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Yeast (ADPribosyl)ation: Revisiting a Controversial Question

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Abstract The controversy about the occurrence of an (ADPribosyl)ating activity in yeast is still standing up. Here we discuss this topic on the basis of results obtained with classic experiments proposed over years as basis to characterize an (ADPribosyl)ation system in any organism. Independent results obtained in two different laboratories were in line with each other and went towards the occurrence of an active (ADPribosyl)ating system in *Saccharomyces cerevisiae*. In fact data collected from nuclear preparations of cultured cells matched those from baker's yeast and lyophilized yeast cells. Yeast (ADPribosyl)ating enzyme is a protein of 80–90 kDa, as determined by electrophoresis on polyacrylamide gel in sodium dodecyl sulphate, followed by immunoblotting with antibodies against anti-poly(ADPribose) polymerase catalytic site. It synthesizes products, that, after digestion with phosphodiesterase, co-migrates mainly with phosphori bosyl adenosine monophosphate after thin layer chromatography on silica gel plate. J. Cell. Biochem. 94: 1258–1266, 2005. © 2005 Wiley-Liss, Inc.

Key words: yeast; (ADPribosyl)ation; NAD^+ metabolism

The dual role of NAD^+ as both coenzyme of pyridinic dehydrogenases and substrate of several nuclear enzymes (DNA ligase, Sir 2, poly (ADPribose)polymerase, etc.) has focused much attention onto its metabolic rate and concentrations within cell compartments [Opphenheimer, 1994; Magni et al., 1999; Frye, 2000; Chiarugi, 2002; Denu, 2003; Rongvaux et al., 2003; Berger et al., 2004]. In eukaryotes NAD^+ synthesis and degradation are strictly related with signal transduction and nuclear events [Rongvaux et al., 2003; Berger et al., 2004]. The metabolic pathway of NAD^+ is rather complex and involves a thick network of reactions leading to different products regulating important cell processes [Rongvaux et al., 2003; Berger et al., 2004]. The final stage of NAD^+ synthesis catalysed by NAD^+ pyrophosphorylase occurs in the nucleus [Magni et al., 1999; Panozzo et al., 2002], and in the human cell line D98/AH2 95% NAD^+ synthesis increases to compensate depletion of the dinucleotide following hyper-poly(ADPribosyl)ation [Hillyard et al., 1977].

(ADPribosyl)ation is a reversible post-translational modification of proteins catalyzed by two main families of enzymes, mono(ADPribose) transferases (ADPRTs) and poly(ADPribose) polymerases (PARPs), whose main member is PARP 1, a chromosomal enzyme [Hayaishi and Ueda, 1977; Shall, 2002; Corda and Di Girolamo, 2003; Rouleau et al., 2004]. Both groups of enzymes use NAD^+ as a substrate to synthesize ADPribose and transfer it to protein acceptors [Hayaishi and Ueda, 1977; Shall, 2002; Corda and Di Girolamo, 2003; Rouleau et al., 2004]. However, only PARPs are able to synthesize polymers of adenosine diphosphate ribose (poly (ADPribose), pADPR) [Hayaishi and Ueda, 1977; Shall, 2002; Rouleau et al., 2004]. PARP 1, also known as genome "guardian," is involved in the regulation of several DNA metabolic processes, particularly DNA repair, and in the expression and propagation of the genetic information (DNA transcription and replication, differentiation, neoplastic transformation) [Shall, 2002; Rouleau

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et al., 2004]. The synthesis of poly(ADPribose) from NAD^+ enormously increases following DNA damage, and can recruit apoptosis inducing factors from mitochondria [Yu et al., 2002]. Upon extensive DNA breakage, hyperactivated PARP 1 consumes NAD^+ subtracted to energy metabolism, and addresses cell fate towards necrotic death [Berger et al., 2004]. Thus intracellular concentrations of NAD^+ are dependent on nuclear enzymes of both synthesis (NAD^+ pyrophosphorylase, NMN adenylyltransferase, etc.) and degradation (Sir 2, PARP, NAD^+ glycohydrolase, etc.) [Opphenheimer, 1994; Magni et al., 1999; Frye, 2000; Chiarugi, 2002; Corda and Di Girolamo, 2003; Denu, 2003; Rongvaux et al., 2003; Berger et al., 2004; Rouleau et al., 2004].

