Phosphorylation of Both Nucleoplasmin Domains Is Required for Activation of Its Chromatin Decondensation Activity*

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Nucleoplasmin (NP) is a histone chaperone involved in nucleosome assembly, chromatin decondensation at fertilization, and apoptosis. To carry out these activities NP has to interact with different types of histones, an interaction that is regulated by phosphorylation. Here we have identified a number of phosphorylated residues by mass spectrometry and generated mutants in which these amino acids are replaced by Asp to mimic the effect of phosphorylation. Our results show that, among the eight phosphoryl group experimentally detected, four are located at the flexible N terminus, and the rest are found at the tail domain, flanking the nuclear localization signal. Phosphorylation-mimicking mutations render a recombinant protein as active in chromatin decondensation as hyperphosphorylated NP isolated from *Xenopus laevis* eggs. Comparison of mutants in which the core and tail domains of the protein were independently or simultaneously “activated” indicates that activation or phosphorylation of both protein domains is required for NP to efficiently extract linker-type histones from chromatin.

The eukaryotic cell genome is packed as a fibrous superstructure known as chromatin. The dynamic structure of chromatin is reflected in the continuous modification of its three-dimensional structure, promoted by the activity of histone chaperones that induce the ordered deposition, removal, or exchange of histones (1–3). Some of these chaperones are involved in histone storage in oogenesis, decondensation of sperm chromatin after fertilization, and nucleosome assembly in early embryonic cells. Among the three chaperone families that participate in these processes (4–6), the nucleoplasmin (NP) family has been shown to play a prominent role (7, 8).

NP is a pentameric protein, built of identical 200 amino acid subunits each organized in two domains. The core domain, corresponding to the 120 N-terminal amino acids, is responsible for oligomerization and confers the protein an extreme thermal stability (9). Its crystallographic structure has been solved (10), as well as those of homologous proteins (11, 12), showing that each monomer folds into an eight-stranded β-barrel with a jellyroll topology, and associates in a 60 × 40Å ring-like pentamer. A continuous ring of conserved apolar residues from each monomer core, interacting through the apolar subunit interface, is most likely responsible for the remarkable stability of the protein (10). The tail domain, which comprises the remaining 80 residues, harbors a segment rich in negatively charged residues ("poly-glu") and a nuclear localization signal (NLS), and it is thought to adopt a native disordered conformation (13).

NP has been proposed to, among other functions, assist the assembly of nucleosomes by modulating the interaction between histones and DNA (7, 8, 14–16, 18). It is the most abundant protein in *Xenopus laevis* eggs, where it binds histones H2A and H2B as a chaperone. The *Xenopus* sperm chromatin consists of a complex mixture of basic proteins known as sp1/2, sp3, sp4, sp5, and sp6, plus histones H3 and H4, whereas it is deficient in H2A and H2B. The NP chaperone activity would provide the H2A and H2B complement to the male pronucleus replacing the sperm nuclear basic protein immediately after fertilization (16, 18). Additionally, NP-mediated chromatin decondensation would be required for replication licensing of quiescent nuclei (19). It has been recently reported that this nuclear chaperone would also regulate chromatin condensation during apoptosis (20) and play a role in epigenetic processes (21).

NP activity is modulated through phosphorylation at multiple residues, both in the core and tail domains (15). The degree of phosphorylation increases during egg maturation, e.g. the average number of phosphate groups per monomer has been estimated to be three and seven to ten in the protein isolated from *Xenopus* oocytes and eggs, respectively (13).

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5 The abbreviations used are: NP, nucleoplasmin; eNP, oocyte nucleoplasmin; eH2A, histone H2A; DHB, 2,5-dihydroxybenzoic acid; ESI, electrospray ionization; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; poly-glu, polyglutamic acid tract; NLS, nuclear localization signal; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
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The increased phosphorylation correlates with a higher decondensation activity: egg NP (eNP) decondenses sperm chromatin with a higher efficiency than oocyte NP (oNP) \textit{in vitro} (13, 22), so that the protein would exhibit its maximal activity at the time of fertilization. The ability of NP to bind and extract basic proteins from chromatin has previously been ascribed to the polyglutamic acidic tract of the tail domain (15). We have recently observed, however, that the phosphorylated core domain isolated from \textit{X. laevis} eggs is able to bind sperm basic proteins and decondense chromatin (23) although less efficiently than full-length eNP. This finding suggests that the presence of both protein domains and/or their phosphorylation may be required for efficient decondensation. The relative contribution of these factors is so far unknown.

