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Identification and characterization of nuclear location signal-binding proteins in nuclear envelopes

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A radioiodinated, photoactivable synthetic nonapeptide corresponding to the nuclear location signal (NLS) of SV40 large T antigen has been used in photolabelling reactions with purified mouse liver nuclei, nuclear envelopes and other cellular fractions, to identify specific NLS-binding proteins which may be involved in selective transport of karyophilic proteins. SDS-polyacrylamide gel analysis of photolabelled products demonstrates that a 60 kDa nuclear protein and four nuclear envelope proteins (67, 60, 53 and 47 kDa) bind specifically to the native NLS and not to a mutant NLS or unrelated sequences. This binding shows saturation kinetics, with highest affinity of the NLS for the 60 and 67 kDa proteins. The nuclear 60 kDa NLS-binding protein is identical to the nuclear envelope 60 kDa NLS-binding protein by two-dimensional gel analysis of labelled proteins. Biochemical fractionation of labelled nuclear envelopes suggests that the 53 and 47 kDa proteins are peripheral membrane proteins whereas the 67 and 60 kDa proteins can be localized to the pore complex. The NLS also binds to solubilized 67, 60, 53 and 47 kDa proteins but with decreased affinity. Our results suggest that one of the early steps in selective nuclear transport of proteins may be the recognition of the NLS by the 60 kDa and/or 67 kDa binding proteins present in the nuclear pore complex.

Introduction

The double membrane of the nuclear envelope partitions the nucleus from the cytoplasm in a eukaryotic cell and is spanned by numerous nuclear pores. The nuclear pore is thought to be the route of most nucleocytoplasmic traffic and is a complex, proteinaceous structure which allows the free diffusion of small molecules but selectively regulates the entry of large molecules [1,2]. Studies on the uptake requirements of nuclear proteins have used approaches based on recombinant DNA techniques or the use of synthetic peptides as probes (reviewed in Ref. 3). For several nuclear proteins, transport into the nucleus has been shown to depend on the presence of a nuclear location signal sequence (NLS) in the mature protein. Although there is no consensus NLS, several known signals contain a common motif. For example, for karyophilic proteins such as SV40 large T antigen [4], nucleoplasmin [5], polyoma T antigen [6], and the adenovirus Ela protein [7], the signal is made up of a short stretch of highly basic amino acids flanked by proline or glycine. However, other signals present in proteins such as the yeast regulatory proteins, MATα2 [8] and GAL4 [9], and yeast ribosomal protein L3 [10], bear little if any homology to the prototype SV40 large T antigen signal or to each other.

The NLS of SV40 large T antigen (Pro-Lys-Lys-Lys-Arg-Lys-Val) shows an absolute requirement for a positively charged amino acid at the second lysine, whereas changes in the flanking residues have lesser effect. Fusion proteins containing the T antigen NLS and a non-nuclear protein are efficiently transported into the nucleus [11], and a synthetic decapeptide NLS covalently linked to non-nuclear proteins such as serum albumin and IgG can induce their nuclear uptake [12]. Thus the seven-residue NLS appears to be essential and generally sufficient for selective nuclear transport of proteins. Transport appears to be a two-step process: karyophilic proteins initially bind to the nuclear envelope and then translocate through the pore complex [13,14]. Thus it is likely that specific nuclear envelope or pore complex proteins may bind to the signal and assist in the selective entry of nuclear proteins across the envelope. Recent chemical cross-linking studies with a synthetic SV40 T antigen NLS suggest the presence of

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both nuclear and cytosolic NLS-binding proteins [15,16]. However, other assays do not indicate an interaction between NLS peptides and cytosolic factors in mammalian or yeast cells [17–20].

In this study, we have used a synthetic photoactivable peptide corresponding to the native NLS of SV40 large T antigen and a mutant signal peptide defective in T antigen transport in which the second lysine is replaced by a threonine [11], in photolabelling experiments with purified nuclei, nuclear envelopes and other cellular fractions. We report that the native NLS binds specifically to a 60 kDa nuclear protein and four proteins on the nuclear envelope, with molecular masses 67, 60, 53 and 47 kDa, but not to any cytosolic proteins. The nuclear 60 kDa protein is shown to be identical to the nuclear envelope 60 kDa protein by two-dimensional polyacrylamide gel electrophoresis.

Materials and Methods

Amino acids and Merrifield's resin (1% cross-linked) were from Sigma Chemical Co. Protected amino acids were synthesized by established procedures. *N*-Hydroxysuccimidyl-4-azidosalicylic acid (NHS-ASA) and Iodogen were from Pierce Chemical Co. $Na^{125}I$ (carrier-free) was obtained from Bhabha Atomic Research Centre, India.

