hSnm1B Is a Novel Telomere-associated Protein

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Artemis, a member of the β-CASP family, has been implicated in the regulation of both telomere stability and length. Promoted by this, we examined whether the other two putative DNA-binding members of this family, hSnm1A and hSnm1B, may associate with telomeres. hSnm1A was found to not interact with the telomere. Conversely, hSnm1B was found to associate with telomeres in vivo by both immunofluorescence and chromatin immunoprecipitation. Furthermore, the C terminus of hSnm1B was shown to interact with the TRF homology domain of TRF2 indicating that hSnm1B is likely recruited to the telomere via interaction with the double-stranded telomere-binding protein TRF2.

The ends of human chromosomes are capped and protected by a DNA-protein structure termed the telomere. The DNA portion of human telomeres is composed of a G-rich repeat (TTAGGG) that extends past the complementary C-rich strand, forming a 3’ extension. This extension has been found by electron microscopy to loop back and invade the double-stranded region, forming a large loop structure termed the t-loop (1). The protein portion of telomeres is composed of a core of six proteins termed the telosome or shelterin (2–4), three of which directly bind telomeric DNA, TRF1 (5), TRF2 (6, 7), and hPOT1 (8), and three that associate with the DNA-binding proteins, Tel2 (9), Tpp1 (10–12), and Rapi (13). TRF1 and TRF2 bind double-stranded telomeric DNA (7), whereas hPOT1 binds the single-stranded region of the telomere (8). In addition to this core complex, many other proteins are known to associate and function at the telomere, albeit at a smaller concentration and often indirectly through binding to TRF1 or TRF2 (4).

One protein that influences the stability of the telomere but is not a member of the described core telomeric complex is Artemis. This protein is a member of the β-CASP family of proteins that contain a unique metallo-β-lactamase domain that hydrolyzes nucleic acids (14). Artemis is well established to play a role in non-homologous recombination where it forms a complex with DNA-PKcs, which cleaves the intermediate hairpin loop structure formed during V(D)J recombination and non-homologous end joining (15). However, Artemis-deficient mice have increased telomere fusions, suggesting that this family of proteins plays a role in telomere structure or function (16). Indeed, Artemis deficient cell lines have increased rates of telomeric shortening as well as rapid accumulation of anaphase bridges (17).

Five mammalian proteins belonging to the β-CASP family have been identified, two of which hydrolyze RNA (18–21), whereas the remaining three, Artemis, Snm1A, and Snm1B, are believed to function solely on DNA substrates (22). Snm1A localizes to DNA double-strand breaks after ionizing radiation; although cells lacking Snm1A are sensitive only to mitomycin C and not strastates (22). Snm1A localizes to DNA double-strand breaks after ionizing radiation (23, 24). Snm1A co-localizes with the DNA damage response protein 53BP before and after exposure to ionizing radiation (23). Snm1A is also highly up-regulated during mitosis and is believed to function as a checkpoint protein during early mitosis (25, 26). Snm1A knock-out mice have increased susceptibility to infection and tumorigenesis (27), further suggesting a role in DNA repair and proper immune function similar to Artemis.

Snm1B is the least well understood of the DNA-binding β-CASP family members. Knock-out of Snm1B in chicken cells and small interfering RNA against Snm1B in human cells both result in mild sensitivity to interstrand cross-linking agents (28, 29). Knockdown of Snm1B by small interfering RNA induces an increase in aberrant metaphase morphology in human cells (29).

The importance of Artemis on telomere stability and telomere length regulation led us to examine whether the other two DNA-binding members of the β-CASP family, human (h)2 Snm1A and hSnm1B associate with telomeres.

EXPERIMENTAL PROCEDURES

Cell Culture—The transformed human embryonic kidney cell line, 293T, was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Plasmids—hSnm1A was PCR-amplified from plasmid clone (ATCC clone 7286032) to include a 5’-FLAG epitope sequence and subcloned into pBabe-Puro. YFP was PCR amplified from pEYFP-C3 (Clontech) and subcloned in frame at the N terminus of FLAG-hSnm1A to generate YFP-FLAG-hSnm1A. The sequence of hSnm1A was verified by DNA sequencing. hSnm1B was obtained by reverse transcriptase PCR amplification of dT primed 293T RNA and subcloned in frame into pEGFP-C1 (Clontech). The sequence of hSnm1B was verified by DNA sequencing. hSnm1A or hSnm1B truncation mutants that result in fragments from amino acids 413–532, 463–532, 496–532, and 363–495 were generated by PCR and subcloned in frame with GFP lacking a stop codon, generating pBabePuro-GFP-hSnm1B or mutants thereof.

pcDNA3-myc-TRF2 was a kind gift from Dominique Broccoli. The following truncation mutants of TRF2 were generated by PCR (numbers refer to the corresponding amino acid region amplified), N-terminally tagged with myc by cloning into pcMV-myc (Clontech): TRF2 (2–100), TRF2 (101–200), TRF2 (301–400), TRF2 (401–500), TRF2 (401–500), and TRF2 (269–445).

