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Tankyrase 1 Interacts with Mcl-1 Proteins and Inhibits Their Regulation of Apoptosis*

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Jeehyeon Bae, Jill R. Donigian‡, and Aaron J. W. Hsueh§

From the Division of Reproductive Biology, Department of Gynecology and Obstetrics, Stanford University School of Medicine, Stanford, California 94305-5317 and the ‡Laboratory for Cell Biology and Genetics, The Rockefeller University, New York, New York 10021

Mcl-1L (myeloid cell leukemia-1 long) is an antiapoptotic Bcl-2 family protein discovered as an early induction gene during leukemia cell differentiation. Previously, we identified Mcl-1S (short) as a short splicing variant of the *Mcl-1* gene with proapoptotic activity. To identify Mcl-1-interacting proteins, we performed yeast two-hybrid screening and found cDNAs encoding tankyrase 1. This protein possesses poly(ADP-ribose) polymerase activity and presumably facilitates the turnover of substrates following ADP-ribosylation. In yeast and mammalian cells, tankyrase 1 interacts with both Mcl-1L and Mcl-1S, but does not bind to other Bcl-2 family proteins tested. Analysis of truncated tankyrase 1 mutants indicated that the first 10 ankyrin repeats are involved in interaction with Mcl-1. In the N terminus of Mcl-1, a stretch of 25 amino acids is sufficient for binding to tankyrase 1. Overexpression of tankyrase 1 antagonizes both Mcl-1L-mediated cell survival and Mcl-1S-induced cell death. Furthermore, coexpression of tankyrase 1 with Mcl-1L or Mcl-1S decreased the levels of Mcl-1 proteins. Although tankyrase 1 down-regulates Mcl-1 protein expression, no ADP-ribosylation of Mcl-1 was detected. In contrast, overexpression of Mcl-1 proteins suppressed the ADP-ribosylation of the telomeric repeat binding factor 1, another tankyrase 1-interacting protein. Thus, interaction of Mcl-1L and Mcl-1S with tankyrase 1 could serve as a unique mechanism to decrease the expression of these Bcl-2 family proteins, thereby leading to the modulation of the apoptosis pathway.

Phylogenetically conserved Bcl-2 family proteins play a pivotal role in the regulation of apoptosis from virus to human (1). Members of the Bcl-2 family consist of antiapoptotic proteins such as Bcl-2, Bcl-x_L, and Bcl-w, and proapoptotic proteins such as BAD, Bax, BOD, and Bok. It has been proposed that anti- and proapoptotic Bcl-2 proteins regulate cell death by binding to each other and forming heterodimers (2). A delicate balance between anti- and proapoptotic Bcl-2 family members exists in each cell and the relative concentration of these two groups of proteins determines whether the cell survives or undergoes apoptosis.

Myeloid cell leukemia-1 (Mcl-1)[1] is a Bcl-2 family protein and was originally cloned as a differentiation-induced early gene that was activated in a human myeloblastic leukemia cell line (3). Mcl-1 is expressed in a wide variety of tissues and cells including neoplastic ones (3–5). We and others recently identified a short splicing variant of Mcl-1 short (Mcl-1S) and designated the known Mcl-1 as Mcl-1 long (Mcl-1L) (6, 7). Mcl-1L protein exhibits antiapoptotic activity and possesses the BH (Bcl-2 homology) 1, BH2, BH3, and transmembrane domains found in the related Bcl-2 proteins (3, 5, 8). In contrast, Mcl-1S is a BH3 domain-only proapoptotic protein that heterodimerizes with Mcl-1L (6). Although both Mcl-1L and Mcl-1S proteins contain BH domains found in other Bcl-2 family proteins, they are distinguished by their unusually long N-terminal sequences containing PEST (proline, glutamic acid, serine, and threonine) motifs, four pairs of arginine residues, and alanine- and glycine-rich regions. In addition, the tissue expression pattern of the Mcl-1 protein is different from that of Bcl-2 suggesting a unique role for Mcl-1 in apoptosis regulation (4, 5).

Tankyrase 1 (TRF1-interacting, ankyrin-elated ADP-ribose polymerase 1) was originally isolated based on its binding to TRF1 (telomeric repeat binding factor-1) and contains the HPS (homopolymeric runs of histidine, proline, and serine) sequence, 24 ankyrin repeats, SAM (sterile α-motif), and the catalytic domain of poly(adenosine diphosphate-ribose) polymerase (PARP) (9). Previous studies have shown that tankyrase 1 promotes telomere elongation in human cells by inhibiting TRF1 through its poly(ADP-ribosyl)ation by tankyrase 1 (9, 10). In addition, tankyrase 1 poly(ADP-ribosyl)ates insulin-responsive amino peptidase (IRAP), a resident protein of GLUT4 vesicles, and insulin stimulates the PARP activity of tankyrase 1 through its phosphorylation by mitogen-activated protein kinase (11). Recently, tankyrase 1 also has been shown to poly(ADP-ribosyl)ate TAB182 (tankyrase-binding protein of 182-kDa protein) (12). ADP-ribosylation is a post-translational modification mechanism that usually results in a loss of protein activity presumably by enhancing protein turnover (13–16). However, little information is available regarding the physiological function(s) of tankyrase 1 other than as a PARP enzyme.

In the present study, we found tankyrase 1 as a specific-binding protein of Mcl-1. Overexpression of tankyrase 1 led to the inhibition of both the survival action of Mcl-1L and the apoptotic activity of Mcl-1S in mammalian cells. Unlike other known tankyrase 1-interacting proteins, tankyrase 1 did not poly(ADP-ribosyl)ate either of the Mcl-1 proteins despite its ability to decrease Mcl-1 protein levels following coexpression. Therefore, tankyrase 1 could regulate Mcl-1-modulated apo-

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§ To whom correspondence should be addressed. Tel.: 650-725-6802; Fax: 650-725-7102; E-mail: aaron.hsueh@stanford.edu.

[1] The abbreviations used are: Mcl-1, myeloid cell leukemia-1; BH domain, Bcl-2 homology domain; Mcl-1L, Mcl-1 long; Mcl-1S, Mcl-1 short; TRF1, telomeric repeat binding factor 1; CHO, Chinese hamster ovary; HA, hemagglutinin; PARP, poly(adenosine diphosphate ribose) polymerase; IRAP, insulin-responsive aminopeptidase.

TABLE I
Primers used for generating Mcl-1 and tankyrase1 mutants

Mutants	Upstream primer (5′ to 3′)	Downstream primer (5′ to 3′)
M1	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTATCCTTGGAAGGCCGTCTC
M2	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTAGGTCTCCAGCGCCTTCCT
M3	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTAGTACAACTCGTCCTCCTC
M4	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTACCCGAGAGGCTCCGGCTC
M5	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTAGCGGCGGGTGGGCGCGAA
M6	ACGACTAGTGAATTCATGGCGCCCACCCGCCGCGCG	CTATAGCTCGAGGGATCCCTAGTACAACTCGTCCTCCTC
M7	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTACCTCCGGGAGTCTGGCGT
M8	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTATCCCCCTATCTCTCGCCG
M9	ACGACTAGTGAATTCATGTTTGGCCTCAAAAGAAAC	CTATAGCTCGAGGGATCCCTAGCCGCTGCCGGCCCCCAA
M10	ACGACTAGTGAATTCATGGGGCCACCCGCCCGGGA	CTATAGCTCGAGGGATCCCTACGCGAAGAAAAGCAGCCT
M11	ACGACTAGTGAATTCATGCCGGGAGGGCGACTTTTG	CTATAGCTCGAGGGATCCCTATCCCCCTATCTCTCGCCG
M12	ACGACTAGTGAATTCATGGGGGAGGCCGGCGCGGTG	CTATAGCTCGAGGGATCCCTACCTCCGGGAGTCTGGCGT
M13	ACGACTAGTGAATTCATGGTCGCGCGGCCGCCGCCC	CTATAGCTCGAGGGATCCCTACGCGAAGAAAAGCAGCCT
T1	CTAAAGCTTACTAGTGATGGCGGCGTCGCGTCGCTCT	CTAAATCTCGAGCTAGCTCACTGCTGGGACCCCTGT
T2	CTAACTAGTGATGGGGGATGGCGCCGCGGGAACA	CTACTCGAGCTAGGTCTTCTGCTCTGCGGC
T3	CTAGGATCCAATGGGGGCCCTACGGGAACTGCTG	CTAAATCTCGAGCTATGCATTGGAGGCTCCTCC
T4	CTAGGATCCAATGGGGGCCCTACGGGAACTGCTG	CTAGAATTCCTATCTGTTCTTTTTAGTTGGATC
T5	CTAGGATCCAATGGGGGCCCTACGGGAACTGCTG	CTAGAATTCCTAGGTGTCCAGTGCATTCATCTT
T6	CTAGGATCCAATGGGGGCCCTACGGGAACTGCTG	CTAGAATTCCTACGACTTTCGCCCATCACTTGC
T7	CTAGGATCCAATGACTCCTTTACATCTAGCAGCG	CTAGAATTCCTAGGTGTCCAGTGCATTCATCTT
T8	CTAGGATCCAATGCTTGGTCAGACTGCTTTGCAT	CTAGAATTCCTATCTGTTCTTTTTAGTTGGATC
T9	CTAGGATCCAATGGATGGAAATACACCTTTGGAT	CTAAATCTCGAGCTATGCATTGGAGGCTCCTCC
T10	CTAGGATCCAATGACTCCTTTACATCTAGCAGCG	CTAAATCTCGAGCTATGCATTGGAGGCTCCTCC

