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The DNA Topoisomerase IIβ Binding Protein 1 (TopBP1) Interacts With Poly (ADP-Ribose) Polymerase (PARP-1)

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Abstract We investigated the physical association of the DNA topoisomerase IIβ binding protein 1 (TopBP1), involved in DNA replication and repair but also in regulation of apoptosis, with poly(ADP-ribose) polymerase-1 (PARP-1). This enzyme plays a crucial role in DNA repair and interacts with many DNA replication/repair factors. It was shown that the sixth BRCA1 C-terminal (BRCT) domain of TopBP1 interacts with a protein fragment of PARP-1 in vitro containing the DNA-binding and the automodification domains. More significantly, the in vivo interaction of endogenous TopBP1 and PARP-1 proteins could be shown in HeLa-S3 cells by co-immunoprecipitation. TopBP1 and PARP-1 are localized within overlapping regions in the nucleus of HeLa-S3 cells as shown by immunofluorescence. Exposure to UVB light slightly enhanced the interaction between both proteins. Furthermore, TopBP1 was detected in nuclear regions where poly(ADP-ribose) (PAR) synthesis takes place and is ADP-ribosylated by PARP-1. Finally, cellular (ADP-ribosyl)ating activity impairs binding of TopBP1 to Myc-interacting zinc finger protein-1 (Miz-1). The results indicate an influence of post-translational modifications of TopBP1 on its function during DNA repair. J. Cell. Biochem. 102: 171–182, 2007.

Key words: protein interaction; ADP-ribosylation; nuclear proteins; post-translational modification; DNA replication

DNA topoisomerase IIβ binding protein 1 (TopBP1) was initially found in a two-hybrid screen with DNA topoisomerase IIβ as a bait [Yamane et al., 1997]. TopBP1 contains eight BRCA1 C-terminal (BRCT) motifs and interacts with several other proteins, including human papilloma virus type 16 (HPV16) transcription/replication factor E2 [Boner et al., 2002], DNA polymerase γ, checkpoint protein hRad9 [Mäkiniemi et al., 2001], Myc-interacting zinc finger protein-1 (Miz-1) [Herold et al., 2002], E2F-1 [Liu et al., 2003], homologous to E6-associated protein (E6AP) C terminus (HECT)-domain ubiquitin ligase human *hyperplastic discs* (hHYD) [Honda et al., 2002] and promyelocytic leukemia protein (PML) [Xu et al., 2003]. It appears to be involved in DNA replication because incubation of an antibody against the sixth BRCT motif of TopBP1 inhibits DNA replication in an in vitro HeLa nuclei replication assay [Mäkiniemi et al., 2001]. TopBP1 shares sequence and structural similarities with the fission yeast Rad4/Cut5 protein. Rad4/Cut5 is a checkpoint Rad protein involved in cellular responses to DNA damage and replication blocks [Yamane et al., 2002, 2003]. In response to ionizing radiation, TopBP1 is phosphorylated by the ataxia-telangiectasia mutated kinase (ATM) [Yamane et al., 2002], implying a role of TopBP1 in DNA damage checkpoint control. This role was directly demonstrated by a later study using TopBP1 antisense oligonucleotides, showing that ionizing radiation-induced G2/M

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checkpoint and checkpoint kinase 1 (Chk1) phosphorylation was partially abrogated in the absence of TopBP1 [Yamane et al., 2003]. Moreover, in a very recent article it was shown that TopBP1 physically interacts with the ATM and Rad3-related (ATR) gene product and greatly enhances ATR's kinase activity [Kumagai et al., 2006]. In addition to its role in DNA damage checkpoint control and DNA replication, TopBP1 is also required for cell survival. Inhibition of TopBP1 expression by antisense Morpholino oligomers or by siRNAs directed against TopBP1 induces apoptosis [Yamane et al., 2002; Kim et al., 2005]. Thus, TopBP1 is involved in several important aspects of growth control [Garcia et al., 2005]. However, the detailed mechanism by which TopBP1 regulates these signaling events remains still poorly understood.

The DNA-repair and protein-modifying enzyme poly(ADP-ribose) polymerase-1 (PARP-1), also called poly(ADP-ribose) synthetase or poly(ADP-ribose) transferase, is an abundant nuclear protein involved in the DNA-base-excision-repair system. It belongs to a large superfamily of proteins including PARP-2, PARP-3, vault PARP, tankyrases and others [Ame et al., 2004]. Each member of the PARP family shares homology on the C-terminal catalytic domain of PARP-1. The primary structure of PARP-1 consists of a N-terminal DNA-binding domain containing two zinc finger motifs, an internal automodification region, which includes a BRCT domain, and the C-terminal catalytic domain. PARP-1 has the activity to catalyze a poly(ADP-ribosyl)ation reaction and to bind to DNA breaks. In response to DNA damage, PARP-1 activity is rapidly increased up to 500-fold. PARP-1 transfers 50–200 residues of poly(ADP-ribose) (PAR) to itself and to acceptor proteins such as histones, DNA polymerases, topoisomerases, high-mobility-group proteins, and transcription factors [Shall and de Murcia, 2000; Smulson et al., 2000]. Activation of PARP-1 regulates cellular repair, transcription and replication of DNA, cytoskeletal organization, protein degradation, and other cellular activities through (ADP-ribosyl)-ation of PARP-1 substrates [Chiarugi, 2002; Virag and Szabo, 2002; Bouchard et al., 2003; Gagne et al., 2003] or through interactions of PARP-1 with various factors and enzymes such as NF-κB [Hassa et al., 2003], AP-2 [Kannan et al., 1999], B-MYB [Cervellera and Sala, 2000], E2F-1 [Simbulan-Rosenthal et al., 2003], YY1 [Oei and Shi, 2001], DNA polymerase α [Dantzer et al., 1998], and topoisomerase I [Yung et al., 2004]. The physiological functions of PARP-1 and the cellular consequence of poly(ADP-ribosyl)ation are still under investigation.

In this work we demonstrate the interaction in vitro and in vivo between TopBP1 and PARP-1. We show that the sixth BRCT domain of TopBP1, that is, aa 807–916, interacts in vitro with a protein fragment of PARP-1 containing the DNA-binding and the automodification domains. More significantly, the in vivo interaction of endogenous TopBP1 and PARP-1 proteins could be shown in HeLa-S3 cells by co-immunoprecipitation. TopBP1 and PARP-1 are localized within overlapping regions in the nucleus of HeLa-S3 cells as shown by immunofluorescence. Exposure to UVB light slightly enhanced the interaction between both proteins. Furthermore, TopBP1 was detected in nuclear regions where PAR synthesis takes place and is ADP-ribosylated by PARP-1. Finally, cellular (ADP-ribosyl)ating activity impairs binding of TopBP1 to Miz-1.

