

## Poly(ADP-ribose) polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with species-specific life span

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**ABSTRACT** Poly(ADP-ribosylation) is a eukaryotic posttranslational modification of proteins that is strongly induced by the presence of DNA strand breaks and plays a role in DNA repair and the recovery of cells from DNA damage. We compared poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) activities in Percoll gradient-purified, permeabilized mononuclear leukocytes from mammalian species of different maximal life span. Saturating concentrations of a double-stranded octameric oligonucleotide were applied to provide a direct and maximal stimulation of PARP. Our results on 132 individuals from 13 different species yield a strong positive correlation between PARP activity and life span ( $r = 0.84$ ;  $P \ll 0.001$ ), with human cells displaying  $\approx 5$  times the activity of rat cells. Intraspecies comparisons with both rat and human cells from donors of all age groups revealed some decline of PARP activity with advancing age, but it was only weakly correlated. No significant polymer degradation was detectable under our assay conditions, ruling out any interference by poly(ADP-ribose) glycohydrolase activity. By Western blot analysis of mononuclear leukocytes from 11 species, using a crossreactive antiserum directed against the extremely well-conserved NAD-binding domain, no correlation between the amount of PARP protein and the species' life spans was found, suggesting a greater specific enzyme activity in longer-lived species. We propose that a higher poly(ADP-ribosylation) capacity in cells from long-lived species might contribute to the efficient maintenance of genome integrity and stability over their longer life span.

Poly(ADP-ribosylation) is a eukaryotic posttranslational protein modification catalyzed by poly(ADP-ribose) polymerase [PARP; NAD<sup>+</sup> ADP-ribosyltransferase, NAD<sup>+</sup>:poly(adenosine diphosphate D-ribose) ADP-D-ribosyltransferase, EC 2.4.2.30], a highly conserved nuclear enzyme that uses NAD as substrate (for review, see refs. 1 and 2). The DNA-binding domain located at the amino terminus of this 116-kDa protein specifically binds to DNA single- or double-strand breaks by the intermediacy of two zinc fingers (3, 4). DNA break binding leads to an immediate and drastic activation of the catalytic center located in the carboxyl-terminal NAD-binding domain; the latter is separated from the DNA-binding domain by a central automodification domain. A large number of studies done in a variety of experimental systems led to the view that poly(ADP-ribosylation) plays a role in DNA repair (5) and other cellular responses to DNA damage, such as cell cycle perturbations (6), DNA amplification (7–9), and malignant transformation (10, 11). Apart from this, poly(ADP-ribosylation) was thought to play a role in DNA replication (12, 13), integration of transfected foreign DNA into the cell genome (14, 15), intrachromosomal homologous recombination (16), differentiation (17, 18), and aging (19–22). In no case, however, have the molecular mechanisms been elucidated so far.

Interestingly, Pero *et al.* (20) described a positive correlation between PARP activities in nucleotide-permeable leukocytes of different mammalian species after high-dose  $\gamma$ -irradiation of the cells and the species-specific life spans. This finding would fit in with the well-known correlation between DNA repair and life span of mammals (23–25).  $\gamma$ -Irradiation, which was used to stimulate PARP activity, however, may not cause the same number of DNA breaks if applied to living cells of different organisms, since many of the breaks are mediated by free-radical mechanisms and/or DNA repair endonucleases (26) whose activities are already known to correlate with the species' life span (23–25, 27). Therefore, it is not clear whether the reported correlation between PARP activity and life span is direct (i.e., due to a higher enzyme content or a greater specific enzyme activity) or indirect (i.e., due to other cellular functions). As another potential source of complication, the NAD concentrations used in the quoted study (25  $\mu$ M) were well below the reported  $K_m$  for polymer synthesis (28).

We therefore set up a method to provide a direct stimulus for PARP in permeabilized cells—i.e., addition of saturating amounts of a double-stranded oligonucleotide (29). We thus could rule out any influence by cellular functions involved in the generation or prevention of DNA breaks and retested PARP activity at saturating NAD concentrations as a function of species-specific life span in mononuclear blood cells (MNC), composed mainly of lymphocytes and monocytes/macrophages.