ptosis by down-regulating the expression of Mcl-1 proteins without the involvement of its ADP-ribosylation activity.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The open reading frame of human Mcl-1S cDNA was fused in-frame with the GAL4-binding domain into the pGBT9 yeast shuttle vector (Clontech, Palo Alto, CA). This vector was used to identify Mcl-1S-interacting proteins by screening 1.5 million transformants from a GAL4 activation domain-tagged ovarian fusion cDNA library prepared from rats primed with equine chorionic gonadotropin (17). Yeast cells were cotransformed with pGBT9-Mcl-1S and cDNAs from the ovarian library, and colonies were selected in plates deficient in tryptophan, leucine, and histidine but containing 30 mM 3-amino-1,2,4-triazole (Sigma). Plasmids were isolated from positive colonies following transformation of *Escherichia coli* cells and then sequenced. Nine independent clones encoded the rat ortholog of human tankyrase 1. Full-length cDNA coding human tankyrase 1 was fused with the activation domain of GAL4 in a yeast shuttle vector, pGADGH. Subsequently, the specific interaction of tankyrase 1 with Mcl-1S was confirmed based on the activation of the GAL1-HIS3 reporter gene.

Assessment of Tankyrase 1 Interactions with Bcl-2 Family Proteins in the Yeast Two-hybrid System—Complementary DNAs encoding tankyrase 1 and various pro- or antiapoptotic Bcl-2 family proteins were subcloned into pGADGH and pGBT9 vectors, respectively. Specific binding of different protein pairs was evaluated based on the activation of the GAL1-HIS3 reporter gene. At least 10 different colonies were tested for each reaction.

In Vivo Binding of Tankyrase 1 with Mcl-1 Proteins—Chinese hamster ovary (CHO) cells (1.5×10^6) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 24 h of culture, cells were transfected with 1.8 μg each of pcDNA3-FLAG-epitope-tagged Mcl-1L, Mcl-1S, or BAD alone or together with 4.2 μg of either HA-epitope-tagged tankyrase 1 or an empty vector using LipofectAMINE (Invitrogen, Carlsbad, CA). CHO cells were harvested at 24 h post-transfection and lysed in prechilled 1% Nonidet P-40 lysis buffer containing 10% of a protease inhibitor mixture (Sigma). Following 30 min incubation in the lysis buffer, lysates were centrifuged at 10,000 $\times$ *g* for 10 min at 4 °C and supernatants were collected. Aliquots of the lysates were precleared by incubation with mouse IgG and Protein-A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4 °C. The precleared lysates were then incubated with 1 μg/ml anti-mouse FLAG M2 monoclonal antibody (Sigma) for 1 h followed by Protein-A-agarose for an additional 1 h at 4 °C. The immune complexes were centrifuged for 5 min at 2,000 $\times$ *g* and washed five times with 1% Nonidet P-40 lysis buffer.

The immunoprecipitates and aliquots of total lysates were boiled in SDS sample buffer for 5 min, subjected to SDS-PAGE, and electroblotted onto 0.22-μm nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline solution with 1% Tween 20 for 1 h followed by incubation with 0.2 μg/ml of the anti-HA monoclonal antibody (Sigma) for 1 h at room temperature. The blot was then incubated for 10 min with 0.1 μg/ml anti-rabbit or mouse IgG-horseradish peroxidase conjugate (Promega, Madison, WI) as a secondary antibody before visualization by enhanced chemiluminescence (Amersham Biosciences). The same membrane was stripped and incubated with 0.2 μg/ml mouse anti-FLAG M2 monoclonal antibody (Sigma), rabbit anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), and rabbit anti-BAD polyclonal antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The blot was exposed to the same secondary antibody and visualized by enhanced chemiluminescence.

For immunoprecipitation of Mcl-1 by tankyrase 1, CHO cells (4.5×10^6) were transfected with equal amounts (7.5 μg) of plasmids encoding

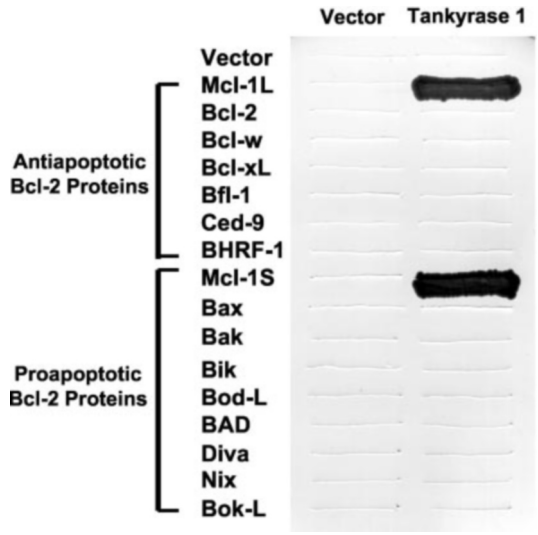


FIG. 1. **Specific interaction of tankyrase 1 with both Mcl-1L and Mcl-1S, but not other Bcl-2 family proteins, in the yeast two-hybrid system.** Yeast cells were grown in selective media containing 30 mM 3-aminotriazole and lacking Trp, Leu, and His. Prominent growth was found in colonies expressing tankyrase 1 fused to the GAL4 activation domain and Mcl-1L or Mcl-1S fused to the GAL4-binding domain. Other Bcl-2 proteins tested include the antiapoptotic Bcl-2, Bcl-w, Bcl-x_L, Bfl-1, Ced-9, and BHRF-1 as well as the proapoptotic Bax, Bak, Bik, Bod-L, BAD, Diva, Nix, and Bok-L. No growth of yeast colonies was found in cells expressing only the Bcl-2 family proteins, thus ruling out self-activation of these constructs. The result is a representative of 10 different colonies tested.

