TANK2, a New TRF1-associated Poly(ADP-ribose) Polymerase, Causes Rapid Induction of Cell Death upon Overexpression*

Received for publication, June 27, 2001, and in revised form, July 13, 2001
Published, JBC Papers in Press, July 13, 2001, DOI 10.1074/jbc.M105968200

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Tankyrase (TANK1) is a human telomere-associated poly(ADP-ribose) polymerase (PARP) that binds the telomere-binding protein TRF1 and increases telomere length when overexpressed. Here we report characterization of a second human tankyrase, tankyrase 2 (TANK2), which can also interact with TRF1 but has properties distinct from those of TANK1. TANK2 is encoded by a 66-kilobase pair gene (TNKS2) containing 28 exons, which express a 6.7-kilobase pair mRNA and a 1166-amino acid protein. The protein shares 85% amino acid identity with TANK1 in the ankyrin repeat, sterile α-motif, and PARP catalytic domains but has a unique N-terminal domain, which is conserved in the murine TNKS2 gene. TANK2 interacted with TRF1 in yeast and in vitro and localized predominantly to a perinuclear region, similar to the properties of TANK1. In contrast to TANK1, however, TANK2 caused rapid cell death when highly overexpressed. TANK2-induced death featured loss of mitochondrial membrane potential, but not PARP1 cleavage, suggesting that TANK2 kills cells by necrosis. The cell death was prevented by the PARP inhibitor 3-aminobenzamide. In vivo, TANK2 may differ from TANK1 in its intrinsic or regulated PARP activity or its substrate specificity.

Telomeres are the repetitive DNA sequences and specialized proteins that cap the ends of linear chromosomes and protect them from end-to-end fusion. In mammalian cells, loss or disruption of a telomere can cause cellular senescence, cell death, or genomic instability, depending on the genotype and cell context. A variety of events can lead to dysfunctional telomeres. Telomeres can be damaged directly by genotoxic agents and/or faulty DNA repair processes. In addition, the telomeric structure can be disrupted by changes in the expression or function of certain telomere-associated proteins. Finally, telomeres can erode owing to the biochemistry of DNA replication, which leaves 50–200 bp1 of 3′-telomeric DNA unreplicated upon completion of each S phase. Thus, in the absence of the enzyme telomerase, or another mechanism to replenish telomeric DNA, proliferating cells progressively lose telomeric DNA and eventually acquire one or more critically short or dysfunctional telomeres (1–3).

Most normal mammalian cells respond to a critically short or dysfunctional telomere by undergoing cellular senescence (4–7). This process results in an irreversible arrest of cell proliferation and striking changes in cell function (8). Dysfunctional telomeres can also induce apoptotic cell death, particularly in cells that harbor mutations in one or more cell cycle or DNA damage checkpoints (9–11). Very little is known about how telomeres signal cells to undergo senescence or apoptosis. However, the recent discovery of a telomere-associated poly(ADP-ribose) polymerase (PARP) (12) provides a potential mechanism by which telomeres transmit signals to cellular proteins that regulate the senescence and apoptotic responses.

PARPs catalyze the formation of branched chains of ADP-ribose polymers on selected proteins, using NAD+ as a substrate (13, 14). Classic PARPs (PARPs 1–3) are activated in response to single- or double-strand DNA breaks, whereupon they ADP-ribosylate a number of proteins, including key regulators of transcription, cell cycle progression, and DNA repair. The ADP-ribosylation is transient and can either stimulate or inhibit the activity of the target proteins. PARP activation provides a rapid, post-translational signal that can halt the transcription and replication machineries and mobilize DNA repair machineries. PARPs are also important for suppressing recombination at DNA ends (15, 16) and participate in anchoring chromatin to the nuclear matrix (17–19), where certain DNA repair and recombination processes appear to occur (20, 21).

Because telomeres are DNA ends that are anchored to the nuclear matrix (19, 22), and appear to elicit a DNA damage response when dysfunctional, PARPs are also thought to participate in telomere maintenance and/or transmitting signals generated by dysfunctional telomeres. Consistent with this view, cells from knockout mice that lack PARP1, a classic PARP encoded by the ADPRT1 gene, have somewhat shorter (30%) telomeres than wild-type cells (15). Interestingly, cell lysates from the knockout mice have residual PARP activity (23). This finding, and the mild telomere phenotype of ADPRT1−/− mice, suggested that one or more PARP distinct from PARP1 may more directly participate in telomere maintenance and/or signaling. Thus far, the strongest candidate for such a PARP is tankyrase (12), referred to here as TANK1.