### MATERIALS AND METHODS

**Cells.** Blood samples of elephant (*Elephas maximus*), pigmy chimpanzee (*Pan paniscus*), gorilla (*Gorilla gorilla*), and donkey (*Equus asinus*) were obtained from a zoo; those of rabbit (*Oryctolagus cuniculus*), pig (*Sus scrofa*), horse (*Equus caballus*), and cattle (*Bos taurus*) were obtained from a slaughterhouse; and blood samples of rat (*Rattus rattus*, laboratory strains Sprague-Dawley and Wistar), guinea pig (*Cavia porcellus*, strain Pirbright/white), marmoset (*Calithrix jacchus*), and sheep (*Ovis aries*) were obtained from local animal research facilities. So as to compare PARP activity between animals of different ages, a cohort of 40 rats (strain BN/BiRj) representing four age groups was obtained from the European Community Concerted Action on Ageing and Diseases central animal care facility (Netherlands Central Organization for Applied Scientific Research, Institute for Experimental Gerontology, Rijswijk, The Netherlands); the animals were 9, 51–54, 93–96, and 163 weeks old, respectively. From this cohort, the data from animals up to 54 weeks old were included in the species comparison shown in Fig. 1. Human blood was drawn from placenta and from volunteers in apparently good health, except for some of

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Abbreviations: MNC, mononuclear blood cells; PARP, poly(ADP-ribose) polymerase; TBS/T, Tris-buffered saline/Tween; TCA, trichloroacetic acid.

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those older than 60 years who suffered from degenerative disorders typical of old age. MNC were routinely isolated by Percoll gradient centrifugation (30). Molt-3, a human T-lymphoma cell line, was maintained as a suspension culture in RPMI 1640 medium (Biochrom, Berlin) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 2 mM glutamine, and 10% heat-inactivated fetal calf serum (Biochrom). Cells were counted in a hemocytometer.

**Cell Permeabilization and PARP Assay.** Cell permeabilization and PARP assays were done as described (29). Briefly, MNC were pelleted, resuspended in ice-cold hypotonic permeabilization buffer (10 mM Tris-HCl, pH 7.8/1 mM EDTA/4 mM MgCl<sub>2</sub>/30 mM 2-mercaptoethanol) at  $2 \times 10^6$  cells per ml and left on ice for 15 min. Then cells were pelleted at  $200 \times g$  at 0°C for 10 min and resuspended in ice-cold permeabilization buffer at  $2 \times 10^6$  cells per 54 µl. Microscopic examination revealed that at least 85% of the cells were rendered permeable to trypan blue. Five micrograms of oligonucleotide (GGAATTCC) dissolved in 13 µl of 15 mM NaCl (except when stated otherwise) and 33 µl of  $3 \times$  reaction mixture [100 mM Tris-HCl, pH 7.8/1 mM NAD (grade V; Sigma, Munich)/120 mM MgCl<sub>2</sub>] containing 18.5 kBq (0.5 µCi) of [adenine-2,8-<sup>3</sup>H]NAD (1.33 TBq/mmol = 36 Ci/mmol; NEN, Dreieich, Germany) were added to samples of  $2 \times 10^6$  cells on ice, yielding a total volume of 100 µl per reaction mixture. Reactions were carried out in triplicate for 10 min at 30°C and stopped by adding 1 ml of ice-cold 10% trichloroacetic acid (TCA)/2% sodium pyrophosphate. TCA precipitates were collected on Whatman GF/C filters, washed three times with 10% TCA/2% sodium pyrophosphate, washed twice with 95% ethanol, and processed for scintillation spectrometry. For each cell sample, triplicate blank determinations were done in parallel by adding TCA immediately while on ice. Mean values of these blanks were subtracted from the respective 10-min reaction mean values. So as to compare enzyme activities that were measured on separate days, using different batches of radioactive substrate, exponentially growing Molt-3 cells ( $10^6$  cells per reaction) were routinely assayed in parallel as an internal standard. Typically, these cells yielded activity values around 10,000 cpm (equivalent to 750 pmol; range between 5000 and 18,000 cpm). For each run, normalization of MNC values was performed by applying the following equation:

$$\text{Normalized MNC cpm} = \frac{\text{crude MNC cpm} \times 10,000 \text{ cpm/Molt-3 cpm}}{\text{Molt-3 cpm}}$$

The data were subjected to linear regression analysis. For interspecies comparisons, rank correlation coefficients were determined according to Spearman (31) rather than standard correlation coefficients, since the life span values did not follow standard distributions.

