Regulation by phosphorylation of *Xenopus laevis* poly(ADP-ribose) polymerase enzyme activity during oocyte maturation

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Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme that is dependent on DNA breaks and nicks for its enzyme activity. These DNA nicks and breaks function as allosteric effectors of the enzyme activity. This reaction is important for efficient DNA base excision repair, although it is not a component of the elementary repair pathway itself. The physiological relevance of this reaction might be to ensure correct and efficient DNA repair. We have examined the enzyme activity of PARP in oocytes and eggs of *Xenopus laevis*. Although both oocytes and eggs contain approximately the same amounts of enzyme protein, there is no detectable enzyme activity in the oocytes, whereas in the eggs the enzyme is active. Enzyme activity appears during oocyte maturation, approx. 4 h after induction by progesterone. This enzyme activation coincides

with the appearance of active maturation-promoting factor. Enzyme activation is accompanied by a shift in the electrophoretic mobility of the polypeptide, from an apparent molecular mass of 116 kDa to 125 kDa. Treatment with either bacterial or potato phosphatase reverses the mobility shift and abolishes enzyme activity. Incubation of maturing *X. laevis* eggs with radioactive inorganic phosphate and subsequent immunoprecipitation demonstrate that the PARP protein is phosphorylated *in vivo*. We show that maturation-promoting factor (Cyclin B/cdc2) cannot itself be responsible for the phosphorylation and activation of PARP in maturing *X. laevis* eggs. Together, these results demonstrate that the enzyme activity of PARP in *X. laevis* oocytes and eggs is regulated by post-translational, covalent phosphorylation.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme that is absolutely dependent on DNA single-strand nicks or double-strand breaks for its enzyme activity [1–5]. On binding to DNA with activating nicks or breaks, this enzyme synthesizes a homopolymer of (ADP-ribose)_n, which can be either linear or branched. The enzyme exclusively utilizes NAD⁺ as its substrate, a co-enzyme that is synthesized in the nucleus but not in the cytoplasm. The protein has been well characterized [6–10] and consists of an N-terminal 42 kDa DNA-binding domain (amino acid residues 1–372), containing two zinc-fingers of an uncommon construction [10]. The function of the zinc-fingers is only partly understood. The second zinc-finger, however, seems to be part of the DNA nick recognition structure [11]. There is a striking similarity between the zinc-fingers in PARP and an N-terminal zinc-finger in DNA ligase III [12]. There is also a C-terminal 55 kDa catalytic domain (amino acid residues 524–1014) to which the NAD⁺ substrate binds. This enzyme can catalyse three apparently different reactions, namely initiation of the chain, elongation and branching. All three catalytic functions are present in the isolated C-terminal catalytic domain. This isolated domain, however, has only 0.2 % of the activity of the complete enzyme. It might be that the first zinc-finger transmits an allosteric activation to the catalytic domain when the enzyme binds to a DNA nick. There is also a central domain of approx. 17 kDa (from residue 372 to residue 524), which is less well characterized and has been variously described as the auto-modification domain, a 'leucine-zipper' dimerization region or a 'hinge' region. Portions of the catalytic domain and the zinc-fingers are highly conserved throughout evolution. This enzyme is found in almost all eukaryotic cells possessing a functional nucleus, including trypanosomes (J. Mhlanga and S. Shall, unpub-

lished work), slime moulds [13], plants [14], and all higher eukaryotes.

The function of PARP is centred on its obligatory requirement for DNA strand breaks [3–5,10]. It seems that whenever there are naked DNA strand breaks or nicks, PARP rapidly binds very tightly to the nick or break [10]. Once the enzyme has bound it immediately starts to synthesize poly(ADP-ribose). This polymer, which is usually covalently attached to a molecule of the enzyme itself, is a substantial polyanion. After a brief period a specific enzyme begins to degrade the polymer, leaving behind only short strands of the polymer [15]. An enzyme molecule that is sufficiently modified is enzymically inactive, probably because it cannot bind to the DNA break. Thus this 'nick-sensor' protein [10] binds to DNA nicks and breaks only very transiently. When the DNA break has been repaired the enzyme loses its activity. These complex enzyme reactions may fulfil several functions ensuring correct and efficient DNA base excision repair. More specifically, for this study, one physiological function of the synthesis of this polymer might be to protect the free end of DNA and to ensure that the free end does not lead to biologically undesirable DNA recombination. This protection would result from the repulsion between adjacent DNA duplexes owing to the high density of negative charges carried by poly(ADP-ribose).

This hypothesis predicts that when biologically relevant DNA recombination is to take place, the enzyme activity of PARP should be suppressed.

We have begun testing this prediction by looking at the regulation of the activity of PARP in developing oocytes of *Xenopus laevis*. These cells provide an attractive system for studying the processes and proteins of DNA replication, repair and recombination because they can be analysed both *in vivo* by micro-injection and *in vitro* by using cell-free extracts. In particular, *X. laevis* oocytes support semi-conservative DNA syn-

Abbreviations used: MPF, maturation-promoting factor; PARP, poly(ADP-ribose) polymerase; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamido-heptan-2-one (tosyl-lysylchloromethane).

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thesis, DNA repair and homologous DNA recombination. In contrast, *X. laevis* eggs that develop from oocytes after progesterone treatment complete their meiotic division and show the additional property of illegitimate DNA recombination and semi-conservative DNA replication [16,17]. Growth without cell division during oogenesis results in the accumulation of extensive stores of components for use during embryogenesis. These stores include active DNA ligase I [18,19], DNA polymerases [20] and PARP [21–23]. Hormonal stimulation of oocytes triggers both new translation and post-translational modifications without transcription (reviewed in [24]). The sequence of these events is still poorly understood, but includes the synthesis of cdk2 [25] and the synthesis of the *c-mos* gene [26], leading to the activation of cdc2 kinase and the mitogen-activated protein kinase [27]. Thus several phosphorylation events and the appearance of new proteins and enzyme activities are correlated temporally with re-entry into the cell cycle, the onset of semi-conservative DNA replication and the potential for illegitimate recombination.

