Mutation of the small acidic tract A1 drastically reduces nucleoplasmin activity

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Abstract ***Xenopus laevis*** **nucleoplasmin is a molecular chaperone that mediates sperm decondensation and nucleosome assembly. Nucleoplasmin has three acidic tracts (A1, A2 and A3) and until recent years the long polyglutamic tract A2 was thought to be the binding site for basic proteins. However, the latest publications in this field show that neither A2 nor A3 is indispensable for histone and sperm-specific protein binding. In this work, we show that the mutation of only four acidic amino acid residues of the small A1 tract drastically reduces nucleoplasmin decondensing activity, pointing out this region as the potential binding site for sperm proteins.**

Keywords: Nucleoplasmin; Nuclear chaperone; Sperm chromatin remodeling

1. Introduction

Xenopus laevis nucleoplasmin was the first molecular chaperone described [1]. Nucleoplasmin is an acidic and thermostable protein that associates into pentamers [2] and participates in both the sperm DNA decondensing and nucleosome assembly that occurs after fertilization [3]. The amino acid sequence of nucleoplasmin, deduced from two slightly different cDNA clones [4,5], shows the presence of three acidic tracts (A1, A2 and A3) in the protein, A2 being the longest one. A2 and A3 are found in the tail domain of nucleoplasmin, while A1 is found in its core domain (Fig. 1A). Although it has been traditionally accepted that nucleoplasmin would interact with basic proteins through its acidic tracts, the latest publications in the field show how nucleoplasmin can still bind to histones and protamines not only in the absence of the acidic tract A3 but also, more surprisingly, in the absence of the long A2 tract [6–9]. However, to date no experimental data have been provided with regard to the role of the small acidic tract A1. In this paper, we show how the change of four of the acidic amino acid residues of the A1 tract drastically reduces the sperm decondensing activity of nucleoplasmin even in the presence of the acidic tracts A2 and A3, thus providing direct evidence of the importance of the A1 tract in nucleoplasmin function.

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Abbreviations: r-NP, recombinant nucleoplasmin; (-A1), mutation in the acidic tract A1; AU–PAGE, acetic acid-urea polyacrylamide gel electrophoresis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Recombinant proteins

Recombinant nucleoplasmins r-NP, r-NP142 and r-NP121, have been obtained from the Bürglin's cDNA [4] as previously described [7]. r-NP represents the full-length recombinant version of nucleoplasmin and contains the three acidic parts of the molecule (A1, A2 and A3). r-NP142 is a C-terminal deleted form, extending from amino acids 1 to 142, which contains the main acidic tract, A2, at the C-terminus of the protein. Finally, r-NP121 (from amino acids 1 to 121) represents the core domain of nucleoplasmin and lacks both the acidic tracts A2 and A3. All the mutations in the acidic tract A1 (-A1) (Fig. 1B) have been produced by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and have been expressed and purified in the same way as their non-mutated counterparts. Protein concentration has been determined from the absorbance at 276 nm [7].

2.2. Isolation of sperm nuclei and protamine purification

Sperm cells from *Dicentrarchus labrax* (sea bass) and its protamine have been prepared as in [7]. Sperm cells from *X. laevis* have been obtained and treated as in [10]. *X. laevis* sperm nuclei are condensed by five different sperm specific basic proteins (SP2-6), which are found coexisting with histones H3 and H4 and small amounts of histones H2A and H2B. Instead, *D. labrax* sperm nuclei are condensed by a single protamine very similar to the protamines from other amphibia, which also possess nucleoplasmin. Thus, *D. labrax* sperm has been accepted as a simple model to test nucleoplasmin activity [7].

2.3. Sperm chromatin decondensation assay

In a first set of assays, 2 μl aliquots containing 3.6×10^5 *D. labrax* or 2×10^5 *X. laevis* demembranated sperm nuclei in EM buffer (0.1 M KCl, 2 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.4) were mixed with 2 μl of 0.1 mg/ml Hoechst 33258 in EM buffer and 5 μl of 5.8×10^{-5} M nucleoplasmin in EM buffer, and the sperm nuclear swelling effect was monitored by fluorescence microscopy ($\lambda = 360$ nm). In a parallel assay, 2×10^6 *D. labrax* demembranated sperm nuclei were incubated with 70 μl of either EM buffer or 5.8×10^{-5} M nucleoplasmin in EM buffer for 30 min at room temperature with gentle agitation. Samples were centrifuged for 5 min at $16\,000 \times g$ in an Eppendorf microfuge and the supernatants were extensively dialyzed against water, using *Slide-A-lyzer mini dialysis units* (Pierce), and speed-vac dried. Then the supernatants were analyzed electrophoretically using acetic acid-urea polyacrylamide gel electrophoresis (AU–PAGE) in order to check the

