Yeast Proteins that Recognize Nuclear Localization Sequences

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Abstract. A variety of peptides can mediate the localization of proteins to the nucleus. We have identified yeast proteins of 70 and 59 kD that bind to nuclear localization peptides of SV-40 T antigen, Xenopus nucleoplasmin, and the yeast proteins Gal4 and histone H2B. These proteins are assayed by the binding of peptide-albumin conjugates to proteins immobilized on nitrocellulose filters. These binding proteins fractionate with nuclei and are extractable with salt but not detergent. Radiolabeled peptide-albumin conjugates also bind to isolated nuclei; the binding is saturable and can be extracted with salt. Different nuclear localization peptides compete with each other, implying that a single class of proteins is responsible for their recognition. The 70- and 59-kD proteins have the properties expected for a receptor that would act to direct proteins to the nucleus.

A distinct set of proteins is localized to the nucleus. By one model, transport of proteins from the cytoplasm into the nucleus is triggered by specific interaction of a short amino acid sequence (termed a nuclear localization sequence [NLS]) within the transported protein and a receptor, perhaps at the nuclear pore. This model is supported by the existence of discrete nuclear localization sequences within nuclear proteins (Dingwall et al., 1982; Kalderon et al., 1984a; Lanford and Butel, 1984; Silver et al., 1984; Hall et al., 1984). These sequences are necessary for specific transport across the nuclear envelope and are sufficient to cause nonnuclear proteins to enter the nucleus.

Recent evidence argues for the existence of an apparatus that would recognize nuclear localization sequences and transport proteins into the nucleus. (a) Uptake of proteins into Xenopus oocyte nuclei is saturable (Goldfarb et al., 1986); (b) Depletion of ATP blocks nuclear protein accumulation both in vivo (Newmeyer et al., 1986a) and in vitro (Markland et al., 1987; Newmeyer and Forbes, 1988), consistent with specific transport requiring energy. (c) Nuclear protein uptake can be separated into at least two steps: binding at the nuclear envelope followed by ATP-dependent translocation through the pore (Richardson et al., 1988; Newmeyer and Frobes, 1988).

Genetically or chemically conjugated peptides (derived from nuclear proteins) direct nonnuclear proteins to the nucleus (for example, see Kalderon et al., 1984b; Silver et al., 1984; Lanford et al., 1986). These peptides contain many basic amino acids, but otherwise have little sequence similarity. One of the best characterized nuclear localization sequences is PKKRRKV, found in SV-40 T antigen between amino acids 126 and 132 (Kalderon et al., 1984b; Roberts et al., 1987). We have previously shown that the SV-40 T antigen NLS functions in the yeast, Saccharomyces cerevisiae, to direct normally cytoplasmic proteins to the nucleus (Nelson and Silver, 1989). Moreover, a single amino acid change in the SV-40 T antigen NLS reduces its function in animal cells (Kalderon et al., 1984a; Lanford and Butel, 1984), as well as in yeast (Nelson and Silver, 1989). From these results, we conclude that the recognition of nuclear localization sequences in yeast is similar to that of mammalian cells.

Other, similarly defined nuclear localization sequences are found in Gal4 (Silver et al., 1984; Nelson and Silver, 1989), Xenopus nucleoplasmin (Dingwall et al., 1988), and yeast histone H2B (Moreland et al., 1987). Each of these sequences has been defined as important for nuclear protein localization by a variety of experiments; gene fusions between the SV-40, nucleoplasmin, Gal4, H2B nuclear localization sequences and nonnuclear proteins such as β-galactosidase encode proteins that are localized to the nucleus (Kalderon et al., 1984a; Dingwall et al., 1988; Silver et al., 1984; Moreland et al., 1987). In addition, the SV-40 T antigen NLS can be chemically linked to nonnuclear proteins and these hybrids are efficiently nuclear localized when introduced into animal cells (Lanford et al., 1986).

