The Telomeric Poly(ADP-ribose) Polymerase, Tankyrase 1, Contains Multiple Binding Sites for Telomeric Repeat Binding Factor 1 (TRF1) and a Novel Acceptor, 182-kDa Tankyrase-binding Protein (TAB182)*

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Tankyrase 1, a human telomeric poly(ADP-ribose) polymerase, was originally identified through its interaction with TRF1, a negative regulator of telomere length. Tankyrase 1 ADP-ribosylates TRF1 in vitro, and its overexpression induces telomere elongation in human cancer cells. In addition to its telomeric localization, tankyrase 1 resides at multiple subcellular sites, suggesting additional functions for this protein. Here we identify TAB182, a novel tankyrase 1-binding protein of 182 kDa. TAB182 displays a complex pattern of subcellular localization. TAB182 localizes to the nucleus in a heterochromatic staining pattern and to the cytoplasm, where it co-stains with the cortical actin network. TAB182 coimmunoprecipitates with tankyrase 1 from human cells and serves as an acceptor of poly(ADP-ribose)ylation by tankyrase 1 in vitro. Like TRF1, TAB182 binds to the ankyrin domain (comprising 24 ankyrin repeats) of tankyrase 1. Surprisingly, dissection of this domain reveals multiple discrete and overlapping binding sites for TRF1 and TAB182. Thus, we demonstrate five well conserved ankyrin repeat clusters in tankyrase 1. Although each of the five ankyrin repeat clusters independently binds to TRF1, only three of the five bind to TAB182. These findings suggest that tankyrase 1 may act as a scaffold for large molecular mass complexes made up of multiple binding proteins. We discuss potential roles for tankyrase 1-mediated higher order complexes at telomeres and at other subcellular sites.

Tankyrase 1 is a member of the growing family of poly(ADP-ribose) polymerases (PARPs).† PARPs catalyze formation of long chains of poly(ADP-ribose) onto protein acceptors using nicotinamide adenine dinucleotide (NAD⁺) as a substrate (reviewed by Ref. 1). The net effect of the negatively charged polymers is to drastically alter the properties of the protein acceptor. Over the last few years multiple members of the PARP family have been identified (reviewed by Ref. 2). PARP family members have unique substrates and display complex patterns of subcellular localization throughout the nucleus and cytoplasm. The homology between tankyrase 1 and the PARPs is limited to the catalytic domain, with no homology outside this region (3). Instead, in addition to its catalytic PARP domain, tankyrase 1 contains a domain of 24 ankyrin (ANK) repeats, making it a member of the ankyrin family of structural proteins (reviewed by Ref. 4). The 33-amino acid ANK repeat motif mediates protein-protein interactions (reviewed by Ref. 5).

Tankyrase 1 was originally identified in a two-hybrid screen with the telomeric DNA-binding protein TRF1 (6), a negative regulator of telomere length (7). TRF1 is believed to act in cis at telomeres to regulate access of telomerase, the reverse transcriptase that maintains chromosome ends (reviewed by Refs. 8 and 9). Tankyrase 1 colocalizes with TRF1 to telomeres in metaphase spreads (3). Tankyrase 1 uses its ankyrin domain to bind TRF1 and its PARP domain to ADP-ribosylate itself and TRF1. Poly(ADP-ribosyla)tion of TRF1 by tankyrase 1 inhibits the ability of TRF1 to bind telomeric DNA in vitro (3). Overexpression of tankyrase 1 in the nucleus releases TRF1 from telomeres and induces telomere elongation, indicating tankyrase 1 as a positive regulator of telomere length (10).

In addition to its telomeric location, tankyrase 1 resides at other sites in the cell. Subcellular fractionation, immunofluorescence, and immunoelectron microscopic analysis revealed tankyrase 1 at the nuclear envelope, specifically on the cytoplasmic fibers of nuclear pore complexes in HeLa cells (11). During nuclear envelope breakdown at mitosis, tankyrase 1 relocalizes to mitotic centrosomes (11). Tankyrase 1 also localizes to GLUT4 storage vesicles in the Golgi complex in adipocytes (12). Consistent with this localization, tankyrase 1 binds to insulin-responsive aminopeptidase, a resident GLUT4 vesicle protein, in vitro (12).

Recently, a closely related homolog of tankyrase 1 (83% overall identity), termed tankyrase 2, was identified (12–17). Tankyrase 2 binds to TRF1 through its ankyrin domain (13, 14) and functions as a PARP in vitro with itself and TRF1 as acceptors of ADP-ribosylation (13). Insulin-responsive amino-

TRF1, telomeric repeat binding factor 1; kb, kilobase(s); GAD, GAL4 activation domain; PBS, phosphate-buffered saline; DAPI, 4,6-diamino-2-phenylindole; HP1γ, heterochromatin protein 1γ; Tx, transfection.

