Nucleosomal Core Histones Mediate Dynamic Regulation of Poly(ADP-ribose) Polymerase 1 Protein Binding to Chromatin and Induction of Its Enzymatic Activity

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Poly(ADP-ribose) polymerase 1 protein (PARP1) mediates chromatin loosening and activates the transcription of inducible genes, but the mechanism of PARP1 regulation in chromatin is poorly understood. We have found that PARP1 interaction with chromatin is dynamic and that PARP1 is exchanged continuously between chromatin and nucleoplasm, as well as between chromatin domains. Specifically, the PARP1 protein preferentially interacts with nucleosomal particles, and although the nucleosomal linker DNA is not necessary for this interaction, we have shown that the core histones, H3 and H4, are critical for PARP1 binding. We have also demonstrated that the histones H3 and H4 interact preferentially with the C-terminal portion of PARP1 protein and that the N-terminal domain of PARP1 negatively regulates these interactions. Finally, we have found that interaction with the N-terminal tail of the H4 histone triggers PARP1 enzymatic activity. Therefore, our data collectively suggests a model in which both the regulation of PARP1 protein and that the N-terminal domain of PARP1 paralog, PARP2, that has no zinc fingers and no direct DNA binding capability, nevertheless exhibits a pattern of chromatin association similar to PARP1 and is able to partially complement PARP1 functions in a PARP1 null mutant (7–9). This suggests that PARP1 and PARP2 both bind chromatin indirectly, through an interaction with one or more DNA-binding proteins. A key aim of this study is to determine the specific mechanisms by which PARP1 protein associates with chromatin in vivo. Considerable evidence now suggests that PARP1 interacts with chromatin by binding to histones (10). For example, histones H1, H2A, and H2B are efficient targets for PARP1 binding in vitro (11) and are enzymatically modified by PARP1 (12–14). This idea is, however, complicated by the fact that Drosophila histone H1 was recently reported as an antagonist of PARP1 binding to chromatin (3). In addition, accumulation of PARP1 interactors, which have to date been identified through in vitro experiments, has resulted in findings suggesting that almost none significantly co-localizes with PARP1 in chromatin. To clarify the many issues involved with PARP1 protein binding activity to chromatin and the activation of its enzymatic activity, we sought an appropriate experimental model.
**Histone H4 Triggers off PARP1 Enzymatic Activity**

**EXPERIMENTAL PROCEDURES**

**Drosophila Strains and Genetics**—Genetic markers are described in FlyBase 1999, and stocks were obtained from the Bloomington Stock Center, except as indicated. pP[w1, UAST::PARP1-DSRed], called UAS::Parp1-DSRed, was described in Ref. 17. The transgenic stock with p(P[w1, UAST::PARG-EGFP], called UAS::Parg-EGFP, was described in Ref. 19 and arm::GAL4 (Bloomington stock no. 1560). Balancer 18. The following GAL4 driver strains were used: 69B-GAL4 (Bloomington Stock Center, except as indicated. pP{w1, Kr-GFP}) (20), was used to identify heterozygous and homozygous Parp<sup>CH</sup> (17).

**Construction of Transgenic Drosophila**—To construct UAS::H2A-ECFP and UAS::H1-EYFP, we generated full-length histone H2A and histone H1 open reading frame using PCR. Primers were used as follows: for H1 cloning, h1d, CACCaagtctgctgccgctt, and h1r, cttttgcaacgttag; and for H2A cloning, h2ad, CACCcattctgcaactgtaaaagg, and h2ar, gcctctttccgtctgcttcttg. We used wild-type *Drosophila* genomic DNA as a template for PCR. The resulting PCR products were cloned through the *Drosophila* Gateway<sup>TM</sup> vector cloning system (Carnegie Institution of Washington) into the corresponding vector for *Drosophila* transformation. Transformation was performed as described in Ref. 21, with modifications (22).

**Fluorescence Recovery After Photobleaching (FRAP) Assay**—FRAP experiments on live *Drosophila* tissues were performed as described in Ref. 23. To conduct these experiments, we used a Leica TCS SP2 confocal microscope with capacity for FRAP. To avoid the oxidative stress and other damage that lasers can cause, we used only the minimal level of laser power. This step extended the “bleaching” phase but did not affect our results. To collect FRAP data, we employed the “FlyMode” program, which allows data collection even during the bleaching phase. The recordings were performed via a 63×1.4 NA oil immersion objective. We found that all the fluorescent epitopes we tested (ECFP, EYFP (Venus), EGFP, and DsRed) were appropriate for FRAP assays, as well as for regular confocal analysis. We did not detect epitope-specific biases in the function, expression dynamics, or localization of any fused moiety. We used transgenic fly stocks that express appropriate fluorescent epitope-tagged proteins. Tissues were dissected in Grace’s medium, and dynamic movement of fluorescent proteins was analyzed for 20–30 min following dissection.

