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Interaction of a nuclear location signal with isolated nuclear envelopes and identification of signal-binding proteins by photoaffinity labeling

(simian virus 40 large tumor antigen nuclear location signal/nuclear protein binding assay)

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ABSTRACT **The nuclear envelope (NE) separates the two major compartments of eukaryotic cells, the nucleus and the cytoplasm. Recent studies suggest that the uptake of nuclear proteins into the nucleus is initiated by binding of nuclear location signals (NLSs) contained within these proteins to receptors in the NE, followed by translocation through the nuclear pore complex. To examine the binding step without interference from intranuclear events, we have used a system consisting of (*i*) purified rat liver NEs fixed onto glass slides and (*ii*) the prototype simian virus 40 large T antigen (SV40 T) NLS conjugated to nonnuclear carrier proteins, and we have visualized the receptor–ligand interaction by indirect immunofluorescence. In this system, incubation of isolated NEs with the wild-type SV40 T NLS conjugate with carrier proteins resulted in binding that was signal sequence-dependent, could be competitively blocked with excess conjugated and unconjugated wild-type peptide, did not require ATP, and was not affected by the transport-inhibiting lectin wheat germ agglutinin. In contrast, only minimal binding was observed with a mutant SV40 T NLS conjugate. These results are consistent with those obtained in other, more complex *in vitro* systems and suggest that binding of the SV40 T NLS is receptor-mediated. Binding is largely abolished by extraction of the NE with the nonionic detergent Triton X-100, suggesting that the receptor is soluble in detergent. We find in the Triton X-100 supernatant four major NLS-binding proteins with apparent molecular masses of 76, 67, 59, and 58 kDa by photoaffinity labeling with a highly specific crosslinker, azido-NLS. The reduced complexity of the system described here should be useful for the functional study of other potential NLSs for the identification and isolation of their binding sites and for the screening of antibodies raised against these binding sites.**

The selective exchange of molecules across the nuclear envelope (NE) is an essential factor in many cellular processes. Recent studies have shown that the regulation of these processes is mediated, at least in part, by the interaction of signal sequences contained within these molecules with receptors localized in the NE.

The four ultrastructurally distinct components of the NE are the outer and the inner nuclear membrane, the nuclear lamina, and the nuclear pore complexes (reviewed in ref. 1). It is generally agreed that the pore complexes provide aqueous channels through which nucleocytoplasmic exchanges, including macromolecular exchanges, take place (1–4), and there is electron microscopic evidence to support this (5–10).

Studies on the movement of molecules from cytoplasm to nucleus have established that pore complexes have channels of about 9–12 nm in diameter available for passive diffusion (11, 12). Globular proteins with molecular masses ≤ 15 kDa will diffuse at roughly the same rate as in free solution, but proteins larger than ≈60 kDa are too big to diffuse through the pore complexes (11). However, certain high molecular mass polypeptides do enter and are concentrated in the nucleus. This suggests that these large nuclear proteins must possess signals for active uptake through the pores and for accumulation in the nucleus (13). Evidence for a karyophilic signal was first demonstrated in studies with the protein nucleoplasmin, a pentamer in which each 22-kDa monomer has a domain necessary for selective transport into the nucleus (14). Subsequently, a small region of basic sequence ($\overset{126}{\text{Pro}}$-$\overset{128}{\text{Lys}}$-$\overset{130}{\text{Lys}}$-Lys-$\overset{132}{\text{Arg}}$-Lys-Val) was identified as a nuclear location signal (NLS) in the large tumor antigen (T) of simian virus 40 (SV40) (15), and mutations of this sequence (especially those involving Lys-128) were shown to drastically reduce nuclear uptake of the respective protein (15, 16). When synthetic wild-type (wt) and mutant SV40 T NLSs were conjugated to a variety of large nonnuclear proteins, only wt SV40 T NLS conjugates were directed to the nuclear compartment (17, 18). Furthermore, nuclear uptake of the wt SV40 T NLS conjugates with nonnuclear proteins was saturable and could be blocked by competition (9, 18), strongly suggesting that the uptake is receptor-mediated. Newmeyer and Forbes (9) and Richardson *et al.* (10) were able to separate uptake into two distinct steps, binding and translocation. Binding localized specifically to the area of the pore complex, was signal sequence-dependent, blocked by competition with excess unlabeled protein, and did not require ATP. When a mutant SV40 T NLS was used, little binding and no transport was observed. Yoneda *et al.* (19) and Adam *et al.* (20) have recently identified two proteins with approximate molecular masses of 60 and 70 kDa as NLS-binding sites.

