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Specific Binding of Nuclear Localization Sequences to Plant Nuclei

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We have begun to dissect the import apparatus of higher plants by examining the specific association of nuclear localization sequences (NLSs) with purified plant nuclei. Peptides to the simian virus 40 (SV40) large T antigen NLS and a bipartite NLS of maize were allowed to associate with tobacco and maize nuclei. Wild-type NLSs were found to compete for a single class of low-affinity binding sites having a dissociation constant (K_d) of ~200 μM. Peptides to mutant NLSs, which are inefficient in stimulating import, were poor competitors, as were reverse wild-type and non-NLS peptides. The NLS binding site was proteinaceous and resistant to extraction under conditions where pores were still associated. In addition, immunofluorescence and immunoelectron microscopy indicated that binding was at the nuclear envelope. Overall, plant nuclei may be an excellent system to identify components of the import apparatus.

INTRODUCTION

The specific transport of macromolecules into and out of the nuclear compartment is an essential process within eukaryotic cells and is mediated by nuclear pores embedded in the nuclear envelope. These large (125 MD) and complex structures are composed of an estimated 75 to 100 different proteins that form, among other features, nuclear and cytoplasmic rings that are attached via spokes to a central transporter (for reviews, see Dingwall and Laskey, 1992; Forbes, 1992). It is thought that the spokes form eight 90 Å channels that permit the passive diffusion of ions and perhaps small proteins (40 to 60 kD; Paine et al., 1975; Breeuwer and Goldfarb, 1990), whereas the central transporter is a regulated channel for the specific transport of proteins and RNAs (Feldherr et al., 1984; Dworetzky and Feldherr, 1988).

The directed import of proteins into the nucleus is mediated by nuclear localization sequences (NLSs), which are short polypeptide regions having a high content of basic amino acids (for reviews, see Roberts, 1989; Garcia-Bustos et al., 1991a; Raikhel, 1992). Although they are found at many locations within proteins, NLSs are not removed following translocation as are signals for chloroplast (Keegstra, 1989), mitochondrial (Hartl and Neupert, 1990), and vacuolar (Chrispeels and Raikhel, 1992) import. The best studied NLSs are those that resemble the import signal of the simian virus 40 (SV40) large T antigen ($PKK_{128}KRKV$). Among the mutations that have been examined, an asparagine or threonine substitution for lysine at position 128 within the NLS greatly reduces the efficiency of nuclear import in terms of rate and final accumulation (Kalderon et al., 1984; Lanford and Butel, 1984). Two additional classes of NLSs have also been identified. These are exemplified by the yeast mating type α-2 NLS (Hall et al., 1984), which possesses hydrophobic and basic amino acids (KIPIK), and bipartite NLSs, which are composed of two short regions of basic amino acids separated by ~10 residues. The bipartite structure may represent the most common structural motif among NLSs (Dingwall and Laskey, 1991).

In animal and yeast cells, it is clear that the process of nuclear import involves an energy-independent binding step followed by translocation, which requires ATP hydrolysis (Newmeyer and Forbes, 1988; Richardson et al., 1988). In addition, at least one receptor-mediated event is thought to be involved because import is saturable and specific only for functional NLSs (Goldfarb et al., 1986). Much attention has recently been focused upon the identification of components of the import apparatus, including receptors that recognize NLSs. Genetic approaches in yeast have yielded some potential receptor mutants (Bossie et al., 1992; Gu et al., 1992), and a number of NLS binding proteins have been identified biochemically in animal and yeast cells based upon association with functional NLSs (for reviews, see Garcia-Bustos et al., 1991a; Nigg et al., 1991; Silver, 1991; Stochaj and Silver, 1992b; Yamasaki and Roberts, 1992). Some of these NLS binding proteins are at least partially cytoplasmic (Adam et al., 1989; Yamasaki et al., 1989; Stochaj and Silver, 1992a); therefore, import receptors may not be located exclusively at nuclear pores. Several studies have also demonstrated that at least one cytoplasmic factor is essential for import in animal cells (Newmeyer and Forbes, 1990; Adam and Gerace, 1991; Moore and Blobel, 1992; Sterne-Marr et al., 1992). With few exceptions (Adam and Gerace, 1991; Stochaj and Silver, 1992a, 1992b), none of the putative import receptors has been shown

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to be functional. Interestingly, Meier and Blobel (1992) have reported that an NLS binding protein may shuttle between the nucleolus and cytoplasm via fibrillar tracks. Several other NLS binding proteins have been identified either as Hsc70 (Imamoto et al., 1992) or components involved in ribosomal RNA processing (Lee et al., 1992). Heat shock protein 70 (Hsp70) and the cytoplasmic cognate Hsc70 may, however, prove to be essential components of import (Shi and Thomas, 1992; see also Dingwall and Laskey, 1992; Goldfarb, 1992).

In higher plants, much less is known about nuclear targeting (for review, see Raikhel, 1992), and most efforts have focused upon defining NLSs of viral and bacterial proteins involved in the nuclear import of nucleic acids during pathogenesis. In these studies, the potyvirus protein NIa (Restrepo et al., 1990) and the Agrobacterium proteins VirD2 (Howard et al., 1992; Tinland et al., 1992) and VirE2 (Citovsky et al., 1992) were found to possess bipartite NLSs. Recently, our laboratory has investigated the nuclear import of several endogenous plant proteins. The maize transcription factor R (Shieh et al., 1993) has been found to possess three functional NLSs: an SV40-type, a mating type α-2, and an unusual type containing three consecutive arginine residues found in some viruses (Garcia-Bustos et al., 1991a). Interestingly, specific combinations of two of the three NLSs are necessary for efficient nuclear localization in onion epidermal cells (Shieh et al., 1993). Another maize transcription factor, Opaque2 (O2), has been shown to localize to the nucleus of both monocot and dicot plant species (Varagona et al., 1991). The protein contains two NLSs: an SV40-type and a bipartite signal, although the bipartite signal was found to be more efficient in directing import (Varagona et al., 1992).

Essentially nothing is known of the nuclear import apparatus of plants. However, some components may be similar among kingdoms; for example, the mammalian virus SV40 NLS has been demonstrated to function in dicot plant cells (Lassner et al., 1991; van der Krol and Chua, 1991). Stochaj and Silver (1992a) have also reported the detection of a 70-kD protein in maize nuclei that is cross-reactive with an antibody to an NLS binding protein of yeast. Significant differences in the apparatuses may also exist, however, given the environmental extremes at which plants must survive and their great genetic diversity.

As an initial step in understanding the import apparatus of higher plants, we examined the specific association of NLSs with purified plant nuclei. Synthetic peptides to the SV40 large T antigen NLS and the bipartite NLS from O2 were found to associate with isolated plant nuclei at low affinity in a specific and saturable manner. Both NLSs competed for a single class of proteinaceous sites that were firmly associated with the nuclei. The association was probably binding rather than accumulation and was not artifactual binding to chaperones. In addition, NLS binding occurred at the nuclear envelope and was associated, although not exclusively, with nuclear pores. These data indicate that plant nuclei possess a specific, low-affinity binding site that recognizes both mammalian viral and plant NLSs. This site may be a nuclear or a partially nuclear import receptor, indicating that a plant system may provide an excellent opportunity to identify an NLS receptor(s) using biochemical and cellular biology approaches.