Changes in nuclear NAD^+ turnover during induction of its synthesis have been demonstrated in some *Saccharomyces cerevisiae* strains, selected as NAD^+ producers [Gulyamova et al., 2001; Panozzo et al., 2002]. The stimulating effect of nicotinamide on intracellular synthesis of NAD^+ was correlated with increased levels of nuclear NAD^+-dependent enzymes, including those catalyzing (ADPribosyl)ation [Gulyamova et al., 2001]. This finding raises again a still controversial question dealing with the occurrence of (ADPribosyl)ation reaction in *S. cerevisiae*. Despite a number of reports have been indicating that yeast lacks the (ADPribosyl)ating system [Kameshita et al., 1985; Scovassi et al., 1986; Lamarre et al., 1988; Simonin et al., 1991; Kaiser et al., 1992; Perkins et al., 2001], Sugimura et al. [1968] described the existence of this reaction in yeast on the basis of preliminary analyses, and previous observations indicated that ribosyl adenosine occurred among phosphodiesterase/alkaline phosphatase digests of yeast tRNA [Hall, 1965]. Later, evidence of endogenous (ADPribosyl)ation of yeast NMN adenylyltransferase was obtained by Ruggieri et al. [1988, 1990]. They characterized this enzyme as a glycoprotein containing 2% sugar, 2 mol of alkali-labile phosphate, and 1 mol of adenine/mol of enzyme. More recently, the induction of NAD^+ synthesis in yeast cell producers was shown to correlate with increased nuclear incorporation of labeled ADPribose [Gulyamova et al., 2001].

These observations prompted us to re-examine the question and to carry on an investigation with classic procedures and experiments. Here we present evidence of an active (ADPribosyl)ation of proteins in yeast cells. The (ADPribosyl)ation system of *S. cerevisiae* was characterized in terms of enzyme activity and expression, protein acceptors, and reaction products.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), β-NAD^+, ADPribose (ADPR), Proteinase K (E.C. 34.21.64), DNase I (E.C. 3.1.21.1), RNase A (E.C. 3.1.27.64), phosphodiesterase I (PDE I; E.C. 3.1.4.1), protease inhibitor cocktail, and yeast lyophilized cells (yeast enzyme concentrate, Y-2875) were purchased from Sigma-Aldrich (Milan, Italy). Prestained molecular mass markers were from Bio-Rad Laboratories (Milan, Italy).

[U-adenine-^{14}C]NAD^+ (280 mCi/mmol), [adenylate-^{32}P]NAD^+ (1000 Ci/nmol), polyvinylidene difluoride (PVDF) transfer membrane were product of the Radiochemical Centre, Amersham-Pharmacia (UK). Polyclonal anti-poly(ADPribose) polymerase (PARP) antibodies (H-250) were from Santa Crutz Biotechnology, Inc. (Santa Crutz, CA). Antirabbit antibody conjugated with horseradish peroxidase (HRP) was from Pierce (Rockford, IL). Thin layer chromatography (TLC) silica gel (60F254) plates were from MERCK, V.W.R. international (Darmstad, Germany).

Yeast Cell Culture

S. cerevisiae strain 913a-1, selected as a NAD^+ producer, was cultivated at 30–32°C for 72 h with shaking [Gulyamova et al., 2001]. Harvested cells were used for nuclei preparation as previously described [Faraone-Mennella et al., 1999].

Preparation of Enzyme Fractions

Homogenate was prepared by gently pottering yeast powder (yeast enzyme concentrate, Y-2875, Sigma), resuspended in 10 nM Tris-HCl buffer, pH 7.5 (1:10, w/v). For DNase digestion the buffer was adjusted to 0.4 nM Tris-HCl, buffer, pH 7.4, containing 100 nM NaCl, 100 nM $MgCl_2$, DNase I (2 mg/ml), 1 nM PMSF, and protease inhibitor cocktail (10 μg/ml) in a final volume of 500 μl. After 30 min at 37°C, RNase A (20 μg/ml) was added and incubation was carried out for further 30 min. The reaction was stopped with 0.2M EDTA on ice. The mixture was centrifuged at 9800*g* for 20 min at 4°C, and the supernatant was collected (DNase supernatant).