To study the binding step of nuclear protein import, we have utilized isolated NEs fixed onto glass microscope slides and have examined binding of the wt SV40 T NLS conjugated to bovine serum albumin (BSA), which we refer to as wt NLS-BSA, by indirect immunofluorescence. Our results show that this binding to the NE is signal sequence-specific and can be blocked by competition with excess ligand. Markedly reduced binding is observed for a mutant SV40 T NLS–BSA conjugate, which we refer to as cT NLS–BSA. Furthermore, extraction of NEs with 1% Triton X-100 prior to incubation with wt NLS–BSA conjugate completely abolishes binding. In an attempt to identify the binding site(s) for the NLS, we synthesized a highly specific, photoreactive,

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Abbreviations: NE, nuclear envelope; T, large tumor antigen; SV40, simian virus 40; NLS, nuclear location signal; wt NLS and cT NLS, wild-type and mutant SV40 T NLS; N_3-NLS, azido-modified NLS; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

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crosslinker containing the wt SV40 T NLS. When subfractions of the NE were allowed to interact with this crosslinker, four major bands with apparent molecular masses of 76, 67, 59, and 58 kDa were found in the Triton X-100 supernatant. We conclude that these bands correspond to proteins that are specific for the binding of the NLS.

This work was presented at the Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology in San Francisco from January 29 to February 2, 1989, and was published as an abstract (21) prior to that meeting.

MATERIALS AND METHODS

Isolation of Nuclei and Nuclear Envelopes. Liver nuclei were isolated from male Sprague–Dawley rats (150 to 200-*g* body weight) by the method of Blobel and Potter (22). Deoxyribonuclease I-treated NEs were prepared by the procedure of Dwyer and Blobel (23) as described (24, 25). All buffers were supplemented with 0.1 mM phenylmethanesulfonyl fluoride and 0.01 mM leupeptin.

Fixation of NEs. Studies with fixed NEs were performed by protocols described for other *in vitro* systems (26, 27). NEs were suspended in 0.25 M STKMC buffer (0.25 M sucrose/50 mM Tris·HCl, pH 7.4/25 mM KCl/5 mM $MgCl_2$/3.3 mM NEs/$CaCl_2$) at 2×10^6 per ml and were spun onto precleaned glass slides at 400 rpm ($\approx 600 \times g$) for 5 min (Shandon Southern Cytospin). Slides were briefly air-dried, fixed in methanol at −20°C for 20 min, rinsed, and stored at 4°C for up to 72 hr in a box containing buffer. Immediately prior to binding studies, the slides were preblocked by immersion in 0.25 M STKMC buffer containing 0.1% hemoglobin for 20 min at 37°C and were then washed with phosphate-buffered saline (PBS) and dried.

Ligand Binding Studies. The NLS conjugates are synthetic molecules in which the wt or mutant (cT) SV40 T NLS is conjugated to 67-kDa BSA or 150-kDa IgG (17). The synthetic peptides are 13-mers and contain, in addition to the SV40 T NLS underlined in Fig. 3*A*, four N-terminal and two C-terminal amino acids. The N-terminal cysteine of the peptide is conjugated to the carrier protein via the heterobifunctional crosslinking agent MBS (maleimidobenzoyl-*n*-hydroxysuccinimide ester; ref. 17). Approximately 13 peptides are added per BSA molecule and 2 peptides per IgG molecule. The properties of these ligands have been described in detail (17, 28). For competition assays, unconjugated wt SV40 T/SV40 T NLS was also used. In the mutant signal sequence, the lysine residue in position 128 is replaced by asparagine (17), and this results in a drastically reduced affinity for nuclear binding.

