Nucleoplasmin Binds Histone H2A-H2B Dimers through Its Distal Face*5

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Nucleoplasmin (NP) is a pentameric chaperone that regulates the condensation state of chromatin extracting specific basic proteins from sperm chromatin and depositing H2A-H2B histone dimers. It has been proposed that histones could bind to either the lateral or distal face of the pentameric structure. Here, we combine different biochemical and biophysical techniques to show that natural, hyperphosphorylated NP can bind five H2A-H2B dimers and that the amount of bound ligand depends on the overall charge (phosphorylation level) of the chaperone. Three-dimensional reconstruction of NP/H2A-H2B complex carried out by electron microscopy reveals that histones interact with the chaperone distal face. Limited proteolysis and mass spectrometry indicate that the interaction results in protection of the histone fold and most of the H2A and H2B C-terminal tails. This structural information can help to understand the function of NP as a histone chaperone.

Nucleoplasmin (NP) or NPM2) belongs to the so called “histone chaperone” or “nuclear chaperone” group of proteins that, together with other chromatin remodeling complexes, are responsible for chromatin remodeling events during cell division, apoptosis, fertilization, DNA damage repair, etc. (1–5).

Histone chaperones shield the positive charge of bound histones, allowing their proper deposition onto DNA in the chromatin assembly process (3, 6). NP regulates the condensation state of chromatin by extracting sperm-specific basic proteins from DNA and depositing H2A-H2B dimers. NP has been isolated in vivo forming complexes with H2A-H2B dimers, and in vitro it also binds H3-H4 tetramers and core histones octamers (1, 7). Moreover, NP interacts with and displaces chromatin-bound linker histones H1, H5 (erythrocytes specific linker histones), and B4 (Xenopus embryonic linker histones) (8, 9). This behavior has also been observed for other histone chaperones belonging to different protein families (NAP-1 and TAF-I) (3, 6, 10, 11).

The nucleoplasmin/nucleoplasmin family of histone chaperones is ubiquitously represented throughout the animal kingdom (4, 12) and includes the following four main groups: nucleoplasmin (NPM1), nucleoplasmin (NPM2 or NP), nucleoplasmin-like (NPM-like), and NPM3. All of them share similarities in their amino acid sequence. They have a protease-resistant N-terminal core, responsible for oligomerization, which includes in most cases a short A1 acidic tract. The C-terminal tail domain usually contains two acidic tracts (A2 and A3) and has variable lengths and specific functional motifs among different family members. The biological activity of NP is controlled by phosphorylation of several residues in both the core and tail domains (13, 14). During egg maturation, the phosphorylation degree of NP increases so that the average numbers of phosphoryl groups per protein monomers estimated for the protein isolated from Xenopus oocytes and eggs are 2–3 and 7–10, respectively (15). Mass spectrometry analysis of eNP has identified eight phosphorylated residues, four located at the flexible N terminus and the other four at the tail domain flanking the nuclear location signal (16). The acidic pentameric N-terminal cores (NP-core; 120 residues) of NP, NPM-like, and NPM1 have been crystallized (17–20), and the structure found is that of a pentamer with each monomer folding into an eight-stranded $\beta$-barrel with a jelly roll topology. Spectroscopic and predictive methods have shown that the NP C-terminal tail (80 residues), which has not been crystallized, has mainly a disordered conformation that might be important for ligand binding (15), in agreement with sedimentation velocity results (21). Based on crystallographic data, three protein surfaces have been defined as follows: the pentamer oligomerization surface, the lateral surface that has been proposed as the binding domain for the core histone octamer, and the distal face that due to its flexibility is not present in the atomic structure (17). Our group has previously suggested that the pentamer distal face is involved in sperm-specific basic proteins and linker his-
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tone binding (14, 16). We have also shown that the interaction of NP with linker-like histones has a strong electrostatic component (22), although core histone binding also involves hydrophobic and stereospecific components such as the histone fold (23).

