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Enzyme characteristics of recombinant poly(ADP-ribose) polymerases-1 of rat and human origin mirror the correlation between cellular poly(ADP-ribosyl)ation capacity and species-specific life span

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Poly(ADP-ribosyl)ation is a posttranslational modification, which is involved in many cellular functions, including DNA repair and maintenance of genomic stability, and has also been implicated in cellular and organismal ageing. We have previously reported that maximum poly(ADP-ribosyl)ation capacity in mononuclear blood cells is correlated with mammalian life span. Here we show that the difference between a long-lived and a short-lived species tested (i.e. man and rat) is directly mirrored by the enzymatic parameters of recombinant poly(ADP-ribose) polymerase-1 (PARP-1), i.e. substrate affinity and reaction velocity. In addition, we have characterized two human PARP-1 alleles and assign their activity difference to their respective initial velocity and not substrate affinity.

Keywords:
Poly(ADP-ribose)polymerase-1
Enzyme activity
Life span
Allele

Evolutionary theory predicts that the ageing process results from accumulation of somatic damage, owing to limited investments in maintenance and repair (Kirkwood, 2008). Genes controlling the activities of protective systems, such as DNA repair and antioxidant defence, should thus regulate longevity. Recent experimental work in mouse models with various deficiencies in DNA repair functions has yielded strong evidence in support of this view (Hoeijmakers, 2009).

We have focused on one biochemical reaction contributing to the DNA base-excision repair pathway, i.e. poly(ADP-ribosyl)ation, a posttranslational modification of proteins. Poly(ADP-ribosyl)ation is catalyzed by the family of poly(ADP-ribose) polymerases (PARPs), using NAD^+ as substrate. The reaction can be dissected in three distinct steps: initiation (transfer of the first ADP-ribosyl residue), elongation (attachment of further ADP-ribosyl residues), and branching. PARPs have been implicated in many aspects of cellular mechanisms like DNA repair (Bürkle, 2006), replication (Simbulan-Rosenthal et al., 1996, 1998), mitosis (Canudas et al., 2007; Chang et al., 2005), telomere maintenance (Beneke et al., 2008; Smith et al., 1998), chromatin organization (Gottschalk et al., 2009; Poirier et al., 1982; Timinszky et al., 2009), and transcription (Hassa and Hottiger, 1999; Meisterernst et al., 1997; Oei et al., 1997). Likewise, regulation of cell death pathways is mediated by PARPs and their product, poly(ADP-ribose) (PAR). Physiological levels of PAR are highly increased by activation of mainly PARP-1 by the presence of DNA strand breaks. Over-activation of PARP-1 can lead to irreversible depletion of energy-rich metabolites (NAD^+/ATP) and subsequently, cell death can occur due to energy failure (necrosis) (Berger et al., 1983) or release of AIF from mitochondria, initiating caspase-independent apoptosis (Koh et al., 2005). PARP-1 and its activity is also involved in cellular ageing, either in complex with interaction partners (von Kobbe et al., 2004) or by its ability to regulate cell death (Beneke and Bürkle, 2007). Poly(ADP-ribose) formation capacity decreases with age in humans and rats (Grube and Bürkle, 1992) but interestingly, centenarian-derived cells display higher poly(ADP-ribosyl)ation capacity than controls (Muiras et al., 1998). Furthermore, fully stimulated PARP activity in permeabilized mononuclear blood cells (PBMC) of mammalian species is directly correlated with species-specific life span (Grube and Bürkle, 1992), with a 5-fold difference between the longest lived and shortest lived species tested, i.e., rat and man. This correlation is in line with the role of DNA repair factors playing an important role in longevity assurance (Kirkwood, 2008; Hoeijmakers, 2009). The correlation, however, was not due to differences in PARP-1 protein levels. Instead we could show that the enzyme of a long-lived species (man) was intrinsically more active that that of a short-lived species (rat) (Beneke et al., 2000), which could in part explain the above mentioned differences in cellular poly(ADP-ribosyl)ation capacity. Recently, allelic substitution of the more common valine