Two parallel approaches have been used herein to address this question. On one hand, phosphorylation of eNP has been analyzed by phosphopeptide TiO\textsubscript{2} enrichment in combination with mass spectrometry (MS). The information obtained by MS together with prediction software has been used in the second approach to generate artificially activated mutants of the protein in which putative phosphorylated residues have been replaced by aspartic acid. Because attempts to phosphorylate recombinant NP \textit{in vitro} have not been successful so far, this approach provides a useful way to understand how phosphorylation activates the protein chromatin decondensation activity. Our results show that both the core and tail domains of eNP are phosphorylated and that phosphorylation-mimicking mutations at both protein domains render a mutant as active as eNP in chromatin decondensation, affinity, and specificity toward basic proteins, in contrast to the wild-type, non-phosphorylated recombinant protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Wild-type and Mutant NPs**

The choice of sites to be mutated in NP was guided by experimental data (see below and Refs. 13 and 23) and prediction software (24). Mutations were made via PCR on a non-fusion clone of wild-type NP in the plasmid pET11b (13) and checked by DNA sequencing. Mutant NP forms as well as recombinant wild-type NP were expressed for 15–20 h at 18 °C in BL21(DE3) cells. Cells were resuspended in 25 mM Tris/HCl, pH 7.5, 120 mM NaCl, 1 mM dithiothreitol buffer containing phosphatase inhibitors (β-glycerophosphate, molybdate, and vanadate) and protease inhibitors, and sonicated (13). Purification involved a 20-min heating step, ultracentrifugation, and a sequence of chromatographic steps with hydroxyapatite, Mono Q, and Superdex 200 columns. Because mutations compromise NP thermal stability, heating took place at different temperatures: 80, 65, 60, and 50 °C for wild-type, NP5D, NP8D, and NP13D, respectively. Mutant NPs were more prone to be degraded as well, thus some preparations contained degradation products (always <10% of the total protein, as estimated by SDS-PAGE and densitometric analysis of the purified samples). Natural NP from \textit{X. laevis} eggs was obtained as described previously (13).

**Mass Spectrometry Studies**

**Digestion of eNP Isolated from \textit{X. laevis}**

\textit{In-gel Digestion—} eNP was visualized in SDS-PAGE by Coomassie Blue staining and in-gel-digested using trypsin. Excised protein bands were cut out and in-gel-digested using trypsin as previously described (25).

\textit{In-solution Digestion in 2 M Urea—} eNP was dissolved in 50 mM NH\textsubscript{4}HCO\textsubscript{3}, pH 7.8, 4 mM urea. Reduction was performed by addition of dithiothreitol to a final concentration of 5 mM and incubation at 25 °C for 1 h. Subsequent alkylation by 15 mM iodoacetamide was performed for 1 h in the dark. The solution was diluted to 2 M urea using NH\textsubscript{4}HCO\textsubscript{3}, pH 7.8, and, after trypsin addition (enzyme:protein, 1:80, w/w), the sample was incubated at 37 °C for 18 h.

\textit{In-solution Digestion in 0.2% Rapigest—} eNP was dissolved in 50 mM NH\textsubscript{4}HCO\textsubscript{3} containing 0.2% Rapigest\textsuperscript{TM} (Waters), followed by reduction, alkylation, and subsequent digestion with trypsin or chymotrypsin (enzyme:protein, 1:80, w/w) at 37 °C for 18 h. The reaction was stopped by addition of HCl (final concentration, 50 mM, pH ≈ 2), and the mixture was incubated at 37 °C for 30 min and finally centrifuged at 13,000 rpm for 10 min. The digest was stored at −20 °C.

**Purification of Phosphorylated Peptides Using TiO\textsubscript{2} Microcolumns**

TiO\textsubscript{2} microcolumns were prepared and used as previously described (25). TiO\textsubscript{2} was packed in a gel loader tip containing C\textsubscript{8} resin to retain the TiO\textsubscript{2} beads, to a column length of ≈3 mm. NP digest was loaded onto the TiO\textsubscript{2} column in 100 mg/ml dihydroxybenzoic (DHB) acid, 80% acetonitrile, 0.1% trifluoroacetic acid. The column was washed with 10 μl of DHB solution and 20 μl of 80% acetonitrile, 0.1% trifluoroacetic acid. Bound peptides were eluted using 2.5 μl of NH\textsubscript{4}OH, pH 10.5. The eluate was acidified by addition of 5% trifluoroacetic acid, and 0.5 μl was spotted onto the MALDI target following addition of 0.5 μl of 20 mg/ml DHB matrix in 70% acetonitrile, 1% phosphoric acid. Inclusion of 1% phosphoric acid in the MALDI matrix solution increases the relative abundance of multiphosphorylated peptides (26). The eluate was acidified with 5 μl of 10% acetic acid for the LC-ESI-MS/MS analysis.