We report here a study on H2A-H2B binding to natural (isolated from Xenopus eggs) nucleoplasmin (eNP). The three-dimensional reconstruction obtained by electron microscopy of eNP reveals five arms protruding from the cup-shaped NP-core, showing for the first time the presence of the flexible C-terminal domains. Our results show that native pentameric eNP is able to bind five H2A-H2B dimers. The three-dimensional reconstruction of the complex shows that the interaction between chaperone and histones takes place at the distal region of NP, through the five arms of the pantameric structure. Docking of the available structures into the EM map of this complex and proteolysis analysis also reveal that the C-terminal domain of NP and the histone fold and C-terminal tails of both histones are involved in the interaction. The structural information thus obtained can help to understand the biological function of NP as a histone chaperone.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Native NPs isolated from eggs (eNP) or oocytes of Xenopus laevis were purified as described previously (15). NP concentration was determined by the bicinchoninic acid assay (Sigma). Native H2A-H2B dimers were obtained from chicken erythrocyte chromatin upon elution of a hydroxyapatite column using a 0.35–2 M NaCl gradient in 20 mM potassium phosphate (pH 6.8) buffer, according to Ref. 24. The extinction coefficient of H2A-H2B dimers in water at 230 nm was determined by amino acid analysis using norleucine as internal standard and was estimated to be 4.35 cm² mg⁻¹. Recombinant full-length (rNP) and truncated mutants ΔC50NP (lacking the last 50 residues) and ΔC80NP (lacking the last 80 residues) were expressed and purified as published previously (15).

Titration of Nucleoplasmin with H2A-H2B Dimers—Native H2A-H2B dimers (3–4 mg/ml) in 20 mM potassium phosphate (pH 6.8) buffer containing 1.6 M NaCl were rapidly mixed with the desired NP concentration. The final buffer composition was 2 mM MgCl₂, 240 mM NaCl, 25 mM Tris-HCl (pH 7.5) (Buffer 1). Molar ratios are expressed as eNP pentamer/H2A-H2B dimers.

Analytical Ultracentrifugation—Sedimentation velocity runs were performed in a Beckman XL-1 analytical ultracentrifuge using an An-55 aluminum rotor, at 20 °C. Samples in Buffer 1 were loaded in double sector cells with aluminum-filled Epon centerpieces (22). UV scans were taken at 230 nm and analyzed by the van Holde and Weischat (Biochem) method using XL-A Ultra Scan II version 6.0 sedimentation data analysis software (Borries Demeler, Health Science Center, University of Texas, San Antonio).

Sucrose Gradients—Sucrose gradients (5–20% sucrose) were prepared in 240 mM NaCl, 12 mM potassium phosphate, 2 mM MgCl₂, 20 mM Tris-HCl (pH 8.8). Histone H2A-H2B dimers at ~3 mg/ml in 20 mM potassium phosphate (pH 6.8), 1.6 M NaCl were rapidly mixed with 2 mg/ml NP solution, in 150 mM NaCl, 25 mM Tris-HCl (pH 7.5) at different molar ratios. After incubating the mixture for 1 h at room temperature, it was loaded onto the sucrose gradients. Gradients were run on an SW 14.1 Beckman rotor at 103,800 × g and 4 °C for 20 h. Fractions from the gradient peak were dialyzed against distilled water, concentrated by speed vacuum, resuspended in sample buffer, and analyzed by SDS-PAGE (12.5% acrylamide). This avoids the different acid-induced precipitation of both proteins that would hamper estimation of their molar ratio in the complex.

Gel Filtration Chromatography—Size exclusion chromatography of NP, histones, and eNP/histone complexes was performed in Buffer 1. The histones, kept at high salt concentration as described before, were mixed with eNP (5.5 μl) solutions at different stoichiometries (pentameric NP/histones). Samples were incubated for 1 h at 25 °C and loaded into a calibrated Superose 6 PC 32/30 analytical column (GE Healthcare). Elution volumes were measured by peak integration using Unicorn software (GE Healthcare). Fractions were precipitated with 20% trichloroacetic acid prior to SDS-PAGE.

Partial Proteolysis by Trypsin—Partial proteolysis of the different nucleoplasmins (eNP, rNP, ΔC50NP, and ΔC80NP) and NP/H2A-H2B complexes (1:5 molar ratio) was carried out in Buffer 1. Isolated proteins or their complexes at 0.38 mg/ml were incubated at 37 °C with trypsin (1:500 w/w). Aliquots were taken at 30 and 60 min, and the reaction was stopped with acetic acid. Proteolytic fragments were analyzed by SDS-PAGE and/or 12.5% Tris-Tricine SDS-PAGE.