* This work was supported by grants from the Ellison Medical Foundation and National Institute on Aging Grant AG09909 (to J. C.) and Training Grant AG00266 (to P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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§ The abbreviations used are: bp, base pair; 3AB, 3-aminobenzamide; BAC, bacterial artificial chromosome; DAPI, 4′,6-diamidino-2-phenylindole; NTD, N-terminal domain; ORF, open reading frame; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RLM, RNA ligase-mediated; PAGE, polyacrylamide gel electrophoresis; TANK, telomere-associated, ankyrin-repeat-containing PARP (tankyrase); non-classical PARP; TANK1, tankyrase 1; TANK2, tankyrase 2; UTR, untranslated region; kb, kilobase pair; Ab, antibody; HA, hemagglutinin; CMV, cytomegalovirus.
TANK1 (encoded by the TNKS gene on human chromosome 8) (12, 24) is a non-classic PARP that interacts with and ADP-ribosylates the telomere-binding protein TRF1 (12). TANK1 lacks a nuclear localization signal and shows a predominantly perinuclear and cytoplasmic distribution, although it is found at the telomeres of metaphase chromosomes in cells that overexpress TRF1. In vitro, ribosylation by TANK1 displaces TRF1 from telomeric DNA (12). This finding, and the phenotype of cells that express a dominant negative TRF1 mutant (25), suggested that TANK1 might be a positive regulator of telomere length in telomerase-expressing cells. Indeed, when overexpressed, a nuclear-targeted TANK1 protein increased telomere length in telomerase-positive tumor cells (26).

Here, we describe the characterization of a second tankyrase-like protein, TANK2, recently identified as a Golgi-associated protein (also referred to as TNKL) (27, 28). TANK2 is encoded by a distinct gene (TNKS2) on human chromosome 10. It shares >80% overall amino acid identity, and a similar intracellular distribution pattern, with TANK1. However, TANK2 contains a unique N-terminal domain, which is also conserved in the murine TNKS2 gene. In contrast to TANK1, however, TANK2, when overexpressed, induced rapid cell death with features of necrosis. Although there is as yet no direct evidence that TANK2 has PARP activity, we further show that TANK2-induced cell death is prevented by the general PARP inhibitor 3-aminobenzamide. Our results raise the possibility that TANK2 may be a more active PARP than TANK1 and/or have unique substrate specificities. Either attribute may enable it to signal cell death.

**EXPERIMENTAL PROCEDURES**

*Cells and Cell Culture*—WI-38 and HT1080 cells were obtained as described (29). 82-6 normal human fibroblasts were from Dr. J. Oshima (University of Washington), and VA13 cells were from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum, as described (29).

**Yeast Two-hybrid Screening**—A partial TNKS2 cDNA was cloned from a human fibroblast yeast two-hybrid library using TRF1 as a bait. The library vectors, and screening methods have been described (30). Briefly, we transfected the library into yeast expressing the pGBT9–TRF1 bait vector and screened 10° transformants on selective media containing 3-aminotriazole. Surviving colonies were tested for expression of the lacZ (β-galactosidase) reporter. One colony (pGAD-TANK2–1.7) that survived selection and expressed β-galactosidase contained a 1.7 kb insert, which we subcloned into pGEM-TA and sequenced. The insert was homologous to a central region of the TNKS cDNA. We named the gene encoding the insert TNKS2 and the corresponding protein fragment encoded by pGAD-TANK2–1.7 interacted using yeast two-hybrid analysis. Briefly, TERF1 cDNA fragments cloned into pGPTB9 (30) were introduced into yeast expressing pGAD-TANK2–1.7, and the level of β-galactosidase reporter activity was quantified as described (30).

**Cloning the Complete TNKS2 Open Reading Frame**—We generated a clone containing the TNKS2 open reading frame (ORF) by 5' RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE) and 3'-RACE. To obtain the 3' end, we used 3'-RACE to screen a human placenta Marathon cDNA library (CLONTECH), and we isolated a 2.7 kb fragment that we subcloned into pGEM-TA and sequenced. This fragment had 1 kb of sequence overlap with the pGAD-TANK2–1.7 insert, an additional 1.5 kb of coding sequence, a stop codon, and 200 bp of the 3'-untranslated region (UTR). To obtain the 5' end, we used a 5'-RLM RACE kit (Ambion) to generate randomly primed cDNA libraries from human placenta (CLONTECH) and the human cell lines HT1080 and VA13. We obtained identical 450 bp products from all three libraries, as determined by sequencing. The combined cDNA fragments generated a continuous 3.4 kb ORF, with ~200 bp each of 5'- and 3'-UTR (GenBankTM accession number AF342982). The fragments were assembled into a single cDNA using the polymerase chain reaction (PCR) and restriction enzyme digestion and ligation. The assembled cDNA was cloned into pBluescript2SK+ (Stratagene) and then subcloned into vectors for epitope tagging or expression. The TANK2 ORF identified by RACE was identical to the ORF contained in three α cDNA clones, isolated from a human 293 tumor cell cDNA library by screening with a 347 bp probe corresponding to the TNKS2 ankyrin repeat region. The clones encompassed 6.1 kb of the TNKS2 cDNA. The partial (450 bp) mouse TNKS2 cDNA was isolated from mouse testis poly(A)+ RNA (Ambion) using 5'-RLM RACE and oligodeoxyribonucleotides corresponding to mouse ESTs with strong homology to human TNKS2. To determine the chromosomal localization of human TNKS2, we screened several thousand clones of the BAC clones.