Synthesis of peptides and derivatives

NLS peptides (for sequences see Fig. 1) were synthesized manually by solid phase methods using Merrifield's resin [21], by previously described procedures [22]. Peptides were purified by fast performance liquid chromatography (Pharmacia FPLC System) on an RP-18 column with gradient elution (A, 0.1% trifluoroacetic acid in water; B, 0.1% trifluoroacetic acid in acetonitrile; 0–50% B in 30 min). Purity of the peptides was confirmed by the correct amino acid analysis. Peptides corresponding to the secretory signal sequences for the precursors of bacteriophage lambda receptor and *Escherichia coli* alkaline phosphatase were a kind gift from R. Nagaraj [22,23]. As these sequences are unrelated to the NLS (see Fig. 1), they were used as non-specific competitors.

Preparation of photoaffinity derivatives was carried out as described in Ref. 24, with minor modifications. All procedures were carried out in the dark. Briefly, 20 μl of NHS-ASA (120 nmol) dissolved in acetonitrile was mixed with 100 nmol of peptide in 100 μl of 25 mM Na_2HPO_4 (pH 7.2), and incubated at room temperature for 1 h. Derivatized peptide was then separated from NHS-ASA and unreacted peptide by fast performance liquid chromatography on an RP-18 column eluted with gradient elution as above. Peptide concentrations were determined by quantitative amino-acid analysis. The extent of photolabelling was calculated by spectroscopic measurements, using $\varepsilon = 20000\ M^{-1}\ cm^{-1}$ at $\lambda_{max} = 310$ nm for NHS-ASA [25]. Values > 80% were consistently obtained. Sequence analysis of the N-terminus of the photolabelled peptides compared with the unmodified peptides confirmed that the photolabel was attached to the N-terminal glycine residue in both peptides. Photolabelled peptide (50–100 μg) was radioiodinated by incubation with 500 μCi of $Na^{125}I$ in an Iodogen-coated tube for 15 min at room temperature. The radiolabelled peptide was separated from unreacted $Na^{125}I$ by gel filtration on a Bio-Gel P-2 column equilibrated with 1% acetic acid. The radioactive peptide fractions were pooled, lyophilized, redissolved in water and counted in a gamma counter (Packard Auto-gamma 5650). Radioiodinated peptides were labelled to a specific activity of approx. $2 \cdot 10^6$ dpm/nmol.

Photolabelling of nuclei, nuclear envelopes and other cellular fractions

Mouse liver nuclei were purified by sucrose density centrifugation and processed to obtain nuclear envelopes by Kaufmann's procedure [26], and characterized in detail for RNA, DNA content and phospholipid/protein ratio as described earlier [27]. The post-mitochondrial supernatant (PMS) obtained after sedimentation of nuclei and mitochondria ($10000 \times g$ supernatant) was also used for initial photolabelling experiments.

Samples containing nuclei, nuclear envelopes, or PMS (approx. 50 μg protein) were mixed with $(2–4) \cdot 10^6$ dpm photolabelled peptide in phosphate-buffered saline, (10 mM sodium phosphate, 150 mM NaCl, pH 7.5), 5 mM $MgCl_2$ (total volume 250 μl) and irradiated at 300 nm for 2 min with a Xenon lamp source. These conditions were sufficient for the complete decay of the 310 nm peak of NHS-ASA as confirmed by UV spectroscopy. Samples were centrifuged (except for PMS) and pellets or an aliquot of PMS were heated in Laemmli's buffer and analyzed by SDS-polyacrylamide gel electrophoresis on 8% gels by Laemmli's method [28]. Gels were stained with Coomassie blue, dried and autoradiographed at −70°C using intensifying screens (for 2–5 days). For experiments requiring preincubation with cold photolabelled peptides, samples were photoactivated for 2 min during the preincubation, followed by the addition of iodinated, photolabelled peptides and a further photoactivation for 2 min. In order to obtain relative intensities of bands for determining affinity constants, autoradiographs were scanned on a soft laser densitometer (model SL2DUV, Biomed).

Fractionation of nuclear envelopes

Nuclear envelopes were fractioned with Triton X-100 and different concentrations of salt or urea according to published methods [29,30]. Nuclear envelopes, after photoactivation with labelled peptides, were centrifuged

at 1600 × *g* for 30 min (4°C) and aliquots of the pellet were resuspended at a concentration of 0.8 mg/ml protein in the following three buffers: Triton/low salt: 2% Triton X-100, 10% sucrose, 20 mM triethanolamine-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol. Triton/high salt: 2% Triton X-100, 10% sucrose, 20 mM Mes-KOH (pH 6.0), 300 mM KCl, 2 mM EDTA and 1 mM dithiothreitol. Triton/urea: 1% Triton X-100, 4 M urea, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF. After 30 min in ice for the first two methods and 10 min at 20°C for the third method, samples were centrifuged at 1600 × *g* for 30 min. Pellet and supernatant fractions from all the procedures were analyzed by SDS-polyacrylamide gel electrophoresis as described before.

