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# Nucleoplasmin associates with and is phosphorylated by casein kinase II

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## SUMMARY

**Nucleoplasmin is a phosphorylated nuclear-accumulating protein. We report herein that the kinetics of its cytoplasm → nucleus transport are affected by its degree of phosphorylation. Therefore, we sought to identify any protein kinase which specifically associates with nucleoplasmin. We discovered that nucleoplasmin co-isolates by two independent methods (immunoabsorption and chromatography) in a complex including a kinase which phosphorylates nucleoplasmin. The co-purifying kinase is casein kinase II-like because: (i) it phosphorylates casein; (ii) its phospho-transferase activity can be competed out by GTP; (iii) it is stimulated by polylysine; and (iv) it is inhibited by heparin. Moreover, a polyclonal antibody to the α (38 kDa) and α′ (36 kDa) catalytic subunits of casein kinase II specifically recognizes 38 and 36 kDa polypeptides in the nucleoplasmin-complex, and a specific inhibitor of casein kinase II inhibits nucleoplasmin's nuclear transport. Additionally, we found that phosphorylation of nucleoplasmin by its associated casein kinase II is strongly inhibited by histones and that, in addition to nucleoplasmin, another protein (p100) in the nucleoplasmin-complex is phosphorylated by casein kinase II.**

Key words: phosphorylation, nuclear transport, kinase

## INTRODUCTION

Many proteins - including structural proteins, enzymes, and transcription factors - are transported through the nuclear envelope more rapidly than can be accounted for by diffusion, some establishing much higher nuclear vis-à-vis cytoplasmic concentrations (reviewed by Feldherr, 1992). Molecular aspects of the responsible transport mechanisms remain obscure. Selective import occurs through the nuclear envelope pore complex (NPC) and is triggered by one or more nuclear localization signals (NLSs) in the proteins' primary structures. During embryogenesis and during the cell cycle in differentiated cells, the nucleocytoplasmic distributions of many NLS-proteins are regulated by phosphorylation/dephosphorylation (reviewed by Boulikas, 1994). In particular, the phosphorylation states of casein kinase II (CKII) and $p34^{cdc2}$ kinase target sites near the NLS can determine the rate of an NLS-protein's nuclear accumulation (Jans et al., 1991; Rihs et al., 1991).

Nucleoplasmin (Np) has been extensively studied as a prototype nuclear-accumulating protein. Its bipartite NLS, consisting of two short clusters of basic amino acids separated by 10 nondescript residues, is flanked by target sites for CKII and $p34^{cdc2}$ (Robbins et al., 1991). We show herein that the rate of Np's nuclear transport is proportional to its overall degree of phosphorylation. Hence, kinases (and phosphatases) are logical candidates for regulators of Np's transport, and we searched for kinases which specifically associate with Np.

We isolated Np from nuclei using a two-pronged experimental approach designed to not disturb intact Np's in vivo associations. First, we generated a highly specific polyclonal antibody to purified Np and coupled this antibody to Sepharose to construct immunoaffinity beads. Second, we immunoabsorbed Np from *Xenopus* oocyte nuclei obtained by manual isolation under oil, a method which minimally disturbs in vivo nuclear composition and functions (Paine et al., 1992). Using these combined methods, we identified one protein which associates with Np as a CKII-type kinase and found that Np serves as a substrate for this associated CKII. The CKII activity also co-isolates with Np by a completely independent purification protocol, one which includes chromatographic fractionations in high ionic strength buffers. These results implied that CKII associates with Np in the living cell and led us to suggest that CKII participates in Np's nuclear transport. Data obtained using a specific inhibitor of CKII support this hypothesis.

## MATERIALS AND METHODS

### Purification of Np, anti-Np antibody, and reduced-phosphate Np

*Xenopus laevis* frogs were maintained at room temperature in dechlorinated water and fed twice weekly. Ovaries were surgically removed into extracellular medium containing (in mM) 82.5 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 1.0 $Na_2HPO_4$, 3.3 NaOH, 5.0 HEPES and 1.0 sodium pyruvate, pH 7.6, and homogenized on ice in (in mM) 20 Tris-HCl (pH 7.5), 120 KCl, 2.0 $MgCl_2$, 2.0 DTT, 1.0 phenylmethylsulfonyl fluoride (PMSF), and pepstatin and aprotinin (each 1 mg/l). Np was purified from the homogenate by an elaboration of the procedure of Dingwall et al. (1982); we specifically avoided heat denaturation for removing other proteins. High-speed (82,000 ***g***) supernatant was extracted (3×) with 1,1,2-trichlorotrifluoroethane to remove yolk

lipoproteins. The clarified supernatant was made 55% in $(NH_4)_2SO_4$ (AS) and allowed to precipitate overnight at 4°C. The 55% AS supernatant was diluted to 1.7 M AS and loaded onto a 15 ml phenyl-Sepharose (Pharmacia) column equilibrated with 1.7 M AS in buffer A (20 mM Tris-HCl, pH 7.4, 1 mM PMSF, 2 mM dithiothreitol (DTT)). Np was eluted in 1.7 and 1.5 M AS, and the fractions were pooled, concentrated and dialyzed against buffer A containing 0.2 M KCl. The dialyzed Np sample was chromatographed on a Mono-Q (HR 5/5; Pharmacia) FPLC column equilibrated in buffer A+0.2 M KCl, and eluted with a linear gradient of 20 ml buffer A+1.0 M KCl. Np-containing fractions (eluted at 0.4-0.5 M KCl) were concentrated and applied to a Superose 12 (Pharmacia) column equilibrated in buffer A+0.2 M KCl. Np eluted in a fraction corresponding to a molecular mass of 190 kDa and was re-chromatographed on a Mono-Q column as described above, except that the column was eluted with a 40 ml linear gradient of buffer A+1.5 M KCl. Fractions containing electrophoretically pure Np (as judged by silver-stained 2D gels) were eluted at 1.0 M KCl, pooled, and dialyzed against phosphate buffered saline (PBS) (in mM, 137 NaCl, 2.7 KCl, 4.3 $Na_2HPO_4.7H_2O$, 1.4 $KH_2PO_4$). (Importantly, this protocol separated Np from all associating CKII, see below.) Np was used as antigen for polyclonal antibody production in rabbits. IgG was purified from whole antiserum and from pre-immune serum. Reduced-phosphate Np (rpNp) was produced by incubating 300 μg of Np with calf intestine alkaline phosphatase (2,000 units; Boehringer) in 20 mM Tris-HCl buffer, pH 8.5, containing 10 mM $MgCl_2$ for 30 minutes at 37°C, and subsequently separating the phosphatase from Np on a Mono-Q column.