**Northern Analysis**—Poly(A) RNA (10 μg) was isolated (Qiagen) from HT1080 cells, separated, blotted onto a nylon membrane, and hybridized to TNKS- or TNKS2-specific probes, as described (33). Probes were generated by PCR using primers to amplify sequences encoding the unique TANK1 and TANK2 N termini. The primers used are CTC CCA ACC AGC GAG CAGT-3′ and GTC AAC TGG ACC CCC AGT ACC-3′, which amplified a 3-kb fragment from 13 out of 83 hybrid clones. These results were submitted to the Stanford RH server, which predicted the initial clone and identified a human bacterial artificial chromosome (BAC) library (Research Genetics) with a 1.7 kb insert used to localize the TNKS2 gene by fluorescence in situ hybridization to 4',6-diamidino-2-phenylindole (DAPI)-based metaphase chromosomes, as described (31). The TNKS2 location was confirmed and refined by radiation hybrid mapping, as described (32). Briefly, we screened the medium resolution Stanford G3 panel using the primers GAT ACA CTC ACC GGA AAG AAG-3′ and GTG AAC TGG ACC CCC AGT ACC-3′, which amplified a 3-kb fragment from 13 out of 83 hybrid clones. These results were submitted to the Stanford RH server, which predicted the initial clone and identified a distinct gene (TNKS2) on human chromosome 10; GenBankTM accession number AL159707, which had sequence gaps. We filled the gaps by sequencing the appropriate PCR fragments of the BAC clones.

**Chromosomal Localization**—The pGAD-TANK2–1.7 insert was used to localize the TNKS2 gene by fluorescence in situ hybridization to 4',6-diamidino-2-phenylindole (DAPI)-based metaphase chromosomes, as described (31). The TNKS2 location was confirmed and refined by radiation hybrid mapping, as described (32). Briefly, we screened the medium resolution Stanford G3 panel using the primers GAT ACA CTC ACC GGA AAG AAG-3′ and GTG AAC TGG ACC CCC AGT ACC-3′, which amplified a 3-kb fragment from 13 out of 83 hybrid clones. These results were submitted to the Stanford RH server, which predicted the initial clone and identified a human bacterial artificial chromosome (BAC) library (Research Genetics) with a 1.7 kb insert used to localize the TNKS2 gene by fluorescence in situ hybridization to 4',6-diamidino-2-phenylindole (DAPI)-based metaphase chromosomes, as described (31). The TNKS2 location was confirmed and refined by radiation hybrid mapping, as described (32). Briefly, we screened the medium resolution Stanford G3 panel using the primers GAT ACA CTC ACC GGA AAG AAG-3′ and GTG AAC TGG ACC CCC AGT ACC-3′, which amplified a 3-kb fragment from 13 out of 83 hybrid clones. These results were submitted to the Stanford RH server, which predicted the initial clone and identified a distinct gene (TNKS2) on human chromosome 10; GenBankTM accession number AL159707, which had sequence gaps. We filled the gaps by sequencing the appropriate PCR fragments of the BAC clones.

**In Vitro Transcription and Translation**—The TNKS2 cDNA in pBluescript2SK+: (0.2 μg of DNA) TNKS2 cDNA in pBK-CMV (provided by T. DeLange) (1 μg), and tERT cDNA in pGRN125 (1 μg) were added to a combined in vitro transcription/translation reaction containing [35S]methionine and rabbit reticulocyte lysate (Promega), as described (30). Reactions were run for 60 min at 30 °C. The translation products were separated by 6.5% SDS-PAGE and visualized by autoradiography. The in vitro precipitated proteins binding TANK1, TANK2, or epitope-tagged TRF1 (HA-TRF1) were translated and in vitro translated HA-TRF1 in 0.5 ml of binding buffer (20 mM Tris (pH 7.5), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, Sigma mammalian protease inhibitors) and incubated for 1 h at 4 °C. Reactions were precleared with 25 μl of protein A/G beads (Pierce), and the supernatant was incubated with 5 μg of anti-HA antibody for 4 h at 4 °C. Protein A/G beads (25 μl) were added, and after 1 h incubation at 4 °C, the beads were collected by centrifugation and washed with binding buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer and separated on 4–15% SDS-PAGE gels. 35S signals were detected using a PhosphorImager.

**Western Analysis**—Western analysis was carried out as described previously (34), using enhanced chemiluminescence (Amersham Pharmacia Biotech) and autoradiography. Mouse monoclonal antibodies were used to detect V5 (R96025; Invitrogen), FLAG (M2 or M5; Sigma), and PARP (33–3100; Zymed Laboratories Inc.). Mouse monoclonal (575400; Calbiochem) or rabbit polyclonal (described below) antibodies detected both TANK1 and TANK2, as described in the text.

