pADPRT-2: a novel mammalian polymerizing(ADP-ribosyl)transferase gene related to truncated pADPRT homologues in plants and *Caenorhabditis elegans*[1]

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Abstract Until recently, poly(ADP-ribosyl)ation was supposed to be confined only to polymerizing(ADP-ribosyl)transferase/(ADP-ribose)polymerase (E.C. 2.4.2.30). Here, we present novel polymerizing(ADP-ribosyl)transferase homologues from mouse and man that lack all of the N-terminal DNA binding and BRCA1 C-terminus domains and will be designated polymerizing(ADP-ribosyl)transferase-2 as distinguished from the classical polymerizing(ADP-ribosyl)transferase (polymerizing(ADP-ribosyl)transferase-1). The murine polymerizing(ADP-ribosyl)-transferase-2 gene shares three identical intron positions with its *Caenorhabditis elegans* (EMBL nucleotide sequence database Z47075) and one with the *Arabidopsis thaliana* homologue ('APP', GenBank database AF069298). Expression of the murine polymerizing(ADP-ribosyl)transferase-2 gene was elevated in spleen, thymus and testis and the corresponding poly(ADP-ribosyl)ation activity might account for most of the residual poly(ADP-ribosyl)ation observed in polymerizing(ADP-ribosyl)transferase-1$^{-/-}$ mice.

Key words: Poly(ADP-ribosyl)ation; Polymerizing(ADP-ribosyl)transferase negative mouse; Genomic organization; Activity gel analysis; Sequence comparison

1. Introduction

Catalytic properties and the domain organization of the nuclear polymerizing(ADP-ribosyl)transferase (pADPRT)-1 enzyme have been studied in detail in a number of diverse organisms [1–4]. The N-terminal zinc finger bearing domain of pADPRT-1 activates the C-terminal catalytic domain upon binding to free DNA ends and consequently, high amounts of the substrate NAD^+ are consumed which is cleaved to yield ADP-ribose that is initially attached to glutamic acid residues of pADPRT-1 (automodification). More ADP-ribose units are used to extend this modification and, finally, to form a long, branched and negatively-charged polymer which repells DNA and attracts nuclear proteins [5–7].

The biological role of pADPRT-1 has been elucidated by creating pADPRT$^{-/-}$ mice which showed increased levels of sisterchromatid exchanges [8], an enhanced sensitivity to X-ray treatment at high doses [9] and a slightly reduced growth rate of cells [10], suggesting a role of pADPRT-1 in maintenance of the genome. pADPRT-1 deficiency also protected from a variety of oxidant-induced cellular injury, e.g. in inflammation, shock, stroke or diabetes mellitus [11]. In both animals and plants, oxidant-induced apoptosis could also be reduced by inhibition of the poly(ADP-ribosyl)ation (PAR) activity [12]. Nevertheless, many cellular functions which were impaired after treatment with PAR inhibitors [13] still appeared normal in pADPRT-1$^{-/-}$ mice.

Only very recently, a substantial level of the remaining PAR activity was demonstrated in cells from pADPRT-1 negative mice [14] and PAR enzymes in addition to pADPRT-1 were detected in plants as well as in human: a 72 kDa enzyme from *Arabidopsis thaliana* ('APP', SWISS-PROT database (sp) Q11207) which has a homologue in maize ('NAP', EMBL nucleotide sequence database (embl) AJ222588) was shown to be a functional pADPRT which was activated by DNA although it lacks the N-terminal domain including the DNA binding domain of pADPRT-1 [4,15]. Another functionally active human pADPRT homologue ('tankyrase') shares only the most C-terminal 139 amino acids (aa) of the catalytic domain with pADPRT-1 and was found to be associated with telomeric repeat binding factor-1 (TRF1) [16].

In this publication, we present the cloning and characterization of a novel mammalian gene (pADPRT-2) which is homologous to the C-terminal catalytic domain of pADPRT-1 and might be responsible for the majority of the residual PAR activity in pADPRT-1 knock out mice.

