Characterization of a *Drosophila* phosphorylation-dependent nuclear-localization-signal-binding protein

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A 94 kDa nuclear-localization-signal (NLS)-binding protein was purified from *Drosophila* embryos. The NLS of the simian-virus-40 T-antigen is specifically bound by the dephosphorylated form of the protein. After phosphorylation, the affinity of the protein for the NLS is sharply decreased. In the dephosphorylated form, p94 (protein of 94 kDa) is the major NLS-binding protein in *Drosophila* embryos. Immunoprecipitation confirmed the ATP-

dependent phosphorylation of p94, and co-precipitation of two additional phosphorylated proteins, indicated that the NLS-binding protein is part of a larger complex in *Drosophila* embryos. In agreement with the immunoprecipitation results, cross-linking experiments demonstrated the interaction of p94 with three additional proteins. These protein–protein interactions were also phosphorylation-dependent.

INTRODUCTION

Nuclear proteins synthesized in the cytoplasm are transported to the nucleus through the nuclear-pore complex by a selective mediated process. The selectivity of the process is ensured by short peptide sequences present in nuclear proteins, which are called ‘nuclear localization signals’ (NLS). Deletion of these sequences from a nuclear protein abolishes its transport to the nucleus, while tagging of these sequences to a cytoplasmic protein ensures nuclear localization for the cytoplasmic protein (reviewed in [1]). A detailed sequence comparison of the different NLSs did not reveal a clear consensus sequence; three different classes of NLS sequences have been identified so far. A high proportion of basic residues is present in a single cluster in the SV-40 large T-antigen-type NLSs [2]. Two clusters of basic residues are separated by a spacer sequence in the bipartite nucleoplasmin-like NLSs [3]. In these NLSs the average length of the spacer is ten amino acids, but both the length and the amino acid sequence of the spacer are fairly mutation-tolerant. In the NLS of the oncoprotein c-Myc [4] a very short cluster of basic residues is flanked by neutral and acidic amino acids (PAAKRVKLD). This unusual arrangement of neutral, basic and acidic amino acids represents the third type of known functional NLSs, because fusing the PAA sequence to the N-terminal end, and the LD dipeptide to the C-terminal end, of the tetrapeptide KKKK (an inactive cluster of basic amino acids derived from the bipartite NLS of nucleoplasmin) generates a functionally active NLS [5]. The relative positions of the fused PAA and LD sequence elements are crucial to the function of the ‘artificial’ NLS.

The existence of an NLS indirectly suggested that a signal-recognition receptor molecule(s) should be involved in the nuclear-protein import. Several candidates for this receptor function have been detected by affinity approaches [6–16], and some of these proteins have been purified to homogeneity. Functional assays obtained from *in vitro* nuclear-transport experiments indicated that only karyopherin α /importin α fulfilled all the criteria of a genuine NLS receptor. It specifically

recognizes and binds the NLS and, after association with importin 90/karyopherin β , mediates the docking of NLS proteins to the periphery of the nuclear-pore complex (for reviews see [17–20]). Although no clear consensus sequence was established for the NLS, the recognition by the NLS receptor is very precise: a single amino acid change in the NLS of the SV-40 virus T-antigen almost completely abolishes the *in vivo* or the *in vitro* transport competence [21,22].

