

Identification of a Human Protein That Interacts with Nuclear Localization Signals

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Abstract. Through a series of label transfer experiments, we have identified a HeLa cell nuclear protein that interacts with nuclear localization signals (NLSs). The protein has a molecular weight of 66,000 and an isoelectric point of ~ 6 . It associates with a synthetic peptide that contains the SV-40 T antigen NLS peptide but not with an analogous peptide in which an asparagine is substituted for an essential lysine (un-NLS peptide). In addition to these peptides, several proteins have been tested as label donors. With the proteins, there is a correlation between nuclear localization (assayed with lysolecithin-permeabilized cells)

and label transfer to the 66-kD protein. The NLS peptide (but not the un-NLS peptide) competes with the proteins in label transfer experiments, but neither wheat germ agglutinin nor ATP has an effect. These results suggest that the 66-kD protein functions as an NLS receptor in the first step of nuclear localization. In the course of this work, we have observed that the *Staphylococcus aureus* protein A is a strongly karyophilic protein. Its dramatic nuclear localization properties suggest that it may have multiple copies of an NLS.

MOST nuclear proteins with molecular weights greater than $\sim 20,000$ are transported into the nucleus through a process that requires the recognition of a signal sequence (for reviews see Dingwall and Laskey, 1986; Newport and Forbes, 1987). Nuclear localization signals (NLSs)¹ have been analyzed from a number of proteins in yeast (Hall et al., 1984; Silver et al., 1984; Moreland et al., 1985, 1987; Pinkham et al., 1987), *Xenopus* (Burglin and De Robertis, 1987), viruses (Kalderon et al., 1984; Richardson et al., 1986; Wychowski et al., 1986; Jones et al., 1986; Lyons et al., 1987), and mammals (Lee et al., 1987; Stone et al., 1987; Picard and Yamamoto, 1987; Dang and Lee, 1988; Greenspan et al., 1988). In general, the signal sequences contain short regions of basic amino acids punctuated by proline residues. The most extensively studied signal is the NLS of the SV-40 T antigen, which has the sequence Pro-Lys-Lys₁₂₈-Lys-Arg-Lys-Val. By appending this sequence to other proteins, either through the construction of chimeric proteins or by the cross-linking of synthetic peptides, proteins that are normally restricted to the cytoplasm are efficiently transported into the nucleus (Lanford et al., 1986; Kalderon et al., 1984; Goldfarb et al., 1986; Yoneda et al., 1987a). Analyses of point mutations (Kalderon et al., 1984; Colledge et al., 1986; Yoneda et al., 1987a) have shown that the central Lys₁₂₈ is essential for this activity.

The mechanism by which proteins are segregated into the

nucleus is poorly understood, but it is clear that the mechanism must be fundamentally different than for protein transport into other cellular compartments. Because mitosis requires the disintegration of the nucleus and its subsequent reformation in the daughter cells, the localization signals associated with nuclear proteins must be retained for more than a single cycle of nuclear entry. Indeed, the NLSs that have been identified are internal and function independently of their position in the primary structure of the protein (Roberts et al., 1987). This contrasts with the segregation of proteins into mitochondria, chloroplasts, or the endoplasmic reticulum, which usually involves the recognition of amino-terminal signal sequences that are removed from the proteins by proteolytic cleavage.

The import of nuclear proteins can be divided into two stages. First, the protein binds to a recognition site on the outside of the nucleus and then, in the second step, it is internalized (Newmeyer and Forbes, 1988; Richardson et al., 1988). The second step requires the hydrolysis of ATP and is temperature sensitive. It is also inhibited by wheat germ agglutinin (WGA), an inhibitor of nuclear transport that binds to nuclear pore complexes (Yoneda et al., 1987b; Finlay et al., 1987; Dabauvalle et al., 1988; Newmeyer and Forbes, 1988; Richardson et al., 1988).

It is generally thought that the transport of proteins into the nucleus as well as the export of RNAs out of the nucleus occurs at nuclear pores (Newmeyer and Forbes, 1988; Richardson et al., 1988). Although a considerable amount is known about the structure of the nuclear pore complex, little is known about the proteins that are actually involved

1. **Abbreviations used in this paper:** ASD, 2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate radical; D-PBS, Dulbecco's PBS; NLS, nuclear localization signal; SASD, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate; WGA, wheat germ agglutinin.

in the transport process or how they function. As a step toward understanding the mechanism of nuclear transport, we have identified a nuclear protein that interacts with NLSs in what appears to be the first step of the transport process. Two proteins with similar properties have recently been identified in rat liver nuclei (Adam et al., 1989).

Materials and Methods

The synthetic NLS peptide (Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly) and the analogous un-NLS peptide (Cys-Gly-Tyr-Gly-Pro-Lys-Asn-Lys-Arg-Lys-Val-Gly-Gly) were synthesized by the peptide chemistry laboratory of the Department of Chemistry, New York University (New York). The plasmid pSKS106 (Casadaban et al., 1983) was a gift from M. Casadaban (University of Chicago, Chicago, IL). The plasmid pNBG, which encodes the NLS- β -galactosidase fusion protein, was constructed by inserting the synthetic oligonucleotide ATCCGCCGAAGAAAACGTAAGGTA between the Bam HI and Kpn I sites of the pUC19 polylinker. The region of the polylinker between the Hind III and Eco RI sites was then inserted between the analogous sites in pSKS106. The resulting plasmid encodes the NLS sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Pro-Ser-Ser. Bacteria carrying either pNBG or pSKS106 were induced as described (Casadaban et al., 1983). The protein was released by sonicating the bacteria in 20 mM Tris-HCl, 10 mM MgCl₂, pH 7.4, followed by a 10-min, 10,000-g centrifugation. The supernate was adjusted to 1.6 M NaCl, 10 mM 2-mercaptoethanol and passed through a *p*-aminobenzyl-1-thio- β -D-galactopyranoside agarose (Sigma Chemical Co., St. Louis, MO) affinity column in a procedure similar to that of Ullmann et al. (1984). After washing the column with the same buffer, protein was eluted with 0.1 M sodium borate, 10 mM 2-mercaptoethanol, 1 mM MgCl₂, pH 10. Fractions containing β -galactosidase activity were detected colorimetrically with *o*-nitrophenyl- β -D-galactopyranoside. The resulting proteins were ~90% pure as judged by SDS-PAGE. Normal *Escherichia coli* β -galactosidase was obtained from Sigma Chemical Co. The IgG binding domain of *Staphylococcus aureus* protein A was obtained by inducing *E. coli* transformed with pRIT2T (Nilsson et al., 1985) and was purified by IgG affinity chromatography (Nilsson et al., 1985). Normal *S. aureus* protein A was obtained from Sigma Chemical Co.