2. Material and methods

Immortalized embryonic fibroblasts from wild-type and pADPRT-1$^{-/-}$ mice [10] were grown in DMEM medium, supplemented with 2 mM glutamine, 10% FCS, 50 U/ml ampicillin and 35 μg streptomycin (all ingredients from PAA Laboratories, Linz, Austria). Standard methods were followed for isolation of DNA, extraction of mRNA and Northern blots. Digoxigenin-labelled probe preparation, hybridization and detection were performed as recommended (Roche Diagnostics, Vienna, Austria)

2.1. Isolation of pADPRT-2 cDNAs and the murine pADPRT-2 gene

First strand cDNA served as a template for reverse-transcribed (RT)-PCR and 3′-rapid amplification of cDNA ends (RACE) and was prepared using 3 μg total RNA, 15 ng dT(17)-Not primer (5′-CACTATAGGGCGGCCGCTTTTTTTTTTTTTTTT) and MuLV reverse transcriptase (Promega, Madison, Wisconsin, USA) at 42°C in a 20 μl reaction volume according to the instructions of the man-

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[1] The human pADPRT-2 cDNA sequence reported in this paper has been deposited in the EMBL database with accession number AJ236876 and the mouse pADPRT-2 cDNA sequence in the GenBank database with accession number AF072521.

Abbreviations: PAR, poly(ADP-ribosyl)ation; pADPRT, polymerizing(ADP-ribosyl)transferase; BRCT, BRCA1 C-terminus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; gb, GenBank database; sp, SWISS-PROT database; embl, EMBL nucleotide sequence database

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ufacturer. The reaction was diluted 30 times with TE (10 mM Tris-HCl pH = 8.0, 1 mM EDTA) and 3 μl was added directly to a 50 μl PCR reaction using the 'high fidelity' system from Roche Diagnostics (Vienna, Austria). 3′-RACE was carried out according to [17] using a reverse primer with the same sequence as above, yet with only a single T at the 3′-end and a forward primer matching both the human and mouse pADPRT-2 gene (m/h4F-5′-CTATACTATGACCTTGCTGGAT). For RT-PCR, the following mouse and human pADPRT-2 gene-specific primers were used additionally: m1F-5′-GCAACAGAAGACGACTCTCCTCCT, m/h2F-5′-CAATCTCCAGTTCAACAACAACAAG, m3F-5′-TCTCAGCTGGATCTTCGAGTC, m5F-5′-GTAGCTCTAGGTCAGTGTAATGAAC, m/h1R-5′-TATGATCAACATTCACCACAGCT, m/h2R-5′-TTGCCCATCCCCTTGGTGCTATG, m/h3R-5′-CTCTTGGAGGACATGTCAG, m4R-5′-CTGGATCCATGCCAGAGCAG, m5R-5′-GATTTGCGCCACTGTCAGCTTTC, m/h6R-5′-GTATTGGCGGCATAGTCCATCTGT, m7R-5′-CTTAAGCTTTGTTGAGGTCACCAGAAC. Primers and template DNA were added at 80°C. After denaturing for 2 min and 30 s at 93°C, 28 cycles with a denaturing time of 15 s at 95°C, an appropriate annealing temperature (between 55°C and 60°C) for 50 s and an extension period of 5 min at 66°C, they were run on a 'Trio-thermoblock' (Biometra, Göttingen, Germany). The same primers and conditions were used for amplification of genomic mouse DNA.

