IJP

INTERNATIONAL JOURNAL FOR PARASITOLOGY

TcPARP: A DNA damage-dependent poly(ADP-ribose) polymerase from Trypanosoma cruzi

Silvia H. Fernández Villamil *, Rodrigo Baltanás, Guillermo D. Alonso,
Salomé C. Vilchez Larrea, Héctor N. Torres, Mirtha M. Flawiá

Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas and Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina

Abstract

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme present in most eukaryotes and has been involved in processes such as DNA repair and gene expression. The poly(ADP-ribose) polymer (PAR) is mainly catabolised by poly(ADP-ribose) glycohydrolase. Here, we describe the cloning and characterisation of a PARP from *Trypanosoma cruzi* (*Tc*PARP). The recombinant enzyme ($M_r = 65$) required DNA for catalytic activity and it was strongly enhanced by nicked DNA. Histones purified from *T. cruzi* increased *Tc*PARP activity and the covalent attachment of [^{32}P]ADP-ribose moieties to histones was demonstrated. *Tc*PARP required no magnesium or any other metal ion cofactor for its activity. The enzyme was inhibited by 3-aminobenzamide, nicotinamide, theophylline and thymidine but not by menadione. We demonstrated an automodification reaction of *Tc*PARP, and that the removal of attached PAR from this protein resulted in an increase of its activity. The enzyme was expressed in all parasite stages (amastigotes, epimastigotes and trypomastigotes). When *T. cruzi* epimastigotes were exposed to DNA-damaging agents such as hydrogen peroxide or β-lapachone, PAR drastically increased in the nucleus, thus confirming PAR synthesis in vivo and suggesting a physiological role for PARP in trypanosomatid DNA repair signalling.
© 2007 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Trypanosoma cruzi; PARP; DNA repair signalling; PARG

1. Introduction

Poly(ADP-ribosyl)ation of nuclear proteins, especially histones, is a post-translational modification that was originally found to be induced by DNA strand breaks. This modification establishes a molecular link between DNA damage and chromatin structure remodelling. Poly(ADP-ribose) polymerase (PARP) catalyses the transfer of an ADP-ribose moiety from NAD^+ to a glutamate, an aspartate or a carboxyterminal lysine residue of target proteins. Subsequently, this enzyme is responsible for the extension and branching of poly(ADP-ribose) (PAR) chains, to produce polymers of a length up to 200 units with multiple branching points (Ueda and Hayaishi, 1985; D'Amours et al., 1999; Ame et al., 2004; Diefenbach and Burkle, 2005; Kim et al., 2005; Hassa et al., 2006; Schreiber et al., 2006). The catabolism of PAR is mediated primarily by poly(ADP-ribose) glycohydrolase (PARG), an enzyme with exo- and endoglycosidase activity which generates free ADP-ribose (Brochu et al., 1994; Davidovic et al., 2001; Bonicalzi et al., 2005).

PARPs constitute a large family of at least 17 protein members in humans, encoded by different genes and showing a conserved catalytic domain. According to the amino acid sequence similarity in this domain, Otto et al. (2005) divided the family into five groups. Other classifications group the members using the functional domains as criteria (Ame et al., 2004; Schreiber et al., 2006). The catalytic

domain comprises a NAD$^+$-binding fold composed of six β-strands and one α-helix. There is a catalytic triad: H (histidine)–Y (tyrosine)–E (glutamic acid), which is conserved in PARP-1, PARP-2, PARP-3, vPARP and Tankyrases 1 and 2 (Otto et al., 2005).

The structure of PARP-1 has been investigated in depth and comprises an N-terminal DNA-binding domain (DBD), a regulatory domain, a WGR domain and a C-terminal catalytic domain (Otto et al., 2005; Schreiber et al., 2006). PARP-2 is shorter and resembles an N-terminal truncated PARP-1 with a short basic amino terminal. This enzyme displays an automodification reaction and properties similar to those of PARP-1 (Ame et al., 1999; Diefenbach and Burkle, 2005). PARP-1 and PARP-2 are the only members of the family that are highly activated upon DNA damage.

The targets of PARP-1 activity include PARP-1 itself, PARP-2, core histones (mainly H2B), the linker histone H1, high-mobility group (HMG) proteins, DNA topoisomerases 1 and 2, and DNA polymerase β. In addition, X-ray repair cross-complementing factor 1 (XRCC1) and a variety of transcription-related factors interact with PARP-1. A prevailing opinion on the mechanisms leading to PARP activation is that the activity requires the binding of the enzyme to damaged DNA. However, DNA hairpins, cruciform and stably unpaired regions are all effective coactivators of poly(ADP-ribosyl)ation of histone H1 and PARP-1 automodification (Lonskaya et al., 2005; Potaman et al., 2005).

The models that have been proposed to explain the role of PARP during DNA repair in living cells were widely reviewed by D'Amours et al. (1999). PARP is one of the first nuclear factors to recognise lesions in DNA and PAR synthesised at the site of a DNA strand break could dissociate histones from DNA, thus granting the DNA repair machinery access to damaged DNA. Several recent studies have extended the knowledge on the roles played by PAR, PARP-1 and some other PARPs in diverse molecular and cellular processes, including DNA damage detection and repair, chromatin modification, transcription, cell death pathways and a structural role in the mitotic spindles (D'Amours et al., 1999; Ziegler and Oei, 2001; Tulin et al., 2002; Kraus and Lis, 2003; Chang et al., 2004; Kim et al., 2004; Diefenbach and Burkle, 2005; Faraone-Mennella, 2005; Hassa et al., 2005, 2006; Malanga and Althaus, 2005; Petermann et al., 2005; Gagne et al., 2006). In addition, different mechanisms were observed in transcriptional activation. The first of those affects many genes located in the same locus and operates by loosening the chromatin structure after histone and HMG protein modification; the second acts on individual genes through the participation of PARP in promoter/enhancer binding complexes functioning as transcriptional coactivators (D'Amours et al., 1999; Kraus and Lis, 2003; Hassa et al., 2005). Several reports also point out that PARP could be involved in the differentiation state of cells, through its chromatin remodelling capacity and genomic regulation (Schreiber et al., 2006).

We previously reported the presence of PARP in the trypanosomatid *Crithidia fasciculata* (Villamil et al., 2001; Podesta et al., 2004). Here, we present the cloning and biochemical characterisation of a PARP from *Trypanosoma cruzi*, the aetiological agent of Chagas' disease in Latin America. In addition, we demonstrate that the enzyme is activated in vivo in response to DNA damage.

2. Materials and methods

2.1. Materials

All radiochemicals used in this work were purchased from Dupont NEN Life Science Products Inc., Boston, MA and restriction endonucleases were from New England Biolabs Inc., Beverly, MA. Bacto-tryptose and liver infusion were from Difco Laboratories, Detroit, MI. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO. β-lapachone: (3-4-dihydro-2,2-dimethyl-2*H*-napho [1,2-*b*] pyran-5,6- dione) was a gift from Dr. Marta Dubin (School of Medicine, University of Buenos Aires, Argentina).

2.2. Parasites cultures and cell extracts

Trypanosoma cruzi amastigotes and trypomastigotes were kindly supplied by Ms. Berta Franke de Cazzulo from the University of San Martin, Buenos Aires, Argentina. *Trypanosoma cruzi* epimastigote forms (CL Brener strain) were cultured at 28 °C for 7 days in liver infusion tryptose (LIT) medium (5 g l^{-1} liver infusion, 5 g l^{-1} bacto-tryptose, 68 mM NaCl, 5.3 mM KCl, 22 mM Na$_2$HPO$_4$, 0.2% (W/V) glucose, and 0.002% (W/V) hemin) supplemented with 10% (V/V) FCS, 100 U ml^{-1} penicillin and 100 mg l^{-1} streptomycin. Cell viability was assessed by direct microscopic examination. Cells were harvested by centrifugation at 750g and 4 °C, washed three times with PBS and resuspended at 10 g ml^{-1} of wet weight in buffer A: 50 mM Tris–HCl, pH 8.0, 1.0 mM EDTA, 10% (V/V) glycerol, 10 mM 2-mercaptoethanol, containing protease inhibitors: 1 μg ml^{-1} *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), 1 mM pepstatine A, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1 mM *Nα-p*-tosyl-L-lysine chloro-methyl ketone (TLCK). Cells were lysed in an Ultrasonic Processor Model W385 Sonicator (Heat Systems-Ultrasonic Inc. Plainview, IL, NY, USA) and centrifuged for 30 min at 27000g and 4 °C. The supernatant thus obtained was used as a protein source for Western blot analysis and activity measurement.