Preparation of HeLa Cell Extracts

HeLa cells grown to a density of 5×10^5 cells/ml in Joklik's modified minimum essential medium supplemented with 5% bovine calf serum were centrifuged at 500 g for 5 min and washed with PBS (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2). For HeLa cell extracts, the cell pellet was resuspended in 20 vol ice-cold resuspension buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl₂, pH 7.4), centrifuged at 500 g for 5 min, resuspended at a concentration of 1.5×10^8 cells/ml in buffer A (resuspension buffer containing 20 μ M cytochalasin B, 20 μ g/ml aprotinin, 0.5 mM PMSF, 0.5 mM DTT), and then disrupted by sonication. Extracts were stored at -70°C. HeLa cell nuclei were prepared by resuspending the cell pellet in buffer A containing 0.3% NP-40 and homogenizing with a homogenizer (Dounce; Kontes Glass Co., Vineland, NJ). They were then sedimented at 500 g for 5 min, washed with buffer A, and resuspended in buffer A at a concentration of 1.5×10^8 nuclei/ml. Sonicated nuclei were obtained by sonicating nuclei prepared as described above until ~95% were disrupted. Nuclear extracts were prepared by centrifuging the disrupted nuclei at 100,000 g for 30 min in an ultracentrifuge (TL-100; Beckman Instruments, Inc., Fullerton, CA).

Preparation of Labeled Proteins and Peptides

Iodogen-coated tubes were prepared by placing 100- μ l portions of Iodogen (Pierce Chemical Co., Rockford, IL) dissolved in chloroform (0.4 mg/ml) at the bottoms of 1.5-ml microfuge tubes and evaporating the chloroform under nitrogen. The tubes were capped and stored at -20°C until use. All further steps were done under dim light. An Iodogen-coated microfuge tube was washed once with 0.2 M sodium phosphate, pH 7.5; excess buffer was removed by pipette; and 5 μ l 0.2 M sodium phosphate, pH 7.5, 0.1 mCi Na¹²⁵I (100 mCi/ml), and 1 μ l of 1 mg/ml sulfo-succinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD; Pierce Chemical Co.) dissolved in dimethylformamide were added rapidly in succession at room temperature. After 30 s, the mixture was removed and immediately added to an ice-cold microfuge tube containing 1 μ l of 5 M NaI and 5 μ l of either

protein (5 mg/ml in 0.1 M sodium phosphate, pH 7.5) or peptide (0.25 nmol in 0.1 M sodium phosphate, pH 7.5). This was incubated on ice for 30 min followed by the addition of 90 μ l of 0.25 M NaI, 50 mM sodium phosphate, pH 7.5. Unreacted iodine and SASD were removed by passing the mixture through a PBS-equilibrated column of Sephadex G-25 superfine (for proteins) or Bio-gel P2 (for peptides). The labeled peptide or protein eluted in the void volume, well separated from free iodine and unreacted SASD.

Label Transfer Experiments

Under dim light, 130 pmol ¹²⁵I-2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate radical (ASD)-labeled peptide or 1 μ g ¹²⁵I-ASD-labeled protein (50,000-100,000 cpm) in PBS was added to the extract to be tested (in PBS, prepared from 6×10^5 cells) in a total volume of 20 μ l. After incubating on ice in the dark for 15 min, cross-linking was achieved by placing the sample, in ice, 2 cm below a 15-W germicidal lamp and irradiating for 1 min. In preliminary experiments, we found that these conditions resulted in the photolysis of 90% of the SASD as determined spectrophotometrically (Vanin and Ji, 1981).

For analysis on one-dimensional gels, one half of the sample was added to an equal volume of 4% SDS, 20% glycerol, 125 mM Tris-HCl, pH 6.8. The other half was added to an equal volume of the same buffer containing 0.1 M DTT. The samples were boiled for 3 min and then electrophoresed through 12% SDS-PAGE gels (Laemmli, 1970). For separations in two dimensions, samples containing whole-cell extracts, nuclei, or sonicated nuclei were first digested with DNase I (50 μ g/ml) for 10 min on ice. To each 10- μ l sample, the following were added: 14.5 mg urea, 1.25 μ l 2 M DTT, 1.25 μ l ampholines (pH 3-10; LKB Instruments, Inc., Gaithersburg, MD), and 2.5 μ l 20% NP-40. In the first dimension, the samples were separated by isoelectric focusing in 130 \times 2-mm tube gels as described (O'Farrell, 1975) except the gels contained only ampholines of pH 3-10 (2%). In the second dimension, the proteins were separated on 12% SDS-PAGE gels (Laemmli, 1970).

Diffusing Proteins into Lysolecithin-permeabilized Cells

For fluorescence studies of nuclear localization, BSC-1 cells grown on coverslips were used. To permeabilize the cells, a coverslip with adhering cells was briefly rinsed by dipping into a beaker containing cold Dulbecco's PBS (D-PBS; Sigma Chemical Co.) and then immersed in a beaker containing 50 μ g/ml lysolecithin (Sigma Chemical Co.) in D-PBS at 4°C. After 2 min, the coverslip was washed twice with D-PBS, and excess solution was removed by blotting. A 10- μ l sample, containing 1-3 mg/ml of FITC-labeled protein and rhodamine B-dextran 70 (Sigma Chemical Co.) in D-PBS was applied, and the coverslip was incubated at 37°C in a humid chamber. After 15 min, the coverslip was washed twice with D-PBS, the side without cells was rinsed free of salt, and the coverslip was mounted on a slide with D-PBS and viewed immediately. No fixation was used.

To permeabilize cells for *in vivo* label transfer experiments, HeLa cells, grown and washed with D-PBS as described above, were resuspended at a density of 3×10^6 cells/ml in ice-cold D-PBS containing 80 μ g/ml lysolecithin. After 2 min, the cells were washed three times by pelleting the cells at 500 g for 5 min and resuspending them in cold D-PBS. Protein labeled with ¹²⁵I-SASD (15 μ g) was then added to 10^7 cells in a total volume of 0.35 ml and incubated on ice in the dark for 30 min. The cells were then irradiated with UV as described above, washed three times with D-PBS, and resuspended in resuspension buffer containing 0.1 mM PMSF, 0.5% Triton X-100, and 100 μ g/ml DNase I. After incubating for 30 min on ice, SDS sample buffer (Laemmli, 1970) was added, and a portion of the sample was analyzed by PAGE.

Results

We have used label transfer experiments to identify a HeLa cell nuclear protein that interacts with NLSs. The bifunctional cross-linking reagent SASD was used in these experiments. This reagent contains a photoreactive azido group that can be labeled by iodination with Na¹²⁵I. The azido group is linked through a disulfide to an *N*-hydroxy-succinimide that can be used for the modification of a peptide or protein. In the experiments described here, we have modified

proteins and peptides, both with and without nuclear localization properties, and have used the modified peptides and proteins as probes for the identification of closely interacting cellular proteins.

Modification of Proteins with SASD

Several procedures have been developed for iodinating SASD and modifying ligands with it (Allen et al., 1986; Sorensen et al., 1986; Knutson, 1987; Shephard et al., 1988). Most of these procedures, however, use nonaqueous solvents and are not appropriate for the modification of proteins. Therefore, as a part of this work, we have investigated several procedures for iodinating SASD and modifying proteins with it. The procedure that we have found to work the best is similar to the procedure recently developed by Shephard et al. (1988). We find that it is necessary to iodinate the SASD before modification of the protein to minimize the labeling of protein tyrosines. This is undesirable since it leads to an excessive amount of background radioactivity in the label transfer experiments. We have used Iodogen-coated microfuge tubes to catalyze the iodination since the reaction can be stopped rapidly by removing the solution from the tube. The use of soluble catalysts is undesirable since reducing agents, which are added to stop the iodination, cleave the disulfide of the SASD. Since the rate of hydrolysis of *N*-hydroxysuccinimide is significant, the iodination must be done rapidly and must be followed immediately by modification of the protein. This makes it impractical to separate iodinated SASD from free iodine before protein modification. It is also important that the volumes be kept to a minimum since the efficient modification of the protein is favored by a high concentration of SASD. Although a pH of 8.5 is better for the modification of proteins, we have used a pH of 7.5 for the modification of the peptides. At this pH, the SASD reacts primarily with the amino terminus rather than with the lysines, which are required for function.