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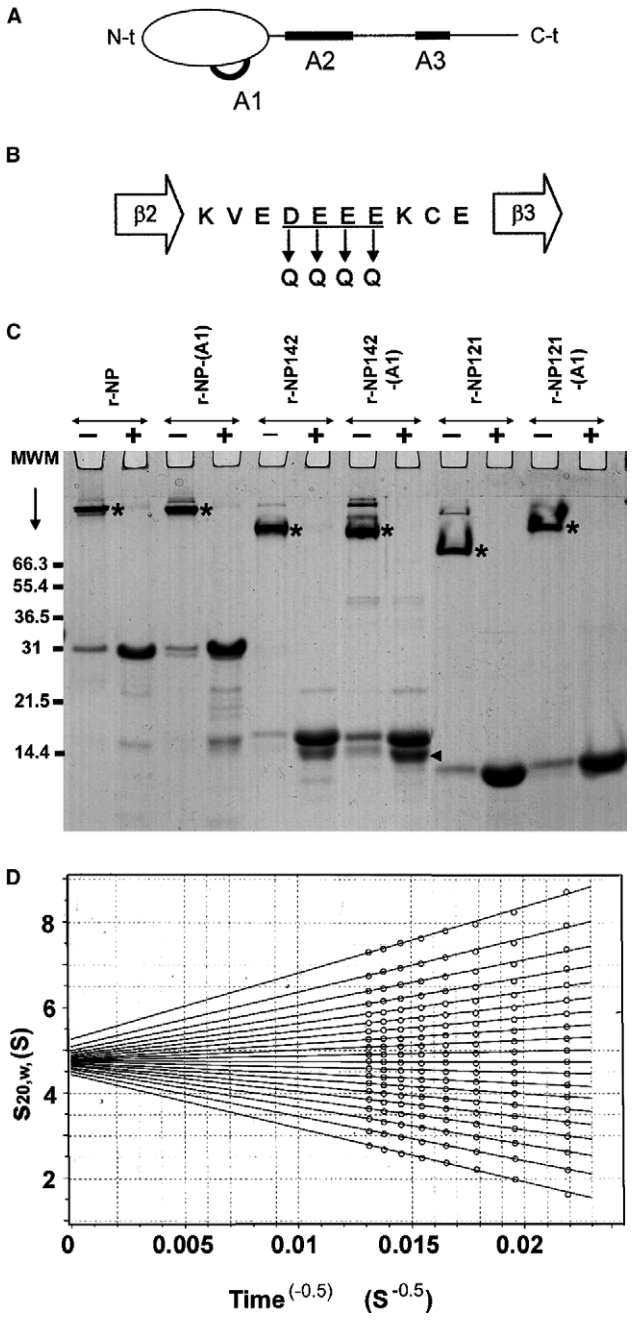

Fig. 1. (-A1) nucleoplasmin mutants. (A) Schematic representation of nucleoplasmin monomer. The acidic tract A1 is found in the core domain of the protein in an external loop between β-sheets 2 and 3. The acidic tracts A2 and A3 are found in the tail domain of the protein. (B) Scheme of the loop mentioned in A showing the amino acid residues that have been mutated to obtain the (-A1) mutants. (C) 15% SDS–PAGE of the mutant (-A1) forms used in this work. (+) Samples boiled or (−) not boiled prior to loading onto the gel. The asterisks show the oligomeric forms of nucleoplasmin. The arrowhead shows a truncated nucleoplasmin form [13,14] that copurifies with the main form. Molecular weight markers (MWM) are shown in kilodaltons. (D) Sedimentation velocity analysis of r-NP121(-A1).

amount of protamine that has been removed from the sperm DNA. This experiment was always carried out by duplicate.

In order to check the effect of nucleoplasmin concentration in these assays, we performed a second set of similar assays but using increasing concentrations of the different nucleoplasmin (-A1) mutant forms. Thus, concentrations up to 3× have been evaluated.

2.4. Miscellaneous methods

Sedimentation velocity analysis, AU–PAGE and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were carried out as in [7]. Comparison of the electrophoretic bands has been carried out by densitometry.

