Vertebrate tankyrase domain structure and sterile α motif (SAM)-mediated multimerization

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Tankyrases 1 and 2 are two highly related poly(ADP-ribose) polymerases that interact with a variety of cytoplasmic and nuclear proteins. Both proteins have been implicated in telomere length regulation, insulin signalling and centrosome function. To learn more about their mode of action, we have isolated the chicken tankyrase homologues and examined their interaction partners and subcellular location. Cross-species sequence comparison indicated that tankyrase domain structure is highly conserved and supports division of the ankyrin domain into five subdomains, which are each separated by a highly conserved LLEAAR/K motif. Glutathione S-transferase pull-down experiments demonstrated that the ankyrin domains of both proteins interact with chicken telomere repeat factor 1 (TRF1). Analysis of total cellular and nuclear proteins revealed that cells contain approximately twice as much tankyrase 1 as tankyrase 2. Although $\geqslant 90\,\%$ of each protein is present in the cytoplasm, both tankyrase 1 and 2 were detected in the nucleus. The nuclear location together with its ability to interact with TRF1, point to tankyrase 2 having a telomeric function. Yeast two-hybrid and cross-linking experiments show that both tankyrases can multimerize through their sterile-α motif domains. These results indicate that tankyrases may be master scaffolding proteins, capable of regulating assembly of large protein complexes.

Key words: chicken, telomere repeat factor 1 (TRF1), telomere, insulin signalling, poly(ADP-ribose) polymerase (PARP).

INTRODUCTION

Tankyrase 1 and tankyrase 2 are members of the poly(ADP-ribose) polymerase (PARP) family, a group of enzymes that catalyse addition of poly(ADP-ribose) chains on to acceptor proteins using NAD as a substrate [1,2]. These poly(ADP-ribose) chains are negatively charged, so their addition can dramatically alter the activity of an acceptor molecule [2]. In addition to the catalytically active PARP domain, tankyrase 1 and 2 contain 19–24 ankyrin (ANK) repeats and a sterile α motif (SAM) [3–5]. The ANK repeats show homology with a 33-amino-acid repeated motif present at the N-terminus of the cytoskeletal protein ankyrin. Similar repeats are found in over 400 proteins [6], where they typically mediate protein–protein interactions. All known target proteins of tankyrases interact with the ANK repeats. The SAM domain (approx. 70 residues) was originally identified as a conserved motif in yeast, when mutations within this region of several proteins were found to cause sterility [7]. SAM domains have since been found in many different proteins that are involved in development and signalling [8,9]. Like ANK repeats, SAM domains mediate protein–protein interactions, but they have a preference for homo- and heterotypic associations with other SAM domains [9–13]. The presence of both SAM and ANK motifs in tankyrase 1 and 2 indicates that these proteins may bind simultaneously to multiple interaction partners. In addition to the ANK, SAM and PARP domains, tankyrase 1 has an N-terminal homopolymeric region of unknown function that contains stretches of histidine, proline and serine (the HPS domain) [3]. This HPS domain appears to be absent in tankyrase 2 [4,14].

Human tankyrase 1 was first isolated from a two-hybrid screen for proteins that interact with the telomere-binding protein TRF1 (telomere repeat factor 1) [3]. TRF1 is a substrate for tankyrase 1 *in vitro*, and the resultant poly(ADP-ribosyl)ation prevents TRF1 from binding to telomeric DNA. *In vivo*, tankyrase 1 clearly has a telomeric function, as it localizes to telomeres and overexpression causes telomere elongation in a telomerase-dependent manner [3,15,16]. However, tankyrase 1 is also found at centrosomes, where it seems to interact with NuMA (nuclear mitotic apparatus protein) [17,18] in association with nuclear pore complexes [17], and at Golgi-associated GLUT4 vesicles, where it appears to be involved in insulin signalling [19]. Additionally, tankyrase 1 binds TAB182, a newly identified protein that co-localizes with heterochromatin in the nucleus, and with the cortical actin network in the cytoplasm [20]. Human tankyrase 2 was initially isolated as a tumour antigen that elicits an antibody response in some cancer patients [21,22], and subsequently as a factor that interacts with TRF1, the insulin-responsive amino peptidase (IRAP), and Grb14, an SH2 domain-containing adaptor protein that binds to the insulin and fibroblast growth factor receptors [4,14,19]. The varied interaction partners of tankyrase 1 and 2, together with their PARP activity, suggest that they may be effector proteins for signal transduction pathways that regulate a wide range of cellular responses. However, the upstream elements of these pathways remain largely unknown, as does the mechanism for directing tankyrase PARP activity

Abbreviations used: ANK, ankyrin; BS^3, bis(sulphosuccinimidyl)suberimidate; GST, glutathione S-transferase; HPS, N-terminal homopolymeric region of tankyrase 1 containing stretches of histidine, proline and serine; IRAP, insulin-responsive amino peptidase; MAP, mitogen-activated protein; MBP, maltose-binding protein; mSARP2, $Met^{1054} \rightarrow Val$ mutant of SARP2; PARP, poly(ADP-ribose) polymerase; PH, polyhomeotic; RSB, reticulocyte standard buffer; SAM, sterile α motif; SARP, SAM-PARP; TEL, translocation Ets leukaemia; TRF1, telomere repeat factor 1; cTRF1, chicken TRF1.

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The nucleotide sequences of the chicken tankyrase 1, chicken tankyrase 2 and chicken telomere repeat factor 1 genes have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY142108, AY142107 and AY237359 respectively.

to specific target proteins, and the purpose of the resultant poly(ADP-ribosyl)ation.

Tankyrase 1 and tankyrase 2 show a significant functional overlap, both *in vitro* and when overexpressed in cells. Both proteins exhibit *in vitro* PARP activity and poly(ADP-ribosyl)ate their binding partners, IRAP, TAB182 and TRF1, but not the related telomere protein TRF2 [3–5,14,16,19,20]. Moreover, when either protein is overexpressed and targeted to the nucleus by a nuclear localization sequence, TRF1 is removed from the telomere [15,16]. These findings suggest that the two proteins may have at least partially redundant functions. However, it is still unclear whether they are present in identical subcellular locations and hence whether they always function in the same signalling pathways. In particular, it has not been possible to determine whether endogenous tankyrase 2 localizes to telomeres, or even to the nucleus, because of the difficulty in generating high quality tankyrase 2-specific antibody. Thus it remains to be determined whether tankyrase 2 actually has a telomeric function *in vivo*.

There are indications that the two tankyrases may be differentially regulated, suggesting that their function is not fully redundant. Tankyrase 1 is a substrate for phosphorylation by mitogen-activated protein (MAP) kinase and the four canonical MAP kinase phosphorylation sites are located in the HPS domain [19]. The only MAP kinase site in tankyrase 2 is in the SAM domain [5]. Interestingly, tankyrase 2 has higher PARP activity than tankyrase 1 [19], and overexpression of tankyrase 2, but not tankyrase 1, causes rapid PARP-dependent cell death [14]. This variation in PARP activity might result from differences in intrinsic catalytic activity, post-translational modification (e.g. phosphorylation), or from association with different regulatory factors.