Identification of a Protein That Interacts with an NLS

To identify proteins that interact with NLSs, the NLS peptide (Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly), which contains the SV-40 T antigen NLS, was synthesized. This peptide has previously been shown to confer nuclear localization properties on proteins to which it is cross-linked (Lanford et al., 1988). Another peptide, the un-NLS peptide (Cys-Gly-Tyr-Gly-Pro-Lys-Asn-Lys-Arg-Lys-Val-Gly-Gly), has an asparagine at position seven in place of lysine and is incapable of directing nuclear localization (Lanford et al., 1988).

For label transfer experiments, the NLS and un-NLS peptides, conjugated to ¹²⁵I-SASD as described above, are mixed with extracts prepared from HeLa cells and cross-linked to associated proteins by irradiation with UV. The transfer of the ¹²⁵I label to the recipient protein is then completed by reducing the disulfide of the ASD. There is no change in the charge and only a small increase in the mass of the labeled recipient protein. In Fig. 1, it can be seen that upon UV cross-linking with the ¹²⁵I-ASD-NLS peptide, a protein with an apparent molecular weight of ~66,000 becomes labeled (lane 5). The label can be transferred to this protein by reducing the disulfide of the ASD with DTT (lane 1). In the absence of UV irradiation, the NLS peptide is not associated

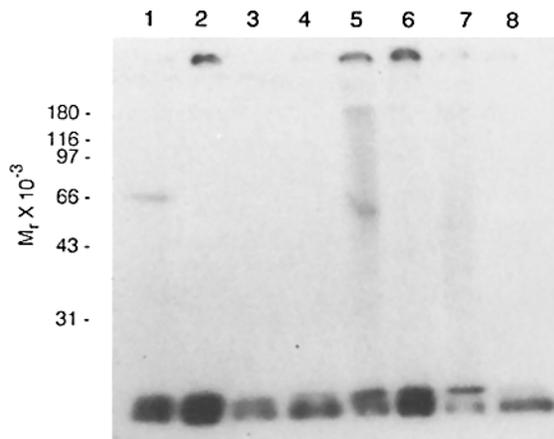


Figure 1. Identification of a protein that interacts with the NLS peptide. ¹²⁵I-ASD-NLS peptide (lanes 1, 2, 5, and 6) or ¹²⁵I-ASD-un-NLS peptide (lanes 3, 4, 7, 8) was mixed with sonicated nuclei and exposed to UV for 1 min (lanes 1, 3, 5, and 7) or kept in the dark (lanes 2, 4, 6, and 8). The samples were mixed with SDS sample buffer either with 100 mM DTT (lanes 1-4) or without (lanes 5-8), and separated on a 12% Laemmli gel (Laemmli, 1970).

with this protein (lane 6), and no label is transferred to it (lane 2). In parallel experiments using the un-NLS peptide, it is seen that the ¹²⁵I-ASD-un-NLS peptide is not cross-linked by UV, nor is label transferred from this peptide to the 66-kD protein.

The DTT-reduced protein migrates slower (lane 1) than the protein in the absence of reducing agent (lane 5). Apparently, the 66-kD protein contains one or more disulfides that constrains the denatured protein. A dramatic effect of the reducing agent is seen in lane 5, where the curved band is due to the presence of DTT in the adjoining lane. Most other proteins, visualized by staining with Coomassie blue (not shown), do not show this difference in mobility. The biological implications of this observation are not clear; since the cell maintains reducing conditions, disulfides that are formed outside of the cell may not be present in vivo.

Results that are qualitatively the same are obtained with sonicated whole HeLa cell extracts, isolated nuclei, sonicated nuclei (shown in Fig. 1), or the supernatant of a high speed (100,000 g) centrifugation of sonicated nuclei. The amount of label transferred is somewhat greater when the whole cell extract is used as judged from the amount of label transferred to the receptor. This may be due to a loss of receptor during the isolation of nuclei.

The protein released from sonicated nuclei sediments through sucrose gradients at <10 S and behaves as a monomer when analyzed by gel filtration or electrophoresis through nondenaturing polyacrylamide gels. These results suggest that, if the protein is associated with the filamentous structures emanating from nuclear pores that have been identified by Richardson et al. (1988), the process of sonication results in their disruption and monomerization.

It is possible to introduce significant amounts of proteins or peptides into living cells that have been permeabilized by lysolecithin (Miller et al., 1985). By carefully titrating the concentration of lysolecithin, conditions can be obtained under which >90% of the cells are permeable to proteins as large as IgG and β -galactosidase (as assayed by immunoflu-

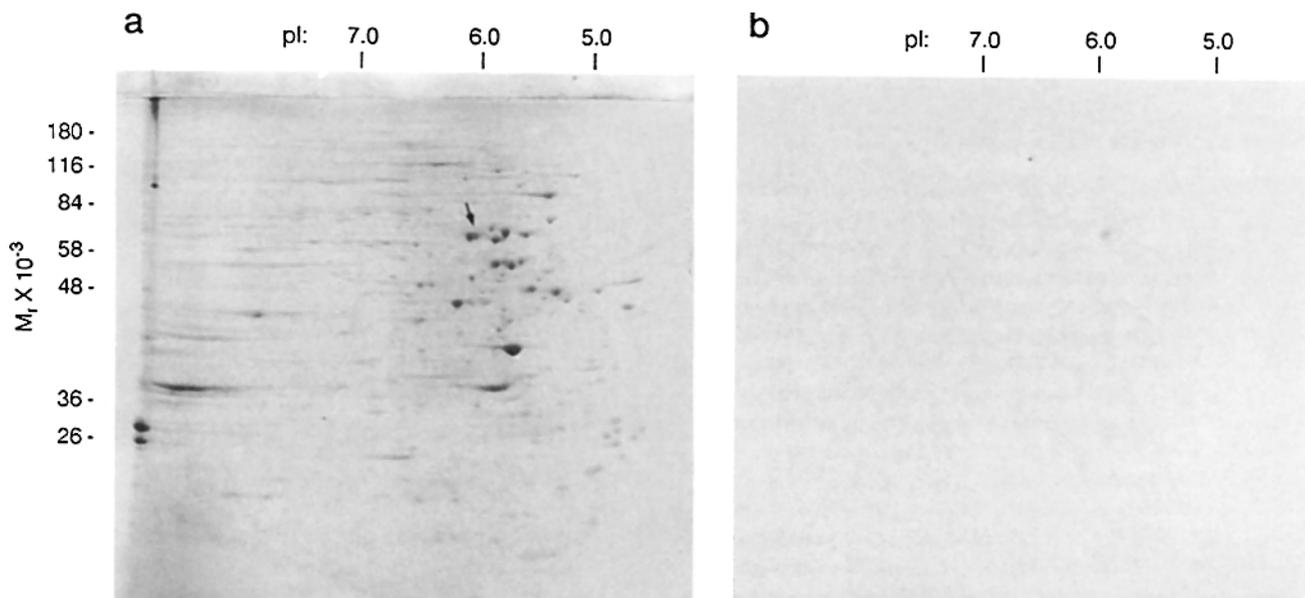


Figure 2. Two-dimensional analysis of a protein that interacts with the NLS peptide. (a) Coomassie brilliant blue-stained gel; (b) an autoradiogram of the same gel. The arrow in a indicates the position of the spot seen in b. Sonicated nuclei were UV cross-linked with ^{125}I -ASD-NLS peptide, reduced with sample buffer containing 100 mM DTT, and separated by isoelectric focusing in the first dimension and SDS-PAGE in the second.