**Nuclei Isolation and Micrococcal Nuclease Digestion**—0.5 g of fresh pupae were homogenized in 10 ml of buffer A1 (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% Triton X-100, 0.1 mM EGTA, 0.5 mM DTT, and Complete<sup>TM</sup> protease inhibitors (Roche Applied Science)), using a Potter homogenizer (Pyrex). The homogenate was filtered through two layers of Miracloth (Calbiochem), homogenized using a Dounce homogenizer (Pestle B) (Kontes Glass Co.) with 10–15 strokes, and centrifuged for 4 min at 4000 × g at 4 °C. The pellet was washed once with 10 ml of the A1 buffer, then resuspended in 6 ml of A1, loaded onto 3 ml of buffer A1/0.3 M sucrose, and centrifuged for 6 min at 1500 × g at 4 °C. The nuclei were washed once with 3 ml of micrococcal nuclease (MNase) digestion buffer (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.3 M sucrose, 0.5 mM DTT, and EDTA-free Complete<sup>TM</sup> protease inhibitors (Roche Applied Science)), diluted by MNase digestion buffer to 1 ml, and incubated with ~200 units of MNase (Worthington) at 37 °C for 3 min, 650 rpm in Thermomixer (Eppendorf). An amount of MNase sufficient for complete chromatin digestion to mononucleosomes was chosen in preliminary experiments for each aliquot of the enzyme. The reaction was stopped by 25 µl of 0.5 M EDTA. After the addition of 200 µl of M buffer (190 mM Tris-HCl, pH 7.5, 25% glycerol, 440 mM NaCl, 5 mM MgCl<sub>2</sub>, 125 mM NaF, 5 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA, 1% Nonidet P-40, 5 mM DTT, and 2× Complete<sup>TM</sup> protease inhibitors (Roche Applied Science)), the nuclei were lysed on a rotating platform at 4 °C for 20 min. The nuclei extract was clarified by centrifugation for 20 min at 17,000 × g at 4 °C.

**Sucrose Gradient**—300 µl of nuclear extract were loaded onto 12 ml of 10–30% linear sucrose gradient in buffer B (30 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.7 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and Complete<sup>TM</sup> protease inhibitors (Roche Applied Science)) and poured into UltraClear ultracentrifuge tubes (Beckman, no. 344059), using Hoefer SG15 gradient maker (Hoefer Scientific Instruments) and Pharmacia Biotech Pump P1. The probes were centrifuged using Sw41Ti rotor (Beckman) (35,000 rpm, 20 h, 4 °C). 1-ml fractions were collected manually through the hole made in the bottom of a tube.

**Analysis of Gradient Fractions**—Proteins were trichloroacetic acid-precipitated from 700 µl of 1-ml gradient fraction, dissolved in 200 µl of 2× Laemmli, and analyzed by Western blot (30 µl for one assay) on 4–12% Bis-Tris NuPAGE Gel (Invitrogen). The primary antibodies used were as follows: mouse monoclonal antibody H1 (Santa Cruz Biotechnology, sc-8030) (1:500), monoclonal antibody H3 (Upstate Biotechnology, Inc., no. 05-499) (1:1000), rabbit polyclonal antibody H2A#618 (1:3000) from Dr. R. Glaser (Division of Genetic Disorders, Wadsworth Center, Albany, NY), polyclonal antibody PAR (Calbiochem) (1:4000), and polyclonal antibody GFP (1:1000 – 1:1500) (TP401, Torrey Pines Biolabs). The remaining 300 µl of each fraction was digested with 100 µg/ml proteinase K in 1% SDS at 50 °C for 2 h, 650 rpm in Thermomixer (Eppendorf). DNA was then recovered by phenol chloroform extraction, followed by ethanol precipitation with glycogen as a carrier. Pellet was dissolved in 40 µl of HB<sub>2</sub> in O, incubated with 2 µg of RNase A for 30 min at 37 °C, and analyzed on 1.2% agarose gel.