Baker's yeast (10 g) was homogenized with Ultraturrax homogenizer (mod. T8.10, IKA Laboratories, Staufen, Germany) at low speed in lysis buffer (0.0125M Na-phosphate buffer/ 61 nM sodium dodecyl sulphate, SDS), until dissolved. The suspension was left on ice for 1 h and centrifuged at 1500*g* for 15 min at 4°C. The supernatant (cytoplasmic fraction) was removed, whereas pellet was gently suspended in Buffer A (10 nM Tris-HCl buffer, pH 7.5, 1 nM EDTA and EGTA, 1 nM β-mercaptoethanol, 0.15 nM spermine, 0.75 nM spermidine, 1 nM PMSF, and protease inhibitor cocktail, 5 μg/ml), containing 2.2M sucrose [Faraone-Mennella et al., 1999]. After centrifugation at 31,000*g* for 1 h at 4°C, the pellet (nuclei) was washed three times in buffer A, containing 0.25M sucrose and 1% Triton X-100.

Protein concentration was determined by a commercial kit for Coomassie assay (Pierce), using bovine serum albumine as a standard.

Enzyme Assay

(ADPribosyl)ating activity was routinely assayed for 30 min at 37°C and, only where indicated, at room temperature (25–30°C), in the presence of 0.64 nM [*adenylate*-^{14}C]NAD^+ or [^{32}P]NAD^+ (10,000 cpm/nmole). The final specific radioactivity (10,000 cpm/nmole) and concentration (0.64 nM) were reached by mixing labeled (see "Materials") and 4 nM unlabeled NAD^+. Two types of reaction mixtures (mixture A and B) were used. Mixture A was composed of 100 nM Tris-HCl buffer, pH 7.8, containing 2.0 nM NaF (final volume 62.5 μl); alternatively the assay was performed in 10 nM Tris-HCl pH 8.0, 10 nM $MgCl_2$ (final volume 50 μl) (mixture B). The reaction was stopped with ice-cold 20% trichloroacetic acid and the radioactivity present in the acid-insoluble material, collected on a HAWP filter (0.45 μm, Millipore, Woburn, MA), determined on a Beckman LS 1701 liquid scintillation spectrometer [Faraone-Mennella et al., 1999].

Specific enzyme activities were calculated from the initial rates and expressed in mUnits/ mg of proteins. One milliunit is defined as the amount of enzyme required to convert 1 nmol of NAD^+/min under standard conditions.

[^{32}P] Labeled Acceptor Proteins

Different yeast preparations (200 μg proteins) were incubated under standard conditions, with ^{32}[P]NAD^+ (40,000 cpm/nmole) in mixture A (final volume 312.5 μl). The reaction was stopped by transferring the mixture on dry ice and lyophilizing. The dried samples were suspended in electrophoretic buffer and loaded on polyacrylammide (12%) gel.

To test ADPR–protein stability aliquots of yeast homogenate (600 μg) from yeast powder (yeast enzyme concentrate, Y-2875, Sigma) were incubated for 30 min at 37°C in the reaction mixture A, in the presence of 0.64 nM [^{32}P]NAD^+ (10,000 cpm/nmole) in a final volume of 1.81 ml. The reaction was stopped with ice cold 20% trichloroacetic acid.

The acid-insoluble material was washed first with 20% TCA followed by two washes with ethanol.

The precipitate from each aliquot was incubated in 0.33M NaOH at 56°C, or in 3M NH_2OH at 37°C, or in 0.1M HCl at 37°C for up to 180 min. At given times, 62.5 μl of every mixture were precipitated with TCA 20% (v/v) and radioactivity in the acid-insoluble material was determined on a Beckman Coulter (LS 1701 Nyon, Switzerland) liquid scintillation counter.

Polyacrylammide Gel Electrophoresis in Sodium Dodecyl Sulphate (SDS) and Autoradiography

Yeast proteins were analyzed on 12% polyacrylamide slab gels in the presence of 0.1% SDS as described previously [Faraone-Mennella et al., 1999]. For immunoblotting electrophoresed proteins were transferred onto PVDF membrane (Bio-Rad) at 200 V for 1.5 h at 4°C in the same buffer used for the electrophoretic run.

Images of stained gels and filters and autoradiographic patterns of labeled proteins were acquired by a phosphor imager (mod. FX, Bio-Rad) or by exposing to HP Hyperfilm (Amersham-Pharmacia) films for the needed time.

For immunoblot experiments procedures and buffers were according to Harlow and Lane (1988). PVDF sheets were treated for 3 h with the blocking solution (50 nM Tris-HCl buffer, pH 8.0, 150 nM NaCl, 0.5% (v/v) Tween 20 and 3% (w/v) gelatine. Incubation with commercial anti-PARP antibodies (Santa Cruz; rabbit anti-human PARP, H-250, 1:2000, v/v) was performed for 30 min at room temperature in the same solution supplemented with 0.3% gelatine). For comparison purified PARPSso from *Sulfolobus solfataricus* was analyzed [Harlow and Lane, 1988; Faraone-Mennella et al., 1998].