Before the work reported here, there was very little evidence that the enzyme activity of PARP could be regulated. We have reported in the past that the synthesis of this enzyme is regulated at the transcriptional level in lymphocytes stimulated with mitogens [28]. This has been confirmed more recently [29,30]. In addition, Taniguchi and co-workers have demonstrated a down-regulation of the transcription of this enzyme induced by interferon γ [31]. All other examples of increases in the enzyme activity are due to activation of preformed enzyme protein by the allosteric effect of DNA breaks or nicks. It has been known for many years that PARP activity is present in *X. laevis* eggs [22,23,32–34]. Rather unusually, NAD⁺ turnover in *X. laevis* oocytes is extremely low; eggs and embryos, in contrast, possess a similar rapid turnover to somatic cells [23]. In our current study we have discovered, rather surprisingly, that there is covalent regulation, by phosphorylation, of the enzyme activity of PARP in developing *X. laevis* oocytes.

MATERIALS AND METHODS

Antibodies and immunoblotting

Two rabbit polyclonal anti-(human PARP) antibodies were used (MAS 1 and MAS 2) prepared by overexpression of human PARP cDNA in *Escherichia coli* and subsequent purification of the protein. Full-length human PARP cDNA cloned into a bacterial expression vector was a gift from Gilbert de Murcia (Strasbourg, France). Immunoblotting was performed as described previously [35] unless otherwise stated.

Chemicals and enzymes

Protein kinase p34^{cdc2}/Cyclin B complex from starfish oocytes, purified as described [36], was a gift from M. Dorée (Montpellier, France). *E. coli* alkaline phosphatase type III was purchased from Sigma and further purified to remove traces of protease activity as follows. The enzyme (3.5 mg/ml) was dialysed overnight against 50 mM Tris/HCl (pH 8.5)/1 mM MgCl₂/0.1 mM ZnCl₂. The enzyme solution was then heated at 90 °C for 10 min, chilled to 0 °C and incubated at 22 °C for 6 h. After centrifugation for 15 min at 10000 g, the supernatant was dialysed overnight at 4 °C against 10 mM Tris/HCl, pH 8.0, and then applied to a small DEAE-Sepharose (Pharmacia) column (5 cm × 1 cm) equilibrated with the same buffer. After washing of the column, the phosphatase was eluted with 300 mM NaCl/10 mM Tris/HCl (pH 8.0) and frozen in aliquots. Potato acid phosphatase was purchased from Boehringer Mannheim; the enzyme preparation was a suspension in (NH₄)₂SO₄ solution

that was pelleted by centrifugation immediately before use and dialysed overnight against 100 mM NaCl/20 mM Mes (pH 6.5)/1 mM MgCl₂. Trypsin (sequencing grade) was from Boehringer Mannheim.

Molecular mass standards

Molecular mass standards for PAGE were myosin H-chain (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (67 kDa) and carbonic anhydrase (29 kDa), all purchased from Sigma.

X. laevis oocytes, eggs and embryos

Xenopus laevis were generously donated by Dr. C. C. Ford (University of Sussex, Brighton, E. Sussex, U.K.). Oocytes were prepared from total ovaries treated with collagenase (1 mg/ml) for 1 h to dislodge the follicle and cultured in modified Barth medium [37]. Fully grown stage VI oocytes, selected in accordance with the description by Dumont [38], were used for the preparation of extracts. Unfertilized eggs were obtained from females injected with 900 i.u. of human chorionic gonadotropin.

For fertilization, the eggs were squeezed from an induced female directly into a Petri dish and fertilized with a fresh sperm suspension that had been prepared from testes crushed with forceps in F1 medium, pH 7.8 (31 mM NaCl/1.7 mM KCl/1 mM CaCl₂/2 mM NaOH/10 mM Hepes). Eggs and embryos staged by the method of Nieuwkoop and Faber [39] were dejellied in 2% (w/v) cysteine in F1 medium and frozen in liquid nitrogen until required.

Oocyte maturation *in vitro*

Stage VI oocytes, recovered as described above, were cultured in modified Barth medium containing 5 mg/ml progesterone at 22 °C to induce maturation. In the course of maturation, the oocytes complete the first meiotic division, expel the first polar body and enter the second meiotic division [40]. Zero time is defined as the time when progesterone was added. Germinal vesicle breakdown, which is a result of maturation-promoting factor (MPF) activity, was monitored by observing white spot formation in the animal pole of the oocytes. Inhibition of protein synthesis during maturation was achieved by adding 150 mg/ml cycloheximide.

Preparation of extracts

Oocytes or dejellied eggs were homogenized in a glass-Teflon homogenizer in one volume of extraction buffer [50 mM Tris/HCl (pH 8.0)/125 mM NaCl/1% (w/v) NP40/2.0 mM EDTA/5% (w/v) sucrose]; in addition, the protease inhibitors PMSF (1 mM), aprotinin (27 mg/ml) and leupeptin, pepstatin, chymostatin and tosyl-lysylchloromethane (Tos-Lys-CH₂Cl) (each at 10 mg/ml) were added. The extract was centrifuged for 15 min at 12000 g at 4 °C. The supernatant was transferred to a clean tube and centrifuged again for 120 min at 47000 g at 4 °C. The final clear supernatant was withdrawn, sampled and then snap-frozen in liquid nitrogen.

Phosphorylation and dephosphorylation of PARP *in vitro*

PARP was immunoprecipitated from oocyte extracts and was subsequently phosphorylated with active MPF in p34^{cdc2} kinase buffer [50 mM NaCl/50 mM Tris/HCl (pH 7.5)/10 mM MgCl₂/1 mM dithiothreitol] containing protease inhibitors (0.17 μ g/ml PMSF, and aprotinin, leupeptin, pepstatin and Tos-Lys-CH₂Cl, each at 10 mg/ml) in the presence of 50 μ Ci of γ -

^{32}P ATP (3000 Ci/mmol; Amersham, Little Chalfont, Bucks., U.K.) in a reaction volume of 50 μl for 30 min at 25 °C. The reaction was stopped by adding SDS sample buffer and heating for 10 min at 90 °C, followed by SDS/PAGE and autoradiography. In all experiments gel electrophoresis was performed essentially as described by Laemmli [41]. Dephosphorylation of egg extracts was performed with bacterial alkaline phosphatase in dephosphorylation buffer [50 mM Tris/HCl (pH 8.5)/1 mM MgCl_2 /0.1 mM ZnCl_2] and protease inhibitors for 30 min at 25 °C. Dephosphorylation with potato alkaline phosphatase was performed similarly, except that reaction mixtures contained 20 mM Mes (pH 6.0) instead of Tris/HCl and ZnCl_2 .

