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Poly(ADP-Ribose) Polymerase-1: Association With Nuclear Lamins in Rodent Liver Cells

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Abstract The distribution of poly(ADP-ribose) polymerase-1 (PARP-1) over different nuclear compartments was studied by nuclear fractionation procedures and Western analysis revealing a prominent role of the nuclear matrix. This structure is operationally defined by the solubility properties of the A- and B-type lamins under defined experimental conditions. We consistently observed that most of the nuclear matrix-associated PARP-1 partitioned, in an active form, with the insoluble, lamin-enriched protein fractions that were prepared by a variety of established biochemical procedures. These PARP-1–protein interactions resisted salt extraction, disulfide reduction, RNase and DNase digestion. An inherent ability of PARP-1 to reassemble with the lamins became evident after a cycle of solubilization/dialysis using either urea or Triton X-100 and disulfide reduction, indicating that these interactions were dominated by hydrophobic forces. Together with in vivo crosslinking and co-immunoprecipitation experiments our results show that the lamins are prominent PARP-1-binding partners which could contribute to the functional sequestration of the enzyme on the nuclear matrix. J. Cell. Biochem. 93: 1155–1168, 2004.

Key words: PARP-1; interaction partners; nuclear lamina; lamins; functional compartmentalization; co-immunoprecipitation; *cis*-DDP; in vivo crosslinking

Poly(ADP-ribose) polymerase-1 (PARP-1) is a member of the ADP-ribosylating family of enzymes that are found in the nuclei of all animal cells. Other members of the family include PARP-2 with a role in DNA base excision repair [Amé et al., 1999; Schreiber et al., 2002], PARP-3 [Johansson, 1999], tankyrase [Smith et al., 1998], and vPARP a component of the Vault ribonucleoprotein complex [Kickhoefer et al., 1999].

PARP-1 is a 113 kDa protein that binds, in a sequence-independent manner, to single-strand interruptions in DNA where it catalyzes the transfer of ADP-ribose moieties from nicotinamide adenin dinucleotide (NAD^+) to over 30 nuclear proteins among which PARP-1 itself is the main acceptor [Rolli et al., 2000]. Repair proteins are recruited by this automodified form. In cases where the damage cannot be repaired, the cell may decide to enter apoptosis in which case PARP-1 is proteolytically cleaved into fragments of 24 kDa (DNA-binding domain) and 89 kDa (automodification and catalytic domain). Other roles of the enzyme have been described for processes like chromatin

Abbreviations used: PARP-1, poly(ADP-ribose)polymerase-1; S/MAR, scaffold/matrix attachment region; *cis*-DDP, *cis*-dichlorodiammineplatinum (II); PCNA, proliferating cell nuclear antigen; NAD^+, nicotinamide adenine dinucleotide; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; DTT, dithiotreitol; MES, 4-morpholineethanesulfonic acid; IAA, iodoacetamide; PMSF, phenylmethylsulfonyl fluoride.

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decondensation, gene expression, DNA replication, malignant transformation, and cellular differentiation [Dantzer et al., 1998; d'Amours et al., 1999]. While many specialized factors involved in these processes have been delineated, the question remains as of the principles according to which these various complexes are formed and targeted to their sites of action.

Several groups have previously reported the existence of PARP-1 in a state of association with the nuclear matrix [Alvarez-Gonzalez and Ringer, 1988; Quesada et al., 1994], a structure considered to constitute the structural protein framework of the nucleus. Recent evidence supports and extends this view in that we are dealing with a dynamic rather than a static supramolecular organization [Heng et al., 2004]. Thereby this entity serves important functions in many nuclear processes [Berezney and Wei, 1998], notably the organization and differential activation of chromatin domains and the functional consequences thereof [Laemmli et al., 1992].

Nuclear matrix association of PARP-1 was first concluded from the enzyme's ability to label nuclear fractions with radioactive NAD^+, as for instance in the experiments by Alvarez-Gonzalez and Ringer [1988] who recovered 55% of the total nuclear poly(ADP-ribose) polymer from the rat liver nuclear matrix. Cardenas-Corona et al. [1987] reported that, regardless of whether high-salt extraction with 2 M NaCl, 250 mM ammonium sulfate or 3,5-diiodosalicilate (LIS) was used, 70–90% of the total nuclear poly(ADP-ribose) polymer remained in the nuclear matrix. This distribution supports the view by Boulikas [1996] according to whom DNA repair is one of the processes that is functionally linked to the nuclear matrix. This property is shared by other enzymes with specialized functions such as type II DNA topoisomerases [Fernandez and Catapano, 1991]; histone deacetylase [Hendzel et al., 1991]; and protein kinase CK2 [Tawfic et al., 1996]. Several of these interact with PARP-1 in a functional manner to enable cell survival and DNA metabolism, such as DNA polymerase α [Dantzer et al., 1998], pRB [Mancini et al., 1994], the Ku protein [Galande and Kohwi-Shigematsu, 1999], and DNA-polymerase α-primase in dividing cells [Dantzer et al., 1998]. It has been shown previously that PARP-1 co-immunoprecipitates with the proliferating cell nuclear antigen (PCNA) and p21 and this kind of interaction could be essential in regulation of DNA replication and repair [Frouin et al., 2003]. The dynamic association/dissociation of these components appears to be an important regulatory mechanism that integrates their function into the activities of the nuclear matrix.

Electron microscopy revealed that the isolated nuclear matrix consists of three major compartments: the peripheral nuclear lamina, an inner fibrogranular network, and residual nucleoli [Nickerson, 2001]. In addition to their established role forming the peripheral lamina, lamins have more recently been suggested also to form nucleoplasmic structures. Although fluorescence recovery after photobleaching (FRAP) studies with green fluorescent protein (GFP) fusions have indicated that this fraction participates in a stable inner network [Goldmann et al., 2002] these findings have remained controversial.

Preparative procedures have been developed to separate nuclear matrix compartments and it has been shown that such biochemical compartments overlap, to a large extent, with the structures observed by electron microscopy [Zackroff et al., 1982; Kaufmann and Shaper, 1984; Stuurman et al., 1990; Belgrader et al., 1991]. Operationally, these entities are defined by their solubility under the given experimental conditions, that is, by the type of protein–protein interactions that are disrupted by chemical agents with defined biophysical actions.

During the cell cycle, the lamins assemble a number of different structures, some of which may act as specialized scaffolds to organize factors for particular nuclear functions [Goldmann et al., 2002], and we will show in this work that they are important PARP-1-interacting partners. In an attempt to define these nuclear matrix anchors, we first applied various classical fractionation schemes to prepare subsets of nuclear matrix proteins. The conclusions from these studies were then supported by in vivo crosslinking and co-immunoprecipitation experiments.

MATERIALS AND METHODS

Materials

Wistar strain male adult rats (2.5 months old, 200–250 g) were the source for various nuclear matrix preparations. The animals were kept under a constant temperature, humidity and controlled lighting schedule with a 12-h dark

period. Food and water were freely available. Before the experiments the rats fasted for 24 h.