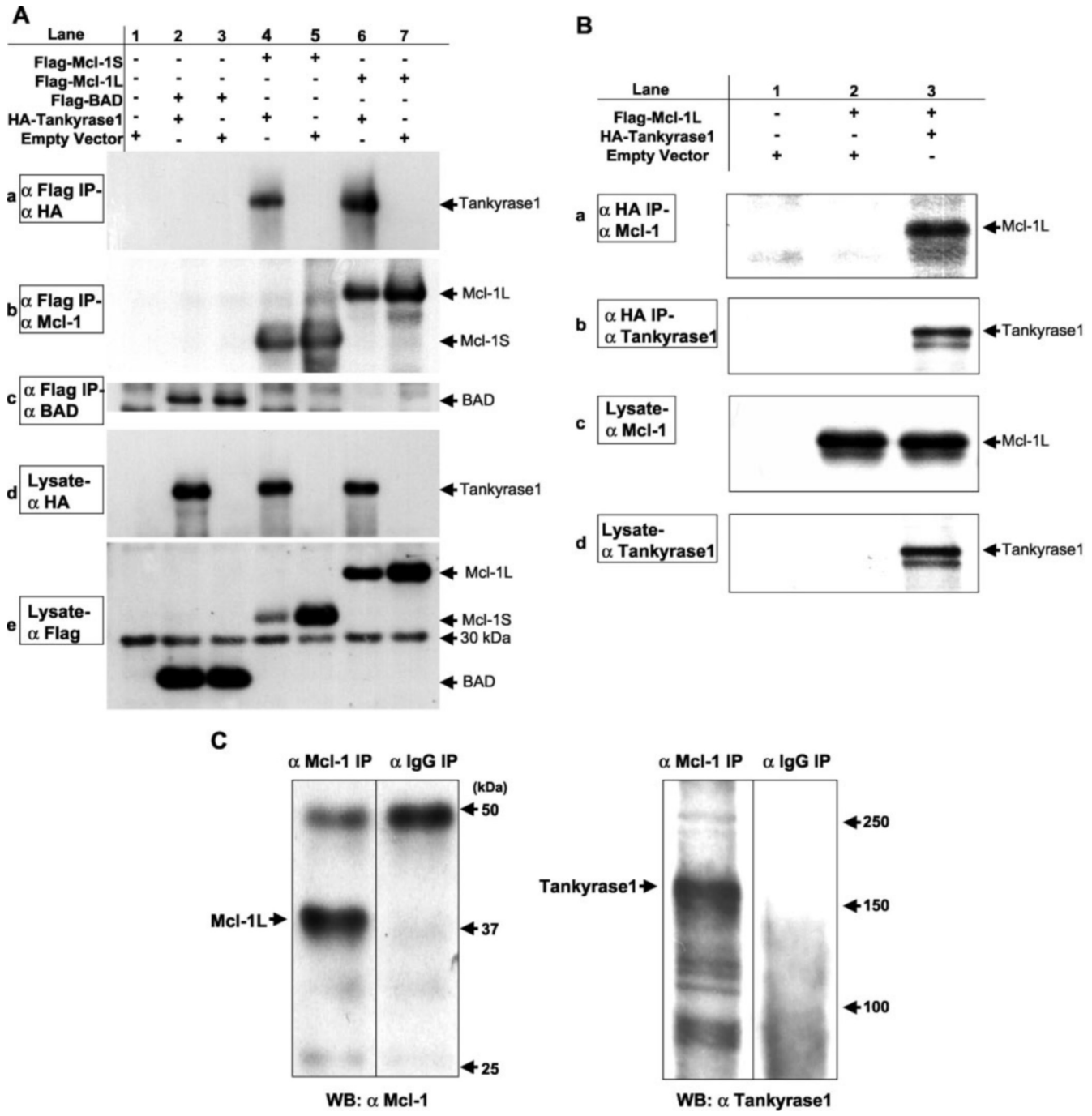


FIG. 2. ***In vivo* dimerization of tankyrase 1 with Mcl-1 proteins in mammalian cells.** *A*, co-immunoprecipitation of tankyrase 1 together with Mcl-1 proteins. CHO cells were transfected with FLAG-tagged Mcl-1L or Mcl-1S with or without HA-tagged tankyrase 1. As a negative control, HA-tagged tankyrase 1 was also coexpressed with FLAG-tagged BAD. Equal amounts of total DNA (6 μg) was used for transfection, including 4.2 μg of HA-tagged tankyrase 1 or the empty vector along with 1.8 μg of plasmid encoding FLAG-tagged Mcl-1L, Mcl-1S, or BAD. At 24 h after transfection, the cell lysate was used for immunoprecipitation tests with the M2 antibodies against the FLAG epitope (*α FLAG IP*). Following precipitation of FLAG-tagged Mcl-1 proteins, immunoblotting with the HA antibody was performed to demonstrate the co-precipitation of tankyrase 1 (*panel a*). The same membrane was blotted using antibodies against Mcl-1 (*panel b*) and BAD (*panel c*) to confirm their identity. Aliquots of the same lysate also were used for the detection of tankyrase 1 (*panel d*), Mcl-1, and BAD (*panel e*). *B*, co-immunoprecipitation of Mcl-1 proteins together with tankyrase 1. CHO cells were transfected with FLAG-tagged Mcl-1L with or without HA-tagged tankyrase 1. Equal amounts of total DNA (15 μg) were used for transfection, including 7.5 μg of HA-tagged tankyrase 1 or FLAG-tagged Mcl-1L. Cell lysates were immunoprecipitated using the HA antibody (*α HA IP*), followed by blotting with the Mcl-1 antibody (*panel a*) or the tankyrase 1 antibody (*panel b*). Aliquots of the lysates were also blotted using the same antibodies (*panels c* and *d*). *C*, interactions between endogenous Mcl-1 and tankyrase 1 in K562 cells. The cell lysate was immunoprecipitated with the monoclonal Mcl-1 antibody or IgG, and the precipitates (*α Mcl-1 IP* or *α IgG IP*) were blotted using the polyclonal Mcl-1 antibody (*left panel*) or the tankyrase 1 antibody (*right panel*).

HA-tankyrase 1 or FLAG-Mcl-1L. Cell lysates were immunoprecipitated using the anti-HA monoclonal antibody, and the precipitates were further blotted by the anti-Mcl-1 antibody or a rabbit anti-tankyrase 1 polyclonal antibody (9).

Binding of Endogenous Tankyrase 1 and Mcl-1L—Human myeloid leukemia cell line K562 (ATCC, Manassas, VA) was cultured in RPMI 1640 medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were lysed and precleared before incubation with 2 μg/ml mouse anti-Mcl-1 monoclonal antibody (BD Pharmingen, San Diego, CA) or mouse IgG for 2 h, followed by incubation with Protein-A-agarose for 2 h at 4 °C. The immune complexes were centrifuged for 5 min at 2,000 × *g* and washed

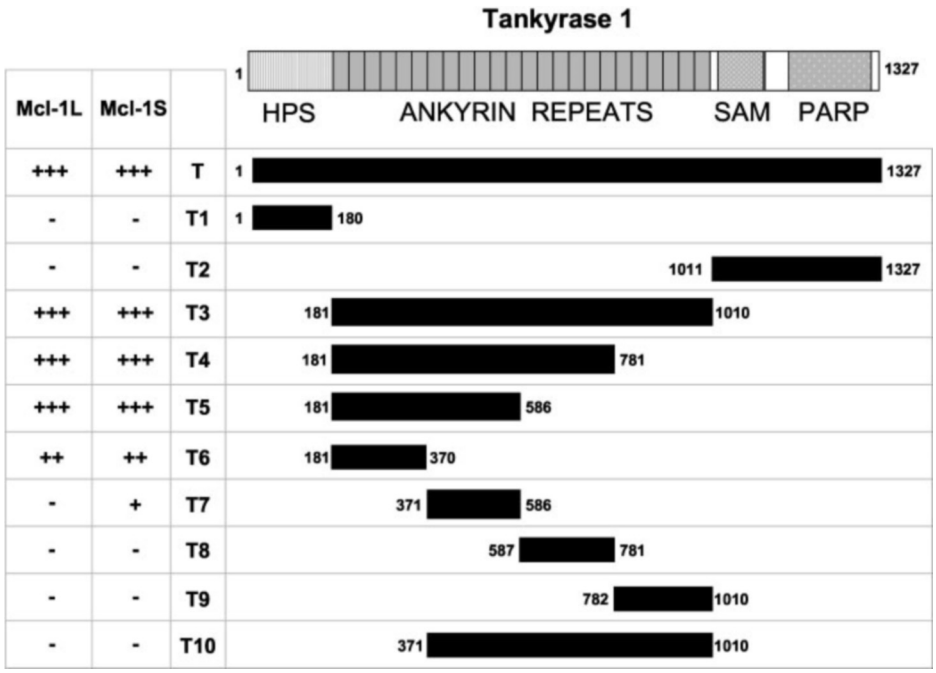


FIG. 3. **Interactions of Mcl-1 proteins with truncated tankyrase 1 mutants in the yeast two-hybrid system: involvement of ankyrin repeats for Mcl-1 interaction.** Yeast cells were grown under the same conditions described in the legend of Fig. 1 and the interactions between tankyrase 1 mutants and the Mcl-1 proteins were examined. Either Mcl-1L or Mcl-1S coding plasmids was fused to the GAL4-binding domain, and wild type (*T*) or mutant (*T1* to *T10*) tankyrase 1 were expressed in the yeast vector containing the GAL4 activation domain. The truncation of the HPS sequence, SAM, and PARP motifs of tankyrase 1 did not affect the interactions between tankyrase 1 and the Mcl-1 proteins. In addition, ankyrin repeats toward the C-terminal end (T3 *versus* T5) are dispensable for tankyrase 1 binding to the Mcl-1 proteins. However, truncation of the N-terminal ankyrin repeats (T7 to T10) of tankyrase 1 abolished its interaction with both Mcl-1 proteins. The absence of detectable yeast growth is indicated as "−", whereas +++, ++, and + depict strong, medium, and weak interactions, respectively. The data is a summary of at least 10 different colonies.

five times with 1% Nonidet P-40 lysis buffer. Immunoblotting was performed using either the anti-Mcl-1 polyclonal antibody or the anti-tankyrase 1 antibody.