RESULTS

Specific Association of NLSs with Plant Nuclei

To detect specific association of NLSs with isolated plant nuclei, a number of NLS peptides were synthesized, as shown in Figure 1. A 31-residue peptide corresponding to the wild-type bipartite import signal of the transcription factor O2 (O2WT) as well as a mutant sequence (O2Mut) were synthesized (Figure 1A). The O2WT sequence (Figure 1A, O2WT) corresponds to the minimum length of polypeptide demonstrated to stimulate import of a reporter protein in transiently transformed onion epidermal cells and cells of transgenic tobacco plants (Varagona et al., 1992). This bipartite NLS has recently been studied in more detail, and specific mutations have been introduced that greatly reduced the capacity of this signal to direct nuclear import in onion cells (M.J. Varagona and N.V. Raikhel, unpublished data). The O2Mut possesses amino acid substitutions at three positions and is one such mutant sequence (Figure 1A, O2Mut). In addition, SV40 large T antigen NLS peptides of 10-residue length were synthesized (Figure 1A). The wild-type SV40 NLS sequence (SV40WT) and a mutant sequence containing a threonine rather than a lysine at protein position 128 (SV40Mut) were similar to peptides used in numerous previous studies of import and NLS binding in animals and yeast (Garcia-Bustos et al., 1991a). As an additional control, an SV40WT NLS was synthesized in the reverse orientation (SV40Rev), thus preserving the overall charge of the wild-type NLS (Figure 1A). Finally, a synthetic peptide was chosen for a control unrelated to NLSs. This nine-residue peptide (non-NLS) has an acidic charged residue and several hydrophobic residues and was judged to be a "random" peptide control (Figure 1A). All peptides contained a cysteine residue at the amino or carboxy terminus to facilitate radiolabeling with carbon-14, whereas the O2 peptide also possessed a naturally occurring tyrosine residue that was useful for labeling with iodine-125.

Nuclei were purified from tobacco suspension–cultured cells by multiple Percoll gradients (see Methods) and found, as in Figure 1, to be at least 90% unruptured by fluorescence microscopy in the presence of the nuclear stain 4′,6-diamidino-2-phenylindole dihydrochloride (Figure 1B, right). In addition, electron microscopy indicated that structures such as the nuclear envelope and pores remained intact (see Figure 6). The nuclei appeared to be free of contaminating organelles such as mitochondria and chloroplasts, although small grains, possibly starch or Percoll, were evident (Figure 1B, left). The preparations did contain endoplasmic reticulum (ER), as demonstrated by the use of antibodies specific for several ER proteins (see Figure 5). Such contamination is probably unavoidable, since the membranes of the ER are contiguous with

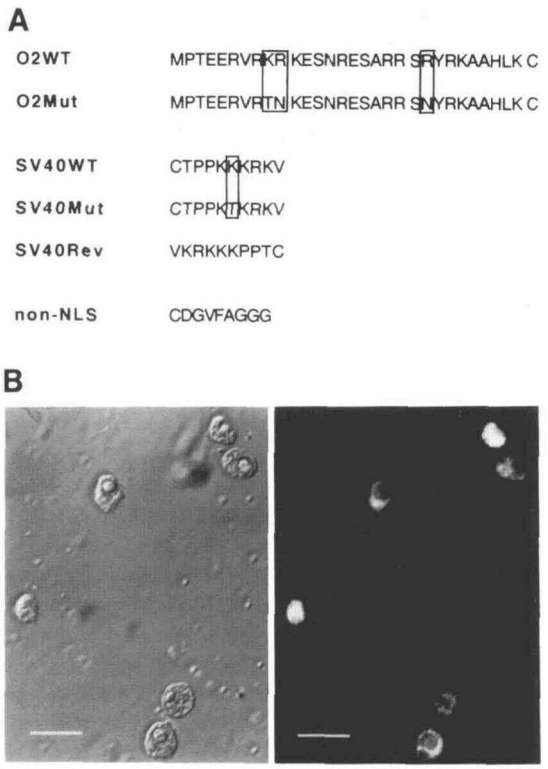

Figure 1. Synthetic Peptides and Purified Nuclei Used in Association Studies.

(A) Amino acid sequences of peptides corresponding to the wild-type (O2WT) and mutant (O2Mut) O2 NLSs and the wild-type (SV40WT), mutant (SV40Mut), and reverse wild-type (SV40Rev) NLSs of SV40 large T antigen are shown in single-letter code. A peptide unrelated to NLSs (non-NLS) was also synthesized. Boxed regions identify amino acids that were substituted in the wild-type NLSs to produce the mutant sequences. An amino- or carboxy-terminal cysteine residue was added to each peptide for radiolabeling and chemical cross-linking.
(B) Purified tobacco nuclei were visualized with fluorescence optics in the presence of the nuclear stain 4',6-diamidino-2-phenylindole dihydrochloride (right) or by Nomarski optics (left). Bars = 32 μm.

the nuclear envelope. Similar nuclear preparations have been shown by light and electron microscopy to be of equivalent purity (Saxena et al., 1985).

To assay for specific association of NLSs with nuclei, the O2WT and SV40WT peptides were labeled with carbon-14 and added to purified nuclei. Binding was allowed to proceed for 5 min on ice, and association was determined by scintillation counting of pelleted nuclei, as shown in Figure 2. The binding of both wild-type peptides was found to be specific and saturable. The displacement curve utilizing ^{14}C-O2WT as a substrate and O2WT as a competitor was sigmoidal in shape with one inflection, indicating a single class of binding sites (Figure 2A, O2WT). Maximum competition (i.e., saturation) occurs at the addition of 3 to 10 mM of O2WT peptide, corresponding to a nonspecific background association of only ~10% of total binding. Saturation experiments confirmed that maximum association occurs at ~3 mM of substrate (data not shown). While the observed association was of low affinity with an approximate dissociation constant (K_d) of 200 μM, it was also specific. The mutant NLS peptide O2Mut competed poorly with ^{14}C-O2WT for nuclear association, displaying competition that was

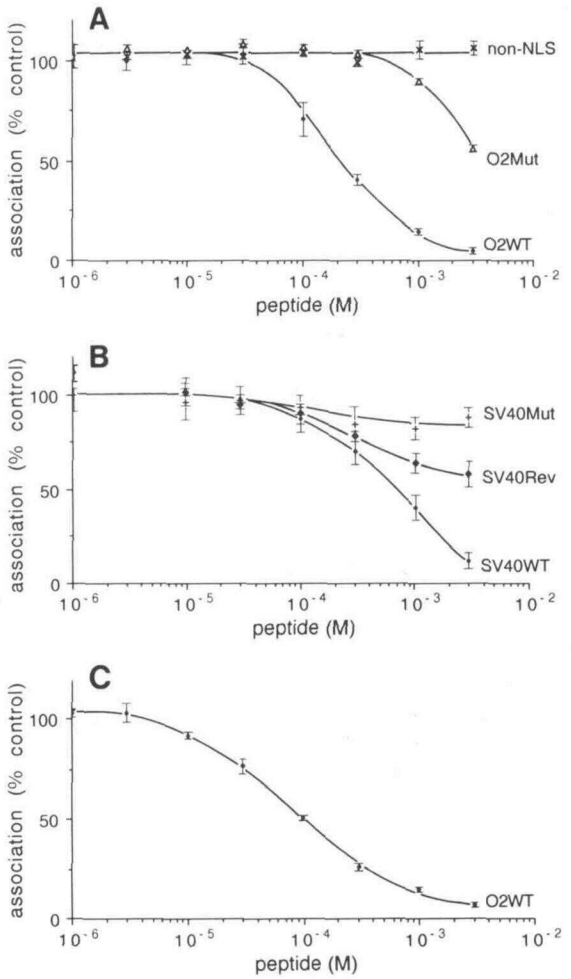

Figure 2. Specific Association of NLSs with Purified Plant Nuclei.

