A Conserved Phosphoprotein That Specifically Binds Nuclear Localization Sequences Is Involved in Nuclear Import

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Abstract. We have purified proteins of 70 kD from *Drosophila*, HeLa cells, and *Z. mays* that specifically bind nuclear localization sequences (NLSs). These proteins are recognized by antibodies raised against a previously identified NLS-binding protein (NBP) from the yeast *S. cerevisiae*. All NBPs are associated with nuclei and also present in the cytosol. NBPs are phosphorylated and phosphatase treatment abolished NLS binding. The requirement for NBPs in nuclear protein uptake is demonstrated in semipermeabilized *Drosophila melanogaster* tissue culture cells. Proper import of a fluorescent protein containing the large T antigen NLS requires cytosol and ATP. In the absence of cytosol and/or ATP, NLS-containing proteins are bound to cytosolic structures and the nuclear envelope. Addition of cytosol and ATP results in movement of this bound intermediate into the nucleus. Anti-NBP antibodies specifically inhibited the binding part of this import reaction. These results indicate that a phosphoprotein common to several eukaryotes acts as a receptor that recognizes NLSs before their uptake into the nucleus.

THE composition of the nucleus requires vectorial transport of macromolecules across the nuclear envelope. Thus far, import of proteins into the nucleus could be divided into several steps. The initial reaction includes specific recognition and binding of nuclear proteins at the nuclear envelope (Newmeyer and Forbes, 1988; Richardson et al., 1988). This is followed by ATP- and temperature-dependent translocation through the nuclear pore complex.

Specific delivery of cytoplasmically synthesized polypeptides destined for the nucleus can be mediated by short basic peptides, termed nuclear localization sequences (NLSs)[1] (reviewed in Garcia-Bustos et al., 1991; Silver, 1991). One of the best characterized NLSs comes from SV-40 large T antigen and consists of the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val. Mutation of the Lys at the position equivalent to amino acid 128 of large T antigen inactivates the nuclear localization activity (Kalderon et al., 1984). The presence of NLSs and the apparent saturability of nuclear protein import (Goldfarb et al., 1986) led to the proposal that certain proteins may recognize NLSs and act as receptors for nuclear protein import. In agreement with this hypothesis, proteins of similar size that specifically interact with NLSs have been identified by affinity-labeling, cross-linking, and ligand-binding assays (Adam et al., 1989; Yamasaki et al., 1989; Silver et al., 1989; Li and Thomas, 1989; Lee and Melese, 1989). In general, these proteins can distinguish between wild-type and mutant forms of NLSs.

Several models exist for how NLS-binding proteins (NBP) could mediate nuclear protein import. By one hypothesis, proteins destined for the nucleus might interact with a cytoplasmic receptor. This reaction would be followed by binding of the complex at the pore and subsequent translocation to the nuclear interior. Transported proteins might remain bound to the receptor during transport and released once inside the nucleus. The receptor would then be recycled to the cytoplasm. Breeuwer and Goldfarb (1990) predicted the existence of cytoplasmic receptors for histone H1 by in situ titration of cytoplasmic H1 binding sites. Subsequently, Adam and Gerace (1991) purified NBPs from erythrocytes and showed that they were important for import of proteins into the nucleus in permeabilized mammalian cells.

Import of proteins into yeast nuclei shares many of the features of the process in mammalian cells. The SV-40 NLS is efficiently recognized (Nelson and Silver, 1989) and the yeast *S. cerevisiae* contains several proteins that specifically bind NLSs such as the one from SV-40 large T antigen (Silver et al., 1989). One major yeast NBP of 70 kD has been purified from salt extracts of yeast nuclei (Stochaj et al., 1991). Immunolocalization and cell fractionation studies indicate that most of this yeast NBP is associated with nuclei with ~20% in the cytosol. Antibodies raised against the purified yeast protein inhibit association of NLS-bearing proteins with yeast nuclei in vitro.

However, our knowledge of the details of the translocation of proteins to the nucleus and subsequent import is still limited. For instance, how do NPBs specifically recognize their substrates? In this study, we show that NBPs from higher eukaryotes have biochemical properties similar to those of yeast. Moreover, antibodies raised against the yeast NLS-binding protein cross react with NLS-binding proteins from

1. *Abbreviations used in this paper*: HSA, human serum albumin; NBP, NLS-binding protein; NLS, nuclear localization sequences.

other species. NBPs require phosphorylation for their binding activity. Finally, we show that in semipermeabilized *Drosophila* cells, these NLS-binding proteins are involved in protein import.

Materials and Methods

Cell Culture and Preparation of Nuclei

Drosophila Schneider S_2 cells were grown in Schneider's tissue culture medium containing 10% FBS supplemented with 100 μg/ml streptomycin and 32 μg/ml penicillin. HeLa cells were kindly provided by Dr. D. Ornelles (Princeton University, Princeton, NJ). Strain ABYS1 (*pral prb1 prc1 cps1 ade2*) was used for preparation of yeast nuclei (Silver et al., 1989). Nuclei from *D. melanogaster* Schneider and HeLa cells were prepared according to Davis and Blobel (1986). Nuclei prepared from *Z. mays* endosperm prepared according to Fusswinkel et al. (1991) were kindly provided by Dr. H. Fusswinkel, University of Cologne, Germany. Protein concentrations were determined with the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

Preparation of Protein Conjugates and Labeling Procedures

Conjugation of peptides to human serum albumin (HSA), radioiodination, and fluorescent labeling of the substrates with TRITC for nuclear import was as described in Silver et al. (1989). For coupling of lissamine-rhodamine (Molecular Probes, Inc., Eugene, OR) to SV-40 HSA, substrates were dialyzed against 0.1 M $NaHCO_3/Na_2CO_3$, pH 9.0, followed by incubation with lissamine-rhodamine in dimethylformamide (0.5 mg lissamine-rhodamine/10 mg protein). After 1 h incubation at 4°C with gentle agitation, NH_4Cl (50 mM final concentration) and glycerol (5% vol/vol) were added and protein conjugates were separated by chromatography on Sephadex G-25 followed by further dialysis. All substrates were further purified by passage over BioBeads SM2 (Bio-Rad Laboratories, Cambridge, MA).

Analysis of NLS-binding Proteins

Dot blot analysis of NLS-binding proteins, salt extraction of nuclei and the NLS gel binding assay were carried out as described (Stochaj et al., 1991).

Purification of NLS-binding Proteins

For affinity purification of NBP70s, the nucleoplasmin NLS (CAVKRPAATKKAGAKKK) was covalently attached to HSA (Silver et al., 1989) and then coupled to *N*-hydroxysuccinimide-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. Isolated nuclei were incubated with 0.3 M NaCl in Buffer A (50 mM Tris-HCl, pH 7.2, 3 mM $MgCl_2$, 2.5 mM $CaCl_2$ containing 1 mM PMSF, aprotinin, antipain, chymostatin, leupeptin, and pepstatin, all at 0.1 μg/ml). Proteins released from nuclei by this treatment were diluted sixfold into Buffer A and subsequently subjected to affinity chromatography. Proteins bound to the affinity resin were eluted with a linear gradient of 50–1,000 mM NaCl in Buffer A. Fractions were assayed for the NLS binding by the dot blot assay and silver staining (Stochaj et al., 1991). The majority of NLS-binding protein was released between 700 and 900 mM NaCl.