5′-RACE involved second strand synthesis using 20 μl of heat-inactivated first strand synthesis (10 min 60°C), 40 U *Escherichia coli* DNA polymerase I (Promega, Madison, WI, USA), 1.5 U RNase H (Promega, Madison, WI, USA), 15 U *E. coli* DNA ligase (Gibco BRL, Gaitherburg, MD, USA) in a 120 μl final volume according to the protocol of Gibco BRL. After phenol extraction, double-stranded cDNA was spun through a S-300 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and a double-stranded linker with a non-matching middle region was ligated onto 5′-phosphorylated cDNA ends by T4-DNA ligase (GeneCraft, Münster, Germany) similar to the 'vectorette' method [18] that will be described in detail elsewhere. 5′-RACE was performed with a linker primer and a gene-specific reverse primer as described above. The correct PCR products were identified by hybridization and if necessary purified from an agarose gel (QIAGEN GmbH, Hilden, Germany) and re-amplified. PCR products were either sequenced directly with gene-specific primers or after cloning into a vector with 3′-T overhangs (Topo-TA-cloning system, Invitrogen, Carlsbad, CA, USA) and 'easy'plasmid preparation [19] with 'Thermosequenase' (Amersham Pharmacia Biotech, Uppsala, Sweden) using biotin-labelled dideoxy nucleotides (GATC GmbH, Konstanz, Germany). Subsequent to blotting onto a nylon membrane using a 'two-step' device (Hoefer Scientific Instruments, San Francisco, CA, USA), the bands were detected with conjugated streptavidin-alkaline phosphatase according to GATC. Three independent clones were sequenced on both strands to eliminate amplification errors.

2.2. PAR activity gel

Total protein from cell cultures and various mouse tissues was prepared by dissolving small pieces of tissue with a syringe fitted with a 22 gauge needle directly in sample buffer and aliquots were loaded onto a 6% polyacrylamide gel. After separation, proteins were re-folded in situ, incubated with [^{32}P]NAD$^+$ (800 Ci/mmol (NEN, Boston, MA, USA) and the bands were detected by autoradiography on Kodak Biomax MR (Eastman Kodak, Rochester, NY, USA) as described [3].

2.3. Computational analysis

BLAST searches [20] of non-redundant, EST and STS databases were performed at NCBI (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform = 0). Alignments were produced with CLUSTAL-W on the PBIL server (http://pbil.ibcp.fr/NPSA/npsa_clustalw.html) at IBCP (Lyon, France) with the gonnet weight matrix, a gap opening penalty of three and a gap extension penalty of 0.005.

3. Results

3.1. Human and mouse pADPRT-2 cDNAs: similarity in sequence and domain organization of pADPRT-2 to other members of the pADPRT multigene family

Using the sequence of the catalytic domain of a pADPRT homologue from *Dictyostelium* [21] for searching the EST databases, we discovered highly similar sequences of both human and mouse origin which we extended by RACE methods to obtain complete cDNAs. The translated pADPRT-2 sequences were homologous to the C-terminal catalytic region of pADPRT-1 enzymes (from aa 485 of mammalian pADPRT-1, Fig. 1) and contained all structural features required for PAR activity [22] but lacked all of the N-terminal DNA binding and BRCA1 C-terminus (BRCT) domains including the bipartite nuclear location signal. The mammalian pADPRT-2 sequences exhibited a much higher homology to each other (83% identity) than to their respective pADPRT-1 counterparts (mouse pADPRT-1/pADPRT-2 42%, human pADPRT-1/pADPRT-2 40%).

The highest homology to other proteins was found to pADPRT homologues of 72 000 *Mr* in plants: 46% with *A. thaliana* 'APP' (sp Q11207) and maize 'NAP' (embl AJ222588), respectively, which are also N-terminally truncated compared to pADPRT-1 enzymes. Nevertheless, they possess an additional domain at their N-termini compared to the protein sequences derived from the mammalian pADPRT-2 cDNAs. Although only distantly conserved in the protein sequence, a *Caenorhabditis elegans* pADPRT homologue (sp Q09525), predicted from the genomic sequence database, has a similar length and the same domain organization as the mammalian pADPRT-2 counterparts.

3.2. The mouse pADPRT-2 gene: conserved introns with C. elegans and A. thaliana pADPRT homologs

We have constructed the complete mouse pADPRT-2 locus (13 kb) from overlapping PCR fragments and determined all of the 15 intron positions (Fig. 2) which were all different to those of the murine pADPRT-1 gene (H. Berghammer, unpublished) which has the same exon/intron organization as the human pADPRT-1 gene [23]. In contrast, three of the intron positions of the mouse pADPRT-2 gene match exactly the corresponding intron positions of the *C. elegans* pADPRT homologue (embl Z47075, Fig. 3) which therefore can be considered as the *C. elegans* pADPRT-2 gene despite some sequence divergence. One of these conserved introns is also found in the *A. thaliana* 'APP' gene (GenBank database (gb) AF069298).