orescence) while >90% of the cells remain viable (as determined by their ability to grow for several generations). The optimal lysolecithin concentration is dependent on the cell type. For HeLa cells grown in spinner culture, optimum results are obtained when the cells are treated with 80 $\mu\text{g/ml}$ lysolecithin as described in Materials and Methods. When ^{125}I -ASD-NLS or ^{125}I -ASD-un-NLS is added to permeabilized cells and UV cross-linked after a 15-min incubation at 37°C, the same results as shown in Fig. 1 are obtained.

In all of the above experiments, it is observed that label is transferred from the ^{125}I -ASD-NLS peptide (but not from the ^{125}I -ASD-un-NLS peptide) to a protein with a molecular weight of $\sim 66,000$. To better characterize this protein, ^{125}I was transferred from ^{125}I -ASD-NLS peptide to interacting proteins present in a nuclear extract, and the mixture was resolved by two-dimensional gel electrophoresis. A gel with isoelectric focusing in the first dimension and SDS-PAGE in the second is shown in Fig. 2, where it is seen that the label is transferred to an acidic protein of 66 kD. No label is seen at this position if the ^{125}I -ASD-un-NLS peptide is used or if UV cross-linking is omitted (not shown). By comparing the migration of the protein in the first dimension with the migration of internal standards added to the sample before isoelectric focusing, we estimate that the protein has a pI of ~ 6 . The position of the radioactive spot seen on the autoradiogram of the gel (Fig. 2 b) coincides with a reasonably prominent spot on the Coomassie blue-stained gel (Fig. 2 a). Although the labeled protein and the protein that is seen on the stained gel may be the same, we can not rule out the possibility that the labeled protein comigrates with another abundant protein on these two-dimensional gels, particularly since the spot is very close to another prominent protein spot. When a narrower range of ampholines (pH 5–8 rather than pH 3–10) is used for isoelectric focusing in the first dimension, the labeled protein still comigrates with the pro-

tein indicated in Fig. 2 (not shown). The narrower range of ampholines, however, gives only a slight enhancement in resolution in this region of the gel.

Label Transfer from Proteins

To examine the possibility that the cross-linking of the ^{125}I -ASD-NLS peptide to the 66-kD protein might be an artifact of the small size or other attribute of the peptide, a β -galactosidase fusion protein containing the SV-40 T antigen NLS near the amino terminus was constructed. A synthetic oligonucleotide encoding this NLS was inserted into the polylinker of the vector pSKS106. This vector expresses β -galactosidase under control of the *lac* promoter. Both β -galactosidase and the fusion protein can be easily purified by passing a sonicated extract of induced bacteria through an affinity column of *p*-aminobenzyl 1-thio- β -D-galactopyranoside agarose in a procedure similar to that described by Ullmann (1984) for an analogous affinity gel.

When label transfer experiments done with β -galactosidase or NLS- β -galactosidase are resolved on one-dimensional gels as in Fig. 1, the data are complicated by the occasional appearance of a degradation product of β -galactosidase that migrates close to the 66-kD receptor protein. For this reason, experiments with β -galactosidase have been analyzed with two-dimensional gels.

The results of experiments in which sonicated nuclear extracts have been probed with the ^{125}I -ASD-modified proteins are shown in Fig. 3. It is evident that after UV cross-linking, the label can be transferred to an acidic protein of 66 kD. This protein migrates at the same position as the one identified through the use of the SASD-modified NLS peptide. A second labeled protein that migrates at the position of β -galactosidase can also be seen on these gels. This arises from the transfer of label to residues on the β -galactosidase