Two-dimensional polyacrylamide gel electrophoreis

Two dimensional electrophoretic analysis of proteins was carried out by the method of O'Farrell [31].

Results

Binding of the native NLS to intracellular fractions

Two nine-residue peptides containing the seven-residue native NLS for SV40 large T antigen and a known mutant NLS (see Fig. 1) were synthesized as described in Materials and Methods. Glycine residues at the N-terminus and C-terminus were added to facilitate synthesis and have been shown by others not to have any effect on transport [32,33]. The synthetic peptides were labelled at the N-terminus by a photoactivable azidosalicylic acid group which could be readily radioiodinated by the Iodogen method [24]. The iodinated, photolabelled native NLS was photoactivated in the presence of purified nuclei, nuclear envelopes or PMS preparations as described earlier and the electrophoretic analysis of the labelled proteins is shown in Fig. 2. In purified nuclei (Fig. 2(a), lane 1), a protein of molecular mass about 60 kDa (including the size of the bound NLS) is prominently labelled and in a specific manner, as demonstrated by competition studies with cold peptides. Binding of the labelled native NLS to the 60

a
Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly
128

b
Gly-Pro-Lys-Thr-Lys-Arg-Lys-Val-Gly
128

c
Thr-Leu-Lys-Lys-Leu-Pro-Leu-Ala-Val-Ala-Val-Ala-Gly-Val-Met-Thr-Ala-Ala-Met-Ala

d
Lys-Glu-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-Val-Thr-Lys-Ala

Fig. 1. Primary structures of synthetic peptides. (a) Native NLS of SV40 large T antigen [4]; (b) a mutant NLS of SV40 large T antigen in which Lys^{128} is replaced by Thr [11]; (c) signal peptide of bacteriophage λ receptor [22]; (d) signal peptide of *E. coli* alkaline phosphatase [23].

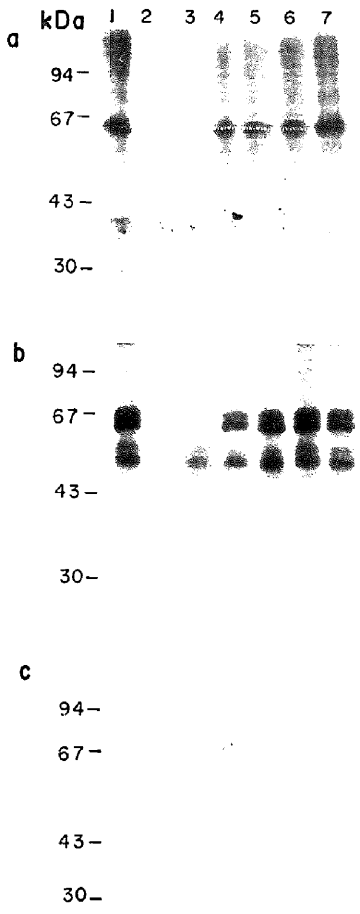

Fig. 2. Intracellular distribution of NLS-binding proteins. Cellular fractions (approx. 50 μg protein) were photoactivated in the presence of $(1–2)\cdot10^6$ cpm of iodinated, photolabelled native NLS and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Autoradiographs of dried gels are presented here. (a) Purified nuclei, labelled with native NLS (lane 1); nuclei labelled with native NLS after preincubation with unlabelled 10-fold excess native NLS (lane 2); 5-fold excess native NLS (lane 3); 10-fold excess mutant NLS (lane 4); 5-fold excess mutant NLS (lane 5); 10-fold excess λ receptor signal peptide (lane 6); and 10-fold excess alkaline phosphatase signal peptide (lane 7). (b) Purified nuclear envelopes, labelled with native NLS (lane 1); envelopes labelled with native NLS after preincubation with unlabelled peptides exactly as for nuclei (lanes 2–7). (c) PMS, labelled with native NLS (lane 1); PMS labelled with native NLS after preincubation with unlabelled 10-fold excess of native NLS (lane 2); mutant NLS (lane 3); λ receptor signal peptide (lane 4); and alkaline phosphatase signal peptide (lane 5). Molecular mass markers are: phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.