MATERIALS AND METHODS

Cell Culture and Treatments

HeLa and HeLa-S3 cells were grown as monolayers in Dulbecco's modified Eagle's medium with GlutaMAX^{TM}I (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom). Cells were maintained in an atmosphere of 5% CO_2 at 37°C. All UV irradiation experiments used 250 J/m^2 UVB light.

Purification of Recombinant TopBP1 Proteins

To produce GST-tagged TopBP1 and GST-tagged Miz-1 protein fragments polymerase chain reaction (PCR) experiments were performed and the resulting PCR products were inserted into the expression vector pGEX-4T-1 (GE Healthcare), encoding glutathione-*S*-transferase (GST). Plasmids containing sequences encoding GST, GST-TopBP1 (aa 1–1435) (kindly provided by W.-C. Lin, AL), GST-TopBP1ctr. (aa 935–1058) as control, GST-TopBP1-BRCT6 (aa 807–916), GST-TopBP1-BRCT6-8 (aa 774–1320), and GST-Miz-1 (aa

1–803) were transformed into *E. coli* strain BL21-CodonPlus-RIL (Stratagene). Cultures were grown in 20 ml LB medium containing 100 μg of ampicillin/ml. After 2 h GST fusion derivates were induced with 1 mM isopropylthiogalactopyranoside (IPTG) and incubation was continued for 2 h. Cells were collected by centrifugation, resuspended in 1 ml phosphate buffered saline (PBS) containing 1% TritonX-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM dithiothreitol, and sonicated. Upon sonification the lysate was cleared by centrifugation and the supernatant was subjected to Glutathione-sepharose affinity chromatography for purification of the GST fusion proteins as described by the manufacturer (GE Healthcare).

In vitro Transcription/Translation of PARP-1 Proteins

To generate pBluescript II KS+-PARP-$1_{\text{full-length}}$ (aa 1–1014), pBluescript II KS+-PARP-$1_{\text{DBD-AD}}$ (aa 1–524, including the DNA binding and automodification domain) and pBluescript II KS+-PARP-1_{DBD} (aa 1–327 containing only the DNA binding domain) the two-hybrid vector pBTM116-PARP-1 was used as a PCR template (a kind gift from G. de Murcia, Illkirch Cedex, France). The PCR fragments were cloned into the BamHI and XhoI sites of pBluescript II KS+ (Stratagene). [^{35}S]methionine-labeled PARP-1-fragments were then synthesized in vitro by coupled T7 RNA polymerase-mediated transcription and translation in a reticulocyte lysate system as described by the manufacturer (Promega).

GST-Pull Down Experiments and Other In Vitro Interaction Experiments

Glutathione-sepharose beads (50 μl) liganded by either GST-tagged Miz-1 (1–803), -TopBP1 (aa 1–1435), -TopBP1-BRCT6 (aa 807–916), -BRCT6-8 (aa 774–1320), -control (aa 935–1058) or GST alone, were washed with HB-buffer (20 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4) and then resuspended in 100 μl HB-buffer containing 0.5% Igepal CA-630 and 10 μl [^{35}S]methionine-labeled PARP-$1_{\text{full-length}}$ (aa 1–1014), PARP-$1_{\text{DBD-AD}}$ or PARP-1_{DBD}. After incubation at 4°C constantly rotating for 2 h, the beads were washed with HB-buffer extensively. Proteins were eluted from the beads using SDS sample buffer. After separation by 8% SDS–PAGE, PARP-1 was revealed by exposure on X-OMAT AR film (Kodak). Alternatively, HeLa-S3 cell extracts (prepared as described below) were used instead of [^{35}S]methionine-labeled proteins. Interacting proteins were detected by immunoblotting as described below.

Western Blot Analysis

Cell lysates or eluted proteins were resolved by 8% SDS–PAGE and transferred to nitrocellulose membrane (Millipore). The membranes were blocked overnight in Tris-buffered saline (pH 7.5) containing 0.2% Tween-20, 5% skim milk (Merck) and incubated with antibodies: anti-TopBP1 (polyclonal, 1:1000, Novus) or anti-TopBP1 (monoclonal, 1:250, BD Biosciences), anti-PARP-1 (1:500, BD Biosciences), anti-GST (1:10,000, Novagen) or anti-PAR (1:500, BD Biosciences) for 1 h. Filters were washed three times each 10 min in Tris-buffered saline (pH 7.5) plus 0.2% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-(mouse IgG) or goat anti-(rabbit IgG), respectively, at room temperature for 1 h. Protein bands were visualized by ECL plus (GE Healthcare) and exposure on X-OMAT AR film (Kodak).

Immunoprecipitation Experiments

HeLa-S3 cells were washed with cold PBS, harvested, and lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% Nonidet-P40, 10 μg/ml leupeptin and aprotinin, 5 μg/ml pepstatin, 1 mM PMSF. The lysate was cleared by centrifugation at 10,000*g* at 4°C. Anti-PARP-1 monoclonal antibody (2 μg/ml, BD Biosciences) or control IgG (anti-FADD polyclonal antibody; 2 μg/ml, Santa Cruz) was incubated with 15 μl (bed volume) protein G sepharose and 3% Perfect Block (mobitec) in lysis buffer overnight at 4°C. The beads were then washed twice with the same buffer, and 300 μl precleared lysate (1.5 mg protein) was added to the antibody-protein-G-sepharose pellet, followed by incubation with constant rotation at 4°C for 2 h. After extensive washing, proteins were eluted from the beads by boiling in SDS sample buffer at 95°C for 5 min. Proteins were detected by immunoblotting as described above.

Alternative of the procedure described above co-immunoprecipitation with μMACS protein G microbeads was performed as described by the

manufacturer (Miltenyi Biotec). Briefly, TopBP1 monoclonal antibody (3 μg/ml, BD Biosciences) and μMACS protein G microbeads were added to the cell lysate. After 1.5 h of incubation on ice, magnetic separation using a μ column and the μMACS separator was performed. After extensive washing, co-immunoprecipitated proteins were eluted from the column with SDS gel loading buffer and detected by immunoblotting as described above.