Regions Responsible for Interactions between Mcl-1 and Tankyrase 1—To construct various truncated mutants of Mcl-1 and tankyrase 1, PCR amplifications were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA). Truncated Mcl-1 and tankyrase 1 cDNAs were generated using primers as described in Table I. Wild type and truncated Mcl-1 cDNAs were subcloned into the pGBT9 vector whereas wild type and mutant tankyrase 1 cDNAs were inserted into the pGADGH vector. Specific interactions between these proteins were determined by the activation of the GAL1-HIS3 reporter gene.

Assessment of Apoptosis in Transfected CHO Cells and Down-regulation of Mcl-1 Proteins by Tankyrase 1—Apoptosis was monitored following transfection of different cDNAs as described previously (18). CHO cells (2×10^5 cells/35-mm well) were cultured for 24 h and transfected with the pcDNA3 expression vector with or without different cDNA inserts using LipofectAMINE. For all experiments, the indicator plasmid pCMVβ (Clontech) was included to allow the identification of transfected cells. Inclusion of a 10-fold excess of expression vectors as compared with the pCMVβ reporter plasmid ensured that most of the β-galactosidase-expressing cells also expressed the protein(s) under investigation. After a 4-h incubation of cells with the transfection mixtures in a serum-free medium, cells were incubated with fresh medium containing 10% fetal bovine serum. Twenty-four hours after transfection, cells were fixed in 0.3% glutaraldehyde and stained with 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside (Invitrogen) to detect β-galactosidase expression. The number of blue cells was counted by microscopic examination. Data were expressed as the percentage (mean ± S.E.) of viable cells as compared with the control group.

To assess Mcl-1 protein levels following tankyrase 1 coexpression, CHO cells were transfected with different cDNAs and cell lysates were prepared at 24 h after transfection. Equal amounts of the lysates were subjected to SDS-PAGE. The same gel was immunoblotted with the anti-FLAG M2, the anti-tankyrase 1, and the anti-β-actin monoclonal antibodies (Sigma).

Purification of Recombinant Mcl-1L, Mcl-1S, and Tankyrase 1 Proteins—N-terminal FLAG-tagged Mcl-1 proteins were expressed in *E. coli* (BL21 codon plus) using the pET-21a Vector (Novagen, Madison, WI) and cell lysates were prepared by a French presser. Recombinant Mcl-1 proteins were purified using an anti-FLAG M2 affinity gel and eluted using the 3× FLAG peptide (Sigma). Purified Mcl-1 proteins were monitored using SDS-PAGE followed by Coomassie Blue staining. Baculovirus-derived tankyrase 1 and TRF1 were prepared as described (9).

In Vitro Interactions between Recombinant Tankyrase 1 and Mcl-1 Proteins—Tankyrase 1 (100 ng) was incubated with either 100 ng of Mcl-1L or Mcl-1S in 100 μl of 1% Nonidet P-40 lysis buffer. After 1 h at 4 °C, the mixtures were subjected to immunoprecipitation using the anti-FLAG M2 affinity gel. The immune complexes were centrifuged for 5 min at 2,000 × *g* and washed five times with the lysis buffer and boiled in the SDS sample buffer. Immunoblotting was done using rabbit antibodies against tankyrase 1 or Mcl-1.

PARP Assay—PARP activity was determined using baculovirus-derived tankyrase 1 as previously described (9). For each reaction, purified tankyrase 1 (2 or 4 μg) was mixed with equal or increasing amounts of the target protein (Mcl-1L, Mcl-1S, and/or TRF1) and incubated for 30 min at 25 °C in an assay buffer containing 50 mM Tris (pH 8.0), 4 mM $MgCl_2$, 0.2 mM dithiothreitol, and 1.3 μM [^{32}P]NAD^+ (4 μCi, 30 Ci/mmol). Reactions were stopped by the addition of trichloroacetic acid to 25%. Acid-insoluble proteins were collected by centrifugation, rinsed in 5% trichloroacetic acid, suspended in the sample loading buffer (1 M Tris base, 12% SDS, 0.2% bromphenol blue, and 0.2 M dithiothreitol), and fractionated on an 10% SDS-polyacrylamide gel. The gel was dried and exposed on a PhosphorImager.

RESULTS

Specific Interactions of Tankyrase 1 with Mcl-1L and Mcl-1S but Not Other Bcl-2 Family Proteins in Yeast and Mammalian Cells—We performed a yeast two-hybrid screening using full-length Mcl-1S as bait to identify interacting proteins from the ovary cDNA library. Sequence analysis revealed that nine strongly interacting clones encode a rat ortholog of human tankyrase 1 with 330 amino acids truncated at the C terminus. The yeast two-hybrid system was used to determine the interactions of tankyrase 1 with Mcl-1 and other Bcl-2 family members. Human tankyrase 1 interacted strongly with both Mcl-1L and Mcl-1S (Fig. 1). In contrast, no interaction was detectable between tankyrase 1 and different Bcl-2 family proteins tested including antiapoptotic Bcl-2, Bcl-w, Bcl-x_L, Bfl-1, Ced-9, and BHRF-1, as well as proapoptotic Bax, Bak, Bik, Bod-L, BAD, Diva, Nix, and Bok-L (Fig. 1).

In vivo interactions of tankyrase 1 with Mcl-1 proteins were confirmed in CHO cells transiently transfected with tankyrase 1 cDNA and Mcl-1 cDNAs. Cell lysate was precipitated using the M2 antibody against the FLAG epitope, followed by blotting using the HA antibodies. As shown in Fig. 2*A* (*panel a*, *lanes 4* and *6*), HA-tagged tankyrase 1 was coimmunoprecipitated with FLAG-tagged Mcl-1L or Mcl-1S. In contrast, tankyrase 1 failed to coprecipitate with FLAG-tagged BAD protein (Fig. 2*A*, *panel a*, *lane 2*), consistent with a lack of interaction observed in the