**Western Blot Analysis.** For Western blots, aliquots of  $10^6$  purified MNC per species, representing pools from 3 to 10 animals, were washed in ice-cold phosphate-buffered saline (18.4 mM Na<sub>2</sub>HPO<sub>4</sub>/10.9 mM KH<sub>2</sub>PO<sub>4</sub>/125 mM NaCl) supplemented with 10 mM sodium bisulfite, 10 µM pepstatin, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cells were pelleted, shock-frozen, and stored at -70°C. During the process of thawing, cells were immediately resuspended in 25 µl of this washing solution, lysed by the addition of 25 µl of  $2 \times$  SDS/PAGE sample buffer (32), immediately heated to 95°C for 5 min, and sonicated. SDS/PAGE was performed as described (32). Proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore) by using a semidry blotting system (33). After staining with Ponceau S and subsequent destaining with phosphate-buffered saline, the blot was blocked in Tris-buffered saline/Tween (TBS/T; 50 mM Tris-HCl, pH 8.0/150 mM NaCl/0.3% Tween 20) containing 5% dry milk and incubated with a rabbit antiserum

(kindly provided by G. de Murcia, Strasbourg, France) directed against the human PARP NAD-binding domain expressed in *Escherichia coli*, at a dilution of 1:1000 in TBS/T containing 5% dry milk. After overnight incubation at 4°C, blots were washed with TBS/T and incubated with goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Sigma) at a dilution of 1:4000 in TBS/T containing 5% dry milk for 2 h at room temperature. The phosphatase reaction was carried out in 50 mM glycine-NaOH, pH 9.7/4 mM MgCl<sub>2</sub>/nitroblue tetrazolium (0.1 mg/ml)/5-bromo-4-chloro-3-indolyl phosphate (0.05 mg/ml). Densitometric scanning of PARP bands was done on a transluent photocopier with a Gilford Response spectrophotometer/densitometer. Signal intensities measured as peak areas were in the linear range, as revealed by serial dilutions of extracts used as standards.

## RESULTS

We measured maximal oligonucleotide-stimulated PARP activity in permeabilized MNC of 132 individuals, representing 13 mammalian species. All donors had completed no more than 37% of their respective species-specific maximal life span, as taken from the zoological literature (34, 35). All blood samples were obtained fresh, since in preliminary experiments we had noticed a decay of PARP activity in our standard assay, with a half-life of about 24 h when heparinized blood had been stored at 4°C (data not shown). As is shown in Fig. 1, regression analysis of the data yielded a strong positive correlation between oligonucleotide-stimulated PARP activities and the species' maximum life spans (rank correlation coefficient  $r = 0.84$ ;  $y = 3.55x + 102.2$ ;  $P < 0.001$ ). A correlation of almost the same strength was obtained when the data were plotted against average life span ( $r = 0.82$ ), whereas the correlation between PARP activity and body weight was clearly weaker ( $r = 0.62$ ).

To study whether there was any relationship with chronological age, we tested 50 human subjects and 40 BN/BiRj rats of different ages. Although the correlations were rather weak, maximal PARP activity showed some decrease with advancing age in both humans ( $r = -0.54$ ,  $P < 0.001$ ; 57% decrease projected over maximal life span by linear regression analysis) and rats ( $r = -0.34$ ,  $P < 0.05$ ; 39% decrease over maximal life span) (Fig. 2). Theoretically, selecting donors of vastly different relative chronological age for a species comparison could thus result in a distorted picture. For the species survey shown in Fig. 1, however, yielding a 5-fold difference in PARP activity between man and rat, we had recruited only young individuals (see above). That activity difference, therefore, cannot be explained by age effects.