Immunofluorescence and Confocal Microscopy

HeLa-S3 cells were grown on coverslips, washed once in PBS and fixed in fresh 3.8% paraformaldehyde for 10 min at room temperature. For neutralization cells were washed twice in 0.1 M glycin/PBS followed by permeabilization in PBS plus 0.5% (v/v) NP-40 for 10 min. Then cells were blocked in PBS containing 0.1% NP-40 and 5% fetal calf serum at room temperature for 1 h and incubated with primary antibody in blocking solution for 1 h, followed by an incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine X-conjugated goat anti-rabbit IgG (all purchased from Dianova). For immunostaining, anti-TopBP1 antibody (1:100, BD Biosciences), anti-PARP-1 antibody (1:100, BD Biosciences), and anti-PAR antibody (1:100, BD Biosciences) were used. To acquire confocal images the thus prepared specimens were subsequently mounted on microscopical slides. Microscopical images were collected with an Axiovert 200 M/LSM 510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The samples were scanned using Plan-Apochromat 63×/1.4 Oil-DIC and Plan-Neofluar 40×/1.3 Oil-DIC oil-immersion objectives (Carl Zeiss) and a scan zoom factor of 1.5. The fluorescein isothiocyanate dye and controls were excited by laser light of a 40 mW argon laser (Lasos, Göttingen, Germany), tuned to a laser line of 488 nm wavelength. Fluorescence signals were detected by using a band pass filter from 505 nm to 530 nm and pseudocolored in green. Rhodamine X (RRX) was excited with a 20 mW helium-neon-laser (Lasos) tuned to 543 nm and detected by band pass filters from 560 nm to 615 nm and pseudocolored in red. The DNA staining dye 4',6-Diamidino-2-phenyleindole-dihydrochlorid (DAPI) was excited by laser light of a 80 mW Enterprise II argon ion laser (Coherent Innova, Santa Clara; CA), tuned to a laser line of 364 nm wavelength. Fluorescence signals were detected using a long-pass filter set from 385 nm upward and were pseudocolored in blue. The confocal microscope was used to create single pictures and optical stacks in z-axis with 50 slices of 0.10 μm thickness. Microscopical images were electronically acquired using the LSM-510-META 3.2 software (Carl Zeiss) and stored as JPEG files. Figures were assembled from the JPEG files with Adobe Photoshop software.

Poly(ADP-ribosyl)ation Assay and Inhibition

Auto-(ADP-ribosylation) of PARP-1 and the modification of substrates was done as described by Niedergang et al. [1997]. Briefly, GST-TopBP1 (aa 1-1435) and GST-TopBP1-BRCT6 were incubated for 30 min at 25°C with 200 ng purified recombinant PARP-1 (Merck) in a reaction mixture (50 μl) containing 50 μM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 3 μg activated calf thymus DNA, and 200 μM NAD^+. The products were subsequently analyzed by SDS–PAGE and immunoblotting experiments with anti-PAR (BD Biosciences) and anti-GST (Novagen) antibodies. Inhibition of poly-(ADP-ribosylation) in HeLa-S3 cells was investigated by the use of the PARP inhibitor 3-aminobenzamide (3-AB, Sigma). Cells were treated with 3-AB (3–10 mM) 30 min before UVB treatment.

RESULTS

TopBP1 is involved in DNA replication and repair processes. However, its precise biological function remains still unclear. It is known to contain one among eight BRCT domains which shows homologies with the auto-(ADP-ribosyl)ation sites of the BRCT domain of PARP-1 (Fig. 1A) [Yamane et al., 1997]. As the protein PARP-1 is also involved in the above processes, we investigated the interaction between TopBP1 and PARP-1 in vitro and in vivo.

PARP-1 Interacts with the 6th BRCT Domain of TopBP1 In Vitro

First, we purified the full-length TopBP1 (aa 1–1435) as a GST-fusion protein from *E. coli* (Fig. 1C) and performed a GST-pull down experiment. The full-length cDNA of PARP-1 (aa 1–1014) was used as a template in a transcription/translation experiment in vitro.

