A Nuclear Localization Signal Binding Protein in the Nucleolus

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Abstract. We used functional wild-type and mutant synthetic nuclear localization signal peptides of SV-40 T antigen cross-linked to human serum albumin (peptide conjugates) to assay their binding to proteins of rat liver nuclei on Western blots. Proteins of 140 and 55 kD (p140 and p55) were exclusively recognized by wild-type peptide conjugates. Free wild-type peptides competed for the wild-type peptide conjugate binding to p140 and p55 whereas free mutant peptides, which differed by a single amino acid from the wild type, competed less efficiently. The two proteins were extractable from nuclei by either low or high ionic strength buffers. We purified p140 and raised polyclonal antibodies in chicken against the protein excised from polyacrylamide gels. The anti-p140 antibodies were monospecific as judged by their reactivity with a single nuclear protein band of 140 kD on Western blots of subcellular fractions of whole cells. Indirect immunofluorescence microscopy on fixed and permeabilized Buffalo rat liver (BRL) cells with anti-p140 antibodies exhibited a distinct punctate nucleolar staining. Rhodamine-labeled wild-type peptide conjugates also bound to nucleoli in a similar pattern on fixed and permeabilized BRL cells. Based on biochemical characterization, p140 is a novel nucleolar protein. It is possible that p140 shuttles between the nucleolus and the cytoplasm and functions as a nuclear import carrier.

PROTEIN import into the nucleus has been studied both in vitro and in vivo. Transport occurs through pore complexes in the nuclear envelope (Feldherr et al., 1984) and can be separated into two steps, binding to the pore complex and ATP-dependent translocation (Newmeyer and Forbes, 1988; Richardson et al., 1988). Import is an active process that does not require intranuclear binding and is limited to nuclei with intact nuclear envelopes (Paucha et al., 1985; Newmeyer et al., 1986; Zimmer et al., 1988). Wheat germ agglutinin (Finlay et al., 1987; Yoneda et al. 1987), which binds to O-linked *N*-acetylglucosamine residues of nuclear pore complex proteins, and monoclonal antibodies directed against these proteins (Featherstone et al., 1988; in vitro: U. T. Meier, unpublished results), inhibit nuclear protein import. Abolishment of transport into nuclei reconstituted with extracts that were depleted of these *N*-acetylglucosamine-containing proteins further underlines their involvement in protein import (Finlay and Forbes, 1990). In addition, a requirement for cytosolic factors in nuclear protein import has been implied by studies performed with the cysteine modifying reagent *N*-ethylmaleimide (Newmeyer and Forbes, 1990).

Nuclear proteins contain specific nuclear localization signals (NLS)[1] that target them to the nucleus (for review see Dingwall and Laskey, 1986). NLS are essential for transport (Lanford and Butel, 1984; Kalderon et al., 1984*a*, *b*) and may function in conjunction with modulatory sequences (Rihs

and Peters, 1989). Generally, NLS are short stretches of predominantly basic amino acids that are often flanked by proline and acidic amino acid residues. NLS of most identified nuclear proteins from different sources share a consensus sequence of four amino acids (Lys-Arg/Lys-X-Arg/Lys; Chelsky et al., 1989). The first and best characterized NLS is the one of SV-40 T antigen (Pro-Lys-Lys128-Lys-Arg-Lys-Val; Lanford and Butel, 1984; Kalderon et al., 1984*a*, *b*). This sequence is sufficient to target nonnuclear proteins to the nucleus when linked to them either by gene fusion (Kalderon et al., 1984*b*) or chemically (Lanford et al., 1986; Goldfarb et al., 1986). Furthermore, increasing numbers of NLS per imported protein enhance accumulation rates (Lanford et al., 1986; Roberts et al., 1987; Dworetzky et al., 1988). The single amino acid change of Lys128 to Thr or Asn in the NLS of SV-40 T antigen almost completely abolishes its nuclear targeting ability. Therefore, nuclear import is a selective process dependent on the NLS of nuclear proteins.

To target proteins to the nucleus, the NLS must interact with a putative NLS receptor on the cytoplasmic side of the nuclear envelope. While this study was in progress, several proteins were identified that specifically recognize the synthetic wild-type NLS peptides of SV-40 T antigen. Four groups reported the identification of NLS binding proteins in mammalian cells. Chemical cross-linking revealed two proteins of 60 and 70 kD that are mainly cytoplasmic but are also associated with the nuclear envelope and found within the nucleus (Adam et al., 1989). Yamasaki et al. (1989) identified two cytoplasmic proteins of 100 and 70 kD and two nuclear proteins of 140 and 55 kD by photoaffinity cross-

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1. *Abbreviations used in this paper:* BRL, Buffalo rat liver; HSA, human serum albumin; NLS, nuclear localization signal.

linking. By a similar method, Li and Thomas (1989) demonstrated the interaction of NLS with a 66-kD nuclear protein, and Benditt et al. (1989) described four NLS binding proteins in detergent extracts of nuclear envelopes. In yeast, Silver et al. (1989) identified two proteins of 70 and 59 kD that on Western blots bind synthetic NLS peptides coupled to human serum albumin (HSA). Lee and Mélèse (1989) described a 67-kD protein in yeast that may be identical to the 70-kD protein. All these proteins have so far been identified and characterized solely on the basis of their ability to interact with synthetic NLS peptides.

We report the immunochemical characterization of one of two NLS binding proteins in rat liver nuclei. Proteins of 140 and 55 kD were identified that bind synthetic NLS peptides coupled to HSA on Western blots. These proteins were extractable from isolated nuclei by either low or high ionic strength buffers. The 140-kD protein was purified and used to raise monospecific antibodies. Immuno- and biochemical characterization shows that p140 is a novel nucleolar protein. We discuss its putative function in nuclear protein import.

Materials and Methods

Peptide Conjugates

Peptides were synthesized at the Rockefeller University biopolymer facility on a model 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). We included two glycines as a spacer, a tyrosine for iodination, and a cysteine for cross-linking purposes at the carboxy terminus of the authentic SV-40 T antigen NLS sequence (Fig. 1 A, italicized residues). Synthetic NLS peptides were coupled to HSA (Calbiochem-Behring Corp., La Jolla, CA) by the heterobifunctional cross-linker m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) as described (Green et al., 1982). To obtain rhodamine-labeled conjugates, HSA was labeled with TRITC (Sigma Chemical Co., St Louis, MO; Newmeyer et al., 1986) before cross-linking the peptides. Approximately 10 peptides were coupled per HSA as judged by mobility shift on SDS-PAGE.

Nuclear Import Assays

In Vitro. We used the assay system developed by Newmeyer et al. (1986). Briefly, 5 μl of rat liver nuclei (∼5 × 10^6; Blobel and Potter, 1966; Newport and Spann, 1987) were added to 100 μl unfractionated *Xenopus* egg extract (Newport, 1987; Newmeyer and Forbes, 1990) containing an ATP regenerating system (2 mM ATP, 10 mM creatine phosphate, and 100 U/ml creatine kinase; all Boehringer-Mannheim Diagnostics, Inc., Indianapolis, IN). After a 30-min preincubation at room temperature, 0.5 μl of rhodamine-labeled peptide conjugate (∼1.4 mg/ml) was added to 9 μl of "healed" nuclei in egg extract. The amount of added conjugate was varied for different preparation depending on the efficiency of rhodamine labeling and peptide substitution of HSA. Import was allowed to proceed for 30 min at room temperature, and accumulation of the rhodamine-labeled conjugates was observed by fluorescence microscopy as described (Newmeyer et al., 1986). Routinely, one-third of the nuclei were intact and import competent when nuclei and egg extracts were used that had been frozen in liquid nitrogen and stored at −70°C. The assay exhibited all the characteristics of authentic nuclear protein import as described by Newmeyer et al. (1986), Finlay et al. (1987), and Newmeyer and Forbes (1988).