**Antibody Production**—We conjugated a peptidic corresponding to amino acids 660–680 of TANK2 to keyhole limpet hemocyanin, and we used the conjugate to produce polyclonal antiserum in rabbits using a commercial service (Covance). The peptide was also used to generate an affinity column (SulfoLink; Pierce). Rabbit serum was applied to the column and washed with 10 ml PBS-Tri (pH 7.5) containing 500 mM NaCl, 1 ml dithiothreitol, 0.1% Tween 20, Sigma mammalian protease inhibitors) and incubated for 1 h at 4 °C. Beads were washed with 1 ml PBS-Tri and 1 ml of 0.2 M glycine (pH 2.8), neutralized with 1 ml Tris (pH 8), dialyzed against 10 ml PBS-Tri (pH 7.5), and stored at ~80 °C.

**Immunocytochemistry**—We immunostained cells as described previously (30, 35). Briefly, cells grown on coverslips were fixed in 10% formalin, blocked in phosphate-buffered saline containing 1% bovine serum albumin, and stained with the mouse anti-V5 or anti-FLAG
antibodies for 2 h at room temperature. Coverslips were washed and stained with fluorescein-conjugated goat-anti-mouse antibody and mounted in Vectashield containing DAPI (Vector Laboratories). Images were obtained using a cooled charged-coupled device camera connected to an epifluorescence microscope.

**Transfections and Cell Viability Assays**—Cells on coverslips were transfected with the TNKS or TNKS2 cDNAs (in pBK-CMV or pcDNA3.1, respectively) or the CMV-b-galactosidase normalization vector, using FuGene 6 (Roche Molecular Biochemicals), as instructed by the supplier. Transfection efficiency was estimated from CMV-galactosidase-transfected cultures by the fraction of b-galactosidase-positive cells, as described (33). Cell death was assessed by observing the number of adherent cells 6–10 h after transfection, and by staining with MitoCapture (Biovision), a fluorescent lipophilic cationic reagent that assesses mitochondrial membrane permeability, according to the supplier’s instructions. Briefly, cells were incubated with the MitoCapture reagent for 15 min at 37 °C and counted by fluorescence microscopy using a wide band pass filter. Cells with intact mitochondria exhibited focal red cytosolic fluorescence, whereas cells with permeabilized mitochondria exhibited diffuse green cytosolic fluorescence. Cells lacking red fluorescence and having green fluorescence were scored positive.

**FIG. 1.** TANK2. A, schematic representation of the human TANK1 and TANK2 proteins indicating the unique N termini (histidine-proline-serine (HPS) domain in TANK1 and N-terminal domain (NTD) in TANK2) and the conserved ankyrin repeat, sterile a-motif (SAM), and PARP domains. B, nucleotide and amino acid sequences of the 5’ ends of the mouse and human TNKS2 genes, revealing 96% amino acid (aa) identity, and >95% nucleotide (nt) identity in the coding region, but <83% nucleotide identity in the 5’-UTR.

**FIG. 2.** Genomic characterization. A, radiation hybrid map localizing TANK2 to the sequence tagged site marker 10S536 on human chromosome 10. B, genomic organization of TNKS2, determined from analyzing and sequencing chromosome 10 BAC clones, showing the exon-intron boundaries and the transcriptional start and polyadenylation sites. The approximate size of the 5’-UTR is 881 bp. The size of the 3’-UTR is 2369 bp, determined by 467 bp present in the cDNA clones and an additional 1.9 kb present in several ESTs in the GenBank™ data base. The position of the cDNA end present in the λ clones is indicated.
Identification of TANK2—We identified TANK2 in a yeast two-hybrid screen (36) of a human fibroblast cDNA library using TRF1 as a bait (30). The screen yielded several positive colonies, one of which harbored a vector (pGAD-TANK2–1.7) containing a 1.7-kb insert with striking sequence homology to TNKS, a gene encoding the TRF1-interacting protein named tankyrase or TANK1 (12, 27). The 1.7-kb insert spanned the region corresponding to the TANK1 ankyrin repeat domain. Sequencing showed that it shared 81% nucleotide identity to TNKS and 85% predicted amino acid identity to the TANK1 protein. This degree of homology, although highly significant, was sufficiently different to suggest that the 1.7-kb cDNA derived from a distinct, albeit related, gene. We refer to this gene as TNKS2 and the protein encoded by it as TANK2. This gene was recently independently identified (and named TNKL) as encoding a protein (TANK2) that, together with TANK1, associates with the Golgi apparatus (27).