Radiolabeled NLS peptides were incubated with tobacco nuclei in the presence of competing peptides, and associated counts per minute were determined by scintillation counting. Results were plotted as percent binding of control (± SE) versus concentration (M) of added competitor.
(A) Association of the ^{14}C-O2WT peptide in the presence of the indicated competitor peptides. Total binding and nonspecific association were, respectively, 15,264 and 1221 counts per min.
(B) Association of ^{14}C-SV40WT in the presence of the indicated competitors. Total binding and nonspecific association were, respectively, 7742 and 2638 counts per min.
(C) Association of ^{14}C-SV40WT in the presence of O2WT indicating competition for the same site. Total binding and nonspecific association were, respectively, 8067 and 1613 counts per min.

at least 10-fold lower in affinity than O2WT (Figure 2A, O2Mut). The non-NLS control peptide did not compete (Figure 2A, non-NLS).

The association of ^{14}C-SV40WT peptide was similar to that of ^{14}C-O2WT. Displacement of ligand with SV40WT peptide resulted in a curve whose parameters were near those of the O2WT peptide (Figure 2B, SV40WT), except that the nonspecific background binding was greater (20 to 50% of total binding). As observed for O2Mut, the SV40Mut peptide competed poorly for association (Figure 2B, SV40Mut). The SV40Rev peptide displayed somewhat stronger association than SV40Mut but was a poor competitor compared to SV40WT peptide (Figure 2B, SV40Rev). This indicates that differences in net NLS charge alone could not account for the observed binding specificity. As with the ^{14}C-O2WT substrate, the non-NLS control peptide did not compete at all for ^{14}C-SV40WT association (data not shown). To demonstrate that the SV40 and O2 NLSs compete for the same class of sites, ^{14}C-SV40WT substrate was allowed to bind in the presence of increasing concentrations of O2WT peptide. This resulted in a curve that was similar to the previously mentioned wild-type NLS displacement curves (compare Figure 2C to Figures 2A and 2B). It should be noted that, although not as extensively examined, purified nuclei from maize were also found to possess specific NLS binding sites of similar affinity (data not shown). In addition, plant nuclei possess only low-affinity sites, since ^{125}I-O2WT NLS peptide, which should have detected binding at nanomolar concentrations, did not detect sites of higher affinity. More importantly, the specific low-affinity binding detected with ^{14}C-O2WT was faithfully reproduced using the ^{125}I-O2WT (data not shown). Overall, the competitive displacement curves indicate that both monocot and dicot nuclei possessed a single class of low-affinity binding sites that recognized two types of NLSs. Furthermore, these sites were specific only for functional NLS sequences.

Binding Site Is Tightly Associated and Proteinaceous

To characterize the NLS binding site, nuclei were treated under a variety of conditions prior to binding of the ^{14}C-O2WT peptide, as shown in Figure 3. Multiple freeze–thaw cycles prior to NLS association had little effect upon binding (Figure 3A). Treatment of nuclei with NaCl, the nonionic detergent Triton X-100, a combination of salt and detergent, or urea prior to binding was mostly ineffective in reducing association (Figure 3B, open bars). The nuclease DNase I, which releases nucleoplasmic proteins, was partially effective in releasing the site of association (Figure 3B). This released component may represent specific binding to a subset of soluble or chromatin-associated proteins. Treatment of nuclei with salt, detergent, or urea after binding indicated that while the binding site per se was firmly associated, the bound peptide was ~75% dissociated from the site by treatment with salt or detergent plus salt and ~30% dissociated by urea (Figure 3B, striped bars). This indicates that ionic interaction may have been a component of the specific NLS association.

Although the site of association was resistant to extraction from nuclei, it was nonetheless proteinaceous because, as shown in Figure 4, binding after trypsin protease treatment was greatly reduced compared to an untreated control (Figure 4, compare tryp and control). Digestion was terminated by the addition of trypsin inhibitor, which was fully active as demonstrated by the coaddition of trypsin and trypsin inhibitor to nuclei (Figure 4, tryp + TI). In addition, trypsin inhibitor alone was not responsible for the dramatic decrease in binding (Figure 4, TI).

Site Is Not a Chaperone

To examine whether O2WT NLS binding activity was associated with proteins that bind peptides with little specificity, such as chaperones, nuclei were extracted with urea. As shown in Figure 3, after this treatment, binding activity remained in the pelleted nuclear fraction (Figure 3B, 6 M urea, open bar). As shown in Figure 5, following extraction and centrifugation, the pellet and supernatant fractions were blotted and probed with antibodies specific for Hsp70 from pea (Figure 5A), tobacco BiP, an Hsp70 homolog found in the ER lumen (Figure 5B),

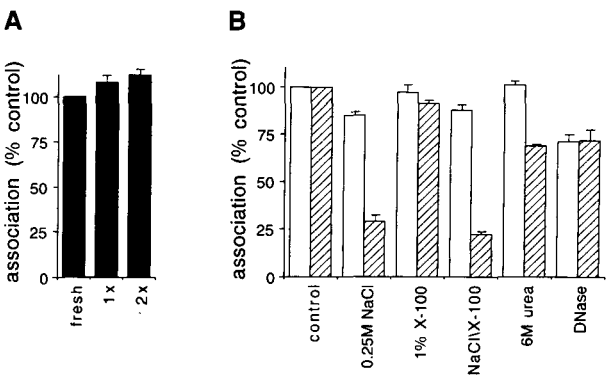

Figure 3. NLS Binding Site Is Firmly Associated.

Purified tobacco nuclei were treated under various conditions either before or after binding of the ^{14}C-O2WT ligand, and the results were plotted as percent binding of control (± SE).

(A) Nuclei were allowed to bind the ligand immediately after purification (fresh) or after one (1×) or two (2×) freeze–thaw cycles. Total binding and nonspecific association were, respectively, 14,612 and 1169 counts per min.

(B) Nuclei were treated as indicated for 15 min on ice before binding of the ligand to determine the strength of binding site association with nuclei (open bars). Nuclei were also treated as indicated for 15 min after nuclei were allowed to bind the ligand, and unbound ligand was removed to examine the nature of NLS association (striped bars). Total binding and nonspecific association were, respectively, 15,251 and 1220 counts per min (open bars) and 5103 and 408 counts per min (striped bars). X-100, Triton X-100.