The blots were washed several times with TBS-Tween and antibody binding was detected

by using HRP conjugated goat anti-rabbit IgG from Bio-Rad. The reaction was revealed by using a kit for chemiluminescence (Super Signal West Dura Extended Substrate, PIERCE) and reading by a phosphor imager (Bio-Rad).

Reaction Products

Yeast proteins (200 μg) were incubated in presence of [^{32}P]NAD^+ (40,000 cpm/nmoles) at 37°C in mixture A (final volume 625 μl). After 30 min, the mixture was cooled on ice and loaded on a Sephadex G-10 column (cm 0.5 × 2) in H_2O. The elution was made with water, collecting 0.1 ml fractions. The [^{32}P] labeled proteins eluted at V_0, were collected, dried, and suspended in 10 nM Tris-HCl buffer, pH 11, 1 nM EDTA, 8 nM NaOH. Under these alkaline conditions poly(ADPribose) was detached from proteins. Proteinase K (50 μg/ml) was added and the mixture was incubated overnight at 37°C [Simonin et al., 1991]. Enzymatic digestion of proteins favored the subsequent extraction of products with chloroform/3-methylbutan-1-ol (49:1, v/v). The products, solubilized in the aqueous phase, were dried and half of them (cpm 12,000) were digested overnight with PDE I (1 mg/ml) in 10 nM Tris-HCl pH 8.8, 0.2 nM EDTA at 37°C, in order to generate the compounds expected when PDE has pADPR as substrate, i.e., AMP and phosphoribosyl-AMP. Both aliquots (with and without PDE) were loaded on TLC silica gel plates. The spots were resolved in isobutyric acid (60 ml)/30% ammonium hydrate (6 ml)/H_2O (15 ml) and exposed to HP Hyperfilm for autoradiography (3 days) [Simonin et al., 1991]. Under these conditions elongated ADPR chains (pADPR) do not migrate, whereas ADPribose/phosphoribosyl-AMP, NAD and AMP have different and specific R*f*.

RESULTS

Enzyme Activity and Protein Expression

Homogenates from baker's yeast and a commercial powder of *S. cerevisiae* cells, as well as nuclear extracts from *S. cerevisiae* strains, selected as NAD^+ producers [Gulyamova et al., 2001], were tested for (ADPribosyl)ating activity in the presence of [^{32}P]NAD. In order to optimize enzymatic assay the different preparations were tested either at room temperature (25–30°C) or at 37°C, in a simple reaction mixture (mixture A), or under conditions described for eukaryotic (ADPribosyl)ating enzyme (mixture B), in the absence of dithiothreitol (DTT). No significant difference of activity was observed by varying incubation mixtures and temperatures (Table I).

In protein extracts obtained after digestion of homogenates with DNase I, 100% activity was recovered under any incubation conditions.

Nuclear extract showed the highest specific activity with 88% recovery of activity from intact nuclei, where enzyme content was about 50% of that measured in the cultured cell homogenate. Enzyme activity was not influenced by fragmented DNA (data not shown).

Table II shows the effect of some compounds and ions on enzyme activity. A very low effect (<20% inhibition) was measured with DTT and

TABLE I. ADPribosylating Activity in *Saccharomyces cerevisiae* Cells

Sample	Temperature (°C)	Reaction mixture	Total mUnits	mUnits/mg protein	mUnits/g cell (dry weight)
Homogenate[a]	37	A	0.185	0.017	1.54
	37	B	0.180	0.017	1.54
	RT	A	0.210	0.019	1.75
	RT	B	0.210	0.019	1.75
(Mean value ± SD)[b]	—	—	—	0.015 ± 0.003	—
DNase supernatant[a]	37	A	0.185	0.123	1.54
	37	B	0.225	0.150	1.88
	RT	A	0.200	0.130	1.67
	RT	B	0.198	0.132	1.65
(Mean value ± SD)[b]	—	—	—	0.15 ± 0.012	—
Nuclear extract[c]	37	A	1.05	0.600	1.05[c]

RT, room temperature.
[a]From a single preparation of commercial powder of *S. cerevisiae* cells.
[b]Mean value of five independent determinations on: three different preparations of yeast commercial powder and two preparations of baker's yeast under any incubation conditions.
[c]Nuclei from *S. cerevisiae* cultured cells (strain 913 a-1; wet weight).