The diversity of sequences that can direct proteins to the nucleus (reviewed in Goodson and Silver, 1989) suggests that multiple receptor proteins might recognize different classes of localization sequences. Alternatively, a single receptor could interact with a diverse set of sequences. We now report the identification of yeast nuclear proteins that specifically bind the SV-40 T antigen NLS, as well as nuclear localization sequences derived from Xenopus nuclear protein and two yeast nuclear proteins.

Materials and Methods

Conjugation and Labeling of Peptides

Peptides corresponding to nuclear localization sequences were chemically conjugated to human serum albumin (HSA) and used as probes for nuclear import and binding to nuclear proteins. All peptides were synthesized by a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City,
To prepare yeast nuclei, a cell lysate was fractionated on a gradient. The pellet contained the nuclei, while the supernatant contained cytoplasm and other organelles as determined microscopically and biochemically for the presence of cytoplasmic and mitochondrial enzymes. Nuclei were prepared from strains ABYSI (MATa lea2 ura3 trpl ade2 his3 cytl::HIS3) or W303 (MATa leu2 ura3 trpl ade2 his3 cytl::HIS3) as described by Allen and Douglas (1989). In brief, cells (2 x 10^8 cells/ml) were hydrolyzed to spheroplasts by treatment with lysozyme 60,000 (ICN Radiochemicals, Irvine, CA) and glusulase (Dupont Co., Wilmington, DE), pelleted, and resuspended in lysis buffer (18% Ficoll/400/20 mM PIPES, pH 6.8/0.5 mM MgCl2/1 mM PMSF/10 mg/ml leupeptin, pepstatin, and 1-Tosylamide-2-phenyl-ethyl chloromethyl ketone (TPCK)). After vortexing at top speed for 3 min, the resulting cell lysate was applied to a glycerol/Ficoll gradient (40, 30, and 20% glycerol cushions) and the nucleus-depleted fraction (Fig. 4A) corresponds to the supernatant from the 23,000 g at 4°C. The resulting nuclear pellet was washed twice and resuspended in 18% Ficoll/buffer A and then viewed by fluorescence microscopy. For experiments with isolated nuclei, nuclei were mixed with 50 mM Tris-Cl, pH 7.5/50 mM NaCl followed by dialysis with 10 mM Na-phosphate, pH 7.4.

Preparation of Yeast Nuclei

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In Vitro Nuclear Localization

To assay nuclear association, the uptake of TRITC-labeled SV-40–HSA by crude or purified nuclei was examined by fluorescence microscopy. Semi-permeable yeast cells from yeast strain ABYSI were prepared by conversion to spheroplasts with zymolyase. The spheroplasts were pelleted and resuspended in 18% Ficoll in buffer A at 30°C, collected by centrifugation at 15,000 g for 10 min, resuspended in 5% BSA/18% Ficoll/buffer A, and transferred to a fresh tube, centrifuged for 10 min at 15,000 g, and the amount of bound and unbound protein determined by counting samples in the gamma counter.

Protein Blots

Interactions between nuclear localization peptides and yeast proteins were detected by a Western blot type assay (Burnette, 1981). Samples of cell lysate and/or purified nuclei were transferred to nitrocellulose, and the resulting protein was probed with 125I-protein HSA. Samples to be analyzed for NLS binding activity were solubilized in gel sample buffer, heated at 90°C for 5 min, and electrophoresed on a 10% SDS polyacrylamide gel (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose with a transfer unit (model PE82; Hoefer Scientific Instruments, San Francisco, CA) in 24 mM Tris/190 mM glycine at 4°C for 12 h. After transfer, the nitrocellulose blot was blocked with 5% BSA in buffer A for 2 h at room temperature and washed once in 0.5% Tween 20 and 1% NP-40. Nuclei-depleted fraction (Fig. 4A) corresponds to the supernatant from the 23,000 g at 4°C. The resulting nuclear pellet was washed twice and resuspended in 18% Ficoll/buffer A and then viewed by fluorescence microscopy. For experiments with isolated nuclei, nuclei mixed with 50 mM Tris-Cl, pH 7.5/50 mM NaCl followed by dialysis with 10 mM Na-phosphate, pH 7.4.

