Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells

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The zinc-finger DNA-binding domain (DBD) of poly (ADP-ribose) polymerase (PARP, EC 2.4.2.30) specifically recognizes DNA strand breaks induced by various DNA-damaging agents in eukaryotes. This, in turn, triggers the synthesis of polymers of ADP-ribose linked to nuclear proteins during DNA repair. The 46 kDa DBD of human PARP, and several derivatives thereof mutated in its first or second zinc-finger, were overproduced in Escherichia coli, in CV-1 monkey cells or in human fibroblasts to study their DNA-binding properties, the trans-dominant inhibition of resident PARP activity, and the consequences on DNA repair, respectively. A positive correlation was found between the in vitro DNA-binding capacity of the recombinant DBD polypeptides and their inhibitory effect on PARP activity stimulated by the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Furthermore, overproduced wild-type DBD blocked unscheduled DNA synthesis induced in living cells by MNNG treatment, but not that induced by UV irradiation. These results define a critical role for the second zinc-finger of PARP for DNA single-stranded break binding and furthermore underscore the importance of PARP to act as a critical regulatory component in the repair of DNA damage induced by alkylating agents.

Key words: base excision/immunofluorescence/microinjection/nucleotide excision repair/zinc-finger protein

Introduction

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is a chromatin-associated enzyme of eukaryotic cell nuclei which, in the presence of DNA breaks, covalently attaches ADP-ribose from nicotinamide adenine dinucleotide (NAD) to various nuclear acceptor proteins and transfers further ADP-ribose units onto this initial adduct to form poly(ADP-ribose). Under conditions of extensive DNA breakage, very large amounts of branched polymers are rapidly synthesized and enzymatically degraded by poly(ADP-ribose) glycohydrolase, resulting in a half-life of < 1 min (Alvarez-Gonzalez and Jacobson, 1987). Although PARP binds to single- or double-stranded DNA whether it is closed circular or linear, its catalytic activity is absolutely dependent on the presence of single- or double-stranded breaks (Benjamin and Gill, 1980). At the molecular level, however, the function(s) of poly(ADP-ribose) synthesis is not clear. Poly(ADP-ribose)ylation has been postulated to influence a number of chromatin functions that involve nicking and rescaling of DNA strands, such as the recovery from DNA damage, cell proliferation, differentiation and genetic recombination (see for review Althaus and Richter, 1987; Shall, 1989).

Poly(ADP-ribose) polymerase is a 113 kDa multifunctional enzyme (Kameshita et al., 1984). Following limited proteolysis of purified protein, three functional domains in the enzyme molecule have been identified: (i) a 46 kDa fragment which acts as a DNA-binding domain (DBD) corresponding to regions A, B and C in Figure 1A. It is located in the N-terminal region which comprises two zinc-fingers involved in DNA strand break recognition (Méniessier-de Murcia et al., 1989; Gradwohl et al., 1990) and a bipartite nuclear location signal (region B) (Schreiber et al., 1992); (ii) a central 22 kDa polypeptide containing several glutamic acid residues which are sites of auto-poly(ADP-ribose)ylation (region D); and (iii) a C-terminal fragment of 54 kDa (regions E and F), encompassing the catalytic domain, which can be cut down to 40 kDa (region F) without losing its basal catalytic activity (Simonit et al., 1990; see for review de Murcia et al., 1991).

It has been shown that PARP needs zinc for enzyme activity (Zahradka and Ebisuza, 1984) and we have determined that each enzyme molecule contains two Zn(II) ions bound to the 29 kDa N-terminal fragment (region A) that is part of the DBD (Mazen et al., 1988, 1989). Disruption of the metal-binding ability of the second zinc-finger (F2) dramatically reduced target DNA-binding in vitro. In contrast, when the Zn(II)-binding residues of the first zinc-finger (F1) were mutated, the DNA-binding activity was only slightly affected (Gradwohl et al., 1990). On the other hand, by exploiting the two properties of the 46 kDa DBD, i.e. its DNA-binding capacity and its nuclear location signal (which ensures rapid and efficient targeting to the nucleus), we demonstrated that overexpression of the human PARP-DBD in transfected cells affects a trans-dominant inhibition of the resident PARP activity (Küpper et al., 1990).