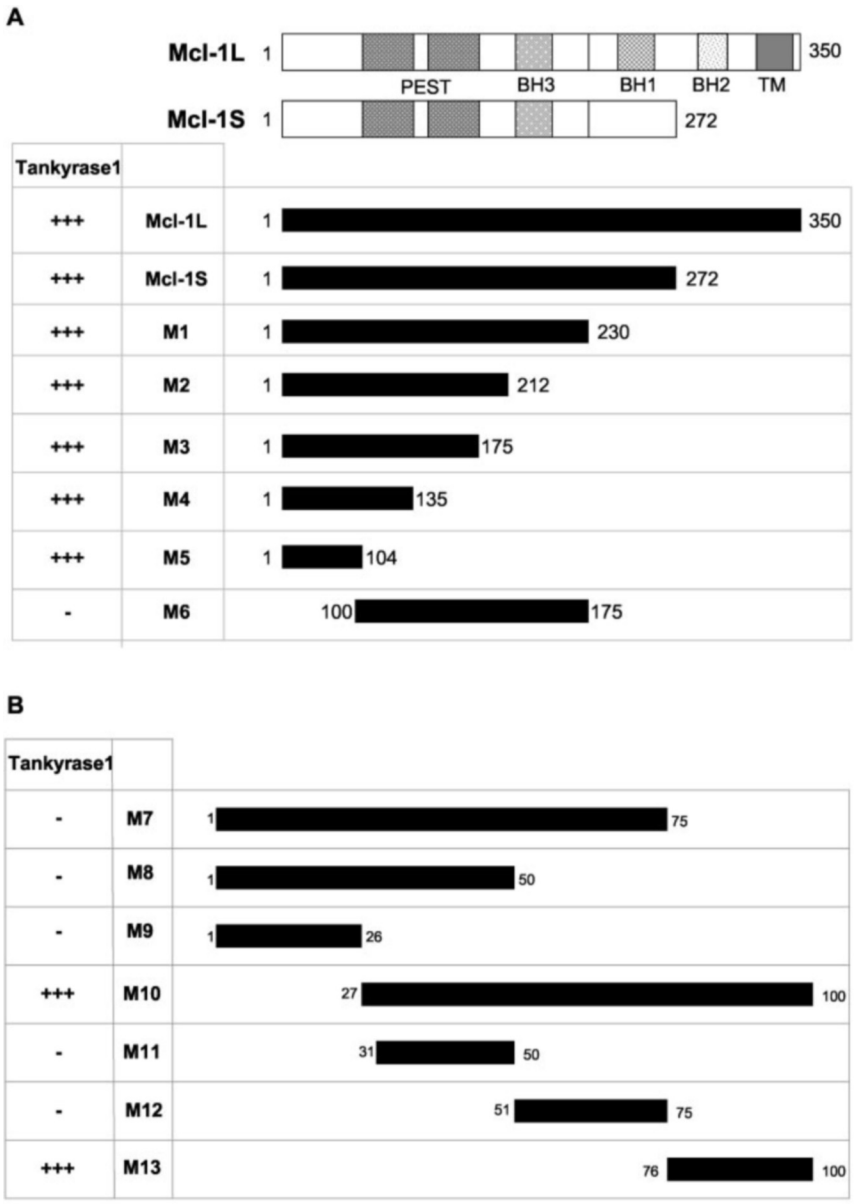


FIG. 4. **Determination of the regions in Mcl-1 that mediate its association with tankyrase 1 in the yeast two-hybrid system.** The interactions between the Mcl-1 mutants (M1 to M13) and tankyrase 1 were determined by expressing truncated Mcl-1 mutants fused to the GAL4-binding domain and the full-length tankyrase 1 fused to the GAL4 activation domain. *A*, truncation of the majority of the C-terminal regions including the PEST, BH1, BH2, BH3, and TM domains of Mcl-1 (*M1* to *M5*) did not affect interaction with tankyrase 1. However, truncation of the N-terminal region of Mcl-1 (*M6*) abolished interactions with tankyrase 1. *B*, the extreme N terminus of Mcl-1 was further truncated (*M7* to *M13*) to identify sequences responsible for tankyrase 1 binding. Mutants missing amino acids 76 to 100 (*M7* to *M9*, *M11*, and *M12*) lost the ability to interact with tankyrase 1, whereas an Mcl-1 mutant possessing only amino acids 76 to 100 (*M13*) exhibited a strong interaction with tankyrase 1 comparable with that of wild type Mcl-1.

yeast two-hybrid system. Of interest, the amount of Mcl-1L or Mcl-1S protein was lower when the Mcl-1 proteins were coexpressed with tankyrase 1 as shown in Fig. 2*A* (*panel e*, *lane 4 versus 5* and *lane 6 versus 7*). The same amount of lysate was loaded in each lane and the nonspecific 30-kDa band served as an internal control. The observed lower expression of Mcl-1L or Mcl-1S protein in the presence of tankyrase 1 suggested an increased turnover of these proteins because of their interactions with tankyrase 1.

In vivo interactions between Mcl-1 and tankyrase 1 were further determined in CHO cells following immunoprecipitation of tankyrase 1. Mcl-1L was coprecipitated with HA-tagged tankyrase 1 using the HA antibody, followed by immunoblotting using the Mcl-1 antibody (Fig. 2*B*, *panel a*, *lane 3*). Because a nonspecific protein reacted to the Mcl-1 antibody and migrated to the same position as the Mcl-1S in the SDS-PAGE gel (data not shown), coprecipitation of Mcl-1S with tankyrase 1 could not be tested.

To test interactions between endogenous Mcl-1 and tankyrase 1 proteins, we used the human leukemia cell line K562. As shown in Fig. 2*C* (*left panel*), endogenous Mcl-1L in these cells was precipitated by an Mcl-1 antibody but not by nonimmune IgG. In addition, tankyrase 1 was found to be coprecipitated with Mcl-1L as evidenced by an immunoreactive band above 150 kDa (Fig. 2*C*, *right panel*). The detection of endogenous Mcl-1L and tankyrase 1 as complexes supports the physiological interactions between these two proteins.

Tankyrase 1 Interacts with the Mcl-1 Proteins through Its Ankyrin Domain—To determine the region of tankyrase 1 responsible for interaction with Mcl-1 proteins, truncated mutants of tankyrase 1 (T1 to T10) were generated and their binding to Mcl-1L and Mcl-1S was tested in the yeast two-hybrid system. Tankyrase 1 protein has four major domains (Fig. 3) including the HPS sequence, 24 ankyrin repeats, SAM motif, and the catalytic domain of PARP (9). The two Mcl-1 proteins did not interact with the HPS (T1), SAM (T2), or PARP (T2) motif of tankyrase 1, whereas the ankyrin domain of tankyrase 1 alone (T3) exhibited a strong interaction to both Mcl-1 proteins (Fig. 3). We further truncated the ankyrin repeats of tankyrase 1 to determine the minimal region responsible for binding to Mcl-1. As shown in Fig. 3, the region spanning from amino acid 181 to 586 of tankyrase 1 (T5), containing 12 ankyrin repeats exhibited the same degree of interaction as wild type tankyrase 1. Additional truncation of

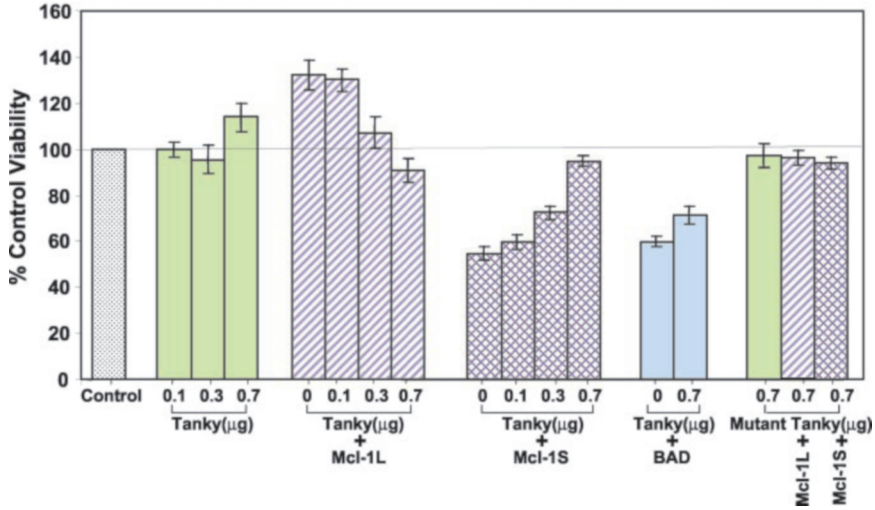


FIG. 5. **Coexpression with tankyrase 1 blocks the antiapoptotic activity of Mcl-1L and the proapoptotic activity of Mcl-1S: lack of involvement of the PARP domain of tankyrase 1.** Quantitative cell viability analysis was performed to test the modulatory effect of tankyrase 1 on the Mcl-1 regulation of apoptosis. CHO cells were transfected with a total of 1.1 μg of plasmid DNA including 1.0 μg of the pcDNA3 expression construct and 0.1 μg of the pCMV-β-gal reporter. The number of β-galactosidase-expressing cells was determined at 24 h after transfection. Data (mean $\pm$ S.E.) are from at least three different experiments in triplicate. For the control group, 1.0 μg of the empty pcDNA3 vector was used. When increasing amounts (0.1, 0.3, or 0.7 μg) of tankyrase 1-expressing plasmid were used for transfection, the empty vector was added to ensure the use of the same amount (1.1 μg) of total plasmid. In all experiments, 0.3 μg of plasmid encoding Mcl-1S, Mcl-1L, or BAD with or without tankyrase 1 plasmid were used. Both cell survival mediated by Mcl-1L and apoptosis induced by Mcl-1S were dose-dependently blocked by tankyrase 1. In contrast, tankyrase 1 was not effective in blocking the cell killing induced by BAD. A mutant tankyrase 1 with the PARP domain deleted (mutant Tanky; 0.7 μg) maintained the ability to inhibit the actions of both Mcl-1L and Mcl-1S, indicating this region is dispensable for the antagonistic actions of tankyrase 1.

ankyrin repeats (T6 to T10) resulted in a lost or weaker interaction.