Results

To generate biochimical probes for nuclear localization in vitro, different nuclear localization peptides and either the radioactive label 125I or the fluorescent label TRITC were chemically linked to HSA. Radioactive labeling of the peptide–HSA conjugate rather than the peptide alone amplifies the signal associated with each substrate molecule. In these experiments, protein conjugates (Fig. 1) are referred to by their source protein: SV-40–HSA refers to the SV-40 large T antigen nuclear localization peptide, SV-40*–HSA to a mutant form of the SV-40 peptide with the lysine converted to threonine, NP–HSA to a peptide derived from the Xenopus nucleoplasm, and H2B–HSA to a peptide from the yeast histone H2B. These NLS–HSA conjugates were used in binding assays to detect the presence of proteins that function in nuclear import.

Nuclear Localization Peptide Conjugates Bind to Isolated Yeast Nuclei In Vitro

The SV-40 T antigen nuclear localization sequence is recognized by yeast nuclei in vitro. A peptide with the sequence CTPPKRRKV (Fig. 1), was chemically cross-linked to HSA (Goldfarb et al., 1986). Most of the resulting population of peptide–protein conjugates had 15–20 peptides conjugated per molecule of albumin as determined by relative radioactivity.
Figure 2. Association of TRITC SV-40–HSA with yeast nuclei in vitro. Semipermeable yeast cells from yeast strain ABYSI were prepared and mixed with TRITC SV-40–HSA as described in Materials and Methods (A–C). Nuclei from strain (W303) were prepared as described in Materials and Methods and mixed with TRITC SV-40–HSA (D–F) or TRITC SV-40*–HSA (G–I) with ATP (2 mM) in buffer A (50 mM Tris–Cl, pH 7.2/25 mM KCl/2.5 mM MgCl₂/3 mM CaCl₂/1 mg/ml leupeptin, pepstatin, and TPCK). A and D, TRITC SV-40–HSA; G, TRITC SV-40*–HSA; B, E, and H, DAPI to visualize nuclei; C, F, and I, Nomarski optics to visualize cells and nuclei. SV-40–HSA and SV-40*–HSA were conjugated with the same amount of TRITC.
Binding of SV-40 Nuclear Localization Peptide to Immobilized Nuclear Proteins

Two major proteins of 70 and 59 kD were recognized by the SV-40 NLS, as judged by the gel binding assay (see Materials and Methods). Yeast nuclei were resuspended in gel sample buffer (Laemmli, 1970), electrophoresed through a 10% SDS polyacrylamide gel, and transferred to nitrocellulose by electroblothing. After blocking in BSA, the nitrocellulose filter was incubated with 125I-labeled SV-40-HSA and exposed to film. Two major bands were observed corresponding to proteins of molecular weights 70 and 59 kD (Fig. 4 A, lane 2). Minor bands corresponding to 95 and 140 kD were also occasionally observed. The 70 and 59 kD bands did not correspond to abundant nuclear proteins as judged by Coomassie staining of the extract (Fig. 4 B, lane 3). Cell lysates depleted of nuclei (Fig. 4 A, lane 4), as well as a similarly treated protein extract from E. coli showed no binding of the 125I-SV-40-HSA (Fig. 4 A, lane 1). All of the binding activity was recovered with the yeast nuclear pellet and none was found in the soluble (cytoplasmic) fraction from lysed cells (Fig. 4 A, lane 5).
Binding to the 70- and 59-kD proteins is specific for the wild-type SV-40 peptide. When the blot containing the nuclear extract was incubated with both 125I-SV-40-HSA and a 100-fold chemical excess of unlabeled SV-40-HSA, the radiolabeled bands were no longer visible (Fig. 5, lane 2). However, when the blot was incubated with [125I-SV-40-HSA and 100-fold chemical excess of unlabeled HSA or SV-40*-HSA (Fig. 1), no diminution in the binding was observed (Fig. 5, lane 3). Moreover, when a blot containing a nuclear extract was incubated directly with 125I-SV-40-HSA and 100-fold chemical excess of unlabeled HSA or SV-40*-HSA, no binding to any proteins was observed (data not shown).