Phosphorylation *in vivo* and immunoprecipitation

Mature oocytes were labelled with 800 $\mu\text{Ci/ml}$ [^{32}P] P_i in phosphate-free modified OR2 medium [82.5 mM NaCl/2.5 mM KCl/1.0 mM MgCl_2 /1.0 mM CaCl_2 /35 mM Hepes (pH 7.8)] for 6 h at 21 °C and then matured by adding progesterone (5 mg/ml). For immunoprecipitation, matured oocytes were homogenized in lysis buffer [50 mM Tris/HCl (pH 8.0)/0.5% NP40/0.1% SDS/150 mM NaCl/100 mM NaF/100 mM sodium pyrophosphate/2 mM Na_3VO_4 /50 mM β -glycerophosphate/2.0 mM EDTA/1.0 mM dithiothreitol/1.0 mM PMSF/27 mg/ml aprotinin/10 mg/ml leupeptin/10 mg/ml chymostatin/10 mg/ml pepstatin/10 mg/ml Tos-Lys- CH_2Cl] and then centrifuged twice for 10 min at 15000 g at 4 °C. The supernatant was incubated with 20 $\mu\text{l/ml}$ of anti-(human PARP) antiserum overnight at 4 °C. The antibody complexes were collected by agitation with Protein A–Sepharose for 2 h at 4 °C and the immunoprecipitate was washed four times with the lysis buffer, eluted with SDS sample buffer and then analysed by SDS/PAGE and autoradiography. Phosphopeptide mapping and phosphoamino acids analysis were performed as described by Prigent et al. [42].

Histone H1 kinase assays during oocyte maturation

Stage VI oocytes were induced to mature with progesterone. At various times oocytes were lysed in histone H1 kinase buffer [80 mM β -glycerophosphate (pH 7.4)/15 mM MgCl_2 /20 mM EGTA/0.3 mM ATP/0.1% NP40/15 mg/ml benzamidine/10 mg/ml leupeptin/10 mg/ml pepstatin/10 mg/ml aprotinin] and then centrifuged at 10000 g for 15 min at 4 °C. For each sample, one part was used for PARP activity and another was used to measure histone H1 kinase activity. For the latter, the equivalent of 0.5 of an oocyte was assayed against 2 mg of histone H1 (Boehringer) in the presence of 1.25 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol) in a reaction volume of 20 μl for 15 min at 21 °C. For quantification of H1 kinase activity, 15 μl of the reaction was loaded on P81 phosphocellulose paper (Whatman) and after three 20 min washes in 150 mM H_3PO_4 (Merck), the paper was dried and the radioactivity estimated by scintillation counting.

Assay of enzymic activity of PARP

The activity of the enzyme was monitored by formation of PARP-ADP-ribose and/or PARP-polymer. The extract was incubated in PARP labelling buffer containing 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1.0 mM dithiothreitol, 0.3% Tween-20, 30 mM ZnCl_2 , 2.0 mM MgCl_2 and 2.0 mg/ml activated DNA in the presence of 4.0 μCi of [^{32}P]NAD $^+$ [30 Ci/mmol; NEN] in a reaction volume of 20 μl . Incubations were at 25 °C for 1 min unless otherwise stated. SDS sample buffer was added, then reaction mixtures were heated at 90 °C for 10 min and analysed by SDS/PAGE. Labelled PARP was detected by autoradio-

graphy. Quantification of PARP activity was estimated by cutting out and counting the radioactive PARP gel bands, which ran just above the apparent molecular mass marker 116 kDa.

RESULTS

PARP activity is present in unfertilized *X. laevis* eggs

The activity of the enzyme was assayed by displaying the formation of poly(ADP-ribose) as described in the Materials and methods section. Radioactively labelled PARP was detected after SDS/PAGE by autoradiography. Under these conditions, in a very short incubation at a low concentration of NAD $^+$, most incorporated ADP-ribose was associated with the PARP protein itself. When the incubation time was increased, the mobility of the PARP decreased markedly, indicating that its apparent molecular mass had increased considerably on attachment to poly(ADP-ribose). We observed considerable PARP activity in unfertilized *X. laevis* eggs (Figure 1A), in agreement with previous reports [23,32,34]. Furthermore, in a quantitative assay of crude

Figure 1 Automodification and quantitative analysis of PARP enzyme activity

(A) Egg extracts were incubated at 25 °C with buffer containing [^{32}P]NAD $^+$ for various times. The reaction was stopped by adding SDS sample buffer and heating for 10 min at 90 °C. Each reaction mixture was analysed by SDS/PAGE [10% (w/v) gel] followed by autoradiography. The positions of molecular mass markers are indicated at the left. Lanes 1 and 2, 20 s; lane 3, 30 s; lane 4, 1 min; lane 5, 5 min; lane 6, 10 min. (B) Relationship between PARP activity and protein content. PARP assays were performed as described in the Materials and methods section with crude protein extracts from unfertilized eggs (1, 10, 20 and 100 mg). The radioactively labelled PARP was analysed by SDS/PAGE and detected by autoradiography. For quantification, the bands containing radioactively labelled PARP were excised from the polyacrylamide gel and the radioactivity was estimated by counting Čerenkov radiation.