**Enzymatic Dephosphorylation of Peptides Using Immobilized Phosphatase Alkaline Magnetic Beads**

Peptides eluted from the TiO\textsubscript{2} microcolumns were diluted in 10 μl of 50 mM NH\textsubscript{4}HCO\textsubscript{3} and incubated with previously washed and equilibrated alkaline phosphatase magnetic beads at 37 °C for 45 min. The beads were then separated from the supernatant using a magnetic rack. The recovered supernatant was acidified by addition of 5% trifluoroacetic acid and loaded onto an R2 POROS microcolumn. The sample was eluted with 0.5 μl of DHAB matrix solution (20 mg/ml DHAB in 70% acetonitrile, 0.1% trifluoroacetic acid). The eluate was spotted directly onto the MALDI target.

**MALDI-TOF MS**

MALDI-TOF MS was performed using a Voyager Elite DE STR instrument (Applied Biosystems, Framingham, MA). All
spectra were acquired in positive ion reflector mode. Ions were generated by irradiation of analyte-matrix deposits with a nitrogen laser (337 nm) and analyzed with an accelerating voltage of 20 kV. Analyte-matrix samples were prepared by the dried-droplet method using DHB matrix solution. Phosphoric acid (1%) was included in the matrix solution for analysis of phosphorylated peptides (27). Mass spectra were externally calibrated in the m/z range of 700 – 4000 using peptides generated by tryptic digestion of bovine β-lactoglobulin. MALDI tandem mass spectra were recorded on a MALDI-QTOF tandem mass spectrometer (Ultima HT, Waters/Micromass, Manchester, UK) equipped with a nitrogen laser (337 nm). Tandem mass spectrometric (MS/MS) experiments were performed using argon as the collision gas and applying a collision energy in the range of 50 – 120 eV to fragment singly protonated peptide ions. Polyethylene glycol was used for external calibration (m/z 400 – 4000).

Data analysis was carried out using either the MoverZ software (Proteometrics) or the software MassLynx 3.5. Sequence analysis and peptide assignment were accomplished with GPMAW software.

**Nano-LC-ESI-MS**

Nano-LC-ESI-MS analysis was performed in a QTOF Ultima mass spectrometer (Waters/Micromass UK Ltd., Manchester, UK) using automated data-dependent acquisition. A nanoflow high-performance liquid chromatography system (Ultimate, Switchos2, Famos, LC Packings, Amsterdam, The Netherlands) was used for chromatographic separation of the peptide mixture prior to MS detection. The peptides were concentrated and desalted on a precolumn (75-μm inner diameter, 360-μm outer diameter, Zorbax® SB-C18 3.5 μm, Agilent, Wilmington, DE) and eluted at 200 nl/min by an acetonitrile gradient (2%/min) onto an analytical column (50-μm inner diameter, 360-μm outer diameter, Zorbax® SB-C18 3.5 μm, Agilent). An MS-TOF survey spectrum was recorded for 1 s. The three most abundant ions present in the survey spectrum were automatically mass-selected and fragmented by collision-induced dissociation (4 s per MS/MS spectrum). The MS/MS data were converted to a pkI file format using the MassLynx 3.5 proteinLynx software, and the resulting pkI file was searched against the NP sequence using an in-house Mascot server (version 1.8, Matrix Sciences, London, UK).

**Functional Assays**

**Chromatin Decondensation**

Demembranated sperm nuclei from X. laevis were prepared as described previously (28). The nuclear decondensation assay was performed as reported (29). Approximately 3 × 10⁵ sperm nuclei (1.4 μg) were incubated in a 33-μl final volume containing 25 mM Tris-HCl, 100 mM KCl, 2 mM MgCl₂, pH 7.5, in the presence of different NP concentrations. Aliquots were taken at 3 and 17 min and diluted with 4’,6-diamidino-2-phenylindole to stain the DNA, and the unfixed samples were immediately photographed in an inverted epifluorescence microscope (Eclipse TS100, Nikon), using a 10× objective and a charge-coupled device camera (CoolSNAPcf, Roper Scientific).

