Reptin and Pontin Oligomerization and Activity Are Modulated through Histone H3 N-terminal Tail Interaction*

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Background: Pontin and Reptin are main components of remodeling complexes responsible for chromatin dynamics. Results: These ATPases interact with the chromatin basic unit, the nucleosome, which regulates Pontin/Reptin enzymatic activities and oligomerization assemblies. Conclusion: By means of this interaction, pontin/reptin generate a loading platform that coordinates assembly of cofactors onto chromatin. Significance: Pontin/Reptin monomeric and multimeric forms control DNA metabolism.

Pontin/RUVBL1 and Reptin/RUVBL2 are DNA-dependent ATPases involved in numerous cellular processes and are essential components of chromatin remodeling complexes and transcription factor assemblies. However, their existence as monomeric and oligomeric forms with differential activity in vivo reflects their versatility. Using a biochemical approach, we have studied the role of the nucleosome core particle and histone N-terminal tail modifications in the assembly and enzymatic activities of Reptin/Pontin. We demonstrate that purified Reptin and Pontin form stable complexes with nucleosomes. The ATPase activity of Reptin/Pontin is modulated by acetylation and methylation of the histone H3 N terminus. In vivo, association of Reptin with the progesterone receptor gene promoter is concomitant with changes in H3 marks of the surrounding nucleosomes. Furthermore, the presence of H3 tail peptides regulates the monomer-oligomer transition of Reptin/Pontin. Proteins that are pulled down by monomeric Reptin/Pontin differ from those that can bind to hexamers. We propose that changes in the oligomeric status of Reptin/Pontin create a platform that brings specific cofactors close to gene promoters and loads regulatory factors to establish an active state of chromatin.

DNA of eukaryotic genomes is packaged into arrays of nucleosome, the basic structural unit of chromatin. For DNA transactions, the DNA template must be made readily accessible. Organization of chromatin structure and loading of transcription machinery and factors are regulated by numerous factors, among them Reptin and Pontin.

Reptin and pontin (also referred to as TIP48/TIP49, TIP49B/TIP49A, RuvBl2/RuvBl1, Rvb2/Rvb1, THH2/TIH1, TAP54/TAP54α, and ECP51/ECP54 (1)) were originally identified as components of multisubunit transcription complexes (2). They were later shown to associate physically with several transcription factors (RNA polymerase II holoenzyme, c-Myc, β-catenin, Ero, E2F1, and ATF2) (3). Depending on the context, they may play antagonistic, repressive, or activating roles during regulation of gene expression and embryonic development (3). Besides interacting with transcription factors, Reptin and Pontin belong to three ATP-dependent chromatin remodeling complexes, TIP60 (4, 5), INO80 (6–8), and SWR1 (9, 10), that intervene in transcriptional processes and in response to double strand breaks. The Reptin and Pontin proteins appear not only as major subunits of these large macromolecular networks but also as necessary elements for their activity.

Reptin and Pontin are closely related and highly conserved eukaryotic proteins. Both are essential for viability in yeast (11), Drosophila melanogaster (12), and Caenorhabditis elegans (13). Both proteins are present in many macromolecular assemblies, but complexes containing only one of the proteins without the other have also been characterized (14).

From a structural point of view, Reptin and Pontin bear the characteristic ATP-binding and hydrolysis motifs of the AAA+ family (ATPases associated with diverse cellular activities) as follows: the Walker A and B, arginine fingers, and sensor domains. The minimal subunit consists of monomers that are made up of three domains (13). The N-terminal domain, domain I, contains the conserved Walker A and Walker B motifs. The C-terminal domain, domain III, associates with domain I to form a nucleotide pocket seen in the three-dimensional structure (13). Between the Walker A and Walker B motifs, an insertion of 170 amino acids represent domain II that is structurally organized as an OB-fold domain and is similar to the three-dimensional structure of the ssDNA4-binding

4 The abbreviations used are: ssDNA, single-stranded DNA; BisTris, 2-[bis(2-hydroxyethyl)amino] -2-[hydroxymethyl]propane-1,3-diol; ERα, estrogen receptor-α; E2, 17β-estradiol; PCNA, proliferating cell nuclear antigen.
domain of the replication factor RPA (13, 15). X-ray and electron microscopy (EM) studies of both human and yeast proteins reported the architecture of Reptin-Pontin complexes as single hexameric rings (13, 16, 17) or as higher order dodecameric rings (18–21). An overall hexameric molecule comprises monomers connected through interactions of DII to DIII domains from adjacent subunits, encircling one ADP unit. Little is known about which forms function in vivo; evidence has been obtained for the existence of hexamers and dodecamers (18, 22, 23) but so far not for monomers.