Whereas the first step of the nuclear transport, the importin α/β -mediated docking of the transport substrate to the nuclear-pore complex, is energy-independent, the second phase of the transport, the translocation of the protein through the central channel of the pore complex, is absolutely energy-dependent. The exact mechanism of this energy-dependent step is not known. Although Ran/TC4, a member of the Ras-like GTPase family plays pivotal role in the translocation, ATP was previously considered to be an indispensable energy donor of the translocation step in *in vitro* nuclear-transport assays. Recently, however, it was shown that a mutant Ran, with altered nucleotide specificity from GTP to xanthosine 5'-triphosphate, alone can support efficient nuclear transport in an *in vitro* assay system with xanthosine 5'-triphosphate as the sole nucleotide. In this assay, protein import could no longer be inhibited by non-hydrolysable ATP or GTP analogues [23]. ATP thus either might participate in the GTPase cycle of Ran/TC4, or might influence the nuclear transport through an ATP-dependent regulatory step. The protein(s) which mediate the effect of ATP is not known. To characterize proteins which may mediate the effect of ATP on the nuclear transport, we have purified and characterized a NLS-binding protein from *Drosophila* embryos. The NLS-binding activity of this protein is ATP-dependent. In the absence of ATP this is the major NLS-binding protein in a *Drosophila* embryonic protein extract. In the presence of ATP, owing to the phosphorylation of the protein, its NLS-binding activity is almost completely lost. This protein is involved in specific protein–protein interactions, and these protein–protein interactions are also phosphorylation-dependent.

Abbreviations used: NLS, nuclear localization signal; SV 40, simian virus 40; DSS, disuccinimidyl suberate; EGS, ethylene glycol bis(succinimidyl succinate).

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EXPERIMENTAL

Synthesis and purification of peptides

The wild-type SV-40 large-T-antigen nuclear localization sequence (Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp), the reverse sequence (Asp-Glu-Val-Lys-Arg-Lys-Lys-Lys-Pro-Gly-Tyr-Gly) and a transport-incompetent mutant NLS sequence (Gly-Tyr-Gly-Pro-Lys-Thr-Lys-Arg-Lys-Val-Glu-Asp), which will be called [Thr¹²⁸]NLS, containing threonine instead of lysine at position 128 in the T-antigen [2,22,24], were synthesized on an Advanced ChemTech model 200 peptide synthesizer according to the manufacturer's recommendations, using well-established solid-phase methods [25]. The protected peptides were cleaved by using anhydrous liquid HF, exchanged to the acetate form on AG-3 (acetate⁻ form) anion-exchange resin, and purified by reverse-phase HPLC on a Delta Pak C18-300 A (Waters) column. The synthetic peptide was iodinated according to standard procedures [26].

Detection and purification of a 94 kDa NLS-binding protein

Crude embryonic extract was prepared from 0–10 h *Drosophila melanogaster* embryos (Canton S strain) as described previously [27]. For the preparation of a partially purified embryonic extract, nucleic acids were removed by the addition of 0.1 vol. of 10% streptomycin sulphate (Sigma), the precipitate was removed by centrifugation [10 000 rev./min (12 000 g) for 10 min] and the supernatant was loaded without dialysis on to a hydroxyapatite column (Bio-Rad) equilibrated with 25 mM potassium phosphate (pH 6.7)/5 mM MgCl₂/5 mM β -mercaptoethanol/5% glycerol. After washing of the column with this buffer until baseline absorbance was attained, the proteins were eluted with a step of 250 mM potassium phosphate, pH 6.7, in the buffer described above. During this purification step only the nucleic acids, low-molecular-mass impurities and the large mass of yolk proteins are removed. Proteins were dialysed against 0.1 M NaCl/20 mM Hepes buffer (pH 7.6)/5 mM MgCl₂/5 mM β -mercaptoethanol/5% glycerol (binding buffer), diluted to a total protein concentration of 20 mg/ml and stored in aliquots at -80°C (hydroxyapatite fraction).

The homobifunctional protein cross-linkers disuccinimidyl suberate (DSS, Pierce) and ethylene glycolbis(succinimidyl succinate) (EGS, Pierce) were dissolved in DMSO.

NLS-binding proteins were detected by a cross-linking reaction. The protein fraction to be tested was incubated in 40 μl of binding buffer with 100 pg of iodinated NLS at 25°C for 15 min. After cooling of the mixture at 0°C for 5 min, 1 μl of 40 mM DSS was added and the cross-linking reaction was performed at 0°C for 30 min. The reaction was stopped by precipitation of the proteins with trichloroacetic acid (10% final concn.). The pelleted proteins were washed with acetone and dissolved in SDS sample buffer. After electrophoresis on SDS/7%-polyacrylamide gel, NLS-binding proteins were detected by autoradiography.