2.3. Histone preparation

Frozen epimastigotes (5 × 10^8 parasites) were resuspended in 1 ml of 10 mM potassium glutamate, 250 mM sucrose, 2.5 mM CaCl$_2$ and lysed by the addition of 0.1% Triton X-100 containing protease inhibitors as described above. The suspension was centrifuged, washed

once with the same buffer without Triton X100 and twice with the buffer lacking sucrose. Parasite lysates were acid-extracted with 0.3 N HCl at 4 °C for 2 h with shaking and acid-soluble proteins were precipitated with acetone, washed and vacuum-dried as described by da Cunha et al. (2005).

2.4. Preparation of T. cruzi DNA and RNA

Genomic DNA was purified as described by Pereira et al. (2000). Total RNA was prepared from 4×10^{10} epimastigotes using the Total RNA Isolation (TRIzol) reagent (Gibco BRL., Life Technologies, Rockville, MD) according to the manufacturer's instructions.

2.5. Cloning of the TcPARP gene

The gene sequence corresponding to *Dictyostelium discoideum* PARP (Accession No. XP629373) was used to screen *T. cruzi* sequence databases (http://www.tigr.org/tdb/e2k1/tca1/ and http://www.genedb.org/genedb/tcruzi/index.jsp) using the WU-Blast2 algorithm. The sequence identified was named *Tc*PARP and oligonucleotides carrying hemi-restriction sites were designed from this sequence: *Tc*PARP-Fw (NcoI) 5′-CCATGGCACCAAAGAAGTTATCAGGAGC-3′; *Tc*PARP-Rv (XhoI) 5′-CTCGAGATGATATTTAAACCCCACATGGAC-3′. PCR amplifications were carried out using 600–800 ng of *T. cruzi* genomic DNA, 100 ng of each primer, 2.5 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphates (dNTPs) and 1–2 U of Taq polymerase (Promega, Madison, WI) in a final volume of 50 μl.

2.6. Northern and Southern blots

For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1.5% formaldehyde–agarose gel, transferred to a Hybond N+ Nylon membrane (Amersham Pharmacia Biotech, Piscataway, USA) and hybridised at 65 °C in Church's buffer [1% (W/V) BSA, 7% (W/V) SDS, 1 mM EDTA pH 8, 0.5% (W/V) Na₂HPO₄] with a specific 1000 bp *Tc*PARP probe carrying the 3′ region (bases 779–1779). Blots were subjected to sequential stringent washes at 65 °C and either exposed to AGFA CP-BU NEW films (AGFA Gevaert N.V., Belgium) or scanned using a phosphoimager STORM 820 (Amersham, Pharmacia, USA). Southern blot analysis was performed with 5 μg of genomic DNA previously digested with restriction endonucleases and hybridised with the same probe as described for Northern blot. All probes were labelled with [α-³²P]-dCTP using the Prime-a-Gene kit (Promega, Madison, WI) following the manufacturer's instructions.

2.7. Expression of recombinant TcPARP in E. coli

The full-length *Tc*PARP gene was amplified using the corresponding primers carrying hemi-restriction sites, cloned into a pGEM T-Easy® plasmid and subcloned into the expression vector pET 22 b(+) (Novagene, Inc.) in fusion with a C-terminal His-Tag. Expression of the recombinant *Tc*PARP was performed in the BL21-Codon plus(DE3)-RIL (*E. coli* B, F-, ompT, hsdS_B, (r_B−, m_B−), dcm⁺, Tetʳgalλ(DE3)endA The, [argU ile Y leuW Cam ᴿ]). Cultures were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 28 °C for 4 h and recombinant *Tc*PARP was purified by metal affinity chromatography on Ni²⁺–NTA columns (Qiagen, Valencia, USA) following the manufacturer's instructions. The purity was analysed by SDS–PAGE and Western blot.

2.8. Sequence analysis

Searches in *T. cruzi* and *Trypanosoma brucei* genome databases (http://www.tigr.org/tdb/e2k1/tca1/ and http://www.tigr.org/tdb/e2k1/tba1/, respectively) were performed with Wu-Blast2. The Sanger Institute Databases (http://www.genedb.org/) were also used to screen *Leishmania major*, *T. cruzi* and *T. brucei* databases. Sequence local alignments were performed with the BLASTP (http://www.ncbi.nlm.nih.gov/blast/index.html). Vector NTI Advance™ (Invitrogen) was used for further sequence analysis. For a multiple sequence alignment either 3DCoffee (Poirot et al., 2004) or T-Coffee webtools (http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) were used. The secondary structures of the crystallised proteins were taken from PDB (http://www.pdb.org/pdb/home/home.do), while secondary structure predictions for *Tc*PARP and *Tb*PARP were generated using DomPred (http://bioinf.cs.ucl.ac.uk/dompred/) and PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). The PARP-1 secondary structure was taken from Otto et al. (2005). Domain predictions for *Tc*PARP and *Tb*PARP were generated using Pfam (http://www.sanger.ac.uk/Software/Pfam/) and InterPro (http://www.ebi.ac.uk/interpro/).

Accession Nos. of human PARP sequences used in the alignment of Fig. 1a were: PARP-1: P09874, PARP-2: CAB41505, PARP-3: AAM95460, vPARP (PARP-4): AAD47250, Tankirase-1 (TANKS): AAC79841, Tankirase-2 (TANKS2): NP_079511, PARP-15: NP_689828, PARP-14: AAN08627, PARP-9 (BAL1): NP_113646, PARP-10: BAB55067, PARP11: AAF91391, PARP-12: NP_073587, PARP-13 (ZAP): NP_064504, TiPARP (PARP-7): NP_056323, PARP-16: AAH31074, PARP-8: NP_078891, PARP-6: CAB59261, *Tc*PARP: DQ061295 and *Tb*PARP: DQ679800.

2.9. PARP activity assay

Enzymatic activity was measured as trichloroacetic acid (TCA)-precipitable radioactivity incorporated from [adenylate-³²P]-NAD⁺ (Zahradka and Ebisuzaki, 1984; Shah et al., 1995). PARP samples (5–10 μl) were incubated at 30 °C for 5 min in a standard assay mixture (50 μl) containing 100 mM Tris–HCl, pH 8.0, 10% (V/V) glycerol, 1.5 mM