Preparation of Antibodies

The generation and affinity purification of antibodies directed against yeast NBP70 were as described by Stochaj et al. (1991). In brief, antisera against NBP70 were preadsorbed to immobilized SV-40 HSA. Immunoglobulins of unbound material were precipitated with $(NH_4)_2SO_4$ and further purified by affinity chromatography on Protein A–Sepharose. Purified immunoglobulins were dialyzed against 20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, and stored at −70°C in the presence of 1 mg/ml HSA.

Electrophoretic Separation of Proteins and Immunoblotting

Proteins were separated by SDS-PAGE using 7.5–15% gradients of acrylamide followed by blotting onto nitrocellulose. Filters were blocked with 5% nonfat dry milk in PBS/0.2% Tween 20/0.005% SDS for 1 h at room temperature, incubated with affinity-purified antibodies overnight at 4°C and then processed as described by Stochaj et al. (1991).

Dephosphorylation of NBP70s

Salt-extracted nuclear proteins were incubated with 50 U/ml calf intestine phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) in 50 mM Tris-HCl, 10 mM EDTA, pH 8.5, for 30 min at 37°C containing antipain, aprotinin, chymostatin, leupeptin, and pepstatin (all at 0.1 μg/ml). Proteins were collected by precipitation with 5% TCA (2 min incubation on ice) and dissolved in gel sample buffer. Proteins were further analyzed by Western blotting with affinity-purified antibodies against yeast NBP70 or by the gel binding assay.

Immunofluorescence

Schneider cells and HeLa cells were grown on multiwell slides, fixed with 2% formaldehyde in PBS (20 min at room temperature) followed by treatment with cold methanol (5 min at 20°C). Fixed cells were pre-incubated for 1 h at room temperature with 2 mg/ml BSA in PBS/0.05% Tween 20 (TPBS/BSA) and subsequently incubated with antibodies against yeast NBP70 (overnight at room temperature). Wells were rinsed five times with TPBS/BSA and incubated with 5 μg/ml FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in TPBS/BSA for 2 h at room temperature. After washing five times in TPBS/BSA, cells were treated with 1 μg/ml DAPI, washed once and mounted in PBS/90% glycerol containing 1 mg/ml *o*-phenylenediamine.

In Vitro Nuclear Import

To study binding of SV-40 HSA, Schneider cells were allowed to attach to polylysine-coated slides overnight and permeabilized with 40 μg/ml digitonin in Buffer B (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, and 1 μg/ml each aprotinin, leupeptin, and pepstatin) followed by incubation with 50 μg/ml fluorescent substrates in Buffer B containing 1 mg/ml HSA. After 30 min incubation at 30°C cells were washed with Buffer B and fixed for 10 min with 4% formaldehyde in Buffer B at room temperature. Slides were rinsed with Buffer B, incubated with 1 μg/ml DAPI, and mounted in PBS/90% glycerol containing 1 mg/ml *o*-phenylenediamine. For treatment with antibodies, permeabilized cells were pre-incubated with purified immunoglobulins in Buffer B containing 1 mg/ml HSA for 30 min at room temperature, washed in Buffer B, and subsequently incubated with fluorescent substrates as described above. To analyze import of SV-40 HSA, permeabilized cells were incubated with Buffer B containing 30 mg protein/ml of cytosol, 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine kinase. Cytosol was prepared from *Drosophila* Schneider cells according to Adam et al. (1990). Cells were incubated for 30 min at 30°C and further treated for viewing with the fluorescence microscope as described above.

Results

Higher Eukaryotes Contain NLS-binding Proteins That Are Similar to Those of Yeast

We previously identified and purified a 70-kD yeast protein that specifically interacts with NLSs. This NLS-binding protein is termed NBP70 and antibodies have been generated against the purified protein (Stochaj et al., 1991). We now show that these antibodies recognize proteins of similar size from cells of higher organisms. Affinity-purified antibodies against the yeast NBP70 were used to probe Western blots of proteins from yeast, *Drosophila*, Hela cells, and *Z. mays* (Fig. 1). Immunocrossreactive proteins of ~70 kD were present in all extracts.

NLS-binding proteins can be purified from different cell types by their affinity for NLSs and similar biochemical properties. To purify the NBP70s from *Drosophila*, HeLa, and *Z. mays* cells, nuclei prepared from the three species were incubated with salt. Numerous proteins were released, including those that cross reacted with the yeast anti-NBP70

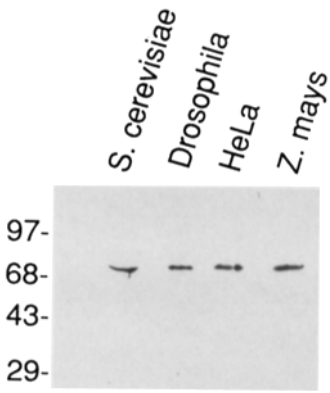

Figure 1. Proteins immunocrossreactive with yeast NBP70 are present in higher eukaryotes. Proteins of isolated nuclei prepared from *S. cerevisiae*, *D. melanogaster* Schneider cells, HeLa cells, and *Z. mays* endosperm were separated by SDS-PAGE followed by electroblotting onto nitrocellulose. Each lane contains 25 μg of protein. Filters were blocked and incubated with affinity-purified antibodies generated against yeast NBP70 as described in Materials and Methods. Molecular weights of marker proteins $\times 10^{-3}$ are indicated on the left side.

(Fig. 2 A). Proteins liberated by the salt treatment were subjected to affinity chromatography on a resin containing immobilized NLSs. We chose the nucleoplasmin NLS because, in our experience, it shows the strongest affinity for NLS-binding proteins. Material bound to the immobilized NLS was eluted with a gradient of 50–1000 mM NaCl. NBP70s from yeast as well as from *Drosophila*, HeLa, and *Z. mays* were eluted between 700 and 900 mM NaCl (Fig. 2 *A*). From ~500 micrograms of salt-released proteins, we purified 2–3 micrograms of NBP70 by this procedure. NLS-binding proteins from different species co-migrated by gel chromatography and were recognized by antibodies against the yeast NBP70 (Fig. 2 *B*). (Occasionally, we obtained co-purification of a 59-kD protein which was eluted in the same fraction as NBP70. This was observed for all the cell types analyzed [data not shown] but we cannot say with certainty that it binds NLSs specifically).