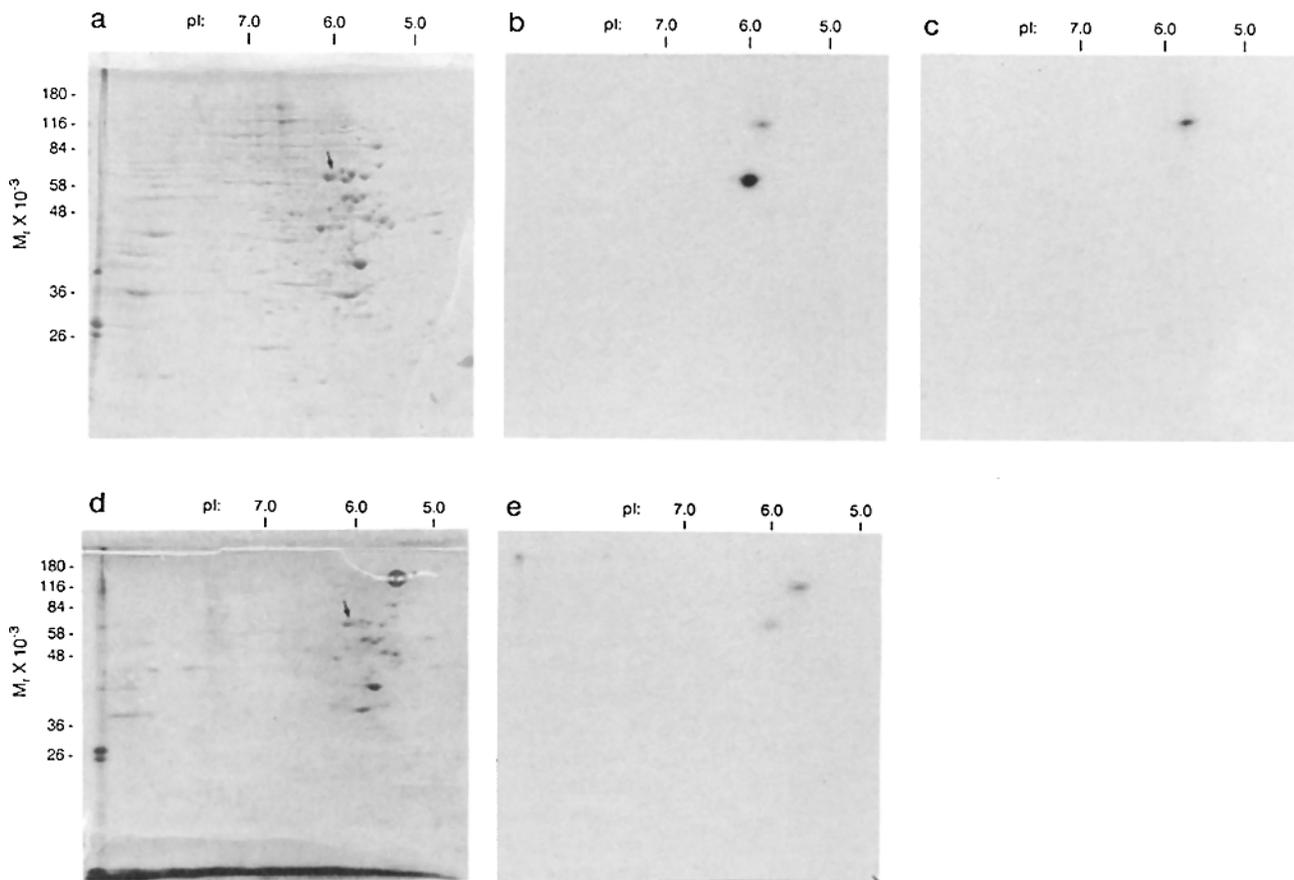


Figure 3. Identification of a protein that interacts with NLS- β -galactosidase. ^{125}I -ASD-NLS- β -galactosidase (*a* and *b*), ^{125}I -ASD-NLS- β -galactosidase that was preexposed to UV for 2 min (*c*), or ^{125}I -ASD- β -galactosidase (*d* and *e*) that was UV cross-linked with sonicated nuclei and separated as described in Fig. 2. Coomassie blue-stained gels are shown in *a* and *d*; autoradiograms of these gels are shown in *b* and *e*. The gel shown in *c* was run simultaneously with the gel shown in *a*. The distribution of proteins was almost identical in the two gels. The arrows in *a* and *d* indicate the positions of the spots seen in the autoradiograms.

and from a small amount of iodination of the β -galactosidase tyrosines during the modification with ^{125}I -SASD. When the label transfer experiment is done with ^{125}I -ASD-NLS- β -galactosidase that has been irradiated with UV for 2 min before its addition to the HeLa cell extract, only the spot at the position of β -galactosidase is seen (Fig. 3 *c*). In the absence of UV cross-linking, no label is transferred, and when the proteins are resolved in the absence of DTT or 2-mercaptoethanol, only high molecular weight products are seen (not shown).

The results of a label transfer experiment in which β -galactosidase was used in place of the NLS- β -galactosidase fusion protein is shown in Fig. 3, *d* and *e*. The β -galactosidase protein expressed by pSKS106 was purified, modified with ^{125}I -SASD, and cross-linked with a portion of the same nuclear extract that was used in the NLS- β -galactosidase experiments shown in Fig. 3, *a-c*. Both experiments were done in parallel under the same conditions. It is seen that a small amount of label is transferred to the 66-kD protein, but the amount is much less than with NLS- β -galactosidase. Densitometry of the autoradiograms shows that about ten times more label is transferred when the fusion protein is used as when normal β -galactosidase is used. The same results are obtained in label transfer experiments with normal *E. coli*

β -galactosidase (not shown). Although there is a striking difference between NLS- β -galactosidase and β -galactosidase, it is curious that any of the β -galactosidase is cross-linked. One possible explanation is that β -galactosidase contains either a very weak NLS or a signal that is masked by secondary or tertiary structure.

In light of the results with β -galactosidase and, as described below, protein A, it was of interest to examine eukaryotic proteins that are not nuclear and therefore would not be expected to interact with the NLS receptor. As shown in Fig. 4, neither wheat germ lectin, bovine carbonic anhydrase, nor hen ovalbumin direct the transfer of ASD to the 66-kD receptor. The proteins were labeled with ^{125}I -SASD by the same protocol as described for NLS- β -galactosidase and β -galactosidase and reacted with a HeLa cell nuclear extract under the same conditions. The radioactivity that is associated with the proteins results from a slight labeling of the tyrosines by residual activated ^{125}I during the modification with ^{125}I -SASD and also from intramolecular transfer of label during UV cross-linking. Even after a long exposure, no transfer of label to the 66-kD receptor can be detected from any of these proteins. These data show that these nonnuclear proteins transfer label at least an order of magnitude less efficiently than the NLS-containing proteins described here.

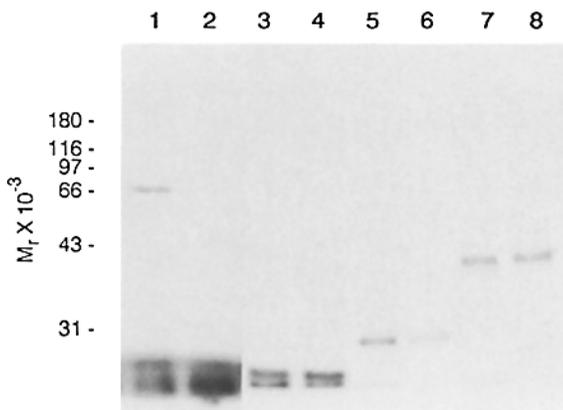


Figure 4. Interactions with proteins that are not nuclear. ^{125}I -ASD-NLS peptide (lanes 1 and 2), ^{125}I -ASD-WGA (lanes 3 and 4), ^{125}I -ASD-bovine carbonic anhydrase (lanes 5 and 6), or ^{125}I -ASD-hen ovalbumin was incubated in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of sonicated nuclei, exposed to UV, reduced, and analyzed by SDS-PAGE as described for Fig. 1, lane 1.

The nuclear localization of NLS- β -galactosidase and β -galactosidase were further investigated by observing FITC-labeled proteins that were either microinjected into cells or transferred into lysolecithin-permeabilized cells. The results of these experiments (Fig. 5) parallel those of the label transfer experiments. As expected, the NLS- β -galactosidase readily enters the nucleus (Fig. 5, *d-f*). However, we often see that some β -galactosidase also accumulates in the nucleus (Fig. 5, *a-c*). Although we have not identified the molecular basis of this phenomenon, it is possible that it involves the recognition of a weak or partially masked signal on β -galactosidase.

In light of the unexpected results obtained with β -galactosidase, the nuclear localization of bovine IgG was investigated. In contrast to β -galactosidase, we very rarely see nuclear localization when FITC-labeled IgG is transferred into lysolecithin-permeabilized cells (Fig. 5, *j-l*) or microinjected. In label transfer experiments with bovine IgG, we find that there is no detectable transfer of label to the 66-kD protein (not shown). This result suggests that the label transfer and nuclear localization observed with β -galactosidase, although much smaller than with NLS- β -galactosidase, is significant.

Protein A Contains an NLS

We were surprised to find that FITC-labeled protein A of *S. aureus* rapidly concentrates in the nucleus when it is diffused into lysolecithin-treated cells (Fig. 4, *g-i*). It also rapidly localizes in the nucleus when microinjected. Since protein A has a molecular weight of 42,000, near the size limit of proteins that are able to diffuse into the nucleus, we explored the possibility that it enters the nucleus by diffusion rather than transport. FITC-protein A-coated colloidal gold was prepared (Slot and Geuze, 1985) and added to lysolecithin-permeabilized cells. As with free protein A, large amounts of the protein A-coated colloidal gold rapidly accumulate in the nucleus. These results indicate that protein A contains one or more NLSs.