A Short Stretch of 25 Amino Acids toward the N Terminus of Mcl-1 Is Sufficient for Binding to Tankyrase 1—Mcl-1L has two PEST motifs together with BH1, BH2, BH3, and a transmembrane domain, whereas Mcl-1S possesses only PEST motifs and the BH3 domain (Fig. 4*A*) (6). Various truncated Mcl-1 mutants (M1 to M13) were generated to determine the region essential for Mcl-1 interaction with tankyrase 1. Because both Mcl-1L and Mcl-1S interacted with tankyrase 1 to the same extent, the interacting region likely resides in the common sequences of Mcl-1L and Mcl-1S. Consistent with this notion, deletion of BH1, BH2, and the transmembrane domains did not affect the interaction of Mcl-1 with tankyrase 1 (Fig. 4*A*, *M1*). In addition, the BH3 domain, the only conserved region found in all Bcl-2 family members, was not involved in tankyrase 1 binding (M2). Deletion of either one or both of the known PEST sequences of Mcl-1 (M4 and M5) also did not alter tankyrase 1 binding; the N-terminal region of Mcl-1 interacted with tankyrase 1 as strongly as the full-length Mcl-1 (M5). However, truncation of the N-terminal region of Mcl-1 from amino acid 76 to 100 (M7) abolished its interaction with tankyrase 1 (Fig. 4*B*). The importance of this region was further supported by a lack of interaction with tankyrase 1 by other Mcl-1 mutants (*M8, M9, M11*, and *M12*) lacking amino acids 76 to 100. In contrast, the Mcl-1 mutant (M13) containing only amino acids 76 to 100 retained a strong binding to tankyrase 1 comparable with that of wild type Mcl-1L or Mcl-1S. Therefore, the short stretch of 25 amino acids (VARPPPIGAEVPDVTATPARLLFFA) of Mcl-1 is sufficient to mediate tankyrase 1 binding. According to the PESTFIND program (Pasteur Institute, France), this interacting region also contains weak PEST sequences (underlined).

Tankyrase 1 Antagonizes Both the Survival Effect of Mcl-1L and the Proapoptotic Action of Mcl-1S—We investigated whether tankyrase 1 modulates the function of Mcl-1 proteins using a cell viability assay. Overexpression of increasing amounts of tankyrase 1 itself did not alter CHO cell viability as compared with controls (Fig. 5). In accordance with previous studies (6–8), overexpression of Mcl-1S induced cell death and overexpression of Mcl-1L prevented the small increases in cell death resulting from transfection *per se*. When tankyrase 1 was coexpressed with either Mcl-1L or Mcl-1S, tankyrase 1 antagonized the actions of both Mcl-1L and Mcl-1S in a concentration-dependent manner. At a higher dose, tankyrase 1 effectively blocked both Mcl-1L-mediated cell survival and Mcl-1S-induced cell death. In contrast, the proapoptotic action of BAD was not antagonized by tankyrase 1. These data suggest that inhibition of Mcl-1 activities by tankyrase 1 is specific, likely resulting from direct interactions of Mcl-1 proteins with tankyrase 1.

To determine the involvement of the PARP catalytic domain of tankyrase 1 in its inhibition of Mcl-1 actions, a mutant tankyrase 1 devoid of the PARP domain (mutant Tanky; amino acids 1 to 1150) was coexpressed with Mcl-1. Of interest, this mutant was still able to antagonize the effects of either Mcl-1L or Mcl-1S on cell viability (Fig. 5). These results suggest that the blockage of Mcl-1 effects by tankyrase 1 does not require ADP-ribosylation of Mcl-1 proteins.

Tankyrase 1 Down-regulates Mcl-1L and Mcl-1S Protein Levels—Because coexpression with tankyrase 1 is associated with decreased levels of the Mcl-1 proteins (Fig. 2*A*), down-regulation of the Mcl-1 proteins by takyrase 1 was further investigated. Coexpression of increasing amounts of tankyrase 1 decreased the expression of both Mcl-1L and Mcl-1S in a concentration-dependent manner (Fig. 6, *panels A* and *B*). In contrast, tankyrase 1 did not alter the expression of BAD. Furthermore, mutant tankyrase 1 devoid of the PARP domain (mutant Tanky) also effectively decreased the expression of both Mcl-1L and Mcl-1S. These data indicate that tankyrase 1-mediated Mcl-1 down-regulation does not require the PARP enzymatic activity of tankyrase 1. Equal loading of samples was demonstrated by Western blotting of the same membrane using the β-actin antibody (Fig. 6, *panel C*), and the nonspecific 30-kDa band that reacted with the FLAG antibody served as an internal control (Fig. 6, *panel A*). Several lower bands found in lanes containing either wild type (*lanes 3–5*, *7–9*, and *11–13*) or

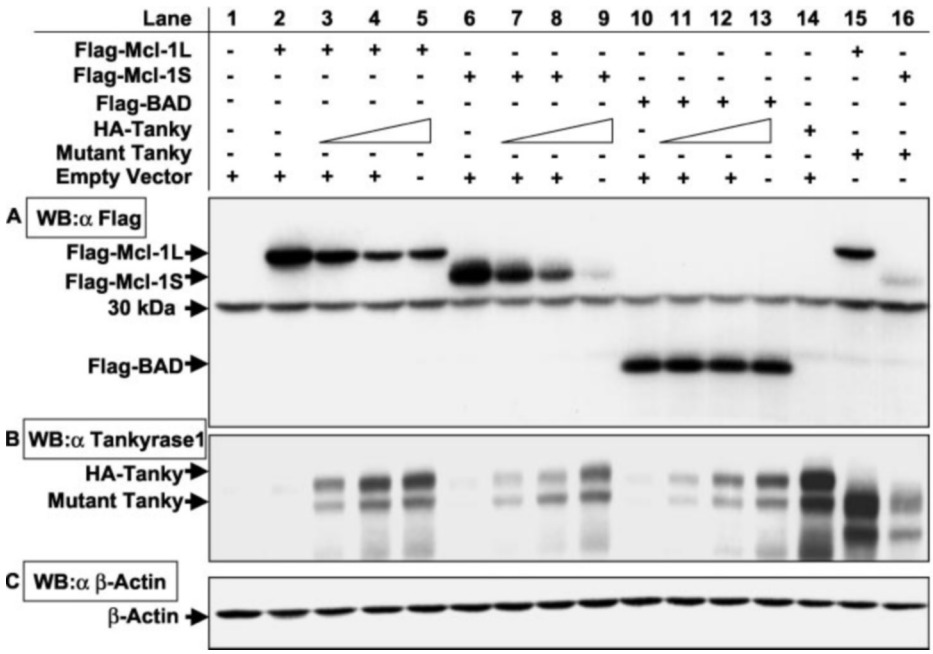
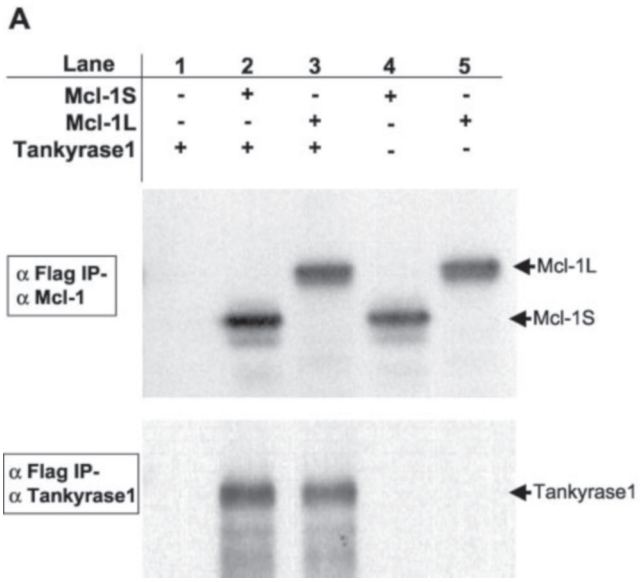


FIG. 6. **Down-regulation of Mcl-1 protein levels by wild type tankyrase 1 and its PARP deletion mutant in transfected cells.** CHO cells were transfected using a total of 1.0 μg of the pcDNA3 expression plasmid and cell lysates were prepared 24 h later. Plasmids (0.3 μg) encoding Mcl-1S, Mcl-1L, or BAD, were used, together with increasing concentrations (0.1, 0.3, or 0.7 μg) of the tankyrase 1 plasmid or a plasmid (0.7 μg) encoding the mutant tankyrase 1 (mutant Tanky) with the PARP domain deleted. Tankyrase 1 decreased the levels of Mcl-1 proteins in a concentration-dependent manner, whereas BAD expression was not altered by tankyrase 1 as demonstrated by immunoblotting using the M2 antibody against the FLAG epitope (*panel A*). *Panel B* is an immunoblot using the tankyrase 1 antibody to demonstrate takyrase 1 expression. Equal loading of lysates is evident in immunoblots using the β-actin antibody (*panel C*).