**Electrophoretic Analysis of Nuclear Decondensation**

To analyze the chromatin proteins solubilized by NP, sperm or chicken erythrocyte nuclei (7 μg) were incubated with 32 μM NP in the above buffer, final volume 50 μl. Upon incubation at room temperature for 1 h, the samples were centrifuged (14,000 × g for 15 min at 4 °C), washed with 50 μl of buffer, and centrifuged once more. The resulting supernatants, containing NP and associated basic proteins, were precipitated with 20% (w/v) trichloroacetic acid. Proteins were extracted from the pellets with 0.4 N HCl and treated as the supernatants. Both fractions were analyzed by acetic acid/urea/Triton X-100 PAGE as previously described (23). When necessary NP13D was denatured in 5 M urea (overnight, 4 °C), and the assay was performed as with native NP at a final urea concentration of 4.5 M. Titrations with different concentrations of NP were performed using this method, and the amount of extracted H5 was quantitated by densitometry of the gel bands with a gel scanner G-800 and the Quantity One software from Bio-Rad. Each data point is an average of at least two experiments.

**RESULTS**

**Mass Spectrometry**—Hyperphosphorylation of NP affects both protein domains.

Proteolytic digestion of eNP following standard in-gel protocols gives poor results (Fig. 1A) most likely due to the high stability and compact structure of the protein that may hinder protease performance. To overcome this problem, NP was digested in the presence of either 2 M urea (Fig. 1B), or Rapigest™ (Waters), a reagent that solubilizes proteins to make them more susceptible to enzymatic proteolysis (Fig. 1C). The results show that more peptides with higher intensities are detected by MALDI-MS when Rapigest™ is used as denaturing agent. Phosphorylated peptide enrichment was achieved by loading the in-solution Rapigest™-treated tryptic peptide mixture onto a TiO₂ microcolumn. Analysis of the retained peptides by MALDI-TOF MS allowed the initial identification by peptide mass fingerprint of 10 phosphopeptide candidates by their 79.96-Da mass increments per phosphate moiety relative to the unmodified peptides (Fig. 2A). Several doubly and triply phosphorylated peptides were associated with NP.
peptides were detected, all of them being assigned to variants of the sequence between residues 168 and 188 (168KKLDKDESSEEDSPTKKGKG188) as a consequence of cleavage sites missed by trypsin (Table 1). The missed cleavage observed at Lys172, occurring in all identified phosphopeptides, could be due to the presence of either acidic residues at both sides of this residue or to adjacent lysines (Lys166-Lys167 and Lys168-Lys169) that could hinder the proteolytic activity of trypsin at those sites.

Although the 168KKLDKDESSEEDSPTKKGKG188 sequence contains four putative phosphorylation sites (Ser176, Ser177, Ser181, and Thr183), only doubly and triply phosphorylated peptides were detected, indicating that distinct populations with different levels of phosphorylation coexist in the purified NP solution. Moreover, it cannot be ruled out that peptides with four phosphoryl groups could also be present but not detected by MS, because they could be suppressed in the ionization process. Due to the low fragmentation efficiency observed in the MALDI-QTOF-MS/MS analysis, the TiO2-enriched fraction was also analyzed by LC-ESI-MS/MS. Six phosphopeptides derived (due to missed cleavages) from the sequence 168KKLDKDESSEEDSPTKKG187, and 3 from the sequence 141EEEEDQESPPKA VK154, not detected by MALDI-TOF analysis, were observed. Fragmentation of peptide 169KLDKDESSEEDSPTK184 allowed the assignment of the phosphorylation sites Ser176 and Ser177. The third phosphorylation site was most plausibly assigned to Ser181, although it was not possible to exclude alternative phosphorylation at Thr183 (data not shown). In addition, fragmentation of the singly phosphorylated peptide 141EEEEDQESPPKA VK154, detected by LC-ESI-MS/MS, revealed phosphorylation at Ser148 (data not shown).

In an attempt to find additional phosphorylation sites, NP was digested with chymotrypsin, and the derived peptides were analyzed as described above (Fig. 2B). Peptide mass mapping allowed initial identification of six candidate phosphopeptides, two doubly phosphorylated, two triply phosphorylated, and two with four phosphoryl groups (Table 2). Furthermore, a mass increase of 42 Da was observed for these phosphorylated peptides, suggesting N-acetylation. Treatment with phosphatase alkaline, which induces a mass decrease of 79.96 Da per phosphate moiety, yielded two peptides, respectively at m/z 1660.9 and at m/z 1702.9, separated by a mass shift of 42 Da (Fig. 3). These two peptides were sequenced by MALDI-Q-TOF MS/MS, confirming the sequences 1ASTVSNTSLKLEKPVSL16 and acetyl-1ASTVSNTSLKLEKPVSL16 (Fig. 3). A missed cleavage in Leu10 suggests that the presence of a glutamic acid in position 11 next to the cleavage site could affect performance of chymotrypsin on Leu10. Peptides at m/z 1339.3 and m/z 1396.3 are observed in the TiO2 eluate and the phosphatase alkaline treated fraction.

