Mutations in the amino-terminal domain of the human poly(ADP-ribose) polymerase that affect its catalytic activity but not its DNA binding capacity

Carlotta Trucco, Eric Flatter, Sébastien Fribourg, Gilbert de Murcia, Josiane Ménissier-de Murcia*

Ecole Supérieure de Biotechnologie de Strasbourg, UPR A9003 du CNRS, Cancérogenèse et Mutagenèse Moléculaire et Structurale, Boulevard Sébastien Brant, F-67400 Illkirch-Graffenstaden, France

Received 27 September 1996; revised version received 7 November 1996

Abstract Poly-ADP ribosylation of nuclear proteins is activated when poly(ADP-ribose) polymerase (PARP), a nuclear zinc-finger enzyme, binds to single-strand DNA breaks. To understand how the signal emerging from its DNA-binding domain (DBD) bound to such breaks is transduced to its catalytic domain, the structure–function relationship of the DBD was investigated. We have used mutagenesis by the polymerase chain reaction (PCR) to generate a random library of PARP mutants. In this work, we describe the identification of catalytically inactive mutants bearing single point mutations, located outside the two zinc fingers in the DBD, that have conserved their full capacity to bind DNA. The results obtained demonstrate that the DNA-dependent activation of PARP requires not only a capacity to bind DNA but also a number of crucial residues to maintain a conformation of the domain necessary to transfer an 'activation signal' to the catalytic domain.

Key words: Random mutagenesis; Colony screening; Zinc finger; DNA binding protein; NAD metabolism

1. Introduction

In living cells, DNA is continuously subjected to genotoxic damage that can lead to DNA strand-breaks, either directly or indirectly following specific recognition and excision of the lesion. To protect the genome from the deleterious accumulation of damage, cells have developed an intricate DNA surveillance network. Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a nuclear protein involved in this process as a molecular nick-sensor [1]. At the site of a DNA strand-break, PARP catalyses the transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD^+), to a limited number of nuclear proteins involved in chromatin architecture, DNA metabolism, and to PARP itself [for a review see [2], [3]]. Although the precise biological role of the enzyme has not been fully elucidated, PARP has emerged in the last decade as a critical regulatory component in the base excision repair (BER) pathway [4–7].

PARP has a modular organization [1] as shown in Fig. 1A. It is a multifunctional, highly conserved enzyme which detects and binds tightly to DNA single-strand breaks via its N-terminal DNA-binding domain (DBD) which subsequentley stabilizes a V-shaped, nicked-DNA conformation [8]. This region encompasses two zinc fingers (domain A), presumably resulting from the duplication of an ancestral motif (exons 1–2 encoding the amino acid sequence 1–97 and exons 3–4 encoding the sequence 98–196) which is involved in the detection of DNA breaks [9–11]. Domain B corresponds to a bipartite, nuclear localization signal [12]. At a site of DNA strand-break, the basal activity of the C-terminal catalytic domain (domain F) is stimulated over 500 times in a DNA-dependent manner [13]. Recently, the crystallographic structure of the chicken PARP catalytic domain has been solved revealing surprising structural homology between its active site (Fig. 1A, residues 859–908) and bacterial mono-ADP ribosylating toxins in spite of a weak sequence homology [14].

To examine the structure–function relationship of the PARP DBD, we and others have previously identified potential critical residues that impair the stimulation of poly-ADP ribosylation by DNA strand-breaks. These residues not surprisingly were the cysteine and histidine ligands of the two zinc ions [10,11] and arginine-138 which is located in a putative α-helix of the second zinc finger [4].

To date, the mechanism of stimulation of the catalytic domain following recognition and binding of the DBD to DNA strand-breaks is not understood. In the present study, we have developed an in vitro PCR random mutagenesis assay to generate a library of PARP DBD mutants that were expressed in *Escherichia coli*. This library of mutants was subsequently screened for: (1) their inability to be catalytically stimulated by DNA strand-breaks and (2) their DNA binding capacity. We report the identification and characterization of four loss-of-function mutants of the PARP DBD, located outside zinc fingers, that are not impaired in the DNA-binding actvity.