The ability to interfere selectively with endogenous PARP by competition for DNA breaks through overproduction of the DBD made it possible to assess the role of the enzyme in different DNA repair pathways. To this aim a series of point mutants of the human PARP-DBD was expressed in Escherichia coli and first characterized with respect to their in vitro DNA-binding properties by South-western blotting. Subsequently, they were introduced into mammalian cells, by transfection of expression constructs, to establish their in vivo DNA-binding and PARP-inhibiting properties. The
Fig. 1. Schematic representation of PARP and the zinc-fingers. (A) Representation of the three different functional domains of poly(ADP-ribose) polymerase obtained after mild trypsin and papain digestion, according to Kameshita et al. (1984). The first box comprises the DNA-binding domain with the zinc-fingers (region A), the nuclear location signal (NLS, region B) and region C. The second box comprises the automodification domain (region D) and the third box the catalytic domain (regions E and F). (B) The primary structure of the two zinc-fingers is shown. Amino acid substitutions are indicated by arrowheads pointing to the mutant amino acids. (C) Helical-wheel representation of the putative α-helices of the FI and FII zinc-fingers.

effect on base excision repair (BER) and on nucleotide excision repair (NER) was investigated by microinjection of the mutant and wild-type DBD protein or DNA constructs into human fibroblasts and determination of the consequences on BER synthesis [induced by the alkylating agent N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG)] or on NER synthesis (induced by UV irradiation). We found that the trans-dominant inhibition of resident PARP, depending upon the integrity of the second zinc-finger, specifically blocks DNA repair induced by alkylation damage but not that induced by UV irradiation.

Results

In vitro DNA-binding properties of recombinant PARP-DBD

The second zinc-finger of PARP (FII) contains a short stretch of amino acids (residues Cys128–Asp145) which is predicted to be in an α-helical configuration (Chou and Fasman, 1974) (Figure 1B). This region contains five basic residues that are potential candidates for DNA interaction. Among them, K131, K134, R138 and K141 are predicted to be on the same face of the putative α-helix (Figure 1C). In view of the preponderant role played by FII in the binding of the enzyme to nicked DNA (Gradwohl et al., 1990; Ikejima et al., 1990), we have systematically mutated these four basic amino acids into Ile; since FI and FII share sequence homology (Uchida et al., 1987), the conserved residues in FI (Lys30 and Arg34) were also mutated into Ile (see Figure 1B). We have subcloned the cDNA encoding the DBD into the prokaryotic expression vector pTG161, overproduced the mutated proteins in E. coli and tested for their DNA-binding activities by South-western blotting. A $^{32}$P-end-labelled 66 bp DNA probe harbouring a single nick at position 33 (Ménissier-de Murcia et al., 1989; Gradwohl et al., 1990) was used for this assay. None of the point
mutations in FI significantly reduced the DNA-binding capacity of the corresponding polypeptide when compared with the wild-type DBD (Figure 2A). This was observed for the C21G mutation affecting one of the Cys residues that coordinates zinc in FI and for the two mutations affecting the basic residues of FI (K30I and R34I). In contrast, some of the FII mutations dramatically decreased the DNA-binding of the corresponding proteins, in particular the C125G mutation, which alters Cys125 (involved in zinc coordination) and R138I (altering one of the basic residues in the putative α-helix). Likewise, the double mutant C21G;C125G did not show any DNA-binding activity. In summary, our results strongly suggest that FII is involved in the interaction with DNA single-stranded breaks, and that Arg138 is crucial for this function.