In Vivo. Nuclear import in living cells was observed after microinjection using a microinjector (Eppendorf, model 5242; Hamburg, Federal Republic of Germany) and a Zeiss Axiovert 10 inverted microscope. Rhodamine-labeled peptide conjugates (∼1.2 mg/ml) were injected into the cytoplasm of Buffalo rat liver (BRL) cells grown on coverslips in DME supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY). The intactness of nuclei was assayed by the exclusion of fluorescein-labeled 148-kD dextrans (0.1 mg/ml; Sigma Chemical Co.) coinjected into the cytoplasm. Transport was observed by fluorescence microscopy, either directly or after formaldehyde fixation of the cells.

Peptide Conjugate Binding

We used a method similar to the one used to identify NLS binding proteins in yeast (Silver et al., 1989). Nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) containing transferred proteins were blocked with 0.5% Tween-20 in PBS and peptide conjugates (10 μg/ml) were incubated overnight in the same buffer. The bound peptide conjugates were detected with rabbit anti-HSA IgG (20 μg/ml; Sigma Chemical Co.) and ^{125}I-protein A (DuPont-New England Nuclear, Wilmington, DE) and visualized by autoradiography. In competition experiments, the indicated amounts of free peptides were included during peptide conjugate incubation.

Purification of p140 from Rat Liver

Rat liver nuclei were prepared as described (Blobel and Potter, 1966) with the following modifications. Solutions were buffered with 10 mM triethanolamine-HCl (pH 7.5) and contained 1 mM DTT, 2 mM PMSF, 2 μg/ml aprotinin, and 1 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A (all from Sigma Chemical Co.). Rat liver homogenate was centrifuged at 10,000 g for 10 min, the supernatant discarded, and the nuclear pellet resuspended in 2 vol of 2.3 M sucrose buffer. Thereafter, we proceeded as described (Blobel and Potter, 1966). The nuclei were either pelleted and frozen (Davis and Blobel, 1986) or extracted as follows.

Nuclei, equivalent to 1,600 OD (1 OD at 260 nm corresponds to 3 × 10^6 nuclei; Aaronson and Blobel, 1974), were resuspended at 20 OD/ml in 25 mM Tris-HCl (pH 8.1) containing 1 mM DTT and all of the above protease inhibitors. After incubation for 1 h on ice, nuclei were centrifuged for 15 min at 20,000 g, the supernatant withdrawn, and the pellets loosened by vigorous vortexing. Subsequently, a second and third low ionic strength extraction was performed identically. The supernatant of the second extraction containing at least half of the p140 was loaded onto a hydroxylapatite (BioGel HTP; BioRad Laboratories, Rockville Centre, NY) column (1.3 × 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 8.1) at 4°C. The column was washed with one column volume of equilibration buffer and subsequently with 500 mM potassium phosphate buffer until the absorption, monitored at 280 nm, declined to baseline. Neither the flow-through nor the 500 mM phosphate wash fractions contained p140 as judged by Western blotting and binding of peptide conjugates or, later on, immunoreaction with anti-p140 antibodies. p140, highly enriched, was eluted with a 1 M potassium phosphate buffer. Some additional p140 was recovered by analogous chromatography of twice the amount of supernatant from the third low ionic strength extraction.

Antibodies to p140

The p140-enriched fraction was subjected to preparative SDS-PAGE and p140 excised from dried gels. As estimated by comparison of known amounts of Coomassie blue–stained molecular weight markers (Sigma Chemical Co.) to p140 on SDS-PAGE, we injected 100–200 μg of protein per animal and injection. Initial immunizations were performed with complete and subsequent injections with incomplete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI). Different injection schedules, route of immunization, and repeated boosting yielded no immune response in three rabbits. Intradermal and subcutaneous immunization of chickens over a period of 5 wk, however, resulted in high titer antisera. These sera were used on Western blots at a 1:10,000 dilution and at a 1:500 dilution for indirect immunofluorescence microscopy. We purified IgG from chicken sera as described (Benedict, 1967). The purified IgG gave qualitatively the same results as the sera, but they appeared to have lost some reactivity: Therefore, all of the presented experiments were performed with sera.

Immunochemical Methods

Western Blots. Proteins were separated on 9 or 10% SDS–polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose filters according to Towbin et al. (1979). Polyacrylamide gels were stained with 0.2% Coomassie blue R 250 and Western blots with 0.1% amido black. Nonfat dry milk (1% in PBS) was used to block free binding sites on nitrocellulose sheets. Chicken sera were incubated for 2 h at room temperature in blocking buffer and detected with rabbit antibodies against chicken IgG (1:1,000, Cappel Laboratories, through Organon Teknika, Malvern, PA) and ^{125}I-protein A (1:1,000, DuPont-New England Nuclear). Nitrocellulose filters were air dried and Kodak XAR films exposed in cassettes with intensifying screens.

Indirect Immunofluorescence Microscopy. This was performed on BRL cells that were grown on coverslips, fixed with 2% paraformaldehyde in PBS for 20 min, and permeabilized with 1% Triton X-100 in PBS for 5 min at room temperature. Incubations with antisera were performed in 10 mM phosphate buffer (pH 7.2) containing 500 mM NaCl and 1% nonfat dry milk for 2 h and with secondary fluorescein-labeled rabbit anti-chicken IgG (Cappel Laboratories) at a dilution of 1:500 for at least 15 min at room temperature with three 5-min washes between each step. In double-immuno-fluorescence experiments, the two primary antibodies were incubated concomitantly as was the pair of secondary antibodies. The coverslips were mounted in a p-phenylenediamine solution (1 mg/ml) in 90% glycerol (pH 8.0; Johnson and Noguiera Araujo, 1981) and viewed under either an Axiophot (Zeiss) or, where indicated, a confocal laser scanning microscope MRC-600 (BioRad Laboratories). T-MAX 400 or T-Max p3200 Kodak films at 1,600 or 6,400 ASA, respectively, were used to take pictures at fixed exposure times. Monoclonal anti-yeast fibrillarin antibodies were used as described (Aris and Blobel, 1988).

Peptide Conjugate Binding to Permeabilized BRL Cells

Cells were fixed and permeabilized as for indirect immunofluorescence microscopy and incubated with rhodamine-labeled peptide conjugates (0.25 mg/ml) in PBS containing 1 mM $MgCl_2$ and 2% BSA for 2 h at room temperature. After one wash with PBS the cells had to be fixed again with 18% formaldehyde in PBS to prevent diffusion of bound conjugates. The conjugate binding appeared to be less tight than that on Western blots where the conjugates remained bound throughout secondary antibody and ^{125}I-protein A incubations. In the case of double-fluorescent labeling, anti-p140 antibodies and fluorescein-labeled secondary antibodies were incubated before the peptide conjugates. Slides were viewed and pictures taken as stated for indirect immunofluorescence microscopy.