The purified NBP70s also recognized NLSs derived from SV-40 large T antigen. To demonstrate this interaction, and the specificity of NLS recognition, the purified NBP70s were adsorbed to nitrocellulose and incubated with ^{125}I-labeled albumin which was conjugated to peptides comprising the SV-40 NLS (SV-40 HSA). All purified polypeptides bound SV-40 HSA in the presence of a chemical excess of unlabeled albumin (Fig. 2 *C*). Unlabeled SV-40 HSA efficiently competed for binding of the radioactive substrate whereas a mutant form of the SV-40 NLS (CTPPK*T*KRKV) coupled to HSA (SV-40* HSA) had little effect. Taken together, these results indicate that we have identified and purified NLS-binding proteins from higher eukaryotes which are similar to the previously identified yeast NLS-binding protein. These proteins bind to two related NLSs and can distinguish between wild-type and mutant forms of the SV-40 NLS. The purified proteins did not cross react with antibodies to a conserved NH_2-terminal peptide found in HSP70s (Chappell et al., 1986). Moreover, anti-NBP70 antibodies did not react with purified yeast HSP70s.

NLS-binding Proteins Are Located at the Nucleus and Are Also Present in the Cytosol

We determined the distribution of NBP70s in tissue culture cells by immunolocalization and cell fractionation. Staining of *Drosophila* Schneider cells and HeLa cells with antibodies directed against yeast NBP70 revealed that the cross-reactive proteins are associated with the nucleus with some also present in the cytoplasm (Fig. 3 *A*). This was consistent with cell fractionation experiments. NBP70 could be detected in nuclear fractions as well as in postmitochondrial high spin supernatants from fly and HeLa cells (Fig. 3 *B*). The relatively large amount of NBP70 found in the cytosol may be a result of its dissociation from nuclei during the preparation of cytosolic fractions.

Phosphorylation Is Required for NLS-binding

Initial experiments using metal-affinity chromatography demonstrated that NBP70s from yeast, *D. melanogaster*, HeLa, and *Z. mays* cells bind under conditions that allow the enrichment of phosphorylated proteins. To further analyze the possible functional role of phosphorylation, NBP70s were liberated from nuclei by salt treatment and incubated with alkaline phosphatase. Pre-incubation with alkaline phosphatase disrupted the ability of NBP70s to bind NLSs; i.e., dephosphorylated NBP70s adsorbed to nitrocellulose no longer bound ^{125}I–SV-40 HSA. Identical results were obtained for proteins from yeast, *Drosophila* Schneider cells, HeLa cells, and *Z. mays* (Fig. 4, *bottom panel*). Treatment with alkaline phosphatase did not affect the reaction of the proteins with antibodies directed against yeast NBP70 (Fig. 4, *top panel*), indicating that the proteins were not degraded. However, NBP70s incubated with alkaline phosphatase did show a slight increase in electrophoretic mobility of ~3 kD as compared with untreated controls, indicating that removal of covalently bound phosphate(s) altered their electrophoretic properties. For the various experiments carried out, similar shifts in electrophoretic mobilities were observed for NBP70s from different organisms (Fig. 4, *top panel*).

To determine directly whether NBP70s are phosphorylated in vivo, yeast cells were grown in the presence of ^{32}P-labeled phosphate. Immunoprecipitation with anti-NBP70 antibody followed by gel electrophoresis demonstrated that radioactive phosphate was incorporated into NBP70, indicating that this protein is a substrate for protein kinase(s) in vivo (data not shown).

Detergent-permeabilized Drosophila Tissue Culture Cells Faithfully Import Nuclear Proteins

Faithful import of proteins into the nucleus can be reproduced in vitro using cells permeabilized with digitonin (Adam et al., 1990). We have used a similar system to analyze the role of the NBPs in the nuclear import reaction. *Drosophila* Schneider cells were grown on multi-well slides and incubated with digitonin. This treatment resulted in selective perforation of the plasma membrane and allowed high molecular weight components to enter the cells. This is demonstrated by the penetration of anti-tubulin antibodies into the detergent-permeabilized cells and their binding to microtubules (Fig. 5). However, under identical conditions, the nuclear envelope remained intact. Dextrans of 70 kD or antibodies to nuclear lamins could not penetrate into the nucleus unless the digitonin-treated cells were further permeabilized with detergents such as NP-40 (Fig. 5). Taken together, these results indicate that digitonin treatment of *Drosophila* Schneider cells permeabilized the plasma membrane, but the nuclear membrane remained intact.