Murine hepatoma cells were grown in Dulbecco's modified Eagle's medium (Sigma, Munich, Germany) supplemented with 10% fetal bovine serum, 1% glutamine, and antibiotics. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and trypsinized when confluent.

Isolation of Rat Liver Nuclei

Rat liver nuclei were isolated and purified by ultracentrifugation in sucrose [Kaufmann and Shaper, 1984]. All buffers contained 1 mM phenylmethylsulfonyl fluoride (PMSF). Unless otherwise indicated, all steps were carried out on ice. Minced rat livers (0.5 g/ml) in 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgSO_4$ (STM) were homogenized by several strokes of a Potter–Elvehjem Teflon–glass homogenizer, centrifuged at 1,000*g* for 15 min, and washed with STM once. The crude nuclear pellet was resuspended in 2.2 M sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgSO_4$ (DS), layered over a 5 ml cushion of the same buffer, and centrifuged at 72,000*g* in a Beckman SW-28 rotor for 60 min. The pellet was resuspended in STM buffer, layered over 5 ml cushions of DS buffer and centrifuged for 30 min at 72,000*g*.

Isolation of Nuclear Matrices

Nuclear matrices were isolated, essentially as described by Belgrader et al. [1991]: freshly isolated purified nuclei were stabilized by incubating at 42°C for 20 min [Ludérus et al., 1992]. The nuclei (10^8 nuclear spheres/ml) were then incubated with 2 mM Na-tetrathionate in 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgSO_4$ for 1 h at 4°C, washed twice with the same buffer without Na-tetrathionate, and incubated with 100 μg/ml of RNase-free DNase I overnight at 4°C. After centrifugation, the post-nuclease supernatant was recovered for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western (immunoblot) analysis. The nuclei were then subjected to consecutive extraction/centrifugation steps: twice with salt buffer: 0.25 M $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 7.4), 0.2 mM $MgSO_4$, once with freshly prepared 1% Triton X-100 in low salt buffer (LS): 10 mM Tris-HCl (pH 7.4), 0.2 mM $MgSO_4$, followed by two washes with LS. The salt wash was recovered and used for SDS–PAGE and Western analysis. Immediately after centrifugation, the obtained nuclear extracts were centrifuged at 10,000*g* in order to remove any nuclear debris Nuclear matrices were resuspended in 0.5 M sucrose, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), 1 mM PMSF to which an equal volume of sterile glycerol was added, and the matrices were kept at −20°C. Determinations of nucleic acids (DNA plus RNA contents estimated from A_{260} readings) and of DNA (fluorescence mediated by the addition of an intercalator) showed reduction to 34 and 10%, respectively. The procedures have been detailed below.

Preparation of Internal Nuclear Matrix Proteins

Internal nuclear matrix proteins were isolated as described previously [Stuurman et al., 1990]. Nuclear matrices were resuspended in LS (2×10^8/ml). An equal volume of 2 M NaCl, 40 mM dithiothreitol (DTT), 10 mM Tris-HCl (pH 7.4), 0.2 mM $MgSO_4$, 1 mM PMSF was added to the suspension and the matrices were incubated for 20 min on ice. Insoluble nuclear matrix proteins were removed by sedimentation at 10,000*g* for 20 min at 4°C. The internal nuclear matrix supernatant was dialyzed against 10 mM ammonium acetate (pH 7.4) with several changes of buffer and stored lyophilized at −70°C.

Solubilization of Nuclear Matrix Proteins

Matrix proteins were solubilized by incubating 3–5 mg of nuclear matrices in deionized 8 M urea, 20 mM 4-morpholineethanesulfonic acid (MES) (pH 6.6), 1 mM EGTA, 1 mM PMSF, 0.1 mM $MgCl_2$, 1% β-mercaptoethanol at 30°C [Zackroff et al., 1982]. Centrifugation at 10,000*g* (room temperature, 30 min) removed proteins that were highly resistant to solubilization. The supernatant was dialyzed overnight at 37°C against 1,000 volumes of assembly buffer: 0.15 mM KCl, 25 mM imidazole hydrochloride (pH 7.1), 5 mM $MgCl_2$, 0.125 mM EGTA, 2 mM DTT, 0.2 mM PMSF. The reassembled filaments were removed by centrifugation at 10,000*g* (room temperature, 15 min) and the supernatant was stored at −70°C.

Isolation of the Peripheral Nuclear Lamina

The peripheral nuclear lamina structures were isolated as described by Stuurman et al. [1990]. In order to prevent the formation of disulfide bonds, nuclei were isolated in the presence of 10 mM iodoacetamide (IAA) instead

of Na-tetrathionate. After the first ultracentrifugation step, the nuclei were resuspended and incubated in STM containing IAA for 1 h on ice. After several washes, purified nuclei were digested with 100 μg/ml DNase I and 250 μg/ml RNase A on ice overnight. Following centrifugation, the post-nuclease supernatant was recovered for SDS–PAGE and Western analysis. After three washes in STM without IAA, the nuclei were resuspended in LS buffer, supplemented with 20 mM DTT and an equal volume of 3.2 M NaCl containing 20 mM DTT was slowly added with stirring. Fifteen minutes later, the peripheral lamina fraction was pelleted by centrifugation at 7,000*g*. The extraction was repeated once. The first salt wash was recovered and used for SDS–PAGE and Western analysis. The peripheral nuclear lamina was subsequently extracted with 1% Triton X-100 in LS buffer and washed once more with LS.

Solubilization of the Isolated Peripheral Nuclear Lamina

The isolated peripheral nuclear lamina was solubilized according to Aebi et al. [1986].

Pre-solubilization wash. Freshly isolated peripheral nuclear lamina was incubated for 30 min (0.25 mg protein/ml) in 10% sucrose, 2% Triton X-100, 20 mM MES-KOH (pH 6.0), 300 mM KCl, 2 mM EDTA, 1 mM DTT, and pelleted at 6,000*g* for 20 min, yielding a supernatant and lamina-enriched pellet.

Solubilization. The pellet was resuspended (0.3 mg protein/ml) in 2% Triton X-100, 20 mM Tris-HCl (pH 9.0), 500 mM KCl, 2 mM EDTA, 1 mM DTT, and centrifuged after 30 min at 200,000*g* for 40 min yielding a supernatant consisting of solubilized lamins and an insoluble pellet. The solubilized lamina proteins were dialyzed against 1,000 vol. of buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, and 1 mM DTT. During this process, lamin assembly was brought about within 4 h. The reassembled lamina filaments were pelleted by centrifugation at 10,000*g* at 4°C for 15 min.

Purification of Lamin B

A total lamin preparation consisting of lamin A, B, and C was isolated from Hepa cells, as described by Aebi et al. [1986]. The lamin preparation was stored at a concentration of 0.2–0.3 mg/ml in lamin buffer (20 mM Tris-HCl, pH 9.0, 500 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 2% Triton X-100).