The recombinant proteins were identified by Western blot experiments using a polyclonal antibody raised against a synthetic peptide corresponding to FII (Simoin et al., 1991) (Figure 2B) as well as monoclonal antibody C10 (Figure 2C), in order to verify that similar amounts of protein were loaded in each experiment. Obviously, the failure of C125G, R138I and the double mutant C21G;C125G polypeptides to bind DNA is not due to lower quantities of the corresponding proteins.

Mutation K131I appeared to abolish binding of monoclonal antibody C5 to the DBD (Figure 2C), indicating that the amino acid K131 is closely related to the epitope recognized by this antibody.

Trans-dominant inhibition of resident PARP activity by overproduction of wild-type or mutant PARP-DBD in CV-1 cells

In order to study the DNA-binding properties of DBD mutants in living cells, we have subcloned the cDNA encoding the wild-type PARP-DBD and different versions mutated in FI (C21G) or FII (C125G and R138I) into the eukaryotic expression vector pECV23 (Belt et al., 1989). These constructs were transfected into CV-1 cells, resulting in a high level of transient expression. We exploited the trans-dominant inhibition of cellular PARP activity in transfected cells overexpressing the PARP-DBD (Kipper et al., 1990) which was apparently mediated by a competition for DNA breaks necessary for the activation of the resident enzyme. The inhibition could be detected in transient transfection assays in which both DBD overproduction and poly(ADP-ribose) formation stimulated by carcinogen treatment were visualized in the same cells by double immunofluorescence. In this study, the pECV series of DBD expression constructs was transfected, and cells were challenged with the alkylating agent MNNG and assessed for trans-dominant PARP inhibition, using a slightly modified immunofluorescence protocol. Repair of MNNG-induced alkylolation damage, by the action of DNA glycosylases and apurinic/apyrimidinic endonucleases, results in the generation of a large number of single-stranded breaks within minutes after exposure of the cells (Lijinsky, 1976). Overproduction of the wild-type or mutant PARP-DBDs (Figure 3A–D) in CV-1 cells is clearly visualized by a strong, nuclear, red fluorescence, whereas in non-transfected neighbouring cells the resident PARP is barely detectable under these conditions. Non-transfected cells can be identified by their non-specific faint cytoplasmic background staining. The lower panel (Figure 3E–I) shows that poly(ADP-ribose) immunofluorescence staining (green) is visible only after cells have been treated with MNNG (50 μM) which induces DNA strand breaks and thus activates PARP. As expected, overexpression of the construct encoding the wild-type DBD (Figure 3F) inhibited resident...
PARP activity completely (within the detection limits of this assay). Complete inhibition was also observed with the mutant affecting FII (C21G; Figure 3G). In contrast, both mutants affecting FII (C125G and R138I; Figure 3H and I) failed to show any inhibition when compared with the neighbouring, non-expressing cells. The double mutant (C21G:C125G) also failed to show any inhibitory effect in this assay (data not shown). Taken together, these results demonstrate that the dominant-negative phenotype of CV-1 cells caused by overproducing the PARP-DBD perfectly matches the in vitro DNA strand break-binding capacity of the respective DBD mutant.