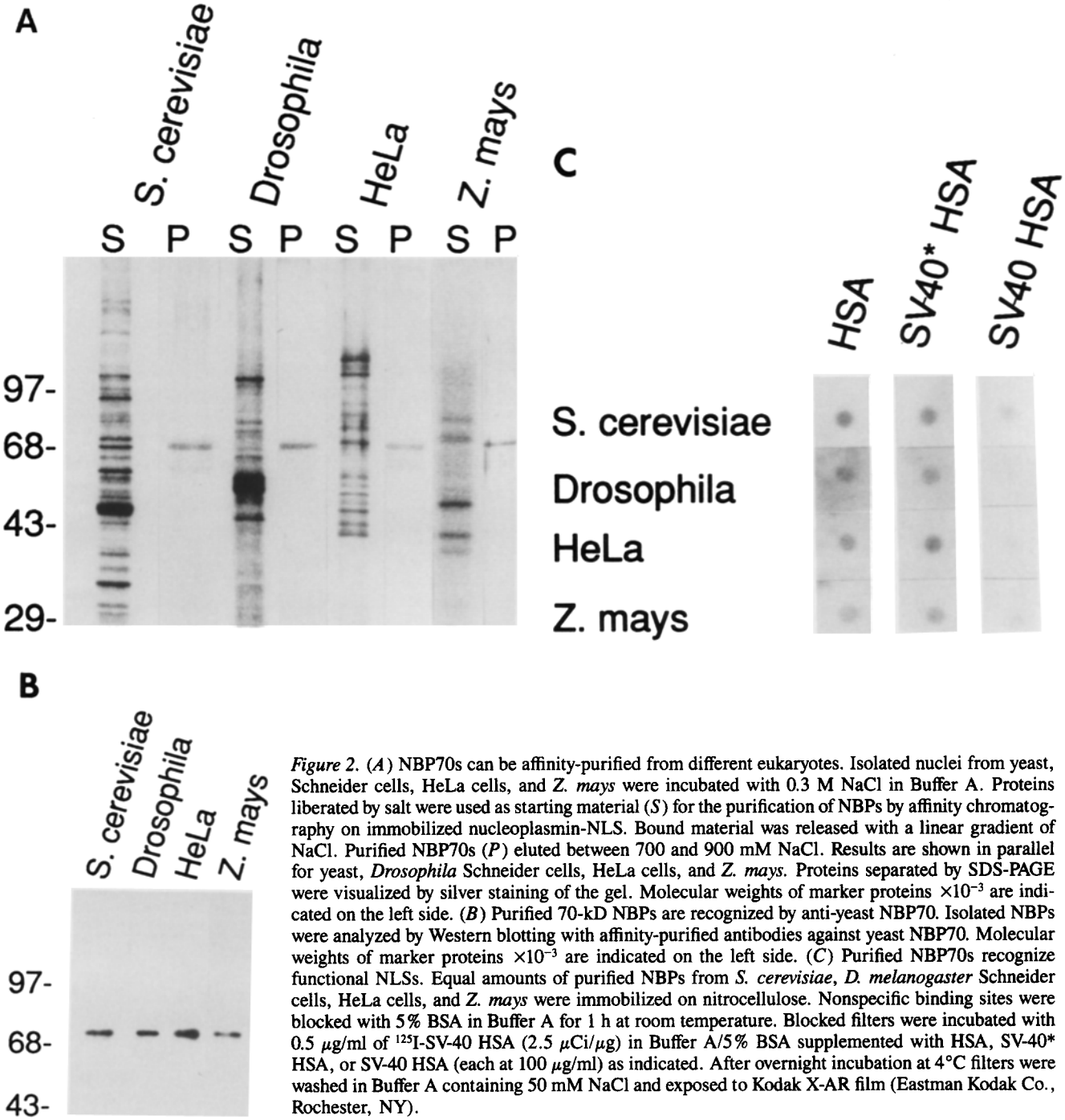

Figure 2. (*A*) NBP70s can be affinity-purified from different eukaryotes. Isolated nuclei from yeast, Schneider cells, HeLa cells, and *Z. mays* were incubated with 0.3 M NaCl in Buffer A. Proteins liberated by salt were used as starting material (*S*) for the purification of NBPs by affinity chromatography on immobilized nucleoplasmin-NLS. Bound material was released with a linear gradient of NaCl. Purified NBP70s (*P*) eluted between 700 and 900 mM NaCl. Results are shown in parallel for yeast, *Drosophila* Schneider cells, HeLa cells, and *Z. mays*. Proteins separated by SDS-PAGE were visualized by silver staining of the gel. Molecular weights of marker proteins $\times 10^{-3}$ are indicated on the left side. (*B*) Purified 70-kD NBPs are recognized by anti-yeast NBP70. Isolated NBPs were analyzed by Western blotting with affinity-purified antibodies against yeast NBP70. Molecular weights of marker proteins $\times 10^{-3}$ are indicated on the left side. (*C*) Purified NBP70s recognize functional NLSs. Equal amounts of purified NBPs from *S. cerevisiae*, *D. melanogaster* Schneider cells, HeLa cells, and *Z. mays* were immobilized on nitrocellulose. Nonspecific binding sites were blocked with 5% BSA in Buffer A for 1 h at room temperature. Blocked filters were incubated with 0.5 μg/ml of ^{125}I-SV-40 HSA (2.5 μCi/μg) in Buffer A/5% BSA supplemented with HSA, SV-40* HSA, or SV-40 HSA (each at 100 μg/ml) as indicated. After overnight incubation at 4°C filters were washed in Buffer A containing 50 mM NaCl and exposed to Kodak X-AR film (Eastman Kodak Co., Rochester, NY).

Digitonin-treated *Drosophila* Schneider cells faithfully imported fluorescently labeled SV-40 HSA into the nucleus (Fig. 6 *A*). On the other hand, import of mutant SV-40* HSA or HSA was not observed (Fig. 6 *A*). Import of SV-40 HSA into the nucleus required the addition of cytosol and ATP. However, in the absence of exogenously added cytosol and ATP, we still observed binding of SV-40 HSA to the nuclear periphery as well as in the cytoplasm (Fig. 6 *B*). Preincubation of permeabilized cells with apyrase did not abolish binding of the substrate (data not shown). This in vitro reaction has properties similar to those reported for nuclear import in permeabilized HeLa cells (Adam et al., 1990). It is temperature dependent (no import occurs at 4°C) and import is inhibited by pre-incubation with 100 μg/ml wheat germ agglutinin.

The association of SV-40 HSA with permeabilized cells was specific as neither HSA nor SV-40* HSA bound to digitonin-treated cells (Fig. 6 *B*). Some NBP70 is still present after treatment with digitonin since cytoplasmic as well as nuclear-associated NBP70 could be detected by immunofluorescence (Fig. 6 *C*). In sum, digitonin treatment of fly tissue culture cells released components essential for protein translocation into the nucleus. However, permeabilized cells still specifically bound a NLS-containing protein,

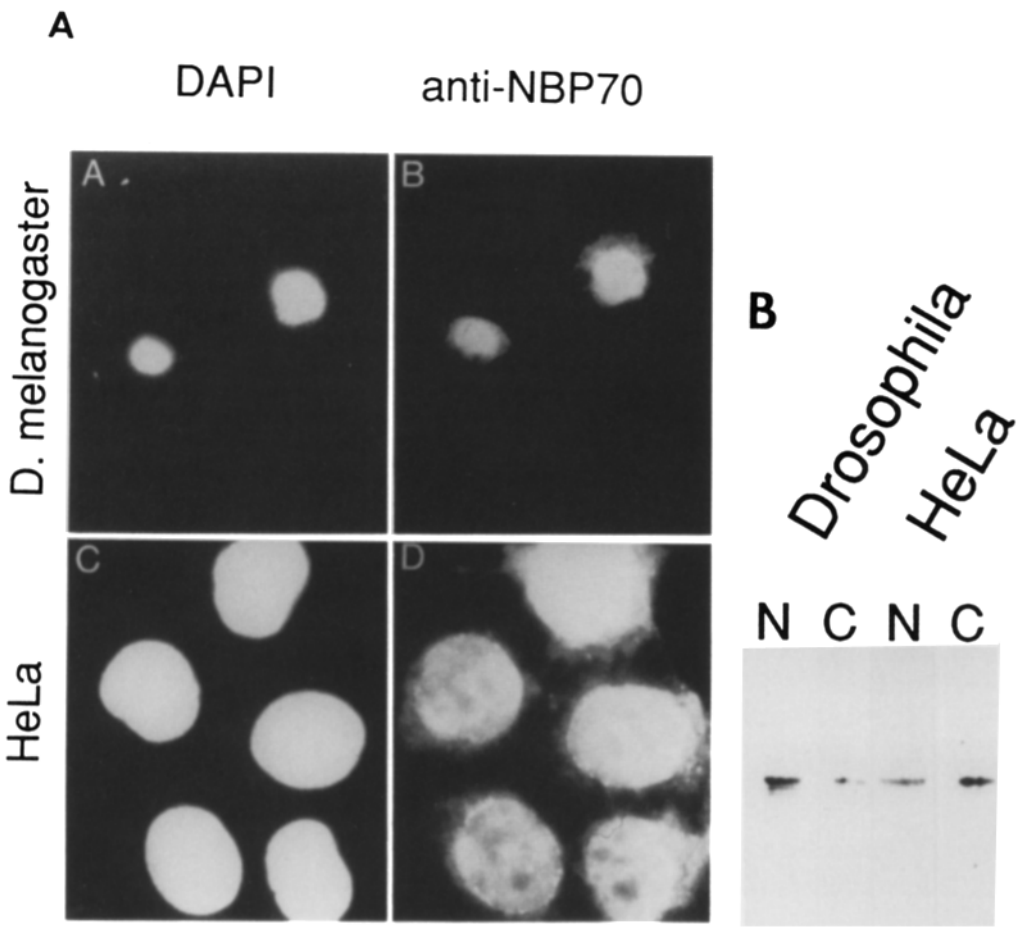

Figure 3. Localization of NBP70s in fly tissue culture and HeLa cells. (*A*) Immunolocalization of NBP70s. *Drosophila* tissue culture cells (panels *A* and *B*) and HeLa cells (panels *C* and *D*) were grown on multiwell slides and processed for immunofluorescence as described in Materials and Methods. Cells were incubated with affinity-purified anti-NBP70. Bound antibodies were visualized with FITC-conjugated secondary antibodies (panels *B* and *D*). Nuclei were located by DNA staining with DAPI (panels *A* and *C*). (*B*) Cell fractionation. *Drosophila* tissue culture cells and HeLa cells were fractionated into nuclei (*N*) and cytosol (*C*). Equal amounts (40 μg per lane) of protein were analyzed by Western blotting with affinity-purified antibodies against yeast NBP70.

thereby providing us with a system to selectively analyze the binding step of nuclear protein import.