TABLE II. Effect of Some Compounds on Yeast ADPribosylating Activity

Compound	mM[a]	mUnits/mg protein[b]	% Inhibition
None	—	0.120	—
3-aminobenzamide	10	0.070	42
Nicotinamide	25	0.096	20
Theophylline	15	0.078	35
DTT	1	0.100	16
NADP	5	0.100	16

[a]Concentration giving the highest inhibition.
[b]The DNase supernatant (20 μg) from yeast cell powder was tested.

NADP. The maximal inhibition (42%) was observed in the presence of 10 nM 3-aminobenzamide (3-ABA), whereas 25 nM nicotinamide and 15 nM theophylline gave 20% and 35% inhibition, respectively.

The enzyme band was revealed after electrophoresis of homogenates on polyacrylamide (12%) gel in sodium dodecyl sulphate and Western blotting, followed by immunorevelation with anti-PARP 1 catalytic site antibodies (Fig. 1). The chemiluminescent signal was localized in correspondence of a molecular mass between 80 and 90 kDa (Fig. 1). A second weak immunosignal was present around 50 kDa. Whether it is a proteolytic fragment of 90 kDa protein or represent a different (ADPribosyl)ating activity has to be investigated.

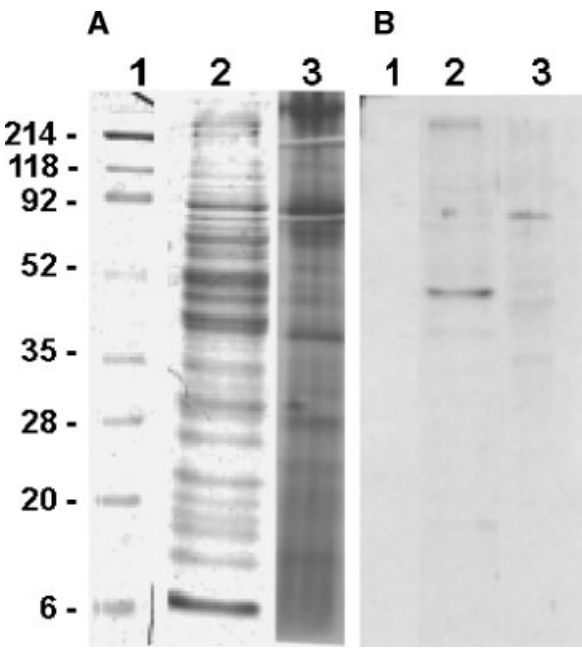

Fig. 1. SDS-polyacrylamide (12%) gel electrophoresis stained in 0.1% Coomassie (**A**) and immunoblot (**B**) of yeast homogenate. 1. Molecular weight markers; 2. DNase supernatant from *S. solfataricus* (20 μg); 3. homogenate from yeast powder (20 μg).

Protein Acceptors

To detect (ADPribosyl)ated proteins different yeast preparations were incubated with [*adenylate*-^{32}P]NAD, and subjected to SDS–PAGE and autoradiography. Protein patterns of homogenate and extract from yeast powder, and nuclei from *S. cerevisiae* strains, selected as NAD^+ producers [Gulyamova et al., 2001], are shown in Figure 2. Nuclei were particularly enriched of low molecular weight proteins, although the electrophoretic pattern reproduced the one of homogenate from dry yeast cells. Most radioactivity was revealed at level of three proteins, in the molecular mass range 40–100 kDa. Minor diffuse labeling was evident in the lowest part of the gel.

Stability of ADPR–Protein Complexes and Reaction Products

Table III shows the resistance of [^{32}P] ADPR–protein complexes to some chemicals known to influence either the N-glycosidic (hydroxylamine) or the carboxy ester bond (alkali). Only in the presence of NaOH [^{32}P] ADPR was dissociated from proteins by nearly 50% as compared with the untreated sample. Hydroxylamine and hydrochloric acid did not affect ADPR–protein stability up to 1 and 3 h incubation, respectively.