2. Experimental

2.1. Construction of the random mutagenesis plasmid library

The internal *Hin*dIII site (position 697) of the human PARP cDNA was mutated by substituting the G residue for an A residue at the third position, conserving the codon for Lys^{233} in the modified cDNA (subsequently referred to as PARP Δ*Hin*dIII). The N-terminal part (domains A, B and C) of human PARP encompassing the DBD was amplified under standard PCR conditions from the plasmid pTGPARPΔ*Hin*dIII under standard conditions using Taq polymerase (Stratagene). The amplified fragment (1100 bp) bearing random mutations encompasses the *Hin*dIII site (nucleotide 21 upstream of the ATG) and the *Bst*EII site (nucleotide 998). Following *Hin*dIII–*Bst*EII restriction, the library of mutated DBD was cloned into the same sites of the prokaryotic expression vector pTG PARP [15], now designated pTG PARP*. This vector was used directly for transformation of the *E. coli* TGE 900 strain [15] from which full-length PARP-bearing mutations in the DBD only (PARP*) was overproduced.

*Corresponding author. Fax: (33) 0388-655-343.
E-mail: demurcia@esbs.u-strasbg.fr

Abbreviations: PARP, poly(ADP-ribose) polymerase (EC 2.4.2.30); PCR, polymerase chain reaction; DBD, DNA-binding domain; FI, zinc finger I; FII, zinc finger II; DTT, dithiothreitol

PII S0014-5793(96)01347-6

2.2. Colony screening for the loss-of-function PARP mutants*

Colonies (grown at 28°C) resulting from the transformation of *E. coli* TG900 strain by pTG PARP* were replica-plated onto LB plates containing 50 μg/ml ampicillin. Nitrocellulose filter lifts were then made and the filters were laid, cells side up, on LB plates containing ampicillin. Incubation was carried out for 5 h at 37°C to induce protein expression. Following this induction period, poly(ADP-ribosylation) activity and immunoreactivity (using an antibody raised against the second zinc finger of the human PARP) were measured on these same filters, as described previously [16].

2.3. Overproduction in E. coli and analysis of the induced proteins

E. coli TGE 900 cells transformed by either the empty vector (pTG 161), the plasmid expressing wild-type PARP (pTG PARP), or by plasmids expressing the mutated PARP*, were grown at 28°C in 2 ml of culture medium. Protein overproduction was obtained following a temperature shift to 37°C and further 5 h incubation.

For DNA binding assays, the culture was centrifuged for 10 min at 7000×*g* and the proteins were dissolved in Laemmli buffer, separated on a 10% SDS-PAGE, and electro-transferred onto nitrocellulose. Following renaturation in 50 mM Tris-HCl, pH 8, 0.1 M KCl, 2 mM $MgCl_2$, 2 mM DTT and 0.1% Nonidet P-40 (NP40) during 30 min, the blot was incubated for 1 h at 4°C in the DNA-binding buffer containing 20 ng of ^{32}P-end-labeled double-strand DNA probe containing a single-strand break [17]. After three washes in binding buffer at 4°C, the blot was subjected to autoradiography to visualize the protein–DNA complexes, as described previously [10]. The relative amount of PARP* was then visualized on the same blots by Western blot technique using a polyclonal antibody, raised against the human recombinant PARP that had been produced in insect cells [18].

Synthesis of ADP-ribose polymers was measured using proteins in cleared lysate as follows: 2 ml of heat-induced bacterial culture was centrifuged and the bacteria were lysed in 1 ml of lysis buffer (0.1 M Tris-HCl, pH 8, 0.1 M NaCl, 1 mM EDTA, 1 mM PMSF and 1 mg/ml lysozyme) for 20 min at 4°C. The lysate was then sonicated and complemented with Tween-20 and NP40 (both at 0.2%) and NaCl at a final concentration of 0.5 M. The crude lysate was centrifuged and the supernatant used for a quantification by Western blot of PARP*. Samples of 200 ng of PARP*, in crude extracts, were used for enzymatic activity measurement according to [19].