For purification of the p94 NLS-binding protein, the hydroxyapatite fraction obtained from 100 ml of embryonic extract was dialysed against 0.17 M NaCl/20 mM Tris/HCl (pH 7.6)/5 mM MgCl₂/5 mM β -mercaptoethanol/5% glycerol, and loaded on to a Q-Sepharose FF (Pharmacia) column (50 ml) equilibrated with the dialysis buffer. At this salt concentration, the p94 protein does not bind to the anion-exchange column. The flowthrough fraction was loaded on to a heparin-agarose column (Sigma, 25 ml) equilibrated with the dialysis buffer. p94 protein, which appeared again in the flowthrough fraction, was loaded on to an aminopentyl-agarose column (Sigma, 25 ml), which was

Figure 1 Purification of the p94 NLS-binding protein from *Drosophila* embryos

Aliquots of the different purification steps were analysed by Coomassie Blue staining after SDS/7%-PAGE. Lanes: 1, crude *Drosophila* embryo extract; 2, hydroxyapatite fraction; 3, flowthrough fraction of the heparin-agarose column; 4 and 5, the aminopentyl-agarose fraction and the Mono Q fraction respectively. Molecular-mass markers are shown in lane M. kD = kDa.

equilibrated with the dialysis buffer. This column was eluted with a linear salt gradient (0.17–1 M NaCl in the dialysis buffer); p94 appeared in the 0.5–0.6 M-salt-concentration interval. The aminopentyl-agarose fraction was dialysed against the dialysis buffer containing only 50 mM NaCl, loaded on to a Mono Q HR 5/5 FPLCTM column (Pharmacia) and eluted with a linear salt gradient (50–300 mM NaCl in the dialysis buffer). p94 was eluted between 70 and 90 mM NaCl. The protein was stored in small aliquots at -80°C . A typical purification is depicted in Figure 1. The purification of p94 was followed by the NLS-binding assay described above. For this assay, the protein fraction should be diluted 10-fold with binding buffer to decrease the free amine concentration to 2 mM.

Covalent cross-linking with EGS was done under the conditions described for DSS. The apparent molecular masses of the different cross-linked p94 products were estimated by gel electrophoresis using multimers of cross-linked phosphorylase b (Sigma) as markers.

Immunoblotting and immunoprecipitation

Polyclonal antibody was raised in rabbits by injecting 200 μg of purified p94 in complete Freund's adjuvant. The rabbits were boosted at 4-week intervals three times with the same amount of protein in incomplete Freund's adjuvant. The IgG fraction was purified on Protein A-Sepharose (Pharmacia) according to the manufacturer's recommendations.

Immunoblotting was performed according to standard procedures [26], using either alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) or the enhanced chemiluminescent detection kit (ECLTM, Amersham).

For immunoprecipitation, 50 μl of embryonic extract was incubated at 25°C for 30 min with 30 μCi of [γ -³²P]ATP (800 Ci/mmol) in the presence of 10 mM NaF and 2 mM sodium β -glycerophosphate as phosphatase inhibitors. Immunoprecipitation was performed in the presence of 1% Nonidet P40 according to standard procedures [26]. Immunoprecipitated proteins were separated on SDS/7%-polyacrylamide gel and revealed by autoradiography. To determine the apparent molecular mass of the phosphorylated bands co-immunoprecipitat-

ing with p94, a molecular-mass-marker mix was run on the same gel parallel with the immunoprecipitated material, the gel was stained with Coomassie Brilliant Blue, dried and autoradiographed. The stained marker pattern was aligned with the autoradiograph.

Proteins were separated on SDS/polyacrylamide gels prepared either from a 29:1 mixture or a 25:1 mixture of acrylamide and bisacrylamide as indicated in the text.

In vitro nuclear-transport assay

Digitonin permeabilization of HeLa or *Drosophila* Schneider S3 tissue-culture cells and the nuclear transport of phycoerythrin-NLS transport substrate was studied as described previously [28].