### Coupling of proteins to Sepharose beads

Anti-Np IgG (or preimmune-IgG) was coupled to 6-aminohexanoic acid *N*-hydroxysuccinimid ester-Sepharose 4B (Sigma). Briefly, proteins were transferred to a coupling buffer (0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl) and mixed with the Sepharose bead suspension in the ratio 2-3 mg of protein/ml beads for 4 hours at 4°C. After the coupling reaction, remaining active coupling sites were blocked by 0.1 M Tris-HCl buffer (pH 8.0) and excess ligands were washed away alternately with 0.05 M formate buffer (pH 4.0) containing 0.5 M NaCl and 0.05 M Tris buffer (pH 8.0) containing 0.5 M NaCl.

### Protein labeling and microinjection, isolation of nuclei, and in vitro transport assay

Np (control or rp) was labeled with $^{35}$SLR-reagent (Amersham) (Vancurova et al., 1993) and was microinjected into the vegetal cytoplasm of living oocytes. Oocyte proteins were labeled in vivo by incubation overnight in a modified Barth's saline (MBS) (Colman, 1984) containing 500 μCi/ml of carrier-free [$^{32}$P]orthophosphate (Amersham). Oocytes were rinsed in fresh MBS, blotted on a filter paper and their nuclei and cytoplasms were cleanly isolated under oil (Paine et al., 1992). In vitro nuclear transport assays were done as previously described (Vancurova et al., 1993). The adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a specific inhibitor of CKII, was purchased from Biomol Research Laboratories.

### Kinase assay of the complex of Np and its associated proteins

Oil-isolated nuclei were solubilized in an immunoabsorption buffer IB (20 mM Tris-HCl, pH 7.6, 150 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 1% Triton X-100, 0.5 mg/ml BSA, 1 mM PMSF, 1 mg/l leupeptin, 1 mg/l aprotinin, 1 mg/l pepstatin) and precleared by incubation with preimmune IgG-Sepharose beads (4°C, 12 hours). The precleared nuclear supernatant was then incubated for 1 hour at 4°C with anti-Np IgG-Sepharose beads. Immune complexes were washed 5× in IB buffer and twice with kinase buffer (KB; 50 mM Tri-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl). The kinase activity was assayed by resuspending the pelleted beads in a total volume of 30 μl of KB. For phosphorylation of exogenous, purified proteins: oocyte Np was expressed in and purified from *Escherichia coli* transformed with an expression vector carrying the Np gene (Kalinich and Douglas, 1989) and further purified as described above; dephosphorylated α casein (Sigma), calf thymus histone mixture (H1, H2A, H2B, H3, and H4) (Fluka), and BSA (Sigma) were purchased. Reactions were initiated by addition of 10 μM ATP (20 Ci/mmol) and incubated at 30°C for 15 minutes. Assays were terminated by adding 7 μl of 5× SB and boiling for 5 minutes. Samples were analyzed by 12% SDS-PAGE and autoradiography. Phosphate incorporation was measured by excising substrate bands from the Coomassie-stained gels and counting the radioactivity by liquid scintillation. To test the influence of histones and other basic macromolecules on the phosphorylation of Np by CKII, a buffer containing physiological salt concentrations (20 mM Tris-HCl, pH 7.4, 120 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT) was used in place of KB buffer. Histone H1 was purchased from Boehringer, core histones (H2A, H2B, H3, and H4) from Worthington, and heparin, ATP, GTP, protamine, and poly-L-lysine from Sigma, and [γ-$^{32}$P]ATP from Amersham.

### SDS-PAGE, autoradiography, and western blot analysis

One-dimensional SDS-PAGE and 2D IEF-SDS-PAGE were performed according to routine procedures, and the gels were stained with either Coomassie Blue or silver (Bio-Rad kit). Autoradiography was done exposing the gels with $^{32}$P to a Hyperfilm-MP (Amersham). For western blots, polypeptides were separated by 1D or 2D SDS-gels and transferred to nitrocellulose (Schleicher and Schuel, 0.05 μm). The blots were washed in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.3, 150 mM NaCl), blocked in TBS containing 1% BSA for 4 hours, and incubated with a primary antibody for 12 hours in TBST (TBS + 0.05% Tween-20) containing 1% BSA. The primary antibody for Np detection was the purified rabbit anti-Np IgG (1 μg/ml). The α subunit of CKII was detected with rabbit anti-human α subunit CKII IgG (2 μg/ml; Upstate Biotechnology, Inc.). Duplicate blots were exposed to pre-immune rabbit IgG at the same concentration, as controls for non-specific staining. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:1,000 dilution) (Sigma) in 1% BSA/TBST for 4 hours, and washed 5× in TBST and 2× in 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. Unless stated otherwise, the secondary antibody was detected with an HRP Color Development Reagent kit (4CN, Bio-Rad). For detection of α subunit CKII, a more sensitive chemiluminescence method (ECL, Amersham) was used.

### Phosphopeptide mapping

Limited proteolysis of phosphorylated Np with *Staphylococcus aureus* V8 protease (Miles Scientific) or trypsin (Sigma) was performed (Cleveland et al., 1977). The phosphorylated Np band was cut out from the gel and placed on a 4.5% polyacrylamide stacking gel. The protein was cleaved in the stacking gel with V8 protease or trypsin for 30 minutes, and the resulting phosphopeptides were separated on a 15% polyacrylamide running gel.