[^{32}P] labeled proteins from yeast powder homogenate and DNase supernatant, and nuclear extract from cultured *S. cerevisiae* cells, in duplicate, were incubated in the presence of proteinase K and one of the two samples was further digested with phosphodiesterase. The digestion products were analyzed by thin layer chromatography (Fig. 3). In the absence of phosphodiesterase most radioactivity was at the origin (H, S), as expected for intact elongated ADPribose chain, and an intense spot (H, S, N) was localized in the middle between the origin and R*f* of ADPribose/iso-ADPribose, which migrate very close each other, under the experimental conditions described. A weak band at level of standard ADPR was evident for yeast powder homogenate and DNase supernatant, but not in nuclei. After PDE digestion (lanes with asterisks) the whole radioactivity corresponded to ADPribose/iso-ADPribose uv spots. No detectable labeling was at level of 5′-AMP. The middle band disappeared, indicating that in this compound PDE-sensitive bonds

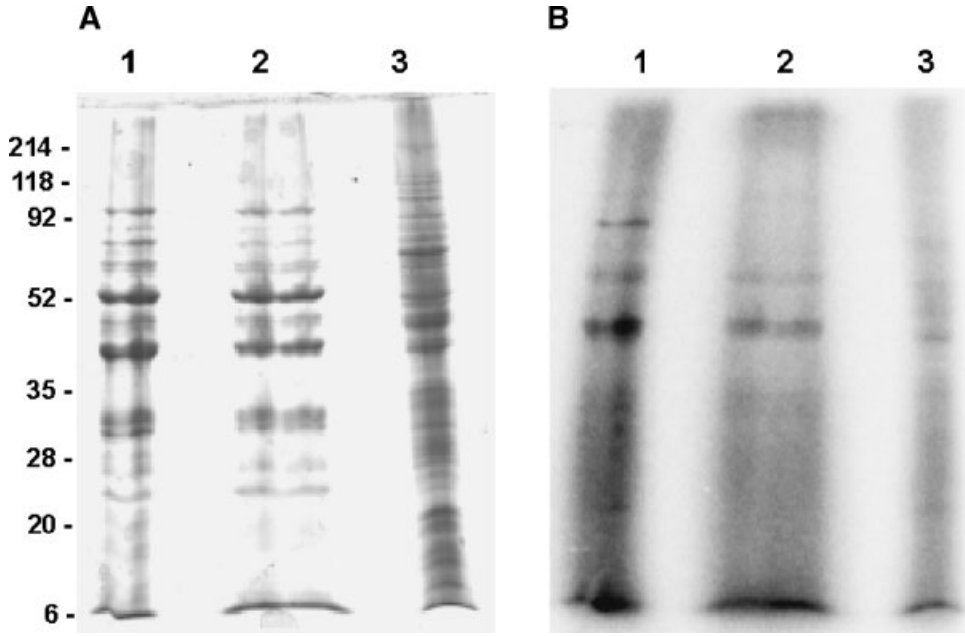

Fig. 2. SDS-polyacrylamide (12%) gel electrophoresis stained in 0.1% Coomassie (**A**) and autoradiography (**B**) of yeast preparations after incubation with [^{32}P]NAD. 1. Homogenate (20 μg) and 2. DNase supernatant (20 μg) from yeast powder; 3. nuclear extract (20 μg) from *Saccharomyces cerevisiae* strains, selected as NAD^+ producers.

were present. It cannot be excluded that this spot corresponds to dimers or trimers of ADPribose.

DISCUSSION

In *S. cerevisiae* the central role of NAD^+ as substrate of many enzymes is well established [Frye, 2000; Chiarugi, 2002; Denu, 2003; Berger et al., 2004]. The unique ADPribose synthesis described till now as certain, is the one catalyzed by Sir deacetylating enzymes, identified from yeast genome on the basis of similarity with other eukaryotic deacetylases [Sandmeier et al., 2002; Rongvaux et al., 2003; Berger et al., 2004].

TABLE III. Stability of yeast ADPR–Protein Complexes

Chemical[a]	M	Incubation time (min)	Bound ADPribose (nmol)
—	—	30–180	0.190–0.185
NaOH	0.3	30[b]	0.097
		180[b]	0.095
NH_2OH	3.0	60[c]	0.180
HCl	0.1	180[c]	0.187

[a][^{32}P] ADPR–protein complexes from yeast powder were incubated in the presence and absence of indicated compounds.
[b]At 56°C.
[c]At 25°C.