For binding studies, ligand was added to final concentrations ranging from 0.7 nM to 7 μM, followed by incubation at 37°C for 20 min. Binding was observed at ligand concentrations exceeding 70 nM. Therefore, all subsequent ligand binding assays were performed with a ligand concentration of 0.7 μM, and lower concentrations were not further examined. After ligand incubation, slides were rinsed in PBS and air-dried. Ligand binding was visualized indirectly by using fluorescein isothiocyanate (FITC)-conjugated antibodies against BSA (ICN). FITC-conjugated anti-BSA was spotted onto the slides and again incubated at 37°C for 20 min. Slides were washed in PBS and then examined with a Nikon Diaphot-TMD fluorescence microscope.

Synthesis of the Photoaffinity Label Azido-NLS (N_3-NLS). The peptide crosslinker N_3-NLS is shown in Fig. 3*A*. It is a 14-mer that contains the wt SV40 T NLS plus three N-terminal amino acids with a tyrosine residue for radioiodination and four C-terminal amino acids serving as spacers. During synthesis, the protected peptide was coupled to *p*-azidobenzoylglycine. To obtain *p*-azidobenzoylglycine, we synthesized *p*-azidobenzoic acid from *p*-aminobenzoic acid (29). The *p*-azidobenzoic acid was then dissolved in thionyl chloride to obtain *p*-azidobenzoyl chloride, which reacted with glycine to form *p*-azidobenzoylglycine.

The peptide was unprotected by incubation with 95% trichloroacetic acid for 2 hr at 50°C, protecting groups were removed by three extractions with diethyl ether, and the neutralized aqueous phase was taken to dryness in a rotating evaporator. The peptide was then redissolved in 10 mM Tris buffer (pH 8.0) to a concentration of ≈30 mM. Peptide modified with *p*-azidobenzoylglycine was separated from unmodified peptide by reverse-phase high performance liquid chromatography (HPLC) using a Vydek C_4 column and a gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. The N_3-NLS-containing fractions were rotated in the evaporator to dryness, redissolved in buffer, and radioiodinated with Iodo-Beads (Pierce) to a specific activity of $\approx 10^6$ cpm/μg. Unlabeled N_3-NLS and monoiodo-N_3-NLS were separated by reverse-phase HPLC in a 0–100% acetonitrile gradient containing 0.1% trifluoroacetic acid. The radioactive fraction, which was eluted at 28% acetonitrile, was rotated in an evaporator to dryness and redissolved in buffer.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE). NE fractions were electrophoretically separated by SDS/10% PAGE by standard procedures (30). After electrophoresis, gels were stained with Coomassie blue, destained, dried, and exposed to Kodak XAR film at −70°C with an intensifying screen.

RESULTS

A System for Nuclear Protein Binding. We reasoned that the least complex system for examining nuclear protein binding and identifying and characterizing NE receptors would consist of isolated NEs and ligands that contain a functioning nuclear location signal but lack any intranuclear binding domains. First, it is generally agreed that nuclear proteins bind to pore complexes for nuclear uptake. Isolated NEs (essentially free of intranuclear components) should permit the examination of this binding without interference from intranuclear events. Second, NLS conjugates do not, like most nuclear proteins, have domains for intranuclear binding that can interfere with signal sequence receptor studies. Therefore, we have used the well-characterized SV40 T NLS conjugates (17, 28) and NEs prepared according to the widely applied procedure of Dwyer and Blobel (23). These NEs appear as nuclear ghosts after resuspension in 0.25 M STKMC buffer (25). In the wt SV40 T NLS conjugates, a 13-mer synthetic peptide containing the SV40 T transport signal is conjugated via a thioether linkage to nonnuclear carrier proteins (17). A mutant signal, cT SV40 NLS, defective in nuclear transport, which contains a single amino acid change (Lys-128 → Asn-128) (17), was used as a control.