Subcellular Fractionation

BRL cells were grown to confluency in 100-cm² petri dishes. The cells from five dishes were washed three times with ice-cold PBS and then scraped into 10 ml PBS. All subsequent manipulations were performed on ice. After pelleting the cells at 1,000 g for 3 min, they were resuspended in 5 ml of homogenization buffer (10 mM triethanolamine [pH 7.4], 0.25 M sucrose, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF, 2 μg/ml aprotinin, and 1 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A) and homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with a tight fitting pestle. The homogenate was centrifuged at 1,000 g for 10 min and the resulting supernatant at 27,000 g for 15 min. The 27,000 g supernatant was then centrifuged at 100,000 g for 1 h to yield a postmicrosomal supernatant. The pellets of these differential centrifugation steps were called nuclear (1,000 g), mitochondrial (27,000 g), and microsomal (100,000 g) fraction. For further fractionation of nuclei, the 1,000 g pellet was resuspended in 5 ml homogenization buffer containing 1 M instead of 150 mM NaCl and incubated for 15 min on ice. Centrifugation at 20,000 g for 10 min resulted in supernatant and pellet of the nuclei after 1 M salt extraction. All pellets were resuspended in homogenization buffer and the protein content determined by BCA assay (Pierce Chemical Co.). Equal amounts of protein (75 μg) of each fraction were TCA (10%) precipitated, subjected to SDS-PAGE (10%), and transferred to nitrocellulose filters. The protein content of the nuclear 1 M salt extract fraction may have been underestimated due to the presence of the bulk of nuclear DNA (Fig. 5 A, compare lane 1 to the other lanes).

Results

Peptide Conjugates

To look for proteins that interact with the NLS of SV-40 T antigen, we used synthetic NLS peptides chemically cross-linked to HSA. The peptides correspond to the wild-type and mutant NLS peptides used by Goldfarb et al. (1986) except that a proline was substituted by two glycines to avoid a labile Asp-Pro bond. The peptide conjugates are depicted in Fig. 1 A. The italicized amino acid residues are not part of the SV-40 T antigen sequence.

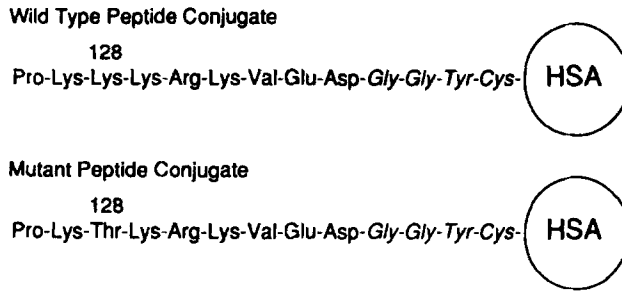HSA was labeled with rhodamine before coupling of the

NLS peptides in order to monitor the functionality of our peptide conjugates in vitro and in vivo. In an in vitro nuclear import assay (Newmeyer et al., 1986), the conjugates were added to isolated rat liver nuclei that had been allowed to "heal" in *Xenopus* egg extract supplemented with an ATP regenerating system. Wild-type conjugates accumulated within intact nuclei (Fig. 1 B, panel 1) while mutant conjugates remained completely excluded (Fig. 1 B, panel 4). Note, that this was not true for broken nuclei (Fig. 1 B, double arrowheads) while blebs (Fig. 1 B, single arrowheads) generated during preincubation of nuclei in egg extract behaved like intact nuclei. Blebs are formed from egg extract material at locations of the nuclear envelope that ruptured during isolation of the nuclei (Newmeyer et al., 1986). Our wild-type peptide conjugates also accumulated in the nucleus under in vivo conditions. Upon microinjection into the cytoplasm of BRL cells, the wild-type conjugate concentrated in the nucleus within few minutes while the mutant conjugate remained excluded (data not shown). Although the wild-type peptide conjugate accumulated in the nucleoplasm, it was not visible in nucleoli (data not shown). Taken together, these data demonstrate that our wild-type peptide conjugate is a functional probe for a putative import receptor and the mutant conjugate a closely related negative control.

Identification of p140 and p55

Because an early and ATP independent step of nuclear import involves binding to the nuclear pore complexes (Newmeyer and Forbes, 1988; Richardson et al., 1988), we tested isolated rat liver nuclei for the presence of a putative NLS receptor. Nuclear proteins were resolved by SDS-PAGE and transferred to a nitrocellulose filter (Fig. 2, A and B). The Western blot was then cut in half and incubated in parallel with wild-type (Fig. 2 A, lane 2) and mutant (Fig. 2 A, lane 3) peptide conjugates. The conjugates were detected by antibodies against HSA followed by incubation with ^{125}I-protein A and autoradiography. Both conjugates bound nonspecifically to many common proteins, the number of which varied from blot to blot depending on protein transfers and peptide conjugate preparations (compare Fig. 2, A and B and Fig. 3 C). However, two bands of 140 and 55 kD were exclusively recognized by the wild-type conjugate (Fig. 2 A, lane 2; Fig. 2 B, lane 2) whereas the mutant conjugate did not interact with them (Fig. 2 A, lane 3; Fig. 2 B, lane 1). These proteins were called p140 and p55. They were exclusively found in the nucleus when subcellular fractions were tested (data not shown, but see Figs. 5 and 7).

To further investigate the specificity of this interaction, we competed the wild-type conjugate binding to p140 and p55 with free NLS peptides. Free wild-type peptides competed the wild-type conjugate binding in a concentration dependent manner (Fig. 2 B, lanes 3–5). At an equivalent high concentration, free mutant peptides also competed but less efficiently than the free wild-type peptides (Fig. 2 B, lanes 6 and 5). This is consistent with the observation that mutant NLS peptides are able also to target albumin to the nucleus when they are coupled at high ratios (Lanford et al., 1988). Binding to proteins that were recognized by both wild-type and mutant peptide conjugates was competed for equally well by both free peptides (Fig. 2 B, lanes 5 and 6, bands below p55).
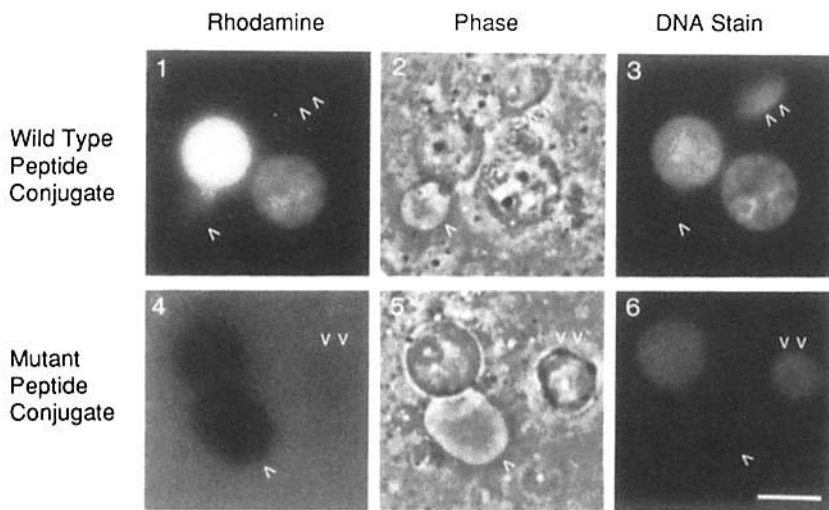
A