**Trans-dominant inhibition of resident PARP activity blocks alkylation-induced DNA repair synthesis**

The PARP-DBD was overproduced in E.coli and purified, in order to evaluate possible consequences of the inhibition of resident PARP activity on DNA repair after its microinjection into cultured human fibroblasts (de Jonge et al., 1983). The PARP-DBD was highly overproduced after heat induction of bacteria, the recombinant protein represented ~20% of the total protein content and migrated at the predicted mol wt. in SDS-polyacrylamide gels (Figure 4, lane b). The recombinant DBD was present in the soluble fraction and was easily recovered in the clear lysate (Figure 4, lane c). Protamine sulfate precipitation was used to remove DNA, followed by ammonium sulfate precipitation (Figure 4, lanes d and e, respectively) to reduce the volume before loading the sample on a hydroxyapatite column. After this purification step the protein was almost pure (Figure 4, lane f) and subsequent single-stranded DNA cellulose column chromatography was used to remove minor contaminants. The final purified material consisted of one major band of 46 kDa as detected on a Coomassie-stained polyacrylamide gel (Figure 4, lane g). About 5 mg of PARP-DBD per litre of bacterial culture were purified using this procedure. The recombinant PARP-DBD was functional as judged by its DNA-binding properties measured by the gel retardation assay (data not shown), by its efficient nuclear translocation and by its PARP-inhibiting properties (see below).
Fig. 5. Microinjection of the 46 kDa PARP-DBD protein into CSRO repair-proficient fibroblasts. Nuclei were visualized by staining with the non-intercalating DNA dye DAPI (panels A and D). Panels C and F show a micrograph of CSRO fibroblast homo-polynarys microinjected with wild-type DBD. UDS was assayed following MNNG treatment (100 μM; panel C) or UV irradiation (10 J/m²; panel F). Panels B and E show the corresponding fields prepared for immunofluorescence with the monoclonal C13 anti-DBD antibody. The arrows point to the injected homo-polynarys.

To investigate the involvement of PARP in BER and in NER, the purified PARP-DBD was introduced by microinjection into the cytoplasm of repair-proficient homopolykaryons generated by fusion of normal (CSRO) human fibroblasts as described by de Jonge et al. (1983). Cells were subsequently subjected either to treatment with MNNG (100 μM), inducing methylation damage which is mainly removed by BER, or to UV irradiation (10 J/m²) which causes formation of photoproducts which are then eliminated by NER. To visualize and quantify repair activity, the cells were—after the genotoxic treatment—assayed for unscheduled DNA synthesis (UDS) by incubation in the presence of [3H]Tdr (for specific conditions see Materials and methods), followed by fixation and processing for autoradiography to detect newly synthesized DNA repair patches. To assess DBD overproduction and UDS in the same cell, we developed a procedure that combines immunofluorescence and autoradiography (see Materials and methods). The injected cells exhibited clearly detectable quantities of the DBD polypeptide in their nuclei as demonstrated by immunofluorescence (Figure 5B and E). Furthermore, we verified that the introduced DBD efficiently inhibited cellular MNNG-induced PARP activity in the injected human fibroblasts, by double immunofluorescence for DBD and poly(ADP-ribose) performed on a separate slide injected and treated in parallel (data not shown). Figure 5 panels C and F demonstrate that both the treatment with MNNG and UV induced significant levels of UDS. To examine whether the UDS seen after MNNG treatment indeed was derived from BER (short patch repair) and was
not caused by spurious lesions eliminated by NER (long patch repair), XP-A cells (which are totally deficient in NER) were subjected to MNNG treatment and similarly assayed for UDS. The number of grains above XP-A and normal fibroblasts was similar (data not shown), thus excluding the possibility that the MNNG-induced repair synthesis was in fact derived from NER. Strikingly, the nuclei of injected homopolykaryons displayed dramatically decreased levels of MNNG-induced UDS when compared with non-injected, neighbouring monokaryons (Figure 5C). In contrast, the UDS derived from UV lesion removal was not significantly affected by the block of PARP activity induced by the injection of the PARP-DBD (Figure 5F). UV irradiation was reported to induce poly(ADP-ribose) formation in living cells, as detected by boronate chromatography of cell extracts and quantification of retained polymers (Jacobson et al., 1983). We have, however, been unable to detect polymer signals by immunofluorescence in CV-1 or human fibroblasts following UV irradiation of 0.5–500 or 2–20 J/m², respectively. This apparent discrepancy might be due to different cell culture conditions and/or the different poly(ADP-ribose) detection method. In any event, relatively low levels of UV-induced poly(ADP-ribose) formation are consistent with the lack of UDS inhibition by overproduced DBD.