## RESULTS

### Nucleoplasmin's transport is influenced by its phosphorylation

Nucleoplasmin (Np) is a nuclear-accumulating protein with five CKII and two p34$^{cdc2}$ consensus sequence target sites, plus eighteen other serine and threonine, and one tyrosine residue (Dingwall et al., 1987). Np exists in vivo as multiple species with different degrees of phosphorylation (Laskey, 1983). To determine whether Np's overall degree of phosphorylation influences its nuclear transport, we incubated Np

with alkaline phosphatase - a treatment which removes 70% of Np's phosphates and characteristically shifts it to a higher electrophoretic mobility on 1D SDS-PAGE (Cotten et al., 1986). We confirmed the higher electrophoretic mobility of this reduced-phosphate Np (rpNp) (Fig. 1A). 2D IEF-SDS-PAGE (not shown) also confirmed that rpNp has a higher pI and less charge heterogeneity than control Np. We labeled Np and rpNp with $^{35}S$ and microinjected them into the cytoplasm of living *Xenopus* oocytes. We subsequently measured the kinetics of the nuclear accumulation of the two forms of the protein by isolating individual nuclei and cytoplasm under oil and measuring the amount of protein in each compartment by scintillation counting. The rpNp accumulated with markedly slower kinetics than control Np (Fig. 1B).

### Np co-isolates by immunoabsorption with a kinase which phosphorylates it

We raised a rabbit polyclonal antibody to purified Np and isolated the IgG fraction. This antibody was highly specific, recognizing Np only on western blots of nuclear lysates (Fig. 2). IgG from the pre-immune serum did not react with any nuclear proteins (not shown). We covalently coupled anti-Np IgG to Sepharose beads (Pharmacia LKB Biotechnology, 1988). We gently isolated nuclei from individual *Xenopus* oocytes under paraffin oil, a procedure which avoids significant cytoplasmic contamination, obviates the loss of nuclear proteins, and leaves undisturbed nuclear structure and functions (Paine et al., 1992). Oil-isolated nuclei were solubilized in immunoabsorption buffer (IB) formulated to minimize disruption of in vivo protein-protein interactions and nonspecific binding of proteins to Sepharose (Springer, 1989). The solubilized nuclei were pre-cleared by incubation with beads coupled to pre-immune IgG and were then incubated with anti-Np IgG-beads. The beads were extensively washed to remove non-specifically bound proteins and then were incubated in kinase buffer (KB) in the presence of [$\gamma$-$^{32}P$]ATP. The immunoabsorbed proteins were separated by 1D SDS-PAGE and analyzed by autoradiography. We found that $^{32}P$ was heavily incorporated into the 30 kDa Np band and into a 100 kDa Np-associated polypeptide (p100) (Fig. 3A, lane 5); no other polypeptides were detectably phosphorylated. We conclude that at least one protein kinase co-isolates with Np by immunoabsorption from the nucleus, and that Np (as well as a co-isolating p100) serves as a substrate for this kinase in vitro.

### The associated kinase may phosphorylate Np in vivo

We investigated whether the associated kinase also phosphorylates Np in vivo. When we labeled intact oocytes with [$^{32}P$]orthophosphate, the nuclear proteins incorporating radiolabel included 30 kDa and 100 kDa bands (Fig. 3A, lane 1). Subsequently, we used anti-Np IgG-beads to extract Np and its co-isolating p100 from nuclei isolated from such in vivo-labeled oocytes; both proteins were found to be $^{32}P$-labeled (Fig. 3A, lanes 2 and 3). *S. aureus* V8 and trypsin phosphopeptide maps of the in vivo-labeled $^{32}P$-Np (Fig. 3B, lanes 1 and 2) were compared with maps of Np labeled with $^{32}P$ by the in vitro assay described above (Fig. 3B, lanes 3 and 4). Radiolabeled fragments of the in vitro-labeled Np comigrated with fragments of the in vivo-labeled protein, indicating that the kinase which co-isolates with Np and phosphorylates it in vitro may also phosphorylate Np within the living cell (although high resolution mapping of Np's phosphorylation sites will be required to prove this point). Some low $M_r$ peptide fragments of in vivo-phosphorylated Np were not detected on the maps of the in vitro-labeled protein, suggesting that within the living cell additional sites on Np are phosphorylated (by the Np-associated kinase or by other kinases).

### The Np-associated kinase is a CK II-type kinase

We investigated the substrate specificity of the Np-associated kinase by adding Np, histones, BSA or dephosphorylated $\alpha$ casein to anti-Np IgG-beads with bound Np complexes and measuring the extent to which the added proteins served as substrates in the phosphorylation assay. Added Np, expressed and purified from *E. coli* so as to not contain any associated CKII (Cozzone, 1988), was the best substrate on a per unit mass basis (Fig. 4, lane 2). Dephosphorylated $\alpha$ casein and core histones were also phosphorylated, but less extensively than Np (Fig. 4, lanes 3,4). No phosphorylation of histone H1 or BSA was detected (Fig. 4, lanes 4,5). No phosphorylation was detected for any proteins nonspecifically absorbed to preimmune IgG-beads (data not shown). These relative substrate specificities indicated that the Np-associated kinase is one of the casein kinases (Tuazon and Traugh, 1991).