Enzymatically, Reptin and Pontin share ATPase and helicase activities. Both activities are DNA-dependent. The isolated DII domain of Pontin has been shown to bind nucleic acids (ssDNA, dsDNA, and RNA) in vitro (13). In addition, we previously reported that the DNA binding capacity of Reptin relies on the monomeric form (22). The limiting step for binding is the initial cooperative loading of the protein onto DNA. This binding cooperativity might attest to the need for a critical concentration of protein subunits close to DNA. DNA binding stimulates a weak ATPase activity; it also supports a slow DNA unwinding activity (22). In contrast, Reptin hexamers are inactive for ATP hydrolysis and DNA unwinding (22). Similar results were observed for Pontin.5

Biological activities of the Reptin and Pontin proteins are generally inferred from the function of factors with which they associate (23–25). Mechanisms through which Reptin and Pontin exercise their activities are far from being understood. A role in the proper assembly of the multicomponent chromatin remodeling complexes, INO80 and Tip60, has been proposed (4, 7, 23), but no link with the enzymatic activities of the Reptin/Pontin has yet been established.

We report here our investigation of how the biochemical properties of monomers and oligomers of the Reptin/Pontin proteins contribute to the function of chromatin remodeling complexes through regulation of DNA binding. We demonstrate a physical interaction between Reptin/Pontin proteins and the nucleosome. We identify a new mechanism showing how the Reptin/Pontin enzymatic capacities are modulated by post-translational modifications of H3 tails. Reptin/Pontin conformational transitions arising from these interactions may coordinate recruitment of different protein partners.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used: anti-Reptin antibody 2E9-5 (SAB42000115, Sigma); anti-TIP49A (Pontin) antibody 2943C1a (ab51500, Abcam); anti-histone H2A (ab13923, Abcam); anti-histone H3 antibody (ab1791, Abcam); ERα antibody (H-184) (sc-7207, Santa Cruz Biotechnology); HDAC1 antibody 10E2 (sc-81598, Santa Cruz Biotechnology); HDAC2 antibody 3F3 (sc-81599, Santa Cruz Biotechnology); anti-MLH1, clone 14 (ab-1, Oncogene Research Product); and anti-PCNA, clone PC10 (ab-1, Calbiochem).

**Protein Purification**—For Pontin and Reptin purification, recombinant *Rattus norvegicus* Pontin was produced following the same protocol but with 3 h of isopropyl 1-thio-β-d-galactopyranoside induction. Each protein was separately purified as monomers and as hexamers (Fig. 1B) as detailed previously and illustrated (22). The purification scheme included an LC-MS/MS analysis confirming the absence of bacterial ATPase contamination.

**Histone Purification**—The coding sequences of *H. sapiens* full-length histones H2A, H2AX, H2A.Z, H2B, H3, and H4 were a kind gift from Dr. A. Hamiche (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The coding sequence of the tail-less H3 was obtained by PCR amplification subcloning of the H3 coding sequence from H40 to the end. To optimize the production of H2AX and H4 histones, the first codons were modified. For histone H2AX, ATG TCG GGC CGC GGC AAG was changed to ATG TCT GGC CGC GGT AAG. This modification does not alter the protein sequence. For H4 histone, the sequence ATG TCT GGC CGC GGT AAG was changed to ATG ATA GGC AGA GGT AAG. This modification replaces S2 by Ile. Recombinant histone proteins were produced in bacteria and purified as described (26) with some modifications. Briefly, H2A.Z, H2B, and H3 histones were expressed in *Escherichia coli* strain BL21 (DE3) and H2A, H2AX, and H4 histones in strain Rosetta (DE3) pLysS. Histones were recovered in inclusion bodies that were washed, solubilized, applied to a strong cation exchange column (HiTrap™ SP-Sepharose FF, 1 ml, GE Healthcare), and eluted with the same step procedure used previously. Purified histones were lyophilized and conserved at −80 °C. Lyophilized histones were used to reconstitute H2A-H2B, H2AX-H2B, and H2A.Z-H2B dimers and the (H3-H4)2 tetramer, purified by gel filtration (HiLoad 16/60 Superdex S-200 PG column, GE Healthcare).

**Mononucleosome Assembly**—The positioning sequence used for nucleosome reconstitution is contained in a pUC18 plasmid (27). A 193-bp DNA fragment (used to assemble Nucl-193) was synthesized by PCR using 5′-biotinylated primers (see Fig. 1A for schematic). A 148-bp blunt-ended DNA fragment (used to assemble Nucl-148) was obtained by enzymatic digestion (ScaI-AluI, on Fig. 1A) of the 193-bp product. The DNA fragments were gel-purified and end-labeled by T4 polynucleotide kinase (Promega) using [γ-32P]ATP. Nucleosome assembly by salt gradient dialysis was performed as described previously (28). The efficiency of nucleosome assembly was monitored by electrophoretic migration in a 6% polyacrylamide gel in 0.5× Tris borate/EDTA, and mononucleosomes were isolated from the gel as described (28).

**Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay**—For EMSA, 0.3–0.9 μM Reptin or Pontin was incubated for 15 min at 4 °C with 32P-end-labeled nucleosome-DNA substrate (DNA equivalent, 75 pg) or 20 bp (7.75 pg) or 40 bp (15.5 pg) of substrate in a 10-μl reaction volume containing 25 mM HEPES-KOH (pH 8.0), 2.5 mM Mg(CH3COO)2, 0.2 mM DTT, 100 μg/ml BSA (Sigma) (RB 1× buffer) supplemented with 2 mM ATP or ADP as indicated. The reaction was loaded onto a 6% polyacrylamide gel in 0.5× Tris borate/EDTA. For competition experiments, DNA competitors were added after the binding reaction for a further incubation of 10 min. For the supershift assay, 2 μM Reptin or Pontin was incubated for 15 min at 4 °C with 140 pg of Nucl-193 in a 10-μl reaction volume
Impact of Nucleosome Interaction on Reptin/Pontin Activities

RESULTS

Reptin and Pontin Bind to Nucleosomes as an Active Complex—

To determine how Reptin/Pontin interacts with chromatin, we reconstituted nucleosome core particles containing the canonical histones, (H2A-H2B) dimers and (H3-H4) tetramers, on a 193-bp DNA carrying a positioning sequence (27). Mononucleosomes can adopt two alternative positions, central or end-positioned, defined after analysis by restriction enzyme accessibility (Fig. 1A). As Reptin/Pontin preparations, we used purified monomeric Reptin or Pontin fractions (Fig. 1B). We have previously demonstrated that the monomeric form obtained in these preparations, rather than oligomeric assemblies, is proficient for DNA binding and enzymatic activity (22). Electrophoretic mobility gel shift assays showed that Reptin or Pontin monomers form complexes with nucleosome-DNA substrates (Fig. 1, C and D, lanes 1–4) in the same range of protein concentrations needed for binding to naked DNA (Fig. 1, C and D, lanes 13–16).

We also prepared mononucleosome-DNA substrates that include H2A variants, because Reptin and Pontin have been associated with H2A.Z exchange (31) and phosphorylated H2AX removal (4). We observed that complex formation on these substrates was as efficient as on canonical H2A nucleosome-DNA substrates (Fig. 1, C and D, lanes 5–12). To make sure that Reptin or Pontin binding preserves nucleosome integrity, we first confirmed the presence of H2A protein in the binding complex. Addition of the corresponding antibody produced a supershift of the pre-formed Reptin/Pontin-nucleosome complexes (Fig. 1E, lanes 4 and 9). Moreover, Western blot analysis of the retarded band confirmed the presence of H2A protein as described previously (29). Samples were sonicated to generate DNA fragments <500 bp. Chromatin fragments were immunoprecipitated using antibodies against H3k9-me2 (ab1220, Abcam), pan-acetyl H3 (ab47915, Abcam), H3 (ab1791, ABCAM), or an irrelevant HA antibody (H6908, Sigma). Anti-Reptin was synthesized by the laboratory from the purified protein (30). The precipitated DNA was amplified by real time PCR, with primer sets designed to amplify the promoter (transcription start site) of the PGR gene (Fig. 5A).

Quantitative RT-PCR primers are as follows: PGR, 5' - GCCTC-GGGTTGTAGATTTCA-3' and 5' - TGGGTGAAGCCTTG-TTGTA-3'. All ChIP data are shown as percent input.

Pulldown Analyses—800 µl (10 mg/ml) of HeLa nuclear extract were pre-cleared for 4 h at 4 °C with 80 µl of previously equilibrated anti-FLAG® M2 affinity gel (Sigma) in IP300 buffer (300 mM NaCl, 25 mM Tris-HCl (pH 8), 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 5 mM MgCl2). Precleared samples (100 µl) were mixed with a monomeric or hexameric form of Reptin or Pontin proteins (10 µg) in 180 µl of reaction buffer containing 25 mM HEPES-KOH (pH 8.0), 2.5 mM Mg(CH3COO)2, 0.2 mM DTT, 100 µg/ml BSA (Sigma) for 1 h at 4 °C. Reptin or Pontin complexes were recovered by incubating the samples with 20 µl of equilibrated anti-FLAG gel overnight at 4 °C on a rotating wheel. Beads were washed four times in IP300 buffer, and Reptin and Pontin complexes were eluted with 100 µl of FLAG peptides (250 µg/ml) in IP300 buffer. Bound proteins were analyzed by SDS-PAGE and Western blotting.
Impact of Nucleosome Interaction on Reptin/Pontin Activities

A) Naked DNA and nucleosome variants

B) Gel electrophoresis for Reptin and Pontin

C) Complexes and nucleosomes for Reptin and Pontin

D) Additional gel comparisons for Reptin and Pontin

E) Supershift complexes and nucleosomes

G) Proteins and complexes

WB: α-H3 and ExtrAvidin
Impact of Nucleosome Interaction on Reptin/Pontin Activities

We next verified the functionality of this complex. We knew that ATP/ADP inhibited the Reptin or Pontin monomer binding to ssDNA, whereas these nucleotide cofactors did not significantly affect the stability of the protein-dsDNA complexes (data not shown). We therefore tested the effect of adding ATP/ADP to the complexes composed of Reptin or Pontin monomers and a reconstructed mononucleosome. As shown in Fig. 1F (lanes 3 and 4), addition of ATP or ADP released a fraction of proteins from the substrates, demonstrating that the preformed Reptin/nucleosome interaction is reversible. A similar result was obtained with the Pontin protein (Fig. 1F, lane 7). ATP/ADP induction of conformational changes of Reptin/Pontin might be correlated with changes in their DNA-binding properties.