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† The abbreviations used are: PARP, poly(ADP-ribose) polymerase; ANK, ankyrin; AR, ANK repeat; ARC, ANK cluster; GAD, GAL4 activation domain; GST, glutathione S-transferase; hTERT, telomerase catalytic subunit; TAB182, tankyrase-binding protein of 182 kDa;
peptidase, the tankyrase 1-binding protein, also binds to the ankyrin domain of tankyrase 2 (12). And finally, GB14, an endosomal adapter protein, was identified as binding to the tankyrase 2 ankyrin domain (16). Subcellular fractionation showed that tankyrase 2 co-fractionated with GB14 in the low density microscope fraction (16). Whether tankyrase 2, like tankyrase 1, localizes to the Golgi complex or to telomeres remains to be determined.

The identification of multiple tankyrase 1- and tankyrase 2-interacting proteins combined with the complicated subcellular localization pattern for tankyrase 1 suggests an underlying complexity for these proteins. Thus, there are likely to be different intracellular pools of tankyrase 1 or tankyrase 2, which may poly(ADP-ribosyl)ate proteins other than TRF1 and exhibit diverse functions depending on the binding partners. Furthermore, because both tankyrase 1 and 2 contain 24 ANK repeats and a sterile alpha module domain (two protein-protein interacting motifs) (5, 18, 19), tankyrase 1 and 2 have the potential to function as bridges forming large molecular mass complexes made up of different binding proteins.

In this study, we have identified a novel tankyrase-binding protein of 182 kDa, (TAB182), which binds to the ankyrin domain of tankyrase 1 or tankyrase 2. Immunofluorescence analysis showed a complex pattern of subcellular localization; TAB182 (like tankyrase 1) resided in the nucleus and cytoplasm. Cytoplasmic TAB182 co-stained with cortical actin. In the nucleus, TAB182 displayed a heterochromatic-like staining pattern and remained associated with chromatin throughout mitosis. Unexpectedly, we found that both TAB182 and TRF1 displayed a nuclear localization pattern and remained associated with chromatin throughout mitosis. TAB182 (like tankyrase 1) resided in the nucleus and cytoplasm. Cytoplasmic TAB182 co-stained with cortical actin. In the nucleus, TAB182 displayed a heterochromatic-like staining pattern and remained associated with chromatin throughout mitosis. Unexpectedly, we found that both TAB182 and TRF1 displayed a nuclear localization pattern and remained associated with chromatin throughout mitosis.

**Experimental Procedures**

Yeast Two-hybrid Screen and Assays—LexA-tankyrase 1 and LexA-tankyrase 2 fusion vectors were constructed by cloning restriction fragments of tankyrase 1 (encoding the amino acids indicated in Fig. 4) and tankyrase 2 (encoding the amino acids 120–518) into BD116 (20). Two-hybrid screens were performed with the yeast reporter strain L40 (21) using the human fetal liver cDNA library (CLONTECH) and the LexA-fused tankyrase 1 (FL, amino acids 1–1327), AR1–21 (amino acids 1–881), or AHPS (amino acids 181–1327) according to CLONTECH Matchmaker protocol. Ten of 22 positive clones contained the same 2.1-kb cDNA, named TAB182C. Of note, TAB182C was the only clone isolated with this bait.

GAL4 activation domain (GAD)-TAB182 fusions were constructed by cloning restriction fragments of ankyrin G (encoding the amino acids 73–681, or 73–881), or AHPS (amino acids 181–1327) according to CLONTECH Matchmaker protocol. Ten of 22 positive clones contained the same 2.1-kb cDNA, named TAB182C. Of note, TAB182C was the only clone isolated with this bait.

**Isolation of TAB182 cDNA**—A DNA database search revealed that two-hybrid isolate TAB182C contained a partial cDNA of a novel gene in genomic clone RP11-872D17 (GenBank accession number AP000781) on human chromosome 11q12. The TAB182C cDNA was used as probe on a Uni-ZAP XR cDNA library made from human placenta (Stratagene). Thirty positive clones were isolated, and the DNA sequence of the longest four clones was analyzed. Among them, the clone BS/TAB182-12 had the most extended 5’ sequence but still lacked the putative first ATG codon. Thus, employing several rounds of 5'-rapid amplification of cDNA ends technology, we obtained the 5’-end of the TAB182 cDNA using a human testis Marathon-Ready cDNA (CLONTECH) and assembled the 5,947-bp full-length cDNA for the TAB182 gene. We isolated the same upstream 239-bp fragment (nucleotides 53–345) containing the first ATG and the upstream in-frame stop codons by PCR from a different source of CDNA (pancreas; data not shown). We confirmed that all clones sequenced identically by two-hybrid screen, λ-ZAP screen, and 5’-rapid amplification of cDNA ends contiguously lay on RP11–872D17.

**Preparation of Glutathione-S-transferase (GST) Fusion Proteins**—For GST vectors for GST-TAB182C and GST-TRF1 were constructed by cloning restriction fragment into pGEX-5X vectors (Amersham BioSciences). Recombinant GST fusion proteins were produced in the bacteria BL21 strain and purified with Glutathione-Sepharose 4B (Amersham Biosciences). For the tankyrase 1 PARP assays, the fusion proteins were eluted by the glutathione elution buffer containing 10 mM glutathione (pH 8.0) and dialyzed against phosphate-buffered saline (PBS) twice. Protein concentration was determined using a protein assay system reagent (Bio-Rad).