RESULTS AND DISCUSSION

During the first 2 h of *Drosophila* embryogenesis there are 14 synchronous nuclear-division cycles, the fastest mitotic rate recorded in the Animal Kingdom. During these divisions, all the proteins required for the assembly of chromatin should be transported to the nucleus in the 4–5 min-long S-phase. Thus cytosolic protein factors involved in the nuclear-transport process should be present in high concentration in early *Drosophila* embryos. To detect NLS-binding proteins in early *Drosophila* embryos, a peptide corresponding to the NLS of the SV-40 large T-antigen was radiolabelled by iodination and incubated with a partially purified *Drosophila* embryonic extract (hydroxyapatite fraction). Chemical cross-linking with DSS and autoradiography of the electrophoretically separated proteins were applied to detect NLS-binding proteins. As shown in Figure 2, several polypeptides had a high enough affinity for the NLS to be cross-linked under the mild conditions applied. Being a highly charged basic peptide, NLS can non-specifically interact with acidic proteins, and thus the specificity of protein–NLS interactions should always be carefully controlled. Competition binding experiments with non-radiolabelled wild-type or a transport-incompetent mutant NLS were previously used to prove the specificity of binding [6,9]. An alternative way to demonstrate the specificity of NLS binding was based on the assumption that phosphorylation of a specific component(s) of

Figure 2 Detection of NLS-binding proteins in a partially purified *Drosophila* embryonic extract (hydroxyapatite fraction) in the presence (lane 1) or absence (lane 2) of ATP

Figure 3 The main NLS-binding proteins in *Drosophila* embryos: p94 and karyopherin α

The hydroxyapatite fraction was size-fractionated on a Superose 6 FPLC column equilibrated with binding buffer. Aliquots of the column fractions were tested by immunoblot assay, using a mixture of anti-p94 and anti-OHO31 antibodies (A) or by the NLS-binding assay (B). Owing to the lower total protein concentration of the column fractions, only 10 pg of radiolabelled NLS was used in the NLS-binding assay.

the transport machinery may be involved in the regulation of the transport process. To test whether phosphorylation can affect the NLS-binding affinity of any *Drosophila* embryonic protein, the hydroxyapatite fraction was dialysed against binding buffer to remove the residual ATP completely or, alternatively, it was dialysed against binding buffer supplemented with 1 mM ATP, and the cross-linking reaction with iodinated NLS was repeated with these extracts. Whereas the cross-linking pattern for the majority of the NLS-binding proteins was not affected by the dialysis conditions, the NLS-binding activity of a 94 kDa protein (p94) was greatly suppressed in the presence of ATP (Figure 2). The NLS-binding activity of p94 in the absence of ATP can be suppressed after preincubation of this fraction at 25 °C for 20 min in the presence of 1 mM ATP. Conversely, the weak NLS-binding activity of p94 in a hydroxyapatite fraction prepared in the presence of ATP was greatly enhanced after removal of the ATP from this fraction by preincubation for 20 min at 25 °C with apyrase (results not shown).

In the absence of ATP, p94 is the main NLS-binding activity present in *Drosophila* embryonic extract. This is demonstrated in Figure 3. In this experiment the ATP was removed from the hydroxyapatite fraction by size fractionation on a Superose 6 FPLC column. The NLS-binding activity of the proteins present in the chromatography fractions was tested by the cross-linking assay using radiolabelled NLS, and the distribution of p94 and karyopherin α proteins was detected by immunoblotting using anti-p94 IgG or an antibody developed against OHO31, the *Drosophila* counterpart of karyopherin α [29].