Of the casein kinases, CKII is abundant in the *Xenopus* oocyte (Leiva et al., 1987; Cormier et al., 1989) and has been shown to phosphorylate Np in vitro (Taylor et al., 1987). CKII is characterized by: (i) high-sensitivity to inhibition by polyanions, including the glycosaminoglycan heparin; (ii) stimulation by polyamines (spermine and spermidine) and basic polypep-

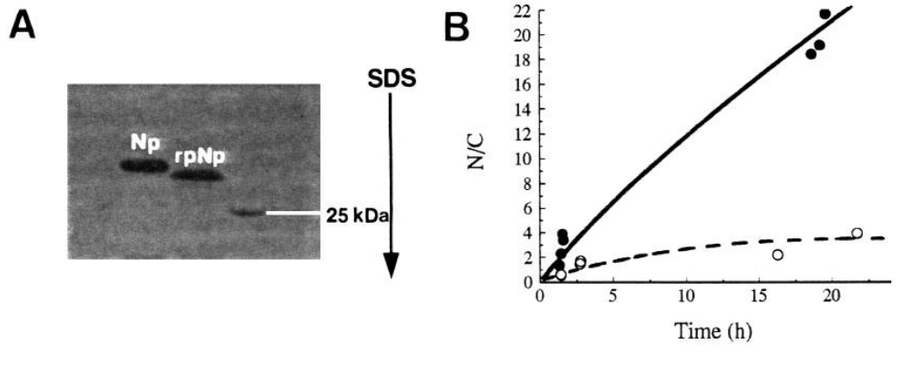


**Fig. 1.** (A) 1D SDS-PAGE; 2 μg control Np and reduced-phosphate Np (rpNp), silver-stained. (B) Np's nuclear/cytoplasmic concentration ratio after microinjection into oocyte cytoplasm. (———) Control Np; (– – – – –) rpNp. The kinetics vary from frog to frog, but in all five animals used rpNp accumulated significantly slower than control Np.

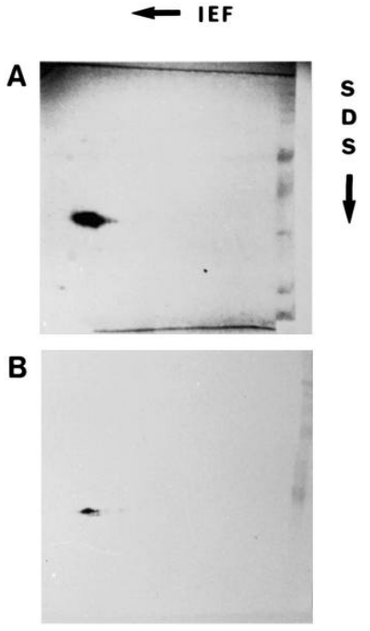


**Fig. 2.** (A) 2D IEF-SDS-PAGE; 2 μg purified Np; silver-stained. (B) Five oocyte nuclei were isolated under oil, solubilized in lysis buffer (still under oil), run on 2D-PAGE, electroblotted, probed with rabbit anti-Np IgG, and visualized with HRP-labeled anti-rabbit IgG.

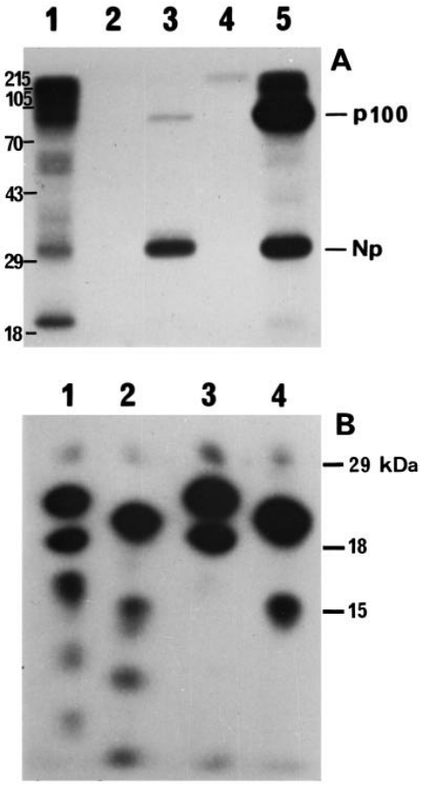


**Fig. 3.** (A) In vivo and in vitro Np phosphorylation. Nuclear extracts of in vivo $^{32}P_i$-labeled oocytes (lane 1) were incubated with preimmune IgG-Sepharose (lane 2) or anti-Np IgG-Sepharose (lane 3) and absorbed proteins were run on 1D SDS-PAGE. Np and the co-isolating p100 are phosphorylated (lane 3). For in vitro phosphorylation, nuclear extracts from unlabeled oocytes were incubated with preimmune IgG-Sepharose (lane 4) or anti-Np IgG-Sepharose (lane 5), and the protein-Sepharose complexes were incubated with [$^{32}$P]ATP. Like in vivo, Np and its co-isolating p100 are phosphorylated. (B) Phosphopeptide mapping of Np phosphorylated in vivo and in vitro. Proteins from oocyte nuclei that were labeled in vivo with $^{32}P_i$ (lanes 1 and 2) or unlabeled (lanes 3 and 4) were immunoabsorbed to anti-Np IgG-beads. The immunocomplexes from unlabeled cells were phosphorylated in vitro by incubation with [$^{32}$P]ATP. Proteins were resolved on 12% SDS-PAGE, and the Np bands were excised and digested with 1 μg of *S. aureus* V8 protease (lanes 1 and 3) or trypsin (lanes 2 and 4). The digested proteins were resolved on 15% polyacrylamide gels and visualized by autoradiography. Molecular mass markers: carbonic anhydrase, 29 kDa; β lactoglobulin, 18 kDa; and lysozyme, 15 kDa.

tides (polylysine, histones and protamines); and (iii) the ability to use either GTP or ATP as a phosphate donor (Pinna, 1990; Tuazon and Traugh, 1991). Therefore, we tested the sensitivity of the Np-associated kinase activity to heparin, polylysine and salmon sperm DNA, as well as its ability to use GTP. Since the effects of heparin and polylysine on protein phosphorylation by CKII are dependent on the substrate used, we used both dephosphorylated casein (Fig. 5A) and *E. coli*-expressed Np (Fig. 5B) as substrates. The casein and Np bands were excised from gels after autoradiography and the incorporation of $^{32}$P was quantitated by liquid scintillation. Incorporation into both Np and casein could be competed away by addition of unlabeled GTP (Fig. 5, A and B, lanes 3). Heparin (10 μg/ml) completely inhibited phosphorylation of casein (Fig. 5A, lane 2), while Np phosphorylation was inhibited by 56% (Fig. 5B, lane 2). Polylysine (100 μg/ml) stimulated casein phosphorylation 22-fold (Fig. 5A, lane 4), but, surprisingly, inhibited phosphorylation of Np by 40% (Fig. 5B, lane 4, and see below). Sonicated salmon sperm DNA (1 mg/ml) had no effect on phosphorylation of either substrate (Fig. 5, A and B, lanes 5). These data indicate that the Np-associated kinase is, or is closely related to, CKII.