a

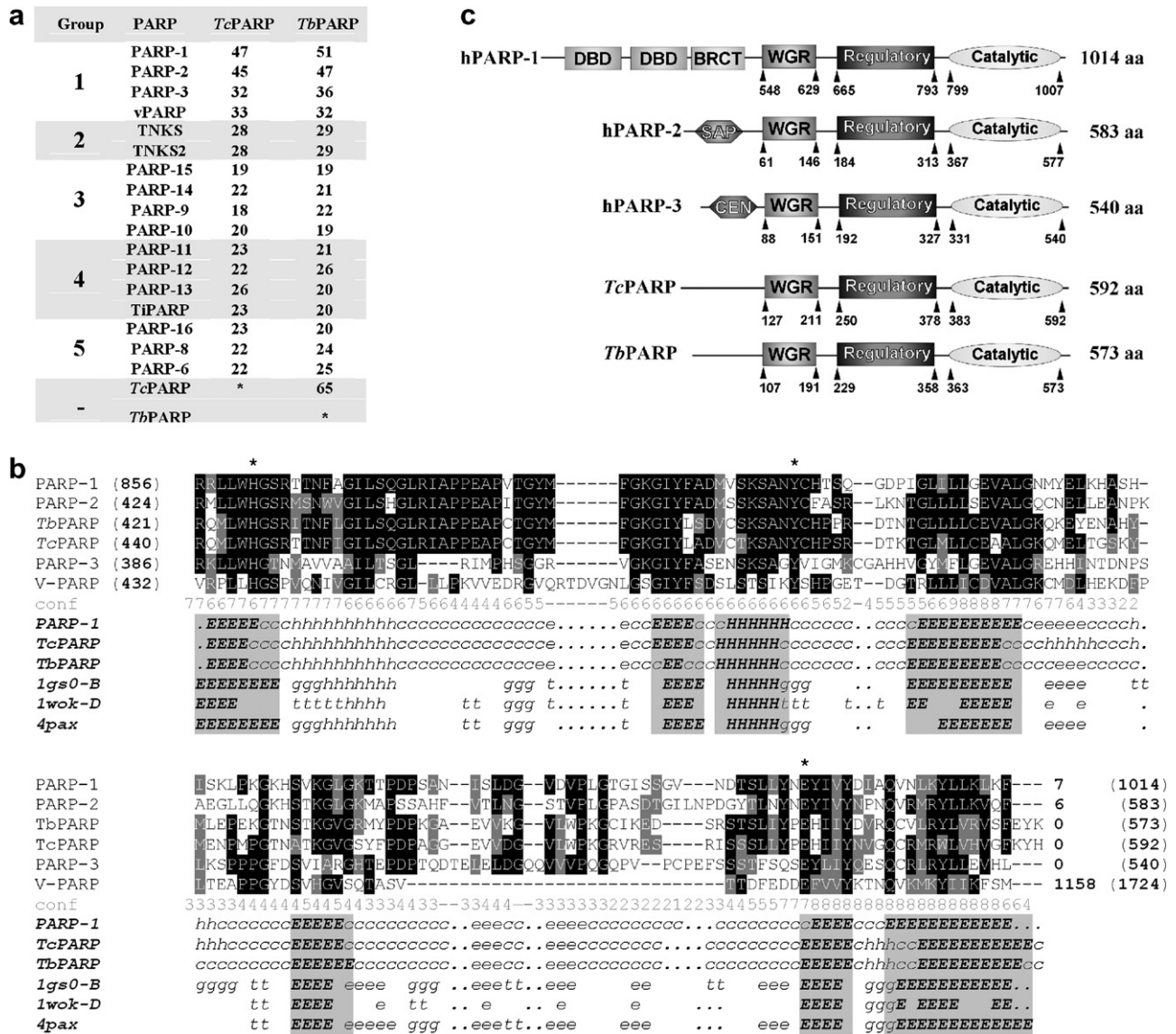| Group	PARP	TcPARP	TbPARP
1	PARP-1	47	51
	PARP-2	45	47
	PARP-3	32	36
	vPARP	33	32
2	TNKS	28	29
	TNKS2	28	29
3	PARP-15	19	19
	PARP-14	22	21
	PARP-9	18	22
	PARP-10	20	19
4	PARP-11	23	21
	PARP-12	22	26
	PARP-13	26	20
	TiPARP	23	20
5	PARP-16	23	20
	PARP-8	22	24
	PARP-6	22	25
-	TcPARP	*	65
	TbPARP		*

Fig. 1. Sequence alignment and structure of poly(ADP-ribose) polymerases (PARPs) from different organisms. (a) Result of a multiple sequence alignment, showing the percentage of sequence identity of the catalytic domains of TcPARP and TbPARP against the 17 human PARPs. Comparisons were performed using the T-Coffee webtool. (b) Multiple sequence alignment between the catalytic domain of human PARP-1, PARP-2, PARP-3, vPARP, TcPARP and TbPARP was performed using the 3DCoffee webtool. 1wok-D, 1gs0-B and 4pax are the crystallised catalytic domains of human PARP-1, murine PARP-2 and chicken PARP-1, respectively. The confidence of the prediction (conf) is indicated in grey numbers (highest confidence = 9). Secondary structures are indicated underneath the alignment: e and E: extended beta strand, h and H: alpha helix, t: turn, g: pi helix, c: not assigned. The secondary structures of the crystallized proteins were taken from PDB, predictions for TcPARP and TbPARP were generated using DomPred and PSIPRED, and the secondary structure for PARP-1 was taken from Otto et al. (2005). Highlighted in grey are the six extended beta strands and the alpha helix that conforms the catalytic nucleus. Asterisks indicate the catalytic triad H–Y–E. Catalytic domain fragments used in the alignment are indicated by numbers between parentheses. (c) Domain architecture of human and trypanosomatid PARPs. Protein interaction domains: BRCT (breast cancer suppressor protein C-terminal), WGR (WGR domain); nucleic acid binding domains: DBD (DNA binding domain), SAP (putative DNA binding domain), CEN (centriole localisation).

dithiothreitol (DTT), 70 μg ml^{-1} activated DNA (nicked DNA) and 70 μg ml^{-1} histone II-A (Sigma). The reaction was started by adding 0.2 mM NAD$^+$ ([adenylate-^{32}P]-NAD$^+$ 1.7 μCi μmol^{-1}), and stopped by the addition of ice-cold 25% (W/V) TCA. BSA (2 mg ml^{-1}) was added and after 20 min standing on ice, samples were filtered through 0.45 μm pore GF/C filters (Millipore), previously saturated with 25% (W/V) TCA, 45 mM H$_3$PO$_4$ and 1 mM NAD$^+$.

Filters were then washed twice with ethanol 96° and dried, and [^{32}P] incorporation into TCA-precipitated protein was measured. PARP activity was expressed as U mg^{-1}protein. One unit (U) is defined as picomoles of ADP-ribose incorporated to proteins per min, under standard conditions. Protein concentration was measured by the Bradford method (Bradford, 1976). Each value corresponds to the average of at least three separate measurements.

2.10. Analysis of the length distribution of ADP-ribose chains

The assay for the synthesis of ADP-ribose polymers was adapted from Mendoza-Alvarez and Alvarez-Gonzalez (1993). Briefly, samples were incubated for 10 min in standard assay mixture, without histone addition, and stopped by adding 10 mM 3-aminobenzamide. After that, mixtures were subjected to proteinase K digestion (20 mg ml^{-1}) at 45 °C for 30 min. The [^{32}P]ADP-ribose chains were chemically detached from proteins with 0.1 M NaOH, 20 mM EDTA at 60 °C for 2 h and neutralised with 0.1 M HCl. Standard DNA loading buffer containing xylene cyanol and bromophenol blue was added and samples were further analysed by 20% SDS–PAGE (15×20 cm) for 20 h at 100 V. Gels were dried on 3 MM paper (Whatman International, Maidstone, UK) and either exposed to AGFA CP-BU NEW films (AGFA Gevaert, Argentina) or scanned using a Phosphorimager STORM 820 (Amersham, Pharmacia, USA).

2.11. SDS–PAGE and Western blot

Procedures for SDS–PAGE of protein samples were carried out as described by Laemmli (1970). Polypeptides were electrotransferred from polyacrylamide gels to Hybond-C membranes (Amersham Pharmacia Biotech, Piscataway, USA). For the reaction with the antibody, the transferred membranes were blocked with a 3% (W/V) non-fat milk suspension for at least 2 h. After incubation for 2 h with a rabbit polyclonal antibody against human PARP (1:800) (Santa Cruz Biotechnology Inc.) or against PAR (1:1600) (Alexis Biochemicals), proteins were visualised with a 1:5000 dilution of the mouse anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, USA) and developed with the Renaissance Western blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, USA).

2.12. Immunodetection of PAR and PARP

Epimastigotes of *T. cruzi* ($2–5 \times 10^7$ cells ml^{-1}) were exposed to either 3 mM H$_2$O$_2$ or 20 μM β-lapachone and washed with PBS. The parasites were fixed for 20 min with 3.8% (W/V) formaldehyde in PBS at room temperature, permeabilised with ice-cold methanol for 5 min and blocked overnight at 4 °C with 5% (W/V) BSA in PBS. Poly(ADP-ribose) polymer was detected with 1:200 rabbit polyclonal PAR antibody followed by 1:600 anti-rabbit Cy3 conjugated antibody. A laser scanning confocal microscope (Olympus FV300, Latin America, Inc. software version 3.3) was used to obtain the fluorescence (laser HeNe G, 543 nm) and differential interference contrast images (DIC). For immunolocalisation of *Tc*PARP, *T. cruzi* epimastigote cells (approximately 1×10^7 parasites ml^{-1}) were fixed and blocked with 5% (W/V) BSA in PBS. Samples were incubated with 1:500 rabbit polyclonal antibody against PARP, washed with PBS and incubated with 1:800 FITC-conjugated goat anti-rabbit IgG and Höechst. Signals were visualised using an Olympus BX 50 (Olympus America Inc. Melville, NY, USA) fluorescent microscope.