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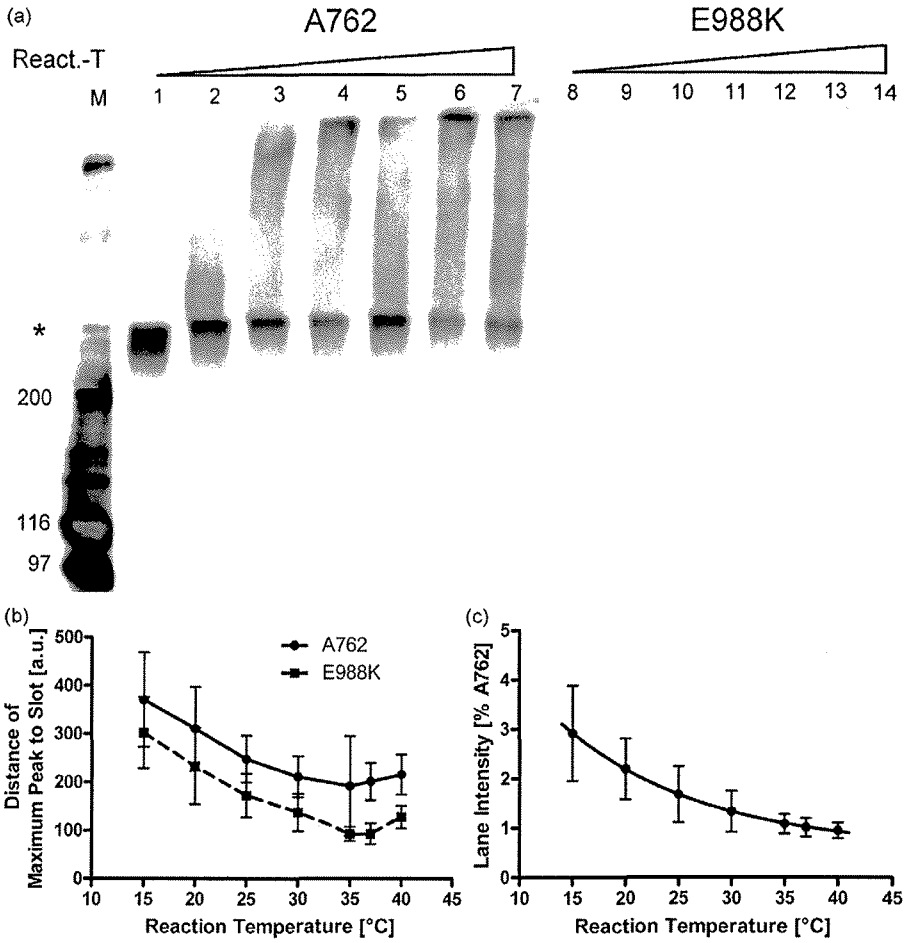

Fig. 1. Temperature dependency of PAR formation by PARP-1. One hundred ng of human PARP-1 A762 variant or E988K mutant proteins, respectively, was subjected to PARP activity assay at 15/20/25/30/35/37/40 °C, as described. Twelve % of A762 and 48% of E988K reaction volume was separated by SDS-PAGE and analyzed by western blotting against PAR. Both proteins show temperature-dependent increase in polymer production up to 30 °C, with a slight drop at 40 °C. (a) Western blot representative of three different experiments. M: Fermentas broad range marker with sizes as indicated; React.-T = reaction temperature; 1–7: reaction of A762 variant at increasing temperature from 15 to 40 °C; 8–14: reaction of E988K mutant at increasing temperature from 15 to 40 °C; *border between stacking and separating gel. Note that the signals smear down from the wells to the start of the separating gel. (b) Evaluation by ImageJ for maximum peak distance from start in arbitrary units in relation to temperature. As the two different proteins had to be analyzed separately due to the highly divergent signal intensities, only the shape of the graphs was compared. There is no significant difference between A762 and E988K in temperature-dependent PAR production. (c) Evaluation of the amount of PAR produced by the two enzymes. For each individual temperature we calculated the amount of PAR formed by E988K relative to A762. Probably due to a lesser degree of polymer complexity at low temperatures, signals are more focused at one spot and therefore PAR is detectable even in small amounts.