**Immunoprecipitation**—For one immunoprecipitation reaction, 300 µl of nuclear extract was incubated with 60 µl of protein G-Sepharose 4B (Sigma P3296-5ML) on a rotating platform for 1 h at 4 °C. The beads were removed by spinning for 5 min at 15,000 × g. 25 µg of anti-GFP polyclonal antibody (Torrey Pines, TP401) were added to the extract and incubated for 2 h or overnight on a rotating platform at 4 °C. Then 50 µl of protein G-Sepharose 4B were added to the extract and incubated for 2 h at 4 °C with rotation. The beads were washed five times for 3 min in 1.2 ml of the buffer (50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% Nonidet P-40, 1.5 mM MgCl<sub>2</sub>B, 25 mM NaF, 1 mM Na<sub>3</sub>BO<sub>4</sub>, 1 mM EDTA, and Complete<sup>TM</sup> protease inhibitors (Roche Applied Science)).
Bound proteins were eluted by 100 μl of 2× Laemmli with heating at 90 °C for 5 min.

**Mass Spectrometry Analysis**—Mass spectrometric identification of proteins was carried out as described in Ref. 24. Complete lanes from protein gels were cut into slices (narrow for specific bands) and analyzed by liquid chromatography-tandem MS. The tandem MS data were analyzed using the SEQUEST™ program. Protein complexes from four purifications were analyzed by liquid chromatography-tandem MS, and a total of 22 proteins were identified. It should be emphasized that it is essential to perform several purifications for a given bait protein to obtain a reliable view of its interaction network, because significant interactors are expected to be reproducibly identified in more than one experiment.

**In Vitro Interaction Assays**—Histones and histone octamers were isolated or assembled according to Ref. 25. Protein coupling to CnBr-activated Sepharose beads (GE Healthcare) and in vitro binding assays were adapted from (26). Brieﬂy, beads coupled to histone octamer (20 pmol), PARP1 (30 pmol, Trevigen), or individual histones (400 pmol) were washed once for 10 min in binding/washing buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 3 mM DTT, and 0.1% Triton X-100). Washed beads were incubated with octamer (22.12 pmol), PARP1 (8 pmol), or rabbit IgG (8 pmol, Sigma) in binding/washing buffer for 20 min. The beads were then washed ﬁve times for 10 min in binding/washing buffer. All of the binding/washing was done at room temperature with gentle rotation. Full-length PARP1 and rabbit IgG were visualized on Western blots with anti-PARP1 (mouse monoclonal, 1:500, Serotec) and anti-rabbit horseradish peroxidase (1:3000, Jackson ImmunoResearch Labs), respectively. Anti-PARP1 C terminus (rabbit polyclonal, 1:1000) and anti-PARP1 N terminus (rabbit polyclonal, 1:1000) were gifts from Dr. Lee Kraus (Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY).

**PARP1 Activity Assay**—0.2 nmol of histones and/or 2.5 μg of endonuclease-digested plasmid DNA were combined with 5× PARP1 reaction buffer (0.05 unit/μl PARP1 enzyme (Trevigen), 500 μM NAD (Sigma), 500 mM Tris, pH 8, 50 mM MgCl₂, and 5 mM DTT) in a ﬁnal volume of 25 μl. PARP1 inhibition was achieved by the addition of 3-aminobenzamide (Sigma) to a ﬁnal concentration of 12 mM. All of the reactions were carried out for 10 min at room temperature.

**RESULTS**

**PARP1 Protein Association with Chromatin Is Dynamic**—To study PARP1 interaction with chromatin, we ﬁrst analyzed the dynamic localization of this protein in vivo by using a FRAP assay. To visualize the PARP1 protein in *Drosophila*, we used the UAS/Gal4 system (27) for transgenic expression of PARP1-DsRed (encoding full-length, catalytically active PARP1) and for a contrasting control, PARP-EGFP (encoding a naturally occurring, catalytically inactive form of PARP1) (Fig. 1A). Previously, we biologically validated those constructs by testing their ability to rescue a ParpCH1 mutation phenotype and by using immunofluorescence to assess recombinant protein localization to chromatin (2, 17). As references, we also made transgenic ﬂies with core histone H2A-ECFP and linker histone H1-EYFP (Fig. 1A), which served as comparative controls defining the protein mobility of chromatin-associated proteins. As an additional reference for the mobility of a nucleoplasmic soluble protein, we made transgenic ﬂies expressing PARG-EGFP (18) (Fig. 1A). To express our transgenic constructs in *Drosophila*, we used the 69B-GAL4 driver (19), which allows expression of recombinant protein ubiquitously without excess overproduction (supplemental Fig. S1). All of the recombinant proteins demonstrated exclusive nuclear localization in all tissues of the fruit ﬂy (supplemental Fig. S1). All of the recombinant proteins except PARG-EGFP were also associated with chromatin. Previously, we reported that PARG is a soluble nucleoplasmic protein (18). H1-YFP and H2A-ECFP histones remain bound to chromatin during all stages of cell cycle, whereas PARP-EGFP and PARP1-DsRed are partially excluded from mitotic chromosomes (supplemental Fig. S2).