The human pADPRT-2 gene is located on chromosome 14 according to records in the STS database (G31064, G36285) and seems to be organized in much the same way as the mouse gene, since some human EST sequences were found to contain intron sequences in the corresponding positions (e.g. AA328303, H22705, N54120, AA595596).

3.3. Multiple transcription initiation and expression levels in wild-type and pADPRT-1$^{-/-}$ mice and in various tissues

We observed 5′-RACE PCR products of two different lengths in human (corresponding to cDNAs of 1797 bp and 1536 bp) and three in the mouse (corresponding to cDNAs of 1766 bp, 1587 bp and 1521 pb). Sequence analysis provided evidence for multiple transcription initiation sites which are indicated in the map of the mouse gene (Fig. 2). All of the observed smaller varieties of the mouse pADPRT-2 cDNAs start close to upstream introns which are likely to harbor promoter regions that will be described elsewhere. Corresponding sizes of mRNAs (1.8 kb–2.0 kb) were detected on Northern blots of mouse embryonic fibroblasts and adult tis-

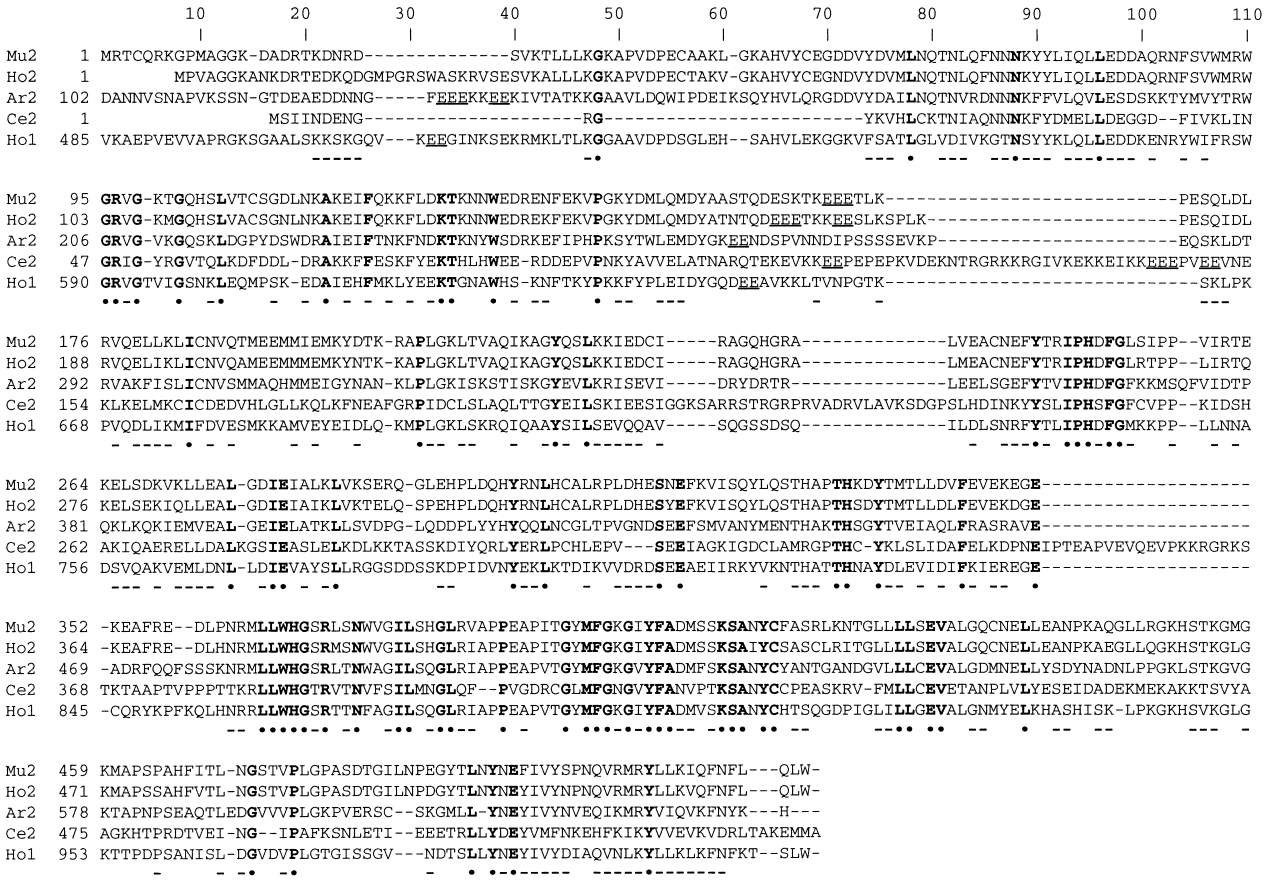

Fig. 1. Alignment of pADPRT protein sequences: mouse pADPRT-2 (Mu2, gb AF072521), human pADPRT-2 (Ho2, embl AJ236876), *A. thaliana* 'APP' (Ar2, sp Q11207), *C. elegans* (Ce2, sp Q09525), human pADPRT-1 (Ho1, sp P09874). Homologous regions are indicated by -, identical amino acid residues are in bold and indicated by ●, putative polyADP-ribose acceptor sites are underlined.

sues, although the different types could hardly be resolved on standard formaldehyde gels (Fig. 3A). Human pADPRT-2 mRNAs of virtually the same sizes were also detected in SV40-transformed human fibroblasts and adenovirus-transformed human fibroblasts (data not shown) using mouse cDNA as a probe (nucleotide homology is 80% between the human and mouse sequence). We have not found any longer