Binding of Nuclear Proteins to Permeabilized Drosophila Cells Is Inhibited by Antibodies to NBP70

We further analyzed the role of the previously identified NBP70s in the import of proteins into detergent-permeabilized cells. Pre-incubation of *Drosophila* cells with antibodies against the yeast NBP70 reduced binding of SV-40 HSA, whereas incubation with pre-immune serum had no effect when compared with the untreated control cells (Fig. 7). Quantitative determination of the degree of inhibition by confocal densitometry revealed that pre-incubation with anti-NBP70 reduced binding of SV-40 HSA to <20% as compared with cells treated with pre-immune serum. Thus, antibodies that recognize a *Drosophila* NBP70 interfered with an early step in nuclear import in semipermeabilized fly tissue culture cells. In addition, when cytosol is combined with anti-NBP70 in a complete import reaction, import is abolished in 50–60% of the cells. Similarly, depletion of NBP70 from the cytosolic fraction reduced import to the same extent. Failure to completely inhibit the reaction may be because of the inability to add saturating amounts of antibody or because of the presence of other proteins with similar function. Addition of a 20-fold excess of purified NBP70 to permeabilized cells instead of cytosol did not support import, suggesting that other cytoplasmic factors are required. Taken together, these results indicate that NBP70s are involved in the recognition and binding of NLS-containing proteins in higher eukaryotes.

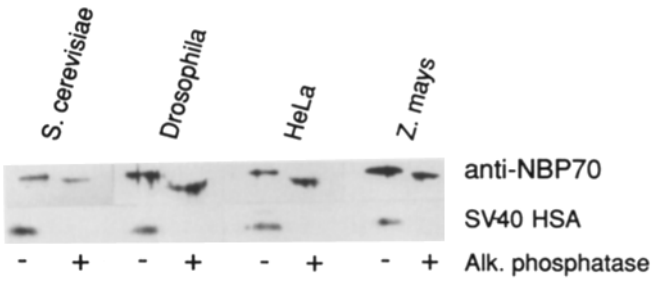

Figure 4. Dephosphorylation of NBP70s eliminates binding to NLSs. Proteins released from nuclei of *S. cerevisiae*, *Drosophila* Schneider cells, HeLa cells, and *Z. mays* by treatment with 0.3 M NaCl were incubated in the absence or presence of alkaline phosphatase for 30 min at 30°C as indicated. Proteins collected by precipitation with TCA were separated by SDS-PAGE and blotted onto nitrocellulose. Blocked filters were probed with affinity-purified antibodies against yeast NBP70 (top panel) or incubated with 1 μg ^{125}I-SV-40 HSA at 2.5 μCi/μg (bottom panel).

To address whether the observed cytoplasmic binding of SV-40 HSA to permeabilized cells is a physiologically "meaningful" reaction, we performed the following experiment. Detergent-permeabilized cells were incubated with SV-40 HSA, and unbound substrate was removed by rinsing cells with buffer. Washed cells were subsequently incubated