To establish the specificity of the inhibition effect of the PARP-DBD on MNNG-induced UDS and to find out whether the inhibition effect was dependent on the DNA-binding properties of the PARP-DBD, mammalian expression vectors encoding the various mutated DBD polypeptides were injected intranuclearly into one of the nuclei of CSRO homo-polykaryons. After an incubation of 24 h to permit expression of the injected DNA, the effect on MNNG-induced BER was analysed. The data are presented as histograms shown in Figure 6. Upon injection of the wild-type construct (Figure 6A) two distinct cell populations can be discerned: (i) one displaying a clearly decreased level of UDS, corresponding with properly injected and DBD-expressing cells as assessed by immunofluorescence, and (ii) a subpopulation with normal UDS in which no DBD expression could be demonstrated probably because the cells were not (properly) injected or did not express the injected gene for other reasons. In contrast, when the double mutant (C21G:C125G) that does not bind DNA was overproduced, no inhibition of UDS was recorded (Figure 6D). Conversely, the corresponding single mutants displayed an intermediate behaviour, with the F1 mutant (C21G) being a more potent inhibitor of UDS than the FII mutant (C125G; Figure 6B and C, respectively).

These results show that the wild-type PARP-DBD, either as a purified polypeptide or overproduced from an expression vector, strongly inhibits UDS induced by an alkylating agent but not by UV irradiation. This blocking property of the DBD is completely lost if both zinc-fingers are mutated, demonstrating the absolute requirement of DNA-break binding for the UDS inhibition to occur; however, either the first or the second zinc-finger alone is sufficient to cause partial UDS inhibition.

Discussion

Mutations affecting the second zinc-finger dramatically reduce binding to nicks in DNA α-Helices are frequently found as recognition elements in the helix-turn-helix motifs of a number of prokaryotic and eukaryotic proteins.
eukaryotic genetic regulatory proteins (Luisi, 1992). This is also true of the three structural classes of zinc-fingers crystallized so far: the Zif 268 protein which belongs to the TFIIBA zinc-finger family (Pavletich and Pabo, 1991), the glucocorticoid receptor (Luisi et al., 1991) and the Gal4 protein (Marmorstein et al., 1992). They have in common, within their DBD, an α-helix which lies in the major groove of the DNA and directs interactions with the bases of the target sequence. In PARP, a putative α-helix in the second zinc-finger might be a candidate for the interaction with single strand breaks in DNA. The five basic residues present in FII, as well as the corresponding basic amino acids of FI, were mutated into Ile. All mutated DBD polypeptides display the same DNA-binding capacity as wild-type, with the exception of three, all of which were altered in the second zinc-finger: C125G, R138I and C21G;C125G. Furthermore, the epitope corresponding to the monoclonal antibody C1 was found to be associated with the Lys131 region, adjacent to the critical residue Arg138. This antibody is the only one that has been reported to inhibit in vitro PARP activity substantially (Lamarre et al., 1988). It is interesting to note that the two basic residues Lys131 and Arg138, which appear to be important for DNA-binding and/or activity, have been conserved during evolution and are located on the same face of the predicted α-helix two turns apart. Although these results do not prove that such an α-helix exists, they indicate that FII, and especially the amino acid Arg138, are crucial for binding of the 46 kDa DBD to nicks, confirming the results described by Gradwohl et al. (1990) in the context of a 29 kDa PARP N-terminal fragment.

Trans-dominant inhibition of nick-induced PARP activity requires the second zinc-finger

The effects of the trans-dominant inhibition of the cellular resident PARP of point mutations affecting FI (C21G) or FII (C125G, R138I) or both (C21G;C125G) were examined in eukaryotic cells overexpressing the wild-type or mutated versions of the DBD cDNA in the pEVC vector. In perfect agreement with the in vitro DNA-binding results shown in Figure 2A, we found a trans-dominant inhibition of the endogenous PARP activity not only in cells overproducing the wild-type DBD, as expected (Küpper et al., 1990), but also in FI-mutated versions. In contrast, the FII-mutant DBD which did not bind DNA in vitro failed to inhibit the resident PARP activity. Thus, point mutations in crucial parts of FII abolish the trans-dominant inhibition of cellular PARP. The most plausible interpretation is that the impaired DNA-binding of the mutated DBD polypeptide no longer permits competition with the endogenous enzyme for single-stranded breaks. Consistent with this notion is the observation that the inhibition is also abrogated by an excess of single-stranded breaks (Küpper et al., 1990). Taken together, our results show that in living cells carrying single-stranded DNA breaks, the trans-dominant phenotype is related to the integrity of FII and that the underlying mechanism is a competition for DNA strand breaks which are a necessary cofactor for activating the enzyme.