kDa nuclear protein was effectively competed out by a 5-fold or 10-fold molar excess of cold, native NLS (Fig. 2(a), lanes 3, 2). However, at these concentrations of cold mutant peptide and two unrelated sequences, there was significant labelling of the 60 kDa protein by the native NLS (lanes 4–7). In nuclear envelopes (Fig. 2(b)), four proteins of molecular masses 67, 60, 53 and 47 kDa specifically bind to the native NLS. With nuclear envelopes also, binding of the native NLS to the four binding proteins was considerably decreased in the presence of cold native NLS (lanes 2, 3) but not after incubation with cold mutant NLS or unrelated peptides (lanes 4–7). The data presented is representative of several sets of experiments that have been carried out. The 53 and 47 kDa proteins are highly unlikely to be proteolytic products of the higher molecular mass binding proteins as proteinase inhibitors were present throughout the nuclear envelope isolation [27], and the stoichiometry of labelling of the four bands did not vary significantly in different experiments.

In order to determine the extent to which the size of the bound NLS would change the actual molecular masses of the binding proteins, we have calculated the approximate ratio of peptide molecules bound to the 60 kDa nuclear envelope protein, considering that the amount of protein per gel lane is about 250 ng (4 pmol). The radioactivity incorporated into the 60 kDa protein (at a peptide concentration of approx. 10 μM, which is below saturation) was determined by cutting out the corresponding gel slice and counting it. An average value of 1200 dpm was obtained from three separate experiments (spec. act. of NLS, $2 \cdot 10^6$ dpm/nmol). This gives an approximate value of one peptide molecule bound per six molecules of the 60 kDa protein and similar values were obtained for the other proteins indicating that there is negligible addition to the actual molecular masses of the proteins. All experiments (except the kinetics of saturation) were carried out at concentrations of peptide below saturation.

The result of the photoactivation of PMS proteins with the NLS peptide is shown in Fig. 2(c). Only a faint labelling of some bands is visible, probably due to the high positive charge on the NLS, but there is no specific binding of the native N LS to any PMS protein as there is no competition with a 10-fold excess of cold native NLS. Similar results were obtained with a high-speed (100 000 × *g*) supernatant and pellet of PMS.

Binding studies with native NLS

Binding studies of the native NLS to nuclei and nuclear envelopes were carried out at different peptide concentrations and samples were analyzed by SDS-polyacrylamide gel electrophoresis as described earlier. Autoradiographs were scanned on a densitometer and intensities of specifically labelled bands were plotted as a function of peptide concentration as shown in Fig. 3.

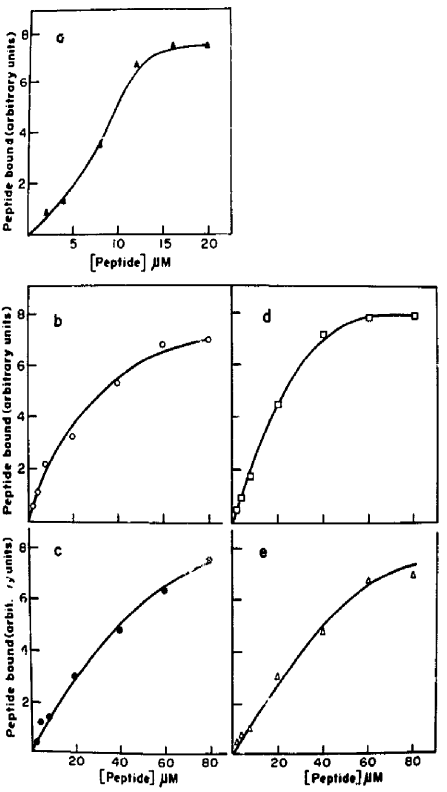

Fig. 3. Kinetics of binding of NLS to nuclear and nuclear envelope proteins. Equal amounts of protein (approx. 50 μg) were labelled with increasing concentrations of native NLS (constant specific activity). Autoradiographs were scanned; peak areas were integrated automatically and used to plot the relative amount of peptide bound for each protein band. Values are an average of three sets of experiments. (a) Nuclear 60 kDa protein; (b) nuclear envelope 67 kDa protein; (c) nuclear envelope 53 kDa protein; (d) nuclear envelope 60 kDa protein; and (e) nuclear envelope 47 kDa protein.

Saturation was achieved at low peptide concentrations (20–80 μM for different bands), indicating high affinity binding. Assuming one binding site per receptor for the NLS-containing nuclear protein for steric reasons (molecular mass of T antigen is approx. 90 kDa), the peptide concentrations at half-maximal binding approximate the dissociation constant or K_d. The nuclear 60 kDa NLS-binding protein has the highest affinity for the NLS ($K_d = 8$ μM), followed by the envelope 60 and 67 kDa proteins ($K_d = 17$ μM and 20 μM) and the envelope 47 and 53 kDa proteins ($K_d = 30$ μM and 40 μM). At present, it is not clear if the slightly sigmoidal kinetics of binding to the nuclear 60 kDa protein as compared to the envelope proteins represents a true difference in binding properties or results from other differences between the fractions.