To isolate the entire TNKS2 coding region, we used 5’/H11032-RLM-RACE and 3’/H11032-RACE. We assembled a 3.8-kb cDNA containing a 3.4-kb ORF with putative translational start and stop codons, and ~200 bp each of the 5’- and 3’-untranslated regions (UTRs). The ORF encoded an 1166-amino acid protein that had 85% identity to the ankyrin repeats, sterile motif, and PARP catalytic domain of TANK1 (Fig. 1A). However, TANK2 contained a unique 25-amino acid N-terminal domain (NTD) that replaced the larger histidine-proline-serine (HPS)-rich domain in TANK1 (Fig. 1A). To verify the position of the initiation codon, we isolated a partial mouse TNKS2 (mTNKS2) cDNA encompassing the NTD and part of the 5’-UTR. The NTD was nearly identical between the human and mouse TANK2 proteins, with only 2 conservative amino acid changes in the first 56 residues (96% identity; Fig. 1B). Both the murine and human TNKS2 cDNAs have an ORF that extends downstream of the putative initiation ATG. They share 95% nucleotide identity throughout the 168 residues that encoded the first 56 amino acids, with 6 of the 8 mismatches occurring in the third position of codons. Upstream of the presumed initiation codon, the nucleotide identity declined abruptly (83%), and the sequence mismatches were randomly distributed among all three codon positions (Fig. 1B). This pattern of conservation strongly suggests that our human TNKS2 cDNA contained the translational start site.

Chromosomal Localization and Genomic Organization—To confirm that TANK2 is not encoded by TNKS, we used fluorescence in situ hybridization to map TNKS2 to human chromosome 10q23-24 (not shown). This location was verified by radiation hybrid mapping, which localized TNKS2 more precisely to 10q23.3, and identified the closest marker as D10S536 (Fig. 2A). This position agrees with a report that localized the gene (TNKL) identical to TNKS2 near D10S2170 (37). Since TNKS is located on chromosome 8 (24), these findings verify that TNKS2 is distinct from TNKS.

To determine the genomic organization of TNKS2, we analyzed the High Throughput Genomic Sequence data base and identified TNKS2 sequences encompassing most of the gene,
sient transfection efficiencies into HT1080 cells were generally
vector backbones were pcDNA3.1 and pBK-CMV, respectively. Tran-
Cells were lysed 6–8 h after transfection. SDS-PAGE, as described under
lanes 1–5 cDNAs, or no
lanes 4
Western blotting using affinity-purified Ab-591 (commercial antibody
a human BAC library, obtained four BACs containing the
TNKS2 gene is
/H11011
/HT1080 transiently transfected with either a
TNKS2 (lane 4) or TNKS (lane 5) expression vector (30 μg) were analyzed by
Western blotting using affinity-purified Ab-591 (lanes 1 and 2) or a
commercial antibody (lanes 4 and 5). The TNKS2 and TNKS expression
vector backbones were pcDNA3.1 and pBK-CMV, respectively. Tran-
sient transfection efficiencies into HT1080 cells were generally >30%.
Cells were lysed 6–8 h after transfection.

albeit with some sequence gaps. To close the gaps, we screened a human BAC library, obtained four BACs containing the
TNKS2 gene, and sequenced the relevant segments. The
TNKS2 gene is ~66 kb in size, organized into 26 introns and 27 exons, all of which contain coding sequences and two of which also contain 5′- and 3′-UTR sequences (Fig. 2B). The exons range from 37 to 484 bp, whereas the introns vary greatly in size, from 89 bp to 14 kb.

TNKS2 mRNA Expression—To determine the expression pattern of TNKS2 among human tissues, we probed a Northern blot containing poly(A) RNA from human heart, brain, plasma-
cent, lung, liver, skeletal muscle, kidney, and pancreas with a
cDNA probe corresponding to a 1-kb region encompassing the
ankyrin domain of TANK2. The probe detected a single 6.6-kb mRNA species (Fig. 3A). TNKS2 mRNA was expressed in all the tissues tested (including liver, evident on longer expo-
ures), albeit to varying levels. In addition, a different multi-
tissue Northern blot showed that the mRNA was expressed in spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes (not shown). Thus, the TNKS2 appeared to be ubiquitously expressed among human tissues.

Because the TANK2 probe used in this analysis shared sig-
ificant sequence similarity to TANK1, we designed cDNA probes corresponding to the unique N-terminal domains of TANK1 and TANK2. We hybridized these probes to Northern
blots of polyadenylated RNA from cultured HT1080 fibrosar-
coma cells. As expected (12), the TANK1-specific probe hybrid-
ized to multiple mRNA species, ranging in size from 2 to 9 kb (Fig. 3B). By contrast, the TANK2-specific probe hybridized to a single 6.6-kb mRNA species (Fig. 5B). This size is consistent with the analysis of human tissue RNA (Fig. 3A) and with the size of the TNKS2 cDNA contained in the λ clones. These results verify that TANK1 and TANK2 are produced from different mRNAs and indicate that TNKS2, in contrast to
TNKS, generates only a single major mRNA species.