To confirm the identification of CKII by an independent, nonenzymatic assay, we probed western blots of 1D SDS-PAGE gels loaded with Np and its co-immunoabsorbing proteins with a polyclonal $IgG_1$ antibody to the CKII α subunit (Fig. 6). Among the polypeptides of the immunoabsorbed Np complex, the antibody detected bands of 38 and 36 kDa (Fig. 6, lane 3), corresponding to the mobilities of the α and α′ subunits of CKII, respectively (Pinna, 1990). We detected no CKII α subunits in parallel control experiments using pre-immune IgG-beads (Fig. 6, lane 2). It was possible that CKII co-isolates with Np via immunoabsorption by immunological cross-reaction with the anti-Np IgG-beads, rather than because it is specifically associated with Np. If this were the case, CKII might also be expected to cross-react with the anti-Np IgG on western blots. However, on blots of gels loaded with whole oocyte nuclei (Fig. 2) or immunoabsorbed Np and its associated nuclear proteins (data not shown), the only protein recognized by the anti-Np IgG was Np itself.

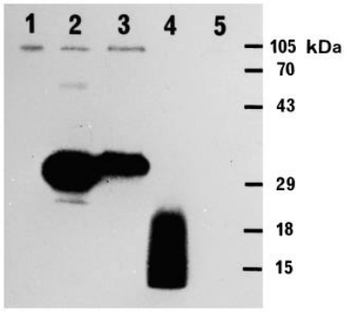


**Fig. 4.** Substrate specificity of the Np-associated kinase. Nuclear proteins were immunoabsorbed on anti-Np IgG-Sepharose and the protein-Sepharose complexes were aliquoted. The following purified proteins were added to individual tubes and tested for their ability to serve as phosphate acceptors: Lane 1, control (no exogenous proteins added); lane 2, *E. coli*-expressed Np (5 μg); lane 3, dephosphorylated casein (5 μg); lane 4, natural mixture of histones H1, H2A, H2B, H3 and H4 (20 μg); lane 5, BSA (5 μg).

### CKII also co-isolates with Np using chromatographic procedures

In search of additional evidence that CKII and Np are specifically associated, we assayed for casein-phosphorylating, heparin-sensitive activity in the Np-containing fractions throughout our chromatographic purification of Np. Np co-eluted with heparin-sensitive casein-phosphorylating activity throughout each of the first three chromatographic columns (phenyl-Sepharose, Mono-Q, and Superose 12 FPLC), which incorporated high ionic strength conditions. The single, symmetrical peak eluting from the Superose 12 column at a molecular mass of 190 kDa included Np (Fig. 7A). Incubation of this fraction in the presence of [$\gamma$-$^{32}$P]ATP revealed heparin-sensitive kinase activity (Fig. 7B). Moreover, this fraction also phosphorylated (in heparin-sensitive fashion) the included p100 (Fig. 7B), detectable as a kinase substrate even though not detectable by staining (Fig. 7A). Longer exposure of the film (Fig. 7B, lane 3) revealed that $^{32}$P was also incorporated into a polypeptide of 38 kDa, the size of the $\alpha$ (catalytic) subunit of CKII (known to autophosphorylate; Tuazon and Traugh, 1991). The fourth chromatographic step of our purification protocol, an additional Mono-Q column under modified conditions (see Materials and Methods), did quantitatively separate the CKII and p100 from Np, as determined by the above kinase assay (providing the pure Np which we used as antigen for antibody production). These results, together with peptide mapping data showing that Np is phosphorylated by CKII in vivo as well as in vitro, indicate that CKII and Np are associated in vivo, rather than simply as an in vitro artifact of the immunoabsorption protocol.

### A specific inhibitor of CKII inhibits Np nuclear transport

We investigated the involvement of CKII in the selective nuclear transport and accumulation of Np using an in vitro transport assay (Vancurova et al., 1993). Briefly, oil-isolated nuclei (OIN) from *Xenopus laevis* oocytes were individually abutted (under oil) to nuclear-size volumes of isolated oocyte cytoplasm (C) which had been preloaded with $^{35}$S-Np, thus forming OIN:C 'transport pairs'. At various times after pair formation, nuclei were separated from their paired cytoplasms, the amounts of $^{35}$S-Np in each compartment were measured by scintillation counting, and the nucleocytoplasmic concentration ratio of $^{35}$S-Np was plotted as a function of time. In some pairs, the cytoplasm was also preloaded with DRB - a specific inhibitor of CKII (Zandomeni and Weinmann, 1984) previously shown to inhibit phosphorylation of Np by CKII (Taylor et al., 1987). Table 1 shows that the OIN accumulates $^{35}$S-Np 16-fold in control transport pairs within 3 hours, and that DRB (150 μM) inhibits this selective transport and accumulation by 65%.