**In Vitro Binding Assay**—One microgram of baculovirus-derived tankyrase 1 protein (3) was incubated for 2 h at 4 °C with the GST fusion protein-bound glutathione-Sepharose 4B (containing 1 μg of fusion protein) in TNE buffer consisting of 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The beads were washed with the TNE buffer five times and subjected to SDS-PAGE.

**Western Blot Analysis**—Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 5% skim milk in PBS containing 0.1% Tween 20 and then incubated with the following primary antibodies: rabbit anti-tankyrase 763 (0.5 μg/ml) (raised against full-length baculovirus-derived tankyrase 1, rabbit anti-MyC (0.8 μg/ml, Santa Cruz Biotechnology #sc-789), mouse monoclonal anti-MyC 9E10 (1:40, supernatant), rabbit anti-TAB182 388 (0.5 μg/ml), or rabbit anti-TRF1 415 (0.3 μg/ml) (13). The blots were washed with PBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham Biosciences, 1:2,500). Bound antibody was detected using the enhanced chemiluminescence kit (Amersham Biosciences).

**Tankyrase 1 PARP Assay**—Tankyrase 1 PARP assays were performed essentially as described previously (3); samples containing each GST fusion protein (0.2–4 μg) and baculovirus-derived tankyrase 1 (1 μg) were incubated for 30 min at 25 °C in the reaction buffer containing 50 mM Tris-HCl (pH 8.0), 4 mM MgCl2, 0.2 mM diethiothreitol, 5-aminolevulinic acid (0 or 1 mM), 1.3 μM [γ-32P]NAD+ (4 μCi), and unlabeled NAD+ (0 or 0.1 mM).

**Transfection—**TAB182 constructs (with a Myc epitope tag at the N terminus) were generated by cloning each restriction fragment of Myc-TAB182 (encoding the amino acids indicated in Fig. 6A) into appropriate sites of the pLFC retroviral vector (26). HeLa.2.11 cells, a subclone of HeLa.2.1, were transfected with TAB182 constructs with the total cell lysate. For subcellular fractionation, PBS-washed cell pellets were further washed with 0.1 M glycine (pH 2.2). After immediate neutralization with 1 M Tris (pH 9.5), the peak fractions were dialyzed against PBS.

**Preparation of Human Cell Lysates—**Cells were washed in ice-cold PBS and lysed in TNE buffer for ice on 30 min. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant was collected as the TNE lysate. These lysates were incubated with protein G-Sepharose (Amersham Biosciences) at 4 °C overnight. The resulting supernatants were incubated with either preimmune sera (mouse or rabbit) or specific antibodies (anti-MyC 9E10, anti-tankyrase 1 763, or anti-TAB182 388) for 1 h at 4 °C and further incubated for 1 h in the presence of protein G-Sepharose. The beads were washed with PBS once and with TNE buffer four times and subjected to SDS-PAGE.

**Generation of Affinity-purified Anti-TAB182 388 Antibody**—A New Zealand White rabbit was immunized with purified GST-TAB182C protein under a standard protocol (Covance). The resulting immune serum, anti-TAB182 388, was collected and passed through GST-coupled Sepharose 4B to remove the anti-GST fraction. The flow-through was further applied onto the GST-TAB182C-coupled Sepharose 4B, and the bound fraction was eluted with 0.1 M glycine (pH 2.2). After immediate neutralization with 1 x Tris (pH 9.5), the peak fractions were dialyzed against PBS.
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Consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, and a 1:40 volume of protease inhibitor mixture (Sigma) containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotonin, and sodium EDTA. The pellet was resuspended in buffer A and homogenized on ice with a Dounce homogenizer. After centrifugation at 1,000 × g for 10 min at 4 °C, the supernatant was collected as the cytoplasmic fraction. The resulting nuclear pellet was then washed with buffer A twice and resuspended in TNE buffer.

Retroviral Cell Lines—Amphotropic retrovirus was generated by transfecting pLP/CAB182FL into Phoenix amphotropic cells using standard calcium phosphate precipitation. HTC75 cells (a clonal cell line derived from HT1080 fibrosarcoma cells) (7) were infected with the retrovirus essentially as described (26). Infected cells were selected with 2 μg/ml puromycin. Immunofluorescence analysis of stable cell lines expressing Myc-TAB182FL revealed a heterogeneous population where all cells displayed a cytoplasmic stain but only a subset of cells displayed a nuclear and cytoplasmic stain (data not shown). Clonal cell lines that maintained dual localization of Myc-TAB182FL to the nucleus and cytoplasm in the entire population of cells were isolated (see Fig. 8A).