Figure 2 Absence of PARP activity from oocytes

Oocyte extracts were prepared as described in the Materials and methods section; PARP enzyme activity was then assayed (**A**), and the presence of PARP protein was investigated by Western blotting with a polyclonal antiserum against human PARP (**B**). Samples in (**A**) and (**B**) were the same. Lane 1, unfertilized egg extract (control); lanes 2 and 3 were two different oocyte extracts prepared from two different females. The positions of molecular mass markers are indicated at the left.

extracts, we observed a dependence of enzyme activity on protein concentration (Figure 1B). The quantification of PARP activity was achieved by estimating the radioactivity at the position of the PARP protein on the acrylamide gel. At short incubation times (1 min) with radioactive NAD^+ the area on the polyacrylamide gel containing the labelled PARP was very limited, allowing an accurate quantification of the activity by cutting out the radioactive PARP gel band, and then estimating the radioactivity in the band.

Absence of PARP activity from oocytes

Oocyte extracts were prepared as described in the Materials and methods section, and PARP activity was assayed. PARP activity was absent from all the extracts that we tested (Figure 2A, lanes 2 and 3), although unfertilized egg extracts assayed in parallel were enzymically active (Figure 2A, lane 1). In contrast, immunoblotting revealed the presence of PARP protein in the enzymically inactive oocytes, as well as in the enzymically active eggs. The absence of enzyme activity was not a result of loss or degradation of protein; this was shown by immunoblotting experiments (Figure 2B), which clearly indicate the presence of PARP protein in the enzymically inactive oocytes. The dilution of extracts with buffer failed to remove this inhibition (Figure 3), indicating that the absence of PARP activity from oocyte extracts is not due to the very concentrated nature of these extracts. Even after dilution by 1:5 to 1:50, the egg extracts contained considerable enzyme activity (Figure 3, lanes 6–8).

Electrophoretic mobility shift of PARP during oocyte maturation

Immunoblotting experiments with *X. laevis* oocytes and unfertilized egg extracts yielded two electrophoretic forms of PARP,

Figure 3 PARP activity in oocytes is not recovered by dilution

Oocyte and unfertilized egg (UFE) extracts were diluted in labelling buffer containing protease inhibitors; the extracts were then assayed for PARP enzyme activity as described in the Materials and methods section. Lanes 1 and 5, undiluted extracts (controls); lanes 2 and 6, 1:5 dilution; lanes 3 and 7, 1:10 dilution; lanes 4 and 8, 1:50 dilution. The positions of molecular mass markers are indicated at the left.

Figure 4 Altered migration of PARP during oocyte maturation and after phosphatase treatment

Unfertilized egg extracts (UFE; 100 μg of protein) were incubated for 30 min at 22 $^{\circ}\text{C}$ with bacterial alkaline phosphatase (BAP) or with potato acid phosphatase (PAP) in the presence or absence of phosphatase inhibitor (100 mM phosphate); or the egg extracts were treated for 90 min at 25 $^{\circ}\text{C}$ with 100 mM KOH/50 mM EDTA (alkali). After treatment, SDS sample buffer was added and the proteins were separated by electrophoresis on a 0.1% SDS/10% (w/v) polyacrylamide gel followed by Western blot analysis with an anti-(human PARP) polyclonal antibody. For detection, horseradish peroxidase-labelled second antibody was used with the enhanced chemiluminescence detection system (Amersham). All the tracks on the gel contained unfertilized egg extracts, except for that labelled oocyte. The different treatments are indicated at the top of the figure. The positions of molecular mass markers are indicated at the left.

corresponding to apparent molecular mass estimates of 116 kDa for the oocyte PARP and in addition, a second form of approx. 125 kDa in unfertilized eggs. To determine whether the additional band with the changed electrophoretic migration had been modified either by ADP-ribosylation or by phosphorylation of the protein, oocyte and egg extracts were treated with either bacterial alkaline phosphatase or potato acid phosphatase in the presence of protease inhibitors. As a control, similar extracts were also incubated with phosphate buffer (and phosphatase enzyme) as an inhibitor of the phosphatases. Dephosphorylation of the unfertilized egg enzyme caused an increase in its rate of migration on gel (Figure 4), corresponding to a decrease in apparent molecular mass from 125 to 116 kDa. Addition of sodium phosphate at pH 8.0 (100 mM final concentration) to some samples inhibited this change in apparent molecular mass, confirming that the observed change in apparent size was caused by dephosphorylation. Alkali treatment of the extracts, to cause the hydrolysis of mono-ADP-ribose to 5'-AMP [43], did not cause any decrease in apparent molecular mass of the 125 kDa

form of PARP. The alkaline treatment should also have released poly(ADP-ribose) from the protein; because there was no change in apparent size after alkali treatment we can also exclude the presence of poly(ADP-ribose) in the more slowly migrating form of the protein. These results are consistent with the conclusion that the mobility change observed between oocytes and unfertilized eggs reflects different phosphorylation states of PARP.

Activation of PARP during maturation and early development

To determine the timing of the appearance of the PARP enzyme activity during oocyte maturation, stage VI oocytes were induced to mature with progesterone as described in the Materials and methods section. At 1 h intervals after induction, oocyte extracts were prepared and divided into two aliquots: in the first, PARP activity was assayed by SDS/PAGE and autoradiography (Figure 5A); the second aliquot was assayed for histone H1 kinase activity (Figure 5B). PARP enzyme activity appeared at approx. 4 h after incubation with progesterone; this activation of the enzyme coincided with the appearance of histone H1 kinase activity. Cycloheximide blocked oocyte maturation induced by progesterone; it also inhibited the appearance of PARP enzyme activity in maturing oocytes (Figure 5A, lanes a and b). These results indicate that PARP activation requires protein synthesis during the maturation process and that the activation of PARP roughly coincides with the appearance of histone H1 kinase activity, which is usually taken as an indication of cdk1 (cdc2) enzyme activity. After fertilization, PARP enzyme activity is detectable for at least 7 h of development (Figure 5C).