Isolated NEs Exhibit Peripheral Binding of NLS–BSA Conjugates. For binding studies, incubation with wt NLS–BSA was performed at a ligand concentration of 0.7 μM; wt NLS–BSA showed binding around the rim of each NE (Fig. 1*A*). In contrast, cT NLS–BSA at a concentration of 0.7 μM displayed greatly reduced binding (Fig. 1*B*). It has been shown that cT NLS is a much less effective signal than wt NLS (9, 15–18). The reduced, but not absent, binding of cT NLS–BSA that we observe in our system supports this finding and implies a diminution in the affinity of the mutated signal for the binding site. These results show that this simple assay system can discriminate between the wt and mutant NLS.

When FITC-labeled anti-BSA (Fig. 1*C*) or FITC alone was incubated with NEs, or when BSA was used instead of wt NLS–BSA, no peripheral binding and only minimal background was observed (data not shown). As a comparison, we

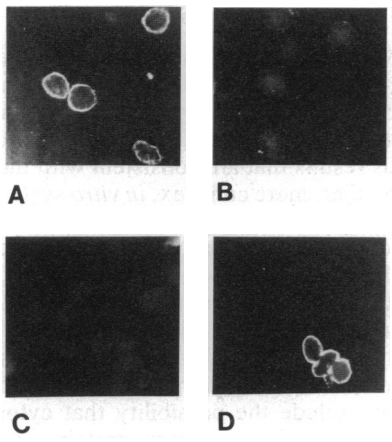


FIG. 1. Specificity of binding of SV40 T NLS–BSA to isolated rat liver NEs. NEs suspended in 0.25 M STKMC buffer were spun onto glass slides at a concentration of 2×10^6 NEs per ml, fixed in methanol, preblocked in 0.25 M STKMC buffer containing 0.1% hemoglobin, and incubated with wt NLS–BSA or mutant cT NLS–BSA at a concentration of 0.7 μM for 20 min at 37°C. After the incubated NEs were washed in PBS, FITC-labeled anti-BSA antibody was added for 20 min at 37°C. The slides were washed in PBS and examined with a Nikon Diaphot-TMD fluorescence microscope. Incubation with wt NLS–BSA resulted in a strong peripheral binding (*A*), while the mutant cT NLS–BSA revealed minimal peripheral binding (*B*). Very little background fluorescence was observed with anti-BSA antibody alone (*C*). As a control for peripheral binding, a FITC-labeled anti-lamin A/C antibody (kindly provided by Frank D. McKeon) was used (*D*).

have visualized the lamina proteins, which are known to be exclusively localized in the periphery of the interphase nucleus (31), with fluorescent antibodies against lamins A and C (kindly provided by Frank D. McKeon at Harvard University). As expected, incubation resulted in a peripheral, rim-like pattern (Fig. 1*D*).

Binding of wt NLS–BSA to Isolated NEs Can Be Competitively Blocked by Unlabeled Ligand and Is Affected Neither by the Transport-Inhibiting Lectin Wheat Germ Agglutinin nor by ATP. To test further the specificity of binding of wt NLS–BSA, competition experiments were carried out with either unconjugated wt NLS or wt NLS conjugated to rabbit IgG (17). Compared with wt NLS–BSA, binding in the absence of competitor (Fig. 2*A*), coincubation of wt NLS–BSA with a 100-fold molar excess of wt NLS–IgG (Fig. 2*B*), or a 1000-fold molar excess of wt NLS (Fig. 2*C*) resulted in markedly reduced binding of wt NLS–BSA. To exclude the possibility that binding of wt NLS–BSA was due to the positive charge of the signal peptide, we coincubated wt NLS–BSA with a 100-fold molar excess of poly(L-lysine) of an average molecular mass of 22 kDa. No difference in wt NLS–BSA binding was noted in comparison with incubation in the absence of poly(L-lysine) (data not shown).

From these studies we conclude that the binding of wt NLS–BSA to isolated NEs fulfills the requirements of a receptor-mediated process. Moreover, they show that this receptor–ligand interaction can occur without the addition of other (e.g., cytoplasmic) factors.

Not unexpectedly, wheat germ agglutinin, which has been shown to inhibit nuclear protein transport (32–35) but not the initial binding step (9), had no effect on the binding of wt NLS–BSA to isolated NEs (Fig. 2*D*). Moreover, ATP, which has been shown to be required for translocation of ligand through the pore (9), did not appear to increase the association of wt NLS–BSA with NEs (data not shown).