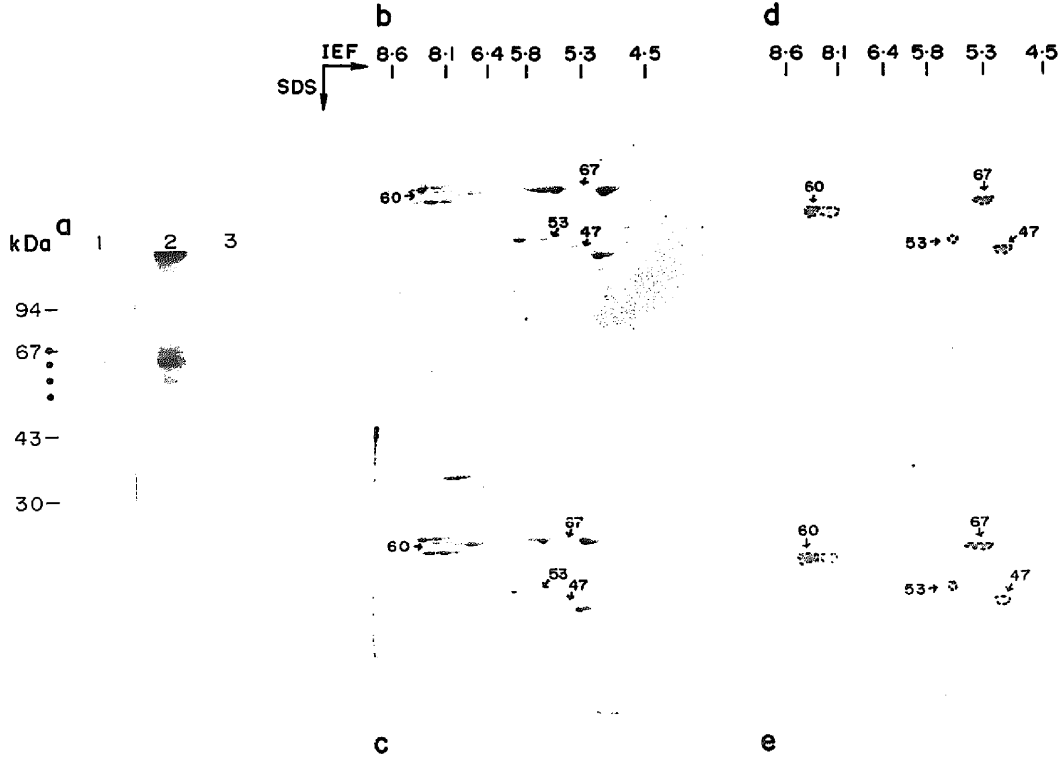

Fig. 4. Comparison of NLS-binding proteins from nuclei and nuclear envelopes. (a) Purified nuclei, labelled with native NLS, were nuclease-treated and salt-extracted to obtain nuclear envelopes. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Lane 1: labelled nuclei; lane 2: extracted nuclear envelopes; lane 3: supernatant after extraction. Positions of 67, 60, 53 and 47 kDa proteins are marked by bold dots (●). (d) Two-dimensional gel analysis and autoradiography of nuclear envelopes labelled with native NLS: and (e) envelopes obtained from nuclei labelled with native NLS. The corresponding Coomassie blue-stained gels are shown in (b) and (c).

Comparison of NLS-binding proteins from nuclei and nuclear envelopes

Since the size of the 60 kDa NLS-binding protein from nuclei coincided with that of one of the NLS-binding proteins from nuclear envelopes, we investigated the possibility that both proteins might be identical. In the first set of experiments, nuclei, after photoactivation with labelled peptide, were processed to obtain nuclear envelopes by Kaufmann's method [26]. Briefly, labelled nuclei were digested with DNAse and RNAse, followed by salt extraction with 1.6 M NaCl which removed most of the intranuclear contents [27]. Labelled nuclei, salt-extracted nuclear envelopes and the supernatant after extraction were analyzed by SDS-polyacrylamide gel