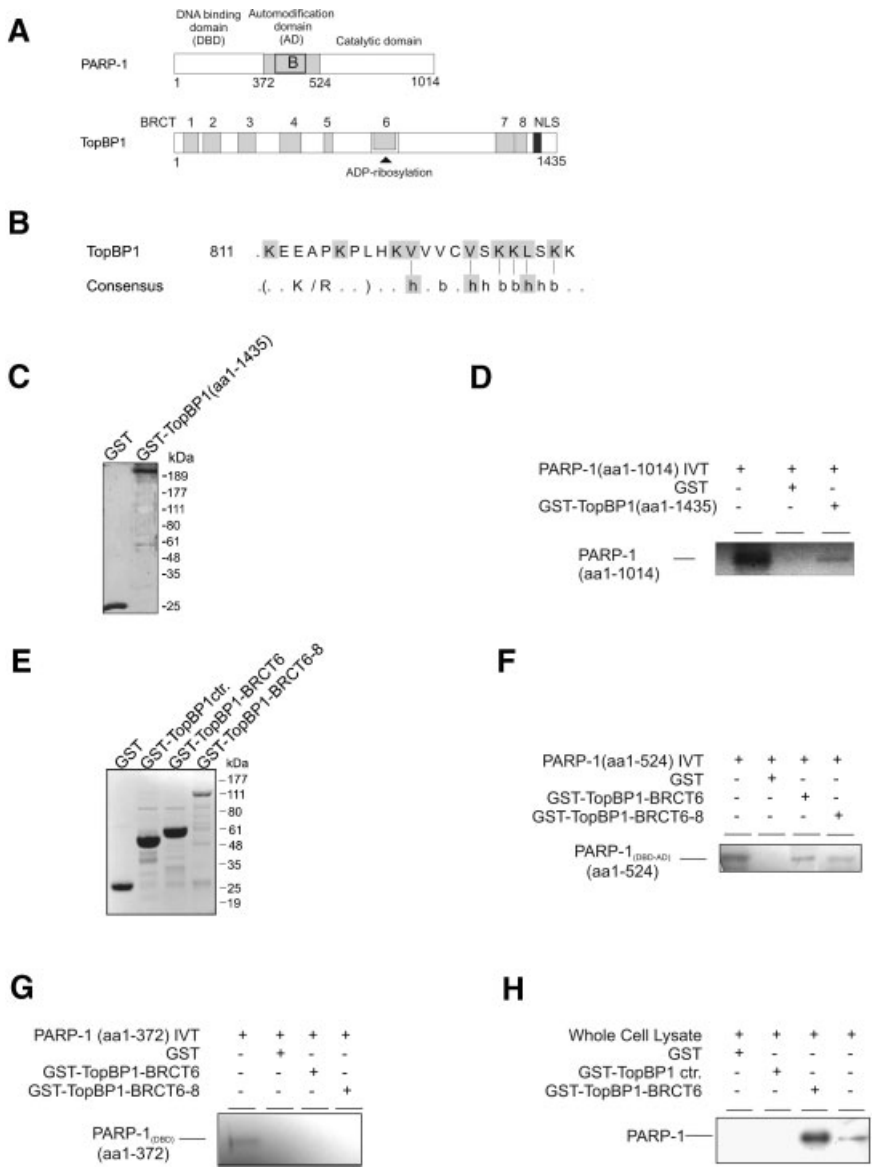

Fig. 1. In vitro interaction of PARP-1 with the 6th BRCT domain of TopBP1. **A**: Modular organization of PARP-1 and TopBP1. **B**: BRCT domain of PARP-1. B: Alignment of a peptide sequence situated within the 6th BRCT domain of TopBP1 with a consensus PAR-binding motif which was found in various DNA damage checkpoint proteins [Pleschke et al., 2000]. **C**: GST and GST-TopBP1 (1–1435) were visualized by Coomassie staining. **D**: Shown are the results obtained by GST-pull down experiments with GST-TopBP1 (1–1435) and GST as a control. Interaction assay was performed with [^{35} S]-labeled full-length PARP-1. PARP-1 was detected by autoradiography. **First lane**: input, 10 µl of the in vitro translate used for the interaction assay. **E**: GST, GST-TopBP1ctr., GST-TopBP1-BRCT6, and GST-TopBP1-BRCT6-8 were visualized by Coomassie staining. **F** and **G**: Shown are the results obtained by GST-pull down experiments with GST-fusions of the 6th BRCT domain of TopBP1 (aa 807–916) and a C-terminal fragment including the 6th, 7th, and 8th BRCT domains (aa 774–1320). GST served as a control. Interaction assays were performed with N-terminal [^{35} S]-labeled PARP-1 fragments containing either both the DNA-binding and the auto-modification domains (PARP-1$_{DBD-AD}$, aa 1-524) (F) or only the DNA-binding domain (PARP-1$_{DBD}$, aa 1–372) (G). PARP-1 was detected by autoradiography. First lane: input, 10 µl of the in vitro translate used for the interaction assay. **H**: Results of GST-pull down experiments with the 6th BRCT domain of TopBP1 and a neighboring control-GST-fusion protein of TopBP1 (aa 935–1058) using a whole cell lysate from HeLa cells as source for PARP-1. The bound proteins were analyzed for PARP-1 by Western-blot analysis. **Lane 4**: 10% input of the quantity of cell lysate used for the interaction assay.

The resulting PARP-1 fragment ([^{35}S]-labeled) specifically interacted with the GST-TopBP1 (aa 1–1435)-fragment but did not interact with the GST fragment as a control (Fig. 1D).

A region within the 6th BRCT domain of TopBP1 is similar to the auto-(ADP-ribosyl)a-tion sites of the BRCT domain of PARP-1 (Fig. 1A) [Yamane et al., 1997]. In addition, a

peptide sequence within the 6th BRCT domain of TopBP1 matches with a consensus PAR-binding motif found in various DNA-damage checkpoint proteins (Fig. 1B) [Pleschke et al., 2000]. Therefore we proposed that the interaction between PARP-1 and TopBP1 may occur between the BRCT and automodification domain of PARP-1 and the 6th BRCT domain of TopBP1. To investigate this we performed two different GST-pull down experiments involving the purified 6th BRCT domain of TopBP1 (aa 807–916), a neighboring fragment as control (aa 935–1058) and a larger C-terminal fragment containing the 6th, 7th, and 8th BRCT domains (aa 774–1320) as GST-fusion proteins (Fig. 1E). First, a DNA-fragment of PARP-1 (aa 1–524) containing the DNA-binding and the auto-modification domains (Fig. 1A) was used as a template in a transcription/translation experiment in vitro. The resulting PARP-1_{DBD-AD} fragment ([^{35}S]-labeled) specifically interacted with both GST-TopBP1-fragments containing the 6th BRCT domain. However, it did not interact with the GST fragment as a control (Fig. 1F). An N-terminal DNA-fragment of PARP-1 (aa 1–372, [^{35}S]-labeled) containing only the DNA-binding domain did not interact with the GST-TopBP1-fragments (Fig. 1G) showing that the automodification domain of PARP-1 was essential for the binding to TopBP1. Second, glutathione-sepharose coupled either to the 6th BRCT domain- or to the neighboring control-GST-fusion proteins were incubated with HeLa cell lysates. The associated proteins were pelleted by centrifugation, washed and analyzed for full length PARP-1 by Western-blot analysis. PARP-1 exclusively bound to the 6th BRCT domain of TopBP1 and did not interact with the TopBP1 control fragment (Fig. 1F), that is, the in vitro interaction studies showed that PARP-1 and TopBP1 interact. The 6th BRCT domain of TopBP1 was sufficient to bind the full-length PARP-1. Furthermore, the region of PARP-1_{DBD-AD} (aa 1–524) containing the DNA-binding and the auto-modification domains was enough to interact with TopBP1.

Co-immunoprecipitation of PARP-1 and TopBP1

Beside our studies in vitro it was crucial to investigate the interaction between the endogenous full-length PARP-1 and TopBP1 proteins in vivo using non-transformed human cervical cancer cells HeLa-S3. To address this, we performed co-immunoprecipitation experiments. Lysates from HeLa-S3 cells were co-immunoprecipitated with anti-TopBP1 (Fig. 2A) and anti-PARP-1 antibodies (Fig. 2B). Associated proteins were detected by immunoblotting. Anti-TopBP1 antibody bound to μMACS protein G microbeads co-precipitated PARP-1 resulting in a 115 kDa band on Western blots thus proving the physiological interaction in vivo (Fig. 2A). In the reciprocal experiment anti-PARP-1 antibody bound to protein G sepharose beads co-precipitated TopBP1 resulting in a 170 kDa band on Western blots. All control experiments, that is beads alone or beads with control serum, confirmed the specificity of the above precipitation. As it was shown that both TopBP1 and PARP-1 are involved in repair processes of UV light-induced DNA damage [Ame et al., 2004; Garcia et al., 2005], we repeated co-immunoprecipitation experiments with UVB irradiated HeLa-S3 cell lysates. Co-immunoprecipitations revealed that exposure to UVB light slightly enhanced the amount of TopBP1 present in PARP-1 immunoprecipitations (Fig. 2B, lane 3). Moreover, UVB light exposure enhanced the efficiency of TopBP1 immunoprecipitation resulting in a higher co-immunoprecipitated PARP-1 amount (Fig. 2A, lane 3). The above co-immunoprecipitations revealed that TopBP1 and PARP-1 interact in vivo and that exposure to UVB light slightly enhanced the interaction between both proteins.