Wild Type Peptide Conjugate

128
Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-*Gly-Gly-Tyr-Cys*-(HSA)

Mutant Peptide Conjugate

128
Pro-Lys-Thr-Lys-Arg-Lys-Val-Glu-Asp-*Gly-Gly-Tyr-Cys*-(HSA)

B

Rhodamine	Phase	DNA Stain

Wild Type Peptide Conjugate

Mutant Peptide Conjugate

Figure 1. Peptide conjugates are functional in nuclear import. (*A*) Peptide conjugates. Peptides corresponding to the wild-type and mutant NLS of SV-40 T antigen were synthesized and coupled to HSA. Italicized amino acid residues are not part of the T antigen sequence (see Materials and Methods). (*B*) In vitro nuclear accumulation of rhodamine labeled wild-type peptide conjugates (panel *1*) and exclusion of mutant conjugates (panel *4*). Phase-contrast pictures (panels *2* and *5*) and Hoechst DNA stain (panels *3* and *6*) are shown of the respective nuclei (panels *1* and *4*). Single arrowheads point to blebs formed from *Xenopus* egg extract during preincubation to seal the nuclei; double arrowheads point to broken nuclei (see text). Bar, 10 μm.
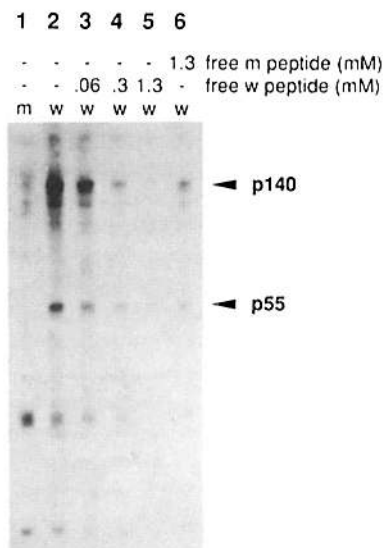
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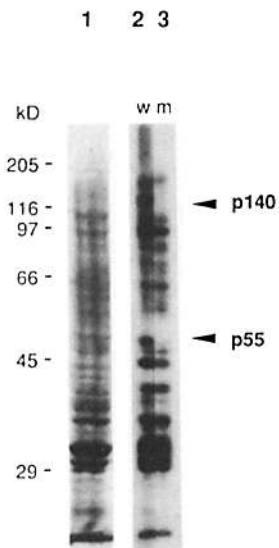
B

Figure 2. Peptide conjugate binding on Western blots of rat liver nuclei. (*A*) Identification of p140 and p55. Lane *1*, amido black stain of rat liver nuclei proteins transferred to nitrocellulose after separation by SDS-PAGE; lanes *2* and *3*, the same strip of nitrocellulose filter as in lane *1* cut in half and incubated in parallel with wild-type (lane *2*) and mutant (lane *3*) peptide conjugate that was visualized by autoradiography after incubation with anti-HSA IgG and ^{125}I-protein A. Relative molecular mass of marker proteins is indicated on the left of lane *1*. (*B*) Autoradiograms of NLS peptide conjugate binding in the presence of free peptides on Western blots of whole rat liver nuclei. Lanes *1* and *2*, controls, mutant (lane *1*) and wild-type (lane *2*) conjugate incubated in the absence of free peptides. Lanes *3–6*, wild-type conjugate incubated in the presence of the indicated amounts of free wild-type (lanes *3–5*) and mutant (lane *6*) peptides. w and m, wild type and mutant, respectively.

A concentration of 1.3 mM free wild-type peptide was required to abolish binding. This concentration is in the same order of magnitude as the free peptide concentration required for ~50% inhibition of nuclear transport of iodinated peptide conjugates in *Xenopus* oocytes (Goldfarb et al., 1986). A millimolar concentration may be needed because of the presence of multiple binding sites for NLS to which the multivalent peptide conjugates could bind stronger than single free peptides. This interpretation is supported by the fact that increasing numbers of NLS per imported protein enhance the rate of nucleocytoplasmic transport of the respective proteins (Lanford et al., 1986; Roberts et al., 1987; Dworetzky et al., 1988).

Purification of p140

We purified p140 in order to raise antibodies and to define the role of this protein in nucleocytoplasmic transport. p140 was given priority because p55 might have been a proteolytic fragment of that protein. Consecutive extractions of rat liver nuclei with DNAse, detergent, and salt (Dwyer and Blobel, 1976; Davis and Blobel, 1986) showed that most of p140 is extracted into the supernatant after the first DNAse incubation step (data not shown). To facilitate column chromatography, we extracted the nuclei with low ionic strength buffer at pH 8.1 in the absence of DNAse (Fig. 3 *A*). This method extracts primarily nuclear proteins but not DNA (Kay et al., 1972) which would interfere with chromatography. Surprisingly, at least half of p140 was found in the supernatant of the second of two identical and consecutive extractions (Fig. 3 *C*, lane *4*). This supernatant was chromatographed on hydroxylapatite. After extensive washing of the column with 500 mM phosphate, p140 was eluted highly enriched by 1 M

phosphate (Fig. 3 *B*, lane *2*). p140 in this eluate fraction was exclusively recognized on Western blot by the wild type but not by the mutant peptide conjugate, emphasizing the specificity of the NLS-p140 interaction (Fig. 3 *C*, lane 6). The binding of the wild-type peptide conjugate to the purified p140 fraction (Fig. 3 *C*, lane *6*) was very strong and revealed some additional bands that resulted most likely from proteolytic degradation of p140. We found that p140 was very susceptible to proteolytic degradation (not shown). Furthermore, neither wheat germ agglutinin nor concanavalin A reacted with the purified protein on Western blots (data not shown). This indicates that p140 is not glycosylated by either *N*-acetylglucosamine, α-D-glucose, or mannose residues.