To check that the oligonucleotide concentration in our standard assay (5 µg/100 µl) was indeed saturating for enzymes of different species, dose-response experiments were performed with a set of three species (rat, pig, and man) that covers the whole range of life spans studied. As is shown in Fig. 3A, in each case PARP was saturated by concentrations as low as 1 µg/100 µl.

To determine whether or not poly(ADP-ribose) synthesized in our assays was subject to degradation, rat, pig, and human MNC were first allowed to produce polymer for 10 min, followed by the addition of 3-aminobenzamide to inhibit further polymer synthesis. Any degradative activities should then be revealed by a loss of acid-insoluble radioactive material previously accumulated. Fig. 3B shows that no polymer instability was detectable under our assay conditions, ruling out that PARP activity data were influenced by catabolic processes [e.g., a putative species-specific poly(ADP-ribose) glycohydrolase activity]. Apart from this, we verified in rat, pig, and human cells that the reaction tem-

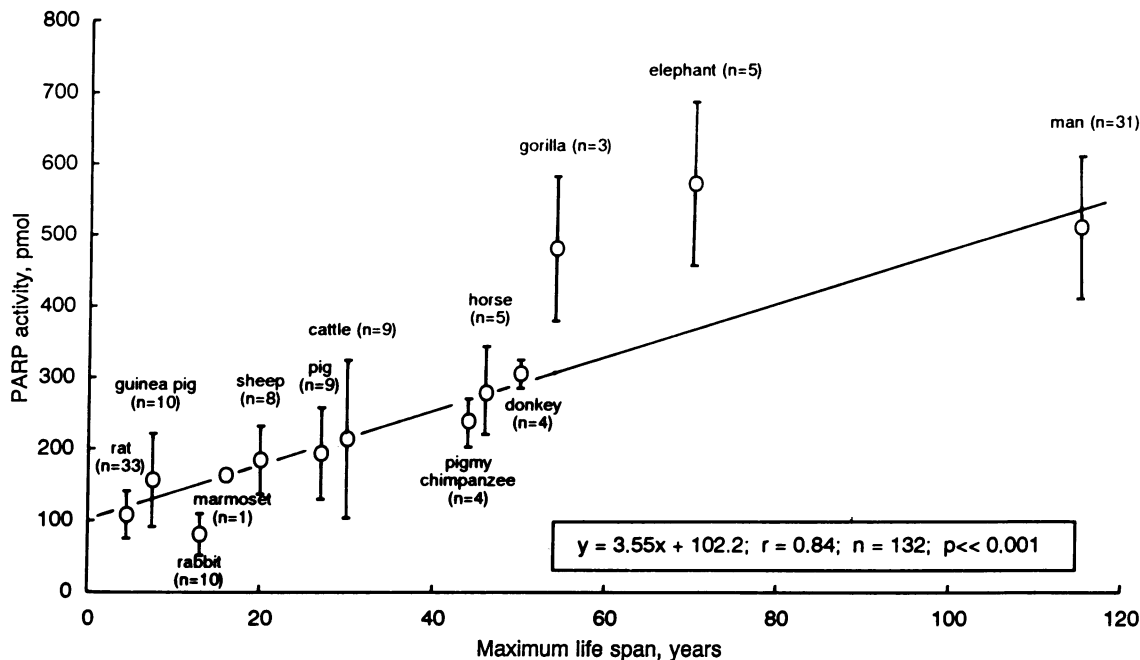


FIG. 1. Correlation between PARP activity and maximal life span of 13 different mammalian species. MNC were assayed for maximal PARP activity as described in *Materials and Methods*. Results are given as mean values of the respective species ( $n$  = numbers of individuals)  $\pm$  1 SD. Data on maximal life spans were taken from refs. 34 and 35 or, for pigmy chimpanzee, were kindly provided by C. Schärpner (Frankfurt Zoo, Frankfurt). The marmoset sample was a pool from eight individuals. Spearman's rank correlation coefficient ( $r$ ) was determined according to ref. 31.

perature (30°C) was optimal for each of these species (data not shown).