PARP interferes with DNA repair induced by alkylating agents

The monofunctional alkylating agent MNNG induces a diverse set of methylation damage including N³-methyl adenine, and N⁵- and N⁷-methyl guanine as the main products, and O⁶-methyl guanine as the major mutagenic lesion. A large fraction of these lesions is removed by base excision repair, involving the action of specific DNA glycosylases that catalyse the excision of free bases, thereby generating abasic sites in the DNA. These are, in turn, the substrate for apurinic/apyrimidin endonucleases which induce single-stranded breaks, followed by limited exonuclease action, resynthesis and ligation. In contrast, UV-induced photoproducts (mainly cyclobutane pyrimidine dimers and 6-4 photoproducts) are recognized and eliminated by a principally different mode of repair: the nucleotide excision pathway. This repair process consists globally of five steps carried out in a complex reaction mechanism by the concerted action of multiple proteins: (i) lesion recognition, (ii) dual incision in the damaged strand at some distance around the lesion (Huang et al., 1992), (iii) excision of the oligonucleotide containing the injury, (iv) gap-filling DNA synthesis and (v) ligation. The first three steps are probably performed by one or more multienzyme complexes [see Friedberg (1985) for a comprehensive review on DNA repair]. In our microinjection experiments, the PARP-DBD, either as injected protein or as the product of an injected expression construct, induced a drastic reduction of BER-associated UDS. In contrast with MNNG inducing UDS, the microinjection of the wild-type DBD had no significant effect on UDS induced by UV exposure. These results indicate that, in vivo, PARP is indeed targeted to sites of DNA strand breaks induced during the repair of alkylation-type lesions, and that the DBD interferes with rather early steps, such as excision and/or polymerization. That the DBD does not inhibit UV-induced UDS is probably related to the apparently low levels of stimulation of cellular PARP activity by UV damage in both CV-1 cells and human fibroblasts. This could be due to the low steady-state level of breaks in DNA following UV treatment of the cells (James and Lehmann, 1982; Cleaver et al., 1983) or to the masking of the repair patch by the human excinuclease (Huang et al., 1992).

Satoh and Lindahl (1992) have recently shown in an essentially histone-free in vitro DNA repair system that NAD-starved (i.e. inactive) PARP present in the cell extract inhibited DNA repair, as compared with the active enzyme. Interestingly, depletion of PARP from the crude extract had the same effect as had providing NAD for enzyme catalysis. This suggests that the nick-binding activity of PARP, residing in its DBD, may block free DNA ends against attack by the repair enzymes, and that this block can be relieved by allowing PARP to become catalytically active and auto-modified. Our data on the effect of the 46 kDa DBD on repair synthesis, obtained in living cells, are fully consistent with those in vitro results, since the introduction of an excess of catalytically inactive DBD molecules led to inhibition of UDS, taken as a measure of in vivo repair (Figure 6).

On the other hand, our data are also entirely consistent with an alternative mechanistic model to explain the function of poly(ADP-ribosyl)ation at the molecular level, i.e. the relaxation or the opening of chromatin superstructure by covalent or non-covalent modification of histones, thus allowing access for the DNA repair machinery (de Murcia et al., 1988; Althaus, 1992). Inasmuch as the PARP-DBD acts as a trans-dominant inhibitor of poly(ADP-ribosyl)ation, of course it would not allow other DNA repair factors to access the damaged site.