The procedure for the purification of lamin B was based on that of Aebi et al. [1986]. All steps were performed at 4°C. For the purification of lamin B, 1 ml of the total lamin preparation was mixed with 24 ml of 6 M urea and 20 mM Tris-HCl (pH 9.0) and passed over a strong cation exchanger (sulfonic acid, Viva Science, Sartorius AG, Germany). The flow-through material, which was enriched with lamin B, was passed over a weak anion exchanger (diethylamine, Viva Science). This column was washed with buffer U_1 (6 M urea, 20 mM Tris-HCl (pH 9.0), 500 mM KCl, 2 mM EDTA, 1 mM DTT). This yielded a pure lamin B fraction with a protein concentration of 0.1–0.2 mg/ml.

Protein–Protein Interaction of Two Component System (Reassociation Experiments)

Purified lamin B (~1 μg) in buffer U_1, was dialyzed for 2 h at room temperature against binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA). Under these conditions aggregation of lamin B takes place. Binding buffer (50 μl) and recombinant PARP-1 (1 μg) (Alexis Biotechnology, Grünberg, Germany) were added, and binding was carried out for 3 h at room temperature. Lamin B-PARP-1 interaction was analyzed by a sedimentation procedure. The content of the dialysis bag was sedimented in a microcentrifuge (30 min at 10,000*g*), and proteins from the pellet and supernatant were separated by SDS–PAGE on a 10% gel, blotted on membrane, and immuno-stained with anti-PARP-1 (H-250, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-lamin B antibodies (M-20, Santa Cruz Biotechnology), respectively.

Immunoprecipitation Experiments Using Complete Cell Lysate

Aliquots of 1×10^6 Hepa cells were lysed for 30 min on ice with 1 ml of cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail (100×)–PMSF, 5 mg (50 μg/ml), aprotinin, 100 μg (1 μg/ml), leupeptin, 100 μg (1 μg/ml), pepstatin, 100 μg (1 μg/ml)), with occasional mixing. Samples were centrifuged for 15 min at 10,000*g* at 4°C, and the supernatant containing the soluble fraction was transferred to a clean tube. For immunoprecipitation experiments, an equal amount of cell lysate (200 μg of proteins), was incubated overnight at 4°C, either with 5 μg of polyclonal anti-PARP-1 (Roche Diagnostics GmbH, Mannheim, Germany) or polyclonal

anti-lamin B (M-20, Santa Cruz Biotechnology) antibody. As a negative control, immunoprecipitation was performed with anti-rabbit IgG (Jackson ImmunoResearch, Hamburg, Germany). After adding protein G-Sepharose (Santa Cruz Biotechnology), samples were incubated for 1 h at room temperature under agitation, centrifuged at 10,000*g*, washed, and finally analyzed by SDS–PAGE. Immunoblot analysis was then performed with the following antibodies: anti-PARP (H-250, Santa Cruz Biotechnology) and anti-lamin B (M-20, Santa Cruz Biotechnology).

In Vivo Crosslinking With *Cis*-Dichlorodiammineplatinum (II) (*cis*-DDP)

Crosslinking with *cis*-DDP was performed, according to Ferraro et al. [1992] as modified by Samuel et al. [1998] in a procedure that activates the agent depending on Cl^- concentrations.

Hepa cells (5×10^8) were washed with ice-cold Hanks buffer (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose). The cells were collected by centrifugation and *cis*-DDP was added to a final concentration of 1 mM. After 1 h incubation at 37°C, the crosslinking was stopped by the addition of Cl^- anions. To this end, the cells were centrifuged and resuspended in cold lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl), added to pre-equilibrated hydroxyapatite (HAP-BioRad) and incubated on an orbitron for 1 h at 4°C. Cells were collected by centrifugation and the pellet was washed three times with lysis buffer. Crosslinks between proteins and HAP-bound DNA were cleaved by addition of reverse lysis buffer (1 M thiourea, 2 M guanidine hydrochloride, 2 M NaCl) followed by incubation on an orbitron for 2 h at 4°C. After centrifugation, the supernatant with the released proteins was dialyzed for 24 h against four to five changes of ddH_2O containing 1 mM PMSF and subjected either to TCA precipitation or to antibody-mediated precipitation experiments (Fig. 6).

Co-Immunoprecipitation Experiments With Crosslinked Protein Fraction

After dialysis, remaining DNA was degraded by digestion with DNase I for 1 h at 4°C. The fraction was concentrated with Vivaspin 20 (Viva Science, 10,000 MWCO). Five hundred microliters of the resulting concentrated protein solution was incubated with 10 μg/ml of either anti-PARP-1 or anti-lamin B antibodies at 4°C overnight. The mixture was then centrifuged for 2 h at 16,060*g* and 4°C. The resulting pellet was resuspended in 2× sample buffer and analyzed by SDS–PAGE and Western blot analysis.

SDS–PAGE

For SDS–PAGE 20 μg of proteins were loaded onto 1 mm 4% stacking/12% separating slab gels [Laemmli, 1970], using a Bio-Rad Mini-PROTEAN II electrophoresis cell. The gels were stained with Coomassie Brilliant Blue R-250. Protein concentrations were determined according to Lowry et al. [1951].

Western Immunoblot Analyses

Twenty micrograms of proteins separated by SDS–PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Western analysis was performed using rabbit polyclonal antibodies to rat PARP-1 (Roche/Santa Cruz Biotechnology-H250) or C/EBPβ, and goat polyclonal antibodies to rat A/C and B lamins, respectively (Santa Cruz Biotechnology N-18, M-20). Staining was performed by the chemiluminescent technique according to the manufacturer's instructions (Santa Cruz Biotechnology). Western immunoblots from at least three separate experiments were analyzed with TotalLab (Nonlinear Dynamics Phoretix, Progenesis gel image-analysis software; Durham, NC) electrophoresis software (v1.10) to determine densitometric units for each band. Data from the same electrophoretogram were used to calculate the ratio between PARP-1 and lamin B in the different nuclear protein fractions. Thus, the PARP-1 and lamin B bands that were obtained after Western analysis were expressed as the percentage change relative to the respective values quantified in the initial preparation which were assumed to be 100%. In some instances, the relative PARP-1 and lamin B content was expressed as the lamin B to PARP-1 ratio.

Poly(ADP-Ribose)Polymerase Assay

PARP activity assays were carried out according to Quesada et al. [2000] with minor modifications. The reaction mixture (final volume 50 μl) contained 100 mM Tris-HCl pH 8, 14 mM β-mercaptoethanol, 10 mM $MgCl_2$, 400 μM (^{14}C)NAD^+ (10,000 cpm/nmole) and, as the enzyme source, an amount of nuclei, nuclear matrices, and different nuclear protein fractions

that corresponded to 100 μg protein each. After 15 min of incubation at 30°C, the reaction was stopped by the addition of ice-cold trichloroacetic acid 40% (v/v), and the radioactivity in the acid-insoluble material was measured in a Beckman LS8100 liquid scintillation counter. One milliunit is defined as the amount of enzyme activity catalyzing the incorporation per minute of 1 nmole of ADP-ribose into acid-insoluble material.