**Binding of Other Peptides to Immobilized Nuclear Proteins**

Different nuclear localization peptides compete with each other for binding to the 70 and 59 kD proteins. Binding of 125I-SV-40-HSA was inhibited by the presence of 100-fold chemical excess of either NP-HSA or H2B-HSA (Fig. 5, lanes 4-6). When 125I-NP-HSA conjugate was incubated with blots containing yeast nuclear extracts, binding to the 70- and 59-kD proteins was observed directly (Fig. 5, lane 7). Additional binding of NP-HSA to the 95- and 140-kD proteins was also often observed. When a radiolabeled protein fragment containing Gal4 amino acids 1-147 (termed Gal4(1-147)) was used to probe a blot containing yeast nuclear proteins, two bands of 70 and 59 kD were observed (Fig. 5, lane 8). The Gal4 binding could also be competed by addition of excess of unlabeled SV-40-HSA, NP-HSA, and H2B-HSA (data not shown). These results suggest that the same yeast proteins are capable of binding to distinct nuclear localization sequences. We refer to these proteins as NLS binding proteins.

**Characterization of NLS Binding Proteins**

The NLS binding activity, assayed by binding to proteins immobilized on nitrocellulose, can be partially solubilized by high salt but not by detergent. When nuclei were incubated with Triton X-100 and collected by centrifugation, all of the binding activity remained associated with nuclei (Fig. 6, lane 10). When nuclei were treated with 0.5 M or 1 M NaCl (Fig. 6, lanes 3 and 4), 50-90% of the activity was released into the supernatant (Fig. 6, lanes 7 and 8). Treatment with 2.5 mM MgCl2 and 3 mM CaCl2 had no effect (Fig. 6, lanes 1, 2, 5, and 6). This binding activity was completely eliminated by protease treatment of nuclei (Fig. 6, lanes II and I2).

**Discussion**

The existence of nuclear localization sequences within proteins suggests that specific receptors may recognize proteins destined for the nucleus. Isolated nuclei from Saccharomyces cerevisiae bind to nuclear localization peptides conjugated to albumin (Figs. 1 and 2). This binding has the hallmarks of a receptor-ligand interaction: the binding is saturable and specific.

From isolated nuclei, we identified proteins of 70 and 59 kD that also bind to nuclear localization peptides. Binding was assayed using a Western blot-type assay. The properties of these proteins suggest that they are receptors for nuclear proteins. Binding to the 70- and 59-kD proteins is competitive, and mutant peptides do not bind or compete. Different peptide conjugates also cross-compete for binding to the 70- and 59-kD proteins, suggesting that they bind to the same sites. Finally, the binding is not a consequence of high peptide concentration on the conjugates, because a fragment of the yeast DNA binding protein, Gal4, binds to the same proteins. (This Gal4 fragment contains sufficient information for nuclear import [Silver et al., 1984; Nelson and Silver, 1989]).