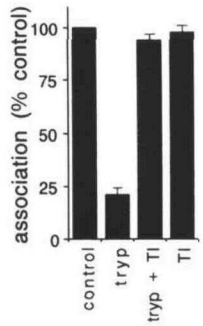

Figure 4. NLS Binding Site Is Proteinaceous.

Prior to association with ^{14}C-O2WT ligand on ice, nuclei were treated with the 120 units of the protease trypsin (tryp) for 15 min at 25°C, and then digestion was terminated by the addition of 20 μg trypsin inhibitor. Nuclei were unaffected when coincubated with trypsin and trypsin inhibitor (tryp + TI), indicating that the trypsin inhibitor was active. For comparison, untreated nuclei (control) and nuclei treated with trypsin inhibitor (TI) only were included. Results were plotted as percent binding of control (± SE), and total binding and nonspecific association were, respectively, 17,854 and 1429 counts per min.

or alfalfa protein disulfide isomerase (PDI; Figure 5C). In all cases, the antibodies detected reactive bands almost exclusively in the supernatant fractions of urea extracted nuclei (Figures 5A to 5C; urea). In fact, significant amounts of antibody-reactive proteins were released from mock-treated nuclei simply by pelleting (Figures 5A to 5C; mock). Similar results were obtained with nuclei that were extracted with 1% Triton X-100 (data not shown). High titers of the antibodies were also added to the nuclei to deplete them of NLS binding activity, but there was no effect upon binding activity (data not shown). These results indicate that the specific NLS binding sites were not Hsp70 or the common ER contaminants BiP or PDI.

Because the binding activity of the nuclei remained following extraction by salt, detergent, or urea (Figure 3B, open bars), it was of interest to determine whether there were identifiable structures. Nuclei were extracted with urea or NaCl and Triton X-100 under conditions that were similar to those previously described and then examined by electron microscopy, as shown in Figure 6. In longitudinal sections, untreated control and extracted nuclei had visible double-membrane structures with identifiable inner and outer nuclear membranes (Figures 6A to 6C; upper sections). In addition, nuclear pore complexes were visible (Figure 6, arrowheads) in both longitudinal (upper) and tangential (lower) sections. Based upon this analysis, it seemed likely that the NLSs were associated with either structural components of the nucleus or nuclear pore complexes.

Binding Occurs at the Nuclear Periphery

To determine the location of NLS binding, a study was undertaken using indirect immunofluorescence microscopy. To facilitate detection of NLS binding, several NLS peptides were chemically cross-linked to human serum albumin (HSA). The peptide–HSA conjugates O2WT-HSA or SV40WT-HSA were allowed to associate with tobacco nuclei for 5 min on ice under conditions similar to those indicated for association of ^{14}C-O2WT (Figure 2A). Nuclei were then washed, and binding of the conjugate was visualized via a rabbit anti-HSA antibody followed by a rhodamine-conjugated anti–rabbit secondary antibody. As shown in Figure 7, optical sectioning of unfixed nuclei by the use of a laser-scanning microscope

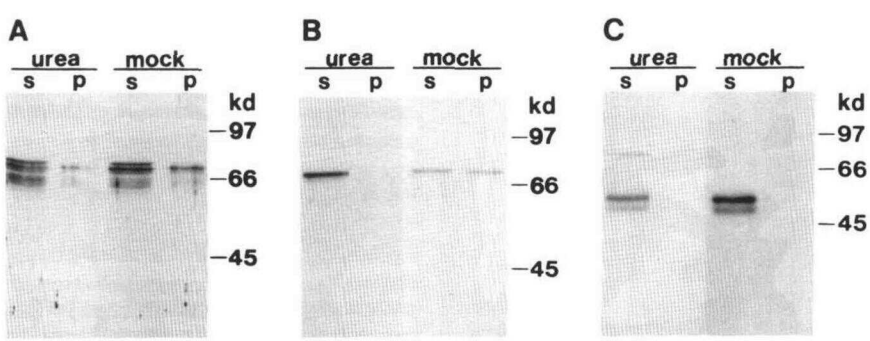

Figure 5. NLS Binding Site Is Not a Chaperone.

Tobacco nuclei were extracted with 6 M urea under conditions used to treat nuclei prior to ligand association (Figure 3B, open bars). Extracted nuclei (urea) and mock-treated controls (mock) were then pelleted, and the resulting supernatants (s) and pellets (p) were subjected to SDS-PAGE and protein blotting.

(A) Immobilized protein probed with antibody specific for Hsp70.

(B) Protein probed with antibody specific for BiP.

(C) Protein probed with antibody specific for PDI.

Antibody-reactive proteins were visualized using an alkaline phosphatase–conjugated secondary antibody. Molecular mass standards (kD) are as indicated.

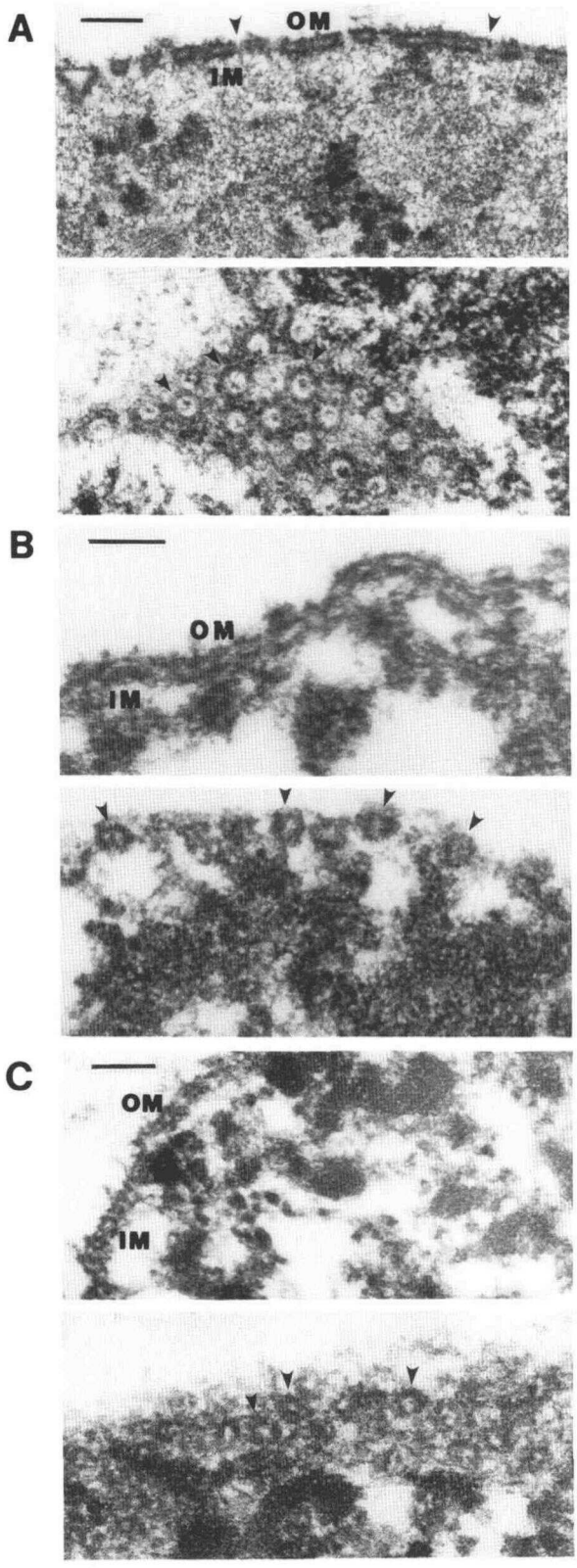

Figure 6. Nuclear Structures Remain following Extraction.