Determination of DNA

Aliquots of turbid nuclear and nuclear matrix suspensions were withdrawn and diluted (1:10) in cold 5.5 M urea and 2.2 M NaCl and the absorbance at A_{260} was determined [Laitinen et al., 1994]. To avoid contributions by RNA and protein, a specific DNA determination was performed in a DyNA Quant 200 fluorometer in the presence of 0.1 μl Hoechst 33258 in 1× TNE buffer. At an excitation wavelength of 356 nm fluorescence was read at 460 nm and referenced to a calf thymus DNA standard.

RESULTS

PARP-1 in the Classical, Salt-Extracted Nuclear Matrix Preparation

The nuclear matrix was isolated from adult rat liver according to procedures that allowed for a better preservation of the internal nuclear matrix fibro-granular network than 1.6–2 M NaCl or LIS [Belgrader et al., 1991], i.e., by extraction with ammonium sulfate, an extracting agent that largely preserves the nuclear morphology and recovers almost twice as many proteins as part of the isolated nuclear matrix (results not shown).

The relative PARP-1 content in soluble and insoluble biochemical compartments that were obtained during nuclear matrix and peripheral nuclear lamina isolation and fractionation was evaluated by Western immunoblot analysis. Figure 1 shows the results for isolated nuclei, post DNase wash, ammonium sulfate extract, and isolated nuclear matrices, revealing distinct protein profiles (SDS–PAGE in Fig. 1A, lanes 1–4, respectively). The relative lamin B content in the nuclear extracts was subsequently determined by Western analysis (Fig. 1B), which was also used to monitor the efficiency of separation of nuclear proteins in the course of the fractionation. Lamin B was chosen since, in contrast to lamin A/C it is constitutively expressed and well separated from the other immunostained bands.

From the Western blot in lane 4, it becomes evident that the nuclear matrix is highly lamin-enriched: image analysis showed that, if data were referenced to the isolated nucleus as a

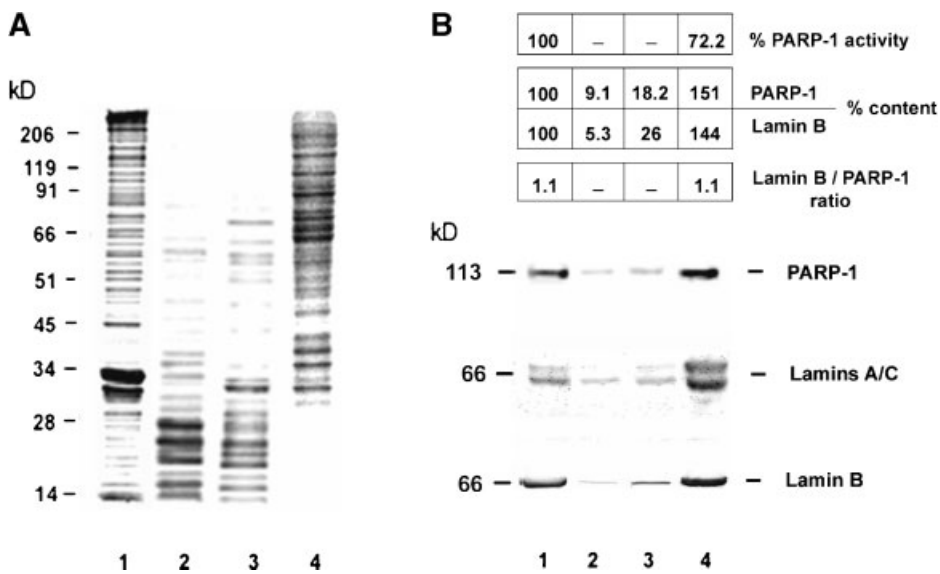

Fig. 1. Classical preparation of the nuclear matrix. Coomassie staining (**A**) and Western analysis (**B**) of proteins obtained during nuclear matrix isolation. Twenty micrograms of proteins per lane were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Isolated rat liver nuclei (**lane 1**); proteins released after DNase digestion (**lane 2**) and during the first extraction step with 250 mM $(NH_4)_2SO_4$ (**lane 3**; see Materials and Methods). Isolated nuclear matrix (**lane 4**). Western analysis was performed with anti-PARP-1, anti-lamin A/C, and anti-lamin B antibodies on the same membrane, in parallel. The activity data and percent contents in the inset are averages from at least three different experiments.

reference (100%), the respective relative content of lamin B in the isolated nuclear matrix was ~144% and that of the 113 kDa PARP-1 protein ~151% (inset in Fig. 1B) showing a significant enrichment for both proteins. The fact that extraction steps do not change the protein ratio which has remained 1.1 in the matrix suggests the possibility that PARP-1 and lamin(s) form a stoichiometric complex.

The balance of protein and DNA contents of nuclear matrix relative to nuclei shows that a typical nuclear matrix sphere retained about 34, 10, and 51% of total protein, DNA and PARP-1, meaning that half of the total PARP-1 protein exists in a soluble state in isolated nuclei. Only part of this is reflected by lanes 2 and 3 in Figure 1, in which only the DNAse I-released fraction and one out of several washing steps has been analyzed (see Materials and Methods).

In addition to its physical presence in the nuclear matrix, we wanted to prove the specific nature of association by determining the biological activity of PARP-1. Although the poly(ARP-ribose)polymerase assay does not distinguish among PARP isoforms it is known that PARP-1 accounts for 70–80% of the activity in nuclei [Rolli et al., 2000]. We have performed enzymatic PARP assays on isolated nuclei and nuclear matrices and we could demonstrate that 72.2% of total nuclear PARP activity was retained in the isolated nuclear matrices (inset in Fig. 1B). This result not only indicates that the nuclear matrix-associated PARP-1 has remained properly folded but also demonstrates that a significant amount of nuclear PARP-1 activity is associated with the nuclear matrix.