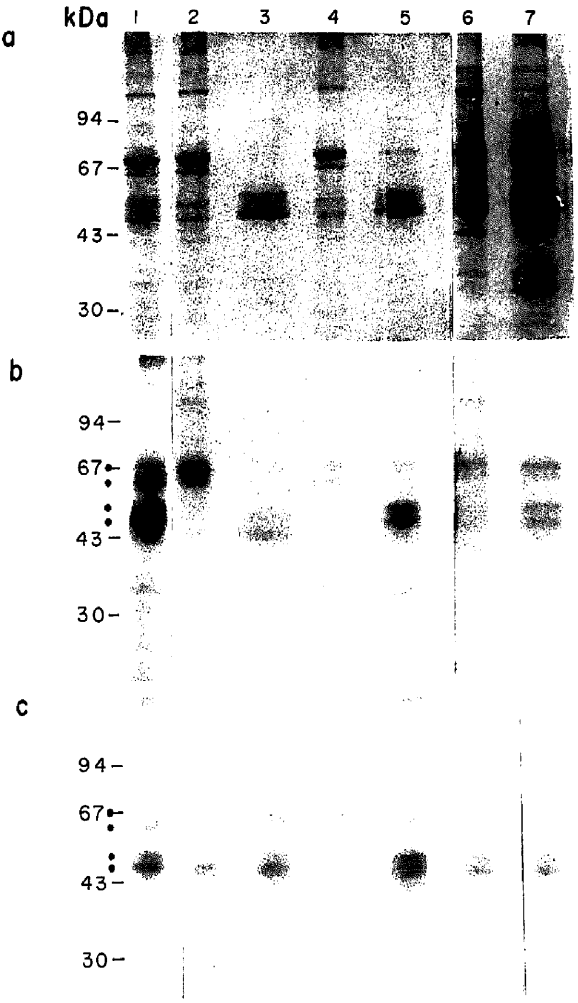

Fig. 5. Biochemical fractionation of NLS-binding proteins in nuclear envelopes. (a) Nuclear envelopes, labelled with native NLS, were fractionated as described in Materials and Methods (Coomassie blue-stained gel). Lane 1: nuclear envelopes; lanes 2 and 3: pellet and supernatant of Triton/low salt method; lanes 4 and 5: pellet and supernatant of Triton/high salt method; lanes 6 and 7: pellet and supernatant of Triton/urea method. Molecular mass markers are as indicated in the legend to Fig. 2. (b) Autoradiograph of identical gel. Positions of 67, 60, 53 and 47 kDa proteins are marked by bold dots (●). (c) Nuclear envelopes were fractionated by the Triton/urea or Triton/low salt method described earlier. Solubilized proteins were labelled with native NLS and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Triton/urea extract: Proteins in supernatant labelled with native NLS (lane 1); protein labelling after preincubation with 25-fold excess of unlabelled native NLS (lane 2) or unlabelled mutant NLS (lane 3). Triton/low salt extract: Proteins labelled with native NLS in pellet (lane 4) and supernatant (lane 5); protein labelling in supernatant after preincubation with 25-fold excess of unlabelled native NLS (lane 6) or unlabelled mutant NLS (lane 7). Positions of 67, 60, 53 and 47 kDa proteins are marked by bold dots (●).

electrophoresis as shown in Fig. 4(a). The 60 kDa NLS-binding protein present in nuclei (lane 1) is clearly retained in salt-extracted nuclear envelopes (lane 2) and is not present in the supernatant after salt extraction (lane 3). In addition, three labelled bands are visible in nuclear envelopes which coincide in size with those obtained by photoactivating nuclear envelopes with labelled peptide (67, 53 and 47 kDa). These envelope proteins may be present in lower concentrations in intact nuclei or represent low-affinity binding sites and become apparent only after enrichment of nuclear envelope proteins.

In order to confirm that the NLS-binding proteins identified in intact nuclei are identical to those present in nuclear envelopes, we have carried out a comparative two-dimensional gel analysis (isoelectric focussing in the first dimension followed by SDS-polyacrylamide gel electrophoresis in the second dimension, by O'Farrell's method [31]). The results are shown in Fig. 4(b)–(e). The labelled nuclear envelope proteins of molecular masses 67, 60, and 47 kDa have apparent p*I* 5.2, p*I* 8.4 (major spot)–p*I* 8.1 (minor spot), and p*I* 5.1 respectively (Fig. 4(d)). The labelled 53 kDa protein was faintly visible with p*I* 5.7. The corresponding labelled 60 kDa protein obtained when nuclear envelopes are isolated from labelled nuclei also has p*I* 8.4–8.1 (Fig. 4(e)). Due to the presence of large amounts of DNA in nuclei, nuclear envelopes and intact nuclei did not give a strictly comparative two-dimensional gel analysis; hence we have compared labelled nuclear envelopes and nuclear envelopes obtained from labelled nuclei.