Purified tobacco nuclei were extracted with 0.25 M NaCl plus 1% Triton X-100 or 6 M urea as was given for treatment prior to ligand association (Figure 3B, open bars). Nuclei were then fixed and sectioned for electron microscopy.

(A) Longitudinal section (upper) and tangential section (lower) of mock-treated control nuclei. Inner membrane (IM), outer membrane (OM), and pores (arrowheads) are visible.

(B) Pores (arrowheads) are also apparent in longitudinal (upper) and tangential (lower) sections of nuclei treated with 0.25 M NaCl plus 1% Triton X-100. IM, inner membrane; OM, outer membrane.

(C) Pores (arrowheads) are visible in longitudinal (upper) and tangential (lower) sections of nuclei treated with 6 M urea. IM, inner membrane; OM, outer membrane.

Bars = 200 nm.

indicated that both O2WT-HSA and SV40-HSA were bound at the nuclear envelope or proximal to it (Figures 7A and 7B, left).

Binding of the conjugate was dependent upon the presence of the NLS, since control nuclei treated with unconjugated HSA displayed little or no peripheral staining (Figure 7C, left). In other experiments, the coincubation of nuclei with O2WT-HSA and 3 mM of free O2WT or SV40WT-HSA and 1 mM of free SV40WT resulted in much reduced peripheral staining, whereas incubation in the presence of the respective mutant peptides had little effect (data not shown).

To more precisely determine the site of NLS binding, immunoelectron microscopy was done following binding of O2WT-HSA to nuclei. As shown in Figure 8, gold label was found to be associated with the nuclear envelope (Figures 8A to 8C). In addition, label was often found associated with nuclear pores. It was unclear if nuclear binding was exclusively at pores because it was difficult to achieve both structural preservation of isolated nuclei and adequate sensitivity to detect O2WT-HSA binding, a possible consequence of low-affinity binding. Interestingly, gold label was often observed in clusters, indicating possible binding to a multivalent site(s). Binding was specific for O2WT-HSA, since control nuclei that were allowed to associate with HSA displayed little or no detectable gold label (Figure 8D). These results, coupled with the immunofluorescence data, indicate that the specific binding to plant nuclei was probably at the pores or some other firmly anchored component(s) associated with the nuclear envelope.

DISCUSSION

We demonstrated the specific and saturable association of several types of NLSs, including an endogenous bipartite signal from maize, to isolated plant nuclei. The binding site was located at the nuclear envelope and was proteinaceous and firmly associated with the nuclear structure, components of which were visible following extraction. Furthermore, the observed binding was not an artifactual association with Hsp70 or ER resident BiP or PDI.

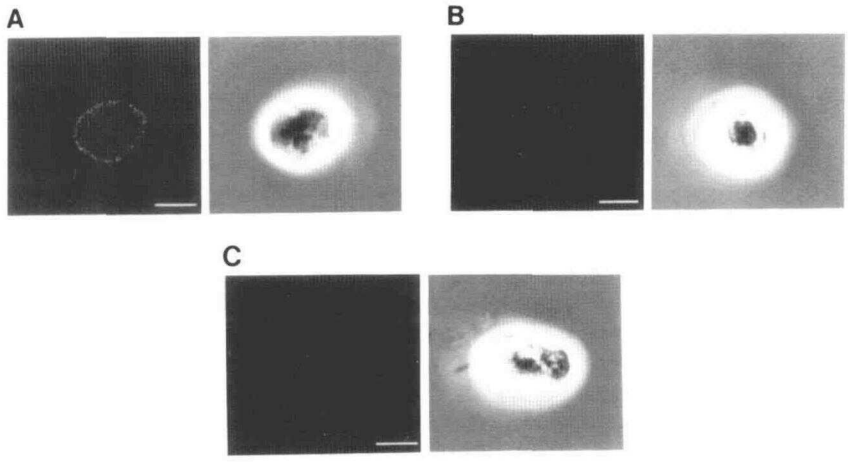

Figure 7. NLSs Bind to the Nuclear Periphery.

Purified tobacco nuclei were incubated with peptide–HSA conjugates, and binding was visualized with an anti-HSA antibody and a rhodamine-conjugated secondary antibody. Nuclei were then optically sectioned using a laser-scanning microscope.
(A) Nucleus incubated with O2WT-HSA and then optically sectioned (left) with matching Nomarski video image (right) shown for comparison.
(B) Nucleus incubated with SV40WT-HSA (left) with Nomarski image (right) shown for comparison.
(C) Control nucleus incubated with unconjugated HSA (left) with Nomarski image (right) shown for comparison.
Note that Nomarski video images are of inherent low quality. Images are shown to orient the reader in matching optical sections. Bars = 5 μm.

In Vitro Binding

Synthetic peptides were used in this study because their low molecular mass and specific binding make them particularly advantageous as probes in molecular tagging approaches such as chemical cross-linking. Thus, competition studies were done with radiolabeled NLS peptides to determine whether specific binding sites could be detected in purified plant nuclei. Inefficient competition for binding by the SV40 and O2 mutant NLS sequences was indicative of specific binding (Figures 2A and 2B). However, by themselves, they were insufficient proof because both mutant sequences have reduced charge due to the replacement of their basic residues. To address this problem, we included a nonfunctional reverse wild-type SV40 NLS peptide as a control because it should possess the same overall charge as the functional NLS. The use of reverse sequences has rarely been reported (Adam and Gerace, 1991), although recent reviews have stressed their importance (Forbes, 1992). In our study, a reverse SV40 peptide, SV40Rev, was an inefficient competitor compared to the wild-type SV40 peptide, SV40WT (Figure 2B). A non-NLS peptide was also included as a control, but it displayed no ability to compete for binding, which indicates that the binding was not a general phenomenon such as association with chaperones.