PARP protein is phosphorylated *in vivo* in *Xenopus* eggs

The enzyme was immunopurified from crude extracts of eggs matured in the presence of [32 P]P_i. Analysis of immunoprecipitates by SDS/PAGE and autoradiography showed a radioactive protein band that was present at the position expected for PARP (Figure 6, middle lane). The first step in the PARP enzyme reaction is the binding of NAD⁺, followed by the formation of an enzyme-ADP-ribose complex, with the release of nicotinamide. Therefore at least some of the PARP molecules can be expected to have incorporated 32 P because of the binding of NAD⁺ and/or polymerization from [32 P]NAD⁺, which would have been formed during metabolic labelling. Thus the radioactivity associated with the PARP protein might be in NAD⁺, covalent phosphate esters, ADP-ribose oligomer or polymer. However, most of the radioactive material was removed by treatment with bacterial alkaline phosphatase (Figure 6, right lane). When 100 mM phosphate was added as a phosphatase inhibitor, the phosphatase did not remove the radioactivity (Figure 6, left lane), indicating that the radioactive phosphate was in the form of covalent phosphate esters. Bacterial alkaline phosphatase does not cleave oligomers or polymers of ADP-ribose, thus confirming that the observed effects were caused by dephosphorylation. These results demonstrate that PARP in *Xenopus* eggs is a phosphoprotein containing one or several modified amino acid residues susceptible to phosphatase treatment.

Timing of PARP phosphorylation during oocyte maturation

To investigate more precisely the timing of the phosphorylation of PARP protein during oocyte maturation, stage VI oocytes were metabolically labelled by incubation with [32 P]P_i. Maturation was induced by progesterone; in these experiments 50% germinal vesicle breakdown had occurred by 4 h and was 95% complete by 7 h. At 1 h intervals after the addition of progesterone, oocyte extracts were prepared and PARP protein was

Figure 5 Time of appearance of PARP enzyme activity during maturation and early development of *Xenopus laevis* oocytes

Stage VI oocytes were matured *in vitro* by incubation with progesterone. At various stages of maturation, oocytes were recovered and protein extracts were prepared and then assayed for PARP activity (A) and histone H1 kinase activity (B). The onset of germinal vesicle breakdown occurred at approx. 4 h after progesterone treatment. Inhibition of protein synthesis during maturation was achieved by adding cycloheximide to oocytes and incubating for 7 h (A; lane a) or by adding it to oocytes 1 h before adding progesterone and then incubating for 7 h (A; lane b). (C) Enzyme activity of PARP during early development. Unfertilized eggs (UFE) and embryos were obtained as described in the Materials and methods section; protein extracts from different stages of embryogenesis were prepared and assayed for PARP enzyme activity. The positions of molecular mass markers are shown at the left (in kDa) in (A) and (C).

immunoprecipitated and analysed by SDS/PAGE and autoradiography (Figure 7A). PARP protein containing radioactive phosphate began to appear approx. 4 h after incubation with progesterone. The radioactivity associated with PARP during oocyte maturation was removed by bacterial alkaline phosphatase (Figure 7B, right lane). An immunoblotting experiment confirmed that the incorporation of radioactivity coincided with the appearance of the more slowly migrating form of the protein (results not shown).

Figure 6 Phosphorylation of PARP *in vivo*

Oocytes were incubated with [32 P] P_i in phosphate-free medium during maturation with progesterone as described in the Materials and methods section. The PARP in these cells was then immunoprecipitated in the presence of proteases and phosphatase inhibitors, with a polyclonal rabbit anti-(human PARP) antibody. The antibody-antigen complex was captured with Protein A-Sepharose and washed. The immunoprecipitate was divided into three aliquots and treated as follows: left lane, control, no treatment; middle lane, treated with 0.5 unit of bacterial alkaline phosphatase (BAP) in the presence of 100 mM phosphate as enzyme inhibitor; right lane, treated only with 0.5 unit (as defined by the manufacturer) of bacterial alkaline phosphatase. The immunoprecipitates were then separated by electrophoresis and the radioactive phosphate was detected by autoradiography.

Figure 7 Time course of labelling of PARP *in vivo* during maturation

Phosphorylation of PARP *in vivo* was achieved by maturation of stage VI oocytes in the presence of [32 P] P_i ; 50% germinal vesicle breakdown was observed at 4 h. (A) PARP was immunoprecipitated at 1 h intervals during maturation; enzyme activity was analysed by SDS/PAGE and autoradiography. (B) Phosphatase treatment of immunoprecipitated PARP from matured oocytes. Lane 1 (BAP—), immunoprecipitate without further treatment; lane 2 (BAP+), immunoprecipitate treated with 0.5 unit of bacterial alkaline phosphatase. The numbers above (A) indicate the duration (h) of progesterone treatment. The positions of molecular mass markers are indicated at the left.

Figure 8 Phosphorylation of PARP by purified cyclin B/p34^{cdc2} *in vitro*

PARP was immunoprecipitated from stage VI oocytes and then incubated with purified, active MPF in the appropriate buffer in the presence of [γ - 32 P]ATP for 30 min at 25 °C, then analysed by SDS/PAGE and autoradiography. Lane 1, immunoprecipitation with pre-immune serum; lane 2, immunoprecipitation with a rabbit polyclonal (MAS 1) antibody against human PARP; lane 3, immunoprecipitation with a different rabbit polyclonal (MAS 2) antibody against human PARP.

Figure 9 Inhibition of enzymic activity of PARP by phosphatase treatment

Unfertilized egg extracts were incubated at 22 °C with a phosphatase in the appropriate buffer and then assayed for PARP enzyme activity. (A) Kinetics of inactivation of PARP by treatment with 0.5 unit of bacterial alkaline phosphatase. Times of incubation (min) with bacterial alkaline phosphatase are indicated above the lanes. (B) Decrease of PARP activity after dephosphorylation with increasing amounts (units; U) of potato alkaline phosphatase (PAP). Incubation time with the phosphatase was 30 min. (C) Aliquots of the extract before (—) and after (+) exposure to 0.5 unit (U) of bacterial alkaline phosphatase (BAP) for 30 min, were analysed by Western blotting with anti-(human PARP) antibody (top panel), anti-(*Xenopus* DNA ligase I) antibody (middle panel) and anti-proliferating-cell nuclear antigen (bottom panel) to monitor the efficiency of the dephosphorylation reaction and the absence of protein degradation respectively. The radioactivity was detected by autoradiography.