Localization of PARP-1 and TopBP1 in the Nucleus

The elucidation of the localization of endogenous TopBP1 and PARP-1 in untreated HeLa cells and cells after exposure to UVB light by three different means of staining, that is fluorescein isothiocyanat (FITC), rhodamine-X, and Dapi conjugated secondary antibodies, revealed a localization of both proteins exclusively in the nuclei as expected for nuclear proteins (Fig. 3). Furthermore, there were large structures in the nuclei, which could not be stained and probably represent nucleoli. In addition, superimposition of the images shown in the first lane of Figure 3 with the images of lane two in Figure 3 revealed that both proteins under investigation were localized in overlapping regions of the nuclei (Fig. 3, third lane). Furthermore, these findings were most prominent shortly after exposure of HeLa cells to UVB

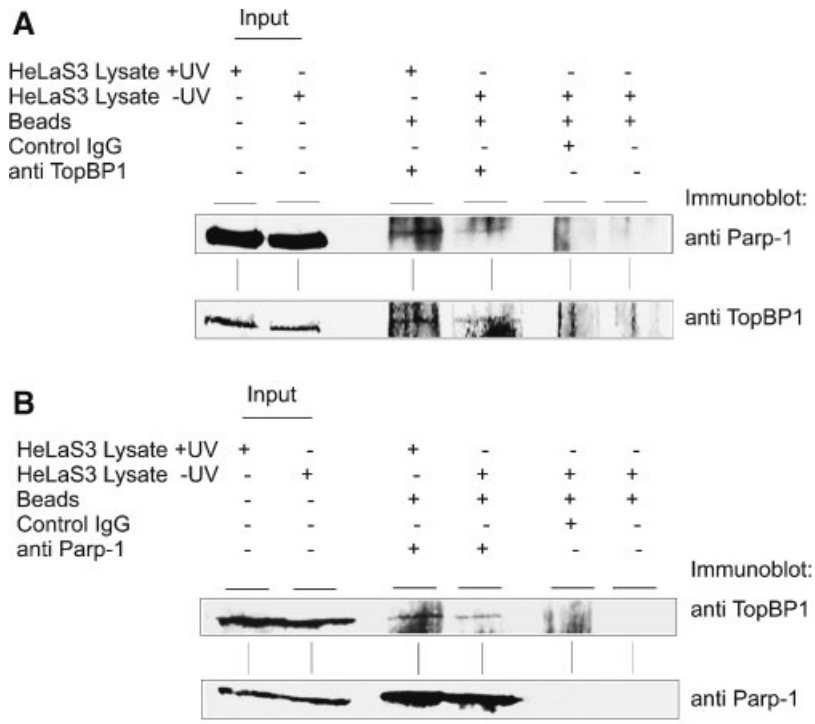

Fig. 2. PARP-1 associates with TopBP1 in vivo. Whole cell lysate (1.5 mg protein) derived from HeLa-S3 cells (untreated [-UV] or 15 min after exposure to 250 J/m^2 UVB light [+UV]) was subjected to co-immunoprecipitation experiments using antibodies against TopBP1 (**A**) and PARP-1 (**B**). Controls were beads alone or anti-FADD antibody, as indicated in Materials and Methods. The precipitates were analyzed by immunoblotting experiments using antibodies against PARP-1 and TopBP1. Input indicates 20% of cell lysate used for co-immunoprecipitation. Co-immunoprecipitations in (A) and (B) are one representative example of three experiments performed in each case.

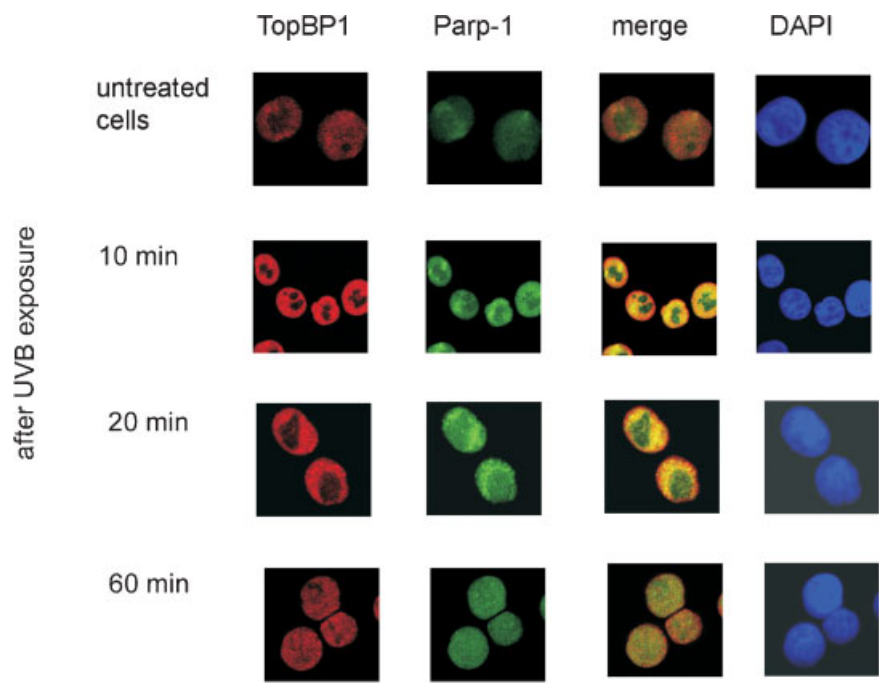

Fig. 3. TopBP1 and PARP-1 are localized within overlapping regions in the nucleus of HeLa-S3 cells. Immunofluorescence staining of endogenous TopBP1 and PARP-1. HeLa-S3 cells (untreated or at the indicated time points after exposure to 250 J/m^2 UVB light) were fixed and stained with anti-TopBP1 (red), anti-PARP-1 (green), and Dapi (blue). Merge: yellow dots indicate the localization of TopBP1 and PARP-1 within overlapping regions in the nucleus of HeLa-S3 cells.

light (Fig. 3, 10 and 20 min) in comparison with untreated cells or cells studied 60 min after UVB treatment (Fig. 3, untreated and 60 min). The immunofluorescence experiments showed that TopBP1 and PARP-1 are nuclear proteins localized within overlapping regions of the nuclei.