To explore whether the catalytic activity of PARP1 inﬂuences the dynamics of PARP1 protein interaction with chromatin, we
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compared the FRAP dynamics of PARPe-EGFP protein with those of full-length, enzymatically active PARP1-DsRed in Drosophila interphase nuclei. We co-expressed both recombinant PARPs in the Parp<sup>CH1</sup> mutant animals (17) using Arm::Gal4 driver. The catalytically active PARP1-DsRed and inactive PARPe-EGFP demonstrated exactly the same localization profiles (Fig. 1B) and the same replacement rate (Fig. 1C and Table 1). This suggested that the catalytic domain of PARP1 is not involved in PARP1 protein interaction with chromatin. Based on this last result, we then used the PARPe-EGFP isoform to remove the potential for artifacts arising from the expression of catalytically active PARP1-DsRed, e.g. hyper-activation of the pADPr reaction targeting nonphysiological substrates. In the early stages of Drosophila development, catalytically inactive PARPe protein is expressed endogenously, and overexpression of it does not affect Drosophila development (17).

We then compared the PARPe-EGFP protein dynamics after photobleaching to those of histone H2A-ECFP, linker histone H1-EYFP, and the soluble nucleoplasmic protein PARG-EGFP (Fig. 1D and Table 1). As anticipated, the soluble PARG-EGFP demonstrated the highest recovery rate: 78% within 5 s. In contrast, the replacement rate for the control core histone H2A in chromatin was close to zero (Fig. 1D), whereas linker histone H1 showed a ~49% replacement rate after 100 s of recovery (Fig. 1D), which is similar to previously reported values (28). These data indicated that the PARP1 protein recovery kinetics was similar to that of H1 histone. A small, but reproducible difference is only observed during the first “fast” phase of recovery (Fig. 1D). During this phase the PARP1 protein recovery is more rapid, which suggested that the pool of soluble nucleoplasmic PARP1 is higher than the pool of soluble H1 protein. The deviation in the binding kinetics may also reflect differences in mechanisms of PARP1 protein and histone H1 interaction with nucleosomal arrays.

Table 1

| Recombinant protein | T<sub>50</sub> |
|---------------------|--------------|
| PARP1-DsRed        | 104 ± 6 s    |
| PARPe-EGFP         | 101 ± 4 s    |
| PARG-EGFP          | 3 ± 2 s      |
| H1-EYFP            | 108 ± 4 s    |
| H2A-ECFP           | >20 min      |

TABLE 1
Quantification of the fluorescence recovery after photobleaching

A 50% recovery time was obtained after analysis of at least an average of 10 single cells/group.

Next, we analyzed PARPe-EGFP protein dynamics in respect to chromatin subdomains, as noted above. We photobleached regions of euchromatin in a giant polyploid cell of Drosophila salivary gland expressing PARPe-EGFP and then recorded the recovery of fluorescence signal in the bleached area by time lapse imaging (Fig. 2A). PARPe-EGFP protein recovery had two distinct phases: 1) a fast phase, in which ~50% of the fluorescent signal was recovered within 100 s after bleaching (Fig. 2A, graph) and 2) a “slow” phase, in which the signal was recovered up to ~97% of starting levels during 15–20 min (not shown).

These results suggested that, in the nucleus, most of the PARPe-EGFP molecules are bound to chromatin at any given time. Following this hypothesis, the pool of free soluble PARPe-EGFP is rapidly depleted for fast recovery, whereas the slow phase recruits PARPe-EGFP, which has dissociated from other chromatin domains. This hypothesis suggests that there is equilibrium of PARPe-EGFP protein association with different domains of chromatin and depletion of PARPe-EGFP protein from one locus leads to redistribution of PARPe-EGFP in the whole nucleus.