3. Results

3.1. Cloning of TcPARP gene and sequence analysis

The screening to identify putative genes of PARP in *T. cruzi* was performed as described in Section 2. One putative open reading frame (ORF) codifying a protein of 592 amino acids was identified. The predicted protein has a calculated molecular mass of 65 kDa and estimated p*I* of 9.5. This protein showed similarity with three typical PARP-1 domains: WGR, regulatory and catalytic. A fragment carrying the putative gene was generated by PCR amplification from *T. cruzi* epimastigote DNA. The sequenced gene of 1779 bp was named "*Trypanosoma cruzi* poly (ADP-ribose) polymerase" (*Tc*PARP) and annotated in the GenBank under the Accession No. DQ061295. This sequence was used to search for homologous sequences in GeneDB *T. brucei* and *Leishmania major* databases, only showing a positive result for *T. brucei*. The sequence identified (*Tb*PARP) was confirmed by sequencing and deposited in GenBank under the Accession No. DQ679800.

Analysis of the amino acid sequence of these proteins revealed some interesting details. A multiple sequence alignment of the catalytic domain sequences from *T. cruzi*, *T. brucei* and the 17 members of the human PARP family showed that *Tc*PARP shares identity with human PARPs of group 1, which includes PARPs 1, 2, 3, and 4 (vPARP): 47% with PARP-1, 45% with PARP-2, 32% with PARP-3, 33% with vPARP and 65% with *Tb*PARP (Fig. 1a).

A structure-guided multiple sequence alignment was performed between *Tc*PARP, *Tb*PARP and group 1 PARPs (Fig. 1b). The predicted secondary structure motifs of *Tc*PARP and *Tb*PARP, as well as PARP-1, and the crystallised catalytic domains of several PARPs (mouse PARP-2, chicken-PARP-2 and human PARP-1) show a conservation of the six β strands and the α helix that conform the catalytic domain. It is important to note the conservation of the H–Y–E catalytic site. This H–Y–E catalytic triad is only found in PARPs from groups 1 and 2 (the latter includes Tankyrases 1 and 2). In this triad, the glutamic acid was shown to be critical for the catalytic activity of many PARPs (Ame et al., 2004; Otto et al., 2005) (Fig. 1b).

Domain architecture and some other characteristics including protein size were also compared (Fig. 1c). *Tc*PARP (592 amino acids) and *Tb*PARP (573 amino acids) are shorter than hPARP-1 (1014 amino acids) and appear to have a length similar to that of hPARP-2 (583 amino acids) and hPARP-3 (540 amino acids). All these polypeptides carry WGR, regulatory and catalytic domains, but in *Tc*PARP and *Tb*PARP both the Zn-finger domains and a BRCT domain characteristic of PARP-1 are absent. In addition, a high percentage of basic amino acids

in the amino-terminal side of the molecules, present in hPARP-2 (27% in the first 64 amino acids) and hPARP-3 (20% in the first 54 amino acids), was also observed in the trypanosomatid members *Tc*PARP (27% in the first 70 amino acids) and *Tb*PARP (28% in the first 90 amino acids). All these data tend to indicate that *Tc*PARP and *Tb*PARP share the same characteristics as those of their PARP-2 and PARP-3 counterparts.

3.2. Genomic organisation and expression of TcPARP in T. cruzi

Southern blot analysis, hybridised with a probe corresponding to the last 1000 nucleotides of the *Tc*PARP coding region, showed that *Tc*PARP is encoded by a single-copy gene (data not shown). A Northern blot of total RNA from *T. cruzi* epimastigotes, using the same fragment as a probe, revealed a hybridisation band of approximately 1800 bp (Fig. 2A). In order to analyse the expression of *Tc*PARP in all developmental stages of *T. cruzi*, cell-free extracts of amastigotes, epimastigotes and trypomastigotes were subjected to Western blot analysis. Antibodies recognised a band with an apparent molecular mass of 65 kDa in the three stages of the parasite (Fig. 2B).

3.3. Expression and characterisation of TcPARP in E. coli

Over-expression of *Tc*PARP in bacteria mostly yielded the protein in inclusion bodies and proteolytic fragments. Improvement of *Tc*PARP expression in *E. coli* was achieved with the construction of a 6 × His-tagged PARP cloned into a pET 22 b(+) vector and BL21-Codon plus(DE3)-RIL, which rendered the full-length active protein. Western blot analysis of purified recombinant protein revealed polypeptides of predicted molecular weight (Fig. 2B).
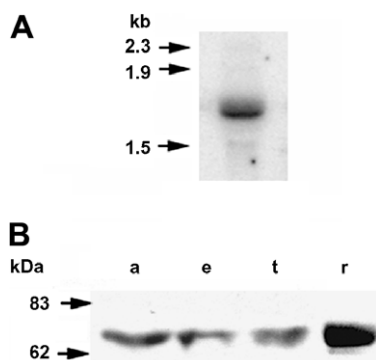

Fig. 2. Expression of *Tc*PARP. (A) Northern blot: total *Trypanosoma cruzi* RNA (15 μg per lane) was electrophoresed under denaturing conditions, transferred and hybridised with a specific probe (*Tc*PARP, bases 779–1779). Molecular masses of rRNAs are indicated. (B) Western blot: native *T. cruzi* poly(ADP-ribose) polymerases (PARP) (80 μg protein) from amastigotes (a), epimastigotes (e) and trypomastigotes (t) or 15 μg protein of recombinant *Tc*PARP (r), were loaded on 10% SDS–PAGE and Western blot as described in the Section 2.

The activation of PARPs by nicked DNA is a well-known property of PARP-1 and PARP-2 from other organisms (Ame et al., 1999; Oliver et al., 1999; Podesta et al., 2004; Diefenbach and Burkle, 2005; Schreiber et al., 2006). Therefore, the activity of *Tc*PARP was tested for DNA dependence. As shown in Fig. 3a, PARP required the presence of DNA for catalysis and the highest activities were observed with nicked DNA (sbDNA). A direct correlation between the rate of poly(ADP-ribosyl)ation and sbDNA concentration was obtained (Fig. 3b). If single-strand DNA (ssDNA) or double-strand DNA (dsDNA) without nicks was used instead, a lower activity was observed (10% and 24%, respectively) (Fig. 3a). When the assay mixture was supplemented with *T. cruzi* histone or commercial bovine II-A histone, the enzymatic activity increased six- to 10-fold (Fig. 3a). Under these conditions both histones were ADP-ribosylated by *Tc*PARP (Fig. 4).

The requirement for divalent cation cofactors was further investigated. *Tc*PARP appeared to have no requirement for exogenously added Mg^{2+} at concentrations up to 10 mM. Moreover, 10 mM Ca^{2+}, 10 mM Mn^{2+}, 1 mM Zn^{2+} or 1 mM Ni^+ were inhibitory on enzymatic activity (58%, 78%, 100% and 100%, respectively). The addition of EDTA (10 mM) failed to inhibit enzyme activity, ruling out the presence of low amounts of Mg^{2+} as contaminant (data not shown).

In order to further characterise the *T. cruzi* recombinant enzyme, a series of inhibitors effective on other well-known PARPs (Rankin et al., 1989; Banasik et al., 1992) were assayed. Theophylline, nicotinamide, 3-aminobenzamide (3-AB) and thymidine, used at 1 mM, inhibited *Tc*PARP enzyme activity. However, 1 mM histamine, capable of inhibiting *C. fasciculata* PARP (Villamil et al., 2001), had a negligible effect on *Tc*PARP activity. Menadione had no effect on *Tc*PARP when tested at 120 μM, a concentration producing 50% inhibition of mono-ADP-ribose transferase activity (Banasik et al., 1992) (Fig. 5). The inhibitory effect of ATP on PARP-1 was described by Kun et al. (2004), as shown in Fig. 5. *Tc*PARP activity decreased approximately 40% in the presence of 10 mM ATP.