To learn more about the function of tankyrase 1 and 2 at their various locations within a cell, we have isolated the chicken tankyrase homologues and have performed a cross-species sequence comparison and biochemical analysis on these proteins. Our analysis supports the division of the ANK domain into 5 sub-domains, separated by a highly conserved motif. When fractionation experiments were used to compare the amount of tankyrase 1 and 2 in cells and nuclei, a significant proportion of each protein was found in the nuclear fraction. Since both proteins also interact with chicken TRF1 (cTRF1), our results indicate that tankyrase 2 is likely to have a telomeric function. Finally, we show that the SAM domains of both tankyrases can self-associate to form high-molecular-mass complexes. Thus both tankyrase 1 and 2 seem to possess the properties needed to act as scaffolding molecules that can regulate formation of a protein lattice.

EXPERIMENTAL

Cell lines

DT40 cells (ATCC CRL-2111) were maintained in RPMI 1640 supplemented with 10 % (v/v) Cosmic Calf Serum (Hyclone; South Logan, UT, U.S.A.), 5 % (v/v) chicken serum, 50 μM 2-mercaptoethanol, 100 units/litre penicillin, 50 μg/ml streptomycin and 2 mM glutamine. LMH cells (ATCC CRL-2117) were maintained in Waymouth medium (Sigma) supplemented with 10 % (v/v) foetal calf serum, 100 units/litre penicillin, 50 μg/ml streptomycin and 2 mM glutamine.

Isolation of chicken tankyrase genes

Chicken tankyrase genes were isolated from a λZAPII chicken embryonic fibroblast cDNA library as recommended by the manufacturer (Stratagene). Sequence alignments were computed with the GCG Wisconsin package (Accelrys, San Diego, CA, U.S.A.). RNA was isolated by lysing DT40 cells in guanidinium thiocyanate and extracting the lysate with acidic phenol. The RNA was reverse transcribed using primer p12-1Rev3 (5′-GCAGAGGAGTGAACTGCC-3′) and Superscript II reverse transcriptase (Gibco/BRL). First strand cDNA was amplified by PCR with primers cTANK2F2 (5′- GTTCCTCCGGGATCCCTC-3′) and cTANK2cDNA (5′ GCCTCCATCATCTCGTGC-3′) using the GC-Melt PCR kit (Clontech). Amplified DNA was gel-purified and sequenced.

Protein expression

Portions of tankyrase 1 and 2 were expressed in *Escherichia coli* BL21 either as glutathione-S-transferase (GST) fusions using pGEX-4T-1 (Amersham–Pharmacia) or as maltose-binding protein (MBP) fusions using pMal-c2 (NEB). Soluble proteins were isolated from 250 ml cultures using 15 ml of B-Per (Pierce) in presence of 5 units of DNase1, 0.5 mM PMSF, 1 mM dithiothreitol (DTT) and yeast protease inhibitor cocktail [6 μg/ml chymostatin, 1 μg/ml E64, 2 μg/ml aprotinin, 0.5 μg/ml phosphoramidon, 1 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml antipain, 0.1 mM benzamidine (Sigma)]. The same concentrations of PMSF, DTT and yeast protease inhibitor cocktail were included in subsequent steps. The fusion proteins were affinity purified with glutathione–Sepharose (for GST-fusions) or immobilized amylose (for MBP-fusions). Protein concentrations were estimated by Coomassie Blue staining and Bradford assays. Factor Xa (NEB) cleavage of the MBP fusions was performed overnight at 4 °C. Cross-linking with bis(sulphosuccinimidyl)suberate (BS^3; Pierce) was performed at 4 °C for 1 h using approx. 10 μg/ml purified SAM domain and 0–1 mM BS^3. The segments of tankyrase 1 and 2 that were expressed as GST, MBP, activation domain or DNA-binding domain fusion proteins comprise the following amino acids: ANK8, amino acids 428–737 of tankyrase 1 and 332–645 of tankyrase 2; SAM-PARP (SARP), 796–1168 of tankyrase 2; SAM, 890–1091 of tankyrase 1 and 796–1000 of tankyrase 2. The relative location of theses domains is indicated at the bottom of Figure 1(C).

GST pull-downs

ANK repeats 10–18 (ANK8) [3] of chicken tankyrase 1 and 2 were expressed in pGEX-4T-1 as described above. GST-fusion proteins were coupled to glutathione–Sepharose 4FF beads (Amersham–Pharmacia), washed sequentially with PBS, wash buffer A [600 mM KCl, 1.5 % (v/v) Triton, 20 mM Tris/HCl, pH 7.4] and PBS, and stored in PBS/glycerol (2:1, v/v) at 4 °C. DT40 nuclear extracts were prepared from 1×10^8 cells. Cells were washed once in PBS and swollen in reticulocyte standard buffer (RSB; 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4) for 10 min on ice. Nuclei were released by Dounce homogenization and washed three times in RSB. Nuclei were extracted with 420 mM KCl, 20 mM Hepes/KOH, pH 7.9, 25 % (v/v) glycerol, 0.1 mM EDTA and 5 mM $MgCl_2$ for 30 min at 4 °C and the extract was dialysed against buffer D [100 mM KCl, 20 mM Hepes–KOH, pH 7.9, 20 % (v/v) glycerol, 0.2 mM EDTA, 0.2 mM EGTA] for 2 h at 4 °C. For GST-pull downs, beads were coupled to approx. 5 μg of fusion protein and then incubated for 90 min with 8 μg of DT40 nuclear extract in a 400 μl reaction. Beads were washed three times with buffer D containing 150 mM KCl, resuspended in 35 μl of SDS/PAGE loading buffer and boiled. Samples were analysed by SDS/PAGE,

followed by Western blotting using anti-cTRF1 polyclonal antibody and horseradish-peroxidase-conjugated secondary. Supersignal West Pico substrate (Pierce) was used for antibody detection.

Antibody production and protein detection

cTRF1 was expressed in *E. coli* as a GST-fusion protein and purified on glutathione–Sepharose 4FF beads (Amersham–Pharmacia). The GST tag was removed by thrombin digestion, followed by gel purification. Polyclonal antibody was raised in rabbits (Covance) and purified over a cTRF1 affinity column. A DT40 cell line stably expressing FLAG-tagged cTRF1 was obtained by electroporating a tetracyclin-responsive cTRF1 expression construct (based on pUHC13-3 [23]) into DT40 cells. The cells were co-transfected with a neomycin resistance construct to allow selection of transformants with G418. To examine Flag-cTRF1 expression, total cellular proteins were extracted in RIPA buffer (150 mM NaCl, 1 % Nonidet P40, 0.5 % deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0, yeast protease inhibitor cocktail, 1 mM PMSF) and analysed by SDS/PAGE followed by Western blotting with anti-cTRF1 antibody or anti-FLAG (M2; Sigma) antibody. Horseradish-peroxidase-conjugated secondary antibodies and Supersignal West Pico and Femto chemiluminescence substrates were used for antibody detection.

The SAM domain from chicken tankyrase 1 was expressed in *E. coli* with a His_6 tag, purified by Ni^{2+}-affinity chromatography and used to raise a polyclonal antibody in rabbits (Covance). The antibody was affinity purified using immobilized SAM domain. To determine the relative affinity of the antibody for chicken tankyrase 1 versus 2, the SAM domains of tankyrase 1 and 2 were expressed as GST fusion proteins (SAM1 and SAM2) in *E. coli*. The amount of each fusion protein was estimated by SDS/PAGE followed by Coomassie Blue staining. The signals from equal amounts of SAM1 and SAM2 were then compared after Western blotting with affinity-purified rabbit anti-tankyrase 1 antibody. To detect endogenous proteins, whole cells or nuclei were lysed in SDS/PAGE loading buffer and loaded directly on a gel. Tankyrase was detected by Western blotting using the tankyrase 1 antibody, horseradish-peroxidase-conjugated secondary antibody and Supersignal West Femto chemiluminescence substrate (Pierce). α-Tubulin was detected with mouse anti-α-tubulin antibody (Sigma). Histone H3 was detected with mouse anti-(histone H3) antibody (Cell Signaling). Tankyrase 1 and 2 were transcribed and translated *in vitro* using a Promega TnT T3/T7 kit.