These results raise the question of which protein kinase is ultimately responsible for the phosphorylation and activation of the PARP enzyme. To determine whether the MPF kinase (cyclin B/cdc2) itself could be directly responsible for the phosphorylation and activation of PARP, we investigated first whether the PARP from oocytes could be phosphorylated by purified active MPF and, secondly, whether such phosphorylation activates the enzyme. Purified, active MPF is able to phosphorylate immunopurified PARP from oocyte extracts *in vitro* (Figure 8, lanes 2 and 3). However, phosphorylation of the PARP protein by active MPF induces neither enzyme activation nor the shift in electrophoretic migration of the polypeptide observed *in vivo* (results not shown).

Inactivation of PARP activity by phosphatase treatment

Egg extracts were incubated in appropriate reaction mixtures with potato acid phosphatase or bacterial alkaline phosphatase

Figure 10 Phospho amino acid analysis of *Xenopus laevis* PARP phosphorylated *in vivo* and *in vitro*

PARP was hydrolysed and mixed with unlabelled standards: phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). Phospho amino acids were separated by electrophoresis on thin-layer cellulose plates and detected by autoradiography. Non-radioactive reference compounds were revealed by ninhydrin staining.

to dephosphorylate the proteins. The activity of the enzyme was then assayed. The ability of PARP to form enzyme-polymer complexes was much decreased or completely abolished after incubation with the phosphatases (Figure 9), as a function both of time (Figure 9A) and of amount of phosphatase added (Figure 9B). Control experiments showed that *Xenopus* DNA ligase I was dephosphorylated under these conditions and that there was no loss of protein with proliferating-cell nuclear antigen as a test protein (Figure 9C). Immunoblotting experiments demonstrated that there was no detectable loss or degradation of the PARP protein during these incubations (Figure 9C), although PARP enzyme activity was obliterated. These results show that dephosphorylation of one or more phosphoamino acids of the *X. laevis* egg PARP protein abolishes the enzyme activity.

Figure 11 PARP is phosphorylated on the same peptide *in vivo* and by MPF *in vitro*

Two-dimensional analysis of tryptic phosphopeptides of *Xenopus laevis* PARP after phosphorylation *in vivo* and *in vitro*. PARP phosphorylated with ^{32}P , labelled *in vitro* or *in vivo* or a mixture of both, was digested with trypsin and peptides were separated by electrophoresis followed by chromatography. Radioactive peptides were detected by autoradiography. The arrow indicates a major peptide obtained after both labelling with p34^{cdc2} *in vitro* and labelling *in vivo*. Note that the three panels are not perfectly aligned: the arrow indicates the same peptide in all three panels.

Formation of phosphoserine *in vivo* and *in vitro*

We next determined the nature of the phospho amino acids generated both *in vivo* and *in vitro*. Labelled PARP was immunoprecipitated from eggs matured in the presence of $^{32}\text{P}_i$ and hydrolysed with 6 M HCl at 110 °C to liberate free phospho amino acids. The phospho amino acids, together with appropriate reference compounds, were separated by electrophoresis on thin-layer cellulose plates. In intact oocytes, phosphoserine was shown to be dominant by autoradiography, and a phosphotyrosine signal appeared after longer exposure, whereas phosphothreonine was not detected (Figure 10). Similar phospho amino acid analysis of oocyte PARP phosphorylated *in vitro* by purified MPF showed that phosphoserine was generated exclusively.

PARP phosphorylation sites *in vivo* and *in vitro*

Immunoprecipitated PARP from eggs metabolically labelled with ^{32}P , or PARP phosphorylated *in vitro* by purified MPF in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, were extensively digested with trypsin. The resulting phosphopeptides were resolved by two-dimensional separation on thin-layer plates, by electrophoresis and ascending chromatography, and detected by autoradiography. The PARP that was labelled *in vivo* generated several distinct, strongly labelled peptides and a few weakly labelled peptides (Figure 11, middle panel). Some of the latter might have been due to partial trypsin cleavage at doubly basic residues. One of the phosphopeptides from the material labelled *in vivo* co-migrated with the major phosphopeptide phosphorylated by MPF *in vitro* (Figure 11, left panel) as shown by analysis of a mixture of the two samples (Figure 11, right panel).

DISCUSSION

There is substantial PARP enzyme activity in unfertilized eggs of *X. laevis*; in contrast, no enzyme activity is apparent in oocytes despite the presence of about the same amount of enzyme protein in the oocytes, as indicated by Western immunoblotting. The absence of enzyme activity is due neither to loss nor to degradation of the protein. Dilution of the oocyte extracts does not restore enzyme activity. These results suggest that we were observing a protein modification, rather than a soluble, reversible inhibitor. Immunoblotting experiments showed that oocytes contain immunoreactive material of an apparent molecular mass of approx. 116 kDa. In unfertilized eggs the antibody recognized in addition a second band, with an apparent molecular mass of 125 kDa. We considered the possibility that the enzyme might be post-translationally modified either by phosphorylation or by ADP-ribosylation. We observed that two different phosphatases were able to alter the apparent molecular mass of the enzyme on Western blots. In contrast, alkali treatment, which should remove mono- or poly(ADP-ribose) from the protein, did not alter the apparent molecular mass of the band recognized by the anti-serum. Furthermore we showed that the immunoprecipitable PARP protein could be labelled by inorganic phosphate in the medium in which the oocytes were matured. We therefore conclude that during maturation of *X. laevis* oocytes to unfertilized eggs, PARP is phosphorylated, which switches on the enzyme activity of the PARP protein.