Peptides derived from the nonyeast proteins, Xenopus nucleoplasmin and SV-40 T antigen, recognize the same proteins as those recognized by yeast nuclear localization sequences. This is based on cross-competition between NP-HSA, SV-40-HSA, and H2B-HSA, as well as direct binding to proteins of the same size. These findings are consistent with our observations that the SV-40 and nucleoplasmin nuclear localization sequences are recognized correctly by yeast in vivo (Nelson and Silver, 1989; Sadler, I., and P. A. Silver, manuscript in preparation).
The biochemical behavior of the proteins suggests that they are associated with nuclei via protein–protein interactions. Binding activity can be extracted from nuclei with salt, but not detergent. Upon treatment with salt, the 70- and 59-kD binding proteins are released (Fig. 6). The amount extracted varies and may reflect populations of protein associated in different ways with the nuclei. Attempts to restore full binding activity by adding back salt-extracted proteins have, thus far, not been successful.

Others (Adam et al., 1989) have identified proteins from rat liver of ~70 and 59 kD that appear to be involved in the localization of proteins to the nucleus. Unlike the yeast proteins described here, the rat liver proteins are found distributed between the nucleus and the cytoplasm. However, the nuclei were treated with higher salt before assaying the NLS binding activity. Our assay also detected proteins of similar size in rat liver nuclei (Seibel, J., and P. A. Silver, unpublished observations). One possibility is that the NLS-binding proteins recognize nuclear-destined proteins in the cytoplasm as they emerge from the ribosome. After binding, the proteins are transported to the nuclear pore where they are subsequently imported. The differences in nuclear association of the binding proteins may represent proteins at different stages of import.

Yoneda et al. (1988) reasoned that since all known nuclear localization sequences are basic, a receptor for these proteins may be acidic. They developed antibodies against the peptide DDDDE and found that these antibodies reacted with rat liver nuclear proteins of 69 and 59 kD. In addition, when the antibodies are microinjected into HEL cells, nuclear import is inhibited. Immunofluorescence with these antibodies revealed punctate staining of rat fibroblast nuclei, suggesting association of the antigen with the nuclear pores.

A high content of basic amino acids is not sufficient to confer nuclear localization activity upon the SV-40 peptide. Removal of positive charge by conversion of lysine 128 to threonine eliminates SV-40 T antigen nuclear localization (Lanford and Butel, 1984; Kalderon et al., 1984a). However, replacement of the neighboring proline by lysine did not restore import (Colledge et al., 1986). This situation is reminiscent of that of other localization sequences: signal sequences for secretion are generally hydrophobic, and those for mitochondrial localization are a combination of basic and hydrophobic residues. Similarly, transcriptionally activating “acid blobs” are all acidic but have little primary sequence homology (Ma and Ptashne, 1987). In each case, there is a semi-specific interaction; many different ligands are allowed, while other potential ligands are excluded. The results in this paper illustrate that semi-specific interactions can be mediated by a single set of proteins.

The NLS binding proteins retain their binding ability despite heating, SDS gel electrophoresis, and electroblotting. GTP binding proteins (Lapetina and Reep, 1987), DNA binding proteins (Lin and Riggs, 1975; Fletcher et al., 1987; Prywes and Roeder, 1987), and the low density lipoprotein receptor (Daniel et al., 1983) also retain activity after similar treatment. One possible explanation for the retention of activity is that only a small region of the protein is responsible for binding to the NLS. Alternatively, some of the protein may renature as the SDS is removed during electroblotting. The partially denatured, electroblotted protein may then only possess a small fraction of the total binding activity of the native protein. In separate experiments, we have detected NLS binding activity in nuclear extracts bound directly to nitrocellulose by filter binding.

The two major binding proteins, distinguished by their mobility on an SDS gel, may be distinct or may be related. While the smaller protein may be a degradation product, in a protease deficient yeast strain (ABYS1), we still observed both binding species. Chemical modifications may also explain altered mobilities.

Nuclear protein import has been proposed to be an active process catalyzed by recognition of nuclear localization sequences and subsequent ATP-dependent transfer through the pore (Feldherr et al., 1984; Newmeyer and Forbes, 1988). We propose that the proteins identified here are part of a recognition apparatus in the yeast S. cerevisiae and may be analogous to proteins identified in mammalian cells (Yoneda et al., 1988; Adam et al., 1989).

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