Fractionation of the Nuclear Matrix Into the Shell and Interior Portions

Next we tried to refine the localization of PARP-1 by separating the internal nuclear matrix from the peripheral nuclear lamina and associated proteins [Stuurman et al., 1990]. The isolated nuclear matrix was incubated with 2 M NaCl, 40 mM DTT, 10 mM Tris-HCl (pH 7.4), 0.2 mM $MgSO_4$, which resulted in the solubilization of the interior part. Figure 2B, lane 2 demonstrates a notable contribution of PARP-1 for the internal nuclear matrix and a more significant one for the nuclear shell which also contained the majority of lamin B (lane 3). These results reveal that a major part of nuclear matrix-associated PARP-1 is resistant to extraction with high-salt and DTT and suggest its localization to the nuclear periphery where the predominant binding partners are the lamins. For the internal portion the share of lamins is

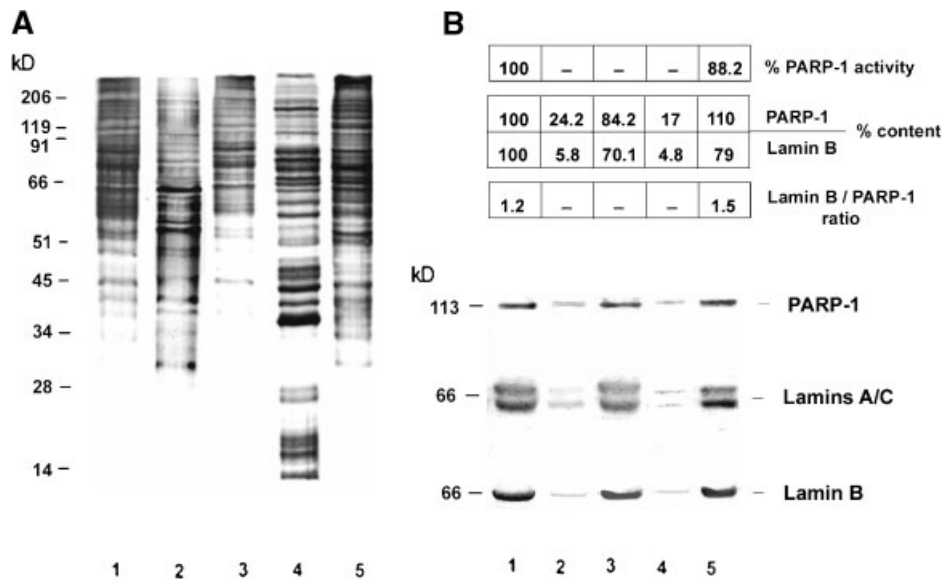

Fig. 2. Fractionation of the isolated nuclear matrix. Coomassie staining (**A**) and Western analysis (**B**) of nuclear matrix protein fractions. Nuclear matrix (**lane 1**), soluble internal nuclear matrix (**lane 2**); pelleted proteins (**lane 3**); proteins soluble in 8 M urea (**lane 4**); proteins reassembled after urea removal by dialysis (**lane 5**). Western analysis was performed with anti-PARP-1, anti-lamin A/C, and anti-lamin B antibodies in parallel. The percent contents in the inset are averages from at least three different experiments.

reduced indicating the possible contribution of different nuclear matrix anchors.

Recovery of PARP-1 After Reconstitution of Lamin Filaments

The importance of hydrophobic interactions in the partitioning of PARP-1 with the lamin-enriched protein fraction was underlined by an alternative procedure in which the isolated nuclear matrix is fractionated into a lamin-enriched insoluble, and a lamin-depleted soluble protein fraction [Zackroff et al., 1982]. To this end, the isolated nuclear matrix was completely solubilized with 8 M urea and β-mercaptoethanol at pH 6.6. Subsequent dialysis against isotonic buffer and β-mercaptoethanol induced protein aggregation that allowed for their separation from soluble nuclear matrix proteins by centrifugation. The process is driven by the inherent capability of the lamins to spontaneously form filaments during urea dialysis, without a requirement for divalent cations and nucleoside triphosphates. Lamins share this property with other intermediate filament proteins and with actin [Lazarides, 1980].

As demonstrated in Figure 2B, lane 5, most of the nuclear matrix-associated PARP-1 partitioned with the reassembled, lamin-enriched proteins. The relative PARP-1 content in the insoluble fraction was ~110% (inset to Figure 2B). This was reflected by a lamin B/PARP-1 ratio of 1.5 in the reassembled protein fraction compared to a ratio of 1.2 in the nuclear matrix (inset in Fig. 2B). Accordingly, the lamin B and PARP-1 contents in the soluble nuclear matrix proteins (lane 4) were ~5 and ~17%, respectively. The retention of 88.2% of the nuclear matrix-associated PARP-1 activity in the reassembled proteins (inset in Fig. 2B) revealed that PARP-1 refolded and reassociated with the lamins in its correct conformation.

Regarding the apparently inherent capability of PARP-1 to reassemble with lamin polymers (Fig. 2B, lane 5), we next wished to establish whether the enzyme could be recovered in the isolated peripheral nuclear lamina itself. In these experiments, the peripheral nuclear lamina was isolated as described by Stuurman et al. [1990]. Rat liver nuclei were prepared in the permanent presence of the irreversible sulfhydryl-blocking reagent IAA. The purified nuclei were then extensively digested with DNase I and RNase A (lane 2), extracted with buffered 1.6 M NaCl/20 mM DTT (lane 3), and washed with buffered 1% Triton X-100 to yield the peripheral nuclear lamina preparation (Fig. 3A,B, lane 4). Image analysis of Western blots performed with anti-PARP-1 antibody

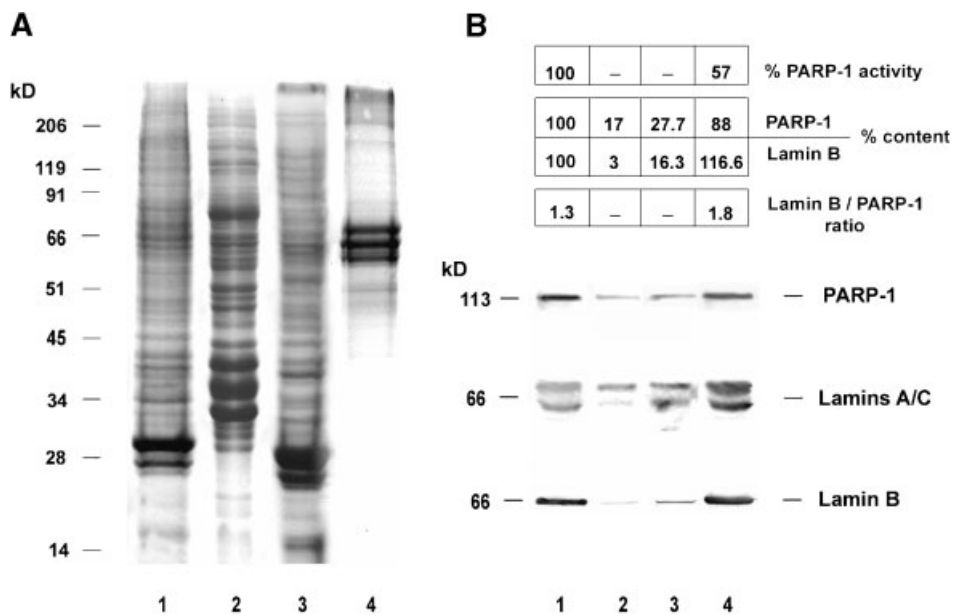

Fig. 3. Isolation of the peripheral nuclear lamina. Coomassie staining (**A**) and Western analysis (**B**) of peripheral nuclear lamina proteins. Isolated nuclei (**lane 1**); proteins released after DNase and RNase digestion (**lane 2**) and extraction with 1.6 M NaCl and 20 mM dithiotreitol (DTT, **lane 3**); peripheral nuclear lamina (**lane 4**). Western analysis was performed with anti-PARP-1, anti-lamin A/C, and anti-lamin B antibodies in parallel. The percent contents in the inset are averages from at least three different experiments.