Reptin and Pontin Preferentially Bind to Nucleosomes with Linker DNA—Because these experiments were carried out with mononucleosomes reconstituted on DNA fragments long enough to leave naked DNA extensions (nucl-193), we wondered whether the DNA by itself could modulate Reptin/Pontin binding to nucleosomes. We used short DNA fragments to reconstitute mononucleosomes with no linker DNA (nucl-148). Association of both monomeric proteins was more efficient on nucl-193 than on nucl-148, suggesting that additional DNA favors formation and/or stability of bound complexes (Fig. 2A).

However, it seemed unlikely that nucleosome association of Reptin/Pontin only relied on the binding to extranucleosomal DNA. First, binding to nucl-148 that completely lacks linker DNA was still effective (more than 30% of bound fraction for both proteins, see Fig. 2A). Second, nucleosome positions on the 193-bp DNA were analyzed by restriction enzyme protection (Fig. 1A) and found to occupy two major positions, relative to the dyad axis: (a) either in a central position leaving symmetric extranucleosomal entry/exit DNA of 15–35 bp (b) or in a distal position, leaving single extranucleosomal entry/exit DNA of 46 bp at the most. For comparison, we used binding substrates of naked dsDNA that were 20 and 40 bp in length (Fig. 2B). Percentages of the bound fraction indicated that the longest DNA (ds-40) is a poorer binding substrate (50 and 24% for Pontin and Reptin binding, respectively) than nucl-193 (85 and 78.5% for Pontin and Reptin binding, respectively).

Finally, we confirmed the stability of the nucl-193 binding complexes by competition experiments, using ds- or ss-oligonucleotides (ds115 and ss115 (22)) which are our usual efficient binding substrates for Reptin/Pontin. These DNA were able to break down band shifting of nucleosome-DNA substrates only when added in at least a 10-fold molar excess to the reaction (Fig. 2C).

Interaction of Reptin/Pontin with the Nucleosome Core Particle Is Modified by Post-translational Modifications of the N Terminus of H3—In view of the nucleosome interaction, we next searched for evidence of the Reptin/Pontin interaction with the histones themselves. It was reported that poly(ADP-ribose) polymerase I protein binds linker DNA and interacts with histones (32, 33). Indeed, Reptin and Pontin proteins specifically co-localized with H3 in far-Western experiments using purified recombinant H2A/H2B dimers and the H3/H4 tetramer (Fig. 3A). This co-localization was specific because no signal was detected when the Reptin/Pontin proteins were omitted from the reaction (data not shown).

The histone tail extensions protrude from the nucleosome core particle and are thought to be largely unstructured and intrinsically flexible (34, 35). Moreover, histone tails are preferential interaction partners, making them promising candidates for interaction with Reptin/Pontin. Preincubating Reptin with an excess of a synthetic peptide corresponding to the H3 N-terminal sequence substantially weakened the interaction signal detected with the tetramer in a far-Western blot (Fig. 3B). In parallel, adding an H2A-tail peptide produced no change in the interaction signal intensity. Finally, to confirm that the H3 tail mediated Reptin/Pontin binding, we found that the interaction was completely abolished when histone H3 lacked its N-terminal extremity (Fig. 3C). Because the histone N-terminal domains are targets for numerous post-translational modifications that occur during DNA metabolism, we asked whether covalent modifications of histone H3 would affect its binding to Reptin/Pontin. We focused on methylation and acetylation of the H3 histone tail. We used synthetic peptides derived from the H3 tail sequence, both unmodified and modified by dimethylation of lysines 4 and 34.

**FIGURE 1. Reptin and Pontin bind to mono-nucleosome. A** characterization of nucleosomes. The extranucleosomal DNAs at one entry/exit of the nucleosome were interpreted by restriction digestion with either Aval (A) or EcoRV (•), or both, as indicated. Samples (6 ng) of the 193-bp DNA without (naked DNA) and with (nuc-H2A) the histone octamer separated by 0% native gel electrophoresis in 0.5%–Tris-glycine. Schematics on both sides of the gel represent the different species as follows: lines used for DNA and gray ovals for nucleosomal particles. EcoRV digestion of the B and D species produced B′ and D′, respectively. Aval digestion of D and D′ species produced D′. The 193-bp DNA template used for nucleosome reconstitutions is drawn below. Rectangle indicates the positioning sequence. Locations of Aval and EcoRV restriction sites (used for cartography) and Scal and Alul (used to produce the 148-bp fragment) are indicated by arrows, B, purified Reptin/Pontin proteins used in this study. Gel filtration profiles of Reptin and Pontin on Superdex 200 column are shown. Aliquots from fractions corresponding to the hexameric and monomeric peaks were analyzed on Blue Native BisTris-PAGE after silver staining. milli-
and 9 or acylation of lysines 14, 18, and 23 (Fig. 3D). The expected interaction between the H3 tail and Reptin/Pontin was verified with dot-blot experiments. Although dimethylation marks allowed us to maintain binding to Reptin/Pontin (Fig. 3E), acetylated marks largely interfered with the interaction between the modified peptide and Reptin/Pontin proteins.
At the same time, no interaction between a H2A-tail peptide and Reptin or Pontin was observed. Reproducibility of the results was confirmed by additional dot-blot experiments (Fig. 3E).