Immunofluorescence Microscopy—Cells were fixed with 2% paraformaldehyde/PBS for 10 min and permeabilized with 0.5% Nonidet P-40/PBS for 10 min. Cells were blocked in PBS containing 1% paraformaldehyde/PBS for 10 min and permeabilized with 0.5% Nonidet P-40. Cells were washed with PBS and incubated with the following primary antibodies: mouse monoclonal anti-Myc 9E10 (1:2000, Jackson ImmunoResearch), rabbit anti-TAB182 388 (0.2 μg/ml), mouse monoclonal anti-heterochromatin protein 1 (HP1γ) (1:40,000 ascites, Chemicon), or mouse anti-nucleolin 7G2 (1:20,000 ascites, kindly provided by Serafin Pinol-Roma) (28). Primary antibodies were detected with fluorescein isothiocyanate- or tetramethylrhodamine B isothiocyanate-conjugated donkey anti-rabbit or anti-mouse IgG (1:100, Jackson ImmunoResearch). Cortical actin was stained with rhodamine-conjugated phalloidin (1:16, Sigma). DNA was stained with 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI). Images were acquired on a Zeiss Axioplan 2 microscope with a Photo metrix SenSyn camera. Photographs were processed and merged using IPLab software.

Characterization of TAB182 Binding by Two-hybrid Analysis—We used two-hybrid analysis to determine the tankyrase 1-interacting domain in TAB182. Our initial two-hybrid screens indicated that TAB182 bound the ankyrin domain of tankyrase 1. Thus, for these experiments a LexA-tankyrase 1 construct containing ANK repeats (AR) 6–24 (see Fig. 4) was tested for binding to various TAB182 deletion mutants fused to the Gal4 activation domain (GAD). As shown in Fig. 2A, a minimal binding site for tankyrase 1 was mapped to the carboxy end of the acidic domain in TAB182 (construct 9, amino acids 1450–1542). Neither the C-terminal fragment lacking this domain (construct 11) nor the N-terminal fragment (construct 12) of TAB182 interacted with tankyrase 1.

By using the yeast two-hybrid system, we also attempted to determine if TAB182 could bind other relevant proteins (Fig. 2B). TAB182 interacted with the ANK repeat domain (ANK repeats 4–15) of the tankyrase 1 homolog, tankyrase 2. Deletion of the tankyrase 1 binding domain (TAB182ΔTBB) completely abolished tankyrase 2/TAB182 binding, indicating that TAB182 bound to the ankyrin domains of tankyrase 1 and tankyrase 2 in the same way. As shown in Fig. 2B and consistent with previous observations, TRF1 bound to the ankyrin domain of tankyrase 1 (3) or tankyrase 2 (13, 14). TAB182 did not interact with other telomere-associated proteins, TRF1, TIN2 (TRF1-interacting nuclear protein 2) (30), or hTERT (telomerase catalytic subunit) (24, 25). Finally, we show that TAB182 binding is specific for the ankyrin domain of tankyrases since TAB182 (like TRF1) (13) did not bind to the ankyrin domain of another ankyrin family member, ankyrin G (23) (Fig. 2B).

Identification of Five Conserved Motifs in the Ankyrin Domain of Tankyrase 1—As shown above, TAB182 is selectively recognized by the ANK repeats of tankyrase 1 or 2 but not of ankyrin G. Although the ankyrin domains of tankyrases and ankyrins (including ankyrin G, -B, -R) show structural similarities, there are a number of distinguishing features between them. Thus, although tankyrases and ankyrins all contain 24 ANK repeats, ankyrins have mostly perfect 33-amino acid repeats in their ANK repeats, ankyrins have mostly perfect 33-amino acid repeats in their ANK repeats (3) (see Fig. 3D). Ankyrins share 67–78% identity in their ankyrin domains, although the tankyrase 1 ankyrin domain shows only 32–39% identity with ankyrins. These comparisons can be illustrated graphically using 2-dimensional homology plots. For example, comparison of the ankyrin domain of ankyrin G and ankyrin R shows high conservation over the entire domain (Fig. 3A). Note the continuous distribution of dots, which reflects identity of more than 50% between two 10-amino acid sequences at respective positions (Fig. 3A). By contrast, comparison of the ankyrin domain of ankyrin G and full-length tankyrase 1 indicated no continuous homology (Fig. 3B).

Interestingly, we noted that despite the lack of continuous homology, tankyrase 1 and ankyrin G shared five discrete, highly conserved regions (Fig. 3B). Comparison of tankyrase 1 to itself indicated that these regions were also homologous to each other (Fig. 3C). More detailed plots between the 24 ANK
repeats of ankyrin G and each individual ANK repeat of tankyrase 1 (Fig. 3D) showed that highly conserved regions in the ankyrin domain of tankyrase 1 corresponded to clusters of 2–3 ANK repeats: ANK repeats 2–3-4, 7–8-9, 12–13, 16–17-18, and 20–21-22. The ANK repeats in these clusters exhibited a number of conserved features. First, the repeat length was the canonical 33 amino acids (5). Second, each contained a critical proline (Pro^{5}) which is important for proper folding or maintenance of ANK-repeat structure (31, 32). And third, each harbored a histidine (His^{7}), which contributes to inter-repeat stabilization (31, 33). Based on these observations, we designated these five regions as ARCs (ANK repeat clusters) I–V. According to these criteria, tankyrase 2 was also found to have five ARCs in the same way except that Pro^{5} at the ANK repeat 24 was not conserved (data not shown). We suggest that each ARC consists of a core of conserved ANK repeats, required for
FIG. 2. Interaction of TAB182 with tankyrase 1 in yeast. A, yeast two-hybrid analysis between various GAD-TAB182 fragments and LexA-tankyrase 1 (ARS–24, shown in Fig. 4). TB, tankyrase 1 binding site. None of the GAD-TAB182 fusions self-activated the lacZ reporter gene when combined with the empty LexA vector, pBTM116 (data not shown). B, TAB182 binds the ANK repeats of tankyrase 1 and 2 but not ankyrin G. Two-hybrid analysis (colony-lift filter assay) was performed between respective LexA fusions and GAD fusions, as indicated. TAB182N, TAB182C, and TAB182CATB are constructs 12, 1, and 11 in A, respectively. TIN2, TRF1-interacting nuclear protein 2. hTERT, telomerase catalytic subunit. The time when blue color development was observed is indicated in parenthesis. Expression of each LexA and GAD fusion was confirmed by Western blot analysis (data not shown).