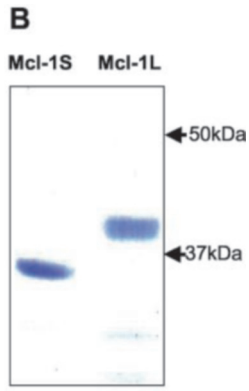


FIG. 7. **Interactions between recombinant Mcl-1 proteins and tankyrase 1 *in vitro*.** *A*, purified Mcl-1 proteins (100 ng) were incubated with tankyrase 1 (100 ng), and the mixtures were immunoprecipitated using the anti-FLAG M2 affinity gel. The immune complexes were fractionated by SDS-PAGE and then immunoblotted using the Mcl-1 polyclonal antibody (*top panel*). The same membrane was further blotted using the tankyrase 1 antibody (*lower panel*). *B*, purified recombinant FLAG-tagged Mcl-1 proteins (1 μg) were subjected to 10% SDS-PAGE and stained with Coomassie Blue.

mutant tankyrase 1 (*lanes 15* and *16*) could result from cleavages of tankyrase 1 by caspases or other enzymes as found for other PARP proteins (19). Because the concentrations of plasmids used were comparable between the experiments shown in Figs. 5 and 6, the effects of both wild type and mutant tankyrase 1 on Mcl-1-regulated apoptosis correlate with their down-regulation of Mcl-1 proteins.

Direct Interactions between Recombinant Mcl-1 Proteins and Tankyrase 1: Tankyrase 1 Does Not ADP-ribosylate Mcl-1 Proteins—We evaluated the interactions between Mcl-1 and tankyrase 1 and the possible ADP-ribosylation of Mcl-1 by tankyrase 1. To assess the functional integrity of the purified recombinant Mcl-1 proteins, their binding to recombinant tankyrase 1 protein was tested *in vitro*. As shown in Fig. 7*A* (*lower panel*, *lanes 2* and *3*), the recombinant Mcl-1 proteins efficiently pulled down tankyrase 1, indicating that the purified Mcl-1 proteins are functionally active. The purity of the Mcl-1 proteins used in this test were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 7*B*).

Tankyrase 1 contains the catalytic domain of PARP, and ADP-ribosylates itself (9) and its interacting proteins such as TRF1 (9), IRAP (11), and TAB182 (12). However, our studies using PARP-deleted tankyrase 1 suggest that ADP-ribosylation is not essential for the modulation of Mcl-1 activities and levels. We further tested whether tankyrase 1 could ADP-ribosylate Mcl-1 proteins. An *in vitro* poly(ADP-ribosyl)ation assay was performed using purified recombinant Mcl-1 proteins and tankyrase 1. Incubation of tankyrase 1 in the presence of [^{32}P]NAD$^+$ resulted in auto-ADP-ribosylation (Fig. 8*A*, *lane 1*) consistent with previous findings (9). Coincubation of increasing amounts of either Mcl-1L or Mcl-1S with tankyrase 1 did not lead to ADP-ribosylation of Mcl-1L or Mcl-1S (Fig. 8*A*, *lanes 5–10*). In contrast, tankyrase 1 ADP-ribosylated its known substrate, TRF1, in a concentration-dependent manner (Fig. 8*A*, *lanes 2–4*) (9). To test the possibility that binding between Mcl-1 proteins and tankyrase 1 could alter the PARP activity of tankyrase 1, the ability of Mcl-1 proteins to modify poly(ADP-ribosyl)ation by tankyase 1 was further examined. As shown in Fig. 8*B*, increasing amounts of either Mcl-1L or Mcl-1S reduced the ADP-ribosylation of TRF1 by tankyrase 1

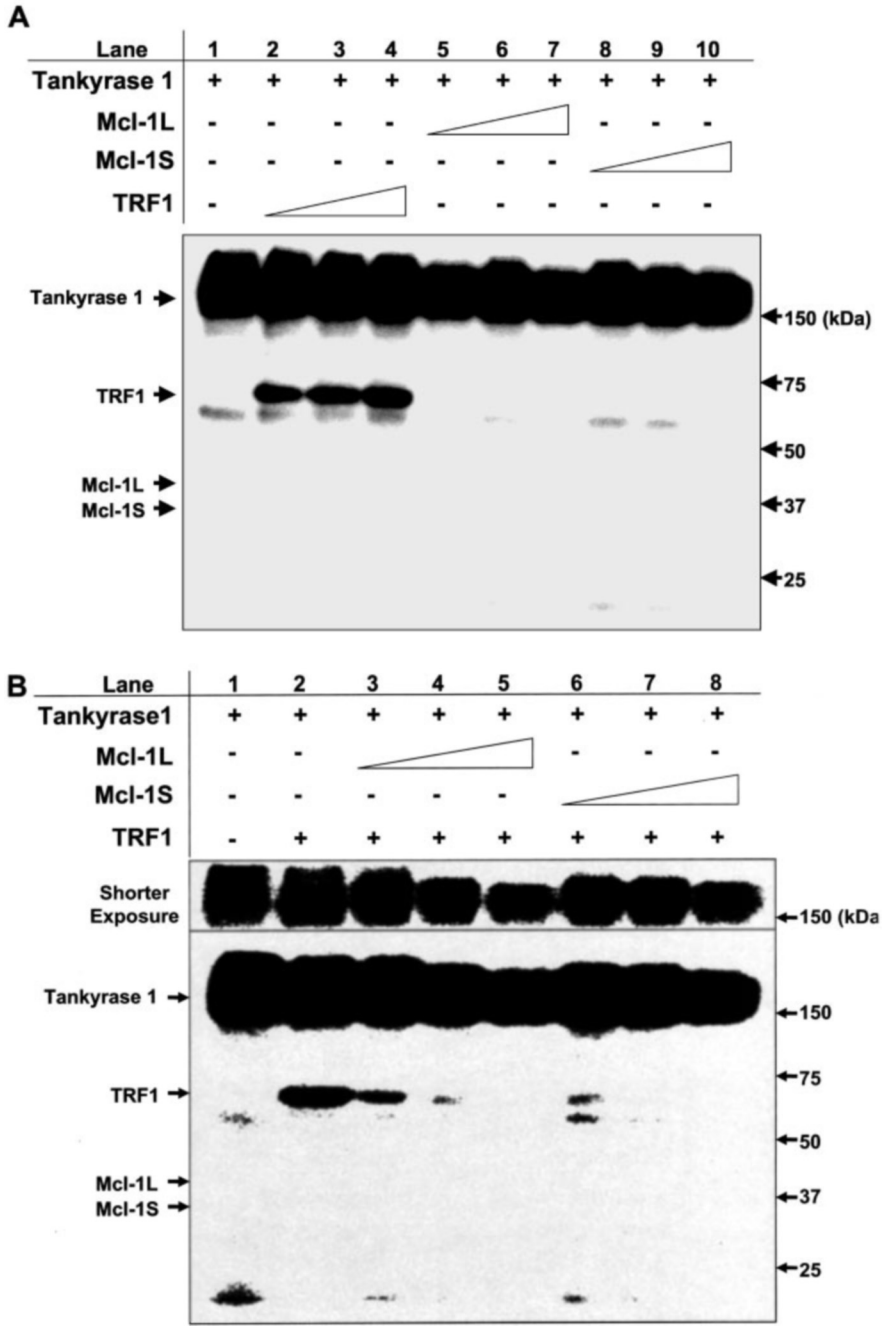


FIG. 8. **Tankyrase 1 does not ADP-ribosylate Mcl-1 proteins whereas Mcl-1 proteins suppress ADP-ribosylation of TRF1 and tankyrase 1.** PARP reactions were performed using [^{32}P]NAD^{+} as the substrate. Samples were fractionated on a 10% SDS-PAGE gel and ^{32}P-poly(ADP-ribosyl)ated proteins were visualized by a phosphoimaging. *A*, equal amounts of tankyrase 1 (4 μg) were incubated without (*lane 1*) or with increasing amounts (1, 2, or 4 μg) of TRF1 (*lanes 2–4*), Mcl-1L (*lanes 5–7*), or Mcl-1S (*lanes 8–10*). Although tankyrase 1 poly(ADP-ribosyl)ated itself and TRF1, it did not ADP-ribosylate either Mcl-1L or Mcl-1S. *B*, the same amount (2 μg) of tankyrase 1 and TRF1 was incubated in the absence (*lane 2*) or presence of increasing concentrations (1, 2, or 4 μg) of Mcl-1L (*lanes 3–5*) or Mcl-1S (*lanes 6–8*). Although Mcl-1 proteins were not ADP-ribosylated by tankyrase 1, inclusion of increasing amounts of Mcl-1 proteins decreased tankyrase 1-mediated poly-(ADP-ribosyl)ation of TRF1 and tankyrase 1 itself in a concentration-dependent manner.

in a concentration-dependent manner. In addition, auto-ADP-ribosylation of tankyrase 1 was also decreased in the presence of increasing amounts of Mcl-1 protein (Fig. 8*B*; shorter exposure). These data suggest that tankyrase 1-mediated ADP-ribosylation of Mcl-1 is unlikely to be the mechanism underlying the down-regulation of the Mcl-1 proteins, and the Mcl-1 proteins could affect tankyrase 1 function by modulating its ADP-ribosylation of itself and its binding protein, TRF1.