Effect of Triton X-100 on the Binding of wt NLS–BSA. The isolated NEs contain the outer and the inner nuclear membrane, the pore complexes, and the lamina (23). To localize

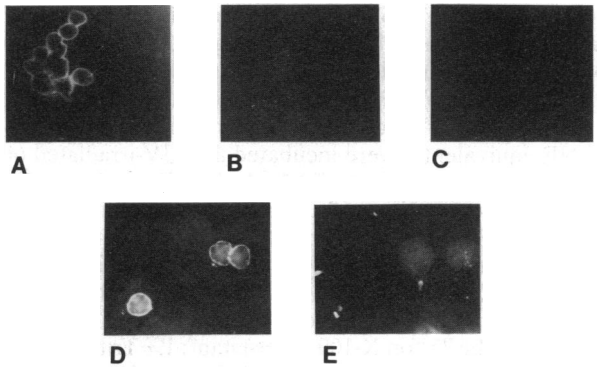


FIG. 2. Competition of binding of wt NLS–BSA to NEs and effects of wheat germ agglutinin and Triton X-100 on binding of wt NLS–BSA to NEs. While incubation of NEs with wt NLS–BSA at a concentration of 0.7 μM in the absence of competitor resulted in a strong peripheral binding (*A*), coincubation with either a 100-fold molar excess of wt NLS conjugated to IgG (*B*) or a 1000-fold molar excess of wt NLS (*C*) resulted in marked diminution of binding. The transport-inhibiting lectin wheat germ agglutinin had no effect on wt NLS–BSA binding at a concentration of 0.1 mg/ml (*D*), whereas incubation of NEs for 15 min at 4°C in 0.25 M STKMC buffer containing 1% Triton X-100 prior to fixation onto glass slides abolished subsequent binding of wt NLS–BSA (*E*).

the site of binding of wt NLS–BSA within the NE, we treated isolated NEs in 0.25 M STKMC buffer with 1% Triton X-100 prior to fixation onto glass microscope slides. In comparison with untreated NEs (Fig. 2*A*), Triton X-100-treated NEs show no binding of wt NLS–BSA (Fig. 2*E*). This suggests that the binding site for wt NLS–BSA can be extracted with detergent, which is consistent with recent data of Adam *et al.* (20), who have found NLS-binding proteins in *n*-octyl glucoside extracts of NEs.

Analysis of NE-Associated NLS-Binding Proteins with a Specific Photoreactive Crosslinker. To identify and eventually isolate proteins in the NE involved in the binding of the SV40 T NLS, we synthesized a photoreactive crosslinker, N_3-NLS (Fig. 3*A*), that contains a sequence identical to that of the wt peptide in the NLS conjugates used in indirect immunofluorescence assays and, in addition, a photoreactive *p*-azidobenzoylglycine covalently attached to the N terminus of the peptide (see *Materials and Methods*).

To test whether both ligands, N_3-NLS and wt NLS, are recognized by the same binding site, isolated NEs were incubated with radiolabeled N_3-NLS at a concentration of 2.5 μM in the absence and presence of a 100-fold excess of unlabeled N_3-NLS and unconjugated wt NLS, respectively. After incubation, NEs were UV-irradiated with an ARC lamp (Oriel, Stamford, CT) at 4 A for 2 min, washed extensively to remove unbound radioligand, and assayed for radioactivity. Competition with unlabeled N_3-NLS and unconjugated wt NLS decreased the ligand binding by 60% (data not shown).

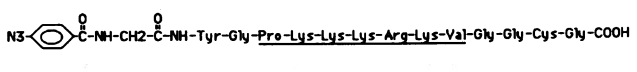
N_3-NLS was also tested for its competition with wt NLS–BSA in indirect immunofluorescence assays. No peripheral fluorescence was observed for wt NLS–BSA in the presence of a 1000-fold molar excess of N_3-NLS (data not shown), which is consistent with the results obtained with a 1000-fold molar excess of unconjugated wt NLS (Fig. 2*C*).