Yeast two-hybrid analysis

The SARP domain of tankyrase 2 (SARP2) was cloned into the LexA DNA-binding domain vector pGILDA and the B42 activation domain vector pJ4-5 of the Duplex-A system (Origene). Constructs were transformed into the yeast strain EGY48/pSH18-34, which harbours a β-galactosidase reporter plasmid. Expression was confirmed by Western blotting of yeast extracts. The $Met^{1054} \rightarrow Val$ mutant (mSARP2) was made using the Stratagene Quick-Change protocol. For β-galactosidase assays, a single colony was grown overnight in 5 ml of inducing medium. A 600 μl aliquot of the culture was used to measure the D_{600}, the remainder of the cells were pelleted and resuspended in 3 ml of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol). After adding 3 μl of 10 % (w/v) SDS to each ml of resuspended yeast, the sample was incubated for 30 min at 30 °C. Reactions were started by addition of 200 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside and stopped with 250 μl of 2 M Na_2CO_3. The samples were centrifuged briefly and the absorbance measured at 420 and 550 nm. Miller units of β-galactosidase were determined according to the following formula: $(1000 \times A_{420})$–$(1.75 \times A_{550})/(t_{min} \times V_{ml} \times A_{600} \times$ concentration factor), where V_{ml} is the volume of the reaction, t_{min} is the time of incubation in minutes, and the concentration factor is volume culture/volume resuspension buffer. Data were acquired from at least nine experiments performed in triplicate. For statistical analysis, data were normalized by log-transformation. Repeated measures analysis of variance was carried out using the SAS (Cary, NC, U.S.A.) procedure PROC MIXED.

RESULTS

Identification of chicken tankyrase genes

Portions of the human tankyrase 1 cDNA were used as probes to screen a chicken embryonic fibroblast cDNA library for chicken tankyrase 1. Because the PARP domains of human tankyrase 1 and 2 are highly conserved, this region of chicken tankyrase 1 was then used as a probe to isolate the tankyrase 2 gene. Sequencing of the chicken tankyrase 1 and 2 cDNAs revealed that the encoded proteins share a high level of sequence identity with their human homologues and have the same overall domain structure (Figure 1A). The 5′ end of chicken tankyrase 1 is highly GC-rich and, like human tankyrase 1, encodes a homopolymeric region that contains tracts of histidines, prolines and serines (the HPS domain). Although the chicken tankyrase 2 gene also has a highly GC-rich stretch at its 5′ end, this region does not appear to be translated and hence to encode an HPS domain. As illustrated in Figure 1(B), the cDNA has a stop codon at nucleotide 120 which is 51 nucleotides upstream of the first AUG at position 171. The apparent lack of an HPS domain in human tankyrase 2 has been somewhat controversial, because the human cDNA has a long stretch of in-frame sequence immediately 5′ of the putative start codon, suggesting that the initiating methionine might be missing [4,14]. We therefore confirmed the presence of the nonsense codon in the chicken tankyrase 2 gene by performing reverse transcriptase-PCR directly on chicken mRNA using primers that flanked both the putative stop codon and translation start site (Figure 1B). Sequencing of the resulting PCR product confirmed the presence of the stop codon in-frame with the start codon, indicating that chicken tankyrase 2 does indeed lack an HPS domain. Given the high sequence identity between the chicken and human genes (see below), our results indicate that an HPS domain is also unlikely to be present in human tankyrase 2.

Conservation of tankyrase proteins

Comparison of the chicken and human tankyrase sequences using the GCG Gap algorithm revealed that chicken and human tankyrase 1 genes are 80 % identical, whereas the tankyrase 2 genes are 81 % identical (results not shown). Most of the sequence differences are at the wobble positions, so the encoded tankyrase 1 proteins are 95 % identical, whereas the tankyrase 2 proteins are 93 % identical (Figure 1C). As telomere-associated proteins are not normally highly conserved ([24,25] and C. Wei and C. Price, unpublished work), this extreme conservation of tankyrase proteins from different species is striking and fits well with tankyrase 1 and 2 being signalling molecules that function not only in telomere biology but also in other cellular processes. Interestingly, the overall identity between the two members of the