Our data indicated that the O2 wild-type NLS bound to plant nuclei with an approximate K_d of 200 μM. Few published estimates of NLS affinity are available for comparison, however, and various methodologies and substrates were used to obtain them. For example, Adam et al. (1989) demonstrated binding of synthetic NLSs by chemical cross-linking to proteins of rat liver nuclei and cytoplasm in the 10 to 100 nM range, and Garcia-Bustos et al. (1991b) reported a K_d of ~0.5 μM for the binding of the transcriptional regulatory protein Mcm1 to purified yeast nuclei. Conversely, Meier and Blobel (1990) required 1.3 mM of free wild-type SV40 peptide to compete for binding of an NLS–HSA conjugate to immobilized proteins from rat liver nuclei. Interestingly, while Goldfarb et al. (1986) estimated a K_m of 1.8 μM for import of an SV40 NLS–BSA conjugate following microinjection into Xenopus oocytes, 1 mM of free wild-type peptide was necessary to cause only a 50% inhibition of transport. Markland et al. (1987) also reported difficulty using as much as 10 mM of free peptide to compete for import in a mammalian in vitro system. Given such a broad range of values, it was impossible to derive an expected K_d for binding. Some possible explanations for low-affinity binding include poor binding of synthetic NLS peptides compared to whole proteins where context can be preserved, alteration of the binding site during nuclei preparation, or an inherent low-affinity binding that reflects transient NLS association during import. Plant nuclei were found to have only low-affinity sites, since peptides labeled with iodine-125 did not detect higher affinity binding. Maize nuclei were also found to have these sites. Thus, the specific binding observed was not a phenomenon unique to tobacco nuclei but was found in the nuclei of both monocot and dicot species.

The specific accumulation of import substrates has been demonstrated for in vitro import systems from animals (Goldfarb et al., 1986; Markland et al., 1987) and yeast (Kalinich and

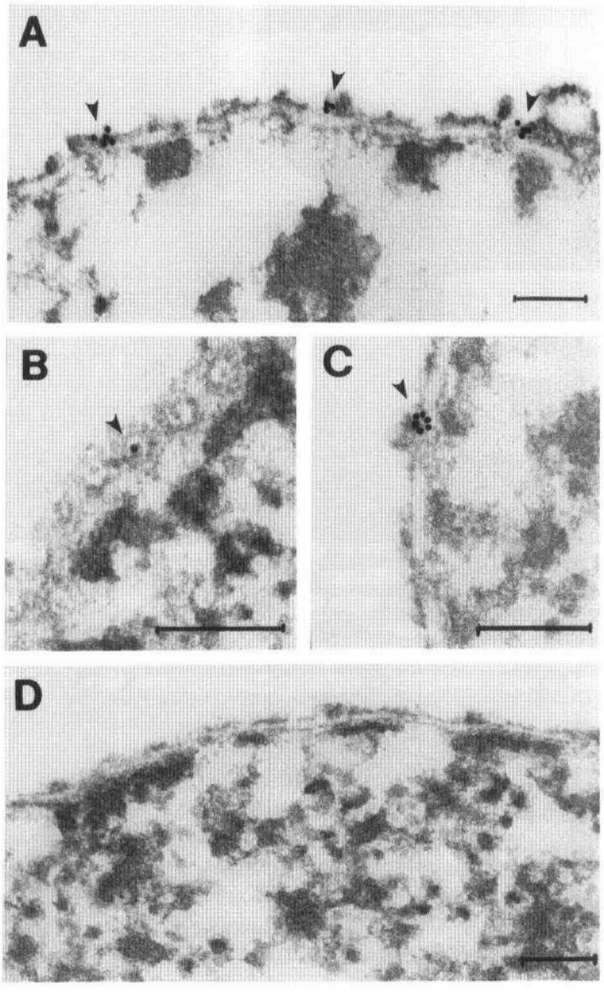

Figure 8. NLSs Bind to Nuclear Pores.

Tobacco nuclei were incubated with 5 μM O2WT-HSA or HSA, and binding was visualized with an anti-HSA antibody and protein-A gold.
(A) Longitudinal section of nucleus incubated with O2WT-HSA showing gold particles (arrowheads) associated with the envelope and pores.
(B) Tangential section showing gold particle (arrowhead) associated with pore.
(C) Longitudinal section with cluster of gold particles (arrowhead) associated with the envelope.
(D) Control nucleus incubated with unconjugated HSA displays little or no gold label.
Bars = 200 nm.

Douglas, 1989; Garcia-Bustos et al., 1991c), and active import has been shown to require ATP hydrolysis and to be inhibited by low temperature, nonhydrolyzable ATP analogs, and the nucleoporin binding lectin wheat-germ agglutinin (for review, see Garcia-Bustos et al., 1991a). We have observed little or no effect of these factors in our system (G.R. Hicks and N.V. Raikhel, unpublished data), which indicates that, in addition to evidence from laser-scanning microscopy (Figure 7) and immunoelectron microscopy (Figure 8), only reversible binding occurred in our system. The results also indicate that plant cells may require cytoplasmic factors for proper import in vitro.

Binding Site

The proteinaceous binding site was stable to freeze–thaw cycles and was only partially released by treatment with salt, detergent, urea, or nuclease (Figure 3). Resistance to 1% Triton X-100 extraction indicates that the binding site, while possibly membrane associated, was tightly bound to the nuclear structure; this was confirmed by the salt and urea treatments. Although salt was ineffective in extracting the binding site, it disrupted a significant amount of NLS association with it (Figure 3B, striped bars). This indicates that ionic interaction participated in specific NLS binding but was not the only determinant because the SV40Rev peptide was an inefficient competitor compared to the SV40WT sequence (Figure 2B). The observed behavior of the NLS binding site is not unique because other groups have reported only partial extraction of NLS binding proteins under similar conditions (Lee and Melese, 1989; Garcia-Bustos et al., 1991b).

Recent work has shown that proteins that bind peptides with low specificity, such as the chaperones Hsp70, BiP, or PDI, may artifactually bind NLS peptides (Forbes, 1992). However, in the case of Hsp70, there is evidence that this chaperone and its cytoplasmic cognate Hsc70 are necessary for nuclear import (Imamoto et al., 1992; Shi and Thomas, 1992). Thus, it was essential to demonstrate that the NLS binding site was not one of these proteins (Figure 5).

Location of NLS Binding

Based upon the results of immunofluorescence microscopy, NLS binding occurs at the nuclear envelope (Figure 7). In addition, the total number of binding sites was estimated based upon the association of ^{125}I-O2WT peptide. By making the simplistic assumption that plant cells, like animal cells, possess 2000 to 4000 pores per nucleus (Stochaj and Silver, 1992b), we estimated that there were 37 to 74 NLS peptide molecules bound per pore. This is in reasonable agreement with electron microscopy observations that multiple nucleophilic proteins (up to 20; Newmeyer and Forbes, 1988) may be associated with a single pore during import (Richardson et al., 1988). Electron microscopy analysis indicated that under our conditions pore complexes remained associated with nuclei following extraction by salt, detergent, and urea (Figure 6). Several groups have previously examined proteins of animal cell pore complexes (Davis and Blobel, 1986; Snow et al., 1987) and have found that, in general, they are also resistant to extraction by high concentrations of salt and detergent. It was possible therefore that the envelope binding we observed occurred at the pores. Immunoelectron microscopy (Figure 8) indicated that NLS binding did, in fact, occur at pores, although it is unclear whether or not the binding was exclusive to this

location. The finding that gold particles were often found in clusters at the nuclear periphery indicates the presence of a binding site that may have been capable of recognizing multiple NLSs.