demonstrated the recovery of PARP-1 in the peripheral nuclear lamina where its relative content was ~88%. Consistent with this was a lamin B/PARP-1 ratio of 1.8 and retention of 57% of nuclear PARP-1 activity in the peripheral nuclear lamina preparation (inset to Fig. 3B). These results suggested that a significant and apparently constant portion of the nuclear matrix-associated PARP-1 was an integral peripheral nuclear lamina component. The internal matrix is disrupted during the RNAse step and was again shown to contain a minor but notable contribution of PARP-1 and lamin B as demonstrated before (Fig. 2, lane 2).

Isolation, Characterization, and Reconstitution of the Peripheral Nuclear Lamina

The view that PARP-1 associates with the peripheral lamina structure was further supported by experiments that examined the biochemical properties of PARP-1–lamin interactions. To this end, the isolated peripheral lamina was subjected to a cycle of solubilization/reassembly [Aebi et al., 1986], namely: (i) a pre-solubilization wash-step with 2% Triton X-100, 10% sucrose, 300 mM KCl, 2 mM EDTA, and 1 mM dithiothreitol at pH 6.0, (ii) solubilization with 2% Triton X-100, 500 mM KCl, 2 mM EDTA and 1 mM DTT at pH 9 when about 80% of all three lamins are rendered soluble [Raymond, 1990], and (iii) repolymerization by dialysis against an isotonic, neutral solution containing DTT. The nonionic detergent Triton forms micelles, which trap and solubilize hydrophobic proteins [Bordier, 1981]. Thereby the more rigorous treatments consisting of denaturation/solubilization with urea were avoided.

These conditions of Triton-solubilization/reassembly are considered to better preserve in vivo interactions than the above procedure since nondenaturing solubilization with Triton X-100 allowed the proteins to maintain aspects of their native state. This in turn reduces the possibility of an adventitious association during lamina repolymerization. DTT was included in the reassociation step in order to exclude the recovery of proteins solely due to the formation of intermolecular disulfide bonds. The relative PARP-1 content in the pre-solubilization wash was ~17% (Fig. 4B, lane 2). More PARP-1 remained associated with the lamin-enriched pellet (lane 3). Its resistance to extraction by Triton suggested interaction with the detergent-insoluble component of the peripheral nuclear lamina. In the next step, the lamina-associated PARP-1 was solubilized together with the lamins (lane 4) resulting in an insoluble and heterogeneous protein fraction, with a negligible contribution of lamins and PARP-1 (lanes 5 in Fig. 4A,B). During subsequent dialysis, which established physiological conditions regarding both ionic strength and pH,

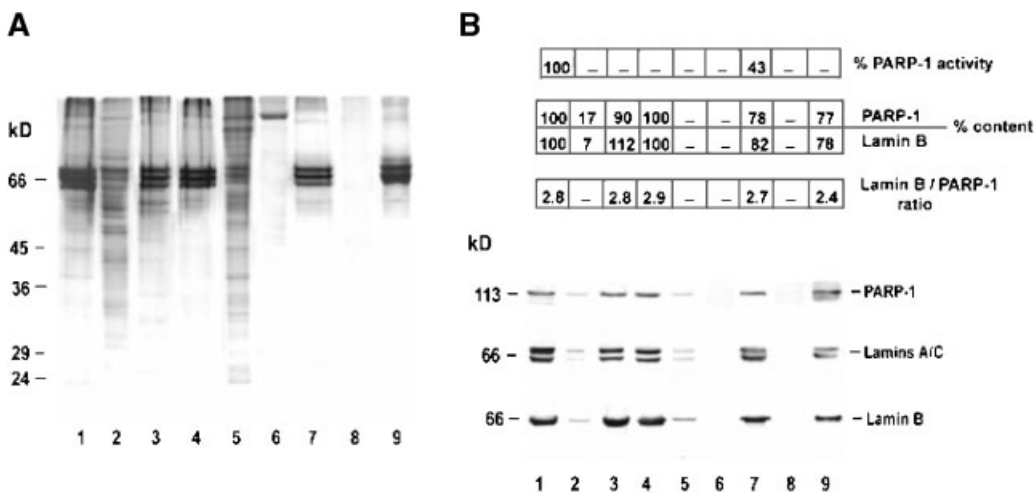

Fig. 4. Solubilization of the peripheral nuclear lamina with 2% Triton X-100. Coomassie staining (**A**) and Western analysis (**B**). Isolated peripheral nuclear lamina (**lane 1**); pre-solubilization wash-step supernatant (**lane 2**); pre-solubilization wash-step lamina-enriched pellet (**lane 3**); solubilized nuclear lamina (**lane 4**); insoluble pellet remaining after lamina solubilization (**lane 5**); supernatant obtained after the reassembly of lamina polymers during dialysis in the presence of 1 mM DTT (**lane 6**); and without DTT (**lane 8**); reassembled lamina polymers obtained after Triton X-100 removal by dialysis in the presence of 1 mM DTT (**lane 7**) and without DTT (**lane 9**). Western analysis was performed with anti-PARP-1, anti-lamin A/C, and anti-lamin B antibodies in parallel. The percent contents and lamin B/PARP-1 ratios in the inset are averages from at least three different experiments.

PARP-1 partitioned with the lamins. The resulting pellet retained 43% of the PARP-1 activity of the peripheral nuclear lamina preparation (inset in Fig. 4B). The reassociation of nearly equal amounts of PARP-1 and lamins occurred regardless of the presence (lane 7) or absence of DTT in the dialysis buffer (lane 9). Together with the observed retention of PARP-1 in the lamin-enriched pellet obtained after nuclear matrix extraction with 2 M NaCl/40 mM DTT (Fig. 2B, lane 3), but contrary to an earlier report [Kaufmann et al., 1991], these results clearly show that the nuclear matrix/lamin-associated PARP-1 is not only present as a component of disulfide linked complexes. Moreover, by image analysis of Western blots (summarized in the inset to Fig. 4B) it was established that the lamin B to PARP-1 ratio remained virtually unchanged in all of the lamin-enriched protein fractions (Fig. 4B, lanes 1, 3, 4, 7, 9).