Antibodies against p140

Three rabbits were immunized with denatured p140 excised from polyacrylamide gels (see Fig. 3 *B*, lane *2*). They gave no immune response, even after repetitive boosting. Immunization of chickens, however, yielded high titer anti-p140 antisera. These anti-p140 antibodies recognized p140 in the enriched fraction (Fig. 4, lane *2*) and reacted specifically with a single protein on Western blots of whole rat liver nuclei (Fig. 4, lane *3*). Preimmune serum did not recognize p140 in the enriched fraction (Fig. 4, lane *1*). Note, that the antibodies did not cross react with any protein of 55 kD. Therefore, it is unlikely that p55 is a proteolytic degradation product of p140. The chicken antibodies exhibited high background binding to nitrocellulose. This is particularly obvious at the top and bottom of the transfers, where the nitrocellulose was not covered by the gel (Figs. 4 and 5). This background was unspecific because it was found as well with
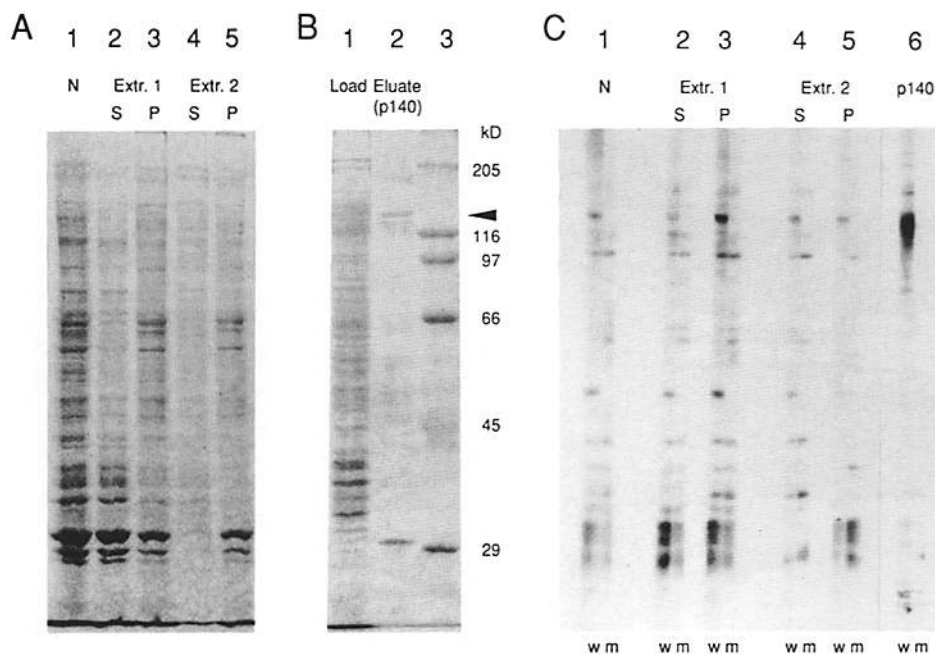

Figure 3. Purification of p140 from rat liver nuclei. (*A*) Coomassie blue profile of proteins from low ionic strength extractions of nuclei separated by SDS-PAGE. Lane *1*, rat liver nuclei; lane *2*, supernatant of first extraction; lane *3*, pellet of first extraction; lane *4*, supernatant of second extraction; lane *5*, pellet of second extraction; the same nuclear equivalents were loaded in each lane (0.5 OD). (*B*) Coomassie blue profile of proteins from hydroxylapatite chromatography fractions separated by SDS-PAGE. Lane *1*, 1/800 (1 OD) of the load fraction (supernatant of the second low ionic strength extraction); lane *2*, 1/100 of the 1 M phosphate eluate fraction (p140). Relative molecular mass of marker proteins is indicated on the right of lane *3*. (*C*) Autoradiograms of the wild-type (*w*) and mutant (*m*) peptide conjugates bound to the fractions shown in *A* and *B* after transfer to nitrocellulose. Lanes *1–5* correspond to lanes *1–5* in *A* and lane *6* contains the p140 enriched eluate fraction as in *B*, lane *2*. Arrowheads point to p140 and p55.
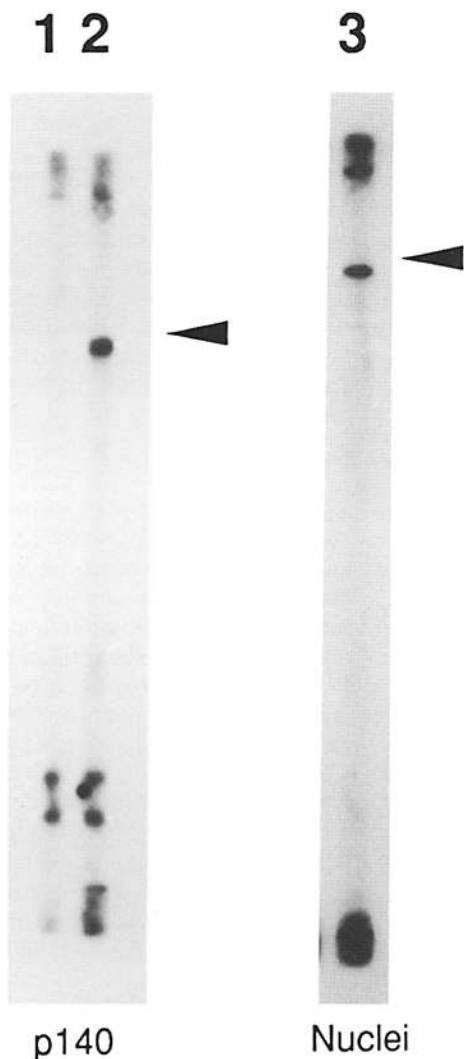
Figure 4. Autoradiograms of Western blots tested with antibodies raised against p140. Lanes *1* and *2* are Western blots of the p140-enriched eluate fraction (see Fig. 3 *B*, lane *2*) incubated with pre-immune (lane *1*) and anti-p140 serum (lane *2*). Lane *3* shows a Western blot of rat liver nuclei incubated with anti-p140 serum. Antibodies were detected by autoradiography after incubation with anti-chicken IgG and ^{125}I-protein A. Arrowheads indicate the position of p140.

the preimmune as with the immune serum (Fig. 4, lanes *1* and *2*). Even purified IgG from anti-p140 serum exhibited the same background staining (not shown).

Subcellular Fractionation

We used these antibodies to study the subcellular distribution of p140. BRL cells were fractionated by differential centrifugation into a nuclear, mitochondrial, microsomal and cytosolic fraction (Fig. 5). The nuclear fraction was further divided into a salt soluble (supernatant; Fig. 5, lanes *1*) and salt-insoluble fraction (pellet; Fig. 5, lanes *2*) by extraction with 1 M NaCl. Probing Western blots of these subcellular fractions with anti-p140 antibodies revealed a single immunoreactive protein in the nuclear fractions (Fig. 5 *B*, lanes

1 and *2*), but no immunoreactive protein was found in the mitochondrial (Fig. 5, lanes *3*), microsomal (lanes *4*), or cytosolic fractions (lanes *5*). p140 was extractable from nuclei by 1 M salt (Fig. 5 *B*, lane *1*) and also by low ionic strength buffer (Fig. 3 *C*, lane *4*). The anti-p140 immunoreactive species and the peptide conjugate binding species were coincident regardless of the nuclear extraction conditions (data not shown). This further establishes the specificity of the antibodies for p140.

Immunofluorescence Localization of p140

To determine the intracellular location of p140, indirect immunofluorescence microscopy was performed on paraformaldehyde-fixed and Triton X-100–permeabilized BRL cells. Surprisingly, anti-p140 antibodies reacted very strongly and in a punctate manner with the nucleolus (Fig. 6 *A*, panel *1*), while preimmune serum exhibited background staining but clearly excluded the nucleoli (Fig. 6 *A*, panel *5*). The nucleolar staining by the anti-p140 serum was the only specific labeling within the cell, because the nucleoplasmic and cytoplasmic staining did not exceed the one seen with preimmune serum. In mitotic cells, the staining was found distributed throughout the cytoplasm (Fig. 6 *A*, right cell in panel *3* is in anaphase). Sometimes dots were visible on chromosomes (Fig. 6 *A*, *arrowhead* in panel *3*) which may represent nucleolus organizing regions around which nucleoli assemble after mitosis.