Localization of nuclear envelope NLS-binding proteins

In order to localize the NLS-binding proteins to different compartments of the nuclear envelope, photoactivated nuclear envelopes were extracted with Triton X-100 and different concentrations of salt or urea by three procedures as described in Materials and Methods. Fractionation of envelopes with 2% Triton X-100 in a low salt buffer (Triton/low salt method) solubilizes the proteins of the outer membrane and peripherally-bound proteins but keeps the pore complex proteins intact [29]. In the Triton /high salt method, most of the pore proteins are solubilized by a combination of 2% Triton X-100, 0.3 M KCl, EDTA and low pH buffer of 6.0 [29]. The Triton/urea method is essentially similar to the Triton/high salt procedure in its effectiveness in solubilizing pore complex proteins, especially in the range of 60–70 kDa [30]. The Coomassie blue-stained gel in Fig. 5(a) illustrates the pattern of protein solubilization by these three fractionation procedures. From the data presented in Fig. 5(b), the labelled 53 and 47 kDa are mostly extracted by the Triton/low salt buffer (lane 3) and may represent outer membrane/peripherally bound proteins. The labelled 60 and 67 kDa are partly extracted by the Triton/high salt method (lane 5) and more effectively by the Triton/urea method (lane 7). Thus the 60 and 67 kDa proteins can be localized in the pore complex fraction of the nuclear envelope.

Binding properties of solubilized nuclear envelope proteins

In order to determine whether the NLS could bind to solubilized binding proteins with the same specificity or whether a membrane location was essential for specific recognition, NLS-labelling experiments were carried out with solubilized nuclear envelope proteins. Nuclear envelopes were extracted by two methods: Triton/low salt and Triton/urea, as described in Materials and Methods and the pattern of solubilized protein is given in Fig. 5(a), lanes 3, 7. The solubilized proteins were photoactivated in the presence of labelled NLS and the samples were analyzed by SDS-polyacrylamide gel electrophoresis as described earlier. The results are shown in Fig. 5(c). The native NLS binds to the 67, 60, 53 and 47 kDa proteins present in the Triton/urea extract (lane 1) and to the 53 and 47 kDa proteins in the Triton/low salt extract (lane 5). However, when the solubilized proteins were preincubated with unlabelled native or mutant NLS followed by photoactivation with labelled native NLS, the labelling of the solubilized proteins was not inhibited (Fig. 5(c), lanes 2, 3, 6, 7; only marginal inhibition is seen in lane 2). This suggests that the labelling of the solubilized proteins by the NLS is non-specific or of low affinity, unlike the specific, high affinity labelling of the membrane-bound receptors by native NLS observed in intact envelopes.

Discussion

We have identified nuclear envelope-bound proteins that specifically recognize a prototype NLS, the SV40 large T antigen NLS. Since short NLS peptides have been shown to target proteins to the nucleus, our strategy has been to use a synthetic nine-residue peptide containing the NLS and to attach at the N-terminal an iodinatable photolabel (NHS-ASA) which produces a relatively short-lived nitrene radical [25], to facilitate specific labelling of binding proteins. The iodinated, photolabelled NLS-peptide was photoactivated in the presence of mouse liver nuclei, nuclear envelopes and PMS and samples were analyzed by SDS-polyacrylamide gel electrophoresis in order to locate proteins covalently linked to the labelled peptide. NLS-binding proteins were identified by demonstrating specific, saturable binding to a native NLS which could not be competed out by a mutant NLS or unrelated peptides.

Properties of NLS-binding proteins

In nuclei, a protein of molecular mass 60 kDa specifically binds to the native NLS. We have also identified four proteins in nuclear envelopes (67, 60, 53

and 47 kDa) that bind to the native NLS specifically. In order to establish that binding of the NLS to envelope proteins is functionally significant and not an artefact, we have demonstrated that: (1) when photoactivated nuclei are processed to obtain nuclear envelopes, the 60 kDa protein is retained on the envelopes and in addition three more proteins of molecular masses 67, 53 and 47 kDa are seen; (2) the 60 kDa NLS-binding protein detected in nuclei is identical to the nuclear envelope 60 kDa protein by two-dimensional gel analysis. We have conclusively shown the identity of the nuclear 60 kDa NLS-binding protein and the nuclear envelope 60 kDa NLS-binding protein, which was not clear from earlier studies [15,17,18].

We have demonstrated that binding of the NLS to nuclear and nuclear envelope binding proteins shows saturation kinetics (K_d range 8–40 μM for different proteins), which strongly indicates specific binding. Moreover, the binding constant for the 60 kDa nuclear protein ($K_d = 8$ μM) is in the same range as that obtained for the kinetics of transport of bovine serum albumin-conjugated synthetic NLS decapeptides ($K_d = 2$ μM) into the nucleus [12], although higher than that obtained for the binding of the 34-residue large T antigen peptide to nuclear proteins (approx. 0.1 μM) [15]. However, since these serum albumin conjugates are specifically localized in the nucleus, the nonapeptide we have used should be sufficient in size for specific binding to proteins which may be required for selective nuclear transport.