To further investigate this, protein A was conjugated with

^{125}I -SASD and used as a probe for NLS receptor proteins as described above. As shown in Fig. 6, label is transferred to the same 66-kD acidic protein that we identified in the experiments with the NLS peptide and NLS- β -galactosidase.

The plasmid pRIT2T (Nilsson et al., 1985) expresses a truncated protein A that contains the five IgG binding domains but lacks much of the amino terminus and the carboxy-terminal cell wall attachment region. This protein, when labeled with FITC and introduced into lysolecithin-treated cells, shows the same strong nuclear localization properties as does protein A (not shown). The pRIT2T-derived protein is also just as effective as protein A in label transfer experiments. These results suggest that the NLS resides in the IgG binding domain which is repeated five times. It has been shown in previous experiments that the nuclear localization properties of a protein increase with the number of NLSs that are present in the protein (Lanford et al., 1986; Dworetzky et al., 1988).

Competition experiments show that the signal on protein A that is being recognized by the 66-kD protein is functionally related to the SV-40 T antigen NLS. As shown in Fig. 7, unlabeled NLS peptide competes with the transfer of label from ^{125}I -ASD-NLS peptide but un-NLS peptide, even at a 240-fold excess has little, if any, effect. When the relative amounts of label transferred in the presence of unlabeled NLS peptide are determined by densitometry of Fig. 7, lanes 1-6, it is seen that about a 30-fold excess of unlabeled NLS peptide over ^{125}I -ASD-NLS peptide is required to reduce the amount of label transferred by half. Since these experiments are done with concentrated solutions of sonicated nuclei (3×10^7 nuclei/ml), a high concentration of nuclear proteins is present. It is likely that these proteins are in competition with the NLS peptide for NLS receptors. The relatively high concentration of unlabeled NLS peptide that must be added to compete with label transfer from the ^{125}I -ASD-NLS peptide is probably a reflection of competition with this complex mixture of NLS-containing proteins for the receptor. It is clear, however, that the un-NLS peptide, which contains a single amino acid difference, is a poor competitor compared with the NLS peptide. From Fig. 7, lanes 10-15, it is seen that the transfer of label from either ^{125}I -ASD-NLS- β -galactosidase or ^{125}I -ASD-protein A is also inhibited by the addition of unlabeled NLS peptide. The addition of the same amount of unlabeled un-NLS peptide has no effect on the transfer of label from any of these proteins.

Label Transfer Is Not Inhibited by WGA and Does Not Require ATP

Nuclear localization requires the hydrolysis of ATP and is inhibited by WGA (Newmeyer and Forbes, 1988; Richardson et al., 1988). However, NLS-containing proteins bind at the nuclear envelope in the absence of ATP or in the presence of WGA. It has been surmised that a step subsequent to the binding of a protein by the receptor is sensitive to these agents.

From Fig. 8 it can be seen that the addition of WGA to nuclear extracts has no effect on the transfer of label to the 66-kD protein. In these experiments, the extract was incubated with WGA for 15 min before the addition of either ^{125}I -ASD-NLS peptide (lane 2) or ^{125}I -ASD-protein A (lane 6) and UV cross-linking. Also shown in Fig. 8, is the lack of effect of ATP on the label transfer experiments. The addition

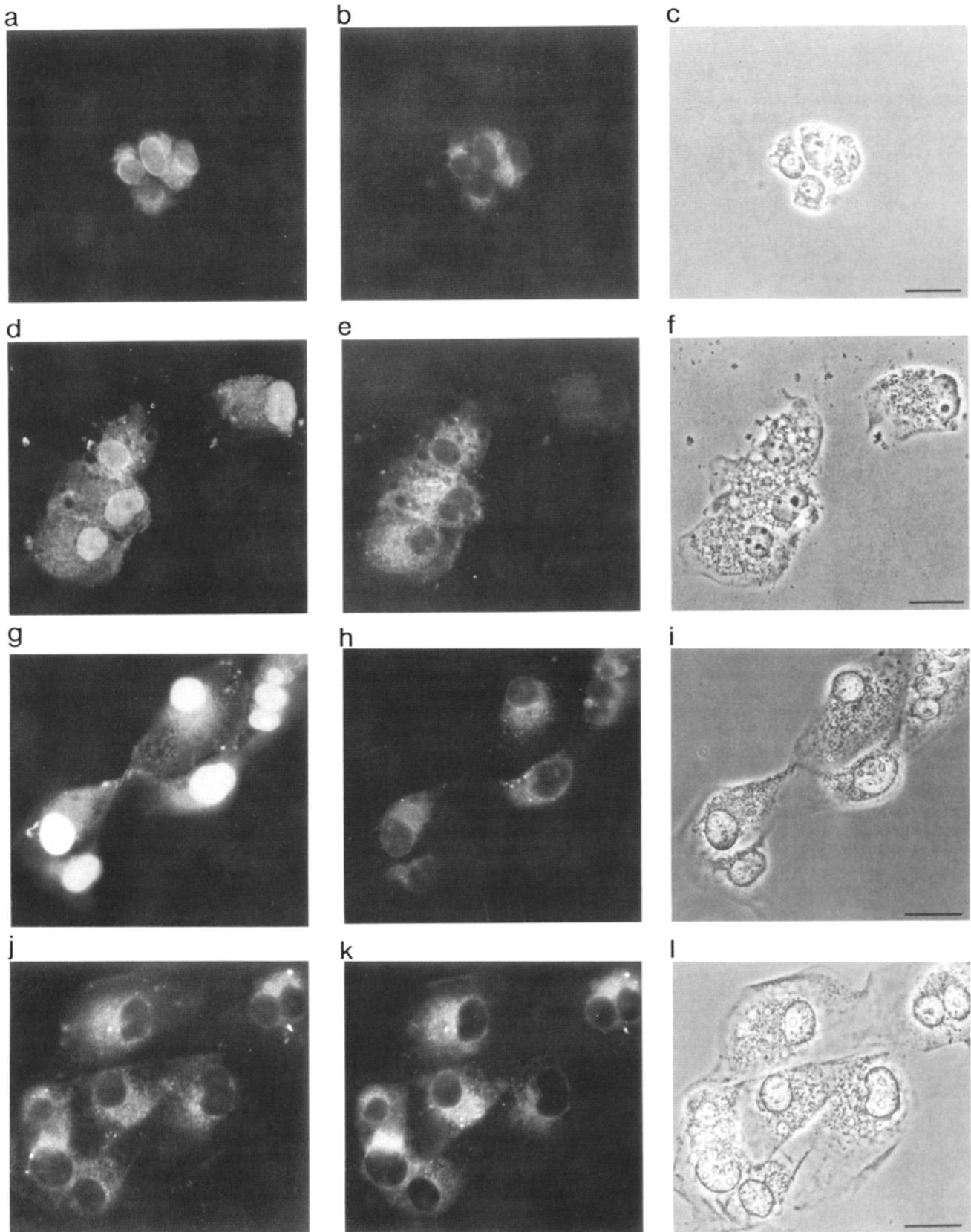


Figure 5. Nuclear localization of proteins applied to lysolecithin-permeabilized cells. Experiments with β -galactosidase (*a-c*), NLS- β -galactosidase (*d-f*), protein A (*g-i*), or bovine IgG (*j-l*) are shown. Localization of FITC-labeled proteins is shown in the first column (*a, d, g, and j*); localization of rhodamine-labeled dextran 70, which was mixed with each protein before adding to the cells, is shown in the second column (*b, e, h, and k*); phase-contrast micrographs of the cells are shown in the third column (*c, f, i, and l*). Bars, 15 μ m.