TopBP1 is ADP-Ribosylated by PARP-1

Due to the homology between the 6th domain of TopBP1 and the auto-(ADP-ribosyl)ation sites of PARP-1 we elucidated whether TopBP1 itself becomes ADP-ribosylated. First of all, we checked whether poly(ADP-ribosyl)ation takes place under our experimental conditions. For this purpose, we used anti-PAR antibody to detect endogenous PAR in untreated and in UVB-treated HeLa-S3 cells. As expected, PAR synthesis was not detectable by immunofluorescence in untreated cells but was induced immediately after exposure of the cells to UVB light (Fig. 4, second lane). Sixty minutes after exposure of the cells to UVB light PAR synthesis was not detectable anymore. More significantly, co-staining of the cells with anti-TopBP1 antibody (Fig. 4, first lane) revealed that TopBP1 was localized in nuclear regions where poly (ADP-ribose (PAR) synthesis takes place (Fig. 4, third lane). Starting from these experiments we wondered whether TopBP1 becomes ADP ribosylated in vitro. Therefore, we incubated the full-length TopBP1 protein and the 6th BRCT domain fused to GST with recombinant PARP-1, NAD^+, and DNA containing strand breaks to allow the activation of PARP-1 upon binding to the strand breaks. GST was used as a control. After separation by SDS–polyacrylamide gel electrophoresis, the protein-associated PAR was visualized by Western blotting experiments using anti-PAR antibody. As shown in Figure 5A,B both full-length TopBP1 and the fragment containing the 6th BRCT domain but not the GST control fragment were oligo(ADP-ribosyl)ated, thus confirming a functional connection between the two interactors.

Cellular (ADP-ribosyl)ating activity impairs binding of TopBP1 to Miz-1. The protein Miz-1 binds to the 7th BRCT domain of TopBP1 [Herold et al., 2002], that is in the immediate neighborhood of the oligo(ADP-ribosyl)ation sites of TopBP1. It was shown that Miz-1 is released from the complex with TopBP1

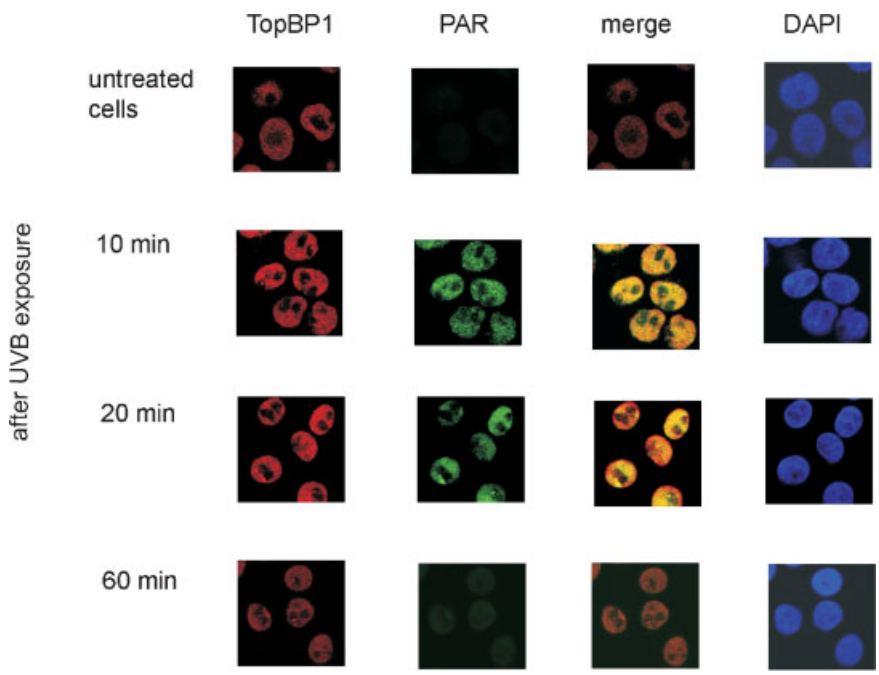

Fig. 4. TopBP1 is localized in nuclear regions where poly(ADP-ribose (PAR) synthesis takes place. Immunofluorescence staining of endogenous TopBP1 and PAR. HeLa-S3 cells (untreated or at the indicated time points after exposure to 250 J/m^2 UVB light) were fixed and stained with anti-TopBP1 (red), anti-PAR (green), and Dapi (blue). Merge: yellow dots indicate localization of TopBP1 in nuclear regions where poly(ADP-ribose (PAR) synthesis takes place.

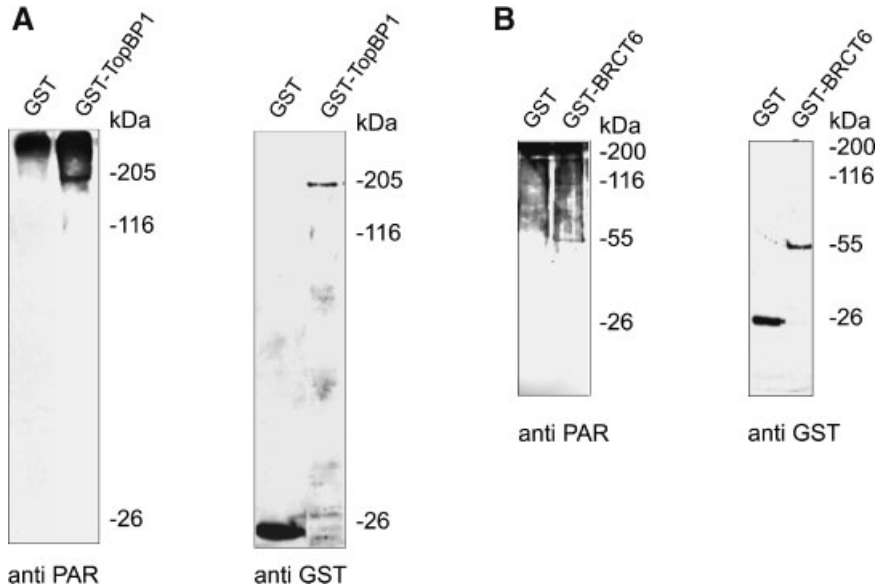

Fig. 5. TopBP1 is modified by PARP-1. PARP-1 (200 ng) was incubated for 30 min at 25°C in the presence of 200μM NAD^+ and 800 ng of GST-TopBP1 (aa 1–1435) (**A**) or GST-TopBP1-BRCT6 (aa 807–916) (**B**). GST was used as control. A: On the left: immunoblot anti-PAR; on the right: the same filter after reprobing with an anti-GST antibody. B: On the left: immunoblot anti-PAR; on the right: the same filter after reprobing with an anti-GST antibody.