Different PARP activities may be due to different amounts of PARP protein, as for example in the case of mitogen stimulation of lymphocytes (36), or due to differences in specific enzyme activities. To address this question, we performed Western blot analyses on MNC from 11 species. To prevent artificial protein degradation, each sample, representing a pool of 3–10 members of a species, was directly lysed in SDS/PAGE sample buffer in the presence of protease inhibitors. To detect PARP of different species with equal efficiency, we used a polyclonal antiserum directed against the human PARP NAD-binding domain, whose primary sequence has been extremely well conserved during evolution (37–42). The blot shown in Fig. 4, together with the results of densitometric scanning of the 116-kDa PARP bands, revealed that there was no obvious correlation between the amount of the protein of different species with life span: signal intensities—e.g., of rat and human cells—were

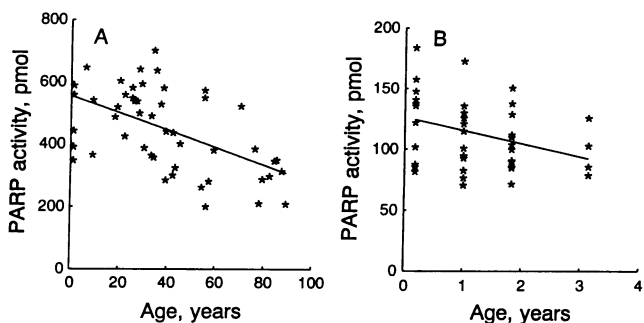


FIG. 2. PARP activity as a function of chronological age. PARP assays were performed in humans (A) and rats (B) of different ages, as described in *Materials and Methods*. (B) A cohort of BN/BiRj rats was used. Each data point represents one individual. Statistical evaluation yielded the following results: for A,  $y = 561 - 2.78x$ ,  $r = -0.54$ ,  $n = 50$ , and  $P < 0.001$ ; for B,  $y = 128 - 11.1x$ ,  $r = -0.34$ ,  $n = 40$ , and  $P < 0.005$ .

almost identical. Probing a parallel blot with an antiserum against the slightly less well conserved second zinc finger, located in the PARP DNA-binding domain, gave a higher degree of variation among different species, but again failed to show any systematic difference that would correlate with life span (data not shown). For unknown reasons, the pigmy chimpanzee signal was surprisingly weak with both antisera, although Ponceau S staining of the blots did show the expected amount of total protein loaded in these lanes. Additional Western blots of independent samples from rat,

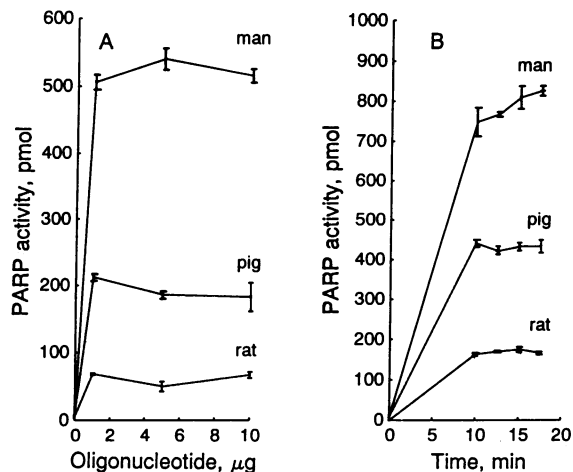


FIG. 3. PARP activity assays in rat, pig, and human MNC to determine saturating oligonucleotide concentrations (A) and poly-(ADP-ribose) stability (B). (A) PARP assays were done as described in *Materials and Methods*, with varying amounts of oligonucleotides per reaction as indicated. Mean values of triplicates  $\pm$  1 SD are given. (B) Assays were performed as described in *Materials and Methods*, except that 2  $\mu$ Ci of [ $^3$ H]NAD (instead of 0.5  $\mu$ Ci) was used per reaction and 3-aminobenzamide (final concentration = 2 mM) was added at 10 min to inhibit further polymer synthesis. In a parallel assay, 3-aminobenzamide, added at 0 min, inhibited enzyme activity completely (not shown).