Visualization of YFP-tagged hSnm1A, GFP-tagged hSnm1B, and TRF2—293T cells grown on coverslips coated with 100 mg/ml poly-L-lysine, M≥300,000 (Sigma), were transiently transfected with either pBabe-YFP-FLAG-hSnm1A or pBabe-GFP-hSnm1B using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s protocols. After 48 h, 293T cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. To visualize TRF2, 293T cells were then treated with 0.5% Nonidet P-40 in 1× PBS and incubated with the anti-human TRF2 monoclonal antibody (Imgenex IMG-124A) at 1:5000 dilution. The primary antibody was detected with the rhodamine (TRITC)-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories). The cells were then washed twice with PBS, mounted, and observed using the 100× objective lens on an Olympus IX70 confocal microscope.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitations were performed as described previously (30) with the following modifications: a Branson sonifier microtip (Branson Ultrasonics) was used for sonication (output 3; duty cycle, 30% for five 1-s bursts), after which insoluble material was pelleted by microcentrifugation (13,000 × g for 5 min at 4 °C), and the remaining lysate was diluted in lysis buffer (1:2). 30 µl of 50% slurry of GammaBind G-Sepharose (Amersham Biosciences) was added to the lystate and incubated at 4 °C for 1 h to preclear the lystate. The lystate was then transferred to new tubes and immunoprecipitated overnight with 4 µg of anti-GFP monoclonal antibody (Roche Diagnostics). Finally, dot blots were hybridized with a 32P-labeled oligonucleotide telomeric probe (TAAGT)6 in Church’s buffer overnight at 50 °C followed by two washes with 4× SSC containing 0.1% SDS. After 5 days of phosphoimaging the dots were then stripped and probed with an α satellite probe (derived from plasmid pBSH2) (31) in Church’s buffer overnight at 60 °C followed by two washes with 0.1× SSC containing 0.1% SDS. Hybridization of the probes was confirmed with 10 µg of total genomic DNA blotted on each membrane.

Immunoprecipitations—293T cells were seeded in 6-cm tissue culture dishes and transfected with 3 µg of plasmid. Nuclear lysates were collected 48 h after transfection and immunoprecipitated overnight with 4 µg of anti-GFP monoclonal antibody (Roche Diagnostics). Bound proteins were then collected on GammaBind G-Sepharose, washed in radioimmune precipitation assay buffer, and denatured by boiling in SDS sample buffer. The proteins were then separated by polyacrylamide gel electrophoresis and immunoblotted with the appropriate antibodies.
ate antibody. Anti-myc antibody (Invitrogen) was used to detect myc-TRF2 and myc-TRF2 truncation mutants. Anti-GFP monoclonal antibody (Roche Diagnostics) and rabbit anti-GFP polyclonal antibody (Santa Cruz Biotechnology) were used to detect YFP-FLAG-hSnm1A, GFP-hSnm1B, and GFP-hSnm1B truncation mutants.

RESULTS

hSnm1B, but Not hSnm1A, Localizes to the Telomere—Perturbing Artemis function in mice leads to telomere defects (16, 17). However, Artemis is only one of three putative DNA-binding proteins containing β-lactamase domains, the remaining two being hSnm1A and hSnm1B (22). hSnm1A and hSnm1B have also been found to localize to discrete punctate nuclear bodies (28), reminiscent of punctate nuclear localization seen with telomeric proteins (7). We therefore tested whether hSnm1A or hSnm1B localized to the telomere. To examine whether these proteins associate with telomeres in human cells, we assayed whether ectopic hSnm1A or hSnm1B co-localize with the known double-stranded telomeric-binding protein TRF2. Specifically, human 293T cells were transfected with either FLAG-hSnm1A or hSnm1B N-terminally tagged with YFP or GFP, respectively, for visualization purposes. The localization of these fluorescent proteins was then compared with that of endogenous TRF2, as measured by immunofluorescence. As reported previously, both YFP-FLAG-hSnm1A and GFP-hSnm1B localized to discrete punctate nuclear bodies (28). However, only GFP-hSnm1B co-localized with endogenous TRF2 at the telomere (Fig. 1). These data support the notion that hSnm1B associates with telomeres.