To test this idea and better evaluate the kinetics of PARPe-EGFP protein exchange between chromatin subdomains, we bleached an extended rectangular area occupying approximately one-third of the
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The table shows the proteins associated with the PARPe-EGFP protein in Drosophila:

| Protein name | Class of protein          | Nominal molecular weight | MASCOT score | MASCOT expect | Sequence coverage % |
|--------------|---------------------------|--------------------------|--------------|---------------|-------------------|
| P17763       | Retroviral genome polyprotein | 138,031                  | 70           | 0.0046        | 18                |
| Fbp1         | Fat body protein          | 119,591                  | 73           | 0.0014        | 23                |
| PARP1        | Poly(ADP-ribose) polymerase | 114,860                 | 63           | 0.0016        | 16                |
| PARG         | Poly(ADP-ribose) glycohydrolase | 87,033                  | 50           | 0.03          | 16                |
| PARPe        | Poly(ADP-ribose) polymerase embryonic (bait) | 64,326                  | 77           | 0.00091       | 56                |
| CG32031      | Arginine kinase           | 61,264                   | 55           | 0.19          | 36                |
| Hrb98OE     | Heteronuclear RNP-binding protein | 39,038                  | 58           | 0.02          | 23                |
| LP23408p     | Unknown                   | 38,716                   | 27           | 2.5           | 11                |
| GAPDH1       | Glyceraldehyde-3-phosphate dehydrogenase | 35,499                  | 60           | 0.028         | 35                |
| RPL22        | Ribosomal protein         | 30,611                   | 27           | 2.5           | 10                |
| RPL30        | Ribosomal protein         | 29,542                   | 29           | 1.9           | 17                |
| RPS4         | Ribosomal protein         | 29,230                   | 55           | 0.009         | 41                |
| RPL23a       | Ribosomal protein         | 28,626                   | 34           | 2.3           | 16                |
| RPS6         | Ribosomal protein         | 28,407                   | 15           | 3.3           | 8                 |
| RPL18a       | Ribosomal protein         | 21,073                   | 66           | 0.00076       | 42                |
| LP11175p     | Ecdysone-induced protein 71Ec | 20,814                  | 28           | 2.4           | 14                |
| RPL14        | Ribosomal protein         | 19,219                   | 63           | 0.0013        | 39                |
| RPL21        | Ribosomal protein         | 18,475                   | 18           | 3.1           | 20                |
| RPS13        | Ribosomal protein         | 17,037                   | 70           | 0.003         | 50                |
| H3 (H3.3)*   | Core histone              | 15,244                   | 34           | 2.2           | 36                |
| H2B          | Core histone              | 13,556                   | 105          | 9.2e-008      | 64                |
| H2A (H2Av)*  | Core histone              | 13,223                   | 42           | 0.19          | 36                |
| H4           | Core histone              | 11,243                   | 72           | 0.00017       | 56                |

* MS analysis could not distinguish between histone variants H2A-H2Av and H3–H3.3.

Total area of the nucleus (Fig. 2B). We compared the fluorescent signal within four distinct euchromatin subdomains, two (RO1 and RO4) localized outside of the bleached area and two bleached subdomains (RO2 and RO3). The recovery kinetics for the two bleached subdomains was similar to that observed in previous experiments. However, unbleached chromatin subdomains lost PARP1-EGFP fluorescence, whereas fluorescent intensity came to equilibrium in all four areas after ~150 s (Fig. 2B). This observation directly demonstrated that PARP1-EGFP is continuously exchanged between chromatin regions in the nucleus.

Based on the rapid exchange rate, our findings further indicate that the PARP1-EGFP protein is dynamic in its association with chromatin. However, the profile of PARP1-EGFP protein distribution among chromatin subdomains was very stable and reconstituted after recovery from bleaching (Fig. 2B). Thus, there must be high affinity landmarks for PARP1-EGFP binding on chromatin that maintains the stability of local PARP1-EGFP concentration in any given domain of chromatin. To identify these landmarks, we performed purification of PARP1-EGFP-containing protein complexes and identification of PARP1-EGFP protein partners using MS analysis.

PARP1-EGFP Protein Co-purifies with Nucleosomal Core Histones—To identify PARP1-EGFP-chromatin targeting proteins, we performed co-immunoprecipitation experiments from a Drosophila stock with ubiquitous expression of the PARP1-EGFP transgenic construct. We purified protein complexes from nuclear extracts prepared from Drosophila pupae. Pupal extracts treated with micrococcal nuclease to produce mononucleosomes were immunoprecipitated with anti-GFP antibodies to collect PARP1-EGFP-associated complexes. As a control, extracts from wild-type flies were immunoprecipitated in parallel reactions to allow identification of proteins specifically interacting with PARP1-EGFP.

Immunoprecipitates were analyzed by MS analysis. Based on this analysis, we identified 22 nuclear proteins that specifically interacted with the PARP1-EGFP protein (Table 2). Among these, only the nucleosomal core histones H4, H3, H2A, and H2B (Fig. 3A) were ubiquitous chromatin components. We did not identify H1 histone among the PARP1-EGFP-interacting proteins in multiple experiments, even though the interaction of PARP1 protein with linker histone H1 has been shown in vitro (29). The last result correlates with the observation that H1 and PARP1 are antagonists in the chromatin in vivo (3).