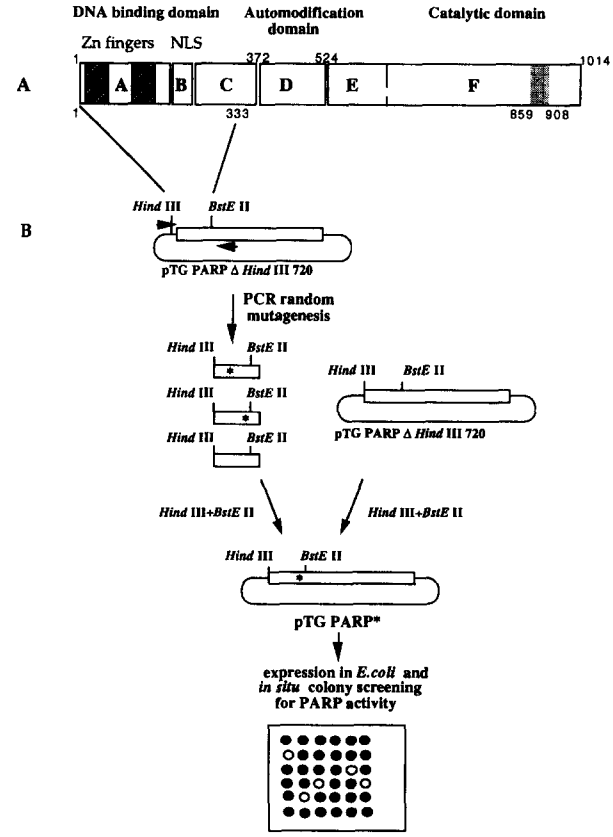

Fig. 1. A: Modular organisation of the human poly(ADP-ribose) polymerase. The DBD encompasses modules A, B and C; module D is referred to the automodification domain, and the catalytic domain is located in modules E and F. The active site is located in region 859–908 [14] B: Protocol used to generate by PCR a random library of PARP* mutated exclusively in the amino-terminal region (residues 1–333).

3. Results and discussion

Random mutagenesis is a powerful method for the identification of critical residues involved in a given enzymatic function. We have previously reported the isolation and characterization of a gain-of-function PARP mutant, L713F, generated by hydroxylamine random mutagenesis of the catalytic domain [20]. Whereas hydroxylamine preferentially modifies GC nucleotides, the errors made by Taq polymerase are more random over the entire genome. In this paper, we have generated mutants of the DBD of PARP, using PCR. To screen for a subsequent loss of function, we have exploited the activity blot procedure described previously [16].

The methodology used for random mutagenesis is illustrated in Fig. 1B. A *Hin*dIII–*Bst*EII fragment of the human PARP cDNA encoding the DBD (module A), the nuclear localization signal [12] (module B) and module C (unknown function), was amplified by PCR, restricted and cloned back into the same sites of the prokaryotic expression vector pTG PARP, giving rise to a library of DBD-mutated PARP* (residues 1–333). The rate of PCR random mutagenesis was about 2%. Screening for PARP activity on replica filters revealed colonies with no enzymatic activity; however, the immunostaining reaction detected PARP protein (data not shown) indicating that these colonies could be expressing loss-of-function mutants.

Polymerizing activity of PARP* mutants in cleared lysate was quantified in vitro according to the standard procedure [19]. As displayed in Table 1, these mutants retain less than 1.5% of the enzymatic activity stimulated by DNA strand-breaks, compared to the wild-type PARP expressed and solubilized under the same conditions.

Table 1
Identification and location of the mutations and residual enzymatic activity of the corresponding PARP* mutants

Mutated amino acid	Mutated nucleotide	Mutated module*	Enzymatic activity** (%)
L77P	T230C	A	< 0.5%
K97R	A290G	A	< 0.5%
K249E	A745G	C	< 0.5%
G313E	G938A	C	1.5%

*See Fig. 1A.
**Percentage of the enzymatic activity of the mutated PARP* compared to the wild-type (see Section 2).