than the above cDNAs in mouse embryonic fibroblasts, although a pADPRT-2 sequence from mouse that extended slightly further upstream was recently deposited into the database (embl AJ007780). A faint band appearing in the Northern blot (Fig. 3A) at about 2.5 kb may therefore rather be due to unspecific cross-hybridization.

The translation products encoded in the largest cDNAs from mouse and human were calculated to an approximate *Mr* of 60 000 (mouse: 522 aa, human: 534 aa), whereas all smaller cDNA variants correspond to a protein of about 53 000 *Mr* (mouse: 462 aa, human: 466 aa). In all cases, additional ATG codons which are not in frame are located upstream of the continuous reading frame which are thought to moderate the translation level [24].

The low frequency of pADPRT-2 sequences in the EST database except for early embryonic stages gave a first indication of expression levels. In agreement with that, we have observed only low transcription levels in most tissues from adult mice (Fig. 4A). Moderate expression levels were found in thymus, spleen, testis and cultured fibroblasts. Generally, we have not noticed an alteration of pADPRT-2 mRNA levels in cells (Fig. 4A) or tissues from pADPRT-1$^{-/-}$ mice compared to wild-type.

The PAR activity remaining in pADPRT-1$^{-/-}$ mice [14] was investigated in more detail by activity gel analysis. When total protein from embryonic fibroblasts was separated on a SDS-polyacrylamide gel and, after refolding of the proteins, was incubated with ^{32}P-labelled NAD^{+}, a second PAR activity in addition to the 116 000 *Mr* pADPRT-1 protein was

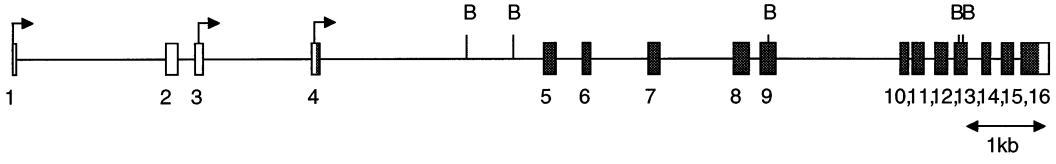

Fig. 2. Organization of the mouse pADPRT-2 gene. Boxes represent exons, transcriptional start sites are indicated by arrows. *Bam*HI cleavage sites are indicated by 'B'. The putative open reading frame is indicated by hatching.