In the past the classic (ADPribosyl)ation reaction in yeast was put in discussion. The first "negative" results were based on immunoblot experiments, performed with either polyclonal or

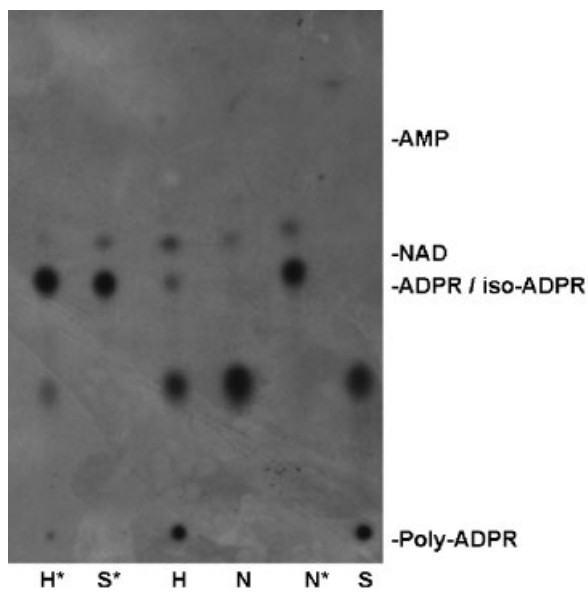

Fig. 3. After thin layer chromatography, the plate was exposed to autoradiography film. Autoradiography of reaction products solubilized after digestion of yeast [^{32}P] ADPribose–protein complexes with proteinase K (H, S, N), and proteinase K and phosphodiesterase (H*, S*, N*). Mobilities of standard compounds are reported. H, homogenate; S, DNase supernatant; N, nuclear extract.

monoclonal anti-PARP antibodies able to recognize specifically the N-terminal DNA-binding domain [Hayaishi and Ueda, 1977; Lamarre et al., 1988; Simonin et al., 1991]. From our experiments it seems that yeast enzyme is smaller than PARP 1 of about 20 kDa, roughly the size of zinc finger motif, and that activity is not influenced by fragmented DNA. At time of first negative results only PARP 1 was known and much attention was payed to the size (116–120 kDa) of immunostained proteins. In fact Kameshita et al. [1985] who used monoclonal antibodies against the whole PARP-1, reported that "... no protein band corresponding to 120 kDa was stained in the case of ... yeast; however, cross-reactive materials of ... 40 kDa were stained ..." The authors conclude that they will investigate "whether the cross-reactive proteins in these species are merely endogenous degradation products of the native enzyme." In the light of these partial observations, weak or negative results of activity blot experiments by others were taken as conclusive, not being supported, once again, by positive immunostaining of yeast preparations with anti-PARP zinc finger antibodies [Scovassi et al., 1986; Simonin et al., 1991].

Thus the statement that yeast lacks (ADP ribosyl)ation reactions starts from the cited articles and has been transmitted up today [Perkins et al., 2001]. Furthermore recently it has been reported that in *S. cerevisiae* genome no DNA sequence similar to known (ADPribosyl) transferase/poly(ADPribose) polymerase genes has been found [Rouleau et al., 2004].

On the other hand, over the last 30 years few articles based on biochemical experiments were in favor of the occurrence of the (ADPribosyl)ation reaction in *S. cerevisiae*, although none of them reported an extensive and exhaustive analysis of this question [Hall, 1965; Sugimura et al., 1968; Ruggieri et al., 1988, 1990; Gulyamova et al., 2001].

In our knowledge, previous negative results have never been revised in the light of the discovery of new, sometime DNA-independent members of PARP family [Kickhoefer, 1999; Smith, 2001].

With the present article the ring we try to add to the chain of previous information includes the evaluation of the question on the basis of results obtained with classic experiments, that until a recent past were considered basic to unequivocally characterize an (ADPribosyl)ating system.

Performing this kind of analyses we got good evidence that (ADPribosyl)ation in yeast is an active reaction and can be distinguished from Sir activity. The latter is highly inhibited by nicotinamide [Anderson et al., 2003], as opposite to the enzyme identified with our analyses (Table II). Yeast (ADPribosyl)ating enzyme is a protein of 80–90 kDa, cross-reacts with antibodies against anti-PARP catalytic site, and is DNA-independent, as determined by activity assay in the presence of the nucleic acid. Yeast enzyme synthesizes products, that, after digestion with PDE, co-migrates with iso-ADPribose (phosphoribosylAMP) on TLC plates, suggesting the presence of ADPR oligomers at least. This result excludes the synthesis of other products like (nicotinic acid adenine dinucleotide phosphate, NAADP), that gives rise to different PDE digestion products as 5′-AMP [Churchill and Galione, 2001; Lee, 2001], and cADPR, improbable substrate of PDE. The behavior on TLC plate is comparable for both crude extracts and nuclear preparations, although mono-ADPribose is evident in yeast homogenate and DNase supernatant after proteinase K digestion. One can hypothesize that the slight immunostained band at 50 kDa might account for a different ADPribosylating activity (mono(ADPribosyl)ation?), but we cannot exclude that it represents a proteolytic fragment of the 90 kDa protein, as suggested by Kameshita et al. [1985]. The present results do not allow to draw a final conclusion.