The effects of post-translational modifications of H3 on direct interactions were also seen in pulldown experiments, in which a biotin tag at the C terminus enabled anchoring to streptavidin-coated beads. Using H3-derived peptides as bait and purified Reptin, we detected stable interactions between proteins and peptides (Fig. 3F). Again, interaction was much stronger with unmodified and methylated H3 peptides than with acetylated H3 peptides. Only a poor signal was detected in the absence of peptides (Fig. 3F, lane 4). Moreover, in the same pulldown experiment, we have included an irrelevant protein (namely Prp43) carrying a His tag. This control does not show any binding to H3 tail peptides.

Physical Interaction between the H3 Tail and Reptin/Pontin Is Functional—To test whether the interaction of Reptin/Pontin monomers with nucleosomes was functionally important for their enzymatic activities, their helicase activity was assayed. We have previously reported that the enzymatic activities relied on the monomeric form of the proteins but not on the hexameric form (22). Purified monomers of Reptin and Pontin possess a 3′ to 5′ DNA-unwinding activity that requires 3′-ssDNA

FIGURE 3. Reptin and Pontin proteins physically interact with the histone H3 through the N-terminal tail. A–C, refolded histones (20 pmol), as H2A/H2B dimers and H3/H4 tetramers, were fractionated on a 16% SDS-PAGE and either visualized on gel by silver staining or subjected to far-Western blotting (WB) analysis in the presence of Reptin- or Pontin-purified proteins (250 pmol). B, binding of Reptin to the H3 histone was challenged by an excess of peptide competitors (8 μM), corresponding to the N-terminal sequences of H2A or H3 histones. C, far-Western experiments were carried out with H3 histone deleted for its tail domain (H3Δt) or full-length H3 histone. D, peptide sequences correspond to the 1–29 amino acids of the H3 N-terminal tail. The positions of the modified residues (di-methylated or acetylated) are shown in boldface. All the peptides carry a biotin labeling at the C-terminal extremity. E, peptides of unmodified (H3), methylated (H3me2/2), and acetylated (H3ac3) H3 tails probed with Reptin or Pontin proteins were tested in dot-blot experiments. H2A tail peptide (H2A) (sequence is given under “Experimental Procedures”) was used as a control. Loading was set up in duplicate with two repeats of the same peptides spotted on the 1st and 2nd lane. Ponceau staining of the membrane validates equal levels for each peptide. Additional results coming from independent dot-blot experiments are presented on the lower panels. F, interaction of Reptin protein with H3 histone tails was examined by pulldown assays using the biotinylated tail peptides immobilized onto magnetic streptavidin beads. Beads prebound to unmodified (H3), methylated (H3me2/2), and acetylated (H3ac3) peptides were incubated with Reptin or Prp43 as an irrelevant protein. At the end of the reaction, proteins bound to the beads were verified by Western blot. Histogram corresponds to the quantification of Reptin protein retained on the membrane. Lane 5 contains 50% of the input protein. IP, immunoprecipitation.
extensions of ≈30 nucleotides (22). Addition of the N-terminal H3 peptide to the reaction completely inhibited the DNA unwinding capacity of Reptin (Fig. 4A). The methylated H3 tail even produced an intermediate inhibition. However, the acetylated H3 tail did not influence helicase activity, as if the acetyl groups added to the H3 tail were sufficient to suppress the inhibitory effect induced by unmodified H3 tails. The capacity of helicase inhibition by the H3 tail follows the binding affinity of Reptin/Pontin for the H3 tail and depends on whether or not the tail carries post-translational modifications.