Electrophoretic Mobility Shift Assay—Mixtures of 27 μM eNP and different histone concentrations in a 12-μl reaction volume were equilibrated at room temperature for 1 h. Complexes were resolved by native PAGE on a Phast System apparatus (GE Healthcare) or by native agarose gel electrophoresis, using a 5–15% gradient Phast gel or a 0.8% agarose gel, respectively. The buffer used in native agarose electrophoresis was 25 mM Tris-HCl, (pH 8.5), 19.2 mM glycin. In both cases the proteins were stained with Coomassie Brilliant Blue as described previously (25).

Mass Spectrometry—Selected tryptic bands were excised manually from the gel and subjected to in-gel tryptic digestion according to Ref. 26 with minor modifications (27). LC-MS/MS analysis was done using a Q-TOF micro mass spectrometer (Waters) interfaced with a CapLC chromatography system (Waters) as described previously (27). Briefly, digested peptides were loaded onto a Symmetry300TM C18 NanoEase Trap (0.18 × 23.5 mm, Waters) precolumn connected to an Atlantis dC18 NanoEase (75 μm × 150 mm, Waters) column. Peptides were eluted with a 30-min linear gradient from 10 to 60% acetonitrile. Data-dependent MS/MS acquisitions were performed on precursors with charge states of 2–4 over a survey m/z range of 400–1500. Collision energies were varied as a function of the m/z and charge state of each peptide. Spectra were processed and searched against SwissProt data base using ProteinLynx Global Server 2.1 (Waters) using standard searching parameters (27).

Electron Microscopy—5-μl aliquots of NP or the NP/H2A-H2B (1:5 molar ratio) complex were applied to glow-discharged carbon grids for 1 min and then stained for 1 min with 2% uranyl acetate. Images were recorded at a 0° tilt under low dose conditions in a JEOL 1200EX-II electron microscope operated...
at 100 kV and recorded on Kodak SO-163 film at ×60,000 nominal magnification.

Three-dimensional Reconstruction and Docking Analysis—Micrographs were digitized using a Zeiss scanner, and 9862 NP and 5557 NP/H2A-H2B particles were selected from the micrographs using XMIPP software (28) at a sampling rate of 2.3 Å per pixel. In the case of NP, several volumes were generated as starting templates for angular refinement methods implemented in EMAN (29). First, images were classified into homogeneous groups using a maximum likelihood approach implemented in the XMIPP platform (supplemental Fig. S1), and selected averages were used to build reference volumes using common lines procedures. Also, the available atomic structure of the NP core filtered at 50 Å and a pentameric gaussian blob with the rough dimensions of the protein were also used as starting models. Projections from all these template volumes were initially confronted to the data set in independent refinements using methods implemented in EMAN; all the reconstructions converged to a unique and stable structural model. This structure was then subjected to the projection matching protocols implemented in the SPIDER package (30) to generate the final volume. In the case of the eNP/H2A-H2B complex, a similar procedure was followed but using as starting models a Gaussian blob, common lines procedures, and the final map of the NP obtained in this work. During all the iterations, 5-fold symmetry was imposed to the volumes. The resolution of the final structures was estimated by Fourier shell correlation to be 21 and 19.5 Å for the eNP and eNP/H2A-H2B complex, respectively (using the 0.5 cross-correlation coefficient criteria).

Docking of the atomic structures of the NP-core domain (PDB 1K5I) and H2A-H2B (extracted from the atomic structure of the nucleosome; PDB 1AOI) into the three-dimensional reconstruction of NP and the NP/H2A-H2B complex was carried out manually and optimized using COLACOR, an off-lattice correlation maximizer distributed with Situs 2.2, based on the local optimization of COLORES (31). The density maps and atomic structures were visualized with UCSF Chimera from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