3.4. Automodification reaction

Down-regulation of PARP activity by auto-poly(ADP-ribosyl)ation is one the best characterised regulatory mechanism for this enzyme (Ferro and Olivera, 1982; Podesta et al., 2004). To determine the automodification process, the purified recombinant *Tc*PARP was incubated with [^{32}P]-NAD$^+$ in the absence or presence of sbDNA and without addition of histone. Western blot assays revealed with anti-PAR antibodies showed that the *Tc*PARP was able to bind ADP-ribose molecules (Fig. 6a). This result is in agreement with the autoradiographic observation of a [^{32}P]-labelled-protein (Fig. 6b). In order to test the chain length synthesised, we analysed the polymer size distribution after those were chemically detached from the protein, followed by gel electrophoresis as described in the Section
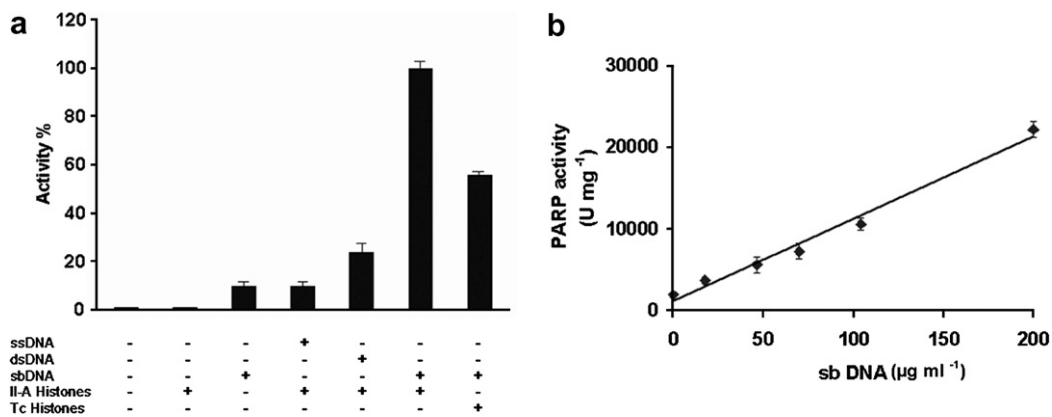
Fig. 3. Requirements for TcPARP activity. (a) The assay mixture contained 100 mM Tris–HCl, pH 8.0, 10% glycerol, 1.5 mM dithiothreitol, TcPARP (15 µg protein), 0.2 mM [^{32}P]-NAD$^+$ and, alternatively, 70 µg ml^{-1} nicked DNA (sbDNA), 70 µg ml^{-1} single-strand DNA (ssDNA), 70 µg ml^{-1} double-strand DNA (dsDNA), 70 µg ml^{-1} commercial type II-A histone (II-A Histones) or 12 µg ml^{-1} Trypanosoma cruzi histone (Tc Histones) according to the figure. Values are means ± SD of three independent measurements. Percentage of activity (Activity %) represents relative activity compared with the sample assayed under standard conditions (70 µg ml^{-1} sbDNA and 70 µg ml^{-1} II-A Histones) taken as 100%. Activity under standard condition was 9076 U mg^{-1}. (b) Effect of sbDNA on poly(ADP-ribose) synthesis. The reaction was carried out under standard conditions, except for the addition of varying amounts of sbDNA, in the presence of 70 µg ml^{-1} II-A Histones and 0.3 mg ml^{-1} TcPARP. Values represent means ± SD, $n = 3$.
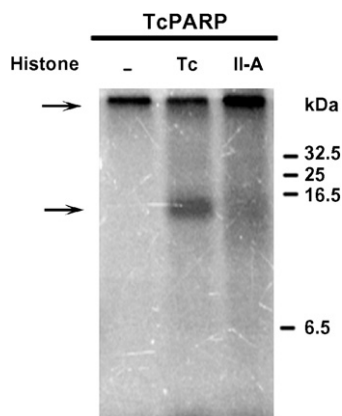

Fig. 4. ADP-ribosylation of Trypanosoma cruzi and II-A Histones by TcPARP. TcPARP (0.3 mg ml^{-1}) was incubated in the absence or presence of 13 µg of T. cruzi purified histones (Tc) or 35 µg commercial type II-A Histones (II-A) and [^{32}P]-NAD$^+$ under standard conditions. The reaction was stopped by heating at 65 °C for 10 min. The samples were analysed by 15% SDS–PAGE and exposed to X-ray film. Arrows indicate ADP-ribosylated histones and automodified TcPARP. Molecular masses are indicated in the figure.
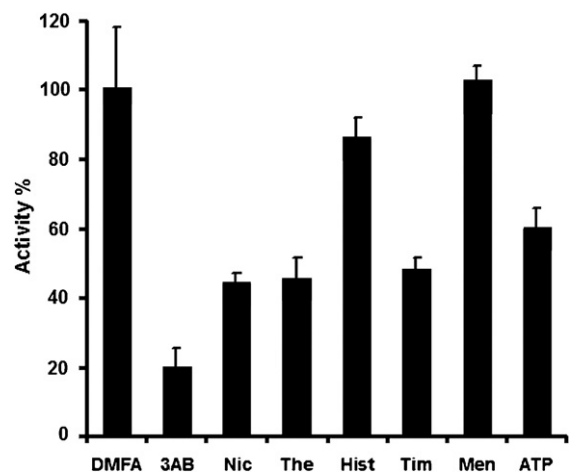

Fig. 5. Effect of inhibitors on TcPARP activity. DMFA, dimethyl formamide; 3AB, 3-aminobenzamide; Nic, nicotinamide; The, theophylline; Hist, histamine; Tim, thymidine; Men, menadione; ATP, adenosine 5′-triphosphate. Standard assay mixtures containing TcPARP and inhibitors (1 mM) except Men (0.120 mM) in 1% V/V solvent (DMFA) or ATP (10 mM) were pre-incubated at 0 °C for 5 min. Then [^{32}P]-NAD$^+$ was added and the activity at 30 °C was measured. Values represent relative activities compared with control samples containing DMFA (100%). Control activity: 1873 U mg^{-1}.

2. As illustrated in Fig. 6c, long polymers were formed under these conditions, as can be observed at the origin of the gel. The presence of short oligomers can also be seen.

As we have previously reported, approximately 65% of ADP-ribose protein linked to C. fasciculata PARP is detached by alkaline treatment at 0 °C for 3 h (Podesta et al., 2004). Taking these data in consideration, we incubated TcPARP with NAD$^+$ and sbDNA to allow the automodification reaction. The modified enzyme was then subjected to 3 h dialysis either against bicarbonate buffer pH 10.0 or against phosphate buffer pH 7.0. We found that after alkaline treatment the activity of TcPARP increased approximately threefold compared with the enzyme dialysed at pH 7.0 (control activity: 2041 ± 81 U mg^{-1}, auto-modified TcPARP after buffer pH 7.0 treatment: 590 ± 18 U mg^{-1}, automodified TcPARP after buffer pH 10.0 treatment: 1778 ± 90 U mg^{-1}).