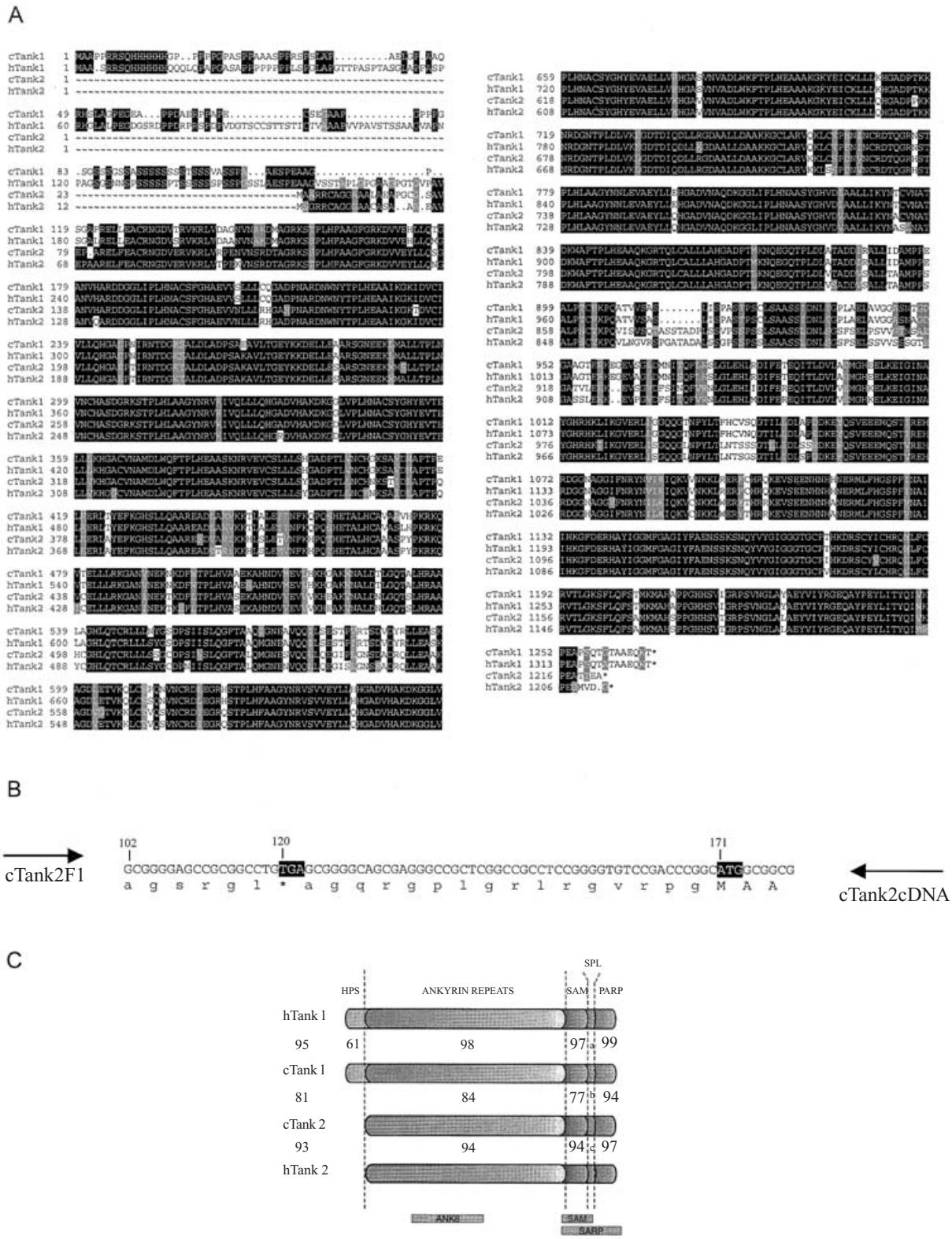


Figure 1 Sequence of chicken tankyrase 1 and tankyrase 2

(**A**) Amino acid sequence comparison of tankyrase 1 and tankyrase 2 from chicken and humans. Dark shading delineates identical amino acids, grey shading denotes similar amino acids. ANK repeat domain, amino acids 120–946 (chicken Tankyrase 1); SAM domain, amino acids 962–1027; SAM-PARP linker, amino acids 1028–1114; PARP domain, amino acids 1115–1155. (**B**) Reverse transcriptase-PCR strategy used to confirm the presence of a stop codon (*) upstream of the putative start codon (M). cDNA was synthesized using the cTank2cDNA primer and then amplified with the cTank2F1 and cTank2cDNA primers. The sequence of the PCR product is shown with the stop and start codons shaded. (**C**) Domain structure and amino acid sequence identity between the domains of chicken and human tankyrase 1 and 2. The numbers show the percentage identity between adjacent pairs of proteins (to the left) or the domains of the adjacent pairs. SPL, SAM-PARP linker domain; a, 98 % identity; b, 74 % identity; c, 94 % identity. Regions used for fusion-protein expression are indicated under hTank2.

tankyrase family is lower (81 % between chicken tankyrase 1 and 2 and 78 % between human tankyrase 1 and 2), with the SAM domains exhibiting the lowest level of identity (77 % between either pair of paralogues). This lower sequence identity, together with the lack of an HPS domain on tankyrase 2, suggests that the two proteins may have acquired different functions during evolution.

BLAST searches of Genbank® and the NCBI Expressed Sequence Tag (EST) database identified sequences from a number of other organisms (mouse, *Xenopus laevis*, pufferfish, zebrafish, catfish, mosquito and *Drosophila melanogaster*) that encoded portions of tankyrase homologues (results not shown). The vertebrate homologues could clearly be identified as tankyrase 1 or 2, based on the level of sequence identity with the human or chicken proteins. For example, a *Xenopus* EST encoded a peptide that was 94 % identical with the PARP domain from chicken tankyrase 2 but only 86 % identical with chicken tankyrase 1, whereas a pufferfish EST encoded a peptide that was 91 % identical with the PARP domain from tankyrase 1 but only 85 % identical with tankyrase 2. Interestingly, only one *Drosophila* tankyrase sequence could be identified, even though the entire genome has been sequenced. The encoded protein shares a significant, but much lower, level of sequence identity (approx. 66 %) with tankyrase 1 and 2 from humans and chickens. Like tankyrase 2, the *Drosophila* protein lacks an HPS domain. However, it does not appear to be more closely related to tankyrase 2 than to tankyrase 1, as the overall sequence identity to both vertebrate proteins is quite similar. The existence of two highly conserved tankyrase genes in vertebrates, but only one more diverged gene in *Drosophila*, suggests that the vertebrate and insect genes evolved from a common ancestor, with a gene duplication occurring during vertebrate evolution to give rise to tankyrase 1 and 2.

Partitioning of the ANK repeats into five subdomains

In addition to providing insight into the evolution of tankyrase genes, the interspecies sequence comparison yielded information about the organization of the ANK repeat domain. When the human tankyrase 1 gene was first identified, sequence analysis suggested that the ANK domain contained 24 ANK repeats [3]. However, a more recent analysis indicated that the domain might instead contain 19 full ANK repeats with two half repeats at either end [5]. This later alignment had fewer deletions and insertions and, most importantly, revealed a four-repeat periodicity. This periodicity results from an approx. 22-amino-acid insertion within every fourth ANK repeat, with each insertion bearing a variant of the sequence LLEAAR/K, a motif that is a poor match to the ANK-repeat consensus [6]. The insertions cause the ANK domain to be subdivided into five approximately equal segments that each contain four repeats. These segments correspond fairly well to the subdomains of ANK repeat clusters that have been identified as separate and redundant binding sites for the tankyrase interacting proteins TRF1 and TAB182 [20]. Comparison of ANK repeat sequences from vertebrate and insect tankyrases indicates that both the sequence and position of the LLEAAR/K motif within the ANK domain are highly conserved (Figure 2). Thus comparative sequence analysis supports division of the ANK repeat domain into five subdomains that provide alternative binding sites for tankyrase 1- and tankyrase 2-interacting proteins.

A database search using all variants of the LLEAAR/K motif (Figure 2) as a query sequence revealed that this motif is present in at least 28 human and 11 *E. coli* proteins, most of which are metabolic enzymes, such as dehydratases, dehydrogenases and epimerases. Strikingly, close to 50 % of these proteins have been

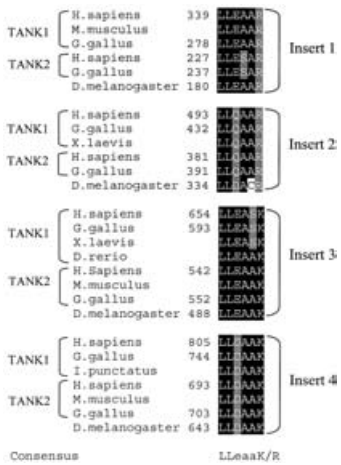


Figure 2 Sequence comparison of the LLEAAR/K motifs from tankyrase 1 and 2

Each sequence alignment corresponds to one of the four LLEAAR/K sequence motifs found in human (*Homo sapiens*), mouse (*Mus musculus*) and chicken (*Gallus gallus*) tankyrase 1, human and chicken tankyrase 2 and *Drosophila* (*D. melanogaster*) tankyrase. The sequence of the equivalent LLEAAR/K motifs from *Xenopus* (*X. laevis*), Catfish (*Ictalurus punctatus*) and Zebrafish (*Dario rerio*) are included where available. Dark shading highlights identical amino acids, grey shading highlights similar amino acids. The consensus sequence for the motif is given at the bottom.

shown to bind NAD^+, ATP or cAMP, and the actual percent may be higher, as little information is available concerning the enzymatic mechanism of the remaining LLEAAR/K-containing proteins. This observation suggests that the LLEAAR/K-containing inserts which subdivide the ANK domain of tankyrase 1 and 2 might provide an NAD^+- or adenosine-interacting domain that is separate from the NAD-binding region of the PARP domain.