3. Results

3.1. Nucleoplasmin (-A1) mutants are thermostable and pentameric

The acidic tract A1 is found within the nucleoplasmin core in an external loop between β-sheets 2 and 3 [6]. In order to eliminate the acidity of this tract without eliminating the polar nature of the loop, we have mutated only four of the acid residues of A1 and have substituted them for four glutamine residues while maintaining the other polar residues of the loop (Fig. 1B). We have mutated the A1 tract not only in the full r-NP form but also in two deletion mutants obtained previously in our laboratory: r-NP142 (which lacks the acidic tract A3) and r-NP121 (which lacks both A2 and A3 tracts) [7], obtaining thus the forms r-NP(-A1), r-NP142(-A1) and r-NP121(-A1).

All the (-A1) mutants were recovered in the thermostable fraction during the purification procedure and retain their pentamerizing ability as shown in Fig. 1C. Thus, as typically happens with nucleoplasmin, when the (-A1) forms are loaded without boiling onto a SDS–PAGE, a high molecular weight band corresponding to the oligomer can be observed (see asterisks in Fig. 1C). However, in the case of r-NP121(-A1) the band corresponding to the oligomer does not present the same electrophoretic migration as its counterpart form r-NP121. In order to check whether or not r-NP121(-A1) exists as a pentamer in solution, we carried out a sedimentation velocity analysis of this form. As shown in Fig. 1D, r-NP121(-A1) presents a sedimentation coefficient of about 4.75 S, in perfect agreement with the 4.8 S value published for the r-NP121 pentamer [7].

3.2. Mutation of the A1 acidic tract drastically reduces nucleoplasmin protamine removal and sperm nuclear swelling ability

In order to test if the (-A1) mutation could affect the sperm decondensing ability of nucleoplasmin, we performed the sperm chromatin decondensation assay described above. For these experiments, we have used the sperm of the fish *D. labrax* as a system because of its simpler chromosomal protein composition (only one protamine present) when compared to *Xenopus* sperm (see also Section 2).

Figs. 2–4 show, respectively, the comparison of r-NP(-A1), r-NP142(-A1) and r-NP121(-A1) with their non-mutated counterparts regarding both their sperm nuclei swelling ability and their protamine removal ability. When equimolar 1× concentrations of control and mutant nucleoplasmins are used, it can be observed that the (-A1) mutation decreases nucleoplasmin activity in the three forms although to different extent. r-NP142(-A1) is less affected, with a decrease in its protamine removal activity of about 40% (Fig. 3A–D), however, r-NP-(-A1) and r-NP121(-A1) are dramatically impaired, with a decrease of about 80% and 90%, respectively, in their protamine removal activity (Figs. 2A–D and 4A–D). There is also a time effect observed during the monitoring by fluorescence microscopy. Thus, r-NP(-A1) and r-NP121(-A1) mutants

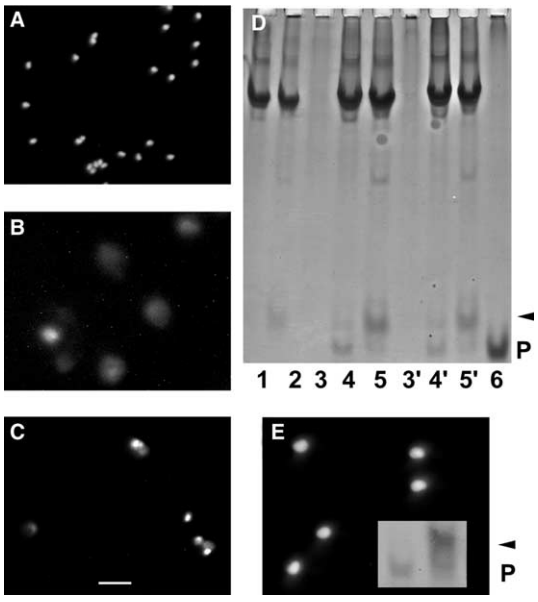

Fig. 2. Comparison of the activity of r-NP and r-NP(-A1). Fluorescence micrographs of *D. labrax* sperm nuclei incubated with either EM buffer, control assay (A), or with an equimolar 1x concentration of r-NP (B) or r-NP(-A1) (C) in EM buffer. The bar corresponds to 10 μm. (D) 15% AU–PAGE of the protamines removed after the incubation of the sperm nuclei with EM buffer (lanes 3 and 3′), or r-NP (lanes 4 and 4′) and r-NP(-A1) (lanes 5 and 5′) at equimolar 1× concentrations. Lanes 1, 2 and 6 correspond to electrophoretic controls of r-NP (1), r-NP(-A1) (2) and protamine (6). The arrowhead shows a fast mobility band that is already found in the r-NP(-A1) sample. (E) Fluorescence micrograph of *D. labrax* sperm nuclei incubated with r-NP(-A1) at a 3× concentration. The amount of protamine removed when r-NP(-A1) 3× is used in the incubation (inset, right) is compared with the amount removed when control r-NP 1× is used (inset, left). The arrowhead shows a fast mobility band that is already found in the r-NP(-A1) sample.