FIG. 3. Identification of ARCs in tankyrase 1. A–C, two-dimensional homology plot between 24 ANK repeats of ankyrin G and ankyrin R (A), 24 ANK repeats of ankyrin G and full-length tankyrase 1 (B), and full-length-tankyrase 1 and itself (C). Plots were made with DNASIS Version 2.2 (Hitachi Software); check size = 10, matching size = 5. Matching between equivalent amino acids (e.g. Ser and Thr) was not considered. D, sequential two-dimensional homology plots between 24 ANK repeats of ankyrin G and each of 24 ANK repeats of tankyrase 1. The square represents a plot between 24 ANK repeats of ankyrin G and each single ANK repeat of tankyrase 1 (numbered 1–24, below). ANK repeats of regular size (consisting of canonical 33 amino acids) are indicated by bold numbers. Carets above (deletion) and below (insertion) squares indicate gaps in comparison with the ANK repeat consensus (numbers of inserted/deleted amino acid residues are indicated). The bridge above two adjacent ANK repeats indicates the presence of a conserved histidine that forms a pair of hydrogen bonds, contributing to inter-repeat stabilization. The underlined ANK repeat number has a conserved proline that is important for proper folding or maintenance of ANK-repeat structure.
ligand binding, flanked by less conserved repeats that may serve as "scaffolds," required for proper folding (33, 34) (see below).

**Tankyrase 1 Has Multiple Binding Sites for TRF1 and TAB182**—We next used two-hybrid analysis to determine whether the TAB182 binding site in tankyrase 1 corresponded to a conserved ARC and to determine whether it overlapped with the TRF1 binding site. First, we examined a series of C-terminal truncations in tankyrase 1 (Fig. 4). Constructs consisting of AR1–14 (#4) or AR1–10 (#5) interacted with TAB182 or TRF1. In contrast, continued truncation to yield constructs containing AR1–8 (#6) or AR1–6 (#7) resulted in proteins that could still bind to TRF1 but not to TAB182. Superimposition of the ARCs onto the two-hybrid analysis reveals two independent binding sites within the first 12 ANK repeats of tankyrase 1. Thus, AR1–6 (#7) contains a complete ARC I, which binds exclusively to TRF1 and AR6–12 (#10) contains a complete ARC II, which binds independently to TRF1 or TAB182 (Fig. 4).

Binding of ARC I (AR1–6) to TRF1 was unexpected since we previously mapped the minimal binding site for TRF1 to AR10–18 (#12) in tankyrase 1 (3). Superimposition of the ARCs indicates that AR10–18 contains a complete ARC III, which can independently bind TRF1 or TAB182. As detailed in Fig. 4, by combining two-hybrid analysis with superimposition of the ARC boundaries, we further demonstrate two more ARCs that act as independent binding sites; they are ARC IV (AR15–21, #15), which can bind to TRF1 and TAB182, and ARC V (AR20–24, #16), which binds exclusively to TRF1. Together these results demonstrate the presence of multiple, discrete binding sites in tankyrase 1 for TRF1 and TAB182. Each of the five ARCs can bind to TRF1, whereas only ARCs II, III, and IV bind to TAB182.

**Tankyrase 1 Poly(ADP-ribosyl)ates TAB182 in Vitro**—Having shown that tankyrase 1 and TAB182 bind by two-hybrid analysis, we next asked if the proteins bind directly by using a GST pull-down assay. A C-terminal fragment of TAB182 (TAB182C; see Fig. 1A) was expressed as a GST fusion protein (GST-TAB182C) in *Escherichia coli* and immobilized on glutathione-Sepharose beads. The beads were incubated with baculovirus-derived tankyrase 1, and the matrix-associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-tankyrase 1 antibody. As shown in Fig. 5A, lane 2, the GST-TAB182C fusion protein specifically bound tankyrase 1. As controls we show that a GST-TRF1 fusion protein bound tankyrase 1 (lane 3), whereas GST protein did not (lane 1). Thus, TAB182 or TRF1 bind directly to tankyrase 1 in vitro.