In immunofluorescence studies, we have shown that incubation of NEs with 1% Triton X-100 in 0.25 M STKMC buffer largely abolishes subsequent binding of wt NLS–BSA (Fig. 2*E*), suggesting that the receptor(s) can be extracted with detergent. Therefore, we subfractionated NEs for receptor labeling studies. By using protocols previously described (23, 24), NEs were first extracted with 1% Triton X-100 in 0.25 M STKMC buffer to remove nuclear membranes and loosely associated proteins. After centrifugation, the pellet was re-

suspended in 0.25 M STKMC buffer and incubated with 1 M NaCl to solubilize the residual chromatin. The resulting pore complex/lamina fraction was then treated with 2 M urea to extract pore complexes. Fractions were dialyzed against 0.25 M STKMC buffer, and aliquots of 50 μg of protein ($\approx$1–3 $\times$ 10^7 NE equivalents) were incubated and UV-irradiated (4 A for 2 min) in a total volume of 0.1 ml with ^{125}I-labeled N_3-NLS at a final concentration of 3 μM in the absence and presence of a 200-fold excess of unlabeled N_3-NLS. After fragments were crosslinked, fractions were electrophoretically separated by SDS/10% PAGE. As shown in the corresponding autoradiograph in Fig. 3*B*, specific N_3-NLS-labeling was only observed in the Triton X-100 supernatant; 1% Triton X-100 in 0.25 M STKMC buffer extracted four major bands and several minor bands with affinity for N_3-NLS, all of which could be blocked by competition with excess unlabeled crosslinker (Fig. 3*B*); the four major bands correspond to proteins with molecular masses of approximately 76, 67, 59, and 58 kDa, whereas the minor bands, which become visible only after longer exposure times, have molecular masses of approximately 130, 52, 38, and 31 kDa. These estimates of molecular masses include, of course, the mass of the crosslinker (2.3 kDa). It is important to point out that none of the four major bands correspond to lamina proteins and that they represent only minor protein species based on their relatively faint Coomassie blue staining (data not shown).

Based on these studies, and in agreement with data reported by others (19, 20), we conclude that the proteins labeled with N_3-NLS are strong candidates for the NLS receptor.

A Cross-linker N_3-NLS



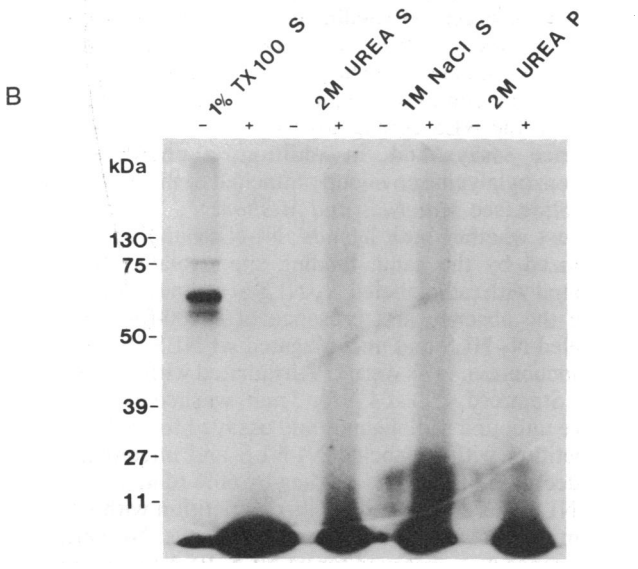


FIG. 3. Cross-linking of NE subfractions with a specific, photoreactive wt NLS, N_3-NLS. (*A*) The crosslinker N_3-NLS was synthesized as described. It is identical in its amino acid sequence to the wt NLS used for conjugation and indirect immunofluorescence studies, with the exception of the addition of a photoreactive *p*-azidobenzoylglycine residue at the N terminus. (*B*) NEs were subfractionated as described (22, 23) and dialyzed against 0.25 M STKMC buffer; fractions corresponding to 50 μg of protein ($\approx$1–3 $\times$ 10^7 NE equivalents) were incubated with radiolabeled N_3-NLS in the absence (lanes −) and presence (lanes +) of a 200-fold excess of unlabeled N_3-NLS. Crosslinked fractions were then directly loaded onto SDS/10% PAGE gels. TX100, Triton X-100; S, supernatant; P, pellet.