The specificity of the NLS binding of the purified p94 was proved in competition binding experiments with wild-type or a transport-incompetent mutant NLS ([Thr¹²⁸]NLS). In the experiment presented in Figure 4, the purified p94 was preincubated with radiolabelled wild-type NLS in the presence of increasing concentrations of unlabelled wild-type or unlabelled [Thr¹²⁸]NLS mutant peptides, and the competition of NLS binding by p94 was monitored by the cross-linking assay. Whereas a 50-fold molar excess of unlabelled wild-type NLS peptide completely competed with the binding of the radiolabelled wild-type NLS, only a moderate competition could be achieved with a 100-fold excess of mutant [Thr¹²⁸]NLS peptide. The specificity of NLS binding is supported by the observation that the purified p94 protein does not bind the radiolabelled reverse peptide, which has the same overall charge as the wild-type NLS, and the

Figure 4 Competition binding experiment with wild-type or transport-incompetent NLS peptides

A portion (50 ng) of purified p94 was preincubated with 20 pg of iodinated NLS for 15 min at 25 °C and DSS cross-linking was performed as described in the Experimental section (lane 1). Similar cross-linking reactions were carried out after preincubation of the purified p94 and the radiolabelled NLS in the presence of a 5-fold (lane 2), 10-fold (lane 3), 50-fold (lane 4) or 100-fold (lane 5) molar excess of non-radiolabelled wild-type NLS, or a 5-fold (lane 6), 10-fold (lane 7), 50-fold (lane 8) or 100-fold (lane 9) molar excess of [Thr¹²⁸]NLS mutant NLS peptides.

unlabelled reverse peptide does not compete with the binding of the radiolabelled wild-type NLS (results not shown).

The purified p94 was used to raise polyclonal antibody in rabbits. As shown by immunoblotting, the antibody recognizes a single 94 kDa polypeptide in a *Drosophila* embryonic extract preincubated either in the presence or in the absence of ATP (Figure 5A, lanes 1). This antibody was employed for immunoprecipitation to prove directly the phosphorylation of p94 in the presence of ATP. *Drosophila* embryonic extract was preincubated with [γ -³²P]ATP in the presence of protein phosphatase inhibitors, and immunoprecipitation was performed with either an affinity-purified anti-p94 IgG or preimmune IgG. As shown in Figure 6, anti-p94 IgG precipitated a phosphorylated 94 kDa polypeptide which was missing in the precipitate of the pre-immune IgG. Besides this 94 kDa phosphorylated band, anti-p94 IgG specifically precipitated two additional phosphorylated polypeptides with apparent molecular masses of 118 and 145 kDa. As anti-p94 IgG recognizes a single polypeptide in the *Drosophila* embryonic extract preincubated in the presence of

Figure 6 p94 immunoprecipitation

Crude *Drosophila* embryonic extract was preincubated in the presence of [γ -³²P]ATP and immunoprecipitated with preimmune IgG (lane 1) or anti-p94 IgG (lane 2). The immunoprecipitated proteins were separated by SDS/7%-PAGE and revealed by autoradiography. Molecular-mass markers were separated on the same gel, the stained marker pattern was aligned with the autoradiograph and the positions of the different marker fragments are shown on the left-hand side.

ATP (Figure 5A), it is unlikely that the two additional precipitated bands were hyperphosphorylated forms of the protein. It was more reasonable to suppose that a p94-mediated protein-protein interaction causes the co-precipitation of these proteins by anti-p94 IgG. If p94 is really involved in protein-protein interactions, an appropriate bifunctional protein cross-linker must readily cross-link the interacting proteins to p94. As the electrophoretic mobility of p94 will be shifted towards the higher-molecular-mass region after cross-linking with a protein, immunoblotting with anti-p94 IgG seemed to be a sensitive assay for the detection of protein-protein interactions. Additionally, this cross-linking approach might permit study of the effect of phosphorylation on the formation of protein-protein inter-

Figure 5 Phosphorylation-dependent protein-protein interactions of p94 detected by a cross-linking/immunoblot technique