RESULTS

Hyperphosphorylated eNP Pentamer Binds H2A-H2B Dimers with a 1:5 Molar Stoichiometry—Complex formation between pentameric eNp and H2A-H2B dimers was analyzed by native electrophoresis (Fig. 1A). As seen by the electrophoretic mobility of the eNP/H2A-H2B complex at a molar ratio of 1:2 (Fig. 1A, lane 2), there is no free NP, and the molecular mass of the complex increases with increasing molar ratios (lanes 3–5) until a charge neutralization effect hampers the complex to enter into the gel. The same applies to the free histones, which being positively charged do not enter the gel, and therefore, although this technique detects complex formation, it cannot be used to estimate the stoichiometry of the complex under saturating conditions. To overcome this limitation, the eNP/H2A-H2B complexes were also run in a 5–30% sucrose gradient. Protein composition of different fractions was analyzed by SDS-PAGE to estimate the eNP/histone molar ratio, using densitometric analysis of the corresponding bands and known amounts of protein as standards. eNP/H2A-H2B complexes give rise to a peak that sediments faster than the free eNP pentamer and H2A-H2B dimers (Fig. 1, B and C). The estimated saturating molar ratio for the eNP/H2A-H2B complex was 1:5.2 ± 0.5, because above this value the complex appears in the same fractions, with the same relative amounts of bound histones, and the peak of free histone becomes detectable.

We next studied complex formation by sedimentation velocity analysis (Fig. 2A). Under the experimental conditions used in this study the native histone H2A-H2B dimer (~28 kDa) has a sedimentation coefficient of 1.6–2.0 s20,w (data not shown), whereas eNP forms a stable pentamer (110 kDa) with a sedimentation coefficient of 6.04 s20,w (Fig. 2A). At eNP/H2A-H2B molar ratios of 1:1 and 1:2, the complex exhibits a narrow distribution of s20,w with midpoint values of 6.7 and 7.0 s20,w, respectively. A further increase in the molar ratio to 1:3 and 1:5
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results in a parallel increase of the $s_{20,w}$ values to 8.0 and 8.7–9.1, respectively. At higher molar ratios (1:7 to 1:10), the integral distribution of the sedimentation coefficients shows a bimodal behavior, with 75–85% (after correction for the different extinction coefficients of nucleoplasm and histone dimer) of the sample sedimenting with a narrow sedimentation coefficient distribution centered around 9.1, and the rest sedimenting as free histones and a very small (less than 5%) aggregated fraction that came out of solution. From these results, the estimated saturated eNP/H2A-H2B molar ratio is also 1:5. The same complexes were analyzed by size exclusion chromatography analysis (Superose 6PC 3.2/30) of eNP/H2A-H2B complexes. Absorbance at 230 nm for eNP control (dotted line), H2A-H2B (gray), eNP/H2A-H2B 1:5 (dashed-dotted line), eNP/H2A-H2B 1:8 (dashed line), and eNP/H2A-H2B 1:10 (black). C, SDS-PAGE analysis of the fractions corresponding to the eNP/H2A-H2B 1:5 and 1:8 complexes, obtained in B, which were precipitated in 20% TCA. The eNP and H2A-H2B positions are indicated. Lane C contains the input sample.

showed that eNP and H2A-H2B dimers coelute as a complex, although by following the same argument mentioned above for isolated eNP, it was not possible to accurately measure its molecular mass with this technique. As expected for a saturating binding process, at eNP/H2A-H2B molar ratios higher than 1:5, besides the peak corresponding to the saturated complex, it is also possible to detect a shoulder that, as shown by SDS-PAGE, contained free histones. Taken together, all these results indicate that the eNP pentamer can stably bind five H2A-H2B dimers, in agreement with previously reported data on recombinant nonphosphorylated NP/H2A-H2B complexes (32).

Three-dimensional Reconstruction of eNP—Aliquots of eNP were negatively stained and observed under the electron microscope. The particles looked square, with some small arms protruding from one of the sides (supplemental Fig. S1A). These images show that the size of the molecules is compatible with the presence of single pentamers, and no double pentamers, like the ones found in the crystallographic structures (17, 18), were observed under our experimental conditions. A three-dimensional reconstruction was carried out with negatively stained particles of eNP, as described under “Experimental Procedures,” imposing a 5-fold symmetry throughout the reconstruction procedure, a symmetry that is clearly present in the particles (supplemental Fig. S1B). The volume generated (Fig. 3A) has the shape of a cup of ~70 Å wide and ~45 Å high. Protruding from the main body, the structure reveals five small arms of ~30 Å high that point outward, parallel to the 5-fold axis. The structure of the main body is very similar to that obtained at atomic resolution for the NP-core (17, 18, 20), and this resemblance is strengthened when the atomic structure of the NP-core is docked into the mass of the reconstructed particle (Fig. 3B). The docking leaves the C-terminal domains of the NP-core pentamer pointing toward the arms, thus suggesting that these protruding masses form part of the C-terminal, histone-binding domain. This domain has been hypothesized to be intrinsically disordered and very flexible (15), contrary to the highly structured N-terminal, NP-core domain, the only amenable one to crystallization. It is therefore very tempting to suggest that these arms are flexible but that the 5-fold symmetrization imposed has generated an average conformation of this part of the pentamer.