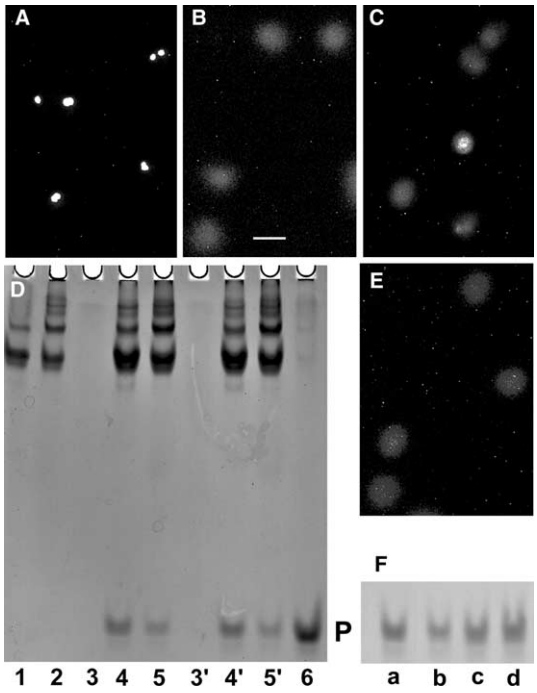

Fig. 3. Comparison of the activity of r-NP142 and r-NP142(-A1). Fluorescence micrographs of *D. labrax* sperm nuclei incubated with either EM buffer, control assay (A), or with an equimolar 1× concentration of r-NP142 (B) or r-NP142(-A1) (C) in EM buffer. (D) 15% AU–PAGE of the protamines removed after the incubation of the sperm nuclei with EM buffer (lanes 3 and 3′), r-NP142 (lanes 4 and 4′) or r-NP142(-A1) (lanes 5 and 5′) at equimolar 1× concentrations. Lanes 1, 2 and 6 correspond to electrophoretic controls of r-NP142 (1), r-NP142(-A1) (2) and protamine (6). The bar corresponds to 10 μm. (E) Fluorescence micrograph of *D. labrax* sperm nuclei incubated with r-NP142(-A1) at a 3× concentration. (F) The amount of protamine removed by control r-NP142 at 1× concentration (lane a) is compared with the amount removed by r-NP142(-A1) at 1×, 2× and 3× concentrations (lanes b, c and d, respectively).

require more time (10–15 min more) to swell the nuclei than their non-mutated couterparts and they do it just to a very limited extent. r-NP142(-A1) is less affected both in the time needed (5–7 min of delay regarding r-NP142) and the extent of nuclear swelling achieved.

In a set of experiments carried out to test the effect of nucleoplasmin concentration, we observed that while both the sperm decondensing ability and the protein removal activity could be soon matched with the control when increasing concentrations of r-NP142(-A1) were used, this was not the case with r-NP(-A1) and r-NP121(-A1). Thus, when the concentration of r-NP142(-A1) was increased 3-fold that of the control (r-NP142), the amount of protamine removed by both proteins was the same (Fig. 3F). Likewise, during the fluorescence microscopy assays, r-NP142(-A1) 3× showed the same activity as control r-NP142 1× both in the extent of decondensation and in the time needed to achieve it (Fig. 3E). However, with r-NP-(-A1) and r-NP121(-A1) only a certain increase in the degree of sperm swelling and protamine removal is observed upon increase of the concentration. However, it does not match that of their respective control activity (see Figs. 2E and 4E).