Keeping in mind that helicase activity requires ATP hydrolysis (22), we then monitored the effect of histone tails on Reptin/Pontin ATPase activity. Literature on Reptin/Pontin ATPase activity may appear as controversial. However, data obtained with defined oligomeric forms of the proteins produced consensual results showing that ATPase activity is...
restricted to the monomeric form of the human (22, 36) or yeast (17, 22) Reptin/Pontin. We found here that the interacting H3 tail peptides hindered the ATPase activity when monomeric purified Reptin/Pontin were used (Fig. 4, B and C). Indeed, addition of increasing amounts of unmodified and methylated H3 peptides to the ATPase reaction resulted in a decrease in Reptin/Pontin ATP hydrolysis, whereas addition of acetylated H3 tails did not affect the reaction (Fig. 4, B and C). Because ssDNA was in large excess (28 μM) over protein (1 μM) or peptides (up to 10 μM), the observed inhibition was unlikely to be due to titration of DNA by the positively charged H3 tail peptide. Indeed, varying the ssDNA substrate concentration (data not shown) demonstrated that H3 peptide addition blocked ATPase activity above the basal activity. Moreover, a control peptide whose charge density was comparable with H3 tail peptide (P21; sequence in “Experimental Procedures”) did not inhibit the ATPase capacity of Reptin or Pontin (Fig. 4, B and C). Thus, the inhibition induced by the presence of unmodified and methylated H3 tails was clearly due to a negative effect on the enzymatic activity.

The presence of dimethylation on both Lys-4 and Lys-9 of H3 has not been described. We therefore tested two peptides, each with a single dimethylated lysine for effects on Reptin/Pontin ATPase activity. Both disturbed the ATP hydrolysis capacity of Reptin and Pontin monomers when added to the reaction (Fig. 4, B and C). Thus, the inhibition induced by the presence of unmodified and methylated H3 tails was clearly due to a negative effect on the enzymatic activity.

In Vivo Dynamics of Reptin on the Progesterone Receptor Gene Promoter Are Associated with Local Changes in H3 Histone Modifications—To evaluate the physiological relevance of the interaction between H3 tails and Reptin/Pontin, we assayed the presence of the proteins on a promoter of an endogenous gene whose expression can be activated. We chose a promoter whose activity depends on estrogen receptor (ER)-α and the progesterone receptor promoter (PgR), in ERα-positive human breast cancer cells (MCF-7). It was recently established that Reptin is required for initiating chromatin reorganization during 17β-estradiol (E2) induction of the ERα target oncogene, cyclin D1 (CCND1) (30). Pontin itself was not included in this study; nonetheless, it usually belongs to Reptin complexes, and in particular, silencing one protein led to depletion of both in MCF7 cells and other tumor cell lines (37). We found Reptin associated with the progesterone receptor (PgR) promoter in conditions under which PgR is not expressed (see “Experimental Procedures” and Fleury et al. (49)) using chromatin immunoprecipitation with an antibody directed against Reptin (Fig. 5A). Addition of E2 to the cell culture for activation of PgR transcription led to a 2.5-fold decrease in the amount of Reptin bound. We then explored the H3 content in the surrounding nucleosomes before and after E2 treatment. Using specific antibodies raised against acetylated lysines of the H3 N-terminal extension, we determined that acetylation levels at the PgR promoter increased 2.2-fold following E2 induction (Fig. 5B). Concomitantly, a 2.8-fold decrease in the H3K9 di-methylation levels was detected (Fig. 5C), although the amount of total immunoprecipitated histone H3 remained unchanged. Thus, variations in H3 post-translational modifications accompany Reptin binding to the endogenous PgR promoter region independently of nucleosome occupancy.

Monomer to Hexamer Transitions of Reptin/Pontin Impact Cofactor Recruitment—Monomeric subunits of Reptin and Pontin can assemble as hexameric rings or as dodecameric double rings (38). We postulated that H3 N termini and specific post-translational modifications may influence the Reptin/Pontin assembly as they regulate the enzymatic activity of these proteins. Each oligomeric state of Reptin/Pontin proteins

![Figure 5](http://www.jbc.org/content/journal/jbc/289/49/fig/fig5)

**FIGURE 5.** Reptin to the PGR promoter inversely correlates with changes of adjacent histone H3 acetylation. MCF-7 cells were cultivated 3 days in steroid-stripped (white) medium and then induced by 10^{-7} M E2 for 45 min. Chromatin was immunoprecipitated using antibodies against Reptin (A), pan Ac-H3 (B), H3K9-me2 (C), or histone H3 (D). Sequences within the PGR-B promoter were amplified by quantitative PCR. Results are shown as percent input. NT, not treated.
Impact of Nucleosome Interaction on Reptin/Pontin Activities

would then be able to recruit a distinct set of cofactors that controls their function.

The conversion of purified monomers to hexamers and even dodecamers was induced by addition of ATP or ADP and monitored by native PAGE analysis (Fig. 6A, lanes 2 and 3). The presence of DNA in tandem with ADP helps the Reptin protein to organize in hexamers (Fig. 6A, lanes 4–6). It was not possible to resolve Pontin migration on native gels as the protein has appeared unable to form detectable oligomers upon nucleotide binding (20, 39). Addition of the H3 tail peptide further promoted the transition from monomers to hexamers, under these experimental conditions (Fig. 6B) as shown on the Western blot corresponding to the native gel. Next we used, on purpose, less favorable hexamerization conditions. In the absence of DNA and at lower ADP concentrations, only a fraction of monomer converted to hexamers and allowed us to quantify the effect of H3 tail peptides (Fig. 6, C and D). Hexamerization was efficient in the presence of the unmodified H3 peptide as well as the methylated H3 peptide (Fig. 6C, lanes 3 and 4). In contrast, hexamerization of Reptin was compromised by adding the acetylated H3 peptide (Fig. 6C, lane 5). Quantification of each oligomer species detected on the Western blot confirmed these results (Fig. 6D). Addition of either nonmodified or methylated peptides stimulated the transition from monomer to hexamer (from 40 to 51.5% or 46.5%). In the presence of acetylated peptide, hexamer formation was largely impeded (from 40 to 34.4%). Furthermore, we note a global reduction (up to 40% for some experiments) in the total amount of proteins in the presence of H3 tail peptides. Possibly, these peptides generate aggregates or some kind of material unable to migrate under our conditions of native gels.