Three-dimensional Reconstruction of the eNP/H2A-H2B Complex—The complex between eNP and the H2A-H2B dimers was prepared as described under “Experimental Procedures,” and aliquots of the complex were negatively stained and subjected to electron microscopy. The particles observed revealed a larger mass than that corresponding to the eNP particles (supplemental Fig. S1C). Because the stoichiometry determined for the interaction between the eNP pentamer and the H2A-H2B dimers is 1:5, a three-dimensional reconstruction was performed with these particles assuming 5-fold symmetry for the complex throughout the reconstruction procedure (supplemental Fig. S2). The three-dimensional reconstruction generated rendered a structure of ~115 Å high and ~85 Å in its widest part (Fig. 4A), with two large masses connected by five linkers. The largest mass clearly resembles the structure of the NP-core, and the docking of the atomic structure of the NP-core into this part of the reconstructed...
complex confirms this assumption (supplemental Fig. S3), pointing to the other large mass as the place where the five H2A-H2B dimers are located. Docking of these dimers into the reconstructed mass is very good (Fig. 4B) and suggests that each H2A-H2B dimer interacts with one of the arms of the eNP pentamer, which corresponds to the C-terminal region that has been postulated to be involved in H2A-H2B binding (32) but also with adjacent dimers forming a five-star structure (Fig. 4B). Although at this level of resolution the orientation of the H2A-H2B dimer is uncertain, charge distribution and proteolysis experiments (see below) support the following docking analysis. Each H2A-H2B dimer would interact with the C-terminal region of one of the NP monomers through the H2B subunit, more specifically with the C-terminal region of α-helix 2 (Arg79–Arg86), which contains several basic amino acids, including Arg86, involved in DNA phosphate-histone interactions in the nucleosome (Fig. 4B) (33). The pentameric star-like arrangement of the H2A-H2B dimers would be generated through the interaction of the C termini of two adjacent H2A subunits (approximately the Gly105–Pro109 sequence of a given H2A subunit with the Asn89–Leu93 sequence of the adjacent one; Fig. 4, B and C). The charge distribution in the surface of the individual H2A-H2B dimers shows a clear complementarity between the putative contact regions defined in the docking (Fig. 4C).

Limited Proteolysis of the eNP/H2A-H2B Complex—The three-dimensional reconstruction of the eNP/H2A-H2B complex described here reveals that the interaction between the chaperone and the histones takes place through the distal face of the pentamer, and it differs from previous reports that point to the lateral face of the chaperone as the histone-interacting region (17, 19, 20, 32). We further characterized this interaction by carrying out limited proteolysis experiments on the eNP/H2A-H2B complex (Fig. 5A). As a control, the H2A-H2B dimers were subjected to trypsin treatment, and it was found that whereas the histone dimer was almost completely degraded by trypsin after 30 min in 0.24 M salt, at 2 M NaCl the digestion produced two stable fragments with molecular masses of 9 and 6.4 kDa (Fig. 5A), as already described (34).
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FIGURE 5. Complex formation protects histones against proteolysis. A, Tris-Tricine gel electrophoresis of the tryptic peptides obtained from the NP/H2A-H2B (1:5 molar ratio) complexes and isolated proteins at two digestion times. The left lane contains molecular weight markers. The bands highlighted within the box for eNP/H2A-H2B (1:5) complexes were excised, in-gel digested, and analyzed by mass spectrometry. ΔC50NP and ΔC80NP bands are marked with an asterisk. B, amino acid sequence of the H2A and H2B histones. Shown in red are the sequences identified by the mass spectrometry analysis. The histone fold of the H2A-H2B dimer is shown in a gray box, and the α-helices are indicated. C, docking of the H2B dimer into the three-dimensional reconstruction of the eNP/H2A-H2B complex. The regions of the H2A-H2B dimer accessible to the trypsin attack are labeled in yellow. The atomic structure of H2A is depicted in red and that of H2B in pink.