The interactions of the PARP1 protein with core histones were confirmed in experiments with immunoprecipitation of protein complexes with PARP1-DsRed protein as bait (not shown). Based on these results, together with our earlier data demonstrating the broad distribution of the PARP1 protein in chromatin, we hypothesized that the PARP1 protein interacts either directly or indirectly with nucleosomal particles.
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core histones; 2) fraction 4 containing auto-modified PARPe-EGFP; and 3) fraction 2 containing free PARPe-EGFP with a mobility shift similar to that previously reported for the phosphorylated form of PARP (30). We confirmed that PARPe-EGFP protein in fraction 4 is automodified by treatment of this fraction with PARP enzyme. After cleavage of ADPPr with PARG, the band of automodified PARPe-EGFP protein disappeared from Western blot (Fig. 3D, inset).

Our data demonstrate that the PARP protein and core histones always co-purify and co-migrate on sucrose gradients after complete digestion of chromatin as a single protein complex. This, in turn, suggests that linker DNA was not required for PARP protein binding to nucleosomes, which implies that PARP interacts either directly or indirectly with core histone particles.

PARP1 Directly Interacts with Core Histones in Vitro—To test whether PARP1 protein interacts with core histones directly, we performed in vitro interaction experiments. We purified core histones (supplemental Fig. S3) and assembled the core histones into octamers (supplemental Fig. S3) as described in Ref. 25. The resulting samples contain core histone octamers and oligomerized octamers, as well as tetramers and dimers (supplemental Fig. S3). Full-length, enzymatically active PARP1 protein coupled to CNBr-activated Sepharose beads was used for an affinity binding assay (26). Beads with PARP1 specifically precipitated histone complexes enriched with core histones H3 and H4 (Fig. 4A), but not IgG protein, which we used as a negative control (Fig. 4A). In a reciprocal experiment, beads with preassembled octamer samples specifically precipitated PARP1 protein from solution, but not IgG (Fig. 4B).

To confirm that histones H3 and H4 mediate PARP1 protein binding, we tested the interaction of individual histones with PARP1. We coupled individual core histones to CNBr-activated Sepharose beads and analyzed their ability to precipitate PARP1 protein from solution. All four of the core histones (H2A, H2B, H3, and H4) were able to bind PARP1 with high affinity in comparison with a Mock control (Fig. 4C). Histone H2B interacted most weakly with PARP1 (titrates 35–40% of PARP1 from solution), whereas H3 and H4 core histones showed highest affinity to PARP1 (precipitated 75 and 60% of PARP protein).

PARP1 preps (Trevigen) typically contain an 80-kDa C-terminal fragment of PARP1, as well as full-length PARP1 (Fig. 4C). In precipitation reactions, the 80-kDa C-terminal fragment of PARP1 protein specifically interacts with histones H3 and H4, whereas H2A and H2B histones bind to full-length PARP1 and the PARP1 C-terminal fragment with similar affinity (Fig. 4C). These data suggest that histones H3 and H4 play the key role in PARP1 targeting to chromatin, whereas the N-terminal domain of PARP1 masks the site of H3/H4 binding on PARP1.

Histone H4 Triggers PARP1 Protein Enzymatic Activity Independently from DNA—To investigate the functional significance of the PARP1 protein interaction with individual histones, we performed a PARP1 activity assay. PARP1 protein was premixed with an equimolar amount of the particular core