**TABLE 1**

| m/z       | Sequence                          | Number of phosphorylated (PO4) sites |
|-----------|-----------------------------------|-------------------------------------|
| 1948.6    | 170LDKDESSEEDSPTK184              | 3                                   |
| 1996.7    | 170LDKDESSEEDSPTKK185/169         | 2                                   |
| 2059.6    | 170LDKDESSEEDSPTKK185/169         | 2                                   |
| 2076.6    | 170LDKDESSEEDSPTKK185/169         | 3                                   |
| 2124.8    | 169KLDKDESSEEDSPTKKG187/146       | 2                                   |
| 2133.7    | 170LDKDESSEEDSPTKKG187/146       | 3                                   |
| 2204.8    | 169KLDKDESSEEDSPTKK185/169       | 3                                   |
| 2261.8    | 170LDKDESSEEDSPTKKG187/169       | 3                                   |
| 2332.9    | 169KLDKDESSEEDSPTKK185/169       | 3                                   |
| 2389.9    | 169KLDKDESSEEDSPTKKG187/169      | 3                                   |
| 2446.9    | 169KLDKDESSEEDSPTKKG187/169      | 3                                   |
indicating that these peptides are not phosphorylated. The assignment of the four phosphorylations in the sequence 1ASTVSNTSKLEKPVSL16 where six potential phosphorylated sites are present, could not be confirmed either by MALDI-QTOF-MS/MS analysis or by LC-ESI-MS/MS, because the fragmentation of the peptide was very poor. Only the four neutral losses corresponding to four phosphoryl groups present in the sequence were clearly detectable.

**Design of the Activated Mutants**—Sequence coverage of the MS analysis using chymotrypsin and trypsin is 71 and 51%, respectively (Fig. 4). This means that 19 putative phosphorylation sites out of the theoretically 26 phosphorylatable residues could be detected. The fact that only 8 phosphorylated sites (9 if we consider phosphorylation of Thr183) are experimentally detected does not rule out the existence of more phosphorylation sites. For instance, residue Tyr123, whose dephosphorylation has been recently associated with apoptosis (20; Tyr124 according to their sequence numbering), is located within a non-covered sequence, and other phosphorylated residues within the covered regions might not be detected considering the highly heterogeneous phosphorylation degree of eNP. Therefore, mutants have been designed based on MS data, prediction software (23, 24), N-terminal amino acid analysis (23), sequence comparison within the NP family, and structural properties of the protein core domain, including solvent accessibility of the predicted phosphorylation sites (10). Previous chemical and MS analyses showing that eNP contains an average of 7–10 phosphoryl groups (13) have also been considered, as well as the fact that at neutral pH the Asp side chain displays one negative charge while the phosphoryl group average charge would be between $-1$ and $-2$ (30).

Three mutants have been generated in this work, in which several threonine and serine residues have been replaced by aspartate: NP5D, with five phosphorylation-mimicking mutations in the tail domain, NP8D with eight mutations in the core, and NP13D, a combination of both sets of mutations. The mutated amino acids are indicated in Fig. 4. Ser148 was identified as a phosphorylation site after generating the mutants containing Thr159, also predicted with high reliability as a phosphorylated residue, instead of Ser148 replaced by aspartate. Mutant NP8D has six substituted Thr and Ser residues located within the highly flexible sequence formed by the first 15 N-terminal residues of the core domain. Of these, MS identifies four phosphorylation sites, and two remaining substitutions affect residues Thr66 and Thr96, which are located at solvent-exposed loops and predicted to be phosphorylated with high reliability (23). Residues Thr66 and Thr96 are relatively well conserved within the NP family, and in an NP homolog, dNLP, Thr and Glu are found instead, suggesting that at least one of these two