TANK2 Protein Expression—To verify that the predicted ORF in the TNKS2 cDNA is correct and to characterize the TANK2 protein, we transcribed and translated the TNKS and
TNKS2 cDNAs in vitro, and we analyzed the radiolabeled translation products by SDS-PAGE (Fig. 4A). Both cDNAs pro-
duced a single labeled protein. As expected (12), TANK1 mi-
grated with an apparent molecular mass of 150–160 kDa. TANK2 migrated somewhat faster, with an apparent molecular mass of 130–135 kDa, consistent with the 127 kDa size predicted from the ORF.

To analyze endogenous TANK proteins, we raised a rabbit polyclonal antisemper against a peptide corresponding to amino acids 660–680, a conserved sequence in the ankyrin domains of TANK1 and TANK2. This antisemper (Ab-591) was affinity-
purified and used to analyze Western blots of cell lysates from normal human fibroblasts (WI-38) and the human tumor cell lines HT1080 and VA13 (Fig. 4B). In all cells, the antibody detected two major proteins, which generated signals of nearly equal intensity. These proteins corresponded in size to the in vitro translation products (Fig. 4A) and to those predicted from the TANK1 and TANK2 ORFs. Similar results were obtained with a commercially available antibody (not shown), except the TANK1 signal was about 5-fold more intense than the TANK2 signal. To confirm the identities of the proteins detected by the antibodies, we transiently transfected TANK1 and TANK2 expression vectors into HT1080 cells, and we analyzed cell lysates by Western blotting (Fig. 4B). The TANK1 expression vector generated a prominent 150–160-kDa band, whereas the TANK2 vector generated a prominent 130–135-kDa band.

Interaction with TRF1—Our yeast two-hybrid screen indi-
cated that TANK2, like TANK1, is a TRF1-interacting protein, although previous reports either did not test (27) or could not demonstrate (28) interaction with TRF1. To verify that TANK2 and TRF1 interact, we produced radiolabeled epitope (HA)-
tagged TRF1 (30), untagged TANK1, and untagged or epitope-
tagged TRF1 interact, we produced radiolabeled epitope (HA)-
tagged TRF1 (30), untagged TANK1, and untagged or epitope-
tagged TRF1 (V5 or FLAG)-tagged TANK2 by in vitro transcription and
translation (Fig. 5A). We then combined the HA-TRF1 with the
TANK1 or TANK2 translation products, immunoprecipitated with anti-HA or control antibodies, and we identified proteins in the immunoprecipitates by SDS-PAGE and autoradiography
(Fig. 5B). As expected, anti-HA (Fig. 5B, lane 1), but not control antibody (not shown), precipitated TANK1. In addition, anti-
HA, but not control antibody, also precipitated unmodified or FLAG-tagged TANK2 by in vitro transcription and translation (Fig. 5A). These results indicate that TANK2, like TANK1, interacts with TRF1. We were unable to co-precipitate TRF1 and TANK2 from cell lysates. However, when cells were lysed in non-denaturing immu-
oprecipitation buffers, the majority of TANK2 was found in the insoluble material (not shown). This result suggests that
TANK2 associates with the relatively insoluble nuclear or cy-
toskeleton matrices and may explain why we could not precip-
itate it with TRF1 from non-denaturing cell lysates.

To identify the domain on TRF1 that interacts with TANK2, we used the yeast two-hybrid system (36) to test TRF1 frag-
ments (30) for their ability to interact with the TANK2 frag-
ment encoded by pGAD-TANK2–1.7. TANK2 interacted with a
52-amino acid domain in the TRF1 N terminus (Fig. 5C). This

FIG. 4. In vitro translated and endogenous TANK2. A, coupled in
vitro transcription and translation reactions were carried out in the
presence of [35S]methionine using the TNKS, TNKS2, or hTERT
(human telomerase catalytic component; positive control) cDNAs, or no
cDNA (negative control), as indicated. The products were analyzed by
SDS-PAGE, as described under “Experimental Procedures.” B, protein
extracts from proliferating WI-38, HT1080, and VA13 cells (60 μg)
(lanes 1–3) or HT1080 transiently transfected with either a
TNKS2 (lane 4) or TNKS (lane 5) expression vector (30 μg) were analyzed by
Western blotting using affinity-purified Ab-591 (lanes 1 and 2) or a
commercial antibody (lanes 4 and 5). The TNKS2 and TNKS expression
vector backbones were pcDNA3.1 and pBK-CMV, respectively. Tran-
sient transfection efficiencies into HT1080 cells were generally >30%.
Cells were lysed 6–8 h after transfection.
TRF1 domain was also shown to interact with TANK1 (12). These results suggest that TANK1 and TANK2 bind the same region in TRF1 and thus may compete for interaction with TRF1.