in response to DNA damage [Herold et al., 2002]. We wondered whether oligo(ADP-ribosyl)ation of TopBP1 in UVB-exposed HeLa cells has any influence on its binding to Miz-1. Therefore, we purified the full-length Miz-1 protein as a GST fusion protein and GST as a control (Fig. 6A) and performed GST-pull down assays using untreated and UVB-exposed HeLa cell extracts. As expected TopBP1 from untreated HeLa extracts bound to Miz-1 (Fig. 6B, lane 3). However, it did neither interact with the GST-fragment nor with the control glutathione-sepharose beads (Fig. 6B, lanes 1 and 2). In contrast, with UVB-treated cell extract containing the same amount like above but oligo(ADP-ribosyl)ated TopBP1, decreased binding of TopBP1 to Miz-1 was detected (Fig. 6B, lane 4). However, this is still not a direct evidence that the (ADP-ribosyl)ation of TopBP1 is inhibiting its interaction with Miz-1. There may be other (ADP-ribosyl)ated proteins which are acting on TopBP1 to compromise Miz-1 interaction. To show that cellular (ADP-ribosyl)ating activity caused by UVB treatment of the cells was the reason of decreased binding of TopBP1 to Miz-1, HeLa-S3 cells were treated with increasing concentrations of the PARP-inhibitor 3-aminobenzamide (3-AB) before UVB treatment. First, we checked whether 3-AB inhibits poly(ADP-ribosyl)ation under our experimental conditions. For this purpose, we used anti-PAR antibody to detect endogenous PAR in untreated and UVB-treated HeLa cells in the presence or absence of increasing concentrations of 3-AB. As shown in Figure 6C poly(ADP-ribosyl)ation was significantly increased after UVB treatment (Fig. 6C, lane 2) when compared to non-irradiated cells (Fig. 6C, lane 1). Addition of increasing concentrations of 3-AB led to an almost complete inhibition of poly(ADP-ribosyl)ation (Fig. 6C, lanes 5–7). GST-pull down assays with UVB and 3-AB treated cell extracts revealed binding of TopBP1 to Miz-1 (Fig. 6B, lanes 5–7). This means that inhibition of cellular (ADP-ribosyl)ating activity restores the interaction between Miz-1 and TopBP1.

DISCUSSION

We aimed to elucidate the physical interaction between TopBP1 and PARP-1. In the present study, we first demonstrated that, PARP-1 interacts with the 6th BRCT domain of TopBP1 in vitro. Second, PARP-1 co-precipitates with a TopBP1 antibody in vivo in HeLa-S3 cells and both proteins are localized within overlapping regions in the nucleus of HeLa-S3 cells. Third, TopBP1 is ADP-ribosylated by