To independently confirm the localization of hSnm1B to telomeres, we tested whether hSnm1B associates with telomeric chromatin in vivo by chromatin immunoprecipitation. GFP-hSnm1B was transiently expressed in 293T cells for 48 h, and cells were cross-linked, lysed, the chromatin sheared, and the tagged protein immunoprecipitated with GFP antibody. To detect associated telomeric DNA, DNA was purified from the immunoprecipitate, blotted on a membrane, and hybridized with a telomeric probe to determine specific protein-telomere interactions. As a control for nonspecific association with telomeric DNA, GFP and YFP-FLAG-hSnm1A, both of which are not found to be associated with telomeric DNA by immunofluorescence (Fig. 1 and data not shown) were similarly expressed in 293T cells and analyzed for association with telomeric DNA in parallel with GFP-hSnm1B. We also stripped and re-hybridized the membranes with a centromeric probe to control for nonspecific protein-DNA interactions. Finally, to ensure that interactions with telomeric DNA were specific, non-cross-linked controls were also subject to chromatin immunoprecipitation (Fig. 2A). We found that hSnm1B co-immunoprecipitated specifically with telomeric DNA (Fig. 2, A and B), as a strong signal was detected when hSnm1B immunoprecipitates were hybridized with a telomeric probe but not the centromeric probe (Fig. 2, A and C), and only background signal was detected with the telomeric probe in the absence of crosslinking. Similarly, immunoprecipitates from control GFP and YFP-FLAG-hSnm1A gave background signals with both the telomeric and centromeric probes (Fig. 2, A–C). We thus conclude that hSnm1B associates with telomeres in vivo.

The C Terminus of hSnm1B Binds TRF2—Given that the immunofluorescence and chromatin immunoprecipitation experiments demonstrate that hSnm1B associates with telomeres in vivo, we sought to determine how hSnm1B is tethered to telomeres. We explored the possibility that hSnm1B localizes to the telomere via protein-protein interaction. Since TRF1, TRF2, and hPot1 are the core proteins directly associating with telomeric DNA, and
these proteins are known to serve as a scaffold for proteins to telomeres (4), we first tested whether they associate with hSnm1B. 293T cells were transiently transfected with GFP-hSnm1B, immunoprecipitated with an anti-GFP monoclonal antibody, and blotted with an anti-TRF1, TRF2, or hPot1 antibody to detect for an association with endogenous TRF1, TRF2, or hPot1, respectively. Of these, only TRF2 appeared to co-immunoprecipitate with hSnm1B (Fig. 3A and data not shown). This interaction was specific to hSnm1B, as transiently expressed YFP-FLAG-hSnm1A did not co-immunoprecipitate with TRF2 (Fig. 3C).

To further explore the association of TRF2 with hSnm1B, we mapped the TRF2-binding domain on hSnm1B. We focused on the C-terminus of hSnm1B, as this region is not conserved with hSnm1A, which does not associate with telomeres. Three progressively larger N-terminal truncation mutants of hSnm1B (413–532, 463–532, 496–532) were generated and N-terminally tagged with GFP (Fig. 3B), transiently expressed in 293T cells, after which the mutant proteins were immunoprecipitated with GFP monoclonal antibody and immunoblotted with an anti-TRF2 antibody to detect endogenous TRF2 and data not shown). This interaction was specific to hSnm1B, as transiently expressed YFP-FLAG-hSnm1A was unable to co-immunoprecipitate with TRF2. We next tested whether the TRF2-binding region of hSnm1B was required for telomere association in vivo by chromatin immunoprecipitation. Specifically, the GFP-hSnm1B-(363–495) was transiently expressed in 293T cells, immunoprecipitated with an anti-GFP monoclonal antibody, and associated telomeric DNA detected by hybridization with a telomeric probe. Unlike full-length hSnm1B, the TRF2-binding mutant did not co-immunoprecipitate with telomeric DNA (Fig. 2). Based on these in vitro and in vivo experiments, we conclude that hSnm1B is localized to telomeres via an interaction with TRF2.

hSnm1B Interacts with the TRFH Domain of TRF2—Because TRF2 is known to interact with a number of telomere-binding proteins, we were interested in determining what domain of TRF2 was required for the interaction with hSnm1B. A panel of N-terminal myc-tagged TRF2 truncation mutants were generated (Fig. 4A) and assayed for association with hSnm1B. Specifically, 293T cells were transiently transfected with wild-type or mutant myc-TRF2 and GFP-hSnm1B, after which hSnm1B was immunoprecipitated with an anti-GFP monoclonal antibody followed by immunoblotting with an anti-myc antibody to detect associated ectopic TRF2. Only full-length hSnm1B, the TRF2-binding mutant did not co-immunoprecipitate with telomeric DNA (Fig. 2). All of these mutants were able to co-immunoprecipitate endogenous TRF2 except mutant GFP-hSnm1B-(363–495). YFP-FLAG-hSnm1A was unable to co-immunoprecipitate TRF2.