Furthermore, independent experiments on various yeast preparations made by authors in two different laboratories were highly comparable. In fact data collected from nuclear preparations of cultured cells, preliminarily reported [Gulyamova et al., 2001] and extended in the present study, were in line with those from baker's yeast and lyophilized yeast cells (Table I). The previous evidence that in nuclei (ADPribosyl)ated proteins are present [Gulyamova et al., 2001], is here supported by SDS–PAGE of acceptor proteins (Fig. 2), similarly labeled and comparably alkali-labile in both nuclei and crude extracts. Once again, the nature of these acceptors needs further investigation. However it is interesting that one of the three major ADPR acceptors has a molecular mass (50 kDa) close to that of yeast NMAT, reported as likely ADPribosylated by an endogenous enzyme [Ruggieri et al., 1990].

Few years ago all these results would have been usually taken as adequate to tag an enzyme as involved in the (ADPribosyl)ating reaction. Nowadays, the availability of yeast genome sequence raises doubts since no nucleotide sequence similar to ADPRT/PARP genes has been found, with understandable statements that no (ADPribosyl)ation reaction occurs in yeast [Herceg and Wang, 2001; Shall, 2002; Rouleau et al., 2004]. This observation has prompted researchers to use yeast cells as an in vivo system to test the PARP inhibiting effect of some compounds [Perkins et al., 2001]. It has been reported that the constitutive expression of human PARP inhibits yeast cell growth, through extensive automodification of PARP with long poly(ADPribose) chains which regulate chromosomal proteins likely by non-covalent interactions [Kaiser et al., 1992]. Suppressing PARP activity restores the normal yeast growth. It is worth nothing that expression of PARP truncated at N-terminus has no influence on cell proliferation at all: in fact the N-terminal DNA-binding domain contains the first zinc finger able to recognize DNA breaks for PARP activation that leads to cell growth inhibition [Kaiser et al., 1992; Collinge and Althaus, 1994].

The fact that yeast (ADPribosyl)ation enzyme is smaller in size than PARP, probably lacking zinc fingers, is independent from DNA breaks, and forms oligomers (5–6 ADPribose residues) are possible explanations for the normal growth of yeast cells.

On the other hand the recent (ADPribosyl)ation history teaches that evolutive distance can determine essential structural differences among similarly functioning enzymes and probably these differences occur in yeast protein. Within (ADPribosyl)ation field the power of searching genome sequence data to find distant homologues was showed by the discovery of >20 new putative bacterial mono-ADPRTs (ARTs) and their human counterparts with new sequence analysis approaches, although this finding waits for experimental evidence that these genes actually encode ART activity [Pallen et al., 2001]. However, growing evidence indicates that in organisms evolutionarily distant, proteins with the same function do not necessarily share high similarity of primary structure. On many occasions the similarity between a protein and its functionally characterized homologs is so low that computational methods for sequence analysis need to be expanded and re-newed to give significant and unequivocal results [Makarova and Koonin, 2003]. A limit of the present study is indeed the unavailability of purified enzyme to get protein sequence and to help understanding the question; but it gives the experimental evidence, which does not exclude the possibility of structurally different, but functionally related proteins, as suggested in other systems under study [Faraone-Mennella et al., 1996, 1998].

On our opinion it is necessary to study better, in available genomes, hypothetical proteins with still unknown functions, perhaps, as stated above, with the help of new computational approaches and programs, to compare them with evolutionary distant counterparts.

In recent years we have learned that scientific "dogmas" can be revisited and remodelled in the light of results from new experimental and computational approaches, as, for instance, the discovery of new ARTs, of a crowded family of PARPs, of a non-nuclear localization of some of them and, sometime, of their independence from DNA-breaks [Kickhoefer, 1999; Shall and de Murcia, 2000; Herceg and Wang, 2001; Pallen et al., 2001; Cohen-Armon et al., 2004; Rouleau et al., 2004].

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