is due to the salt-induced stabilization of the histone fold that diminishes the accessibility of protease cleavage sites (35). Several NP variants (eNP, rNP, and the deletion mutants ΔC50NP and ΔC80NP) were also subjected to trypsin proteolysis. Whereas no proteolysis was observed for the deletion mutants, suggesting that there are no cleavage sites for trypsin in the N-terminal compact NP-core (supplemental Fig. S4), trypsin treatment of both rNP and eNP produced several proteolytic fragments with apparent molecular masses between 17 and 15 kDa. rNP was degraded more slowly than eNP due to the more compact conformation of nonphosphorylated recombinant NP (15, 36, 37).

NP/H2A-H2B complexes were subsequently formed with the NP variants described above, using a 1:5 molar ratio (Fig. 5A). H2A-H2B binding to all NP variants, except ΔC80NP, protected histones against protease attack. Using the same protein/peptidase ratio in all samples, the histone tryptic fragments obtained from the complexes are similar to those seen after protease treatment of isolated H2A-H2B dimers in the presence of 2 m salt (Fig. 5A). This suggests that H2A-H2B dimers are less accessible to enzymatic cleavage when bound to NP, most likely due to a direct interaction with the chaperone that hinders cleavage sites and/or to a conformational change brought about by electrostatic stabilization of the complex that compacts the histone structure. The comparison of the relative intensities of the histone-protected bands also reveals the following protection hierarchy eNP > ΔC50NP > rNP. The tryptic fragments between 14.4 and 6.5 kDa generated upon proteolysis of the eNP/H2A-H2B complex were further in-gel digested with trypsin and analyzed by mass spectrometry. With one sequence coverage of 64 and 54% for H2A and H2B, respectively, all of the identified peptides came from H2A-H2B and none from NP. The noncovered part of the sequence corresponds to the C- and N-terminal tails of the histones, very rich in Lys and Arg residues and therefore difficult to be detected by mass spectrometry analysis after proteolysis (Fig. 5B). This finding indicates that the globular core (histone fold) of both histones, and most of their C-terminal tails are protected against protease attack when complexed with NP. The protection pattern agrees very well with the docking of the five H2A-H2B dimers into the three-dimensional reconstruction of the eNP/H2A-H2B complex shown in Fig. 5C, because the regions of the H2A-H2B dimer that in the docking model are involved in the interaction with either eNP (Arg⁷⁹–Arg⁸⁶ in H2B) or with the other H2A-H2B dimers (Asn⁸⁹–Leu⁹³ and Gly¹⁰⁵–Pro¹⁰⁹ in H2A) are indeed protected from proteolysis (Fig. 5B). These results show for the first time the involvement of the H2A-H2B dimer histone fold in nucleosomal histones-NP interaction, in agreement with the finding that the core of the histone octamer interacts with NP (23).

Negative Charge of the Chaperone at Its Distal Face Modulates the Stoichiometry of the Complex—If H2A-H2B dimers interact with the distal face of NP, then the overall negative charge at this protein face should modulate complex formation. To test this possibility, the interaction between natural (eNP and oNP) and nonphosphorylated recombinant (rNP, ΔC80NP) NPs and H2A-H2B was followed by changes in their electrophoretic mobility (Fig. 6). The overall negative charge of the complex is progressively neutralized as the amount of bound histone increases, resulting in a reduced complex migration. Above certain NP/H2A-H2B molar ratios, different for each NP variant, the corresponding complexes do not enter the gel as a consequence of charge neutralization and not to protein aggregation. Aggregation can be ruled out because analysis of the same samples by sucrose gradients demonstrated that they did not contain a significant amount of aggregated material (see Fig. 1B for eNP/H2A-H2B complexes). The good correspondence between the eNP/H2A-H2B molar ratio at which charge
Based on the atomic structures of *Xenopus* nucleoplasmin, *Drosophila* NPL, and nucleophosmin (NO38) cores (17, 19, 20), it has been proposed that histone octamers bind to the lateral surface of the chaperone, formed by β-hairpins. This model assumes that five octamers could dock around the periphery of an NP decamer. Under our experimental conditions, we have not been able to detect the presence of NP decamers, neither for NP nor for the NP/H2A-H2B complexes. The three-dimensional reconstruction of the eNP/H2A-H2B complex presented herein demonstrates that the distal face of nucleoplasmin interacts with H2A-H2B dimers and that each eNP monomer has one binding site for an H2A-H2B dimer. The atomic structure of the Asf-1 core domain bound to truncated recombinant histones H3-H4 (38) also shows that each Asf-1 monomer binds one H3-H4 dimer and that complex formation involves the H3 histone fold and the H4 C-terminal β-sheet. Recent NMR data indicate that Chz.1 binds one H2A-H2B dimer per protein core (39), through clusters of oppositely charged residues.