### CKII is highly accumulated in the oocyte nucleus

Accurate, quantitative determination of a protein's in vivo nucleocytoplasmic distribution is rarely possible. Cell-fractionation and immunocytochemical studies have indicated that in vivo CKII is predominantly located in the cell nucleus (Krek et al., 1992), although some studies have identified it in cytosolic fractions after cell disruption (Edelman et al., 1987). Importantly, there are also reports that the localization of CKII, as well as regulation of its kinase activity, are cycle dependent (Yu et al., 1991; Carroll and Marshak, 1989). Co-isolation of CKII by immunoabsorption with Np from nuclei which have been cleanly isolated under oil demonstrates the presence of this enzyme in the nucleus of the prophase-arrested *Xenopus* oocyte. We sought to determine whether CKII is also present

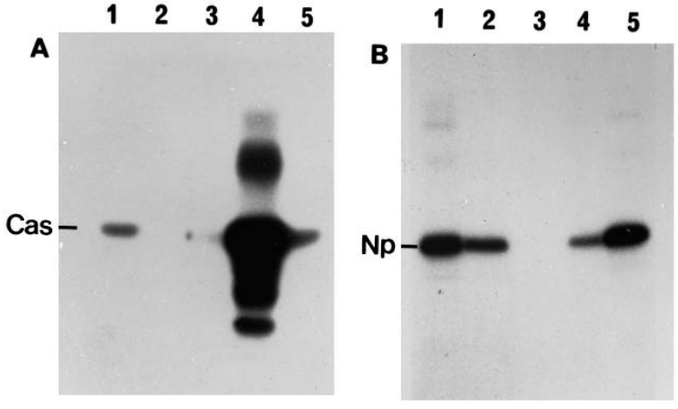


**Fig. 5.** Effects of heparin, GTP, polylysine and salmon sperm DNA on Np-associated kinase activity. Nuclear proteins were immunoabsorbed on anti-Np IgG-beads and aliquoted, and the following compounds were added to the individual tubes to test their effects on the Np-associated kinase: lanes 1, control (nothing added); lanes 2, heparin (10 μg/ml); lanes 3, unlabeled GTP (100 μM); lanes 4, poly-L-lysine (100 μg/ml); lanes 5, salmon sperm DNA (1 mg/ml). Experiments were done using either casein (Cas, 5 μg, panel A) or *E. coli*-expressed Np (5 μg, panel B) as phosphate acceptors.

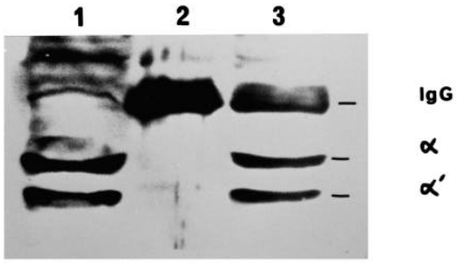


**Fig. 6.** Detection of the α subunit of CKII on a western blot of nuclear proteins co-immunoabsorbed with Np. Immunoabsorbed protein complexes were separated on 12% gels, transferred to nitrocellulose membrane and probed with anti-human CKII α subunit rabbit IgG. The blot was probed with HRP-labeled goat anti-rabbit IgG and detected by enhanced chemiluminescence. The heavily stained bands of 55 kDa are the heavy IgG chains released from the Sepharose beads by boiling and recognized by the secondary antibody. Whole nuclei (lane 1); preimmune IgG-Sepharose absorbed proteins (lane 2); anti-Np IgG-Sepharose absorbed proteins (lane 3).

in the oocyte cytoplasm. Using the anti-CKII α antibody and a sensitive chemiluminescence assay to probe western blots of oil-isolated oocyte nuclei and cytoplasm, we could detect a signal from a single nucleus, but could not detect CKII in gels loaded with up to 5 enucleated oocytes (data not shown). Since the oocyte nucleus contains ~10% of the oocyte water, these data establish a lower limit of 50:1 for the nucleus/cytoplasm concentration ratio of the CKII catalytic subunit in the prophase arrested oocyte.

### CKII phosphorylation of Np is inhibited by basic macromolecules

Np contains an unusual stretch of glutamic acid residues (128-145), consistent with postulated roles in histone-binding and chromatin assembly (Burglin et al., 1987; Dingwall et al., 1987). Because of the unusual inhibitory effect we observed for basic polylysine on CKII's phosphorylation of Np, we tested the influence of histones and other basic macromolecules on phosphorylation of Np by CKII. To better address the issue of whether histone modulation of Np phosphorylation by CKII is physiologically important, these studies were done using a physiological salt concentration rather than the kinase buffer. We extracted Np and its associated proteins from unlabeled oocyte nuclei by immunoabsorption, and assayed the complex for its ability to incorporate $^{32}P$ into *E. coli*-expressed Np (added to the assay in >50:1 molar excess over the endogenous oocyte Np) or casein, in the presence of (i) polylysine, (ii) histone H1, (iii) core histones (H2A, H2B, H3, and H4, mixed), or (iv) protamines. The proteins of the $^{32}P$-incorporation assay were resolved by SDS-PAGE, the Np and casein bands were visualized by Coomassie staining, excised from the gels, and counted by liquid scintillation. Phosphorylation of casein by the CKII was markedly stimulated by polylysine and histone H1, while phosphorylation of Np was inhibited by both compounds (Table 2). Even greater inhibition of Np phosphorylation was observed when the core histones were present. Protamine (1 mg/ml) had no effect on Np phosphorylation by the associated CKII.

## DISCUSSION

The selective transport of NLS-containing proteins must include their specific interactions with other intracellular proteins. Indeed, NLS-mediated transport exhibits saturation and competition effects (Goldfarb et al., 1986; Newmeyer and Forbes, 1988; Michaud and Goldfarb, 1993; Gulizia et al., 1994), and several cell-fractionation, immunohistochemical, and affinity-binding approaches have identified NLS-specific binding proteins in the cytoplasm, the NPC, and the nucleus (Yamasaki and Lanford, 1992). In addition to NLS-binding proteins, still other proteins, e.g. heat shock proteins (Shi and Thomas, 1992; Okuno et al., 1993) and GTP-binding proteins (Melchior et al., 1993; Moore and Blobel, 1993), are involved, presumably associating with transported proteins directly, or indirectly via complexes with NLS-binding protein(s).