Localization of NLS-binding proteins

We have localized the NLS-binding proteins by biochemical fractionation of envelopes. The 53 and 47 kDa proteins appear to be peripherally bound to the outer membrane whereas the 60 and 67 kDa proteins are most probably part of the pore complex. Although the nuclear lamins also have molecular masses in the range of 60–70 kDa, we have clearly shown that the 60 and 67 kDa NLS-binding proteins are not lamins as they can be solubilized from the envelopes with low concentrations of salt or urea and their p*I* values are different from those of the lamins [26]. We have also observed that an antilamin B antibody does not block the binding of the NLS to these proteins (data not shown). Although the solubilized NLS-binding proteins retain the ability to bind to the native NLS, their affinity for the native NLS is lower when compared to that in intact nuclear envelopes. This suggests that the location of the NLS-binding proteins in the nuclear envelope is an important feature for specific signal recognition. Competition data from other studies also suggest that the solubilized receptors have a lesser affinity for the T antigen NLS [18]. In a recent article [17], the authors have inferred the presence of NLS-binding proteins of molecular masses 59 and 69 kDa in nuclear pores and 65, 54 and 50 kDa in nuclear envelope extracts by an indirect method, using antibodies raised to putative receptor sequences (anti-DDDED). In another study [18], NLS-binding proteins of 76, 67, 59 and 58 kDa have been detected on nuclear envelopes by an assay based on indirect immunofluorescence. Our results are consistent with these findings. At present, on the basis of their interaction with monoclonal antibodies, the proteins that have been proposed to be part of the pore structure have molecular masses of 190 kDa [34]; 62 kDa [35]; 210, 180, 145, 100, 63, 58 and 45 kDa [29]. The available evidence on the ultrastructure of the nuclear pore suggests that it is a highly complex proteinaceous structure, composed of several different polypeptides [2].

Additional requirements for nuclear transport

Recently, Adam et al. [15] have used a synthetic large T antigen NLS-peptide and a cross-linking reagent, disuccinimidyl suberate, to identify a high-affinity 60 kDa binding protein in nuclei, which is also present in cytosol, though to a lesser extent, and have argued for a role of the cytosolic protein in nuclear transport. Although we have identified a similar protein in nuclei and nuclear envelopes, our results on the binding of the native NLS to PMS proteins indicate there are no proteins which specifically bind to the NLS in this fraction. The cytosolic 60 kDa protein in [15] may have a weak affinity for the NLS which we are unable to detect. Yoneda et al. [17] were also unable to detect any cytosolic binding proteins by immunoprecipitation with anti-DDDED antibodies. In an in vitro nuclear transport system using proteins translated from SP6 plasmid-generated RNAs, which we have recently described [36], we do not observe any requirement for cytosolic factors for specific transport of nuclear proteins. The requirement for cytosolic factors for nuclear transport in in vitro systems with purified nuclear proteins has been shown only in the case of nucleoplasmin [37] and not for SV40 large T antigen and other nuclear proteins [18,38]. Cytosolic proteins which bind nonspecifically to the native NLS of T antigen as well as to a modified NLS with poor transport function have also been described, but their role in nuclear transport is unclear [16,33].

Although a requirement for an energy source has been shown for translocation of proteins across nuclear pores, ATP does not appear to be required for the initial binding of karyophilic proteins to the pore complex (which can occur at 0°C and in the absence of ATP) [13,14]. In our studies, addition of ATP did not enhance binding of the NLS to nuclear proteins (data not shown), as observed by other investigators also [15,18]. Similarly wheat germ agglutinin, which has been shown to bind certain nuclear envelope proteins [29,37] but not to the same sites to which the SV40 T antigen

NLS binds [13], did not affect the binding of the NLS peptides to envelope proteins in our study (data not shown).

Our data is compatible with a model for selective nuclear transport of proteins in which the NLS of the protein initially binds with high affinity to one or two proteins at the nuclear pore (60, 67 kDa). If these sites are occupied, more molecules of the karyophilic protein may bind with lesser affinity to adjacent proteins (53, 47 kDa). This is consistent with the rapid accumulation of nuclear proteins at the pores observed in recent ultrastructural studies on nuclear transport [13,14]. In the second stage, recognition of the NLS at the nuclear pore may lead to the subsequent translocation of the nuclear protein through the pore in an energy-dependent manner, and the concomitant 'gating' of the channel [1].

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