**TABLE 2**

| m/z | Sequence | Number of phosphorylated (PO4) sites |
|-----|----------|-------------------------------------|
| 1820.4 | 1ASTVSNTSKLEKPVSL16 | 2 |
| 1862.8 | 1Acetyl-ASTVSNTSKLEKPVSL16 | 2 |
| 1900.8 | 1ASTVSNTSKLEKPVSL16 | 3 |
| 1942.8 | 1Acetyl-ASTVSNTSKLEKPVSL16 | 3 |
| 1980.7 | 1ASTVSNTSKLEKPVSL16 | 4 |
| 2022.8 | 1Acetyl-ASTVSNTSKLEKPVSL16 | 4 |
| 2200.0 | 1ASTVSNTSKLEKPVSLIW18 | 3 |
| 2279.9 | 1ASTVSNTSKLEKPVSLIW18 | 4 |

**FIGURE 3.** Phosphatase alkaline-treated TiO2-enriched fraction of chymotryptic digest of eNP. Insets show the MS/MS spectra of peptides at m/z 1660.9 and m/z 1702.9.
positions might be negatively charged in other members of the NP family. In mutant NP5D, the substituted residues, except Thr159, flank the NLS, as shown by MS. The functional characterization of these mutants will help to understand the role of phosphorylation of each protein domain in NP function. From the structural point of view, phosphorylation-mimicking mutations do not significantly modify the secondary structure of the protein, as seen by infrared spectroscopy, although they reduce the stability of the mutants against the thermal challenge (data not shown). A similar behavior was found when these properties of wild-type recombinant NP and hyperphosphorylated eNP were compared (13).

Functional Characterization of NP Mutants—The affinity and specificity of hyperphosphorylated eNP for histone proteins can be artificially mimicked through replacement of experimentally detected and/or predicted phosphorylated residues by aspartic acid. Fig. 5 shows the ability of the mutants to decondense Xenopus demembranated sperm nuclei. NP mutants can, in contrast to recombinant wild-type NP, decondense sperm chromatin with varying efficiencies. The decondensing activity of NP13D at the shortest time assayed is clearly higher than that of NP5D or NP8D and resembles that of eNP (Fig. 5). More importantly, NP13D is the only recombinant form that, like eNP, elicits decondensation shortly (3 min) after addition of the protein (Fig. 5A), indicating that accumulation of phosphorylation mimicking mutations throughout both domains of the protein renders a fully functional NP. At longer incubation times (17 min) all the mutants are able to decondense chromatin, although NP13D remains the most active species (Fig. 5B).

Chromatin decondensation is due to NP-mediated retrieval of specific basic proteins that keep DNA in a highly packed state. To compare the ability and specificity of the different NP mutants to extract basic proteins, we used a “solubilization assay” in which NP is incubated with chromatin, the mixture is centrifuged, and electrophoresis of supernatant and pellet allows one to distinguish extracted (or solubilized) proteins from those remaining chromatin-associated. The interaction of those mutants with demembranated mature sperm nuclei and permeabilized chicken erythrocyte nuclei is shown in Fig. 6. Wild-type NP hardly binds any sp3, -4, and -5, as already reported (13). In contrast, NP5D extracts sp6 and approximately one-half of the available sp3, -4, and -5, whereas NP8D binds almost all the available sp3, -4, and -5 and about one-half of the sp2. Removal of sperm chromatin proteins by NP13D is significantly stronger and resembles the effect of eNP, because both proteins extract the majority of sp proteins.

Lanes “N” in Fig. 6 represent the different NP samples without chromatin and allow the assignment of minor bands (due to NP degradation during the experiment) that migrate differently...
from sperm nuclear basic proteins and from linker histones H1 and H5.

When the solubilization assay is performed with chicken red blood cell chromatin, as a model of a quiescent nucleus, essentially the same results were obtained (Fig. 6B). The recombinant wild-type protein and NP5D are unable to bind chromatin-associated histones, whereas NP8D and NP13D extract around 30 and 70%, respectively, of linker histone H5. NP13D can extract as much H5 as eNP, but it does not remove core histones associated histones, whereas NP8D and NP13D extract around 30 and 70%, respectively, of linker histone H5. NP13D can extract as much H5 as eNP, but it does not remove core histones (H2A, H2B, H3, and H4), as reported for eNP (29). When the same experiment is carried out at a higher concentration (72 μM), eNP is able to extract some of the less abundant H1 (29). Only NP13D among the mutants is able to do so (Fig. 6C). Binding to DNA-associated H1 is characteristic of fully activated NP, e.g. eNP, and it has not been observed for partially phosphorylated oNP (29). Denatured NP13D (incubated in 4.3 M urea) does not extract any histone H5 (Fig. 6C), indicating that binding to the chromatin-associated basic proteins is not merely determined by the net charge of the protein but requires the specific spatial charge distribution of natively folded phosphorylated NP. Note that at this urea concentration NP13D is denatured and histones remain DNA-bound in the chromatin fiber.