PARP phosphorylated *in vivo* yielded phosphoserine and, to a smaller extent, some phosphotyrosine. The phosphorylation of PARP *in vitro* (purified from egg extracts) by purified MPF led only to phosphoserine; no phosphotyrosine was observed. The purified enzyme, after phosphorylation *in vitro* by purified MPF, was digested with trypsin and then gave a dominant phospho-

peptide; when PARP phosphorylated in intact cells was purified and subjected to tryptic digestion, it gave the same phosphopeptide plus a second prominent phosphopeptide. Taken together these experiments clearly demonstrate that the radioactive labelling of PARP is due to phosphorylation and that this phosphorylation event coincides in time approximately with the activation of MPF. Activation of MPF is completed when there is 50 % germinal vesicle breakdown and is required for oocyte maturation in *Xenopus*. This conclusion is also strongly supported by the results presented in Figure 6. Thus the formation and activation of cyclin B/p34^{cdc2} kinase is required for both the phosphorylation and mobility shift of PARP during the maturation of *Xenopus* oocytes. In addition, we have shown that PARP is phosphorylated *in vivo*.

Although purified MPF was able to phosphorylate immunopurified PARP from oocytes, this phosphorylation failed to induce the appearance of the enzyme's activity or to produce the increase in apparent molecular mass as observed *in vivo*. Thus these results, and the absence of an obvious MPF consensus phosphorylation site in *X. laevis* PARP protein, suggest that MPF itself is not the protein kinase directly responsible for the phosphorylation and activation of PARP *in vivo* during oocyte maturation. Rather, MPF seems to be part of a kinase cascade *in vivo*, leading to the activation of a more specific PARP kinase. However, another possibility is that the phosphorylation by MPF kinase is required, but by itself is not sufficient for the activation of the enzyme, which might require additional phosphorylations; this possibility could also explain the apparent 9 kDa shift in the electrophoretic mobility of the protein. These results seem to exclude MPF as the only PARP kinase *in vivo*. Nevertheless, MPF might still be part of the activation pathway.

There are two aspects of the present report that seem a little unusual. First, the active form of PARP in mature *Xenopus* eggs seems to have a molecular mass of approx. 125 kDa. In general, PARP from most species seems to show an apparent molecular mass of approx. 116 kDa in electrophoresis, although the deduced amino sequence indicates a mass of 113 kDa. The nucleotide sequence of the *Xenopus* cDNA does indicate a higher molecular mass than in other species, of approx. 116 kDa. Thus we are not too surprised that we observe a molecular mass somewhat higher than is reported for the mammalian proteins. We also draw attention to the well established observation that very basic proteins of which this is an example, and the histones are another, often run anomalously on electrophoretic gels. The second aspect on which we would remark is the rather large change in the molecular mass on phosphorylation of the protein, from approx. 116 to 125 kDa. The explanation for this unexpectedly large change is not known; it might suggest additional additions to the phosphate residues or it might indicate multiple phosphorylations.

The specific PARP kinase is therefore not yet identified. However, several other protein kinases under investigation are possible candidates for the PARP kinase, including the Mos protein, which is a serine/threonine kinase that is specific to germ cells; it is absent from immature oocytes but is synthesized from stored mRNA in response to progesterone. Other possible candidates include mitogen-activated protein (MAP) kinases and MAP kinase kinase, all of which are activated when *Xenopus* oocytes complete meiosis.

It is noteworthy that only one fraction of PARP in *X. laevis* eggs is activated by phosphorylation. This would imply a very economical and efficient way for the cell to activate the necessary amount of PARP needed from the large stock present in the nucleus. Furthermore it is now clear that PARP enzyme activity is apparently actively suppressed in meiotic oocytes. The reason for

this is unknown but we would draw attention to the fact that meiotic DNA recombination occurs in oocytes. Perhaps the suppression of PARP activity is related to the occurrence of DNA recombination in these cells. This would be consistent with the notion that this enzyme participates in some aspect of DNA recombination in eukaryotic cells [44]. One specific possible function might be to prevent biologically inappropriate DNA recombination. A more detailed analysis of the time course of the suppression of the PARP activity during oocyte development would be illuminating. Alternatively, poly(ADP-ribose) might serve to bring to the site of recombination other proteins that contribute to the process of DNA recombination. This would be consistent with recent work, originally in the Laboratory of Dr. G. de Murcia (Strasbourg), and subsequently by Caldecott et al. [45], which shows that, in mammalian cells, the PARP protein is part of a multiprotein complex including several proteins involved in DNA repair and recombination ([45]; G. de Murcia, personal communication).

The nature of the signal that leads to the phosphorylation of the PARP protein and its activation is unknown. However, we note that maturation of the oocyte and appearance of PARP enzyme activity are associated in time with the appearance of a novel enzyme activity, namely the ability to execute non-homologous (illegitimate) DNA recombination [46,47]. This novel and somewhat unusual activity can be inhibited by PARP inhibitors and seems to require NAD⁺ (S. Aoufouchi, S. Shall and C. C. Ford, unpublished work). Consequently there might be a connection between our present results demonstrating the phosphorylation and activation of PARP, and the appearance of the new ability to catalyse non-homologous DNA recombination.

The results presented here concern only *X. laevis* oocytes and eggs. We have yet to determine whether somatic cells of these amphibians also display the same phosphorylation and enzymic activation reactions. It is also not known whether these reactions are peculiar to amphibians or whether mammalian organisms also show this form of regulation. If such regulation were to be found in mammalian germ cell development, it would indicate a quite novel component of regulation in the genetics of mammalian development.