3.5. Poly(ADP-ribose) synthesis in T. cruzi epimastigote cells induced by DNA damage

In higher eukaryotes, PARP-1 and PARP-2 are molecular sensors of DNA breaks (de Murcia and Menissier de Murcia, 1994; Ame et al., 1999; Schreiber et al., 2006) and they have a key role in organisation of the repair
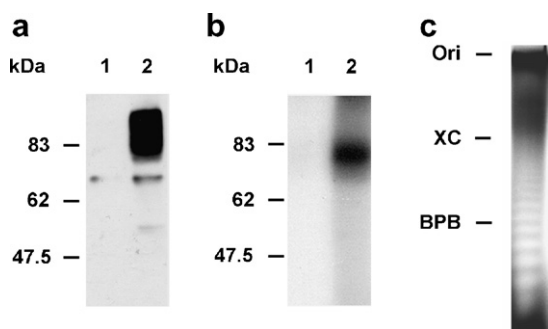
Fig. 6. Analysis of *Tc*PARP automodification reaction. Purified *Tc*PARP was incubated with NAD^+ in either the absence (control) or presence (automodified) of activated DNA to allow the auto-poly(ADP-ribosyl)ation reaction. The reaction was stopped by the addition of 10 mM 3-aminobenzamide and analysed on 10% SDS–PAGE. Other conditions are described under Section 2. (a) Western blot of control (lane 1) and automodified (lane 2) *Tc*PARP, recognised by an anti-PAR antibody. (b) The reaction was carried out in the presence of $[^{32}P]$-NAD^+. The samples (control, lane 1 and automodified, lane 2) were analysed by 10% SDS–PAGE and subjected to autoradiography by exposure to X-ray film. (c) Size distribution of $[^{32}P]$ADP-ribose polymers on a 20% denaturing polyacrylamide gel. The relative electrophoretic migration of xylene cyanol (XC) = (ADP-ribose)$_{20}$, bromophenol blue (BPB) = (ADP-ribose)$_8$ and ORI (beginning of the sequencing gel), are indicated to the left of the autoradiograph. Representative data shown ($n = 3$).

mechanism (Malanga and Althaus, 2005). The possibility that poly(ADP-ribose) synthesis may be triggered in *T. cruzi* by DNA-damaging agents was also explored. *Trypanosoma cruzi* epimastigote cells were exposed to either 3 mM H_2O_2 or 20 μM β-lapachone, with the aim of evaluating this response. Fig. 7 shows that in H_2O_2-treated cells a strong nuclear signal was detected with an anti-PAR antibody. The same result was obtained with β-lapachone (data not shown). No ADP-ribose polymers were detected in untreated cells. This result is in agreement with the immunolocalisation of PARP developed with an antiserum against the catalytic domain (Fig. 7).

4. Discussion

This article reports the identification and characterisation of a PARP from the flagellate protozoan *T. cruzi*, the causative agent of Chagas' disease in Latin America. Southern blot analysis showed that *Tc*PARP is a single-copy gene, which is consistent with the information provided by the *T. cruzi* genome databases. In addition, Northern blot analysis indicated the presence of a specific mRNA in the epimastigote form. The expression of this enzyme has been demonstrated in the three different biological cycle stages of *T. cruzi*: amastigotes, epimastigotes and trypomastigotes.

The molecular mass of the recombinant enzyme, estimated by Western blot analysis, was approximately 65 kDa. This data is similar to that obtained for the *C. fasciculata* PARP (Podesta et al., 2004), mammalian type-2 or type-3 PARP (Ame et al., 1999; Augustin et al., 2003), the thermophilic archaeon *Sulfolobus sulfactaricus* PARP

(Faraone-Mennella et al., 1998) and other PARP proteins identified in *Arabidopsis thaliana* (Lepiniec et al., 1995). On the other hand, these proteins have significantly lower molecular weights than that of PARP-1 characterised in mammalian cells (Ame et al., 2004).

The structure analysis of *Tc*PARP showed the presence of a C-terminal catalytic domain, homologous to human PARP-1 to PARP-4, *T. brucei* PARP and PARPs of other organisms from divergent taxonomical orders. This indicates that the PARP catalytic domain is conserved during evolution. Furthermore, the secondary structure of this domain is similar in all the above-mentioned organisms, including the H–Y–E triad where the glutamic acid was shown to be critical for the catalytic activity of many PARPs (Otto et al., 2005).

The N-terminal Zn-finger DNA-binding domain that is involved in the DNA regulation of human PARP-1 activity is absent in *Tc*PARP. Nevertheless, we found an N-terminal region rich in basic amino acids which could be a potential DNA binding site responsible for modulation of its activity. This basic amino acid domain has been demonstrated to bind DNA in hPARP-1 and hPARP-2 (Buki et al., 1995; Ame et al., 1999; Oliver et al., 2004).

The yield of *Tc*PARP in *E. coli* was significantly improved when the construction was cloned into a pET 22 b(+) vector and expressed in BL21-Codon plus(DE3)-RIL. Accordingly, the predicted *Tc*PARP mRNA has a high proportion of two codons of arginine AGG (0.143 versus 0.003) and CGG (0.229 versus 0.011) compared with their frequency in *E. coli* (Novy et al., 2001). It is important to point out that some preparations contain a 37 kDa peptide as a contaminant, which is recognised by antibodies against the C-terminal region of PARP. Similar results have been described for native protein from *C. fasciculata*, *D. discoideum* and calf thymus, and these have been attributed to partial proteolysis of PARP (Zahradka and Ebisuzaki, 1984; Kofler et al., 1993; Podesta et al., 2004).

*Tc*PARP activity was dependent on the presence of DNA. Under standard assay conditions, sbDNA strongly activated the enzyme in a concentration-dependent manner. Histones partially purified from *T. cruzi* were substrates for *Tc*PARP. The role of the histone poly(ADP-ribosyl)ation in *T. cruzi* could be the prevention of a recombination of the exposed DNA strand breaks between chromosomes. It has been suggested that the increase in the negative charge in the PARylated zone of chromatin could avoid this recombination (D'Amours et al., 1999). To address this point, further in vivo studies should be performed.

*Tc*PARP activity exhibited no metal ion requirement, as has been described for *C. fasciculata* PARP (Podesta et al., 2004). Neither magnesium, manganese or calcium stimulated enzyme activity and chelating agents failed to inhibit its activity, ruling out possible contamination of low amounts of Mg^{2+}. It has been reported that bovine thymus PARP is magnesium-dependent (Yoshihara et al., 1978) and other authors have shown that Mg^{2+} is a positive
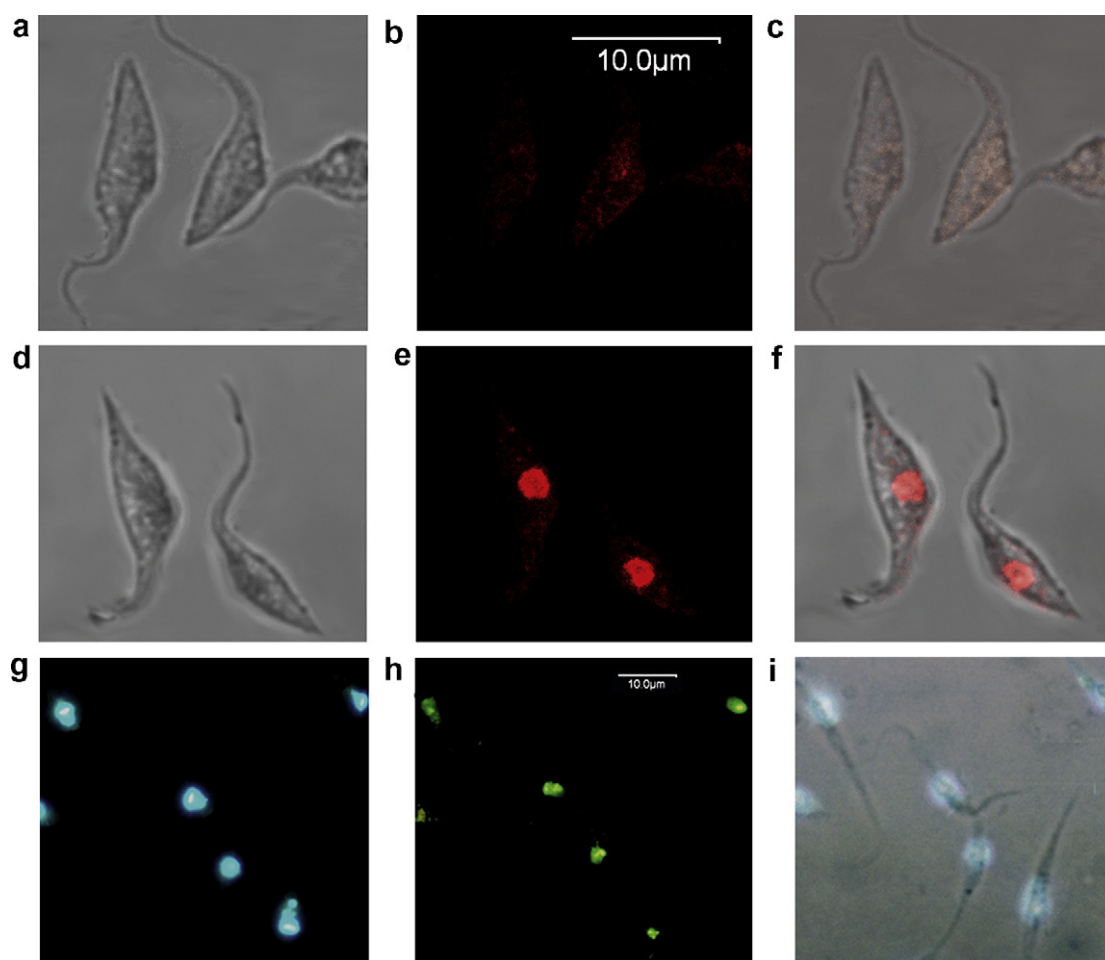
Fig. 7. PAR synthesis in *Trypanosoma cruzi* epimastigotes in response to DNA-damaging agents and PARP immunolocalisation. Cells were either untreated (a, b and c) or exposed to 3 mM H_2O_2 for 10 min (d, e and f). Poly(ADP-ribose) polymer (PAR) was detected with a polyclonal antibody against PAR followed by treatment with Cy3-conjugated anti-rabbit secondary antibody. Differential interference contrast (DIC) images (a and d), fluorescence images (b and e), merge of PAR labelling (red) and DIC images (c and f). For PARP immunocytochemical localisation epimastigotes were fixed, treated with anti-PARP antibody followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody and then Höechst stained. (g) Cells illuminated with UV light showing the fluorescent Höechst-stained DNA structures (330–385 nm). (h) The same cells, showing the FITC-linked antibody labelling (510–555 nm). (i) Merge of transmittant and fluorescent images showing the nuclear localisation of *Tc*PARP.