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                204.. A   P   L       G   K   L  ...209
Mu2 exon 8     698..GCC CCG CTT G | GA AAG CTG ...715 exon 9
Ce2 exon 1    5052..AGA CCA ATT G | AT TGC CTC ..5113 exon 2
                183.. R   P   I       D   C   L  ...188

                426... L   S   E     V   A   L ...431
Mu2 exon14     1364...CTG TCA GAG | GTA GCT CTA..1381 exon15
Ce2 exon 3     5964...CTT TGC GAG | GTT GAA ACT..6030 exon 4
                442... L   C   E     V   A   L ...447
Ar2 exon16    60342...CTC TGC GAG | GTT GCT TTG.60446 exon17
                545... L   C   E     V   A   L ...550

                467. F   I   T   L       N   G  ...472
Mu2 exon15    1487.TTC ATC ACC CT | G AAT GGG ..1504  exon16
Ce2 exon 4    6136.ACT GTT GAA AT | C AAT GGA ..6200  exon 5
                483. T   V   E   I       N   G  ...488
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Fig. 3. Conserved introns in the pADPRT-2 sequence of mouse (Mu2, gb AF072521, numbers refer to the position in the cDNA sequence) and genomic sequences of the corresponding genes in *C. elegans* (Ce2, Z47075) and *A. thaliana* (Ar2, gb AF069298).

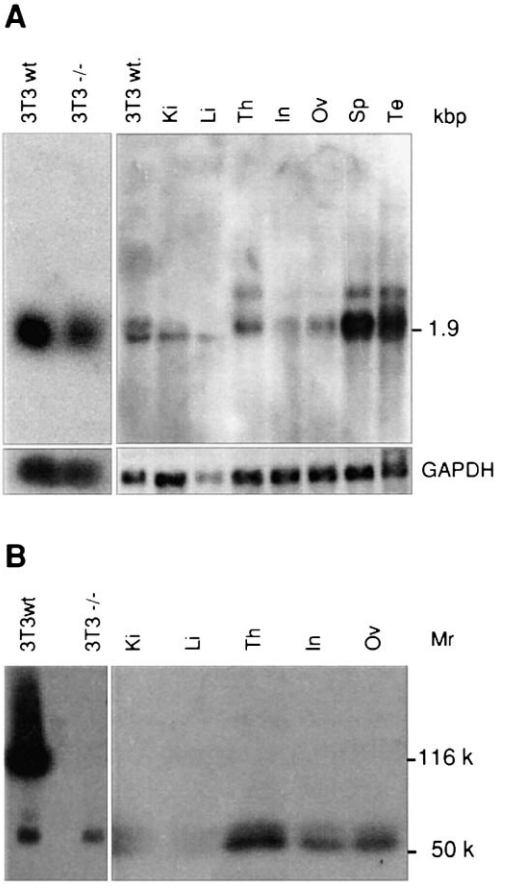

Fig. 4. Northern blots and activity gel analysis of mammalian pADPRT-2. (A) 3 μg of mRNA from mouse 3T3 cells and tissue (Ki: kidney, Li: liver, Th: thymus, In: intestine, Ov: ovary, Sp: spleen, Te: testis) were hybridized with complete murine pADPRT-2 cDNA. As a loading control, the blot was re-hybridized with murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. (B) Aliquots corresponding to 3 mg of cell mass from mouse 3T3 fibroblasts and the indicated tissues from pADPRT negative mice were run on 6% SDS polyacrylamide gels and the in situ PAR activity was determined by incorporation of [^{32}P]NAD^{+} and autoradiography.