FIGURE 2. hSnm1B binds telomeric DNA in vivo. A, 293T cells were transiently transfected with GFP alone, YFP-FLAG-hSnm1A, GFP-hSnm1B, or GFP-hSnm1B-(363–495) and either treated with formaldehyde to crosslink proteins and DNA or left untreated as a control. The cells were then subjected to chromatin immunoprecipitation with an anti-GFP monoclonal antibody followed by Southern blotting with a telomeric probe. As a control, membranes were also stripped and re-hybridized with a centromeric probe to determine nonspecific DNA interactions. B and C, graphical quantification of telomeric (B) or nonspecific centromeric hybridization (C) signals of the indicated chromatin-immunoprecipitated proteins. Only hSnm1B specifically immunoprecipitated telomeric DNA.

FIGURE 3. TRF2 Interacts with the C Terminus of hSnm1B. A, 293T cells were transiently transfected with GFP-hSnm1B and immunoprecipitated with GFP monoclonal antibody. The resultant immunoprecipitates were separated on a polyacrylamide gel and blotted with either GFP monoclonal antibody (top) or TRF2 monoclonal antibody (bottom). GFP-hSnm1B was found to co-immunoprecipitate with endogenous TRF2. Control mock-transfected cells were unable to co-immunoprecipitate TRF2. B, to elucidate the TRF2-binding domain, the indicated GFP-tagged hSnm1B truncation mutants were generated. C, 293T cells were transiently transfected with YFP-FLAG-hSnm1A, GFP-hSnm1B, or a GFP-tagged truncation mutant of hSnm1B, followed by immunoprecipitation with GFP monoclonal antibody, separation on a polyacrylamide gel, and blotting with either GFP polyclonal antibody (top) or TRF2 monoclonal antibody (bottom). All of these mutants were able to co-immunoprecipitate endogenous TRF2 except mutant GFP-hSnm1B-(363–495). YFP-FLAG-hSnm1A was unable to co-immunoprecipitate TRF2. (Fig. 3C). All three progressively shorter C-terminal fragments co-immunoprecipitated with TRF2, including GFP-hSnm1B-(496–532), the polypeptide encompassing the last 37 amino acids of the protein. This suggests that the most terminal amino acids are responsible for the TRF2 interaction. In agreement, a mutant lacking these C-terminal 37 amino acids, GFP-hSnm1B-(363–495), was not capable of binding TRF2.

We next tested whether the TRF2-binding region of hSnm1B was required for telomere association in vivo by chromatin immunoprecipitation. Specifically, the GFP-hSnm1B-(363–495) was transiently expressed in 293T cells, immunoprecipitated with an anti-GFP monoclonal antibody, and associated telomeric DNA detected by hybridization with a telomeric probe. Unlike full-length hSnm1B, the TRF2-binding mutant did not co-immunoprecipitate with telomeric DNA (Fig. 2). Based on these in vitro and in vivo experiments, we conclude that hSnm1B is localized to telomeres via an interaction with TRF2.
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**FIGURE 4.** The TRF2 homology domain is required for interaction with hSnm1B. A, to elucidate the hSnm1B interaction domain on TRF2, the indicated N-terminal myc epitope-tagged TRF2 truncation mutants were generated. E. coli cells were transiently transfected with GFP-hSnm1B and either myc-TRF2 or a myc-tagged TRF2 truncation mutant, followed by immunoprecipitation with GFP monoclonal antibody. The immunoprecipitates were then separated on a polyacrylamide gel and blotted with either GFP monoclonal antibody (top) or myc antibody (middle). **5% of the nuclear extracts were bottom.** hSnm1B was found to strongly interact only with wild-type TRF2 and the region of TRF2 containing the complete TRF2 homology domain.

hSnm1B or produces a conformational change within hSnm1B that fosters immunoprecipitation.

**DISCUSSION**

We have shown that hSnm1B is a novel telomere-associated protein. In addition, we have shown that hSnm1B co-immunoprecipitates with the double-stranded telomere-binding protein TRF2. This interaction occurs via a TRF2 interaction domain on the C terminus of hSnm1B. We speculate that hSnm1B is recruited to the telomere via the interaction with TRF2, although we have not yet determined whether this interaction is direct or indirect. What hSnm1B does at the telomere can only be speculated at this point. On one hand, since Artemis can cleave single-stranded DNA loop structures (15), it is possible that hSnm1B may play a role in processing the telomeric t-loop structure. Alternatively, as the budding yeast SNM1 possesses 5’-exonuclease activity *in vitro* (33, 34), perhaps hSnm1B generates the single-stranded 3’ overhang at the end of the telomere. Nevertheless, the association of hSnm1B with telomeres argues that this protein is likely performing some function critical to telomere structure or function.

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