PARP activity effector (Petzold et al., 1981; Ushiro et al., 1987; Faraone Mennella et al., 2000). On the other hand, Mn^{2+}, Ni^+ and Zn^{2+} proved to be inhibitors of *Tc*PARP activity. The inhibition observed with the two latter cations may be due to the ability of these metals to bind to essential sulphydryl groups (Petzold et al., 1981).

In agreement with our previous findings using *C. fasciculata* PARP (Villamil et al., 2001), purified *Tc*PARP was inhibited by nicotinamide, 3-aminobenzamide, theophylline and thymidine. On the other hand menadione, a known mono-ADP-ribose transferase inhibitor, did not affect enzyme activity. The effect of ATP on purified PARP was also tested. This nucleotide has been implicated in the regulation of PARP activity, particularly at the level of the automodification reaction (Kun et al., 2004). The results showed that *Tc*PARP is partially inhibited by 10 mM ATP. Since previous evidence has indicated that at this concentration the nucleotide completely inhibits the auto-(ADP-ribosyl)ation reaction this may indicate that the

remaining activity observed in the presence of 10 mM ATP could be due to a *trans*-(ADP-ribosyl)ation reaction.

The evidence presented here indicates that *Tc*PARP is capable of carrying out an automodification reaction and that, in turn, it can decrease enzyme activity. The effect can be reversed by alkaline hydrolysis of the attached poly(ADP-ribosyl) chains. This provides an important mechanism for the regulation of PARP activity. A possible explanation for the inhibition is that the large increase in net negative charge eventually causes repulsion between sbDNA and automodified PARP (Ferro and Olivera, 1982; Diefenbach and Burkle, 2005).

A significant finding described here is that well-known DNA-damaging agents such as hydrogen peroxide or β-lapachone (Goijman and Stoppani, 1985; Ame et al., 1999) induce PAR synthesis in the parasite nucleus, thus suggesting that this enzyme is involved in the signalling of this phenomenon. Furthermore, we have found the catabolic counterpart of PARP, poly(ADP-ribose) glycohydrolase in

T. cruzi (GenBank Accession No. DQ679799) which could indicate the conservation of this pathway through evolution.

PARP enzymes from a wide range of organisms are implicated in many vital processes such as DNA repair and replication, transcription regulation, apoptosis and necrosis. It has been suggested that, by using specific inhibitors in trypanosomatids, ADP-ribosylation is involved in *T. cruzi* differentiation (Williams, 1983; Isola et al., 1987). However, the mechanism underlying this process remains obscure. Several recent reports have suggested that PARP may regulate chromatin structure through modification of associated proteins, mainly histones, HMG proteins and insulators. This in turn may affect the access of RNA polymerases to the template. *Trypanosoma cruzi* shows substantial variation from the paradigms of eukaryotic gene expression. These include, in most of the protein-coding genes, the absence of regulation at the level of transcription initiation, a scarce number of known transcriptional factors and a polycistronic transcription pattern. Since the mechanism that controls the expression of developmentally regulated genes in trypanosomatids remains unclear, chromatin remodelling by protein modification, including poly(ADP-ribosyl)ation, may be important for the regulation of gene expression in this organisms. This and other functional studies should be considered in order to fully understand the role of poly (ADP-ribosyl)ation in this parasite.

Acknowledgements

References

Ame, J.C., Rolli, V., Schreiber, V., Niedergang, C., Apiou, F., Decker, P., Muller, S., Hoger, T., Menissier-de Murcia, J., de Murcia, G., 1999. PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. J. Biol. Chem. 274, 17860–17868.

Ame, J.C., Spenlehauer, C., de Murcia, G., 2004. The PARP superfamily. Bioessays 26, 882–893.

Augustin, A., Spenlehauer, C., Dumond, H., Menissier-De Murcia, J., Piel, M., Schmit, A.C., Apiou, F., Vonesch, J.L., Kock, M., Bornens, M., De Murcia, G., 2003. PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. J. Cell. Sci. 116, 1551–1562.

Banasik, M., Komura, H., Shimoyama, M., Ueda, K., 1992. Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J. Biol. Chem. 267, 1569–1575.

Bonicalzi, M.E., Haince, J.F., Droit, A., Poirier, G.G., 2005. Regulation of poly(ADP-ribose) metabolism by poly(ADP-ribose) glycohydrolase: where and when? Cell. Mol. Life Sci. 62, 739–750.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brochu, G., Duchaine, C., Thibeault, L., Lagueux, J., Shah, G.M., Poirier, G.G., 1994. Mode of action of poly(ADP-ribose) glycohydrolase. Biochim. Biophys. Acta 1219, 342–350.

Buki, K.G., Bauer, P.I., Hakam, A., Kun, E., 1995. Identification of domains of poly(ADP-ribose) polymerase for protein binding and self-association. J. Biol. Chem. 270, 3370–3377.

Chang, P., Jacobson, M.K., Mitchison, T.J., 2004. Poly(ADP-ribose) is required for spindle assembly and structure. Nature 432, 645–649.

da Cunha, J.P., Nakayasu, E.S., Elias, M.C., Pimenta, D.C., Tellez-Inon, M.T., Rojas, F., Munoz, M.J., Almeida, I.C., Schenkman, S., 2005. *Trypanosoma cruzi* histone H1 is phosphorylated in a typical cyclin dependent kinase site accordingly to the cell cycle. Mol. Biochem. Parasitol. 140, 75–86.

D'Amours, D., Desnoyers, S., D'Silva, I., Poirier, G.G., 1999. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. 342 (Pt 2), 249–268.

Davidovic, L., Vodenicharov, M., Affar, E.B., Poirier, G.G., 2001. Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. Exp. Cell Res. 268, 7–13.

de Murcia, G., Menissier de Murcia, J., 1994. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem. Sci. 19, 172–176.

Diefenbach, J., Burkle, A., 2005. Introduction to poly(ADP-ribose) metabolism. Cell. Mol. Life Sci. 62, 721–730.

Faraone Mennella, M.R., Castellano, S., De Luca, P., Discenza, A., Gambacorta, A., Nicolaus, B., Farina, B., 2000. Comparison of the ADP-ribosylating thermozyme from *Sulfolobus solfataricus* and the mesophilic poly(ADP-ribose) polymerases. FEMS Microbiol. Lett. 192, 9–14.

Faraone-Mennella, M.R., Gambacorta, A., Nicolaus, B., Farina, B., 1998. Purification and biochemical characterization of a poly(ADP-ribose) polymerase-like enzyme from the thermophilic archaeon *Sulfolobus solfataricus*. Biochem. J. 335 (Pt 2), 441–447.