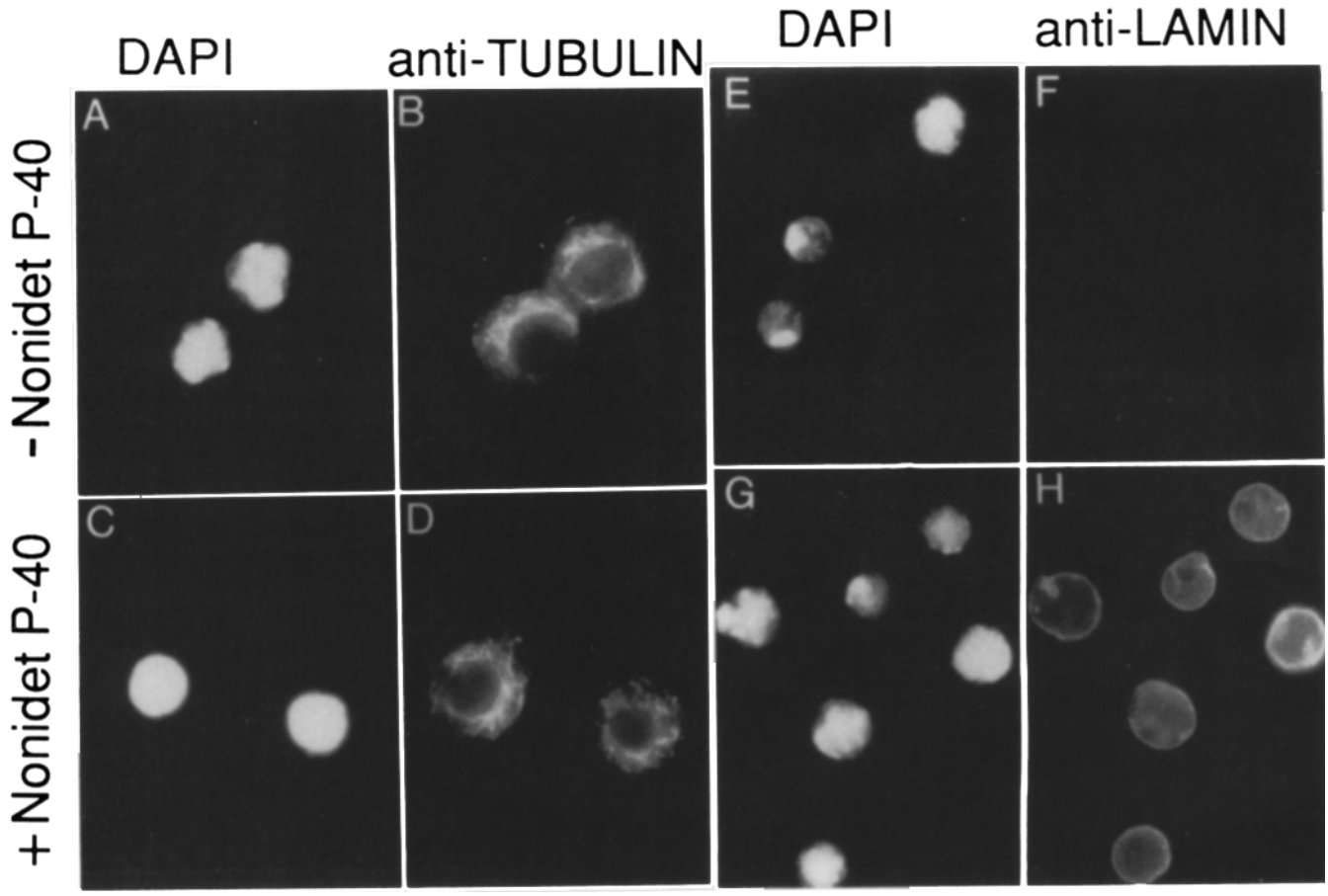

Figure 5. The nuclear envelope remains intact in detergent-permeabilized Schneider cells. *Drosophila* Schneider cells grown on multiwell slides were incubated for 5 min with 40 μg/ml digitonin in Buffer B, rinsed with Buffer B, and incubated in Buffer B for 30 min at 30°C. Cells were rinsed with Buffer B and fixed in Buffer B/4% formaldehyde (20 min, room temperature). Wells were washed with PBS and treated with PBS in the absence (panels *A*, *B*, *E*, and *F*) or presence (panels *C*, *D*, *G*, and *H*) of 0.2% NP-40 (6 min, room temperature). Cells were rinsed with PBS/1% BSA and incubated with antibodies directed against tubulin (panels *B* and *D*) or against lamins (panels *F* and *H*). After 30 min at room temperature wells were washed with PBS/1% BSA followed by incubation with FITC-conjugated secondary antibodies for 30 min at room temperature. Wells were rinsed with PBS/1% and treated with 1 μg/ml DAPI (2 min, room temperature), and cells were viewed with a fluorescence microscope at a magnification of 1,000. Immunofluorescence is shown in panels *B*, *D*, *F*, and *H* and DAPI staining of nuclei in panels *A*, *C*, *E*, and *G*.

in the presence of fresh cytosol and ATP and analyzed for import of substrate. Under these conditions, prebound substrate was, at least in part, imported into the nucleus (Fig. 8), thus indicating that binding of SV-40 HSA represents an intermediate in the import reaction.

Discussion

We have identified a yeast protein, termed NBP70, by its ability to bind to nuclear localization sequences (Silver et al., 1989; Stochaj et al., 1991). We now show that NBPs with similar properties are common to all eukaryotes analyzed including plants. These proteins are modified by phosphorylation and when dephosphorylated, they no longer recognize NLSs. In addition, our results indicate that NBP70 is essential for proper import of nuclear proteins in vitro. We show by antibody inhibition that NBP70 is required for the first step, i.e., specific binding of NLS-containing proteins to a cytoplasmically localized receptor.

Higher Eukaryotes Contain NBPs Similar to That of Yeast

The process of nuclear protein import thus far appears to be conserved among all eukaryotes. For example, NLSs from nuclear proteins of mammalian cells are properly recognized by the nuclear import apparatus of the yeast *S. cerevisiae* (Nelson and Silver, 1989). Components of the nuclear pore complex also show conservation between mammals and yeast (Davis and Fink, 1990; Carmo-Fonseca et al., 1991). We have previously identified a 70-kD yeast protein by its ability to specifically bind NLSs (Silver et al., 1989). In particular, we prepared antibodies against the purified protein (Stochaj et al., 1991). With these antibodies, we asked whether similar proteins existed in higher eukaryotes and, if so, did they play a role in nuclear protein import.

The first indication that the yeast NBP70 is a member of a conserved group of proteins is that antibodies against the yeast NBP70 recognized proteins of identical molecular weights in cells from *D. melanogaster*, human, and *Z. mays*.