Chicken tankyrases interact with cTRF1

The prominent cytoplasmic localization of human tankyrase 1 [16,19] and tankyrase 2 [4,5], together with initial reports that human tankyrase 2 does not interact with hTRF1 [4], led us to ask whether chicken tankyrase 1 and 2 interact with cTRF1. In order to detect cTRF1 in pull-down experiments, it was necessary first to characterize the cTRF1 gene and generate antibodies to the protein. Analysis of a cTRF1 cDNA sequence [kindly provided by D. Broccoli (Fox Chase Cancer Center, Philadelphia, PA, U.S.A.), B. Li (Laboratory of Molecular Parasitology, The Rockerfeller University, New York, NY, U.S.A.) and T. de Lange (Laboratory of Cell Biology and Genetics, The Rockerfeller University, New York, NY, U.S.A.)] showed that the human and cTRF1 proteins are 46 % identical (56 % similar) and share the same domain structure (Figure 3A) [26]. In contrast, cTRF1 and cTRF2 are only 22 % identical (30 % similar). We used the cDNA to produce recombinant cTRF1 in *E. coli* and then made antibody to the purified protein. As shown in Figure 3(B), the affinity-purified antibody detected only one band in nuclear extracts from chicken DT40 cells and this band had the same mobility as bacterially-expressed cTRF1 (lanes 1–4). The apparent molecular mass of approx. 39 kDa corresponds well with the predicted size of the protein (354 amino acids or 41 kDa). To confirm further the specificity of the cTRF1 antibody, we transfected DT40 cells with FLAG-tagged cTRF1 and performed Western blots on nuclear extracts from the transfected cells. As shown in Figure 3(B), lanes 6 and 8, the anti-FLAG and anti-cTRF1 antibodies detected a protein of

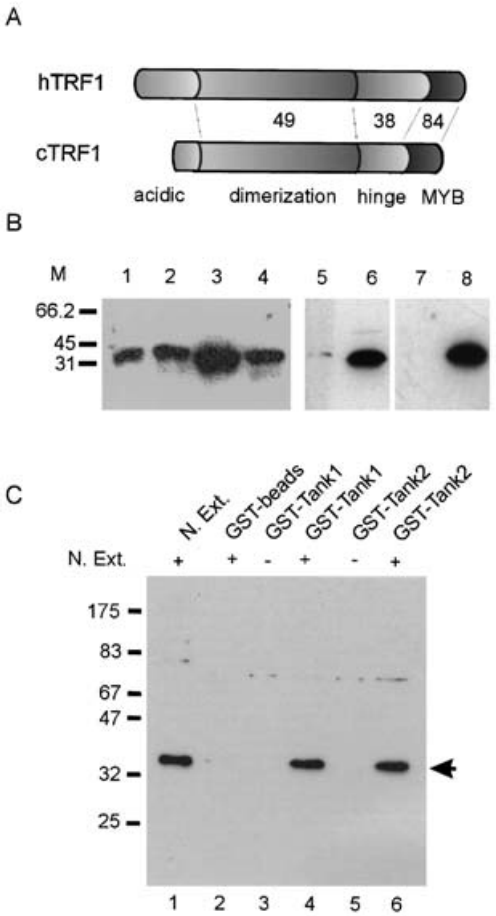


Figure 3 Interaction of chicken tankyrase 1 and 2 with cTRF1

(**A**) A schematic showing TRF1 domain structure and sequence identity between the chicken and human proteins. The numbers show percent amino acid identity between each domain. (**B**) Western blots showing specificity of cTRF1 antibody. Lane 1, nuclear extract from 0.25×10^6 DT40 cells; lane 2, purified recombinant His-tagged cTRF1 expressed in *E. coli*; lane 3, 1×10^6 DT40 nuclei; lane 4, mix of DT40 nuclear extract and recombinant cTRF1; lanes 5–8, 2×10^4 DT40 cells probed with anti-cTRF1 antibody (lanes 5 and 6) or anti-FLAG antibody (lanes 7 and 8). Lanes 5 and 7, wild-type DT40; lanes 6 and 8, DT40 cells expressing FLAG-tagged cTRF1. (**C**) Western blot showing cTRF1 associated with glutathione beads coupled to GST or GST-tankyrase fusion protein after incubation with DT40 nuclear extract. Lane 1, input DT40 nuclear extract (N. Ext); lane 2, GST-beads with nuclear extract; lanes 3 and 4, GST-tankyrase 1 (GST-Tank1) fusion protein (ANK repeats 10–18) incubated without (lane 3) or with (lane 4) nuclear extract; lanes 5 and 6, GST-tankyrase 2 (GST-Tank2) fusion protein (ANK repeats 10–18) incubated without (lane 5) or with (lane 6) nuclear extract. Positions and sizes of molecular-mass markers are shown to the left. cTRF1 is marked by the arrowhead.

identical mobility. cTRF1 is significantly smaller than its human counterpart (354 versus 439 amino acids), with most of the size difference concentrated in the N-terminal tankyrase-interacting domain and the hinge domain (Figure 3A) [27]. We suspect the cDNA clone is missing the extreme 5′-end of the gene, but since recombinant and endogenous cTRF1 have essentially the same mobility in an SDS gel, the missing region probably amounts to only a few amino acids.

Because the cTRF1 clone may be missing part of the tankyrase-interacting domain, we chose to look for an interaction between cTRF1 and tankyrase using the endogenous cTRF1 and recombinant GST-tankyrase fusion proteins. The fusion proteins contained a section of the chicken tankyrase 1 or 2 ANK repeats that corresponded with the TRF1-interacting domain in human tankyrase 1. Each fusion protein was bound to glutathione beads, the beads incubated with nuclear extracts from chicken DT40 cells, washed and analysed by Western blotting with cTRF1 antibody. As shown in Figure 3(C), cTRF1 was pulled down by both the GST-tankyrase 1 and the GST-tankyrase 2 beads (lanes 4 and 6), but not by control beads bound to GST alone. These results show that both chicken tankyrase 1 and 2 interact with cTRF1 through the ANK repeat domain. This has since been shown to also be the case for human tankyrase 2 [14,16].

Subcellular distribution of tankyrase 1 and 2

A key step towards determining whether tankyrase 2 has a telomeric function is to find out whether any of the endogenous protein is present in the nucleus. Although previous fractionation experiments demonstrated that a large percentage of tankyrase 2 is present in low-density microsomes [4], they did not determine whether any of the protein was present in purified nuclei. To address this question, we purified nuclei from chicken DT40 cells and assayed for the presence of tankyrase 2 by Western blotting using an affinity-purified antibody raised against the SAM domain of tankyrase 1. As shown below, this antibody cross-reacts with the SAM domain of both tankyrase 1 and 2. We also used the SAM domain antibody to quantify the relative amounts of tankyrase 1 and 2 in cells, as this information is needed to assess which is the most prevalent poly(ADP-ribosyl)ating activity.

To demonstrate that the tankyrase antibody cross-reacts with both tankyrase 1 and tankyrase 2, the corresponding cDNAs were subject to *in vitro* transcription/translation and the products analysed by Western blotting. As shown in Figure 4(A), lanes 1 and 3, the antibody detected both proteins. When total cellular proteins and proteins from purified nuclei were separated by SDS/PAGE and blotted with the tankyrase antibody, two bands in the 120–140 kDa range were detected in both preparations (Figure 4A, lane 2, and Figure 4B). These ran at the sizes expected for tankyrase 1 (139 kDa) and tankyrase 2 (129 kDa) and aligned with products obtained after *in vitro* transcription and translation of the chicken tankyrase 1 and 2 cDNAs (Figure 4A, lanes 1–3). The 129 kDa band was unlikely to be a degradation product of tankyrase 1 that fortuitously ran with the same mobility as tankyrase 2, because it was observed when whole cells were lysed directly by boiling in SDS. Although the same relative amount of tankyrase 2 was observed in multiple nuclear preparations, the amount was quite low. Consequently, we were concerned that the tankyrase 2 in our preparations might merely reflect cytoplasmic contamination. As control experiments, preparations of cellular and nuclear proteins were probed with antibodies to tubulin, histone H3 and tankyrase (Figure 4C). No tubulin could be detected in samples containing 3×10^6 nuclei (Figure 4C, lane 3), even though tankyrase and histone H3 were readily apparent. Moreover, tubulin could be detected in samples containing 100-fold fewer cells (Figure 4C, lane 1), indicating that there was less than 1 % cytoplasmic contamination in the nuclear samples. Thus our results indicate that a fraction of the endogenous tankyrase 2 is most likely present in the nucleus.