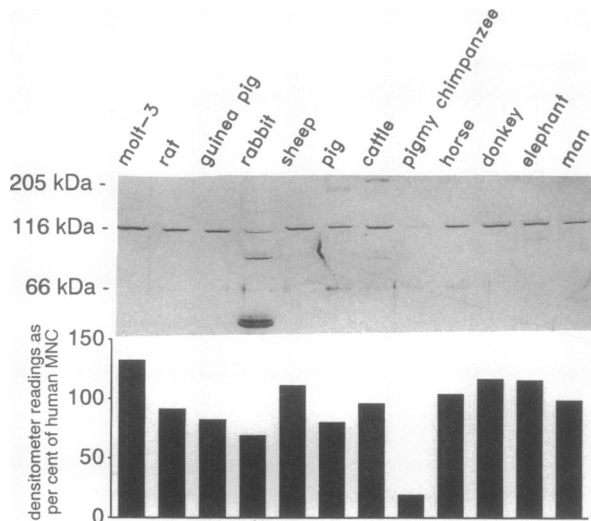


FIG. 4. Western blot analysis of PARP protein from 11 mammalian species. Each lane represents  $10^6$  MNC (except for the reference cell line Molt-3, where only  $5 \times 10^5$  cell equivalents were loaded) from species as indicated. The antiserum used detects the highly conserved PARP NAD-binding domain. Note that the strong reaction of rabbit proteins below 116 kDa is due to a direct recognition of immobilized rabbit immunoglobulins from the mononuclear cell lysate by the secondary antibody used as a detection system for this blot. Bars below each lane represent the results of densitometric scanning of the respective 116-kDa PARP bands, given as percentage of the signal intensity of human MNC.

rabbit, pig, and man, probed with the anti-NAD-binding domain antiserum, gave results similar to those shown in Fig. 4. The sensitivity of our procedure was such that we were able to detect reliably a 2-fold difference in the amount of PARP, as determined by serial lysate dilutions (data not shown). This is also reflected in the comparison of the human cell line Molt-3 with human MNC in Fig. 4: the relative signal intensities correspond well with our standard PARP activity assay, where Molt-3 yielded  $\approx 150\%$  of the mean value of MNC. (It should be noted that for both the activity assay and the Western blot only half the number of Molt-3 cells were applied with respect to MNC.)

A point of concern was that during the process of cell permeabilization and/or the activity assay, proteolysis of PARP could preferentially affect short-lived species, resulting in artificially low PARP activities. Western blot analysis, however, of permeabilized cells and of cells that in addition underwent incubation in reaction buffer without NAD at  $30^\circ\text{C}$  for 10 min failed to show proteolysis that would differentially affect rat or human cells (data not shown). In conclusion, our Western blotting data indicate that qualitative rather than quantitative differences in PARP proteins of different species should account for the correlation of maximal PARP activity with life span.

## DISCUSSION

In this study, we established a strong correlation between the maximal PARP activity stimulated by a double-stranded oligonucleotide in MNC of 13 mammalian species and maximal species-specific life span. Our work was stimulated by the paper of Pero *et al.* (20), in which a similar correlation of PARP activity in leukocytes stimulated by  $\gamma$ -radiation with life span was described. The target tissue of our study was Percoll gradient-purified MNC, since these are primary cells that are readily available and need no culturing before being assayed. This rules out any cell culture artifacts due to, for example, different rates of proliferation, which are likely to have an influence on PARP expression and activity (36). A

major assay modification was the use of oligonucleotides as stoichiometrically defined PARP stimulators that act directly, without the intermediacy of other cellular functions (29).