DISCUSSION

We have developed a simple assay system for nuclear protein binding that employs isolated rat liver NEs essentially free of intranuclear components and synthetic ligands in which the NLS is conjugated to nonnuclear carrier proteins. This system yields results that are consistent with those obtained *in situ* and in other, more complex, *in vitro* systems. We have found by indirect immunofluorescence that binding of the wt NLS–BSA conjugate is signal sequence-dependent, can be competitively blocked, does not require ATP, and is not affected by the lectin wheat germ agglutinin. In contrast, only minimal binding is observed with the mutant cT NLS–BSA conjugate. In agreement with other *in vitro* systems, these results suggest that binding of the NLS is receptor-mediated.

We cannot exclude the possibility that cytoskeletal elements (36, 37) or other auxiliary proteins, contained for example in the egg extracts used by Newmeyer *et al.* (9, 38), may affect receptor–ligand interaction. Nevertheless, this simple *in vitro* system should be useful as a method to screen proteins for their affinity for the nuclear periphery and to functionally test potential NLSs.

The reduced complexity of the system also facilitates the subnuclear localization, identification, and purification of NE proteins involved in binding. As shown in Fig. 2*E*, incubation of the NEs with 1% Triton X-100 in 0.25 M STKMC buffer abolished peripheral binding of ligand as monitored by indirect immunofluorescence, suggesting that the NLS-binding protein is extracted under these conditions. Using a photoreactive crosslinker containing the wt SV40 T NLS, we have identified four major specific NLS-binding proteins of 76, 67, 59, and 58 kDa in this Triton X-100 supernatant (Fig. 3*B*). None of the other fractions examined contained any NLS-binding proteins (Fig. 3*B*). It is possible that two of these bands, corresponding to the 76- and 67-kDa proteins, are identical to proteins described by Yoneda *et al.* (19) and Adam *et al.* (20). Based on the assumption that the NLS-binding region of the putative receptor may be electrostatically complementary to the NLS, Yoneda *et al.* (19) have generated antibodies against a negatively charged synthetic peptide. These antibodies were found to block transport of nuclear proteins into the nucleus and recognized two proteins of 69 and 59 kDa in rat liver nuclear lysates. Adam *et al.* (20) have chemically crosslinked a synthetic 34-mer containing the wt SV40 T NLS to detergent-solubilized subcellular fractions and have identified two proteins of 70 and 60 kDa in a postmitochondrial supernatant, in nuclei, and in purified NEs. However, their study provides no functional assays for the involvement of these two proteins in nuclear protein binding. A relationship between the proteins identified by Adam *et al.* (20) and those described in this communication is supported by our finding that incubation with ^{125}I-labeled N_3-NLS of a 1% *n*-octyl glucoside/300 mM KCl extract of NEs prepared according to the procedure of Adam *et al.* (20) results in labeled bands with the same electrophoretic mobility as those extracted from NEs with 1% Triton X-100 in 0.25 M STKMC buffer (Fig. 3*B*) or 1% Triton X-100 in 0.25 M STKMC buffer containing 300 mM KCl (data not shown). In contrast to the data by Adam *et al.* (20), we have found that binding of the two proteins of 59 and 58 kDa can be competitively blocked by excess crosslinker (Fig. 3*B*). Antibodies against the purified proteins will be needed to study their exact relationships and to examine whether some or all of the NLS-binding proteins identified originate from proteolytic cleavage of a larger protein. We cannot exclude the possibility that the minor N_3-NLS-binding proteins shown in Fig. 3*B* are also important in nuclear protein binding. The system described here should permit the rapid functional testing of antibodies raised against these proteins to establish their role in nuclear transport.

Note. After this paper was submitted for review, Yamasaki *et al.* (39) reported the identification of four NLS-binding proteins (140, 100, 70, and 55 kDa) by UV crosslinking techniques.

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