To compare the amounts of tankyrase 1 and tankyrase 2 in whole cells, we first determined the relative affinity of the antibody for the SAM domains of the two proteins. When Western blots were performed with various amounts of bacterially expressed SAM domain from tankyrase 1 (SAM1) or tankyrase 2 (SAM2), a 1 : 2 ratio of SAM1 : SAM2 was required to give the same signal intensity (Figure 4D). We therefore estimate that the antibody has approximately twice the affinity for tankyrase 1 compared with tankyrase 2. When we compared the number of cells required to give equivalent tankyrase 1 and 2 signals, we found that about four times more cells were required to give a tankyrase 2 signal

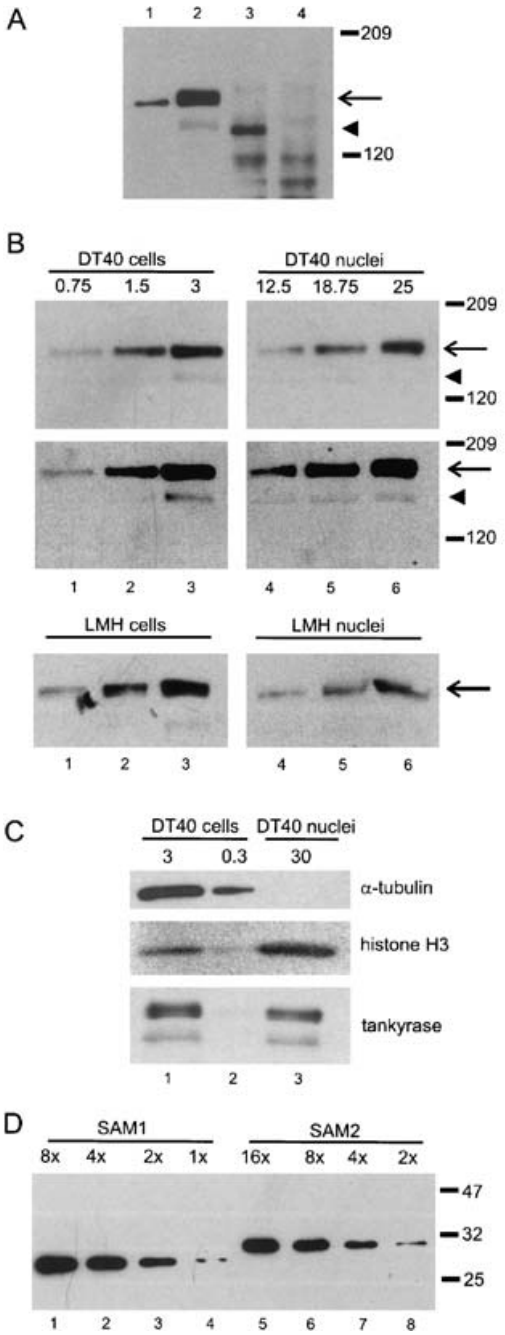


Figure 4 Relative abundance of tankyrase 1 and 2 in whole cells or nuclei.

(**A**) Demonstration of tankyrase antibody specificity. Western blots probed with tankyrase antibody. Lane 1, tankyrase 1 *in vitro* transcription/translation product; lane 2, DT40 nuclear extract; lane 3, tankyrase 2 *in vitro* transcription/translation product; lane 4, luciferase control *in vitro* transcription/translation reaction. (**B**) Detection of tankyrase 1 and 2 in chicken cells and nuclei. Upper panel; Western blots of total cellular or nuclear proteins from DT40 cells probed with tankyrase antibody. The number of cells or nuclei ($\times 10^5$) loaded in each lane is given at the top of each blot. Middle panel; longer exposure of the upper panel. The arrow marks tankyrase 1, the arrowhead marks tankyrase 2. Lower panel; Western blots of chicken LMH hepatocellular carcinoma cells or nuclei probed with the tankyrase antibody. The same number of cells or nuclei were loaded as in the upper panels. (**C**) Assay for tubulin contamination in purified nuclei. Western blots probed with antibody to α-tubulin, histone H3 or tankyrase. Lane 1, 3×10^5 DT40 cells; lane 2, 3×10^4 cells; lane 3, 3×10^6 nuclei. (**D**) Affinity of chicken tankyrase antibody for the SAM domains of tankyrase 1 and 2. Western blot showing reaction of the tankyrase antibody with decreasing amounts of bacterially expressed SAM domains from tankyrase 1 or 2 (SAM1, SAM2 respectively). The relative amount of protein loaded is shown above each lane; equal amounts were loaded in lanes 1 and 6, lanes 2 and 7, and lanes 3 and 8.

that was of a similar intensity to the tankyrase 1 signal (Figure 4B, compare lane 1, Tank1 signal with 0.75×10^5 cells with lane 3, Tank2 signal with 3×10^5 cells). Given the two-fold difference in antibody affinity for tankyrase 1 versus tankyrase 2, this result indicates that there is approximately two-fold more tankyrase 1 than tankyrase 2 in DT40 cells.

Comparison of the tankyrase 1 signals obtained with whole DT40 cells versus nuclei indicated that approx. one-tenth of the total protein is present in the nucleus (Figure 4B, compare lanes 2 and 5 or lanes 3 and 6). A similar result was obtained when we examined the amount of tankyrase 1 in whole cells and nuclei from the chicken LMH hepatocellular carcinoma cell line (Figure 4B, lower panel), indicating that this distribution was a general phenomenon and not specific to the DT40 B-cell line. We were not able to get an accurate estimate for the relative amount of tankyrase 2 in the nucleus versus whole cells, because the signal from the nuclei was too weak to quantify. However, it appears that the fraction of tankyrase 2 in the nucleus is lower than the fraction of tankyrase 1.

SAM domain mediated oligomerization

Although GST pull-down experiments have shown that human tankyrase proteins can interact *in vivo*, to form homo- and heterotypic complexes [5], it has not been determined whether complex formation is mediated by direct association between tankyrase molecules or via other interacting partners. Since SAM domains are known to form both dimers and longer oligomers, we sought to determine whether tankyrase molecules can associate via a direct interaction between SAM domains. In initial experiments, we used a yeast two-hybrid approach to assay for tankyrase 2 self-association. A section of tankyrase 2 encompassing the SARP2 domain was cloned into the DNA-binding domain (LexA-SARP2) and activation domain (AD-SARP2) vectors of the Duplex-A system. However, expression of this fragment killed the yeast cells. As yeast does not have any PARP homologues, we attributed this lethality to poly-(ADP-ribosyl)ation of yeast proteins by the SARP2 polypeptide. We therefore mutated $Met^{1054} \rightarrow Val$, a mutation known to inactivate PARP activity of human tankyrase 2 [5]. The mutated construct (mSARP2) was not lethal and expressed well (results not shown), so it was used in all further experiments.