Whatever the molecular mechanism of action of
poly(ADP-ribosylation) ultimately turns out to be, the striking ability of the PARP-DDB to block MNG-induced nicks and thus to prevent DNA repair synthesis highlights the potential for PARP to act as a critical regulatory component in DNA repair of living cells.

Materials and methods

Cell lines, culture conditions and cell fusion

CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator with a 5% CO₂ atmosphere.

CSRO primary human fibroblasts were cultured in Ham’s F10 medium supplemented with 11% fetal calf serum and penicillin/streptomycin. Homopolykaryons to be injected were generated by fusion of cells using inactivated Sendai virus, as described by de Weerd-Kastelein et al. (1972). The fused cell population was seeded onto 0.6 cm × 0.8 cm areas of a microscope slide provided with a 2 mm grid and cultured for at least 3 days prior to microinjection.

Construction of PARP-DDB expression vectors

A stop codon at position 1119 and a PstI site at position 1124 in the PARP cDNA were introduced by PCR. The resulting PCR product was cloned into the PstI site of the E.coli expression vector pT1G161 (Transgène; Strasbourg) resulting in plasmid pTGT46, or in the XhoI site of the eukaryotic expression vector pECV23 (Bei et al., 1989) using PstI-XhoI linkers, resulting in pEPCV46.

Site-directed mutagenesis

The 1124 bp PstI cDNA fragment encoding the PARP-DDB was purified from pTGT46 and cloned into the polylinker site of plasmid vector M13mp18. Oligonucleotides (15–20mers) were used in Amersham’s oligonucleotide-directed in vitro mutagenesis system under the conditions described by the supplier. After mutagenesis the cDNAs were sequenced and cloned back into the PstI site of pTGT161 or into the XhoI site of pEPCV23.

Analysis of bacterially expressed proteins

E.coli TGE 900 cells (Transgène, Strasbourg) transformed with recombinant plasmids were grown at 28°C to an absorbance at 600 nm of 0.8 in LB medium with 100 μg ampicillin/ml, and then heat-induced at 37°C for 5 h as described by Courtney et al. (1984). Crude extracts were prepared as described by Gradwohl et al. (1990). The bacterial protein samples were electrophoresed in SDS–12% polyacrylamide gels and electrotransferred onto nitrocellulose sheets (BASE 83, Schleicher & Schuell) according to Towbin et al. (1979) at 4°C in a miniblot apparatus for 1 h at 200 mA.

For DNA-binding assays, the blots were treated according to the protocol of Méniérist-de Murcia et al. (1989) with a 66 bp, 32P-end-labelled DNA probe (107 c.p.m. µg) harbouring a nick at position 33. Immunoreactive polypeptides were revealed as described by Mazen et al. (1989).

Purification of the 46 kDa DNA-binding domain

Cultures of plasmid-containing TGE 900 cells were grown and heat-induced as described by Courtney et al. (1984). The culture was centrifuged for 10 min at 7000 g. All subsequent steps were carried out at 4°C. The cells were homogenized in buffer A (50 mM Tris–HCl pH 8.1, 1 mM EDTA, 10% glycerol, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM PMSF) at 10 ml/2 g cells. TWEEN 20 (0.2%) and NP40 (0.2%) were added to the solution and stirred for 30 min. The cell lysate was cleared by centrifugation at 20 000 g for 10 min. Prohibitamine sulfate (Sigma, St Louis, USA) was added to the cleared lysate at a final concentration of 5 mg/ml, and protein-DNA interactions were precipitated with ethanol, washed twice with 70% ethanol, redissolved in 10 mM Tris–HCl pH 7.5 buffer, and snap-frozen in liquid nitrogen. The thawed material was resuspended in buffer B (2 mM K₂HPO₄, 1 mM KH₂PO₄, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF pH 7.3). This sample was loaded on a hydroxylapatite column (HAP Ultrogel). HAP-B was washed with buffer B and eluted with a linear gradient of 3–500 mM phosphate in the same buffer. The elution fractions containing the polypeptide were then dialysed against buffer A and concentrated on a Centricon apparatus using a YM10 filter (Amicon Division, W.R. Grace & Co.-conn., Beverly, USA). The sample was then loaded on a single-stranded DNA cellulose column (Sigma), washed with buffer A and eluted with a linear gradient of 0.1 M to 1.2 M NaCl in the same buffer. The elution fractions containing the protein were pooled, dialysed and concentrated on a Centricon apparatus in 50 mM Tris–HCl pH 8, 20% glycerol. A sample of each step was stored for further analysis on SDS gel and quantification of protein by the absorbance method. The final concentration of the 46 kDa protein was 6–mg/ml.