In a subsequent experiment, we increased the stringency of this reassociation approach by performing the procedure with a well-defined two-component system using lamin B and recombinant PARP-1 that had both been purified to homogeneity. This approach addresses two of the remaining questions:

i. whether or not a mediator-like DNA or a bridging protein is needed for PARP-1/lamin interaction, and
ii. whether the association depends on the presence of a polyADP-ribose tail that is recognized by the lamins [Gagne et al., 2003]. Such a tail would be a consequence of PARP-1 automodification. Any contribution of this kind would be ruled out under the present conditions, i.e., in the absence of NAD^+.

Once again, we initiated the assembly of lamin B by dialysis and added PARP-1 at the point a lamin B meshwork became apparent in the dialysis tube. Figure 5 (lane 2) demonstrates the quantitative occurrence of both proteins in the precipitate; if only PARP-1 was subjected to a parallel dialysis, it remained in the supernatant (lane 5).

The same solubilization/reassembly cycles were applied, in parallel experiments, to the CAAT-enhancer-binding protein beta, C/EBPβ. Although, we have previously observed the reassociation of C/EBPβ with the lamins during a urea-solubilization-dialysis protocol [Uskoković et al., 2002] we found that, in contrast to PARP-1, the lamin-associated C/EBPβ moiety failed to reassemble with the lamins during the Triton-solubilization-dialysis cycle indicating that the criteria of reassociation may be different in both protocols.

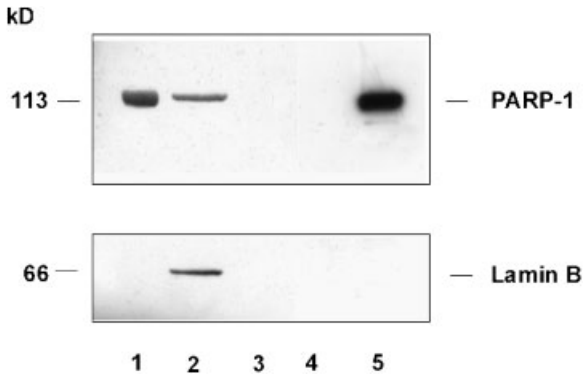

Fig. 5. PARP-1 binding to purified lamin B. Purified lamin B in buffer U_1 was dialyzed against binding buffer and induced to aggregate. Aggregated lamin B (1 μg) was further incubated with 1 μg of recombinant PARP-1. After binding, the content of the dialysis bag was centrifuged and the proteins in the pellet (**lane 2**) and supernatant (**lane 3**) analyzed by immuno-staining with anti-PARP-1 (Roche) and anti-lamin B antibody (M-20, Santa Cruz Biotechnology), respectively. As a control of the binding reaction, recombinant PARP-1 was dialyzed and centrifuged under the same conditions, but without prior addition of purified lamin B and the contents of the pelleted fraction (**lane 4**) and corresponding supernatant (**lane 5**) were immuno-stained with anti-PARP-1 and anti-lamin B antibody. **Lane 1** represents 1 μg of recombinant PARP-1.

Co-Immunoprecipitation of PARP-1 and Lamin B

After excluding mediator functions of a third type of molecule, the remaining question was whether or not PARP-1 and lamin B can form a complex also in the soluble state. To this end, we performed co-immunuprecipitation experiments, first on a cell lysate, and subsequently on a fraction that was part of the in vivo nuclear matrix. In both cases, lamin concentrations had to be kept <0.25 mg/ml which is the threshold at which aggregation starts. Below this threshold concentration the lamins exist as head-to-tail dimers indicating that the interactions demonstrated here do not depend on higher degrees of polymerization.

Experiments on the cell lysate are shown in Figure 6A, which analyzes the precipitates obtained either with an anti-PARP-1 antibody or an anti-lamin B antibody. In both cases

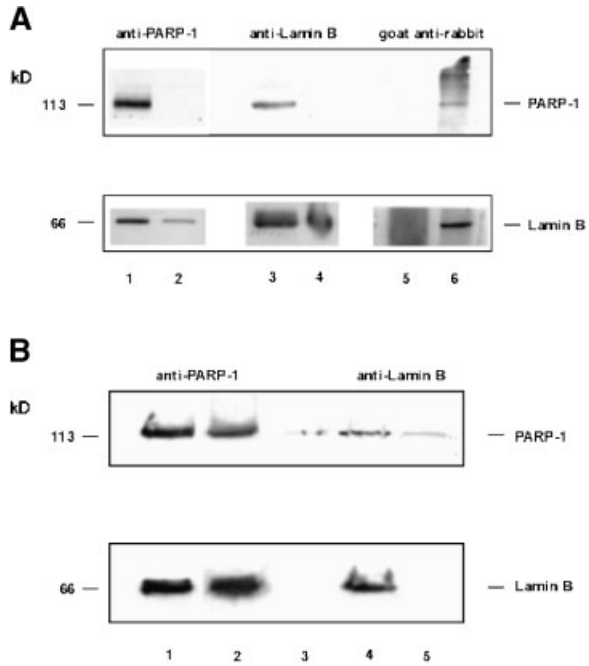

Fig. 6. Co-immunoprecipitation of PARP-1 and lamin B from a Hepa cell lysate. **A**: PARP-1 and lamin B in the pellet (**lane 1**) and supernatant (**lane 2**) after immunoprecipitation with anti-PARP-1 antibody, and in the pellet (**lane 3**) and supernatant (**lane 4**) after immunoprecipitation with anti-lamin B antibody. As a negative control immunoprecipitation was performed with goat anti-rabbit IgG: the pelleted fraction (**lane 5**) and corresponding supernatant (**lane 6**). *Cis*-dichlorodiammineplatinum (II) (*cis*-DDP) mediated crosslinking in living cells. **B**: Hepa cells were incubated with 1 mM *cis*-DDP at low Cl^- concentrations. Prior to lysis the process was stopped by raising the Cl^- concentration. The crosslinked protein fraction contains both PARP-1 and lamin B as demonstrated for a TCA precipitate (**lane 1**). The following two lanes show the results of an immunoprecipitation experiment using an anti-PARP antibody. The pellet (**lane 2**) demonstrates the near quantitative co-precipitation of both components (compare with the supernatant in **lane 3**). Conversely, immunoprecipitation could also be carried out with an anti-lamin B antibody: again the precipitate (**lane 4**) contains both components, while trace amounts of nonprecipitated PARP-1 are left in the supernatant indicating a slight stoichiometric excess of the enzyme.

PARP-1 and lamin B (lanes 1, 3) are exclusively found in the precipitate whereas some excess of lamin B partitions into the supernatant (lanes 2, 4). As a control we applied a nonspecific antibody upon which both components remained in the supernatant (lane 6).

Cis-DDP is an established crosslinking agent by which contacts between nuclear matrix proteins and scaffold/matrix attachment regions (S/MARs) can be trapped in the living cell [Ferraro et al., 1996]. The marked specificity of *cis*-DDP is provided by structural features, which are an exclusive property of S/MAR–DNA [Bubley et al., 1996]. It involves amino acid side chains that are not present in histones. After reversal of the crosslinks the components can be characterized separately [Ferraro et al., 1992, 1996].