variant at amino-acid position 762 of human PARP-1 population with alanine was reported to result in diminished enzymatic activity (Wang et al., 2007) and increased predisposition to certain cancers (Li et al., 2007; Lockett et al., 2004). Other groups, however, were unable to detect any influence on PARP-1 activity by this polymorphism (Zaremba et al., 2009). In order to clarify this issue and to re-assess the question of life span related PARP-1 function, we set out to determine activity parameters of recombinant PARP-1 of human and rodent origin.

Using the well-established baculoviral system for protein overexpression and our previously published purification protocol (Beneke et al., 2000), we purified recombinant PARP-1 to near-homogeneity. All preparations tested negative for automodification by western blotting with 10H antibody. By utilizing a modified immuno-slot-blot technique, we established a sensitive method for detection and quantification of PAR. We routinely used 30 s reaction time at 30 °C with 2.5 nM PARP-1 in 30 μl of reaction buffer (200 μM NAD^+, 25 mg/ml GGAATTCC activator oligonucleotide, 1 mM DTT, 100 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$) if not indicated otherwise. These conditions had proven most suitable for analyses with wild-type (wt) and EGFP-fused PARP-1 (data not shown). Briefly, reaction mix was pre-incubated for 2 min at 30 °C and assay was started by addition of 1/10 volume of PARP-1 or NAD^+, respectively. Reaction was stopped by adding an equal volume of 20% trichloroacetic acid (TCA). Slot-blotting in a 72-well manifold was performed as follows: 3 sheets of Whatman paper and PBS-soaked Hybond-N+ membrane (GE Healthcare) were assembled according to manufacturer's instructions. Each solution was applied to slots in a 50 μl volume. Before application of samples, PBS was aspirated through the slots. Five % of each reaction, diluted with 10% TCA, was transferred to the membrane, respectively. After transfer, all wells were washed once with 10% TCA and once with 70% ethanol. Thereafter, membranes were dried for 1 h at 90 °C and blocked overnight at 4 °C in TNT (0.05% Tween 20, 150 mM NaCl, 10 mM Tris–HCl pH 8.0)/5% dry milk. One-hour incubation with the first antibody (anti-PAR mouse monoclonal 10H) in TNT/5% dry milk was followed by 3× 10 min washes in TNT, 1-h incubation with the secondary antibody (goat-anti-mouse HRP, dako cytomation) in TNT/5% dry milk, and three TNT