To determine whether TAB182 was an acceptor for ADP-ribosylation, a PARP assay was carried out on the fusion proteins in the presence of the radiolabeled substrate, [*32P*]NAD<sup>+</sup>. In this assay, [*32P*]-labeled ribose is transferred from [*32P*]NAD<sup>+</sup> to a protein acceptor. First, as controls, the GST-TRF1 fusion protein or GST was subjected to a PARP assay in the presence...
Tankyrase Binding to TRF1 and TAB182

**Fig. 5.** Tankyrase 1 poly(ADP-ribosyl)ates TAB182 in vitro. **A**, tankyrase 1 and TAB182 bind directly in vitro. The indicated GST fusion proteins bound to beads were incubated with baculovirus-derived tankyrase 1, pelleted, and fractionated by SDS-PAGE. The bead-bound tankyrase 1 was detected by Western blot (WB) analysis with anti-tankyrase 1 antibody 763. **B**, tankyrase 1 poly(ADP-ribosyl)ates GST-TRF1 but not GST. Samples containing either GST or GST-TRF1 (4 μg) and tankyrase 1 (1 μg) were subjected to an in vitro PARP assay containing 1.3 μM [32P]NAD⁺ in the presence (+) or absence of (−) 1 mM 3-aminobenzamide (3AB). **C**, TAB182 is poly(ADP-ribosyl)ated by tankyrase 1. Samples containing GST-TAB182C (0.2–4 μg) and baculovirus-derived tankyrase 1 (1 μg) were subjected to an in vitro PARP assay containing 1.3 μM [32P]NAD⁺ (+). Two reactions contained unlabeled NAD⁺ (triangle, 0.1 mM lane 5) and 1 mM (lane 6)). One reaction contained 1 mM 3-aminobenzamide (lane 7). B and C, reactions were fractionated on SDS-PAGE and visualized by Coomassie Blue staining (left) or autoradiography (right). A–C, molecular mass markers (kDa) are indicated at the left.

of tankyrase 1. The reaction products were analyzed by SDS-PAGE, followed by Coomassie Blue staining or autoradiography. As shown in **Fig. 5B**, right panel, tankyrase 1 ADP-ribosylated itself (lane 1) or GST-TRF1 (lane 4) but not GST (lane 2). The reaction was specifically inhibited in the presence of 1 mM 3-aminobenzamide (3AB), an inhibitor of PARP activity (lanes 3 and 5).

Having established the PARP assay using a GST-TRF1 fusion protein, we next subjected the GST-TAB182C fusion protein to the same analysis. As shown in **Fig. 5C**, right panel, GST-TAB182C was poly(ADP-ribosylated by tankyrase 1 (lanes 2–4). Inclusion of increasing concentrations of unlabeled NAD⁺ in the PARP reaction resulted in a smear of slower migrating products, indicating longer extension of poly(ADP-ribose) chains on tankyrase 1 and GST-TAB182C (lanes 5 and 6). The reaction was inhibited by 3-aminobenzamide (3AB; lane 7). These observations indicate that TAB182 is a novel acceptor of poly(ADP-ribose)ylation by tankyrase 1.

**TAB182 binds Tankyrase 1 in Human Cells.—**We have shown thus far that TAB182 binds tankyrase 1 in *vivo* using a two-hybrid assay and directly in *vitro* using a GST pull-down assay. We next asked if tankyrase 1 and TAB182 are complexed in human cells. A series of TAB182 expression vectors containing an N-terminal Myc epitope tag (Fig. 6A, transfection (Tx) Tx I–7) were transfected into HeLa cells. Immunoblot analysis of transfected cell lysates confirmed expression of the Myc-TAB182 constructs (Fig. 6B, top panel) and showed that (as expected) endogenous tankyrase 1 was present in all extracts (Fig. 6B, bottom panel). Transfected cell extracts were immunoprecipitated with anti-Myc antibody followed by Western blot analysis with anti-tankyrase 1 antibody. As shown in Fig. 6C, endogenous tankyrase 1 co-immunoprecipitated with full-length TAB182 (Tx 4) or with the C-terminal fragment (Tx 6) of TAB182 that contained the tankyrase 1 binding site. Consistent with the two-hybrid analysis shown in 2A, Myc-TAB182 deletion mutants that lacked the tankyrase 1 binding domain did not co-immunoprecipitate endogenous tankyrase 1 (Fig. 6C, Tx 1–3, 5, and 7). As shown in Fig. 6D, reverse combination of antibody usage (i.e. immunoprecipitation with anti-tankyrase antibody followed by Western blot analysis with anti-Myc antibody) also immunoprecipitated TAB182FL (Tx 4) but not TAB182ΔTB (Tx 5). These findings demonstrate that exogenously expressed TAB182 binds tankyrase 1 in human cells and that this binding requires the tankyrase 1 binding domain.