We next assessed whether different cofactors were recruited by distinct oligomerization states of Reptin/Pontin. Pulldown experiments were performed using individually purified tagged monomer or hexamer forms of Reptin or Pontin as baits to complement HeLa nuclear extracts (Fig. 6E). We identified a set of proteins known to interact with Reptin or Pontin (24), such as HDAC1, HDAC2, MLH1, and PCNA. In addition, new potential partners were found, such as ERα. Notably, the profile of proteins that pulled down with Reptin or Pontin monomers was different from those precipitating with the hexameric forms. For example, ERα was found to interact with hexameric Pontin, although HDAC2, MLH1, and PCNA bound to the monomeric form of Reptin. HDAC1, in turn, precipitated with both the monomeric and the hexameric Reptin fraction (Fig. 6E). The exclusive presence of ERα in the Pontin hexamer complex was confirmed using a second antibody raised against the C-terminal part of the protein (data not shown).

As we were concerned with monomer to hexamer transitions, we evaluated the binding affinity between our two oligomeric species. We compared monomer or hexamer binding to naked DNA fragments or to nucleosome substrates by EMSA. Reptin or Pontin monomers efficiently bound a 115-bp dsDNA (Fig. 7A, lanes 2 and 7), whereas hexamers were unable (Reptin) or poorly competent (Pontin) to form complexes on this substrate, even at high protein concentrations (lanes 3–5 and 8–10). However, both monomers and hexamers associated with reconstituted nucleosomes (Fig. 7B). These observations suggest that monomer preferentially may act on nucleosome-free DNA, although hexamers would assume specific functions on nucleosome core particles.

DISCUSSION

The AAA + family members, Reptin and Pontin, are essential components of several high molecular weight protein complexes. Although their structural features and contribution to the organization of these complexes have been extensively studied, the function of Reptin/Pontin still remains unclear. In this study, we provide evidence for physical interaction and functional interplay between Reptin/Pontin and chromatin. We propose a model in which the Reptin/Pontin-nucleosome complex creates a platform for the assembly of a variety of enzymatic complexes in a multistep process (see model Fig. 8). Monomer subunits of Reptin/Pontin are first recruited to DNA in nucleosome-free regions. In the vicinity of nucleosomes, protruding histone H3 tails contact and stabilize this DNA-protein complex. Post-translational modifications of H3 may modulate the physical interaction with Reptin/Pontin, which is lost by H3 tail acetylation but maintained following methylation. The interaction with the nucleosome seems to influence the oligomeric state of Reptin/Pontin, either indirectly by regulating enzymatic activities or directly by modulating subunit assembly. The oligomeric organization of Reptin and Pontin determines binding to different subsets of factors. Newly formed hexamers tend to dissociate from DNA and re-load directly on the proximal nucleosomal core particle, possibly in a “ring to ring” structure.

Our main finding is that Reptin and Pontin are able to bind to nucleosomes. We and others (13, 22) previously demonstrated that monomeric subunits of Reptin and Pontin are able to bind naked DNA. DNA binding determines their biochemical ATPase and helicase activities (22). Here, we show that Reptin and Pontin are also able to form complexes with nucleosomes. The relevance of this interaction is consistent with the presence of the proteins in remodeling and transcription factor networks. Interestingly, our results show that Reptin/Pontin behave in a similar manner to INO80 in that efficient binding to nucleosomes depends on extranucleosomal DNA (40). Moreover, through a combination of electron microscopy, cross-linking, and mass spectrometry, Tosi et al. (41) have determined the position of Reptin/Pontin within a reconstituted INO80 chromatin remodeling complex and mapped interactions with the nucleosome. Among them was Reptin/Pontin cross-linked to the N terminus of H3. The study also mentions cross-links of Reptin to H2B and H4, although these would require partial unwrapping of the DNA. We did not detect complexes with H2B or H4 histones, probably because of their instability. In addition, the H3 N termini were shown to protrude from both sides of the nucleosomal dyad axis and are positioned on DNA at the entry-exit site of the nucleosome (42), at just the place where a protein like Reptin/Pontin bound to free/linker DNA would meet the nucleosome.