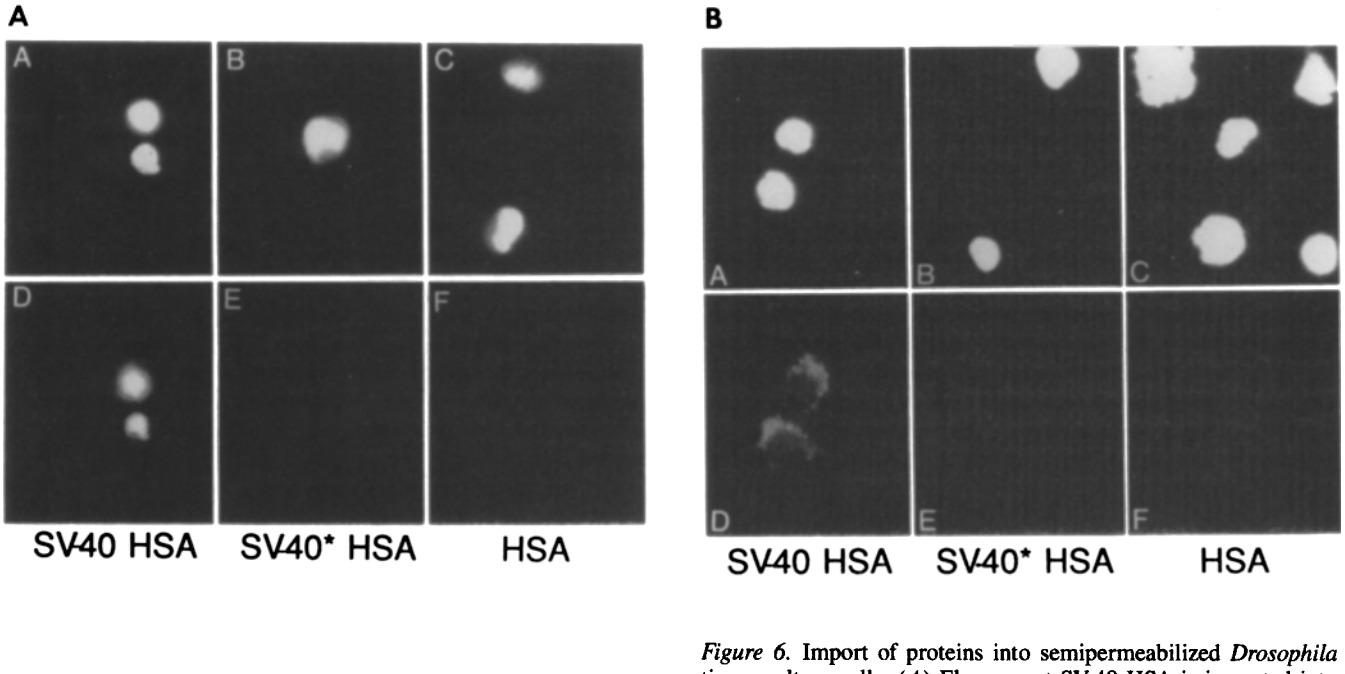

Figure 6. Import of proteins into semipermeabilized *Drosophila* tissue culture cells. (*A*) Fluorescent SV-40 HSA is imported into nuclei. Semipermeabilized fly cells were incubated with TRITC-labeled SV-40 HSA (panels *A* and *D*), SV-40* HSA (panels *B* and *E*), or HSA (panels *C* and *F*) in the presence of cytosol, ATP, and an ATP regenerating system. After 30 min at 30°C, cells were washed in Buffer B, fixed, and incubated with DAPI. The distribution of TRITC-labeled substrate is shown in panels *D*, *E*, and *F*. DNA was visualized with DAPI (panels *A*, *B*, and *C*). (*B*) Binding of SV-40 HSA in the absence of cytosol and ATP. Detergent-permeabilized *Drosophila* Schneider cells were incubated with TRITC SV-40 HSA (panels *A* and *D*), SV-40* HSA (panels *B* and *E*), or HSA (panels *C* and *F*) in Buffer B containing 1 mg/ml HSA but with omission of cytosol, ATP, and an ATP regenerating system. Conditions for incubation and fixation of cells were identical to those described for Fig. 6 *A*. Binding of fluorescent substrates is shown in panels *D*, *E*, and *F* and DAPI staining in panels *A*, *B*, and *C*. (*C*) NBP70 is present in detergent-permeabilized cells. Schneider cells were pretreated with Buffer B in the absence (panels *A* and *B*) or presence (panels *C* and *D*) of digitonin, rinsed with Buffer B and fixed in Buffer B/4% formaldehyde for 20 min at room temperature. Cells were washed with Buffer B, rinsed with PBS, and incubated with PBS containing 0.5% NP-40 (5 min at room temperature). Wells were rinsed with PBS and nonspecific binding sites were blocked in TPBS/BSA. Cells were further processed for immunofluorescence with affinity-purified antibodies against yeast NBP70 as described in Materials and Methods. The localization of NBP70 in shown in panels *B* and *D*, and DAPI staining in panels *A* and *C*.

These proteins in purified form could distinguish between the wild-type and mutant NLSs from SV-40 large T antigen. Immunolocalization revealed that NBP70s are located at the nucleus as well as present in the cytoplasm. This is consistent with the idea that NBP70s act as receptors for nuclear imported proteins and, as such, shuttle between the nucleus and cytosol. In sum, all eukaryotes tested contain proteins of similar molecular weight with conserved antigenic epitopes that specifically recognized functional NLSs.

NBPs have been identified in a number of eukaryotic cell types (Yoneda et al., 1988; Adam et al., 1989; Yamasaki et al., 1989; Silver et al., 1989; Li and Thomas, 1989; Lee and Melese, 1989; Meier and Blobel, 1990). Some of these are indeed of similar size to the proteins we have identified. It is possible that the proteins we have characterized are identical to ones previously described from rat fibroblasts, rat liver, and HeLa cells (Yoneda et al., 1989; Adam et al., 1989; Yamasaki et al., 1989). For yeast, Lee et al. (1991) identified a 68-kD nucleolar protein, termed Nsr1, that binds NLSs from histone H2B. Yeast cells missing Nsr1 are still viable and show no abnormalities in localization of nuclear proteins (M. Bossie, unpublished results). It remains to be shown whether or not Nsr1 indeed has a direct role in the import process. However, Nsr1 and the yeast NBP70 we describe are different proteins based on their biochemical behavior and intracellular distribution. Additionally, yeast

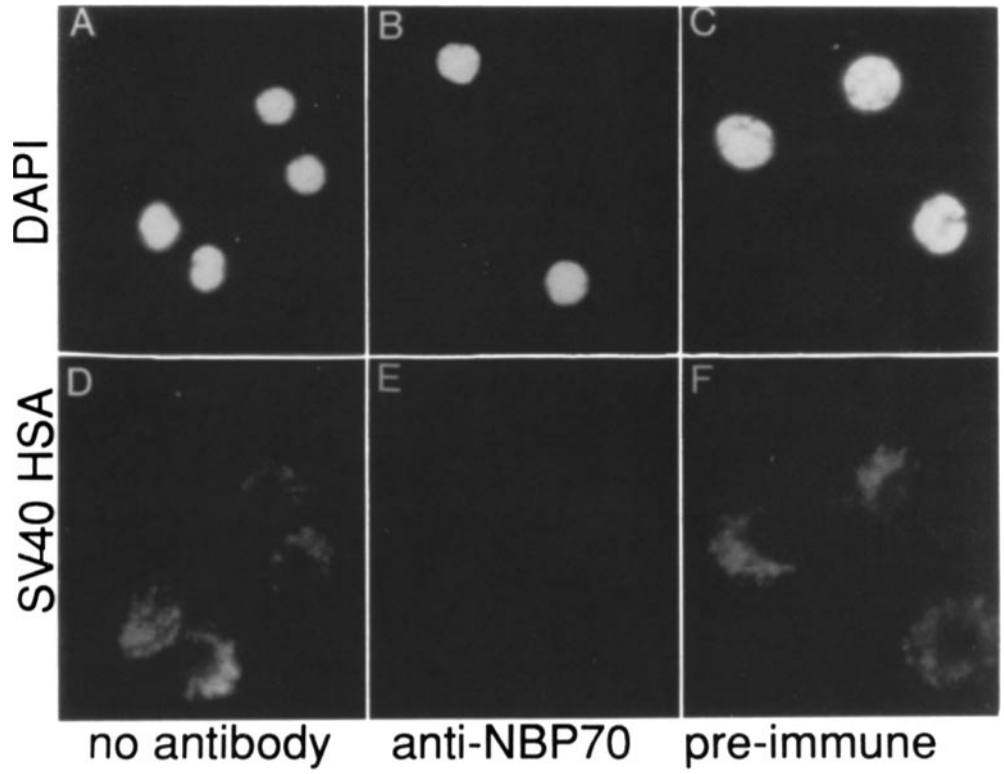

Figure 7. Inhibition of SV-40 HSA binding to detergent-permeabilized cells. *Drosophila* Schneider cells were permeabilized with digitonin followed by treatment with Buffer B/1 mg/ml HSA containing 250 μg/ml anti-NBP70 (panels *B* and *E*), or 250 μg/ml protein A-purified pre-immune serum (panels *C* and *F*), or in the absence of antibodies (panels *A* and *D*). After 30 min preincubation at room temperature, cells were washed in Buffer B followed by incubation with TRITC SV-40 HSA in Buffer B/1 mg/ml HSA (30 min 30°C). Wells were rinsed with Buffer B and cells were subsequently fixed and incubated with DAPI. Binding of TRITC SV-40 HSA is shown in panels *D*, *E*, and *F*, DAPI staining in panels *A*, *B*, and *C*.

