in the induced bacterial culture lysate (Fig. 4). Hence, the 25-kDa protein is udTRF2. The immune serum also recognized a 70-kDa protein in the human skin fibroblast lysate. The apparent molecular weight of this protein corresponds to the documented molecular weight of TRF2 [6].

The ability of anti-udTRF2 antibodies to bind endogenous TRF2 in human skin fibroblasts was tested by immunofluorescent staining (Fig. 5). Preimmune serum was used as a negative control (data not shown). All experiments were performed in triplicate and repeated at least twice. It was shown that the immune serum could reveal native TRF2 in normal human cells.

4. Discussion

In the current work, we obtained the recombinant protein udTRF2, which represents the still uncharacterized linker region (udTRF2) of the telomere-binding protein TRF2 and used it to generate domain-specific polyclonal antibodies. The antibodies were shown to recognize udTRF2 in the induced bacterial lysate on western blot and the wild-type TRF2 in human cells both on western blot and immunostained preparations.

The telomere-binding proteins TRF1 and TRF2 are far homologs; they bind double-stranded TTAGGG telomeric repeats and have similar domain structure, but they significantly differ by their N-terminal domains and linker regions located between the homodimerization and DNA-binding domains. The TRF2 linker region (udTRF2) differs from that of TRF1 both in amino-acid sequence and length, the TRF2 linker region being nearly 100 amino acids longer. This region is still uncharacterized. Some lines of evidence have shown that this region could be involved in TRF2 interaction with nuclear lamina.

In frog oocytes, protein MTBP/TRF2 appeared to be tightly bound to the nuclear envelope [9]. Antibodies generated against a complex of frog MTBP/TRF2 with telomeric DNA displayed cross-reactivity with keratins, which belong to intermediate filament proteins, as well as nuclear laminas. In further studies, it was found that in mouse embryonic fibroblasts, TRF2 co-localized with lamin B in the nuclear envelope remnants during mitosis [3]. Computer analysis of the TRF2 sequence has shown that the linker region has a limited similarity to several sequences of rod domains of human neurofilament and rat lamin A. This similarity with the rod domain may provide a mechanism for the telomere association with laminas. It has been shown that TRF2 can oligomerize in the presence of telomeric DNA, and this oligomerization is driven by the linker region [10]. Because the rod domain participates in lamin dimerization and network formation [11], oligomerization of TRF2 may be partly specified by sequences resembling the rod domain. Wood et al. [12] have shown that the wild-type TRF2 can interact directly with lamin A, whereas TRF2ΔΔM, a mutant allele lacking the DNA-binding domain of TRF2, cannot. As TRF2 binding to telomeric DNA is indispensable for oligomerization, it is possible that TRF2 oligomerization plays some role in the interaction between lamin A and TRF2.

The interaction between nuclear lamina and telomeres is of great interest in the light of genetic disorders called progerias. Some of these diseases, such as HGPS [13] and atypical Werner syndrome (AWS) [14], are caused by mutations in the LMNA gene and characterized by premature aging phenotype and nuclear envelope abnormalities. In HGPS, a mutation in the LMNA gene leads to the production of progerin, the mutant form of lamin A. It is noteworthy that progerin is expressed in small amounts in healthy individuals [15]. HGPS patients’ cells exhibit altered nuclear morphology (blebbings), loss of peripheral heterochromatin and premature senescence [4]. Furthermore, fibroblasts isolated from patients with HGPS exhibit reduced replicative capacity and rapid telomere attrition in comparison with age-matched controls [5]. Moreover, there seems to be a reciprocal relationship between progerin production and altered telomere biology. For example, induction of telomere dysfunction by expression of a dominant negative TRF2 protein (TRF2ΔΔM) leads to elevated progerin production. Replicative senescence of normal human fibroblasts is also accompanied by an increased level of progerin mRNA, while this effect is not observed during telomere-independent senescence [16].

One of the mechanisms by which lamin A can affect telomere biology is the formation of interstitial telomeric loops (ITLs) when telomeric DNA associates with interstitial telomeric sequences (ITSs) found within nontelomeric DNA [17]. It was shown that interaction between TRF2 and lamin A facilitates ITLs’ formation. In contrast with lamin A, progerin does not interact with TRF2, and HGPS patients’ fibroblasts exhibit a reduced number of ITLs [12]. Thus, further investigations of interaction between TRF2 and lamin A may provide new insights into the molecular mechanisms of aging.

In this study, we generated the recombinant protein udTRF2 and antibodies to it. Both protein and antibodies will provide a useful tool for investigation of the udTRF2 domain role in the interaction between TRF2 and nuclear lamina. For example, co-immunoprecipitation experiments with lamin A will show whether udTRF2 is responsible for the interaction between TRF2 and lamin A. The products obtained in this work will be used to uncover some other possible functions of the udTRF2 domain. In our future investigations, we are planning to test the anti-udTRF2 antibodies by immunoprecipitation and perform co-immunoprecipitation experiments of recombinant udTRF2 with cellular lysate, nuclear lysate and nuclear lamina extract to find proteins that specifically bind udTRF2. The proteins bound to the peptide could be identified using different approaches, such as MALDI and western blot. The antibodies can also be used in a variety
of studies to visualize native TRF2 in cells by immunostaining and western blot.

Ethical approval

All animal procedures were performed according to the EU Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes.

Conflict of interests

The authors declare that they do not have potential conflict of interests.

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