Several studies have established that cytoplasmic factors are important for both NLS binding at the nuclear envelope and translocation through the pores in amphibians and mammals (Newmeyer and Forbes, 1990; Moore and Blobel, 1992). In our study, NLS binding to purified plant nuclei occurred in the absence of added cytoplasm. However, it is possible that the nuclear binding sites are a subset of receptors that function in both the nucleus and the cytoplasm. In fact, an import receptor has been found to have NLS binding activity in both postmitochondrial and nuclear envelope fractions (Adam et al., 1989). Several additional reports also indicate that NLS binding proteins may be associated with both compartments (Yamasaki et al., 1989; Stochaj and Silver, 1992a).

We have begun to examine the nuclear import machinery of higher plants. The specific low-affinity binding that we observed may be a unique feature of plant nuclei because, to our knowledge, similar low-affinity in vitro binding has not been reported for animals or yeast. Thus, plants may offer an excellent system for molecular tagging experiments aimed at identifying import components. In addition, our results may aid in the development of in vitro import systems in higher plants for functional examination of putative receptors. It will be interesting to compare other differences between animal, yeast, and plant systems, particularly given the broad genetic diversity and adaptability of plants.

METHODS

Materials

All chemicals were obtained from Sigma Chemical Company unless otherwise noted. All peptides were synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). A peptide unrelated to nuclear localization sequences (NLS) was also synthesized; this peptide (non-NLS) corresponds to a carboxy-terminal signal sequence of barley lectin that is defective in specifying vacuolar targeting (Dombrowski et al., 1993). Antibodies to pea heat shock protein 70 (Hsp70; DeRocher, 1993), tobacco BiP (Denecke et al., 1991), and alfalfa protein disulfide isomerase (PDI; Shorrosh et al., 1993) were generously supplied as gifts.

Cell Culture and Nuclei Purification

Nicotiana tabacum suspension–cultured cells were maintained and subcultured, and protoplasts were prepared from 3- to 4-day cell cultures essentially according to the method of Bednarek et al. (1990). Maize Black Mexican Sweet (BMS) suspension–cultured cells were maintained in Murashige and Skoog (1962) medium (4.3 g/L Murashige and Skoog salts [GIBCO BRL], 1 mg/L thiamine, 0.1 g/L myoinositol, 0.18 g/L KH_2PO_4, 30 g/L sucrose, adjusted to pH 5.7) and 2 μg/L 2,4D. Cells were passaged weekly by 1/5 dilution. Protoplasts were prepared from 3- to 4-day BMS cell cultures essentially as described by Fromm et al. (1987).

The purification of tobacco and BMS nuclei was based upon the methods of Saxena et al. (1985) and Willmitzer and Wagner (1981). For tobacco nuclei, protoplasts from two 30-mL cultures were pelleted at 50*g* for 4 min at room temperature, and the pellet was suspended in 10 mL of nuclei isolation buffer (NIB) (10 mM 2-(*N*-morpholino)-ethanesulfonic acid-KOH, pH 5.6, 0.2 M sucrose, 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 2.5 mM DTT, 0.1 mM spermine, 0.5 mM spermidine) and placed on ice for 7 min. All subsequent steps were performed on ice. Protoplasts were ruptured by passage through a 25-gauge needle five times. The lysate was centrifuged for 3 min at 4°C onto a cushion of 67% Percoll in NIB at 1000*g*, and the cushion and interface materials were diluted with 30 mL 0.6% Triton X-100 (Boehringer Mannheim) in NIB. The lysate was incubated on ice for 20 min and filtered through a 20-μm nylon mesh prior to being divided and layered onto two step gradients consisting of 1 mL of 67% Percoll in NIB and 4 mL 20% Percoll in NIB. The gradients were centrifuged at 1000*g* for 3 min, and the interface between the 67 and 20% Percoll phases was combined and diluted with 0.6% Triton X-100 in NIB. The step gradient was repeated.

The interface material was then loaded onto another step gradient consisting of 0.5 mL 67% Percoll in NIB and 43% Percoll in NIB and centrifuged at 1000*g* for 5 min. The interface containing purified nuclei was diluted with 0.6% Triton X-100 in NIB, and the gradient was repeated. Finally, the purified nuclei were diluted with nuclei storage buffer (20% glycerol in NIB), pelleted at 750*g* for 3 min at 4°C, and suspended in 1 mL of storage buffer. Nuclei were quantitated in the presence of the fluorescent stain 4′,6-diamido-2-phenylindole dihydrochloride with a hemocytometer and visualized using an Axiophot microscope (Carl Zeiss, New York) and fluorescence optics (Varagona et al., 1991). Yields were $\sim 10^8$ total nuclei.

An abbreviated version of this protocol, as described by Varagona et al. (1992), was used for tobacco and maize nuclei purification, except that 0.01% Triton X-100 was included at all steps requiring the detergent. For both procedures, the following concentrations of protease inhibitors were present during all steps: 0.4 mM phenylmethylsulfonyl fluoride, 5 μg/mL caproic acid, 5 μg/mL pepstatin, 5 μg/mL antipain, 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Light microscopy and protein blot analysis were done with tobacco nuclei purified via the more involved procedure. The abbreviated procedure provided essentially identical results in association assays and was thus used in all assays and electron microscopy work.

Radiolabeling of Peptides

The NLS peptides were radiolabeled with carbon-14 by carboxymethylation of cysteine residues in the presence of ^{14}C-iodoacetamide (Nusgens and Lapiere, 1979). Typically, 3 mg of the wild-type bipartite import signal of the transcription factor Opaque2 (O2WT) or 1 mg of the wild-type import signal of the simian virus 40 large T antigen (SV40WT) was suspended in 100 μL 6 M guanidine-HCl, 0.2 M NH_4HCO_3 and added to 50 μCi solid ^{14}C-iodoacetamide (21.1 mCi/mmol; Amersham Corp.). The reaction was allowed to proceed in the dark for 4 hr, at which time unlabeled iodoacetamide was added to a final concentration of 0.5 mM. After 15 min in the dark, the reaction was terminated by the addition of DTT to 1 mM. The unreacted ^{14}C-iodoacetamide was removed by gel filtration through a prepacked PD-10 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 0.2 M NH_4HCO_3. The fractions containing the radiolabeled peptide

were pooled and lyophilized. Lyophilization was repeated until no NH_4HCO_3 remained. The radiolabeled peptide was suspended in 500 μL of 10 mM Tris-HCl, pH 7.3, and stored at −70°C. Specific activities were typically 30 to 40 μCi/μmol. The ^{125}I-O2WT peptide was prepared using the iodination reagent Iodogen (Pierce Chemical Co.) according to the manufacturer's directions. Typically, 5 μg of O2WT peptide in 10 mM phosphate buffer, pH 7.4, was iodinated in the presence of 1 mCi ^{125}I-NaI (17.4 Ci/mg; Du Pont). Specific activities were 30 to 60 mCi/μmol.

Association Assays

For association of NLSs, 10^6 tobacco or maize nuclei were diluted to 70 μL with binding buffer (50 mM Tris-HCl, pH 7.3, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 20% glycerol). To begin the assay, 40,000 cpm of ^{14}C-O2WT or 60,000 cpm of ^{14}C-SV40WT was diluted to 30 μL with binding buffer, and this was added to the nuclei. Association was allowed to proceed for 5 min on ice, at which time the samples were centrifuged for 2 min at 12,000*g*. The supernatant was removed, and the counts per minute associated with the nuclei were assessed by *suspension in 100 μL of binding buffer followed by scintillation counting* in Ready-Solve scintillation cocktail (Research Products International, Mount Prospect, IL). This is referred to as the standard assay.