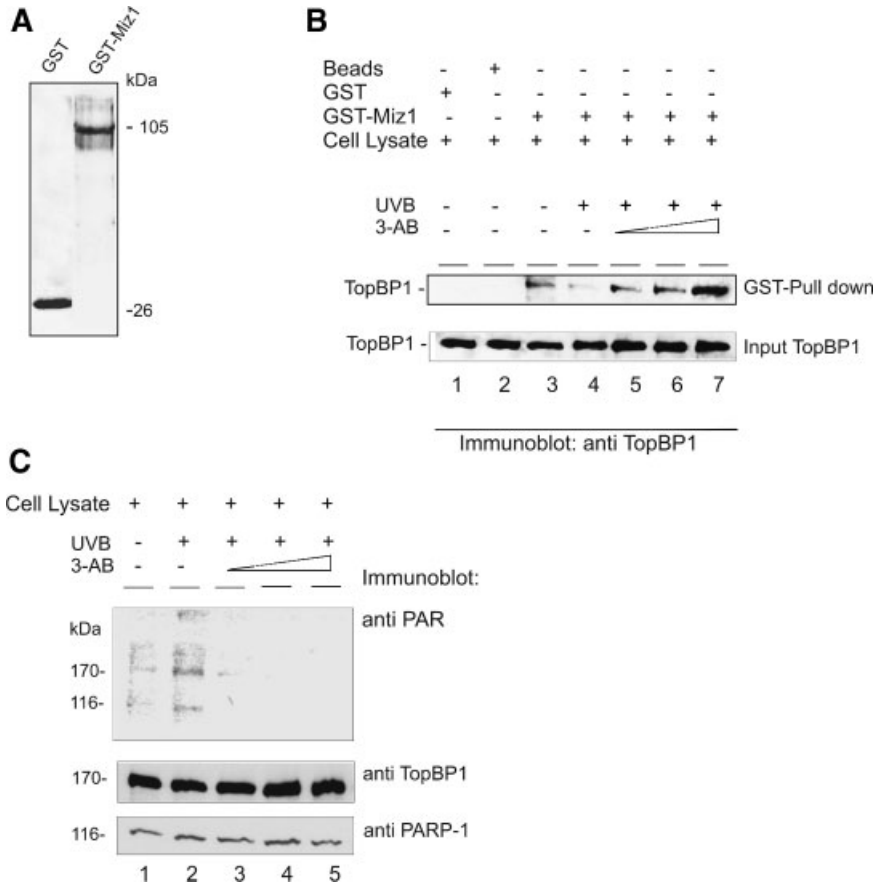

Fig. 6. UVB exposure of HeLa-S3 cells impairs binding of TopBP1 to Miz-1. **A**: GST and GST-Miz-1 (30% of the protein amount used for GST-pull down experiments [15 μg protein]) were visualized by Coomassie staining. **B**: Results of GST-pull down experiments with GST-Miz-1-fusion protein and GST as control (see Fig. 6A) using a whole cell lysate from HeLa-S3 cells (**lanes 1–3**, untreated cells; **lane 4**, cells 20 min after exposure to 250 J/m^2 UVB light; **lanes 5–7**, cells treated for 30 min before irradiation with increasing concentrations of 3-AB (3, 5, and 10 mM) and lysed 20 min after exposure to 250 J/m^2 UVB light) as source for TopBP1 are shown. The bound proteins were analyzed for TopBP1 by Western-blot analysis. Input indicates 50% (65 μg protein) of the cell lysate used for GST-pull down experiments. **C**: HeLa-S3 cell lysates (65 μg protein) (lane 1, untreated cells; lane 2, cells 15 min after exposure to 250 J/m^2 UVB light; lanes 3–5, cells treated for 30 min before irradiation with increasing concentrations of 3-AB (3, 5, and 10 mM) and lysed 20 min after exposure to 250 J/m^2 UVB light) were resolved by 8% SDS–PAGE and transferred to nitrocellulose membrane. The bound proteins were subsequently analyzed for PAR, PARP-1, and TopBP1 by Western-blot analysis.

PARP-1. Fourth, cellular (ADP-ribosyl)ating activity impairs binding of TopBP1 to Miz-1.

It was previously shown [Mäkiniemi et al., 2001; Boner et al., 2002; Herold et al., 2002; Honda et al., 2002; Liu et al., 2003; Xu et al., 2003] that TopBP1 interacts with various proteins involved in different cellular processes. One of the proteins interacting with TopBP1 is Miz-1 which mediates the transcriptional repression by the oncogene product *c*-Myc. Particularly it is involved in the negative regulation of the mammalian UV response by Myc [Herold et al., 2002]. It was shown that upon UV irradiation of human keratinocytes Miz-1 is released from a TopBP1–Miz-1 protein complex thus allowing Miz-1 to contribute to the cell-cycle arrest that occurs in response to DNA damage [Herold et al., 2002]. However, little is known about the mechanism of disruption of the TopBP1–Miz-1 protein complex in response to UV irradiation. Here we show that post-translational oligo(ADP-ribosyl)ation of TopBP1 or of other proteins which may act on TopBP1 by PARP-1 might support the release of Miz-1 from the above mentioned complex. PAR is formed in possibly all multicellular organisms by a family of poly(ADP-ribose) polymerases (PARP) [Ame et al., 2004]. PARP-1 was until recently the only known member of the PARP family. It is a DNA damage signal protein catalyzing its

automodification with multiple, variably sized ADP-ribose polymers. Through these polymers, PARP-1 interacts non-covalently with other proteins, for example, p53 [Malanga et al., 1998], p21 [Pleschke et al., 2000], and DNA-dependent protein kinase [Ruscetti et al., 1998] and is known to alter their properties, for example, DNA binding [Pleschke et al., 2000]. The co-immunoprecipitation of TopBP1 and PARP-1 in untreated HeLa-S3 cells showed that the interaction between the two proteins does not require any oligo(ADP-ribosyl)ation. Further examples of proteins interacting with unmodified members of the PARP family are the proteins X-ray repair cross-complementing 1 (XRCC1) [Masson et al., 1998] and myeolid cell leukemia-1 (Mcl-1) [Bae et al., 2003]. Nevertheless, after exposition of the HeLa-S3 cells to UVB light, leading to immediate PAR synthesis, in vivo interaction of TopBP1 and PARP-1 was slightly increased (see Fig. 2). This slightly enhanced interaction between TopBP1 and PARP-1 after UVB exposure may be caused by the non-covalent interaction of PARP-1-ADP-ribose polymers with the PAR-binding motif within the 6th BRCT domain of TopBP1.

Upon DNA damage by for example UV irradiation or genotoxic agents, PARP-1 binds to the resulting single and double DNA strand breaks followed by a rapid activation of the enzyme. PARP-1 gets auto-(ADP-ribosyl)ated with up to 200 residues of PAR thus allowing a complex formation with various acceptor proteins. It is assumed that the binding to oligo(ADP-ribosyl) chains of PARP-1 brings the above proteins which are mainly involved in DNA repair, replication and transcription, into the vicinity of the DNA single strand breaks and therefore allows their participation in the repair process [Hong et al., 2004]. A similar scenario can be supposed for TopBP1. TopBP1 has been shown to be oligo(ADP-ribosyl)ated by PARP-1 in the presence of nicked DNA in vitro and both proteins have been shown to be localized within overlapping regions in the nucleus of HeLa-S3 cells. Therefore, we propose that PARP-1 is activated upon DNA damage thus resulting in its auto-poly(ADP-ribosyl)ation and the simultaneous oligo(ADP-ribosyl)ation of TopBP1. We further showed that the 6th BRCT domain of TopBP1 is sufficient for binding to PARP-1 and for its oligo(ADP-ribosyl)ation. The protein Miz-1 binds to the 7th BRCT domain of TopBP1 [Herold et al., 2002], that is, in the immediate neighborhood of the above mentioned 6th domain. It was shown that Miz-1 is released from the complex with TopBP1 in response to DNA damage [Herold et al., 2002]. One can imagine that both the oligo(ADP-ribosyl) chains on the surface of the 6th BRCT domain of TopBP1 and the bound oligo(ADP-ribosyl)ated PARP-1 cause a steric hindrance for Miz-1 to bind to the neighboring 7th BRCT domain. This possibly leads to a disruption of the Miz-1–TopBP1 complex in response to DNA damage. Indeed, we could show that cellular (ADP-ribosyl)ating activity impairs binding of TopBP1 to Miz-1 and that its inhibition restores the TopBP1–Miz-1-interaction (see Fig. 6B). However, the ultimate decision whether oligo(ADP-ribosyl)ation of TopBP1 or the modification of other proteins which may act on TopBP1 is the reason for the impaired binding of TopBP1 to Miz-1 needs further experiments. Thus, PARP-1 and TopBP1 perhaps cooperate in regulating the functions of both proteins during DNA repair, replication, and other relevant cellular processes.

The complex formation between TopBP1, PARP-1, and Miz-1 (and other proteins) and the disruption of these complexes under different conditions, for example, DNA damage, raise the question whether TopBP1 acts as a scaffold protein that binds or releases proteins under varying physiological conditions. TopBP1 knockdown cells derived with the help of siRNAs will allow to investigate whether apoptosis, found after inhibition of TopBP1 expression by antisense Morpholino oligomers [Yamane et al., 2002], is caused by one of the released interaction partners of TopBP1 such as PARP-1.

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