FIGURE 3. The PARP-EGFP protein interacts with mononucleosomal arrays in vivo. A, immunoprecipitation assay using PARP-EGFP-expressing Drosophila. Purified nuclei were treated with MNase to obtain complete chromatin digestion, followed by nuclear extract preparation and immunoprecipitation with anti-GFP antibody. Total proteins obtained after immunoprecipitation were subjected to PAGE, and the proteins were detected with Coomassie staining. The left lane is the extract from animals expressing PARP-EGFP recombinant protein, and the right lane is the extract from wild-type Drosophila. The area which corresponds to core histones is labeled on the left side. B, the micrococcal nuclease titration experiment, where nuclei were purified from animals expressing PARP-EGFP protein, is shown. The sample was split into five equal fractions and treated with different amounts of MNase (0, 5, 15, 25, and 50 units/ml) for 3 min, followed by DNA extraction and gel electrophoresis. DNA was visualized by ethidium bromide staining. C and D, sucrose gradient protein-complex fractionation after partial (C) and complete (D) digestion of chromatin is shown. Also shown is the nuclear extract purified from PARP-EGFP animals after nuclei treatment (C) with 5 units of MNase/ml or (D) with 200 units/ml. Treatment was followed by sucrose gradient fractionation. Eleven fractions were collected for each experiment (fractions 10 and 11 are not presented because of the absence of material in those fractions). DNA and proteins were extracted and analyzed using agarose gel (top) and Western blot hybridization (bottom). Specific antibodies were used: RAb anti-GFP (to detect PARP-EGFP); monoclonal antibody anti-H1, rabbit antibody (RAb) anti-H2A and RAb anti-H3 were used to detect linker histone H1 and core histones H2A and H3. DNA molecular weight markers are the same for experiments presented in C and D. Inset, two aliquots of fraction 4 from experiment presented in D were treated with PARG enzyme (+) or incubated with PARG enzyme reaction buffer only (−). The band that corresponds to automodified PARP protein disappeared after incubation with PARG.
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Histones H3 and H4 without N-terminal tails (gift from Ken Zaret Lab) could not activate PARP1 (Fig. 4D, lanes 9 and 10). This result suggested that the N-terminal tail of H4 is critical for H4-dependent PARP1 activation. Surprisingly, we found that our sample of core histone octamers could not stimulate PARP1 enzymatic activity. Moreover, the interaction with octamers inhibited basic activity of PARP1 (Fig. 4D, lane 8). The last result may be explained by the inhibitory effect of the H2A/H2B dimers, which are presented in our octamer samples (Fig. S3B) and which are the PARP1 protein inhibitors (Fig. 4D, lanes 4 and 5). To confirm that PARP1 activation by histones is not due to the presence of DNA contamination in our core histone samples, we repeated the experiment described above, but prior to NAD addition, each sample was treated with MNase. We found that DNA-dependent PARP1 activation was completely abolished in MNase-treated samples, but the H4 sample still stimulated PARP1 activity at the same high level (not shown). These data confirm that the interaction with specific domains of histone H4 could activate PARP1 even without broken DNA.

Next, we analyzed the effects of co-regulation of the PARP1 protein enzymatic activity by individual core histones. Histones H2B and H3 inhibit DNA-dependent PARP1 activation (Fig. 4E, lanes 2 and 3), whereas the H2A histone blocks H4-dependent pADPr-ribosylation (Figs. 4E, lane 9). We also detected that histones H3 and H2B have weak but reproducible inhibitory effects on the H4-dependent PARP1 activation (Fig. 4E, compare lane 6 with lanes 7 and 8).

Based on our results, we conclude that histone H4 triggers PARP1 protein enzymatic activation, which is mediated by the interaction of the N-terminal tail of H4 with C-terminal part of PARP1. Taken altogether, our findings provide the first molecular explanation for DNA-independent PARP1 protein regulation via interaction with different domains of nucleosomal core particles (Fig. 5). The biological significance of the DNA-independent PARP1 activation is also supported by the fact that enzymatic activation of PARP1 protein is involved in transcriptional regulation of inducible genes independent from genotoxic stress response (3, 31).

histone or core histone octamer sample, followed by the addition of NAD to reaction mixture. Upon completion of the reaction, we analyzed the accumulation of the product of PARP1 enzymatic activity, pADPr. The reaction mixtures were subjected to PAGE followed by Western blot analysis using monoclonal antibody against pADPr. PARP1 protein without co-activators showed very low basic activity (Fig. 4D, lane 1). However, DNA digested with endonucleases induced a pADP-ribosylation reaction (Fig. 4D, lane 2). Chemical inhibitor 3-amino-benzosamide completely blocked DNA-dependent PARP1 activity (Fig. 4D, lane 3). Core histones H2A and H2B inhibited, whereas histone H3 stimulated basic activity of PARP1 (Fig. 4D, lanes 4–6). Strikingly, we found that the histone H4 alone stimulated PARP1 four times stronger than randomly broken DNA (Fig. 4D, lane 7).
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FIGURE 5. Model of the regulation of PARP1 protein enzymatic activity in chromatin. Step 1, PARP1 protein is broadly distributed in chromatin because of interaction with core histones in the context of nucleosome. PARP1 is inactive in this state because of inhibitory effect of histone H2A. Step 2, genotoxic stress-dependent PARP1 activation. The N-terminal domain of PARP1 protein serves as a sensor of the double-stranded breaks or nicks in genomic DNA. Upon binding of damaged DNA, it mediates conformational changes, which leads to disruption of interaction with histones and consequently to the activation of PARP1 enzymatic reaction. Step 3, DNA-independent PARP1 activation. Developmental or environmental signals induces local changes in the "histone modification core" and subsequently expose the N-terminal tail of histone H4 and/or hide histone H2A followed by H4-dependent PARP1 activation.