To characterize the properties of endogenous TAB182 protein, we generated a rabbit anti-TAB182 antibody against the C-terminal domain of TAB182 (see “Experimental Procedures”). Mock or TAB182-transfected HeLa cell lysates were analyzed by Western blot analysis using affinity-purified anti-TAB182 antibody. As shown in **Fig. 7A**, anti-TAB182 antibody detected a polypeptide of greater than 200 kDa in mock-transfected HeLa cell lysates (lanes 3 and 5). This protein co-migrated with transfected Myc-TAB182FL detected with anti-Myc (lane 2) or with anti-TAB182 antibodies (lane 4). Note that although the predicted molecular mass for TAB182 is 182 kDa, both endogenous and exogenous TAB182 migrate at a slightly higher molecular mass in SDS-PAGE (greater than 200 kDa). This anomalous migration in SDS-PAGE may be due to the acidic nature of TAB182. Finally, as expected, the exogenous C-terminal fragment, Myc-TAB182C, was also detected by anti-TAB182 antibody (Fig. 7A, lane 6). Detection was specific for TAB182 because preimmune serum did not detect the TAB182 polypeptides (data not shown).

To determine whether endogenous TAB182 and tankyrase 1 were complexed in human cells, we performed immunoprecipitation analysis. As shown in **Fig. 7B**, left panel, anti-TAB182 specifically coimmunoprecipitated endogenous tankyrase 1 from HeLa cell lysates. Reverse combination of antibody usage (i.e. immunoprecipitation with anti-tankyrase followed by Western blot with anti-TAB182) also coimmunoprecipitated TAB182 (Fig. 7B, right panel).

**TAB182 Localizes to the Nucleus and Cytoplasm.—**To investigate the subcellular localization of TAB182, Western blot analysis was performed on fractionated HeLa cells. As shown in **Fig. 7C**, TAB182 (like tankyrase 1) was present in both the cytoplasmic and nuclear fractions at similar amounts. As expected, TRF1 was found exclusively in the nuclear fraction (35). Interestingly, an additional high molecular mass polypeptide was reproducibly detected with anti-TAB182 antibody in the nuclear fraction. The nature of this high molecular mass protein is unknown, but it was resistant to alkaline phosphatase treatment and was not detected by anti-poly(ADP-ribose) West-
ern blot analysis, suggesting that it does not correspond to a phosphorylated or poly(ADP-ribosyl)ated form of TAB182.

We next used indirect immunofluorescence analysis to determine the subcellular localization of TAB182. First, we determined the localization of exogenously expressed TAB182. Myc epitope-tagged TAB182 was introduced into HTC75 cells by retroviral infection, and stable clonal cell lines were selected. As shown in Fig. 8A, indirect immunofluorescence analysis of a stable clonal HTC75 cell line expressing Myc-TAB182 showed staining of both the nuclear and cytoplasmic compartments. Note, although some clonal lines exhibited dual staining of the nucleus and cytoplasm (Fig. 8A), others showed only a cytoplasmic pattern (data not shown). As shown in Fig. 8A, Myc-TAB182 localized to the cytoplasmic borders, where it co-stained with cortical actin, a sheet of F-actin beneath and juxtaposed to the plasma membrane and concentrated at sites of cell-cell contact. In addition, Myc-TAB182 showed a homogeneous nuclear staining pattern. The nuclear and cytoplasmic staining pattern observed for Myc-TAB182 was corroborated using anti-TAB182 antibody to stain the endogenous protein in HTC75 or HeLa cells. As shown in Fig. 8B, endogenous TAB182 displayed a more punctate nuclear staining pattern than the exogenously expressed protein. We therefore further characterized the nuclear localization of endogenous TAB182.

Comparison of the nuclear staining pattern of TAB182 with DAPI staining of the nucleus indicated that TAB182 co-stained with the DAPI-bright regions of the nucleus including the region surrounding the nucleoli. TAB182 did not localize directly to the nucleolus because co-staining with nucleolin revealed no overlap (Fig. 8C, bottom panel). To determine whether TAB182 was indeed a chromatin-associated protein, we analyzed its localization pat-
tern across the cell cycle in a synchronized population of HeLa cells. As shown in Fig. 8D, TAB182 associated with chromosomes throughout mitosis. A similar staining pattern was observed for HP1γ and other heterochromatic proteins (36) (data not shown). These findings suggest that TAB182 associates with heterochromatin in vivo.

**DISCUSSION**

In this study, we have identified a novel tankyrase 1-binding protein, TAB182. TAB182 binds to both tankyrase 1 and tankyrase 2 by two-hybrid analysis. We show that TAB182 binds directly to tankyrase 1 and that it serves as an acceptor of poly(ADP-ribosyl)ation by tankyrase 1 in vitro. Coimmunoprecipitation analysis indicates that endogenous TAB182 and tankyrase 1 are complexed in human cells. Immunofluorescence analysis reveals a complex pattern of subcellular localization; TAB182 (like tankyrase 1) resides in the nucleus and cytoplasm. Cytoplasmic TAB182 co-stains with cortical actin and concentrates at cell-cell contact sites. In the nucleus, endogenous TAB182 displays a heterochromatic-like staining pattern and remains associated with chromatin throughout mitosis. The complex pattern of subcellular localization of TAB182 is reminiscent of the complexity observed previously for tankyrase 1 (i.e. localized to telomeres, nuclear pore complexes, mitotic centrosomes, and the Golgi complex). Although the function of TAB182 is not yet known, its complex localization suggests that it could be a multifunctional protein, like tankyrase 1.