Subcellular Localization—Because Ab-591 and the commercial antibody did not distinguish TANK1 from TANK2, we transfected a V5 (C-terminal) epitope-tagged TANK2 expression vector into cells, and we determined the subcellular localization of the V5 epitope by indirect immunofluorescence (Fig. 6). In both normal human fibroblasts (82-6 in Fig. 6A and WI-38, not shown) and human fibrosarcoma cells (HT1080 in Fig. 6B), TANK2 was detected as punctate perinuclear staining in the cytoplasm and staining at the nuclear boundary. This distribution is similar to that reported for TANK1 (12). It is consistent with localization to Golgi vesicles, as recently reported (27), and the nuclear membrane and pores, as reported for TANK1 (38). Interestingly, TANK2 was more diffusely perinuclear in normal cells, in contrast to a "capped" perinuclear distribution in fibrosarcoma cells (Fig. 6), suggesting possible differences between normal and tumor-derived cells. In mitotic cells, TANK2 localized to the pericentriolar matrix, as observed for TANK1 (Fig. 6C). Fig. 6B shows the staining pattern in HT1080 cells that overexpress TRF1 (30), but an identical staining pattern was seen in unmodified HT1080 cells (not shown). Thus, in contrast to TANK1, TANK2 remained largely perinuclear in TRF1-overexpressing cells.

Overexpression of TANK2 Causes Cell Death—When overexpressed, TANK1 gradually lengthens telomeres in telomerase-positive cells, presumably by inhibiting TRF1 function (26). To determine whether TANK2 affected telomeres, we attempted to stably overexpress it using recombinant retroviruses or DNA transfection. These attempts failed, or yielded clones with low...
expression, in both normal and tumor-derived cells. This result raised the possibility that TANK2 overexpression is lethal to cells.

To test this possibility, we transiently transfected control, TANK1, and TANK2 expression vectors into cells and monitored viability using the MitoCapture probe. This reagent produces red punctate (mitochondrial) fluorescence when the mitochondrial membrane potential is intact (negative with respect to the cytoplasm), and green diffuse cytoplasmic fluorescence when mitochondrial membrane permeability is compromised and the membrane potential collapses (39) (Fig. 7A). The TANK2, but not the TANK1 or control, vector caused a rapid loss of mitochondrial membrane potential within 7 h after transfection (Fig. 7, A and B). At this time, TANK1 and TANK2 were both abundantly expressed (see Fig. 4B). At later times (24–36 h after transfection), virtually no TANK2-expressing cells were detected, as judged by immunostaining for the epitope tag (not shown). These results suggest that overexpression of TANK2, but not TANK1, causes rapid loss of mitochondrial membrane potential, followed by cell death.

We determined the efficiency of TANK2 cell killing by transfecting parallel cultures with a CMV-galactosidase vector and staining for β-galactosidase. Transfection efficiencies were about 10% for normal fibroblasts and 30–40% for HT1080 fibrosarcoma cells (not shown). When normalized for transfection efficiency, overexpressed TANK2 collapsed the mitochondrial membrane potential in >60% of transfected cells within 7 h. At this time, Western analysis showed that TANK1 and TANK2 were highly expressed (Fig. 4B). There was no difference between normal and malignant cells in their susceptibility to killing by overexpressed TANK2 (Fig. 7B). Moreover, TANK2 killed cells regardless of whether it was untagged (Fig. 7) or epitope-tagged at the N (FLAG) or C (V5) terminus (not shown).

To determine whether TANK2-induced cell death had features of apoptosis or necrosis, we assessed the integrity of PARP1, which is cleaved shortly after apoptotic cell death is initiated (40, 41). PARP1 remained intact up to 24 h after HT1080 cells were transfected with the TANK2 expression vector (Fig. 7C), indicating that cell death probably occurs by necrosis.

PARP activity can deplete intracellular NAD+ and subsequently intracellular ATP, thereby causing necrotic cell death (42). Moreover, high PARP activity can cause cells undergoing apoptosis to switch to necrotic cell death, whereas PARP inhibition can cause cells undergoing necrosis to switch to apoptotic cell death (42, 43). To obtain an indication as to whether TANK2-induced lethality depends on its putative PARP activity, we transiently expressed TANK2 in HT1080 cells in the presence of 3-aminobenzamide (3AB), a general PARP inhibitor (44). 3AB substantially diminished TANK2-induced cell death, as indicated by the MitoCapture assay (Fig. 8A). Even 24 h after transfection, very few cells that received the TANK2 expression vector in the presence of 3AB had detached from the culture dish, in contrast to those that did not receive 3AB. 3AB did not interfere with expression of the transfected vector because Western analysis showed that TANK2 was expressed to the same extent in the absence or presence of 10 mM 3AB and that 20 mM 3AB only slightly suppressed expression (not shown). Moreover, the ability of 3AB to protect cells from death was not complete or permanent. TANK2-expressing cells cultured in the presence of 10 mM 3AB eventually died, generally within a few days after transfection (Fig. 8B). These results indicate that inhibition of the putative PARP activity only partially protected cells from TANK2-induced death.