When the sperm decondensing assay was carried out using *X. laevis* sperm nuclei, a similar sperm swelling activity behavior was observed, i.e., the (-A1) mutation affects mostly the decondensing sperm nuclei activity of r-NP and r-NP121, while r-NP142 is little affected (Fig. 5). Sperm nuclei incubated with r-NP(-A1) (Fig. 5C) and r-NP121(-A1) (Fig. 5G) retain a similar appearance as that of control nuclei (Fig. 5A) while the nuclei incubated with r-NP142(-A1) (Fig. 5E) are longer and thicker, highly resembling the nuclei that had been incubated with the corresponding control nucleoplasmin form (Fig. 5D). It is also worth noting that the swelling activity of r-NP (Fig. 5B) and r-NP121 (Fig. 5F) is lower than that of r-NP142 forms (Fig. 5D-E). Similar results have been reported with r-NP obtained from the Dingwall's clone [11], although these authors only observed noticeable decondensing activity with the equivalent form to our r-NP142 (which possess the acidic tract A2 virtually at the end of the molecule) but not with the equivalents to our r-NP and r-NP121 forms. The differences observed between both studies are only quantitative but not qualitative and may be due to the different experimental conditions or to the nature of the nucleoplasmin clone used.

4. Discussion

Contrary to what was expected, a number of articles have appeared in recent years which provide evidence that the long

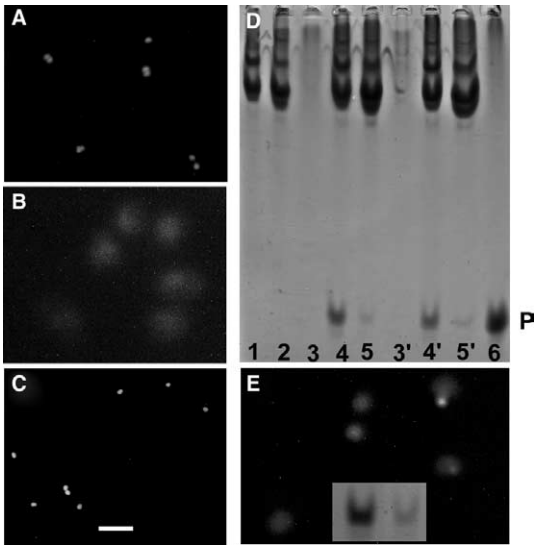

Fig. 4. Comparison of the activity of r-NP121 and r-NP121(-A1). Fluorescence micrographs of *D. labrax* sperm nuclei incubated with either EM buffer, control assay (A), or with an equimolar 1× concentration of r-NP121 (B) or r-NP121(-A1) (C) in EM buffer. The bar corresponds to 10 μm. (D) 15% AU–PAGE of the protamines removed after the incubation of the sperm nuclei with EM buffer (lanes 3 and 3′), or r-NP121 (lanes 4 and 4′) and r-NP121(-A1) (lanes 5 and 5′) at equimolar 1x concentrations. Lanes 1, 2 and 6 correspond to electrophoretic controls of r-NP121 (1), r-NP121(-A1) (2) and protamine (6). (E) Fluorescence micrograph of *D. labrax* sperm nuclei incubated with r-NP121(-A1) at a 3× concentration. The amount of protamine removed when r-NP121(-A1) 3× is used in the incubation (inset, right) is compared with the amount removed when control r-NP121 1× is used (inset, left).

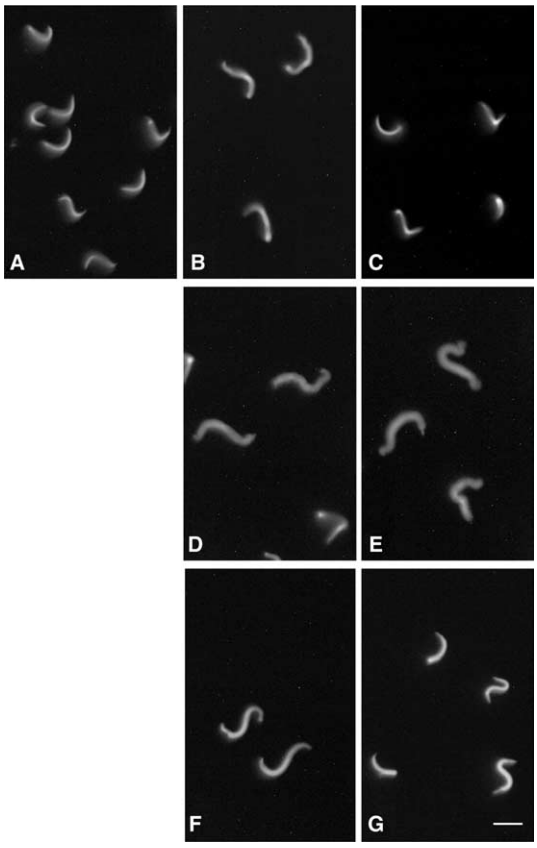

Fig. 5. Decondensing activity of (-A1) nucleoplasmin mutants on *X. laevis* sperm nuclei. Fluorescence micrographs of *X. laevis* sperm nuclei incubated with either EM buffer (A), or with equimolar concentrations of r-NP (B), r-NP(-A1) (C), r-NP142 (D), r-NP142(-A1) (E), r-NP121 (F) and r-NP121(-A1) (G). The bar corresponds to 10 μm.