(A) The hydroxyapatite fraction was preincubated for 20 min at 25 °C either with 10 units/ml apyrase or with 1 mM ATP. Cross-linking with 2 mM EGS was performed at 0 °C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 40 min (lane 6) or 50 min (lane 7). Proteins were separated by SDS/6%-PAGE and analysed by the immunoblot technique with anti-p94 IgG. The position of p94 is indicated by an arrow on the right side. (B) The apparent molecular masses of the cross-linked p94 products were estimated by gel electrophoresis using multimers of cross-linked phosphorylase *b* as molecular-mass markers. The positions and the molecular masses of the phosphorylase *b* multimers are shown on the left side of the immunoblot; the estimated molecular masses of the cross-linked p94 derivatives are shown on the right-hand side.

actions, which cannot be studied by immunoprecipitation, which is always performed in the presence of ATP. Preliminary experiments revealed that EGS, a homobifunctional protein cross-linker with a long spacer arm, is the most suitable cross-linker to detect the protein-protein interactions of p94. To study the effect of phosphorylation on protein-protein interactions, 50 μ l of the hydroxyapatite fraction was preincubated for 20 min at 25 °C either in the presence of 1 mM ATP or with 10 units/ml apyrase. Tris/HCl (1 mM, pH 7.6) was added to the apyrase-treated sample to compensate for the free amine concentration derived from ATP. After this preincubation, the fractions were cooled on ice for 2 min and the cross-linking was initiated by the addition of EGS (2 mM final concn.). The cross-linking reaction was performed at 0 °C for increasing times, and aliquots of the reaction mixture were removed and precipitated with trichloroacetic acid. Proteins were separated on SDS/6%-polyacrylamide gel and analysed by immunoblotting using the anti-p94 IgG. As Figure 5(A) shows, in the absence of ATP three strong cross-linked immunoreactive bands appear. While the intensities of these cross-linked bands increase on prolonged incubation with the cross-linker, no other immunoreactive bands appear, indicating that the cross-linking is non-random and that it represents genuine protein-protein interactions of p94. Characteristic changes were noted in the cross-linking pattern if the hydroxyapatite fraction was preincubated in the presence of ATP. Whereas the intensity of the cross-linked product with the highest mobility did not decrease, or even increased slightly, the cross-linked product with medium mobility almost completely disappeared, and the cross-linked product with the lowest mobility decreased slightly in intensity. The molecular mass of the cross-linked products was estimated by gel electrophoresis using multimers of cross-linked phosphorylase *b* as markers (Figure 5B).

The formation of, and the phosphorylation-dependent changes in, the cross-linking pattern may be due to the interaction of p94 with a multisubunit protein, in which either the conformation of the subunits, or the specific interaction of the different subunits with p94, is changed after phosphorylation. These assumptions can explain the appearance of several p94 cross-linked bands and the specific disappearance of one of these bands in the presence of ATP. The cross-linking pattern can also be interpreted by assuming that p94 interacts with three independent polypeptides, and the interaction of one of these polypeptides is inhibited after phosphorylation. Although the high-molecular-mass cross-linked bands correspond in size quite closely to multimers of a 94 kDa polypeptide, this cross-linking pattern cannot be the trivial consequence of the covalent cross-linking of a tetrameric p94 protein. The importin α/β heterodimer is eluted ahead of p94 on a size-fractionating Superose 6 FPLC column (Figure 3), indicating that p94 is a monomer. Nevertheless, one can assume that the monomeric p94 has a tendency for self-association, and this can be detected after cross-linking. In this case, however, it is difficult to explain why trimer formation is inhibited specifically after phosphorylation, leaving the dimer and tetramer formation unaltered. The co-immunoprecipitation of two different phosphorylated polypeptides with p94 makes the first or the second alternative more plausible.

To study the role of p94 in the nuclear protein transport it was tested in an *in vitro* transport assay using digitonin-permeabilized tissue-culture cells. Neither the rate of the transport nor the total nuclear accumulation of the transport substrate changed after the addition of purified p94 to the cytosolic protein extract used in the *in vitro* transport assay (results not shown).