When β-galactosidase assays were performed with LexA-mSARP2 and AD-mSARP2, a small but reproducible activation of the β-galactosidase gene was observed (Figure 5). Although the level of activation was approx. 140-fold less than that observed for control proteins supplied by the company that exhibit a strong interaction (results not shown), it was greater than that

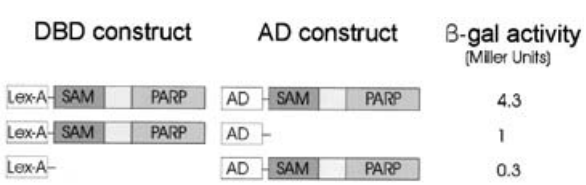


Figure 5 Yeast two-hybrid assay for interactions between the SARP domains of tankyrase 2

The SARP domain of tankyrase 2 ($Met^{1054} \rightarrow Val$ mutation) was expressed as a LexA DNA-binding domain or B42-activation-domain fusion protein and assayed for activation of β-galactosidase activity in the presence of the corresponding activation domain/DNA binding domain fusion protein, or the activation domain/DNA-binding domain alone. The pairs of DNA-binding domain (DBD) and activation domain (AD) constructs are illustrated on the left, while the resulting β-galactosidase (β-gal) activity (in Miller units) is indicated at the right.

observed with some other proteins that are known to interact via their SAM domains. Moreover, statistical analysis by analysis of variance using the SAS procedure PROC MIXED indicated that signal obtained with the LexA-mSARP2 and AD-mSARP2 constructs was significantly higher than the signal obtained in control experiments using the parental DNA-binding domain or activation domain vectors ($P < 0.001$). These results suggested that the SAM or PARP domains of tankyrase 2 mediate a direct self-association. The low level of β-galactosidase expression could reflect a very weak interaction between the SAM domains. Alternatively, it could be caused by self-association of the SAM portions of the activation domain or DNA-binding domain fusion proteins. This would reduce productive activation domain/DNA-binding domain interactions and hence prevent efficient activation of the β-galactosidase gene.

To further investigate tankyrase self-association, we determined whether the bi-functional cross-linker BS^3 could cause intermolecular cross-linking of mSARP2 molecules. mSARP2 was expressed as a MBP fusion protein in *E. coli*, affinity purified and incubated with several concentrations of BS^3. When the cross-linked MBP–mSARP2 was analysed by SDS/PAGE, it did not run as a monomer of 83 kDa (Figure 6A). Instead, most of the protein ran as high-molecular-mass complexes that barely entered the separating gel, and the rest remained in the well of the stacking gel. These high-molecular- mass forms could be detected by both Western blotting and Coomassie staining (Figure 6A, lanes 2–3 and 5–6, and Figure 6B, lanes 3–4). To ensure that intermolecular cross-linking was not caused by the MBP domain of the fusion protein, we cleaved the MBP–mSARP2 with factor Xa to separate the MBP and mSARP2 domains (Figure 6A, lanes 4–6 and 10–12). After cross-linking, all the mSARP2 ran as high-molecular-mass complexes, whereas most of the MBP remained monomeric. At the highest concentration of cross-linker, some of the MBP was observed in the well of the stacking gel. However, a similar phenomenon was observed with BSA when it was present during cross-linking of MBP–mSARP2 (results not shown), therefore we attribute this impaired mobility to the MBP becoming trapped in the well, rather than to intermolecular cross-linking. To further examine whether MBP played a role in the formation of the MBP–mSARP2 complexes, we determined whether purified MBP was cross-linked with BS^3. As shown in Figure 6(B), lanes 1 and 2, all of the MBP remained monomeric, indicating that the protein did not self-associate, whereas the MBP–mSARP2 clearly oligomerized to form high-molecular-mass complexes.

The above results indicate that the SAM and/or PARP domains of tankyrase 2 can mediate oligomerization. As PARP domains do not usually mediate either dimerization or polymerization, we next sought to determine whether the SAM domain alone is sufficient for self-association. The SAM domains of both tankyrase 1 and tankyrase 2 were expressed as MBP fusions (SAM1 and SAM2), purified and incubated with various concentrations of BS^3. As shown in Figures 6(B), 6(C) and 6(D), BS^3 caused intermolecular cross-linking of the SAM1 and SAM2 fusion proteins, indicating that tankyrase 1 and 2 self-association is mediated by the SAM domain. The amount of cross-linking depended on the BS^3 concentration, with low concentrations giving rise to dimers, whereas higher concentrations resulted in dimers, trimers and larger oligomers. Overall, BS^3 cross-linking of SAM domain fusion proteins resulted in less of the high-molecular-mass oligomers than cross-linking of the equivalent SAM–PARP-domain fusion proteins. This could be because the SAM-domain fusion proteins folded less well than the corresponding SARP domains. Alternatively, the PARP domains might promote SAM domain interactions.

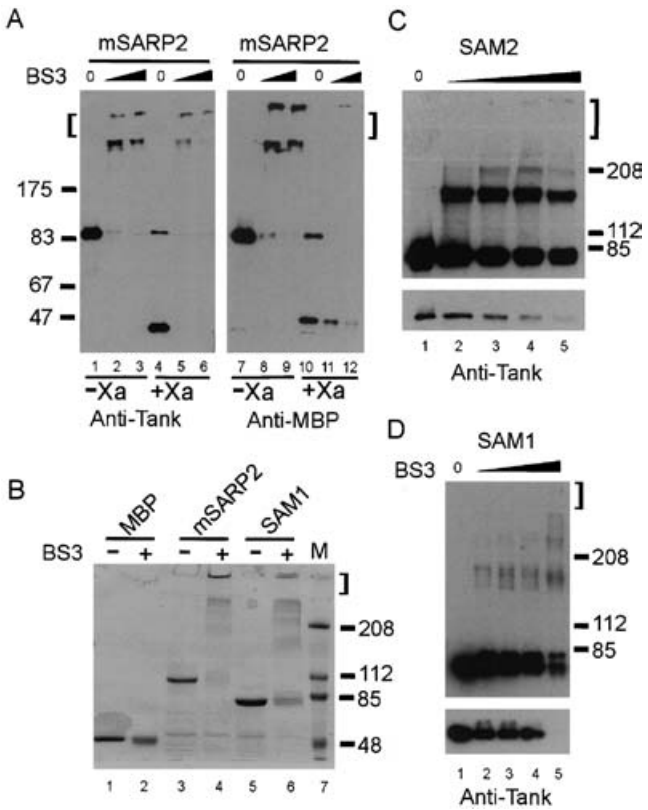


Figure 6 Oligomerization of tankyrase 1 and 2

Purified tankyrase 1 and 2 SARP or SAM domain MBP fusion proteins were cross-linked with BS^3 and separated on SDS/PAGE gels. (**A**), (**C**) and (**D**) show Western blots probed with antibody to tankyrase or MBP and (**B**) shows Coomassie staining. Positions of molecular mass markers are shown to the side of each gel. (**A**) $Met^{1054} \rightarrow Val$ tankyrase 2 mSARP2 domain after incubation with 0, 0.5 or 1 mM BS^3. Right panel, anti-MBP; left panel, anti-tankyrase (anti-Tank) antibody. Samples in lanes 4–6 and 10–12 were cleaved with factor Xa. (**B**) Products obtained after incubating purified MBP (lanes 1–2), mSARP2 domain (mSARP; lanes 3–4) or tankyrase SAM1 domain (lanes 5–6) with (+) or without (−) 2 mM BS^3. (**C**) Products obtained with tankyrase 2 SAM domain (SAM2) after incubation with 0, 0.1, 0.25, 0.5 and 1 mM BS^3. The lower panel shows a shorter exposure of the 72 kDa monomer band. (**D**) Products obtained with tankyrase 1 SAM domain (SAM1) after incubation with 0, 0.01, 0.05, 0.1 and 0.5 mM BS^3. The lower panel shows a shorter exposure of the 72 kDa monomer band.