In the following experiment, we subjected cultured hepatoma cells to *cis*-DDP treatment which is effective in the absence of chloride anions while its action can be precisely terminated by adding an NaCl-containing lysis buffer. Crosslinked protein–S/MAR complexes and DNA (but not free proteins) are then absorbed to hydroxyapatite (HAP). Subsequently, the crosslinks were reversed by thiourea, a step, which releases proteins to the supernatant where they can be analyzed.

Figure 6B (lane 1) demonstrates that both lamin B and PARP-1 are recovered by the procedure confirming S/MAR-binding activities for both partners. For the lamins such an activity has been shown before [Ferraro et al., 1996] and for a complex from PARP-1 and DNA-PK it has recently been described, based on S/MAR affinity chromatography [Galande and Kohwi-Shigematsu, 1999].

Finally, we could also demonstrate an association of both proteins from this source, i.e., the fraction that was associated with S/MAR DNA in vivo. Lanes 2 and 4 in Figure 6B again show the co-immunoprecipitation of both components using either an anti-PARP-1 antibody or an anti-lamin B antibody. Also in this case, the formation of precipitates was strictly dependent on the presence of the respective antibody ruling out any mechanism that is driven by lamin precipitation.

Together, these data confirm the specific association of PARP-1 with lamin B overcoming arguments that the joint recovery during matrix isolation and reconstitution protocols might be a consequence of solubility properties both partners have in common.

DISCUSSION

Although, it has been well-established that PARP-1 interacts with, modifies and modulates a large number of proteins, little is known about the way it interacts with its partners [Frouin et al., 2003]. So far, attempts to narrow down the candidates by immunofluorescence showed a more or less homogenous staining of the nucleus including all nucleoli, consistent with the view

that PARP-1 presents a housekeeping protein that is only modified by processes like DNA damage and apoptosis [Alvarez-Gonzalez et al., 1999]. The observation that a significant part of PARP-1 is associated with the nuclear matrix [Alvarez-Gonzalez and Ringer, 1988; Quesada et al., 1994, 2000] has indicated that the enzyme participates in the DNA metabolism occurring in this nuclear compartment. In order to refine such a broad description of its function, we decided to examine the localization of PARP-1 on the nuclear matrix more precisely and have identified lamins as major and highly specific binding partners. To this end, we started with a number of established classical methods that have been developed for the characterization of nuclear matrix components. We then continued by applying more recent co-precipitation and co-immunoprecipitation approaches to confirm these results. Initial observations regarding the colocalization of the critical partners by FISH- and Halo–FISH methods are in agreement with our conclusions and will be reported elsewhere.

For all nuclear fractionation procedures performed here, the localization of PARP-1 was assessed by examining its relative content in soluble and insoluble biochemical compartments that were obtained during nuclear matrix and peripheral nuclear lamina isolation and fractionation. Thereby we could demonstrate that the insoluble nuclear protein fractions are invariably lamin-enriched, and that the partitioning of nuclear matrix-associated, enzymatically active PARP-1 correlated closely with the presence or absence of the lamins. This effect was consistently found regardless of the protocol used and it could also be seen after LIS-extraction at physiological strength (data not shown) ruling out any co-precipitation artefact.

Similarly, the peripheral nuclear lamina preparation (Fig. 3, lane 4) and the lamin-enriched fractions obtained after of solubilization with Triton X-100 and dialysis (Fig. 4, lanes 7 and 9) showed an elevated PARP-1 content and a virtually unchanged lamin B/PARP-1 ratio (inset to Figs. 4 and 5). Thus, these solubilization/dialysis experiments document the inherent ability of PARP-1 to spontaneously reassemble with the lamins in vitro. Such a finding is only in apparent contradiction to the above-mentioned even nuclear distribution, which comprises all pools of PARP-1 whether matrix-associated or not. In this context, it is revealing that Dantzer et al. [1998] observed a functional PARP-1/DNA polymerase/a-primase complex at the nuclear periphery where it initiates DNA replication in a cell-cycle dependent manner. Although a specific association with the lamina has not been demonstrated in that case, our observations suggest the intriguing possibility that the nuclear lamina is involved in this compartmentalization.

Further experiments demonstrated that the specific association of PARP-1 with the lamins does not require a mediator molecule like a bridging protein or a DNA template and that it is also a property of the molecules in solution. Both binding partners are parts of the nuclear matrix and both show a propensity to associate with S/MARs [see Ludérus et al., 1992; Galande and Kohwi-Shigematsu, 1999]. Due to a highly specific crosslinking agent, *cis*-DDP, S/MAR-binding proteins can be trapped in the living cell. According to this criterion, a subpopulation of PARP-1 and lamin B could be recovered that also showed this type of association.

At least two factors can be considered to contribute to the PARP-1-lamin interaction(s) observed here. First, the specific PARP-1-lamin interaction might be driven by poly-(ADP)-ribosylation. According to Gagne et al. [2003] lamins belong to a group of proteins that, via a specific binding motif, interact with the PARP-1-linked poly(ADP-ribose) tail. This motif consists of a pattern of hydrophobic amino acids interspersed with basic residues [Pleschke et al., 2000]. According to these considerations, the association of PARP-1 with the peripheral lamina implicates it in lamin-associated functions and vice versa. For the present series of experiments, we consider this contribution rather unlikely since we have neither introduced DNA damage nor apoptotic stimuli.

Alternatively, the process of reassembly might be driven by the formation of a coiled-coil involving α-helical regions of both proteins. In this model, PARP-1 would co-assemble with the lamins as a result of forming an amphipathic helix of related structure. In contrast to C/EBPβ, a selective co-assembly could then be explained by its participation in the polymerization process (Fig. 4). Such a conclusion gains support by the finding that PARP-1 is a constituent of the isolated peripheral nuclear lamina, independent of S–S cross-links (Fig. 3, lane 4), which further resists extraction by Triton X-100 (Fig. 4, lane 3). By this criterion, the protein is similar to p54, a protein of the

inner nuclear membrane that is linked to the nuclear lamina [Bailer et al., 1991]. Accordingly, we might view a major portion of the nuclear matrix-associated PARP-1 as an integral component of the peripheral nuclear lamina.

In addition to its structural role in maintaining nuclear structure, it appears that the nuclear lamina anchors and organizes chromatin functions [Cohen et al., 2001]. With regard to the growing number of nuclear lamina-associated proteins that have been reported, the lamina can now be viewed as a dynamic structure where each component interacts with multiple partners forming a network of attachments between the nuclear membrane and chromatin [Gruenbaum et al., 2000; Goldmann et al., 2002]. The fact that PARP-1 activity is maintained upon the interaction with lamins at various states of their oligomerzation seems to indicate that the lamin network serves as a neutral scaffold by which the enzyme is organized in the appropriate form and nuclear compartment.

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