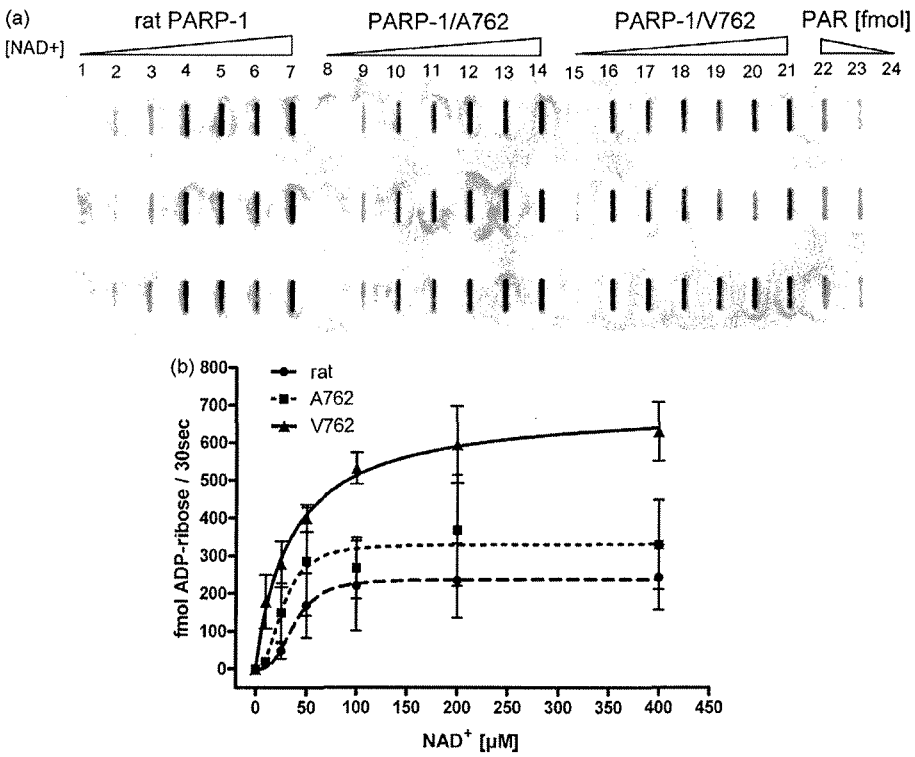

Fig. 2. Poly(ADP-ribose) production with increasing NAD^+ concentrations. Different PARP-1 enzymes (rat, human A762 and V762 alleles) were subjected to PARP-1 activity assay in three replicates with increasing NAD^+ concentrations as described in the text. Five % of reaction material was slot-blotted as technical triplicates on membranes, along with three different amounts of purified PAR and detected by immunoblotting and subsequent ECL reaction. Signals were detected by a Fuji-LAS1000 chemoluminescence reader and analyzed by Aida software. Using the signal intensities from purified PAR as a standard, the amounts of PAR produced (fmol) in the reactions were calculated by averaging the signals from technical triplicates. (a) Representative slot-blot from one of three different experiments. Five % of reaction mixtures from seven different concentrations of NAD^+ (0/10/25/50/100/200/400 µM) were slot-blotted for each of the three proteins, respectively. 1–7: rat PARP-1, increasing NAD^+ concentrations; 8–14: human PARP-1 A762 allele, increasing NAD^+ concentrations; 15–21: human PARP-1 V762 allele, increasing NAD^+ concentrations; 22: 200 fmol purified PAR; 23: 100 fmol purified PAR; 24: 50 fmol purified PAR. (b) Michaelis–Menten kinetics from rat (dots/dashed line), human A762 (squares/dotted line) and V762 (triangles/continues line). Note the second order kinetics (sigmoidal) readily visible in the least active PARP-1 from rat.

washes as above. For ECL reaction, Advanced ECL (GE Healthcare) was used and visualized by Fuji-LAS1000. Evaluation of signal intensities was done with Aida3.5 or ImageJ (MacBiophotonics) software. Production of graphs and statistical analysis was performed using Prism5 and Instat3 software (GraphPad).

In order to determine optimal conditions for PARP-1 activity, we first tested in triplicates polymer production at different temperatures by standard western blotting (including stacking gel) with antibody 10H. To detect suitable amounts of PAR, we used 100 ng (17.7 nM) PARP-1 (A762 variant) and 1 min incubation time, stopping the reaction by addition of the PARP inhibitor PJ34 (10 µM; Alexis Biochemicals). As a presumed negative control, a PARP-1 mutant with substitution of glutamate at amino-acid position 988 to lysine (E988K) was chosen, which has been described as a mono-ADP-ribose transferase lacking polymerizing activity (Rolli et al., 1997), thus catalyzing covalent protein modification with a single ADP-ribose unit, which is not detected by 10H antibody. Surprisingly, we did detect low level of PAR upon activation of the E988K mutant with a similar temperature dependency as the A762 variant and an optimum between 30 and 37 °C (Fig. 1a and b). This polymerizing function apparently had gone unnoticed so far when using radioactively labeled NAD^+, as we calculated that E988K activity is only 1–3% of A762 variant, depending on reaction temperature (Fig. 1c). To exclude contamination with insect PARP during the purification process, we infected *Sf*9 cells with either a PARP-1 cDNA-carrying virus or an unrelated wt virus and performed purification side by side. The dialyzed solution from control preparation did not contain any detectable PARP activity (Supplement Fig. 1). Therefore, the E988K mutant is the source not only of the reported mono-ADP-ribose transferase activity but also the polymerizing activity, which has to

Table 1
Enzymatic parameters of different poly(ADP-ribose) polymerase-1 versions.