Binding of the antibodies to the nucleolar antigen required the presence of 500 mM salt and was undetectable under physiological salt conditions (150 mM). Preincubation of the permeabilized cells with high salt was not sufficient for antibody recognition. Therefore, the salt was most likely needed to make epitopes accessible. Unfortunately, the high salt requirement also prevented the application of the antibodies in any physiological system, in particular the nuclear import systems.

Monoclonal antibodies (D77) directed against a yeast homologue of fibrillarin (Fig. 6 *B*, panel *2*; Aris and Blobel, 1988) colocalized with the anti-p140 antibodies (Fig. 6 *B*, panel *1*) in double-labeling experiments. This was visualized by confocal microscopy (Fig. 6 *B*) and confirmed by superimposition of the two images (not shown). Fibrillarin is found in the fibrillar regions of the nucleolus (Lischwe et al., 1985; Ochs et al., 1985). Thus, p140 is located in the fibrillar rather than the granular component of the nucleolus.

Peptide Conjugate Binding to Permeabilized BRL Cells

Because p140 exhibited such a distinct location, we tested whether the wild-type peptide conjugate would recognize the same structure within the cell. We fixed and permeabilized BRL cells identically as for indirect immunofluorescence microscopy but then incubated them with rhodamine-labeled peptide conjugates. The wild-type conjugates bound to the nucleoli in a punctate pattern (Fig. 7 *A*, panel *1*) while the mutant conjugate did not interact with a cellular component (Fig. 7 *A*, panel *3*). As revealed in double-labeling experiments, the nucleolar staining pattern of the wild-type conjugate (Fig. 7 *B*, panel *1*) is similar to that obtained by the anti-p140 antibodies (Fig. 7 *B*, panel *2*). While antibody binding required the presence of 500 mM salt (see above), peptide conjugate binding was abolished under conditions exceeding physiological salt concentrations.
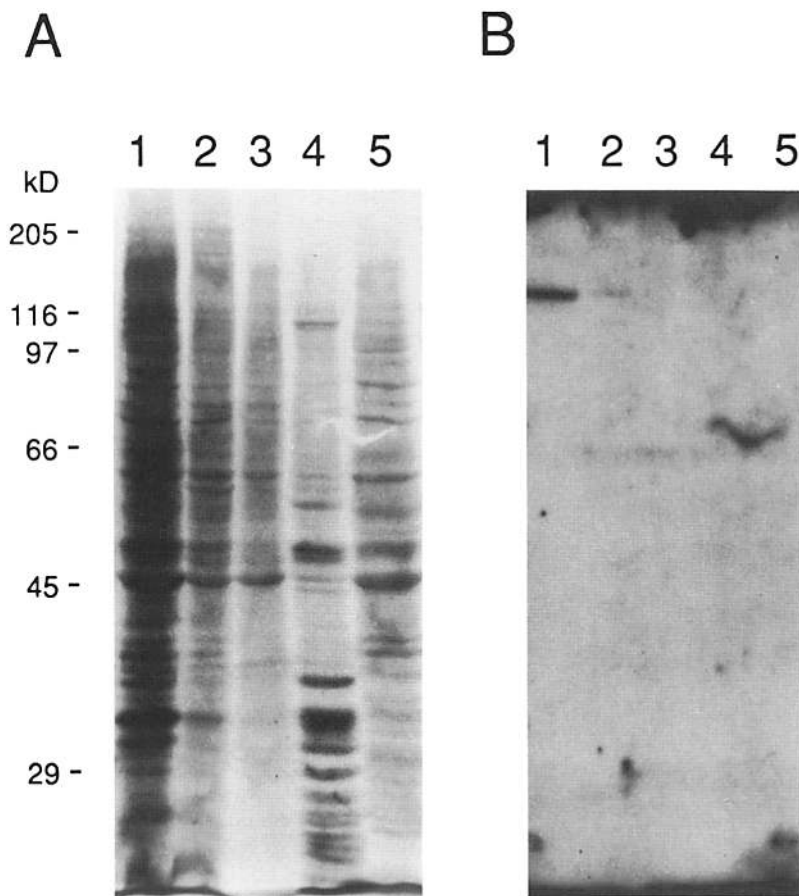
A   B

Figure 5. Subcellular fractionation of BRL cells. (*A*) Amido black stain of subcellular fractions transferred to nitrocellulose after separation by SDS-PAGE. Lane *1*, supernatant of the nuclear fraction after 1 M NaCl extraction; lane *2*, pellet of the nuclear fraction after 1 M NaCl extraction; lane *3*, mitochondrial fraction; lane *4*, microsomal fraction; lane *5*, cytosolic fraction. (*B*) Autoradiogram of the same Western blot as in *A* after incubation with anti-p140 antibodies and detection by secondary antibodies and ^{125}I-protein A.

Discussion

In our attempts to identify putative NLS receptors, we found two proteins of 140 and 55 kD (p140 and p55). These two proteins specifically bind wild-type but not mutant NLS peptides that differ by a single amino acid. We purified p140 and characterized it with the help of polyclonal antibodies. p140 is located in the fibrillar regions of the nucleolus.

To date, the following nucleolar proteins have been described: RNA polymerase I (Scheer and Rose, 1984), a 180-kD protein of the dense fibrillar component (Schmidt-Zachmann et al., 1984), a 145-kD karyoskeletal protein (Franke et al., 1981), nucleolin/C23 (Prestayko et al., 1974; Lapeyre et al., 1987), No38/B23 (Prestayko et al., 1974; Schmidt-Zachmann et al., 1987), ribocharin (Hügle et al., 1985*a*), fibrillarin (Lischwe et al., 1985; Ochs et al., 1985), and ribosomal proteins (S14 and L4: Chooi and Leiby, 1981; S1: Hügle et al., 1985*b*). Of these, only the 145-kD karyoskeletal protein exhibits a molecular mass close to that of p140. This protein however, is insoluble in low and high salt buffers (Franke et al., 1981), while p140 is readily extracted by either. Therefore, p140 is clearly distinct from the 145-kD protein. The punctate nucleolar staining and the labeling of the putative nucleolus organizer regions in mitotic cells by anti-p140 antibodies is reminiscent of the staining observed with anti-RNA polymerase I antibodies (Scheer and Rose, 1984). However, antibodies to RNA polymerase I (Rose et al., 1981; kindly provided by K. M. Rose, University of Texas, Houston) do not cross-react with p140 on Western blots (data not

shown). Thus, p140 is unlikely to be a subunit of RNA polymerase I. However, it might be one of the five proteins that are precipitated in association with RNA polymerase I from [35S]methionine-labeled HeLa cells with anti-RNA polymerase I antibodies, e.g., P3 (155 kD) or P4 (130 kD; Reimer et al., 1987). Finally, the internal sequence of 18 amino acid residues of p140 (unpublished results) revealed no homology to any protein in the screened data bases. Taken together, therefore, these data suggest that p140 is a novel nucleolar protein.