Immunodepletion of the cytosol with anti-p94 IgG did not influence the nuclear transport (results not shown). The failure of

Figure 7 p94 is present in two distinct electrophoretic forms and one of these forms is retained in digitonin-permeabilized cells

(A) Proteins of a *Drosophila* embryonic extract (lane 1) or Schneider S3 tissue-culture cell extract (lane 2) were fractionated by conventional SDS/7%-PAGE (gel prepared from a 29:1 mixture of acrylamide and bisacrylamide) and analysed by immunoblotting. The same two protein extracts were fractionated by SDS/7%-PAGE (gel prepared from a 25:1 mixture of acrylamide and bisacrylamide) and analysed by immunoblotting. Lane 3, *Drosophila* extract; lane 4, S3 extract. (B) Schneider S3 cell extract (lane 1), cytosolic proteins solubilized during digitonin-permeabilization of S3 cells (lane 2) and cytosolic proteins which were not solubilized during the digitonin-permeabilization step (lane 3) were analysed by immunoblotting after SDS/7%-PAGE (gel prepared from a 25:1 mixture of acrylamide and bisacrylamide).

p94 to influence the *in vitro* nuclear transport may be due to an inefficient removal of the endogenous p94 protein during the digitonin permeabilization step. To test this assumption *Drosophila* Schneider S3 tissue-culture cells were permeabilized with digitonin and the cytosolic proteins solubilized during the permeabilization were collected. After the permeabilization the plasma membrane was disrupted completely by incubating the cells in the presence of 0.2% Nonidet P40, the nuclei were removed by centrifugation, and the cytosolic proteins which were not solubilized during the digitonin-permeabilization step were collected. The distribution of p94 between the solubilized and the unsolubilized fractions of the cytosol was detected by the immunoblotting technique. To increase the chance of separating the phosphorylated and non-phosphorylated forms of p94, proteins were separated in these experiments on SDS/polyacrylamide gels prepared from a 25:1 mixture of acrylamide and bisacrylamide. As shown in Figure 7(A) under these conditions p94 can be separated into two electrophoretically distinct forms. During digitonin permeabilization the upper band is almost quantitatively solubilized and washed out of the cell, while only a small amount of the lower band is released. This lower band is solubilized only after a complete disruption of the plasma membrane (Figure 7B). Thus this second form of p94 is either anchored to a structural element of the cytosol, or it is part of a larger complex which cannot escape from the cytosol through the digitonin-generated holes of the plasma membrane. It is reasonable to suppose that the observed two bands correspond to the phosphorylated and non-phosphorylated forms of p94, although bacterial or calf intestine alkaline phosphatase treatment did not change the pattern (results not shown).

Our results demonstrate that we have purified and characterized a new NLS-binding protein from *Drosophila melanogaster*. This 94 kDa protein specifically recognizes and binds the wild-type NLS of the SV-40 T-antigen. The specificity is documented by a competition binding experiment with a transport-incompetent mutant NLS peptide. Whereas the wild-type non-radiolabelled NLS peptide efficiently competed with the binding of the radiolabelled NLS to p94, the mutant peptide, in which a single lysine residue was replaced by threonine, was a much weaker competitor (Figure 4). A weak competition by the mutant

NLS peptide is not unexpected, since it can function as a weak NLS [22,24,30]. The NLS binding is not due to a non-specific electrostatic interaction, as the reverse peptide, which has the same overall basic charge, is not bound by the protein and does not compete with the binding of the wild-type NLS. In its unphosphorylated form, p94 is the major NLS-binding protein present in a *Drosophila* embryonic extract.

Owing to the selective retention of the lower electrophoretic form of p94 in digitonin-permeabilized cells, its role in the nuclear transport cannot be tested directly in an *in vitro* assay. The strong NLS binding by the unphosphorylated form of p94 and the almost complete reversal of the binding activity after phosphorylation suggest that p94 may be involved in the regulation of the nuclear transport by counteracting the effect of importin α . In competition with importin α , p94 may modulate the actual transport of nuclear proteins, and this competition may be regulated through the phosphorylation of p94. This negative regulation may operate in growth-arrested cells where there is an almost 80-fold decrease in the *in vivo* nuclear-transport rate compared with actively proliferating cells [31].

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