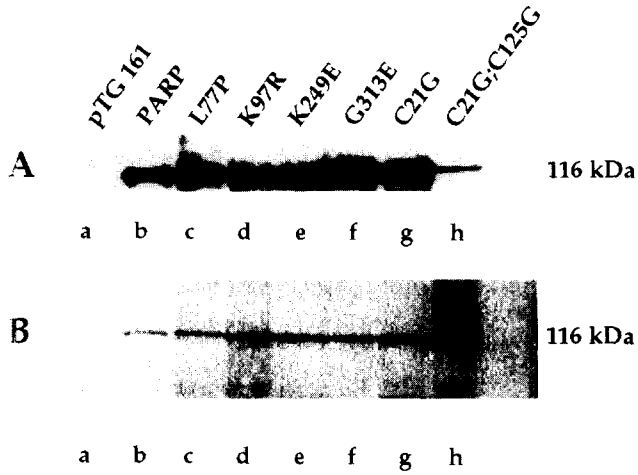

Fig. 2. DNA-binding capacity of the mutated PARP* overproduced in *E. coli* compared to the wild-type PARP; pTG161 indicates transformation of bacteria with the empty vector, C21G and C21G; C125G are site directed point mutants [10]. (A) Southwestern blotting using a ^{32}P-end-labelled nicked DNA probe; (B) Western blot analysis using a polyclonal antibody raised against the human PARP. The same nitrocellulose sheet was used for both experiments.

The corresponding cDNAs were sequenced on both strands; in each case a single nucleotide change was found. Table 1 summarizes the location and the nature of the mutations. Two mutations were located in domain A between the two zinc fingers. L77P is a non-conservative mutation that may induce strong changes in the secondary structure of the protein; however, the second mutation, K97R, is conservative. This mutation strongly suggests that although the positive charge of the amino acid is conserved, modification of the nature of the basic side chain cannot be tolerated. One hypothesis could be that the spatial conformation of residue K97 is modulated by the interaction between the zinc fingers and the DNA breaks. This residue could be a relay informing the catalytic domain that the DBD is bound to an interruption of the sugar-phosphate backbone in the DNA. The two others mutations (K249E and G313E) fall in domain C whose function is unknown, underlying the importance of this region for the stimulation of the catalytic activity by DNA strand-breaks. One cannot rule out the contribution of these mutations to a possible inhibition of the formation of a catalytic dimer which constitutes an essential step in the automodification reaction [21].

It is interesting to note that both amino acids mutated in domain A are strictly and evolutionary conserved in all PARP sequences as well as in the N-terminal part of the DNA ligase III which encompasses a putative zinc finger-sharing homology with those of PARP [22]. This finding reinforces the hypothesis that an ancestral motif encompassing exactly the two first exons has been duplicated and integrated into PARP and DNA ligase III sequences.

Given that PARP activity is strongly dependent upon the presence of DNA strand-breaks [23], PARP* mutants were tested for their DNA-binding capacity using a Southwestern blot assay as described in [10]. Additionaly, site-directed point mutants were tested in parallel: the mutation C21G affecting the structure of the first zinc finger retains a full capacity to bind to nicked DNA whereas the double mutant (C21G; C125G) mutated in the zinc ligands of both fingers displays a strongly reduced DNA-binding capacity [10]. As shown in Fig. 2A, all PARP* mutants obtained by PCR mutagenesis surprisingly retain a full capacity to bind to nicked DNA as compared to the wild-type protein. The same blot was immunostained with an antibody raised againt the full-length human PARP (Fig. 2B) to monitor the amount of protein loaded.

In conclusion, we have shown that a simple relationship between binding to a DNA interruption and PARP activation does not exist. Our data strongly suggest that PARP* mutants may bind to a nick in an inactive form even though the conformation of the DBD is slightly altered. This may be of biological importance since association of the DBD with interacting protein(s) could induce structural changes that mimick the structural alterations observed in the mutants we have generated, serving to negatively modulate PARP activity at the site of a DNA break. Using the 2-hybrid system, we have fished out genes encoding proteins that at the same time interacts with the DBD and impair PARP function (unpublished data). Further work is in progress to test this hypothesis.

Acknowledgements: We thank K. Benton for critical reading of the manuscript. This work was supported by the Association pour la Recherche Contre le Cancer (ARC) and by the Ligue Nationale Contre le Cancer. C.T was supported by a EU fellowship (Human Capital Mobility).