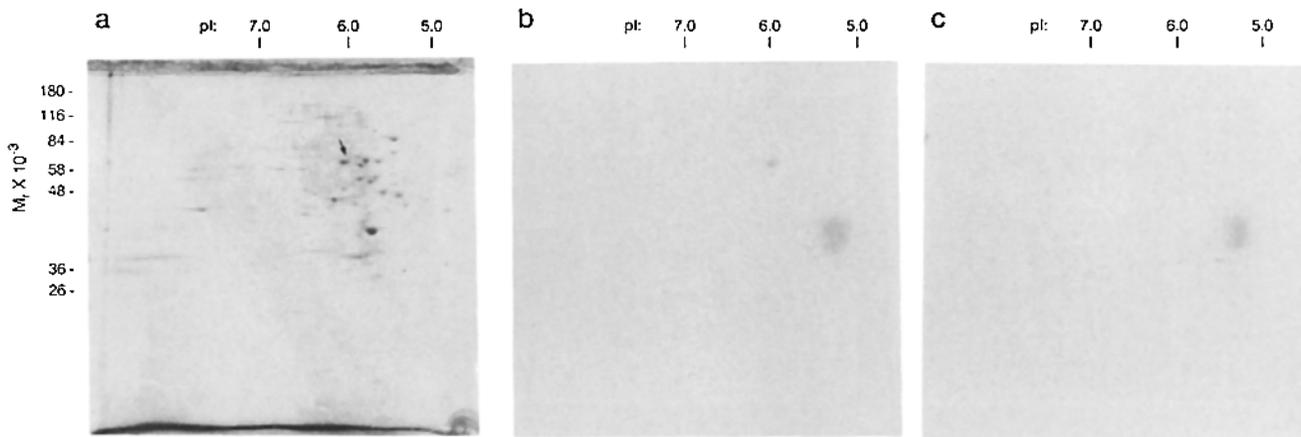


Figure 6. Identification of a protein that interacts with protein A. ^{125}I -ASD-protein A (*a* and *b*) or ^{125}I -ASD-protein A that was preexposed to UV for 2 min (*c*) was cross-linked with a sonicated nuclear extract. The arrow in *a* indicates the position of the spot seen in *b*. The samples were prepared as in Fig. 3.

of ATP with an ATP-regenerating system (Fig. 8, lanes 3 and 7) actually reduces the extent of label transfer. This reduction, however, may be due to the presence of a protease contaminant in the creatine kinase that is added as a part of the regeneration system. The high lysine content of the NLS peptide would make it particularly susceptible to trypsin activities. There is no effect when ATP is added without the regeneration system. Magnesium is also included in these experiments, but as shown in separate studies (not shown), it has no effect. The depletion of ATP by the addition of glucose plus glucokinase also has no effect on label transfer to the 66-kD protein (lanes 4 and 8).

Discussion

Through a series of label transfer experiments, we have identified a HeLa cell nuclear protein with a molecular weight of 66,000 that closely associates with NLSs and has properties expected of protein involved in nuclear transport. Recently, Adam et al. (1989) have identified two proteins from

rat liver nuclei that interact with the SV-40 NLS. The proteins, when conjugated to a reporter peptide, have molecular weights of 60,000 and 70,000; the smaller protein is much more abundant than the larger one. Since these proteins have been irreversibly cross-linked to a peptide with a molecular weight of $\sim 4,500$, the molecular masses of the proteins in the absence of peptide are 56 and 66 kD, respectively. In the experiments that are reported here, the receptor is identified by label transfer experiments so the peptide is not associated with the protein during electrophoresis. The HeLa cell protein that we have identified is ~ 66 kD, as judged by the fact that it comigrates with BSA, which was used as a molecular weight marker. This is the same molecular weight as the larger of the two rat proteins identified by Adam et al. (1989). We have seen no evidence for the presence of a smaller protein of ~ 56 kD. However, we occasionally detect a protein of $\sim 45,000$ molecular weight in sonicated nuclear extracts that interacts with the NLS peptide. We believe that this protein is a product of proteolysis since the amount increases with the age of the extract and PMSF inhibits its formation.

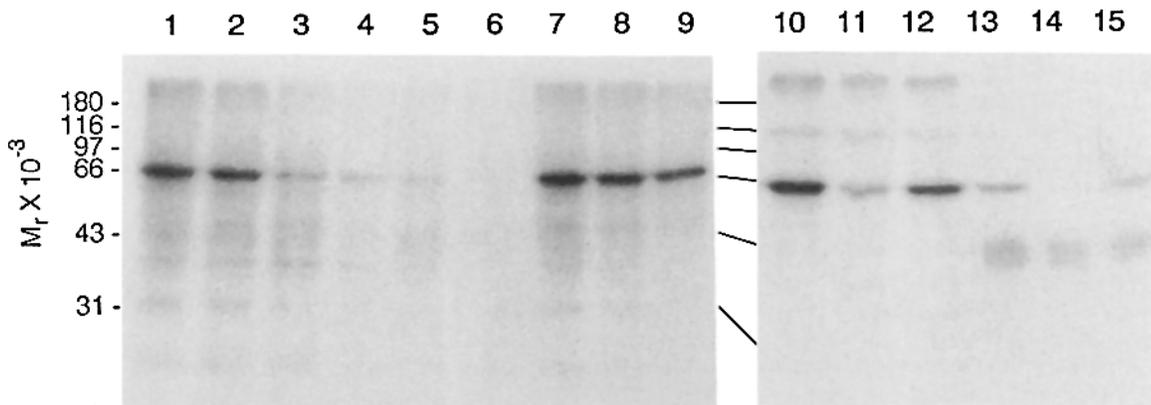


Figure 7. Competition experiments with the NLS peptide. ^{125}I -ASD-NLS peptide (90 pmol) and either unlabeled NLS peptide (0, 1.1, 3.6, 5.5, 11, or 22 nmol in lanes 1-6, respectively) or unlabeled un-NLS peptide (5.5, 11, or 22 nmol in lanes 7-9, respectively) were used in label transfer experiments with sonicated nuclei from 6×10^5 cells in a total volume of $40 \mu\text{l}$ as described in Fig. 1. $1 \mu\text{g}$ ^{125}I -ASD-NLS- β -galactosidase (lanes 10-12) or ^{125}I -ASD-protein A (lanes 13-15) was mixed with either no peptide (lanes 10 and 13), 15 nmol NLS peptide (lanes 11 and 14), or 15 nmol un-NLS peptide (lanes 12 and 15), then cross-linked with sonicated nuclei from 6×10^5 cells in a total volume of $20 \mu\text{l}$, and analyzed as described for Fig. 1, lane 1.