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REFERENCES

- Althaus, F. R. and Richter, C. (1987) *Mol. Biol. Biochem. Biophys.* **37**, 1–237
- de Murcia, G., Menissier-de Murcia, J. and Schreiber, V. (1991) *Bioessays* **13**, 455–462
- Shall, S. (1984) *Adv. Rad. Biol.* **11**, 1–69
- Shall, S. (1984) *Nucleic Acids Symp. Ser.* **13**, 143–191
- Shall, S. (1989) *Biochem. Soc. Trans.* **17**, 317–322
- Nishikimi, M., Ogasawara, K., Kameshita, I., Taniguchi, T. and Shizuta, Y. (1982) *J. Biol. Chem.* **257**, 6102–6105
- Kameshita, I., Matsuda, Z., Taniguchi, T. and Shizuta, Y. (1984) *J. Biol. Chem.* **259**, 4770–4776
- Shizuta, Y., Kameshita, I., Ushiro, H., Matsuda, M., Suzuki, S., Mitsuuchi, Y., Yokoyama, Y. and Kurosaki, T. (1986) *Adv. Enzyme Regul.* **25**, 377–384
- Kameshita, I., Matsuda, M., Nishikimi, M., Ushiro, H. and Shizuta, Y. (1986) *J. Biol. Chem.* **261**, 3863–3868
- de Murcia, G. and Menissier-de Murcia, J. (1994) *Trends Biochem. Sci.* **19**, 172–176
- Molinete, M., Vermeulen, W., Burkle, A., Menissier-de Murcia, J., Kupper, J. H., Hoeijmakers, J. H. J. and de Murcia, G. (1993) *EMBO J.* **12**, 2109–2117
- Wei, Y.-F., Robins, P., Carter, K., Caldecott, K., Pappin, D. J. C., Yu, G.-L., Wang, R.-P., Haseltine, W. A. and Lindahl, T. (1995) *Mol. Cell. Biol.* **15**, 3206–3216
- Brightwell, M. D., Leech, C. E., O'Farrell, M. K., Whish, W. D. J. and Shall, S. (1975) *Biochem. J.* **147**, 119–129

- 14 Chen, Y. M., Shall, S. and O'Farrell, M. (1994) *Eur. J. Biochem.* **224**, 135–142
- 15 Satoh, M. S., Poirier, G. G. and Lindahl, T. (1994) *Biochemistry* **33**, 7099–7106
- 16 Pfeiffer, P. and Vielmetter, W. (1988) *Nucleic Acids Res.* **16**, 907–924
- 17 Blow, J. J. and Laskey, R. A. (1986) *Cell* **62**, 855–862
- 18 Aoufouchi, S., Hardy, S., Prigent, C., Phillipe, M. and Thiebaud, P. (1991) *Dev. Biol.* **152**, 199–202
- 19 Hardy, S., Aoufouchi, S., Thiebaud, P. and Prigent, C. (1991) *Nucleic Acids Res.* **19**, 701–705
- 20 Zierler, M. K., Marini, N. J., Stowers, D. J. and Benbow, R. M. (1985) *J. Biol. Chem.* **160**, 974–981
- 21 Saulier-le Drean, B. M. (1992) Doctoral thesis, Université de Rennes I
- 22 Williams, G. T., Shall, S. and Ford, C. C. (1983) *Biol. Rep.* **3**, 461–467
- 23 Williams, G. T., Shall, S. and Ford, C. C. (1983) *Biochim. Biophys. Acta* **762**, 272–280
- 24 Smith, L. D. (1989) *Development* **107**, 685–699
- 25 Paris, J., Le Guellec, R., Couturier, A., Le Guellec, K., Omilli, F., Camonis, J., MacNeil, S. and Philipe, M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1039–1043
- 26 Sagata, N., Daar, I., Oskarsson, M., Showalter, S. D. and Vande Woode, G. F. (1989) *Science* **245**, 643–646
- 27 Nebreda, A. R. and Hunt, T. (1993) *EMBO J.* **12**, 1979–1986
- 28 Lehmann, A. R., Kirk-Bell, S., Whish, W. J. D. and Shall, S. (1974) *Exp. Cell Res.* **83**, 63–72
- 29 McNerney, R., Tavasolli, M., Shall, S., Brazinski, A. and Johnstone, A. (1989) *Biochim. Biophys. Acta* **1009**, 185–187
- 30 Menegazzi, M., De Prati, A. C., Miwa, M., Suzuki, H. and Libonati, M. (1992) *Biochem. Int.* **26**, 69–77
- 31 Taniguchi, T., Yamauchi, K., Yamamoto, T., Toyoshima, K., Harada, N., Tanaka, H., Takahashi, S., Yamamoto, H. and Fujimoto, S. (1988) *Eur. J. Biochem.* **171**, 571–575
- 32 Burzio, L. and Koide, S. S. (1977) *Ann. N.Y. Acad. Sci.* **286**, 398–407
- 33 Farzaneh, F. and Pearson, C. K. (1979) *Dev. Biol.* **72**, 254–265
- 34 Burzio, L. O., Zuvic, T., Phillips, D. M. and Koide, S. S. (1981) *Molecular Approaches to Gene expression and Protein Structure*, pp. 149–171, Academic Press, New York
- 35 Aoufouchi, S., Prigent, C., Thezen, N., Phillipe, M. and Thiebaud, P. (1991) *Nucleic Acids Res.* **19**, 4395–4398
- 36 Labbe, J. C., Capony, J. P., Caput, D., Cavadore, J. C., Derancourt, J., Kaghad, M., Lelias, J. M., Paicard, A. and Doree, M. (1989) *EMBO J.* **8**, 3053–3058
- 37 Gurdon, G. P. and Wickens, M. P. (1983) *Methods Enzymol.* **101**, 370–386
- 38 Dumont, J. N. (1972) *J. Morphol.* **136**, 153–180
- 39 Nieuwkoop, P. D. and Faber, J. (1967) *Normal Table of Xenopus laevis* (Daudin), Elsevier/North-Holland, Amsterdam
- 40 Gerhart, J., Wu, M. and Kirschner, M. (1984) *J. Cell Biol.* **98**, 1247–1255
- 41 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 42 Prigent, C., Lasko, D. D., Kodama, K., Woodgett, J. R. and Lindahl, T. (1992) *EMBO J.* **11**, 2925–2933
- 43 Goebel, M., Stone, P., Lengyel, H. and Hilz, H. (1977) *Hoppe Seyler's Z. Physiol. Chem.* **358**, 13–21
- 44 Shall, S. (1994) *Molec. Cell. Biochem.* **138**, 71–75
- 45 Caldecott, K. W., Aoufouchi, S., Johnson, P. and Shall, S. (1996) *Nucleic Acids Res.* **24**, 4387–4394
- 46 Pfeiffer, P. and Vielmetter, W. (1988) *Nucleic Acids Res.* **16**, 907–924
- 47 Goedecke, W., Vielmetter, W. and Pfeiffer, P. (1992) *Mol. Cell. Biol.* **12**, 811–816