polyglutamic tract A2 of nucleoplasmin is neither essential for histone binding nor for sperm-specific protein binding. The same is true for the A3 acidic tract. The recently crystallized nucleoplasmin core domain obtained from the Dingwall's clone only presents one small acidic tract (A1 tract) and can still bind to histones [6]. Similarly, an equivalent form expressed from the Bürglin's clone was also able to bind to histones [8]. Furthermore, the core domain of the nucleoplasmin-like protein from *Drosophila melanogaster* (dNLP), which has a crystal structure very similar to the nucleoplasmin pentamer, can also bind to histones in spite of containing only the A1 acidic tract [12]. Also, the sperm chromatin decondensing activity of nucleoplasmin can be performed in the absence of both A2 and A3 tracts, although the A2 tract seems to enhance this activity [7].

These results suggest that the short A1 acidic tract of nucleoplasmin may be much more important for the binding to basic proteins than it had been previously anticipated. However, no direct proof has ever been provided for the involvement of A1 in this function. Our work represents the first attempt to directly answer this question and substantiates the experimental evidence regarding the implication of the acidic tract A1 in the binding and removal of sperm-specific proteins from DNA that results in the sperm nuclei decondensation. We have observed that the mutation of four amino acid residues of the A1 tract (-A1 mutation) drastically reduces not only the decondensing activity of r-NP121 (which possess no other acidic tract but A1) but also reduces that of the full-length form r-NP (which still has the acidic tracts A2 and A3). Hence, r-NP(-A1) and r-NP121(-A1) achieve a lower degree of nuclear swelling than their control counterparts even if 3-fold concentrations are used. The decrease in the sperm-basic protein removal activity does not seem to be due to global changes in the conformation of the molecule, since the (-A1) mutation has no influence in either the thermostability of the protein or its pentameric conformation.

It has been proposed that electrostatic interactions at the C-terminal domain of nucleoplasmin can modulate its chromatin decondensing activity by a mechanism that involves the shielding of the A2 polyglutamic tract by the positively charged amino acids at the C-terminal end of the molecule [11]. This would be in agreement with the finding that r-NP(-A1) activity is drastically diminished in spite of the presence of the A2 acidic tract. It has been further proposed [11] that phosphorylation would modify these interactions and expose the A2 polyglutamic region enhancing nucleoplasmin activity. Again, this is also consistent with our observation that r-NP142(-A1) is the least affected of the (-A1) mutants, as the A2 tract is fully exposed in this mutant.

The acidic tract A1 is found in the nucleoplasmin core in the flexible loop connecting strands $\beta 2$ and $\beta 3$ that is found in the distal face of the pentamer. Although the A1 tract is variable in the nucleoplasmin family, containing from four to twelve acidic amino acid residues, this acidic feature is conserved in this protein family [6,12], adding to the importance of this

region. It has recently been proposed [9] that phosphorylation of certain amino acid residues, in conjunction with the A1 tracts, could generate a strong localized negative potential at the distal face of the nucleoplasmin pentamer, which could be a good candidate for the binding site of sperm specific nuclear basic proteins. We have now provided direct evidence that mutation of the A1 region strongly affects the decondensing activity of nucleoplasmin. Unpublished results from our laboratory with a r-NP142 mutant, that is not able to pentamerize, also indicate that the integrity of the nucleoplasmin pentamer is needed to successfully carry out the protamine removal activity. The non-pentamerizing r-NP142 mutant has a very limited capacity to swell the sperm nuclei when an assay similar to that of Fig. 3 is carried out.

Although the results described in this paper can help to elucidate the working mechanism of nucleoplasmin, there are still many questions that remain unanswered. For instance, there is no agreement yet regarding the stoichiometry of the complexes formed between nucleoplasmin and basic proteins; we still do not know for certain which amino acid residues of nucleoplasmin are phosphorylated during the in vivo activation of this protein; and we do not even know if there is a unique or different binding sites for sperm proteins and histones. In this regard, we are currently investigating how the mutation of the small A1 acidic tract can affect nucleoplasmin binding to histones.

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