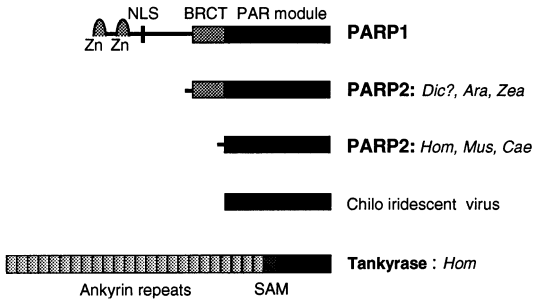

Fig. 5. Schematic comparison of the protein structure of the members of the pADPRT multigene family.

detected at approximately 53 000 *Mr* (Fig. 4B) which was also present in pADPRT-1 negative cells. A faint band at approximately 59 000 *Mr* was also visible in various tissues using a higher resolving low percentage activity gel. The activity was low in liver and brain, moderate in the lung, small intestine and ovary and highest in thymus which coincides well with the transcription levels observed above.

4. Discussion

Besides the mammalian pADPRT-2 genes presented here, pADPRT genes lacking the N-terminal DNA binding domain including the two zinc fingers (Fig. 5) have also been described recently in plants [4,15]. In addition to these putative plant pADPRT-2 genes, truncated pADPRT-1 homologous sequences are also found in the genomes of *C. elegans* (embl Z47075) and the *Chilo iridescent* virus (gb AF003534). In particular the *C. elegans* gene seems to be a true cognate of the mammalian pADPRT-2 genes, since it has the same length and domain organization and shares three of its four intron positions with the murine pADPRT-2 gene. One of these introns is also conserved in the highly homologous *A. thaliana* 'APP' gene (gb AF069298). In contrast, the pADPRT homologous sequence encoded in the genome of the *C. iridescent* virus (gb AF003534) contains two reading frames (035L and 034L) which are separated by a short sequence, most likely representing an intron which is in a position clearly different to those in the pADPRT-2 genes and thus probably represents a separate line of evolution.

The 72 kDa pADPRT homologues from plants (gb AF069298, embl AJ222588) show an additional domain at their N-termini containing additional putative DNA binding helix-turn-helix motifs as well as a putative nuclear location signal which are absent from the mammalian pADPRT-2 genes. These motifs could define another pADPRT family distinct from pADPRT-2 which is either not present in animals or still awaits its discovery. However, it should be noted that these small plant pADPRTs share the highest sequence conservation with the mammalian pADPRT-2 enzymes and thus are likely to represent pADPRT-2 genes.

Plant pADPRT homologues were recently shown to exhibit a DNA-dependent PAR activity despite the lack of the DNA binding domain [4]. This holds also true for tankyrase, a human pADPRT homologue containing only the C-terminal half of the catalytic domain fused to ankyrin repeats [16]. The translation products of the pADPRT-2 cDNAs from both mouse and human are entirely homologous to the

C-terminal catalytic region of pADPRT-1 enzymes with all structural features conserved which are required for catalytic activity [22]. Although they are encoded by separate genes with an exon/intron structure completely different to the pADPRT-1 genes, they should be expected to function as N-terminally truncated forms of pADPRT-1 like the plant pADPRT-2s. Indeed, an activity gel analysis revealed a PAR activity of the size predicted for the pADPRT-2 cDNA translation products which is likely to result from automodification analogous to pADPRT-1 and tankyrase activity [16]. Clusters of consecutive glutamic acid residues present in the mammalian as well as the *C. elegans* and plant pADPRT-2 sequences at conserved positions might serve as potential acceptor sites for the ADPribose polymer. This PAR activity is also present in cells from pADPRT-1 negative mice where it accounts for nearly all of the residual PAR, detected by activity gel analysis. However, pADPRT-2 is not induced in pADPRT-1 negative cells and hence is not used to compensate for pADPRT-1 deficiency indicating that pADPRT-2 might have a different biological role in the cell than pADPRT-1. In contrast, pADPRT-2 mRNA levels and the corresponding activities in various tissues resemble the expression pattern of pADPRT-1 well[10], pointing to a similar role of pADPRT-1 and pADPRT-2. Isolation and analysis of the genomic locus provide the basis for inactivating the pADPRT-2 gene in the mouse by homologous recombination which will be an important step in defining the biological role of pADPRT-2.

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