As mentioned in the introduction, four groups have identified NLS binding proteins in mammalian cells (Adam et al., 1989; Yamasaki et al., 1989; Li and Thomas, 1989; Benditt et al., 1989). Based on biochemical properties, all these proteins appear to be different from each other and their involvement in nucleocytoplasmic transport remains to be proven. To identify putative NLS binding proteins Yoneda et al. (1988) raised antibodies to negatively charged peptides that were "electrostatically complementary" to the NLS of SV-40 T antigen. These antibodies blocked nucleocytoplasmic transport when microinjected into the cytoplasm of tissue culture cells and recognized two proteins of 69 and 59 kD on Western blots of rat liver nuclei. These two proteins may be the same as the 70- and 60-kD NLS binding proteins identified by Adam et al. (1989). It is likely that p140 and p55 described here are identical to p140 and p55 identified by Yamasaki et al. (1989). In both reports, the two proteins were shown to be specifically recognized by SV-40 T antigen wild type but not mutant NLS peptides. Moreover, in both reports
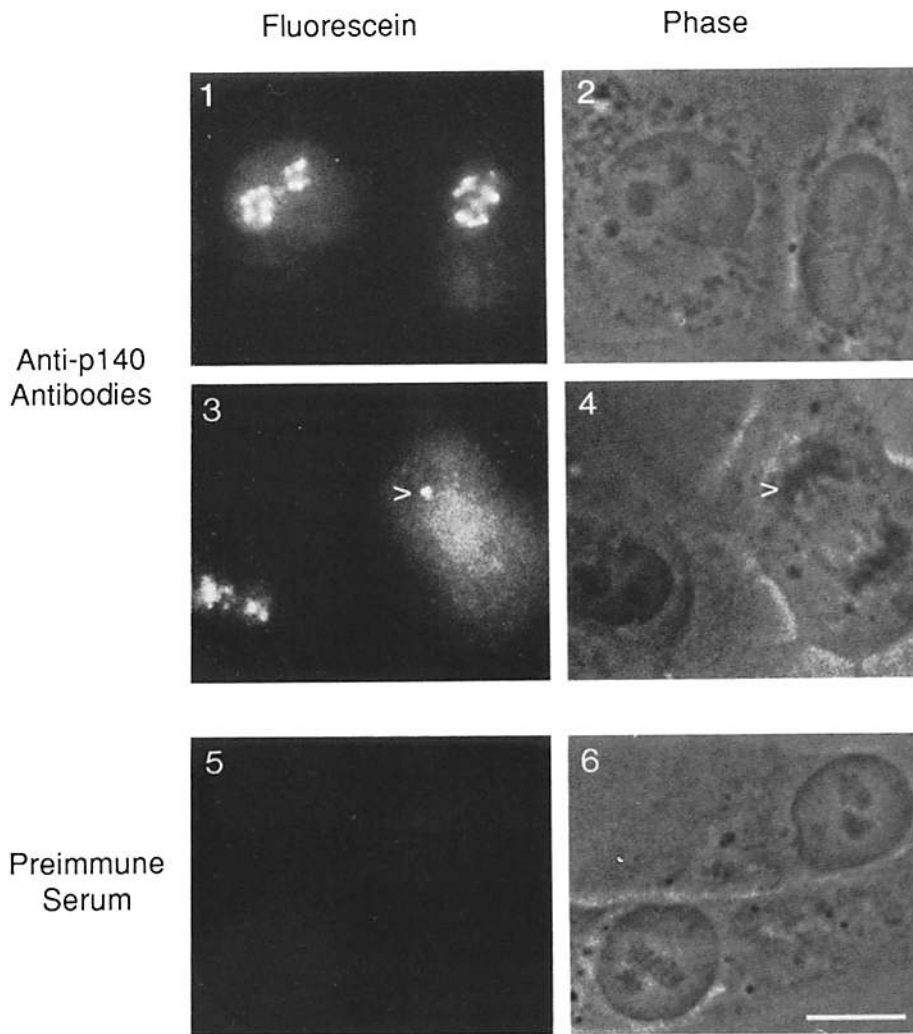
A

Fluorescein   Phase

Anti-p140
Antibodies

Preimmune
Serum

Figure 6. Indirect immunofluorescence of antibodies on fixed and permeabilized BRL cells. (*A*) Panels *1* and *3*, incubated with anti-p140 serum; panel *5*, incubated with preimmune serum. Panels *2*, *4*, and *6* are the phase-contrast images of panels *1*, *3*, and *5*, respectively. The arrowhead in panels *3* and *4* points to chromosomes of a cell in anaphase. (*B*) Double immunofluorescence of anti-p140 serum (panel *1*) and with monoclonal antibodies against the yeast homologue of fibrillarin (D77; panel 2). Anti-p140 antibodies were detected by fluorescein and the monoclonal antibodies by rhodamine-labeled secondary antibodies. Images in *B* are from confocal microscopy. Bars, 10 μm.

B

Anti-p140   Anti-fibrillarin
(Fluorescein)   (Rhodamine)
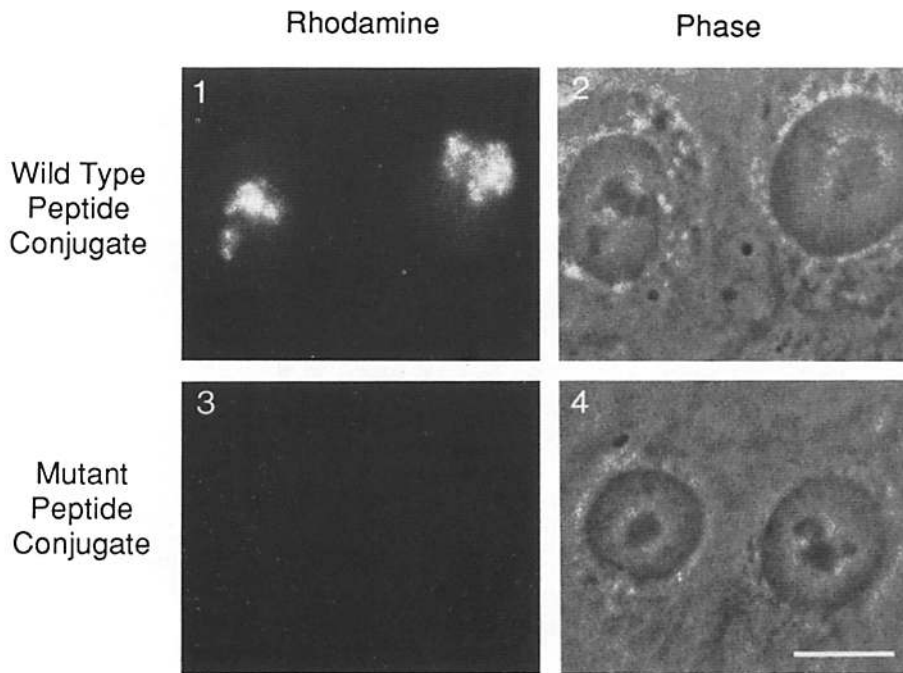
Double
Immuno
fluorescence
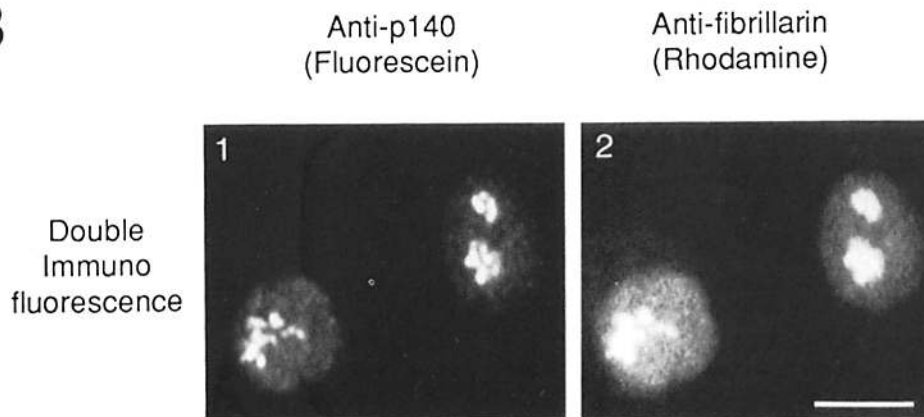
the two proteins were localized to the nucleus and were extractable by either low or high ionic strength buffers. Therefore, using different approaches, Yamasaki et al. (1989) and we have independently identified what appear to be the same proteins. In addition, Yamasaki et al. (1989) demonstrated that the synthetic NLS peptides of nucleoplasmin and adeno-