We have shown that the ankyrin domain of tankyrase 1 contains multiple binding sites for TAB182 and TRF1. Studies suggest that ANK repeats do not fold independently but rather require multiple repeats to form folded domains (33, 34). Our work suggests that the 24 ANK repeats of tankyrase 1-fold into five ARCs, each of which contains 2–3 highly conserved ANK repeats with adjacent scaffolds at both ends. Because other ANK repeat-containing proteins (apart from the ankyrins) usually have only 4–7 ANK repeats (5), it is likely that each single ARC in tankyrase 1 can independently recognize its ligands. In fact, TRF1 was recognized by each of five ARCs of tankyrase 1. It remains to be determined if a single tankyrase 1 molecule can bind multiple TRF1s simultaneously via its multiple ARCs. However, an intriguing implication for the presence of multiple binding sites is that one tankyrase 1 molecule may recognize multiple TRF1 molecules that are tandemly aligned on telomeric DNA (Fig. 9A). In this model, tankyrase 1 could act as a scaffold to generate a higher order structure at telomeres.

Although highly speculative, another potential role for the
multiple TRF1 binding sites in tankyrase 1 could be in meiosis. In mammalian cells during prophase of meiosis I (in a process that may be essential for the pairing and subsequent recombination of homologous chromosomes), telomeres cluster at one pole of the nucleus to form a structure known as the bouquet (reviewed by Scherthan (38)). By tethering multiple TRF1 molecules from different chromosome ends, tankyrase 1 could contribute to telomere clustering during meiosis (Fig. 9B). Indeed, studies indicate that tankyrase 1 and TRF1 are found at meiotic telomeres (39). Potential involvement of tankyrase 1 in meiotic telomere clustering is also suggested by other features of tankyrase 1; tankyrase 1 gene expression is abundant in testis (3), and tankyrase 1 is localized at mitotic centrosomes (11), the region where telomeres cluster during prophase of meiosis I (reviewed by Dernburg et al. (40)).

According to our two-hybrid analysis, three of the ARCs in tankyrase 1 (ARCs II-IV) can bind to either TAB182 or TRF1. Thus, one interesting possibility is that TAB182 and TRF1 could compete for tankyrase 1 binding. For example, binding of TAB182 could block TRF1 binding (Fig. 9C). Alternatively, a single ARC might bind TRF1 and TAB182 without competition via different parts of the ARC surface. So far, due to aggregation of the input proteins, we have been unable to determine whether tankyrase 1/TAB182 binding is competitively inhibited by TRF1.3 Regardless of the mechanism, the presence of multiple binding sites in tankyrase 1 suggests that TRF1 and TAB182 could bind simultaneously to tankyrase 1 (Fig. 9D). In this way tankyrase 1 could act as a scaffold to generate multi-protein complexes. It will be interesting to determine whether a TAB182-tankyrase 1-TRF1 ternary complex can be formed in vitro or if it can be isolated from intact cells. Finally, one would like to know if other tankyrase 1- or tankyrase 2-binding proteins, such as insulin-responsive aminopeptidase (12) and Grb14 (16), are recognized by ARCs. If, in fact, ARCs function as binding units for these proteins, then the results of Lyons et al. (16) indicate that Grb14 (like TAB182) is recognized at least by ARC III but not by ARC V of tankyrase 2.

TAB182 was detected in similar amounts in cytoplasmic and nuclear fractions of HeLa cells. The amount of TAB182 protein in total or nuclear extracts did not differ across the cell cycle, suggesting that each pool of TAB182 might have a discrete function. Cytoplasmic TAB182, which colocalized with cortical actin, could be involved in the signaling or trafficking roles previously suggested for cytoplasmic tankyrase 1(12) or tankyrase 2 (16).

TAB182 localized to the nucleus in a heterochromatic staining pattern and bound to chromosomes throughout mitosis. One striking feature of TAB182 is its highly acidic nature (pI 4.6). This acidity may allow TAB182 to associate with chromatin through binding to basic chromatin proteins, such as histones. One potential function of heterochromatic TAB182 could be to tether tankyrase 1 to chromosomes during mitosis. Although tankyrase 1 has been localized to telomeres (3), the protein does not contain a nuclear localization signal, and its mechanism of localization to telomeres is not known. Thus, tankyrase 1 could bind to chromosomes at the start of mitosis.

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**Fig. 9. Possible implications of multiple TRF1 and TAB182 binding sites in tankyrase 1 (see “Discussion”).** 
A. Tankyrase 1 could act as a scaffold to generate a higher order structure at telomeres where multiple TRF1s are tandemly aligned on the telomeric DNA. B. Tankyrase 1 could contribute to telomere clustering during meiosis by tethering multiple TRF1s from different chromosome ends. C. TAB182 could block the binding sites in tankyrase 1 for other ligands (TRF1). This could negatively regulate functions of tankyrase 1. D. Tankyrase 1 could act as a bridge for TRF1 and TAB182. Similarly, tankyrase 1 could contribute as a scaffold for larger molecular mass complexes made up of different, multiple binding proteins.
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