**DISCUSSION**

The classic PARPs (PARPs 1–3) play important roles in the cellular responses to DNA damage. Although many proteins stimulate PARP activity, it is not yet known how PARPs sense DNA damage. PARPs bind DNA, protect free DNA ends, and modify chromatin by ADP-ribosylating proteins such as histone H1 (14). Activated PARPs ADP-ribosylate many proteins, including key components or regulators of DNA replication, tran-
scription, and repair. PARP substrates include DNA polymerases, topoisomerases, and ligases; high mobility group proteins and transcription factors such as FOS; and p53, XRCC1, proliferating cell nuclear antigen, and the DNA-dependent protein kinase catalytic subunit (14, 45). ADP-ribosylation can activate or inhibit protein function, depending on the substrate. Thus, PARPs transduce signals from damaged DNA to cellular machineries that regulate gene expression, cell cycle progression, and DNA repair.

The TANKs and classic PARPs share a number of differences and similarities regarding function. Like the classic PARPs, the non-classic PARPs TANK1 and TANK2 may transduce signals from dysfunctional telomeres and thus play a role in regulating cellular senescence and genomic stability. On the other hand, because TANKs localize predominantly outside the nucleus and interact with Golgi proteins (27, 28), they may have distinct non-nuclear functions. Likewise, TANK1 and TANK2 share both differences and similarities. First, PARPs have not been shown to interact directly with telomeres or telomere-binding proteins, although PARP deficiency causes telomere shortening (15, 46). By contrast, both TANK1 (12) and TANK2 (Fig. 5) interact with TRF1. Although a recent report found no interaction between the TANK2 ankyrin domain and TRF1 in a Gal4/LexA two-hybrid assay (28), we cloned TANK2 from a Gal4/Gal4 two-hybrid screen for TRF1-interacting proteins. We also used two-hybrid analyses to show that TANK2 interacts with the same TRF1 domain that binds TANK1. Moreover, in vitro translated TANK2 and TRF1 co-immunoprecipitated, indicating that these proteins can interact.

Second, PARPs are almost entirely nuclear, having a diffuse nucleoplasmic distribution (40). By contrast, both TANK1 (27, 38) and TANK2 (Fig. 6) localize predominantly to the perinuclear cytoplasm and nuclear boundary, with only a small fraction evident in the nucleus. Both TANKs lack a known nuclear localization motif, and TANK1 is largely extra-nuclear unless TRF1 is overexpressed (38). TANK2, in contrast, did not localize to the nucleus in TRF1-overexpressing cells, despite its ability to bind TRF1. TRF1 may have a higher affinity for TANK1, relative to TANK2, or TANK2 may interact more strongly with perinuclear or nuclear membrane proteins than TRF1. Whatever the case, in the absence of overexpressed TRF1, both TANKs localize predominantly outside the nucleus, suggesting that only a small fraction can associate with telomeres. Immuno-labeling and electron microscopy showed that TANK1 associates with nuclear pores (26). TANK2 had not been identified when this study was performed, and the antibodies might also have recognized TANK2. Until specific antibodies are developed, it is not clear whether TANK1, TANK2, or both localize to nuclear pores.

Recent data (27) suggest that TANKs also localize to Golgi vesicles. Both TANKs interacted with the insulin-responsive aminopeptidase, a component of Glu4 Golgi vesicles. In addition, TANK2 was identified as an interacting partner of GRB14, an Src homology 2 domain-adapter protein, and showed some, but not complete, co-localization with Golgi vesicles (28). We also observed TANK2 in perinuclear foci, consistent with localization to Golgi and possibly other cytoplasmic vesicles. The significance of the localization is not yet known. TANKs may participate in Golgi or endosome vesicle trafficking (28) or may bring proteins to the nuclear membrane for import.

Despite their dissimilar intracellular localization during interphase, both TANK1 and PARP1 were observed at centrosomes during mitosis (38, 47). TANK2 also localized to mitotic centrosomes. Classic and non-classic PARPs may share a role
in centrosome or spindle function or a common storage site during mitosis. In addition, both PARP1 and TANK1 can, under at least some circumstances, influence telomere length. Germ line inactivation of PARP1 in mice modestly reduced telomere length (15), whereas overexpression of nuclear target-ED TANK1 in human tumor cells modestly increased telomere length (26). Finally, both PARP1 and TANK2 cause necrotic cell death when overexpressed or, in the case of PARP1, activated by DNA damage (42). PARP1 is thought to kill cells by depleting intracellular ATP, a consequence of its utilization of activated by DNA damage (42). PARP1 is thought to kill cells by depleting intracellular ATP, a consequence of its utilization of activated by DNA damage (42).

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Acknowledgments—We thank Mieczyslaw Piatyszek, Melissa Greco, Dawne Shelton, Peter Whittier, and Mike Lombardi for assistance in assembling the full-length TANK2 cDNA clone; Joel Bolonick for bioinformatics assistance; and Titia de Lange for the TANK1 cDNA.