Faraone-Mennella, M.R., 2005. Chromatin architecture and functions: the role(s) of poly(ADP-RIBOSE) polymerase and poly(ADPribosyl)ation of nuclear proteins. Biochem. Cell Biol. 83, 396–404.

Ferro, A.M., Olivera, B.M., 1982. Poly(ADP-ribosylation) in vitro. Reaction parameters and enzyme mechanism. J Biol Chem 257, 7808–7813.

Gagne, J.P., Hendzel, M.J., Droit, A., Poirier, G.G., 2006. The expanding role of poly(ADP-ribose) metabolism: current challenges and new perspectives. Curr. Opin. Cell Biol. 18, 145–151.

Goijman, S.G., Stoppani, A.O., 1985. Effects of beta-lapachone, a peroxide-generating quinone, on macromolecule synthesis and degradation in *Trypanosoma cruzi*. Arch. Biochem. Biophys. 240, 273–280.

Hassa, P.O., Haenni, S.S., Buerki, C., Meier, N.I., Lane, W.S., Owen, H., Gersbach, M., Imhof, R., Hottiger, M.O., 2005. Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. J. Biol. Chem. 280, 40450–40464.

Hassa, P.O., Haenni, S.S., Elser, M., Hottiger, M.O., 2006. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? Microbiol. Mol. Biol. Rev. 70, 789–829.

Isola, E.L., Lammel, E.M., Gonzalez Cappa, S.M., 1987. *Trypanosoma cruzi*: differentiation to metacyclic trypomastigotes in the presence of ADP-ribosyltransferase inhibitors. Exp. Parasitol. 64, 424–429.

Kim, M.Y., Mauro, S., Gevry, N., Lis, J.T., Kraus, W.L., 2004. NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. Cell 119, 803–814.

Kim, M.Y., Zhang, T., Kraus, W.L., 2005. Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev. 19, 1951–1967.

Kofler, B., Wallraff, E., Herzog, H., Schneider, R., Auer, B., Schweiger, M., 1993. Purification and characterization of NAD+:ADP-ribosyltransferase (polymerizing) from *Dictyostelium discoideum*. Biochem. J. 293 (Pt 1), 275–281.

Kraus, W.L., Lis, J.T., 2003. PARP goes transcription. Cell 113, 677–683.

Kun, E., Kirsten, E., Mendeleyev, J., Ordahl, C.P., 2004. Regulation of the enzymatic catalysis of poly(ADP-ribose) polymerase by dsDNA, polyamines, Mg2+, Ca2+, histones H1 and H3, and ATP. Biochemistry 43, 210–216.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lepiniec, L., Babiychuk, E., Kushnir, S., Van Montagu, M., Inze, D., 1995. Characterization of an *Arabidopsis thaliana* cDNA homologue to animal poly(ADP-ribose) polymerase. FEBS Lett. 364, 103–108.

Lonskaya, I., Potaman, V.N., Shlyakhtenko, L.S., Oussatcheva, E.A., Lyubchenko, Y.L., Soldatenkov, V.A., 2005. Regulation of poly(ADP-ribose) polymerase-1 by DNA structure-specific binding. J. Biol. Chem. 280, 17076–17083.

Malanga, M., Althaus, F.R., 2005. The role of poly(ADP-ribose) in the DNA damage signaling network. Biochem. Cell. Biol. 83, 354–364.

Mendoza-Alvarez, H., Alvarez-Gonzalez, R., 1993. Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular. J. Biol. Chem. 268, 22575–22580.

Novy, R., Drott, D., Yaeger, K., Mierenhof, R., 2001. Overcoming the codon bias of *E. coli* for enhanced protein expression. inNovations 12, 1–3.

Oliver, A.W., Ame, J.C., Roe, S.M., Good, V., de Murcia, G., Pearl, L.H., 2004. Crystal structure of the catalytic fragment of murine poly(ADP-ribose) polymerase-2. Nucleic Acids Res. 32, 456–464.

Oliver, F.J., Menissier-de Murcia, J., de Murcia, G., 1999. Poly(ADP-ribose) polymerase in the cellular response to DNA damage, apoptosis, and disease. Am. J. Hum. Genet. 64, 1282–1288.

Otto, H., Reche, P.A., Bazan, F., Dittmar, K., Haag, F., Koch-Nolte, F., 2005. In silico characterization of the family of PARP-like poly(ADP-ribosyl)transferases (pARTs). BMC Genomics 6, 139.

Pereira, C.A., Alonso, G.D., Paveto, M.C., Iribarren, A., Cabanas, M.L., Torres, H.N., Flawia, M.M., 2000. *Trypanosoma cruzi* arginine kinase characterization and cloning. A novel energetic pathway in protozoan parasites. J. Biol. Chem. 275, 1495–1501.

Petermann, E., Keil, C., Oei, S.L., 2005. Importance of poly(ADP-ribose) polymerases in the regulation of DNA-dependent processes. Cell. Mol. Life Sci. 62, 731–738.

Petzold, S.J., Booth, B.A., Leimbach, G.A., Berger, N.A., 1981. Purification and properties of poly(ADP-ribose) polymerase from lamb thymus. Biochemistry 20, 7075–7081.

Podesta, D., Garcia-Herreros, M.I., Cannata, J.J., Stoppani, A.O., Fernandez Villamil, S.H., 2004. Purification and properties of poly(ADP-ribose)polymerase from *Crithidia fasciculata*. Automodification and poly(ADP-ribosyl)ation of DNA topoisomerase I. Mol. Biochem. Parasitol. 135, 211–219.

Poirot, O., Suhre, K., Abergel, C., O'Toole, E., Notredame, C., 2004. 3DCoffee@igs: a web server for combining sequences and structures into a multiple sequence alignment. Nucleic Acids Res. 32, W37–W40.

Potaman, V.N., Shlyakhtenko, L.S., Oussatcheva, E.A., Lyubchenko, Y.L., Soldatenkov, V.A., 2005. Specific binding of poly(ADP-ribose) polymerase-1 to cruciform hairpins. J. Mol. Biol. 348, 609–615.

Rankin, P.W., Jacobson, E.L., Benjamin, R.C., Moss, J., Jacobson, M.K., 1989. Quantitative studies of inhibitors of ADP-ribosylation in vitro and in vivo. J. Biol. Chem. 264, 4312–4317.

Schreiber, V., Dantzer, F., Ame, J.C., de Murcia, G., 2006. Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol. 7, 517–528.

Shah, G.M., Poirier, D., Duchaine, C., Brochu, G., Desnoyers, S., Lagueux, J., Verreault, A., Hoflack, J.C., Kirkland, J.B., Poirier, G.G., 1995. Methods for biochemical study of poly(ADP-ribose) metabolism in vitro and in vivo. Anal. Biochem. 227, 1–13.

Tulin, A., Stewart, D., Spradling, A.C., 2002. The *Drosophila* heterochromatic gene encoding poly(ADP-ribose) polymerase (PARP) is required to modulate chromatin structure during development. Genes Dev. 16, 2108–2119.

Ueda, K., Hayaishi, O., 1985. ADP-ribosylation. Annu. Rev. Biochem. 54, 73–100.

Ushiro, H., Yokoyama, Y., Shizuta, Y., 1987. Purification and characterization of poly (ADP-ribose) synthetase from human placenta. J. Biol. Chem. 262, 2352–2357.

Villamil, S.F., Podesta, D., Molina Portela, M.D., Stoppani, A., 2001. Characterization of poly(ADP-ribose)polymerase from *Crithidia fasciculata*: enzyme inhibition by beta-lapachone. Mol. Biochem. Parasitol. 115, 249–256.

Williams, G.T., 1983. *Trypanosoma cruzi*: inhibition of intracellular and extracellular differentiation by ADP-ribosyl transferase antagonists. Exp. Parasitol. 56, 409–415.

Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H., Kamiya, T., 1978. Bovine thymus poly(adenosine diphosphate ribose) polymerase. J. Biol. Chem. 253, 6459–6466.

Zahradka, P., Ebisuzaki, K., 1984. Poly(ADP-ribose) polymerase is a zinc metalloenzyme. Eur. J. Biochem. 142, 503–509.

Ziegler, M., Oei, S.L., 2001. A cellular survival switch: poly(ADP-ribosyl)ation stimulates DNA repair and silences transcription. Bioessays 23, 543–548.