A comparison of H2A-H2B binding to natural eNP and oNP and to recombinant rNP and ΔC80NP with increasing amounts of H2A-H2B (chaperone pentamer/histone dimer molar ratio) is shown. The position of the different NPs is seen in the lane without histones. Basic histones do no enter the gel on their own.

neutralization (Fig. 6) and saturation (Fig. 2) are observed suggests that saturation occurs at higher molar ratios as the overall negative charge of the chaperone increases by phosphorylation (eNP > oNP > rNP). Data also indicate that the affinity of ΔC80NP toward H2A-H2B is lower than that of the other NP variants and points to the NP C-terminal tail involvement in the stabilization of the complex, in good agreement with a recent study on the interaction of recombinant NP with H2A-H2B (supplemental Fig. S5) (32). Therefore, the overall negative charge at the pentamer distal face modulates the amount of H2A-H2B dimers that the chaperone can bind, as suggested by the EM model.

**DISCUSSION**

The interaction of NP with nucleosomal histones involves electrostatics as well as stereospecific constraints (17, 23, 32). Our results show that H2A-H2B binding to hyperphosphorylated NP is a saturable process with a 5:1 stoichiometry, as found for the interaction with nonphosphorylated recombinant NP (32).

**FIGURE 6. Distal face charge of the chaperone modulates the stoichiometry of the complex.** Complex formation is shown between the different natural and recombinant NP variants and H2A-H2B dimers. Gel shift assay (0.7% agarose) of eNP, oocytes from NP, rNP, and ΔC80NP with increasing amounts of H2A-H2B (chaperone pentamer/histone dimer molar ratio) is shown. The position of the different NPs is seen in the lane without histones. Basic histones do no enter the gel on their own.

Our data also indicate that the interaction between NP and H2A-H2B has a strong electrostatic component, as evidenced by the effect of the C-terminal domain deletion and phosphorylation on the stability of the complex. Complex formation results in charge neutralization, which in turn would stabilize it by diminishing the repulsion due to the proposed accumulation of negatively charged residues at the distal face of the NP pentamer (14, 16, 40). This negative patch would be formed by the acidic tracts A1, A2, and A3, and by the phosphoryl groups at the C- and N-terminal regions of the protein, not resolved in the x-ray structure of the core domain (17, 20). These highly charged protein regions would provide a flexible binding site for H2A-H2B histones. Nevertheless, the importance of hydrophobic interactions in complex formation also has to be considered, as reported recently (32).

During the first stages of fertilization in *X. laevis*, NP is responsible for the decondensation of the sperm chromatin and the assembly of new nucleosomes upon removal of SPs (sperm-specific basic proteins) and addition of H2A-H2B dimers to the pre-existing H3-H4 tetrasomes (5). Our previous work (14)
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suggested that binding of the sperm-specific basic proteins takes place at the distal face of the NP pentamer. Data presented herein conclusively show that the interacting surfaces of NP with H2A-H2B are also placed at the same face of the protein particle and therefore suggest that competition of both types of ligands for common binding sites might be part of the mechanism that facilitates their exchange during chromatin remodeling. The distal face of NP would provide a flexible and phosphorylatable negatively charged region, formed by the intrinsically disordered C-terminal domain and the N terminus of NP, where ligands can bind. The flexibility of this region could also confer this domain with the ability to adapt to different types of histone ligands.

The three-dimensional EM maps have been deposited in the EM Data Base (http://www.ebi.ac.uk/pdbe/emdb/) with accession numbers EMD-1777 and -1778. The atomic coordinates of the docking have been deposited in the Protein Data Bank (http://www.rcsb.org/) with accession code 2QXL.

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