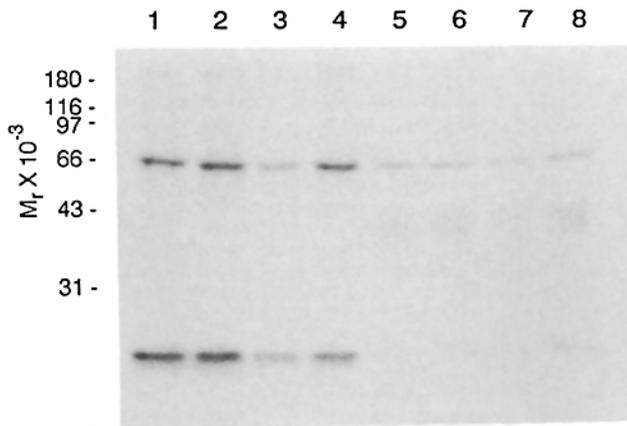


Figure 8. The effect of ATP and WGA. Sonicated nuclei were treated for 15 min with nothing additional (lanes 1 and 5); WGA (0.1 mg/ml; lanes 2 and 6); ATP (1 mM), phosphocreatine (10 mM), and creatine kinase (20 U/ml) (lanes 3 and 7); or with glucose (20 mM) and hexokinase (40 U/ml) (lanes 4 and 8). ^{125}I -ASD-NLS peptide (lanes 1-4) or ^{125}I -ASD-protein A (lanes 5-8) was then added and treated as for Fig. 1, lane 1.

It is possible that the two proteins identified by Adam et al. (1989) may also be related by proteolysis, although protease inhibitors were included in isolation. It is also possible that the differences that we observe simply represent differences between the two species.

Several observations support the idea that the 66-kD protein identified in this report is directly involved in the process of nuclear localization. We have shown that the un-NLS peptide, in which the lysine that corresponds to Lys_{128} is replaced by an asparagine, is completely inactive in the label transfer experiments. It is well documented that Lys_{128} of the SV-40 T antigen is essential for the nuclear localization properties of this protein (Kalderon et al., 1984; Colledge et al., 1986; Yoneda et al., 1987a) and that the substitution of asparagine for Lys_{128} abolishes the nuclear localization of the peptide (Lanford et al., 1988). We have also shown that while the NLS peptide competes with ^{125}I -ASD-NLS- β -galactosidase and ^{125}I -ASD-protein A for label transfer, the un-NLS peptide has no competitive effect. Thus, a specific alteration of the NLS has the same dramatic effect on both nuclear localization properties of the peptide and on its interaction with the 66-kD protein.

The results obtained with the *S. aureus* protein A are also interesting in this respect. We have made the observation that protein A rapidly accumulates in the nucleus when it is either diffused into lysolecithin-permeabilized cells or microinjected. The nuclear localization is dramatic, both in terms of the rate of transport and the amount of protein that enters the nucleus. This suggests that protein A contains one or more NLSs. Although we have not identified the signal, it is present in the fragment that is expressed by pRIT2T. This fragment contains five IgG binding domains with similar sequences (Nilsson et al., 1985). Lanford et al. (1986) and Dworetzky et al. (1988) have shown that increasing the number of NLSs contained in a protein leads to an increase in its nuclear uptake. It is possible that the dramatic nuclear localization properties of protein A are due to the presence of an NLS in each of its five domains.

In addition to its nuclear localization properties, we find through label transfer experiments that protein A interacts with the same 66-kD protein identified by cross-linking with the ^{125}I -ASD-NLS peptide. Also, the competition experiments suggest that the 66-kD receptor is recognizing structures in protein A that are homologous with the SV-40 T antigen NLS. An examination of the sequence of pRIT2T, however, fails to show any striking homology with the SV-40 T antigen NLS sequence. It is possible that the nuclear localization machine does not interact with a particular amino acid sequence but recognizes a higher-order structure that can be assembled from any one of a number of different primary sequences. This is supported by the fact that although the NLSs that have been elucidated so far are rich in basic amino acids, a well-defined consensus sequence is not apparent.

The results of experiments done with β -galactosidase also support this conclusion. As shown by immunofluorescence, β -galactosidase with no inserted NLS sequence enters the nucleus of lysolecithin-treated cells, although to a far lesser extent than does the NLS- β -galactosidase fusion protein. Under the same conditions, bovine IgG is completely excluded from the nucleus. Others have also observed that a small amount of β -galactosidase is transported into the nucleus of mammalian cells (Stacey and Alfrey, 1984; Kalderon et al., 1984), although this does not appear to be the case in yeast (Hall et al., 1984; Silver et al., 1984; Moreland et al., 1985, 1987; Pinkham et al., 1987) or in *Xenopus* oocytes (Burglin and De Robertis, 1987). The results obtained from label transfer experiments using these proteins parallel those obtained from the nuclear localization experiments: IgG is inactive; NLS- β -galactosidase transfers label efficiently; and some label can be transferred from β -galactosidase, but the efficiency is about tenfold less than with NLS- β -galactosidase. These results again demonstrate a correlation between nuclear localization and interaction with the 66-kD protein.

Since β -galactosidase and protein A are both bacterial proteins, there is no apparent function for an NLS. However, the presence of such a signal should not be particularly deleterious to a prokaryote and, apparently, has not been selected against. NLSs may, in fact, be statistically rather common and may be widespread in bacteria and in proteins that are exported. In eukaryotes, these apparently common signals would be deleterious if present in cytoplasmic proteins; it is probably just as essential for the cell to exclude NLSs from cytoplasmic proteins as it is to encode them in proteins destined to become nuclear.

Recent evidence suggests that there are at least two events in the process of nuclear localization (Richardson et al., 1988; Newmeyer and Forbes, 1988). In the first step, a protein containing an NLS binds to the periphery of the nucleus. This step does not require the hydrolysis of nucleoside triphosphates and is not inhibited by WGA. In the second step, the protein is transported into the nucleus. This step requires the hydrolysis of ATP and is inhibited by WGA. The mechanism by which these two events are related is not known. The interaction between ligands and the NLS receptor that we have described here does not require ATP and is not inhibited by WGA, suggesting that the receptor may be involved in the first step of the translocation process.

We have also shown that the receptor can be solubilized

by sonication. The soluble protein behaves as a monomer and, therefore, is not tightly associated with large complexes such as nuclear pores or fragments of the nuclear envelope. A fibrillar structure extends from both sides of the nuclear pore complex into the cytoplasm and nucleus (Richardson et al., 1988). The fibers are ~3 nm in diameter and extend ~200 nm from the nuclear pores. These fibers have been implicated as being involved in nuclear transport since nucleoplasmin-coated gold particles have been shown to associate with them (Richardson et al., 1988). Although any relationship between these fibers and the 66-kD protein identified in this paper remains to be demonstrated, it is possible that sonication could cause their disruption and solubilize the receptor.

In the work reported here, label transfer experiments are used extensively. This is a well-documented approach that has been used for the identification of a number of receptor proteins (Allen et al., 1986; Sorensen et al., 1986; Knutson, 1987; Shephard et al., 1988). In most of the previous studies, small ligands or peptides were modified with the label transfer reagent using nonaqueous solvents. In this paper, we present experiments in which proteins are used as the label donor. In the course of this work, several methods for preparing the ¹²⁵I-SASD-modified proteins were investigated, with optimum results being obtained with the procedure described here.

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