Enzyme	Velocity (fmol/30 s; 2.5 nM)	Velocity (pmol/min µg)	K_M (µM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)
Rat PARP-1	237.7[a]	1121.9[a]	59.11	2.113	3.575×10^4
Human A762 PARP-1	331.3[b,a]	1563.7[b,a]	38.23[b]	2.945	7.703×10^4
Human **V762** PARP-1	701.9[b]	3313.0[b]	33.93[b]	6.239	18.388×10^4

Significance was tested by two-tailed *t*-test for V_{max} and K_M. Bold face: prevalent allele in human population.
[a] Significantly different from V762.
[b] Significantly different from rat enzyme.

be taken into account if this mutant is used in experiments designed to dissect enzymatic functions.

In order to check if the correlation between life span and PARP activity in permeabilized PBMC is reflected in the recombinant PARP-1 of the respective species, we used rat and human PARP-1, and in the latter case the two different alleles A762 and V762. Recently, recombinant V762 was shown to produce twice as much polymer compared to A762, which was reflected in a slightly higher affinity (1.2×) to NAD^+ (lower K_M) (Wang et al., 2007). Those authors had used a commercially available kit to determine substrate affinity, but maximum velocity (V_{max}) had not been reported.

We tested recombinant rat and human PARP-1 as described above, and varied NAD^+ concentrations (Fig. 2). Transferring to the same blot a known amount of purified PAR (Supplement Fig. 2), we were able to calculate Michaelis–Menten constant K_M as well as maximum velocity V_{max}. We could show that rat PARP-1 has a nearly 2-fold higher K_M compared to human enzymes, whereas K_M of A762 and V762 is almost identical (1.1-fold difference), in line with previous results (Table 1). In contrast, V762 shows a 2.1-fold higher velocity compared to A762, with the latter one being slightly more active than rat PARP-1 (1.3-fold). The turnover number k_{cat} (s^{-1}) for rat, A762 and V762 mirrors the differences in V_{max} values (2.1, 2.9, and 6.2, respectively), and the calculated enzyme efficiency k_{cat}/K_M ($s^{-1}\,M^{-1}$) for rat and V762 PARP-1 (3.58×10^4 and 18.39×10^4, respectively) perfectly reflects the 5-fold activity difference detected in permeabilized PBMC (Grube and Bürkle, 1992).

In summary, we were able to confirm PARP activity data from PBMC with purified recombinant proteins. Thus, activity difference between the common V762 version of human PARP-1 and rat PARP-1 can easily be explained by different enzymatic characteristics of PARP-1 itself and no differential interaction of PARP-1 with other proteins needs to be postulated. This also implies that the cellular background (lymphocytes) is not accounting for the activity difference published before. Additionally, we could show for the first time that there is a quality difference between the activities of different PARP-1 proteins, either divergence in substrate affinity (rat vs. human) or in maximum velocity (human alleles). We also showed that velocity and not K_M is the main parameter differing in the A762 variant compared to the more frequent V762. Unexpectedly, we also identified the PARP-1 E988K mutant as a low-level poly(ADP-ribose) polymerase, in contrast to previous reports. As is evident from the signal distribution in western blots (Fig. 1a) as well as silver-staining of size-separated purified PAR (Suppl. Fig. 3), complexity of the polymer seems to be identical between A762 allele and E988K mutant. This has to be taken into account in experiments either aiming at dissection of the three different steps in PARP-1 activity, or in attempts of replacing polymerizing function of PARP-1 by mono-transferase activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2010.04.003.

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