Transfection of CV-1 cells and immunocytochemistry

The eukaryotic expression vector pEPCV encoding the 46 kDa wild-type or mutated human PARP-DDB was digested with Clal and Sall and purified using a GeneClean II kit (BIO 101, La Jolla, CA). Five micrograms of each of these cDNAs, each combined with 15 μg pBluescript (Stratagene) as carrier, were transfected into CV-1 monkey cells by electroporation exactly as described by Küpper et al. (1990). Cells were plated on coverslips and, after 44 h, treated with 50 μM MNGN (Serva, Heidelberg, Germany) for 15 min at 37°C. Coverslips were rinsed twice with PBS and fixed with ice-cold 10% trichloroacetic acid, 2% tetrodiamid pyrophosphate for 10 min, followed by washing in 70%, 90% and absolute ethanol for 3 min each at −20°C. Indirect double immunofluorescence was performed as described by Küpper et al. (1990), with some modifications. Briefly, cells fixed on coverslips were successively incubated with the following antibodies: (i) a rabbit antiserum against the second zinc-finger of PARP (Simonin et al., 1991; diluted 1:100 in 1% BSA in PBS); (ii) tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit immunoglobulin (Nordwald, The Northwich, England) (diluted 1:100); (iii) a chicken anti-β-actin cell culture supernatant containing mouse monoclonal antibody 10H against poly(ADP-ribose) (Kawamitsu et al., 1984); and (iv) fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Bioyeda, Rehovot, Israel; diluted 1:50). Incubations were carried out in a humid chamber at 37°C for 45 min (for primary antibodies) or for 30 min, respectively. Following each antibody incubation coverslips were washed 3 × 5 min in PBS. Photomicrographs were taken using a Leitz Dialux 22 EB microscope.

Microinjection and UDS experiments

Purified 46 kDa protein (2 µg/ml) was microinjected with a glass microneedle into the cytoplasm of fused human fibroblasts (CSRO cells). Thirty minutes later, the cells were exposed to UV light (10 J/m²; 254 nm) or MNGN (final concentration 100 μM) and incubated with [3H]dR (60 µCi/ml, specific activity 40–100 Ci/mmol) for 1 h to increase the sensitivity of the UDS assay. Some modifications to the standard procedure were introduced as described by Vermeulen et al. (1986). The cells were then fixed with 100% cold ethanol for 10 min at −20°C. Immunofluorescence was carried out as described above. Cells were incubated with monoclonal antibody C2 against the 46 kDa protein (primary antibody), then with a FITC-conjugated goat anti-mouse immunoglobulin (secondary antibody). The cells were counter-stained with a photographic emulsion (Kodak NTB2), exposed for 3 days, developed and stained with Giemsa solution. The pEPCV expression plasmids (DNA concentration 0.5 µg/µl) were injected into one of the nuclei of homo-polykaryons. Cells were incubated for 24 h to permit expression of the injected DNA and treated in the same way as after the protein injection.

In each experiment more than 60 homo-polykaryons and monokaryons (non-S-phase) were evaluated for their thymidine incorporation. The data obtained are displayed as histograms, each bar representing the number of nuclei with a given grain-per-nucleus count.

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