virus E1A protein bind to p140 and p55. Thus, p140 and p55 seem to interact with NLS containing proteins in general and are therefore likely to be involved in nucleocytoplasmic transport.

How can a nucleolar protein be involved in nuclear protein import? A nuclear import receptor would be expected to act
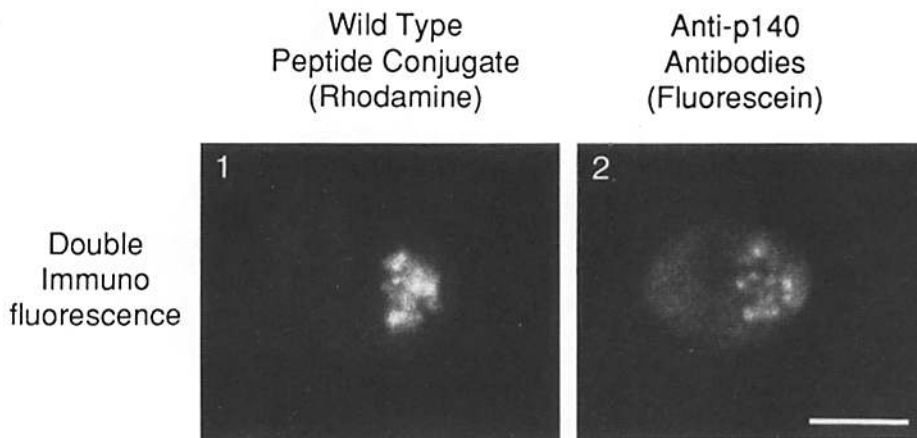
A

Rhodamine Phase

Wild Type
Peptide
Conjugate

Mutant
Peptide
Conjugate

B

Wild Type
Peptide Conjugate
(Rhodamine)

Anti-p140
Antibodies
(Fluorescein)

Double
Immuno
fluorescence

Figure 7. Binding of rhodamine-labeled peptide conjugates on fixed and permeabilized BRL cells. (*A*) Panel *1*, incubated with wild-type conjugate; panel *3*, incubated with mutant conjugate. Panels *2* and *4* show the phase-contrast images of *1* and *3*, respectively. (*B*) Double immunofluorescence of cells incubated with anti-p140 serum and fluorescein-labeled anti-chicken IgG (panel *2*) and followed by wild-type conjugate (panel *1*). Bars, 10 μm.

on the cytoplasmic rather than the nucleoplasmic side of the nuclear envelope in order to target nuclear proteins to their destination. This is supported by data showing that the first step in nuclear import is binding of NLS containing proteins to the cytoplasmic side of the pore complexes (Newmeyer and Forbes, 1988; Richardson et al., 1988). A NLS binding protein should therefore be situated in or near the nuclear pore complexes unless the interaction with pore complex components occurs indirectly through a soluble factor. p140 may be such a soluble NLS binding protein that shuttles between nucleus and cytoplasm functioning as a nuclear import carrier.

The p140 localized in the nucleolus may represent a reservoir and its NLS binding site would have to be inaccessible. The association with NLS sequences would otherwise tie up

NLS containing proteins in the nucleolus and interfere with their function in the nucleoplasm. Indeed, T antigen of SV-40–transformed cells is localized throughout the nucleoplasm except for the nucleoli (Pope and Rowe, 1964) even though p140 is able to bind its NLS sequence. Furthermore, when rhodamine-labeled NLS peptide conjugate was microinjected into the cytoplasm of living cells, it was rapidly taken up into the nucleus but remained excluded from nucleoli (not shown). In paraformaldehyde fixed and Triton X-100–treated cells however, the NLS binding site of nucleolar p140 may have been made accessible (Fig. 7, panel *1*). Nucleolar p140 may also be involved in ribosomal biogenesis and be exported to the cytoplasm in association with preribosomal particles. These particles are exported from nucleoli to the cytoplasm at a high rate and their exact composition

has not been resolved. Preliminary data showed that p140 in nuclear low ionic strength extracts exhibits a sedimentation coefficient >30S (unpublished results). Once in the cytoplasm, p140 would bind the NLS of newly synthesized proteins and carry them through the nuclear pores into the nucleus.

Interestingly, studies of Borer et al. (1989) indicate that No38 and nucleolin shuttle between the nucleolus and the cytoplasm. Both proteins, like p140, are not detectable in the cytoplasm by indirect immunofluorescence microscopy or on Western blots after subcellular fractionation. In addition, No38 was found to bind to immobilized wild-type NLS peptides (Goldfarb, 1988). In this case, however, the absence of binding to the mutant NLS peptide was not tested. No38 contains extended stretches of negatively charged amino acids (Schmidt-Zachmann et al., 1987), which may unspecifically interact with the positively charged NLS. Nucleocytoplasmic shuttling has been described for a number of other proteins like the nonhistone chromosomal protein HMG 1 (Rechsteiner and Kuehl, 1979), a number of unidentified [^{35}S]methionine-labeled nuclear proteins (Goldstein and Ko, 1981), the human nuclear proteins IEF 8Z30 and 8Z31 (Madsen et al., 1986) and the La antigen (Bachmann et al., 1989). It also has been shown that proteins can be transported into the nucleus through association with other proteins. For example, one intact subunit of the nucleoplasmin pentamer containing a NLS is sufficient to carry into the nucleus the residual four subunits after truncation of their NLS (Dingwall et al., 1982). Moreover, histone H1 and other small NLS-containing proteins seem to be associated with a cytoplasmic receptor that prevents their diffusion through nuclear pores in energy depleted or chilled cells (Breeuwer and Goldfarb, 1990). Under these conditions, small proteins lacking a NLS freely diffuse into the nucleus upon cytoplasmic injection.

At this point, we cannot exclude the possibility that the interaction of p140 with wild-type NLS peptide conjugates may be fortuitous and solely due to the association of the positively charged NLS with negatively charged nucleolar proteins. However, this is unlikely because of the specific binding of the wild-type but not the mutant NLS peptide conjugates to p140, and the identification of what appears to be the same protein by an independent approach that was based on the specific interaction of p140 with diverse NLS (Yamasaki et al., 1989). In conclusion, we have identified a novel nucleolar protein that binds the nuclear localization signal sequence of SV-40 T antigen.

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