To gain a more quantitative insight into the ability of the NP mutants to interact with chromatin-bound H5, we have challenged chicken red blood cell chromatin with different concentrations of these proteins. The titration data clearly show that neither recombinant wild-type NP nor NP5D can dissociate chromatin-associated H5 in the protein concentration range (2–65 μM) used in the experiment (Fig. 7). The solubilizing activity of NP8D starts to be significant >18 μM and reaches a plateau at 32 μM, dissociating ~30% of the available H5. In contrast, the activity of NP13D increases with protein concentration and achieves its maximum (dissociating ~60% H5 from DNA) at 18 μM. It is noteworthy that similar percentages of H5 were extracted from chromatin using eNP (Fig. 7), indicating that NP13D closely mimics the capacity of fully phosphorylated eNP to compete with DNA for linker histone binding. It is striking that the H5 solubilization activity of both eNP and NP13D is remarkably higher than those of the other NP mutants below 32 μM, a protein concentration that may be close to the in vivo situation (23, 31).

DISCUSSION

Previous studies have shown that the nuclear phosphoprotein nucleoplasmin participates in the first stage of chromatin decondensation of Xenopus sperm at fertilization. It binds and removes sperm nuclear basic proteins, replacing them with histones. This activity depends on massive phosphorylation of the protein that occurs when oocytes mature into eggs (15). Casein kinase II (32) and mitosis promoting factor (33) have been involved in the post-translational modification of NP. Despite the importance of this and other processes in which NP participates, e.g. putative role in transcription and chromatin decondensation associated with epigenetic modifications (21), the identity of the phosphorylation sites has remained largely unknown. There is only one recent report linking dephospho-

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rylation of Tyr^{123} to apoptotic chromatin condensation (20). The stability of eNP that could hinder its proteolysis and therefore its analysis by MS, and the presence of heterogeneous protein populations with different degrees of phosphorylation and N-acetylation might explain the lack of information on the phosphorylation sites in eNP.

It has been proposed that the chromatin decondensing activity of NP involves a strong electrostatic component regulated by the massive phosphorylation of the protein, in contrast to its chaperone activity that, besides the ionic component, it also seems to involve stereospecific recognition of the nucleosomal histones (mainly H2A and H2B) (29). An important finding of this work is that activation of the NP chromatin decondensation ability requires extensive phosphorylation of both protein domains. This suggestion had been previously derived on the basis of the comparative activities of non-phosphorylated (recombinant NP), partially (oNP), and fully (eNP) phosphorylated NP (13). However, the heterogeneous phosphorylation status of both types of naturally occurring proteins and the lack of information regarding the identity of the phosphorylation sites hampered its direct demonstration. MS-based analysis has allowed us to prove the coexistence of heterogeneous NP populations regarding both the degree of phosphorylation and N-acetylation. Furthermore, we have also demonstrated that mutant NP13D mimics fully active, hyperphosphorylated eNP, because both show similar (i) time dependence of their decondensation activity, (ii) ability to compete with DNA for histone binding, and (iii) specificity to dissociate “linker” histones from chromatin.

Analysis of the decondensation time course reflects the ability of these proteins to compete with DNA for histone binding, which will finally result in slow/fast chromatin decondensation. When Xenopus oocytes mature into eggs, their cytoplasm acquires the ability to decondense chromatin rapidly, reflecting the requirement for rapid sperm chromatin decondensation at fertilization. It has been previously observed that eNP decondenses sperm chromatin at the rate observed in fertilization, whereas oNP does not (22). Our data, showing that both eNP domains are phosphorylated and only NP13D mimics the fast chromatin decondensation activity of eNP, indicate that this is due to hyperphosphorylation of both protein domains. This observation is further supported by the fact that mutants with phosphorylation-mimicking substitutions in only one protein domain, although able to unfold chromatin at long incubation times, show a defective capacity to decondense it within a short timeframe. The time course of the decondensation activity was not taken into account in previous studies, and has allowed us to distinguish a functional important aspect of fully activated NP.