DISCUSSION

The PARP1 protein is required for chromatin assembly, gene silencing, and gene expression (2, 31, 17). Considerable evidence suggests that PARP1 is a regular constituent of chromatin and the nucleoplasm (32, 33). Immunofluorescence studies of mammalian and Drosophila nuclei indicate that PARP1 is widespread and abundant (2, 5, 34). In this paper, we provide the first insight into the nature of the association of the PARP1 protein with chromatin in vivo and in vitro. We have characterized the dynamics between free and chromatin-bound PARP1 protein and suggest an additional mechanism for these interactions. We also demonstrated that PARP1 associates with chromatin on a mononucleosomal level in vivo. More specifically, we demonstrated that H3 and H4 are preferential binding sites for the C-terminal domain of PARP1 and that DNA is not required for this association in vitro. Histone H4 works as a strong DNA-independent activator of pADPr enzymatic reaction, whereas other histones (especially H2A) inhibit H4-dependent PARP1 activation.

Our observations directly show that the PARP1 protein is exchanged rapidly between chromatin regions in the nucleus. We detect no difference between the recovery rate of enzymatically inactive PARPe-EGFP protein and active PARP1-DsRed protein isoforms. Therefore, we propose that PARP1 enzymatic activity is not required for steady-state dynamics. However, PARP1 inactivation followed by due automodification of PARP1 molecules has been shown to be critical for PARP1 protein removal from chromatin (35). We confirmed the existence of two distinct mechanisms controlling PARP1 interaction with chromatin as a result of our sucrose gradient purification experiments. That is, unmodified PARP1 molecules co-purified with nucleosomes, as well as other fractions (Complex I), whereas PARP1 molecules modified with pADPr were segregated to a separate fraction (Complex II) (Fig. 3D). Based on this finding, we conclude that, indeed, two distinct mechanisms conjoin to control PARP1 molecule interaction with chromatin. One involves a protein-equilibrated binding via association-dissociation, and the other involves irreversible removal of PARP1 from chromatin after automodification. Based on an accepted model, the existence of Complexes I and II was expected (10, 36, 37). PARP1 protein is associated with chromatin in its inactive state (Complex I), and upon activation it becomes automodified, loses contact with chromatin, and establishes interactions with pADPr-binding proteins (Complex II). Interestingly, the fraction with Complex II also contains a significant amount of unmodified PARP1. This may suggest that there is a nucleoplasmic pool of unmodified PARP1 that can reversibly bind to pADPr.

Similar to H1, PARP1 controls the establishment of silenced chromatin (17). Recently, it has been shown that PARP1 and H1 work independently. Moreover, they antagonize each other in chromatin (3). This antagonistic interaction strongly suggests competition for the same binding sites. The site of linker histone binding is known to be the linker DNA in the context of nucleosomal array (38–41). We found that, unlike H1, linker DNA is not crucial for PARP protein binding. This, in turn, suggests that if H1 and PARP compete for binding sites, they recognize different but overlapping, epitopes.

The ability of PARP1 to bind chromatin via nicks in double-stranded DNA, as well as noncanonical DNA structures, has been demonstrated in vitro (31). Still, the broad PARP1 localization in chromatin in vivo suggests an alternative mechanism for PARP1 protein binding. Histones H2A and H2B have been reported as preferential targets for PARP1 binding in vitro (11) and for enzymatic modification by PARP1 (12–14). In our experiments, unmodified PARP1 protein always co-purified with core histones, even after DNA digestion to mononucleosomes (Fig. 3). We also found that the C terminus of PARP1 preferentially binds histones H3 and H4 of histone octamers lacking DNA. The PARP1 C terminus contains the catalytic domain and the sequence required for homodimerization and thus activation. PARP1 C terminus binding to H3/H4 may serve to sequester the domains in PARP1 that are required for activation, and this could account for the broad localization of PARP1 in chromatin. We demonstrated that histone H4 activates, whereas histone H2A completely inhibits, PARP1 protein. These findings support the conclusion that the PARP1 protein is generally silent (enzymatically inactive) in chromatin, although a number of developmental and environmental stimuli could still activate it at specific loci. This activation is required for chromatin decondensation and transcriptional activation in these loci. PARP1 activation always correlates with changes of local histone modification (e.g. phosphorylation of histone H3 co-localized with pADPr in Drosophila puffs (2)). Therefore, we hypothesize that changes in histone modification code promote conformational alteration of nucleosomes and...
therefore expose (or hide) specific domains of histones, which activate (or inhibit) PARP1 (Fig. 5).

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