Because: (i) the transport of NLS-proteins requires the hydrolysis of ATP (or GTP) (Feldherr, 1992); (ii) the nucleo-cytoplasmic distributions of many proteins are modulated by phosphorylation and dephosphorylation (Dahmus, 1981;

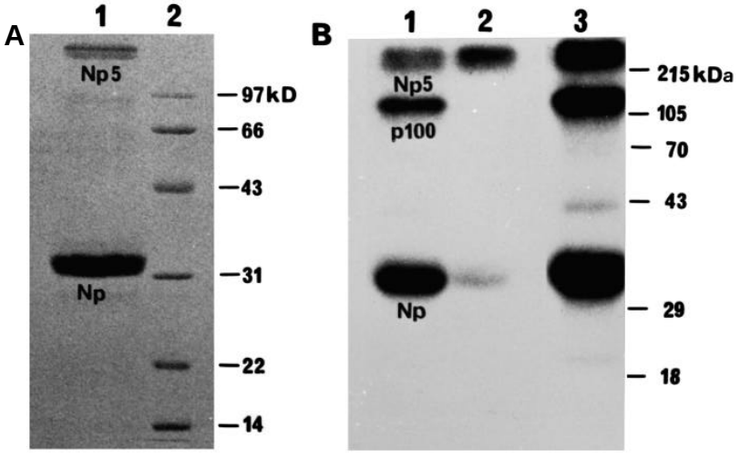


**Fig. 7.** (A) Coomassie-stained 12% SDS-PAGE of purified Np. Lane 1, Np purified on phenyl-Sepharose, Mono-Q and Superose 12 FPLC. Lane 2, molecular mass markers (from top): phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme (BioRad). Np, nucleoplasmin monomer; Np5, nucleoplasmin pentamer (Earnshaw et al., 1980). (B) Biochemically/chromatographically purified Np fraction contains heparin sensitive Np-phosphorylating activity. A 10 µl sample of the Np-containing Superose 12 fraction was incubated in a total volume of 20 µl consisting of 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM KCl, and 1 mM DTT. Reaction was started by addition of 10 µM ATP (20 Ci/mmol), and after 15 minutes of incubation at 37°C it was stopped with 5 µl 5× SB, boiled for 5 minutes, run on a 12% gel, and exposed to Hyperfilm-MP (Amersham). The molecular mass markers are prestained markers from BRL. Lane 1, without heparin. Lane 2, 10 µg/ml heparin. Lane 3, as lane 1, but longer exposure.

**Table 1. DRB inhibition of Np transport in OIN:C pairs**

| Time (h) | [N]/[C] of $^{35}$S-NP | |
|---|---|---|
| | Control | +DRB |
| 1 | 4.0±0.7 | 1.0±0.2 |
| 2 | 9.9±2.3 | 2.8±1.1 |
| 3 | 16.0±2.7 | 5.6±1.9 |

DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol, a specific inhibitor of CKII, was loaded into the cytoplasm (before pair formation) to yield a final concentration of 150 μM in the pair.

Data are means±s.e.m. of measurements for individuals OIN:C transport pairs.

Ackerman et al., 1985; Lüscher et al., 1989; Meek et al., 1990; Tuazon and Traugh, 1991; Li and Roux, 1992; Bojanowski et al., 1993); and (iii) Np's nuclear accumulation kinetics are influenced by its degree of phosphorylation (shown above), we were prompted to search for protein kinase(s) which complex with Np. We generated a polyclonal antibody to purified Np and covalently coupled anti-Np IgG to Sepharose to produce immunoaffinity beads. (The Np antigen was pure as assayed by 2D SDS-PAGE and contained no associated kinase activity; the antibody produced was highly specific, recognizing only Np on western blots of nuclear lysates.) Using the immunoaffinity beads to absorb Np from oil-isolated nuclei, we found that the proteins co-isolating with Np do include a kinase and that this kinase phosphorylates Np, both in vivo and in vitro.

We identified the Np-associated kinase as a CKII-type kinase by a panel of standard criteria: (i) it prefers casein to histone H1 as an artificial substrate; (ii) it can use GTP as well as ATP; (iii) its phosphorylation of casein is specifically inhibited by low concentrations of heparin; and (iv) its phosphorylation of casein is stimulated by basic polypeptides such as polylysine and histones. Importantly, a polyclonal antibody raised against the α and α′ catalytic subunits of human CKII specifically identified these subunits among the oocyte nuclear proteins which coimmunoabsorb with Np. Several properties of the Np-associated kinase are not characteristic of CKII kinases: (i) core histones were phosphorylated by the Np-coisolated kinase in vitro; and (ii) no autophosphorylation of the β CKII subunit was observed in the absence of exogenously added polybasic effectors. However, these two properties may not be intrinsic to the kinase. Rather, its tight association with Np, whose carboxy terminus contains regions rich in lysine residues (Burglin et al., 1987; Dingwall et al., 1987), may be responsible for these atypical characteristics. The rather low inhibition (56%) of Np phosphorylation by 10 μg/ml heparin is in accord with a previous report that Np phosphorylation by CKII is unusually resistant to heparin inhibition (Taylor et al., 1987).

An interesting feature of Np's phosphorylation by its associated CKII is inhibition of the process by polybasic compounds (Table 2). Polyamino compounds such as polylysine, histones and polyamines usually stimulate CKII (Tuazon and Traugh, 1991). Responsiveness of CKII activity to these molecules depends upon the protein substrate and the subunit composition of the CKII holoenzyme (Meggio et al., 1992). We found that polylysine and histones stimulated casein phosphorylation by the Np-associated CKII; but inhibited Np phosphorylation. This is the first demonstration that phosphorylation of another protein by CKII is inhibited by polybasic peptides (although polylysine was described to inhibit autophosphorylation of the β subunit of CKII; Meggio and Pinna, 1984). What is the nature of this inhibition of Np phosphorylation? Does the primary sequence of Np show any overlap or other obvious spatial relation between the CKII target sites and the putative histone-binding sites? Of the five potential CKII phosphorylation sites on the Np molecule, one (Ser-Trp-Ala-Glu; residues 121-124) is immediately adjacent to the putative histone-binding region of Np, which includes residues 124-142 (Dingwall et al., 1987). Thus, if Glu-124 is involved in histone binding, residues 121-124 may be unavailable for association with CKII. Involvement of histones in modulating Np's phosphorylation by CKII is consistent with the predominant nuclear localization of this protein kinase (Krek et al., 1992).