One should keep in mind that our data refer to a subcellular system in which no significant polymer catabolic activities could be detected (Fig. 3B). It remains to be studied whether *in vivo* the higher maximal PARP activities of long-lived species indeed lead to higher levels of poly(ADP-ribose). Alternatively, the rate of poly(ADP-ribose) turnover could be increased due to an enhanced activation of poly(ADP-ribose) glycohydrolase *in vivo* (43). Furthermore, it remains to be investigated whether in other tissues the same differences in enzyme activity exist between different species.

We restricted our analysis to mammals because they provide a large spectrum of different life spans while they are rather closely related genetically. This is particularly evident at the level of available PARP protein sequences of mouse (37), rat (41), cattle (42), and man (38–40), which share a striking degree of homology (e.g., 92% overall sequence similarity at the protein level between mouse and man; ref. 37). This excellent degree of homology, which for most of the NAD-binding domain is close to 100%, allowed for a comparison of enzyme quantities across different species by Western blotting (Fig. 4).

As far as life span data are concerned, one should note that both the average and the apparent maximal life span that human beings can enjoy nowadays may be strongly biased with respect to all other species due to cultural and medical influences. Perhaps this might explain why human PARP activity is not higher than that of gorilla or elephant, although according to the literature a large difference exists in maximal life span.

Looking at maximal PARP activity as a function of chronological age within two species, we found a moderate decline with advancing age, but the correlation was rather weak. Possible relations between aging and poly(ADP-ribosylation) have been studied by several laboratories. When investigating a different tissue, Jackowsky and Kun (19) reported that PARP activity was lower in cardiocytes from 90-day-old rats than in those from 5-day-old rats, despite an increase in the number of DNA breaks. Bizec *et al.* (21) studied PARP activity in bovine eye lens epithelial cells and reported an increase in basal activity with age, most likely due to a parallel increase in DNA strand breakage. Quesada *et al.* (22) examined basal PARP activity in cell nuclei from rat ventral prostate during aging. A large decline was noted with advancing age, along with a changing pattern of modified acceptor proteins. However, in all these studies maximal stimulated activity was not compared. Dell'Orco and Anderson (44) found a decline of unstimulated PARP activity in aging human fibroblast cultures, but surprisingly not so after DNase I stimulation. As the experimental design of their study was different from ours in many respects, there is no easy explanation at hand for this apparent inconsistency.

Our species survey by Western blot (Fig. 4) failed to show a correlation at the level of PARP quantity. Ludwig *et al.* (45) have performed immunoquantitation of PARP from cell lines of different mammalian species as well as from rat liver. Their results did not show any differences in PARP quantity either. However, immortalized and/or transformed cells were studied in which PARP expression might be regulated in a way different from that of normal cells. Viewed together, our data suggest that differences in specific enzyme activity are likely. At least three mechanisms are conceivable: (i) subtle but functionally important differences in the primary structure of this highly conserved enzyme, (ii) putative differences in posttranslational modifications of PARP, or (iii) putative different accessory chromatin factors that could result in different specific activities. Which of these mechanisms

prevails remains to be seen. In any case, further studies on the molecular basis of increased PARP activity in long-lived species could possibly yield important new insights into the structure–function relationships of this enzyme.

Which role could a higher maximal PARP activity play for longer-lived species as compared with short-lived ones? In a whole variety of organisms studied, aging is clearly associated with genetic instability (46, 47), although the causative factors and mechanisms involved have not been elucidated so far. Likely candidates for inducers of genetic instability, however, are any kinds of DNA damages generated by ubiquitous endogenous and exogenous agents, such as oxygen radicals, reducing sugars and other physiological cell metabolites, environmental carcinogens, or irradiations. This view is entirely consistent with the reported correlation between mammalian life span and certain DNA repair functions (23–25) that would antagonize the accumulation of such damages more efficiently in longer-lived species. Since poly-(ADP-ribosyl)ation is involved in DNA repair and the cellular recovery from DNA damage, in such a way that PARP inhibitors potentiate many biological consequences of DNA damage (1, 2, 5–9, 11), we propose that, in turn, a higher poly(ADP-ribosyl)ation capacity in cells from long-lived species might contribute to the efficient maintenance of genome integrity and stability over their longer life span.

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