DISCUSSION

Mcl-1, a unique Bcl-2 family protein, is transiently induced by various growth factors and shows a rapid turnover rate (20, 21). Earlier studies demonstrated that Mcl-1 expression is up-regulated by epidermal growth factor, elk-1, granulocyte-macrophage colony-stimulating factor, gonadotropins, stem cell factor, interferon-α, and different interleukins in diverse cell lineages (22–26). Despite numerous studies on the induction of Mcl-1, no information is available regarding the suppression of the rapidly induced Mcl-1 proteins. Based on yeast two-hybrid screening, we identified an Mcl-1-interacting protein, tankyrase 1, that could provide a novel mechanism to decrease the levels and to suppress the actions of both Mcl-1L and its splicing variant Mcl-1S.

We demonstrated that overexpression of tankyrase 1 suppressed cell survival induced by Mcl-1L. In addition, tankyrase 1 also effectively blocked apoptosis induced by Mcl-1S. In contrast, overexpression of tankyrase 1 itself exhibited no significant effect on CHO cell viability, consistent with a recent observation (34). Furthermore, immunoblot studies showed that coexpression of tankyrase 1 resulted in lower levels of both Mcl-1L and Mcl-1S proteins. Thus, tankyrase 1-mediated changes in cell viability may be related to its ability to down-regulate Mcl-1 proteins. The finding of endogenous interactions between Mcl-1L and tankyrase 1 in a human leukemia cell line further supports the physiological importance of the present findings.

More than 20 known mammalian Bcl-2 family proteins share common regulatory mechanisms to modulate apoptosis. They dimerize with other Bcl-2 family members (1) or interact with specific interacting proteins. For instance, BAD binding to 14-3-3 proteins suppresses the apoptotic activity of BAD (18, 27), whereas Bim/BOD binding to the LC8 dynein light chain leads to its sequestration to the microtubule-associated dynein motor complex (28). Likewise, Bmf interacts with the dynein light chain 2 before being sequestered to the myosin V actin motor complex (29), leading to apoptosis suppression. Here, we have demonstrated that both Mcl-1L and Mcl-1S are strongly associated with tankyrase 1 in yeast and mammalian cells. Interactions of tankyrase 1 with Mcl-1 proteins were specific because none of the other members of the Bcl-2 family that were tested bound to tankyrase 1.

The antiapoptotic Mcl-1L protein consists of two consensus PEST sequences as well as the BH3, BH1, BH2, and a transmembrane domain. However, the proapoptotic splicing variant, Mcl-1S, possesses only the PEST sequences and the BH3 domain. The common N-terminal end of both Mcl-1 proteins contain the two PEST motifs and four pairs of arginine residues found in rapidly degraded proteins such as c-Myc and p53 (30). Although the deletion of these two PEST sequences in Mcl-1 does not affect the half-life of the mutant protein (20), the extreme N-terminal end of Mcl-1 has a stretch of residues with a weak PEST homology. We demonstrated that the binding of Mcl-1 proteins to tankyrase 1 involves this short stretch of 25 amino acids (76 to 100). Of interest, the same region also contains a RPPPIG sequence that resembles the consensus tankyrase-binding motif (R*XX*PDG) found in other tankyrase 1-interacting proteins (31).

Tankyrase 1 possesses four distinct motifs: the HPS module, 24 ankyrin motifs, SAM, and the catalytic domain of PARP (9). Tankyrase 1 is a unique protein with structural features found in both ankyrin and PARP family genes. Ankyrin family proteins are linkers that couple diverse membrane proteins via ankyrin motifs to the underlying cytoskeleton (32, 33). All known tankyrase 1-interacting proteins (TRF1, IRAP, and TAB182) bind to tankyrase 1 through ankyrin repeat regions (9, 11, 12). Likewise, our data indicated that interactions between Mcl-1 proteins and tankyrase 1 also involved the ankyrin motifs, and the first half of the ankyrin repeats (1 to 12) was sufficient for dimerization. Of particular interest, TRF1 and Mcl-1 are capable of binding to overlapping ankyrin repeats in tankyrase 1, suggesting potential competition between these tankyrase 1-interacting proteins.

Tankyrase 1 contains the catalytic domain of PARP and is known to poly(ADP-ribosyl)ate its interacting proteins, TRF1, IRAP, and TAB182 (9, 11, 12). PARP family proteins catalyze the attachment of the poly(ADP-ribose) moiety onto a protein acceptor using the substrate NAD^+ (nicotinamide adenine dinucleotide), and ADP-ribosylation of proteins usually leads to protein inactivation (13–16). However, our study demonstrated that tankyrase 1 does not poly(ADP-ribosyl)ate either of the Mcl-1 proteins. Furthermore, a tankyrase 1 mutant with the PARP domain deleted is still capable of decreasing the Mcl-1 protein levels and blocking the actions of the Mcl-1 proteins. These data suggest that the ability of tankyrase 1 to suppress both anti- and pro-apoptotic actions of the Mcl-1 proteins is not mediated by ADP-ribosylation of these proteins. The findings also suggest that tankyrase 1-interacting proteins are not always the substrates for tankyrase 1. Consistent with the unique structural features and subcellular localization of tankyrase 1, the current study suggests that tankyrase 1 is different from other PARP enzymes (9, 11, 35, 36) and could have nonenzymatic functions. Of interest, tankyrase 2, a gene with 83% sequence identity to tankyrase 1, has recently been isolated (37, 38) and found to induce cell death in different cell lines (34).

In addition to the tankyrase 1 regulation of Mcl-1 functions, Mcl-1 proteins could reciprocally regulate the function of tankyrase 1. When Mcl-1L or Mcl-1S are coincubated with tankyrase 1, auto-ADP-ribosylation of tankyrase 1 decreases in a Mcl-1 concentration-dependent manner. Although the physiological significance of the apparent decrease in poly(ADP-ribosyl)ation of tankyrase 1 is still unclear and one cannot completely rule out the nonspecific effects of unknown contaminants in the purified Mcl-1 preparation, Mcl-1 proteins caused a more profound suppression of the poly(ADP-ribosyl)ation of TRF1 in the same *in vitro* test. Because both Mcl-1 proteins and TRF1 interact with the ankyrin repeats in tankyrase 1, Mcl-1 proteins could compete with TRF1 for binding to tankyrase 1, leading to lower ADP-ribosylation of TRF1. Because tankyrase 1-mediated telomere extension is dependent on ADP-ribosylation of TRF1 (10), inhibition of the ADP-ribosylation of TRF1 by Mcl-1 could prevent telomere elongation and facilitate cell senescence. This is consistent with the ability of tankyrase 1 to suppress the survival action of Mcl-1L, thus leading to the prevention of cell immortalization.

Recent studies indicated that Mcl-1L is localized to the nucleus (39, 40). and is involved in cell cycle regulation by interacting with the proliferating cell nuclear antigen (39). Mcl-1 also serves as a nuclear chaperone for another antiapoptotic protein, fortilin (40). Together with our observation of interactions between Mcl-1 and tankyrase 1, these findings suggest an important role of Mcl-1 in the regulation of diverse nuclear events including cell cycle regulation and telomere elongation. In conclusion, the observed interactions between Mcl-1 proteins and tankyrase 1 represent a novel mechanism for the regulation of the apoptosis function of Mcl-1 proteins. Because Mcl-1 could also regulate the function of tankyrase 1 and other nuclear proteins, Mcl-1 could play an important role in the coordinated regulation of cell survival, proliferation, and immortalization.

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