The Reptin/Pontin proteins do not contain domains implicated in histone recognition. The binding of the Reptin/Pontin to H3 tail residues involves a novel motif. In that sense, Reptin/Pontin resemble the poly(ADP-ribose) polymerase I protein,
Impact of Nucleosome Interaction on Reptin/Pontin Activities

FIGURE 6. In the presence of H3 tail peptide, proteins stand in monomeric or hexameric assemblies, which can recruit differential partners. A, oligomeric products resulted from incubation of recombinant monomer of Reptin (500 ng) (lanes 1–6), with ATP or ADP (2.85 mM) (lanes 2, 3, 5, and 6), in the presence or not of DNA (525 mM) (lanes 4–6), were separated on Blue Native BisTris-PAGE and revealed by silver staining. Purified hexamer (h, lane 7) is included as a marker of migration. B, under identical conditions of ADP and DNA as in A, H3 tail peptide (8 μM) (lane 3) was added to the monomer. The panel corresponds to the detection of the proteins through their His tag by Western blot from native gel with HRP-coupled anti-His. The products were compared with the hexameric form (h, lane 4). C, when DNA is omitted and ADP concentration is lowered (0.3 mM), monomeric (0.75 mM) to hexameric transitions are evaluated in the presence of H3-t peptides (5 μM) as follows: unmodified H3-t (lane 3), methylated H3me2/2-t (lane 4), and acetylated H3ac3-t (lane 5), by Western blot from native gel with HRP-coupled anti-His. D, results from quantification analysis of Western blots from C (three experiments) are expressed as percentages of total signal per gel lane for the hexamer (left) or monomer (right) species. E, HeLa nuclear extracts were complemented with purified monomers (referred to as mono, lanes 2 and 4) or hexamers (referred to as hexa, lanes 3 and 5) of each FLAG-tagged protein, as indicated. Immunoprecipitations (IPs) were performed with either Pontin or Reptin as bait. The pulled down proteins were detected by 4–20% SDS-PAGE followed by silver staining. Asterisk shows the position of precipitating IgG1 heavy chains. Lane 1 is a negative immunoprecipitation control, carried out in the absence of exogenous bait proteins Pontin or Reptin. Presumed interacting proteins were identified by Western blot (WB) analysis with specific antibodies raised against the following: ERα, HDAC1, HDAC2, mHMLH1, and PCNA. Western blot with anti-Reptin and anti-Pontin serve as positive controls for Pontin-IP and Reptin-IP, respectively. The double asterisk on the PCNA blot points out precipitating IgG1 light chains.

FIGURE 7. Binding substrate affinity of monomers and hexamers of Reptin/Pontin. EMSA experiments were performed with either purified monomeric or hexameric forms of the proteins. A, binding substrate was a duplex oligonucleotide of 115 bp in length. The protein concentration was 0.6 μM for the monomer (lane 2) and 0.4, 0.6, and 0.9 μM for the hexamer (lanes 3–5, respectively). B, binding substrate was the reconstituted mononucleosome (nucl-193) used in Fig. 2. The increasing concentration of proteins 0.3, 0.6, and 0.9 μM were used for both monomeric and hexameric fractions.

which interacts with both linker DNA and histone tails independently of any identified consensus sequence (32, 33).

Histone tails serve as binding sites for protein domains and target sites for post-translational modifications to regulate chromatin structure. Interaction of Reptin/Pontin with H3 tails would mask and shield some of these preferential sites, hence controlling H3 tail accessibility. In this regard, we can mention the direct interaction of Tip60 with H3 tri-methylated at Lys-9, which activates its acetyltransferase activity (43). Moreover, the H3 tail, depending on its post-translational modifications, could regulate exchanges of Reptin/Pontin by interfering with the oligomeric state of the proteins. Relocation of Reptin has been observed on inducible promoters (this study and Ref. 30).5 In the repressed state, Reptin is associated with promoter sequences. Its presence possibly masks cognate response elements from specific factors like transcription factors, blocking gene activation. Upon induction, Reptin dissociates from DNA (22).

Although purified hexamers of Reptin/Pontin do not bind efficiently to naked DNA, they do form complexes with nucleosome-DNA substrates (Fig. 8). Thus, a structural shift from monomer to hexamer might be accompanied by shifting the interaction from linker DNA to the adjacent nucleosome. Interestingly, the diameter of both hexamer and nucleosome are similar and would be compatible with a ring to ring interaction. The ATPase domain of Reptin/Pontin could regulate the spatial organization, from monomers lying on the extranucleosomal DNA to hexameric or dodecameric rings spanning the proximal nucleosome. In this case, both topology of linker DNA and histone marks would direct Reptin/Pontin to the −1 or +1 nucleosome, in the same way that they could induce the formation of a ring structure. Consequently, targeting the −1 or +1 nucleosome would expose the surface from either Reptin or Pontin. Such a scenario could explain why Reptin and Pontin were found to play an antagonistic role on the same promoter, because of interaction with different transcription factors (3). The idea that the nucleosome-free region could play a predominant role in targeting components to the proper nucleosome was recently evoked for SWR-C (44).
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