References

[1] De Murcia, G. and Ménissier de Murcia, J. (1994) TIBS 19, 172–176.
[2] Althaus, F.R. and Richter, C. (1987) Mol. Biol. Biochem. Biophys. 37, 1–125.
[3] Lautier, D., Lagueux, J., Thibodeau, J., Ménard, L. and Poirier, G. (1993) Mol. Cell. Biochem. 122, 171–193.
[4] Molinete, M., Vermeulen, W., Bürkle, A., Ménissier de Murcia, J., Küpper, J.H., Hoeijmakers, J.H.J. and de Murcia, G. (1993) EMBO J. 12, 2109–2117.
[5] Satoh, M.S., Poirier, G.G. and Lindahl, T. (1993) J. Biol. Chem. 268, 5480–5487.
[6] Malanga, M. and Althaus, F.R. (1994) J. Biol. Chem. 269, 17691–17696.
[7] Schreiber, V., Hunting, D., Trucco, C., Gowans, B. Grunwald, D., de Murcia, G. and Ménissier de Murcia, J. (1995) Proc. Natl. Acad. Sci. USA 92, 4753–4757.
[8] Le Cam, E., Fack, F., Ménissier de Murcia, J., Cognet, J.A.H., Barbin, A., Sarantoglou, V., Révet, B., Delain, E. and de Murcia, G. (1994) J. Mol. Biol. 235, 1062–1071.
[9] Ménissier de Murcia, J., Molinete, M., Gradwohl, G., Simonin, F. and de Murcia, G. (1989) J. Mol. Biol. 210, 229–233.
[10] Gradwohl, G., Ménissier de Murcia, J., Molinete, M., Simonin, F., Koken, M., Hoeijmakers, J.H.J. and de Murcia G. (1990) Proc. Natl. Acad. Sci. USA 87, 2990–2994.
[11] Ikejima, M., Noguchi, S., Yamashita, R., Ogura, T., Sugimura, T., Gill, D.M. and Miwa, M. (1990) J. Biol. Chem. 265, 21907–21913.
[12] Schreiber, V., Molinete, M., Boeuf, H., de Murcia, G. and Ménissier de Murcia, J. (1992) EMBO J. 11, 3263–3269.
[13] Simonin, F., Ménissier de Murcia, J., Poch, O., Muller, S., Gradwohl, G., Molinete, M., Penning, C., Keith, G. and de Murcia, G. (1990) J. Biol. Chem. 265, 19249–19256.
[14] Ruf, A., Ménissier-de Murcia, J., de Murcia, G.M. and Schulz, G.E. (1996) Proc. Natl. Acad Sci USA 93, 7481–7485.
[15] Courtney, M., Buchwalder, A., Tessier, L.H., Jaye, M., Benavente, A., Balland, A., Kohli, V., Lathe, R., Tolstoshev, P. and Lecocq, J.P. (1984) Proc. Natl. Acad. Sci. USA 81, 669–673.
[16] Simonin, F., Briand, J.P., Muller, S. and de Murcia, G. (1991) Anal. Biochem. 195, 226–231.

[17] Ménissier-de Murcia, J., Molinete, M., Gradwohl, G., Simonin, F. and de Murcia, G. (1989) J. Mol. Biol. 210, 229–233.
[18] Giner, H., Simonin, F., de Murcia, G. and Ménissier-de Murcia, J. (1992) Gene 114, 279–283.
[19] Simonin, F., Poch, O., Delarue, M. and de Murcia, G. (1993) J. Biol. Chem. 268, 8529–8535.
[20] Miranda, E.A., Danzer, F., O'Farrell, M., de Murcia, G. and Ménissier de Murcia, J. (1995) Biochem Biophys. Res. Com. 212, 317–325.
[21] Mendoza-Alvarez, H. and Alvarez-Gonzalez, R. (1993) J. Biol. Chem. 268, 22575–22580.
[22] Wei, Y.F., Robins, P., Carter, K., Caldecott, K., Pappin, D., Yu, G.L., Wang, R.P., Shell, B.K., Nash, R.A., Schar, P., Barnes, D.E., Haseltine, W.A. and Lindahl, T. (1995) Mol. Cell. Biol. 15, 3206–3216.
[23] Benjamin, R.C. and Gill, D.M. (1980) J. Biol. Chem. 255, 10502–10508.