Aside from its coimmunoabsorption with Np from minimally disturbed, oil-isolated nuclei, three additional lines of evidence imply that CKII is associated with Np in the living nucleus. First, CKII remains associated with Np throughout the first three stages of our biochemical purification - a protocol which differs grossly from the immunoabsorption protocol and exposes Np to a completely different sequence of forces. Second, our peptide mapping data indicate that the kinase which phosphorylates Np in vivo shares similar specificity to the co-isolating CKII-like activity. Third, the in vivo nucleocytoplasmic distribution of the CKII in the oocyte is highly asymmetric, paralleling that of Np.

Our data show that removing phosphate groups from Np with alkaline phosphatase reduces Np's rate of nuclear accu-

**Table 2. Inhibition of Np phosphorylation by polybasic compounds**

| Mg/ml | Compound (% of $^{32}$P incorporation)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Polylysine | | H1 histone | | Core histones | | Protamine | |
| | Np | Cas | Np | Cas | Np | Cas | Np | Cas |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.1 | 60 | 2230 | 101 | 688 | 6 | 2138 | 145 | n.d.† |
| 0.5 | 18 | 1237 | 16 | 491 | 1 | 100 | 100 | n.d. |
| 1 | 0 | 1070 | 18 | 474 | 0 | 55 | 100 | n.d. |

*Nuclear proteins were immunoabsorbed on anti-Np IgG-beads and aliquoted into individual tubes. Basic compounds were added to each tube and tested for their effect on $^{32}$P incorporation. Casein (5 μg) or *E. coli*-expressed Np (5 μg) was used as phosphate acceptor.

†Not determined.

mulation, but it remains to be determined which specific phosphorylation sites on Np are affected by this treatment. Np contains five target site motifs for CKII (S/TXXD/E) (Burglin et al., 1987; Dingwall et al., 1987). In view of the previous demonstration (Rihs et al., 1991) that phosphorylation of the CKII site near the NLS of the lg T antigen greatly enhances the nuclear transport of this protein, Np's three CKII sites (S121, S169, S170) near its NLS are likely candidates to be involved in regulating Np's transport. The findings that CKII is complexed with (and likely phosphorylates) Np in vivo and that DRB inhibits Np's transport (Table 1) support a regulatory role for CKII.

In addition to CKII, another oocyte nuclear protein, p100, remains associated with Np, both during immunoabsorption and during our chromatographic purification protocol. We found that p100, like Np, is phosphorylated by the associated CKII in vitro, suggesting that Np, CKII and p100 exist as a multiprotein complex in vivo. (The difference in p100 phosphorylation in vivo and in vitro (Fig. 3A, lanes 3 and 5, respectively) may have several causes. For example, the phosphorylated form of p100 may have a lower affinity for Np than the non-phosphorylated form, or p100 may be dephosphorylated in vivo by a specific phosphatase.) Many proteins in the oocyte nucleus have a molecular mass of 100 kDa, and the identity of p100 is currently under investigation. It may be the previously identified NLS-binding protein, Nopp 140, which has been proposed to shuttle between the nucleolus and the cytoplasm and to function as a chaperone for nuclear import and/or export of proteins. Nopp 140 contains abundant consensus sites for CKII phosphorylation and its dephosphorylated form was shown by electrophoresis on SDS-gels to be 100 kDa (Meier and Blobel, 1992). Two other proteins - each implicated in the nuclear transport/accumulation of specific proteins - are also candidates for p100: (i) the 90 kDa heat shock protein (HSP90), a component of nuclear steroid receptor complexes (Pratt et al., 1992) and a regulator of various protein kinases (Rose et al., 1989); and (ii) nucleolin, a 100 kDa phosphoprotein which is involved in the organization of nucleolar chromatin and in ribosome biogenesis (Olson, 1990) and shuttles between the cytoplasm and nucleolus (Borer et al., 1989). Indeed, both HSP90 and nucleolin are phosphorylated by CKII (Caizergues-Ferrer et al., 1987; Lees-Miller and Anderson, 1989) and copurify with it (Caizergues-Ferrer et al., 1987; Miyata and Yahara, 1992). And recently, nucleolin and CKII were shown to both co-purify with the nuclear FK506-binding protein (FKBP25) (Jin and Burakoff, 1993); analogous to our observations with Np, FKBP25 and nucleolin serve as substrates for CKII in this complex.

To summarize: (i) the rate of Np's transport into the nucleus is a function of its degree of phosphorylation and is reduced by a specific inhibitor of CKII; (ii) Np is present in vivo in a heterocomplex which includes CKII; and (iii) Np serves as a substrate for its associated CKII. Significantly, Np is not unique in this third regard: CKII phosphorylates a number of nuclear-accumulating proteins, including nucleolin (Schneider and Issinger, 1988; Belenguer et al., 1990), RNA polymerase (Cisek and Corden, 1989), nuclear lamins (Lüscher et al., 1989; Li and Roux, 1992), and SV40 large T antigen (Grasser et al., 1988; McVey et al., 1989). It is known that selective nuclear protein accumulation is a multi-step transport process. In light of our present findings, we suggest that the kinase activity of CKII facilitates one or more of the steps involved in transport to the NPC, translocation through the NPC, and/or subsequent transport and binding of NLS-proteins within the nucleus (Vancurova et al., 1993). (A similar model has been previously proposed in which HSP90, HSP70 and HSP56 are components of a heterocomplex or 'transportosome' which functions as a trafficking vehicle to transport the glucocorticoid receptor protein into the nucleus (Pratt, 1993).) Since CKII is one component of a Np-complex, phosphorylation of Np by the CKII, in particular at Np's S177, S178 CKII sites adjacent to its NLS, may be involved in the transport process. Alternatively, CKII phosphorylatiion of other protein components of the Np-complex (e.g. the p100) or the transport machinery may be phosphorylated by CKII. In this context, the transport machinery must be broadly considered to include cytoplasmic, NPC, and intranuclear proteins which might transiently interact with Np or its complex during transport.

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