Chromatin unfolding is due to removal of DNA-bound basic proteins (7, 8, 16, 29), and therefore the kinetic comparison between these mutants discussed above should be paralleled by their concentration-dependent ability to extract these proteins from chromatin, as well as by their capacity to select specific ones. The early decondensation activity of the mutants is well reflected in their ability to dissociate basic proteins, specifically linker-type proteins from chromatin. The comparison between the different mutants underscores the effect of independent or simultaneous activation of the protein domains. Although the independent activation of the protein core or tail domains increases the amount of histones removed from chromatin, the linker histones sp2 and H5 are only effectively extracted when both protein domains are phosphorylated or activated by phosphorylation-mimicking mutations. The fact that histone H5 is removed more efficiently than histone H1 has been recently reported for eNP (29), and might be due to either a different accessibility of linker histones in the erythrocyte chromatin fiber or to a distinct affinity for eNP and NP13D. Furthermore, the fully activated NP13D mutant does not significantly extract nucleosomal histones, as reported for eNP (29) and NAP1 (34, 35). This reflects that the set of chromatin-bound linker histones that can be extracted by NP is regulated by phosphorylation of both protein domains and that interaction with some of these linker histones (sp2 and H5) needs a highly charged NP pentamer to occur. In a previous work it was shown that phosphorylation of the core domain of NP, in the absence of the tail domain, rendered a protein able to interact with basic proteins and therefore to decondense chromatin. This suggested that binding sites other than the long poly-glu tract A2 existed in the core domain (23). We demonstrate here that, although this is the case, the simultaneous presence of both protein domains as well as their phosphorylation enhance the chromatin decondensation activity of the protein. This synergism between phosphorylated protein domains confers the protein the ability to effectively extract the linker histones from chromatin without significant dissociation of nucleosomal histones.

From the structural point of view this observation can be rationalized considering that the phosphorylated N-terminal segment, the acidic tract A1, and possibly the tail domain containing both the poly-glu tract (A2) and at least four phosphoryl groups, point to the same structural region, known as the distal face of the protein. As modeled in the NP activated core domain (23), the negatively charged regions of the protein (poly-glu tracts) and the phosphoryl groups would contribute to an increase in the negative electrostatic potential in this protein region, so that it could act as a binding site efficiently competing with DNA in linker histone binding. It has been proposed that the core domain binds histones through the pentamer lateral face (10, 36). Recombinant NP activated through mutations in the distal face suggests an involvement of this protein surface in the binding process, as recently proposed (18). Both models are not incompatible, because NP might use its lateral face to interact with core histones H2A and H2B, phosphorylation converting the distal face into the main binding site for linker type basic proteins.

A finding that might be relevant for NP nucleocytoplasmic transport is that several of the phosphorylated residues (Ser^{148}, Ser^{176}, Ser^{177}, Ser^{183}, and Thr^{183}) are flanking the NLS. In many cases, nuclear transport is regulated by phosphorylation, as described for p53 (37), signal transducer and activator of transcription 1 (38), and Pho4 (17). Indeed NP has also been observed to accumulate faster in the nucleus upon phosphorylation by casein kinase II (32). The four phosphorylation sites that have been identified by MS increase the negative charge around the NLS and, therefore, could weaken the electrostatic interaction with opposite charged regions of the tail domain.
(poly-glu tract) that has been proposed to modulate protein decondensation activity and stability (9). This, in turn, would unmask both the poly-glu A2 tract, enhancing binding of basic ligands (9), and the NLS, favoring the interaction of nucleoplasmin with the nuclear import machinery. A similar activation mechanism has been recently proposed for nucleosome assembly protein 1 export from the nucleus. In this case, most of the nuclear export signal sequence is shielded from the solvent by an accessory domain, an interaction that could possibly be regulated by phosphorylation (39). Phosphorylation-coupled unmasking of specific protein motifs may also be important in the case of NP for the coordinated control of both the nucleocytoplasmic transport of the protein (and protein ligands) and its chromatin decondensation activity.

Our results also demonstrate that the mutants characterized in this work might be a useful tool for the in vitro characterization of homogeneous complexes of NP with different types of histones, to analyze the effect of modification of specific protein regions on complex formation and stabilization, and to perform biophysical studies on a functionally relevant protein state. In summary, our data with phosphorylation-mimicking mutants indicate that complete activation of NP requires accumulation of negative charge, probably on the distal face of the pentamer, throughout both domains of the protein. Partial phosphorylation of NP (e.g. in oocyte stage) could confer the protein the ability to bind core histones, but only hyperphosphorylation of specific regions of both protein domains would determine the transition from a histone-storage function to a state in which it could extract linker-type chromatin-associated proteins.

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