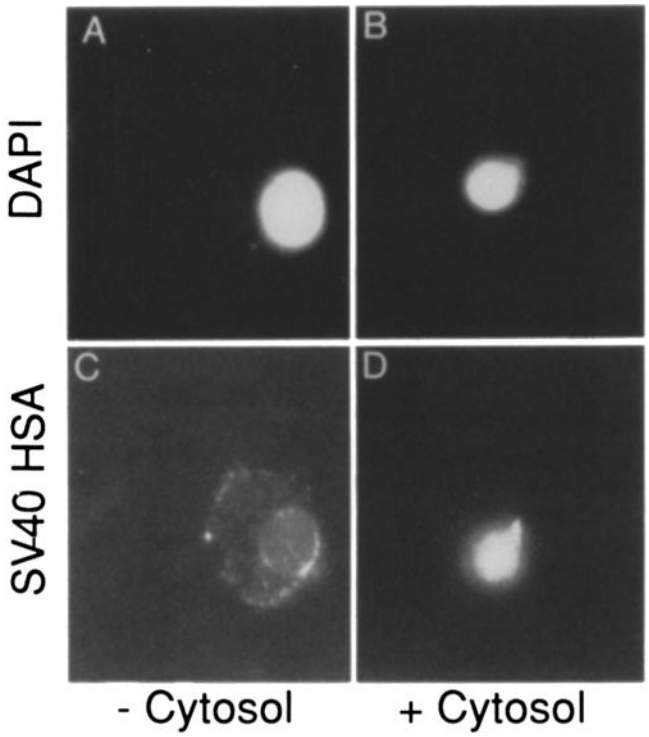

Figure 8. Bound SV-40 HSA is imported into nuclei in the presence of cytosol and ATP. Detergent-permeabilized Schneider cells were incubated with 100 μg/ml lissamine-rhodamine SV-40 HSA in Buffer B/1 mg/ml HSA (5 min at room temperature). Cells were washed with Buffer B and incubated with Buffer B, ATP, and an ATP regenerating system in the absence (panels *A* and *C*) or presence (panels *B* and *D*) of cytosol for 30 min at 30°C. After rinsing with Buffer B, cells were fixed and stained with DAPI. The distribution of lissamine-rhodamine SV-40 HSA is shown in panels *C* and *D*, DAPI staining in panels *A* and *B*.

cells deleted for *NSR1* still contain a 70-kD NBP that reacts with the antibodies raised against purified yeast NBP70 (our own unpublished results). It remains a formal possibility that there are different types of NBPs involved in the import process.

Adam et al. (1991) recently described 56- and 54-kD NBPs from bovine erythrocytes which can stimulate nuclear import in normal rat kidney cells. We do not know whether these proteins are related to the NBP70s described here, i.e., they could be proteolytic degradation products. Alternatively, they may belong to a different group of NLS-receptors which have been stably retained within anucleated cells. Adam et al. (1989) originally indicated that NBPs of 70 and 60 kD fractionated with both the nuclei and post-mitochondrial supernatants of rat liver cells. These data are consistent with our observations that NBP70s are present in nuclear as well as high spin supernatants of fly and HeLa cells. Moreover, we are able to localize NBP70s by immunofluorescence to both the nucleus and the cytoplasm. The staining pattern obtained for fly and HeLa cells might indicate that some of the NBP70 is translocated into the nucleus.

Phosphorylation of NBPs

A common way to regulate enzyme activity, protein interactions, and receptor function is reversible modifications such as phosphorylation. We now demonstrate that dephosphorylation of NBP70s from several different sources prevented their interaction with NLSs. We do not know whether this reflects a direct modification of the NLS binding site or a change in protein conformation, which subsequently alters its activity.

Since NBP70s can become phosphorylated in vivo, phosphorylation might be a way to regulate the number of functional receptors in the cell. Furthermore, dephosphorylation

may play a role in the release of nuclear proteins bound to NBP70s during the process of import.

NBP70 Is Involved in Nuclear Protein Import

To study the role of NBP70s in nuclear protein import, we developed an in vitro reaction using detergent-permeabilized fly tissue culture cells. We chose tissue culture cells derived from *D. melanogaster* because there is the possibility of applying genetics to this system. Moreover, several interesting examples of regulated nuclear import during early embryo development have emerged recently (Steward, 1989; Roth et al., 1989) and this system should provide a ready means for studying some of these reactions in vitro.

Detergent-permeabilized fly tissue culture cells faithfully reproduced the steps of nuclear protein import described so far. After treatment with the detergent digitonin, the plasma membrane was permeabilized such that macromolecules could enter the cells. However, the nuclear envelope remained intact unless further permeabilization was carried out. In the presence of cytosol and ATP, fluorescently tagged SV-40 HSA was efficiently taken up into the nuclei. When the NLS was mutated to an inactive form, no association with the nuclei of the semipermeabilized cells was observed.

In the absence of ATP and cytosol, we did observe binding of the SV-40 HSA to the nuclear envelope as well as in the cytoplasm. This binding was ATP independent, required functional NLSs, and was most likely a true intermediate in the reaction since bound substrate could be subsequently imported into the nucleus upon readdition of cytosol and ATP. These results agree with those previously reported by Newmeyer and Forbes (1990), who could observe protein binding to the envelopes of reconstituted nuclei in vitro. On the other hand, our observation that binding of NLS-containing proteins can be studied in detergent-permeabilized cells contradicts experiments described for similarly permeabilized HeLa cells (Adam et al., 1990). For the HeLa cell system, binding of nuclear proteins could not be demonstrated although permeabilized cells accumulated substrate within the nucleus if provided with cytosol and ATP. The failure to detect nuclear protein binding may be explained by the different substrates used. Substrates used in our assays contain 2–5 times more NLSs than those of Adam et al. (1990). Increasing the number of functional NLSs is known to improve the efficiency of nuclear protein import and may thus stabilize the interaction with NLS receptors (Lanford et al., 1986; Dworetzky et al., 1988). Binding of nuclear proteins in HeLa cells may have escaped detection because of a more labile interaction with NLS receptors. Taken together, our results demonstrate that permeabilized cells specifically bound nuclear imported proteins in the absence of exogenously added factors. This enabled us to study selectively the interaction of proteins with NLS receptors. Substrate prebound to permeabilized cells could be imported into nuclei if cells were provided with cytosol and ATP. Thus, the binding reaction studied by us most likely represents an intermediate in the nuclear import reaction.

Since the experiments presented here avoid the reconstitution of nuclear import with components from heterologous sources, we were able to analyze the role of NBPs authentic to the cells being studied. We have demonstrated that binding of nuclear proteins to permeabilized fly tissue culture cells is, at least in part, mediated by NBP70s. Our experiments do not address the question of whether additional NBPs present in the cytosol may participate in nuclear protein import. Such proteins may have been removed by detergent treatment and, if re-added, might improve the efficiency of the reaction. However, our results do demonstrate that NBP70 is sufficient to allow binding of nuclear proteins that contain NLSs such as the sequence derived from SV-40 large T antigen. Since addition of purified NBP70 will not substitute for cytosol in the import reaction, other cytosolic factors are undoubtedly required for in vitro nuclear import to occur.

The following model summarizes the data presented here. NBP70s present in the cytoplasm specifically recognize NLSs and, as such, act as receptors for nuclear protein import. NLS binding requires phosphorylation of the receptor. We then speculate that the complex of receptor and imported protein binds to the nuclear envelope at the pore complex. Proteins are translocated into the nucleus either complexed to their receptors or after dissociation. The receptor would then recycle and participate in another round of import. During this process, dephosphorylation/rephosphorylation might be involved in discharging and reactivation of the NLS receptors.

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