DISCUSSION

When tankyrase 1 was first identified, its presence at telomeres, together with its enzymically active PARP domain, strongly implicated this protein as an effector molecule in some aspect of telomere regulation. However, the subsequent isolation of tankyrase 2, the identification of the wide variety of tankyrase 1 and 2 interaction partners, and the detection of tankyrase 1 and 2 at multiple unrelated subcellular localizations, suggested a more complex, but poorly understood, multifunctional behaviour. By characterizing tankyrase 1 and 2 from non-mammalian cells, we have been able to identify conserved aspects of tankyrase domain structure, including the five subdomains within the ANK repeats. We have also obtained fundamental biochemical information that sheds light on the function of tankyrase 1 and 2 at their various locations within the cell. We have shown that chicken cells contain approximately twice as much tankyrase 1 as tankyrase 2, and although the majority of each protein resides in the cytoplasm, a significant fraction appears to be present in the nucleus. Since tankyrase 2 binds to both human and chicken TRF1, this nuclear localization indicates that tankyrase 2, like tankyrase 1, is likely to have a telomeric function. We have also shown that both

tankyrase 1 and 2 can self-associate to form large high-molecular-mass complexes. This oligomerization has led us to suggest that, despite being present at such a wide range of locations with a cell, tankyrase 1 and 2 perform a universal task at each location by acting as master scaffolding molecules, which anchor cellular components or assemble signaling complexes

Tankyrase protein–protein interactions

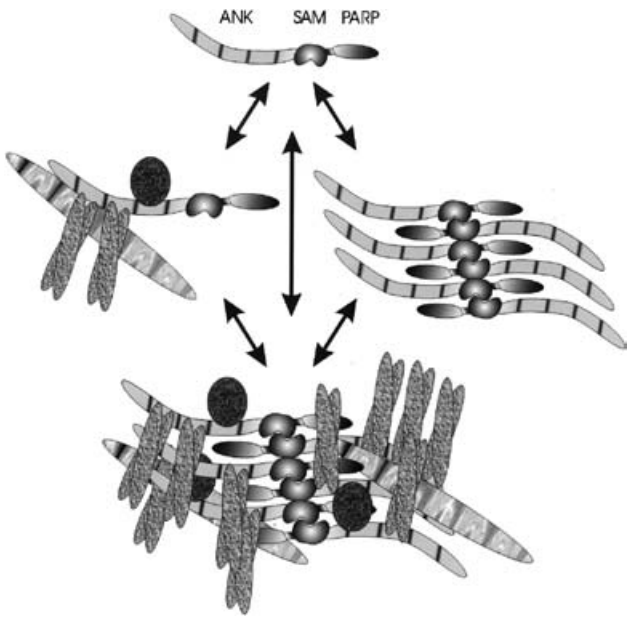
Tankyrase 1 and 2 have two key features that enable them to form large multi-protein complexes: the modular ANK domain that allows binding to multiple interaction partners, and the SAM domain, which allows interaction between multiple tankyrase molecules. Although a wide variety of proteins are known to interact with the ANK domain, it is unclear whether these proteins merely compete with each other for tankyrase binding, or if their interaction is somehow regulated. Our finding that the LLEAAR/K motif, which interrupts the ANK repeats, is highly conserved in tankyrase proteins from different species, and is present in a variety of metabolic enzymes that bind ATP or NAD, suggests that this motif may do more than simply serve to separate the tankyrase ANK repeats into sub-domains. One possibility is that the motif acts as a regulatory element by responding to adenosine-containing nucleotides. If so, it might control which factors interact with the ANK sub-domains.

The SAM domain is a critical feature of both tankyrase proteins, because the unique structure of this domain allows proteins to self-associate to form not only dimers but larger oligomeric complexes [9–13]. Crystal structures of SAM domains from the EphB2 receptor and the TEL (translocation Ets leukaemia) and the PH (polyhomeotic) transcriptional repressors have revealed two separate interaction surfaces that can associate to form long oligomers [28–30]. In the case of the TEL and PH proteins, the two surfaces interact in a head-to-tail manner to form long helical polymers [29,30]. In contrast, the EphB2 SAM domains seem to oligomerize via alternating head-to-head and tail-to-tail interactions [28,31]. Since the manner of SAM-domain association is so variable, structural studies will be required to determine whether the tankyrase SAM domains associate in a head-to-tail or head-to-head/tail-to-tail array. However, regardless of the mode of self-association, the SAM domain provides a means to form very large tankyrase complexes.

At present, we cannot tell the extent to which tankyrase multimerization occurs *in vivo*, as in our experiments even transient associations could be captured by the chemical cross-linking. The solubility of the SAM–PARP-domain fusion proteins suggests that *in vitro* oligomerization was limited to short chains. However, the *in vivo* situation could be quite different. EphB2 must be present at a high concentration to polymerize *in vitro*, but there is evidence that *in vivo* oligomerization may result from ligand-induced receptor binding [28,31]. An equivalent situation might occur for tankyrase, with multimerization promoted by tankyrase interacting factors or post-translational modification.

Tankyrases as master scaffolding proteins

Our proposal that the tankyrase proteins act as master scaffolding molecules is based both on the division of the ANK repeat domain into sub-domains that act as independent binding sites for interacting proteins, and the capacity of the SAM domains to multimerize. These properties mean that both tankyrase 1 and 2 are likely to simultaneously interact with multiple proteins and to self-associate so that a lattice of protein–protein interactions is formed (see model in Scheme 1). Since poly(ADP-ribosyl)ation is associated with the disruption of interactions [2], lattice dissociation may be triggered by PARP activity. The tankyrase PARP domain may therefore provide a way to regulate the association or dissociation of large protein complexes. During insulin signalling, such complexes may be important for regulating release of GLUT-4 vesicles, whereas at telomeres, they may promote packaging of the telomeric DNA into a compact nucleoprotein structure.



Scheme 1 Model for tankyrase 1 and 2 as master scaffold proteins

Tankyrase can interact with multiple proteins via the ANK repeat subdomains and form multimers through the SAM domain. The combination of these two associations leads to the assembly of a protein lattice with tankyrase acting as the scaffolding molecule.

Interestingly, there is a precedent for proteins that contain both SAM and ANK domains acting as master scaffolding proteins. The Shank proteins are a family of related proteins that are found at post-synaptic sites of excitatory synapses [32]. They contain multiple protein-interaction domains, including several ANK repeats, a PDZ domain, a proline-rich region and a SAM domain. The interaction partners identified to date indicate that the Shanks are master scaffold molecules that hold together the NMDA-, mGluR- and AMPA receptor complexes in the post-synaptic region. They are also thought to link the various receptor complexes to the cytoskeleton. Unlike tankyrases, Shank proteins do not appear to contain an enzymic activity that could modulate complex formation. Also, the subcellular location of the Shank complexes is much more restricted, suggesting that tankyrase complexes regulate a more diverse array of cellular processes.

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