For competitive displacement curves, free peptides were added to the standard assay from concentrated stocks made in binding buffer. Since binding affinities were low, nonspecific backgrounds were estimated from the addition of 10 mM of free SV40WT or O2WT peptides. All assay points were the average of duplicate samples, and all experiments were done at least twice. Saturation experiments were done as described for the standard assay except that 0.1 μM to 3 mM ^{14}C-O2WT peptide was added to 10^5 nuclei in a final volume of 10 μL. For freeze–thaw experiments, 10^6 nuclei in 70 μL of binding buffer were used in the standard assay either fresh or after freezing at −70°C for 15 min and thawing to room temperature. For extraction of nuclei prior to ^{14}C-O2WT binding, 10^6 nuclei were incubated on ice for 15 min in the presence of a 100-μL volume of 0.25 M NaCl, 0.25 M NaCl plus 1% Triton X-100 or 6 M urea in binding buffer. For DNase I treatment, nuclei were incubated at 25°C for 15 min in the presence of 300 μ/mL DNase I (Boehringer Mannheim). Treated samples were then pelleted for 2 min at 12,000*g* and suspended in 70 μL of binding buffer for the standard assay.

For extraction of nuclei after ^{14}C-O2WT binding, a standard assay was performed, except the nuclei pellets were suspended in 100 μL 0.25 M NaCl, 0.25 M NaCl plus 1% Triton X-100 or 6 M urea in binding buffer and incubated on ice for 15 min. Samples treated with DNase I were suspended in 300 units/mL enzyme and incubated at 25°C for 15 min. All samples were then pelleted at 12,000*g* and processed as was done in the standard assay. For trypsin digestion, 10^6 nuclei in binding buffer plus 0.05% Triton X-100 were treated with 120 units trypsin, 120 units trypsin plus 20 μg trypsin inhibitor, or 20 μg trypsin inhibitor in a final volume of 70 μL. After a 15-min incubation at 25°C, 20 μg of trypsin inhibitor was added to the noninhibitor-treated sample, and all samples were processed for ^{14}C-O2WT binding as was done in the standard assay. For the freeze–thaw, extraction, and protease experiments, nonspecific association was assumed to be 8% of total binding as was determined from competitive displacement curves.

Antibody inhibition of NLS association was done by adding a 1/50 dilution of antibody against pea Hsp70 or tobacco BiP or a 1/500 dilution of antibody against alfalfa PDI to 10^6 nuclei. Antibodies were incubated for 30 min on ice with nuclei, and then samples were processed for ^{14}C-O2WT binding as done in the standard assay.

Protein Blot Analysis

Nuclei (4×10^5) were extracted with 6 M urea or 1% Triton X-100 in binding buffer exactly as described for samples extracted prior to ^{14}C-O2WT binding, except the nuclear pellet was suspended in SDS-sample buffer, and the extracted supernatant was precipitated overnight at −20°C following the addition of 1 mL acetone-ethanol (1:1). The extracted proteins were pelleted at 12,000*g* for 15 min at 4°C and then dried for 5 min under vacuum. The pellet was suspended in SDS–sample buffer, and the nuclear pellet and extracted proteins were electrophoresed through 10% SDS-polyacrylamide gels, blotted to nitrocellulose (Hybond C; Amersham Corp.), and blocked with nonfat dry milk according to standard methods (Sambrook et al., 1989). Filters were incubated overnight with antisera (1/1000 dilution of anti-Hsp70, 1/5000 dilution of anti-BiP, or 1/5000 dilution of anti-PDI), washed, and then developed using a 1/7500 dilution of goat anti–rabbit alkaline phosphatase–conjugated IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Microscopy

For electron microscopy, nuclei (10^6) were extracted with 0.25 M NaCl plus 1% Triton X-100 or 6 M urea exactly as was done for samples extracted prior to ^{14}C-O2WT binding, except that the pellet was suspended in 50 μL of *binding buffer for electron microscopy fixation*. Fixation of purified nuclei was performed as described by Schroeder et al. (1993). Peptides were coupled to human serum albumin (HSA) through the cysteine residue with the coupling agent maleimidobenzoyl *N*-hydroxysuccimide ester (Pierce, Rockford, IL) as described by Lee and Melese (1989).

For immunoelectron microscopy, 10^6 nuclei were diluted to 50 μL with binding buffer, and O2WT-HSA or HSA was added to a final concentration of 5 μM. After a 5-min incubation on ice, nuclei were pelleted, suspended in 20 μL of binding buffer, and fixed according to the method of Grimes et al. (1992) with the following modifications. Nuclei were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde, 50 mM Pipes, 2 mM $CaCl_2$, pH 7.2, for 30 min at room temperature and then post-fixed in 0.5% OsO_4 in 50 mM Pipes, pH 7.2. For immunolocalization, fixed nuclei were treated with 5% sodium periodate for 30 min at room temperature, three 6-min washes with H_2O, 100 mM HCl for 10 min, three 6-min washes with H_2O, and then blocked with blocking buffer (0.1% Tween-20, 2% BSA in PBS, pH 7.2) for 1 hr.

Sections were incubated with an affinity-purified rabbit anti-HSA antibody (Cappel, West Chester, PA) at a dilution of 1:100 in blocking buffer for 1.5 hr at room temperature. Sections were washed four times for 5 min each in wash buffer (0.5% Tween-20 in PBS, pH 7.2) and reblocked with blocking buffer as given above. Sections were then incubated in a 1:50 dilution of protein-A gold (15 nm) in 0.1% Tween-20, 1% BSA in PBS, pH 7.2, for 1 hr at room temperature. Samples were washed as above with wash buffer and the anti-HSA antibody, and protein-A gold was reapplied as described previously. Following three 10-min washes with wash buffer and three 10-min rinses with H_2O, sections were stained with 5% uranyl acetate for 40 min and $Pb(NO_3)_2$ for 5 min for visualization by electron microscopy.

For indirect immunofluorescence microscopy, 0.25 μM O2WT-HSA or SV40WT-HSA was incubated with 2×10^5 tobacco nuclei on ice

for 5 min in a final volume of 100 μL as described for the standard assay. Following association, the nuclei were diluted by the addition of 100 μL of binding buffer and pelleted by centrifugation for 2 min at 12,000*g*. Nuclei were suspended in 200 μL of a 1/250 dilution of rabbit anti-HSA antibody in binding buffer and incubated on ice for 30 min. The nuclei were diluted by the addition of 200 μL of binding buffer and pelleted. After suspension in 200 μL of a 1/2000 dilution of rhodamine-labeled goat anti-rabbit IgG (Pierce), incubation on ice for 30 min, and pelleting, nuclei were suspended in 30 μL of binding buffer and wet mounted for microscopy. Samples were examined using a laser-scanning confocal microscope (model 10; Carl Zeiss) with phase contrast and fluorescence optics and optically sectioned with a 514-nm argon laser.

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