Identification of Cytosolic Factors Required for Nuclear Location Sequence-mediated Binding to the Nuclear Envelope

Ermoné J. H. Adam and Stephen A. Adam
Department of Cell, Molecular and Structural Biology, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. Nuclear protein import can be separated into two distinct steps: binding to the nuclear pore complex followed by translocation to the nuclear interior. A previously identified nuclear location sequence (NLS) receptor and a 97-kD protein purified from bovine erythrocytes reconstitute the binding step in a permeabilized cell assay. Binding to the envelope is specific for a functional SV-40 large T antigen NLS and is not ATP or temperature dependent. Modification of p97 with N-ethylmaleimide (NEM) decreases binding to the pore, but interestingly, NEM treatment of the NLS receptor does not. Nuclear envelope binding is inhibited by wheat germ agglutinin suggesting a possible mechanism for the inhibition of transport by the lectin.

MACROMOLECULAR traffic between the cytoplasm and nucleus is mediated by large proteinaceous structures in the nuclear envelope known as nuclear pore complexes. Selective mediated mechanisms regulate the transport of proteins and RNA through the pore complex (reviewed by Forbes, 1992; Panté and Aebi, 1993; Hurt, 1993; Newmeyer, 1993). The localization of proteins to the nucleus is mediated by short amino acid sequences present in most nuclear proteins known as nuclear localization sequences (NLSs) (García-Bustos et al., 1991). Although no strong consensus sequence has emerged from the identification of NLSs in a number of proteins, two classes of NLSs may exist (Dingwall and Lükey, 1991). The first class, characterized by the SV-40 large T antigen NLS, consists of a stretch of three to five basic residues conforming to the weak consensus Lys-Arg/Lys-X-Arg/Lys (Chelsky et al., 1989). The second class of sequences, characterized by the Xenopus nucleoplasm NLS, is bipartite consisting of two basic regions of three to four residues each separated by a spacer of approximately 10 amino acids (Robbins et al., 1991). A NLS can function at various positions within a protein but the entire protein context may affect its activity (Roberts et al., 1987). Phosphorylation of sites adjacent to the NLS (Rih et al., 1991; Hennekes et al., 1993) and the number of NLSs in a protein (Dworetzky et al., 1988) may play important roles in the regulation of nuclear localization. Using a variety of affinity techniques, cytoplasmic and nuclear NLS-binding proteins have been identified from a number of sources from yeast to human (Yamasaki and Lanford, 1992). The role of most of these proteins in the protein import process is still unclear.

Materials and Methods

Cell Culture

Madin-Darby bovine kidney cells (MDBK) were grown in high glucose Dulbecco’s modified Eagle’s medium containing 10% calf serum (BioCell...
In Vitro Nuclear Envelope Binding Reaction

Digitonin permeabilization of MDBK cells grown on glass coverslips was as previously described (Adam et al., 1990). 2-4 h before permeabilization, the cells were aspirated from a 15cm dish and replaced with fresh medium. Allophycocyanin coupled to a 12-residue synthetic peptide corresponding to the SV-40 large T antigen NLS (APC-NLS) was prepared as previously described (Adam et al., 1990). For the binding reaction, the coverslips were rinsed briefly in import buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 5% glycerol, 2 mM DTT, 0.01% Brij 35, and developed at 0.5 ml/min. The fractions were assayed for envelope-binding activity, pooled, and concentrated as before. The Superose 12 column was repeated and the active fractions pooled. The pooled fractions were loaded on a Mono Q HR5/5 column equilibrated in TEA, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.01% Brij 35, and 2 mM DTT. The column was eluted with a linear gradient of 200–600 mM NaCl. Fractions containing envelope-binding activity were pooled and concentrated by vacuum dialysis in a celloidin apparatus (Schleicher & Schuell, Keene, NH). Aliquots were frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined using the micro BCA protein assay reagent (Pierce Chem. Co., Rockford, IL).

NEM Treatment of Purified Proteins

Purified NLS receptor and 97-kD protein were diluted in import buffer without DTT (0.2 µg in 40 µl). NEM dissolved in import buffer was added to a final concentration of 5 mM. The samples were incubated for 60 min on ice and the reaction terminated by the addition of DTT to 5 mM and BSA to 20 mg/ml. The proteins were then dialyzed for 2 h against import buffer containing 2 mM DTT. As controls, the proteins were treated with 5 mM DTT, and dialyzed as described. After dialysis, 20 µl of each protein mixture was loaded to a final volume of 40 µl and APC-NLS was added before application to the permeabilized cells.

Wheat Germ Agglutinin Inhibition of Envelope Binding

To assess the effects of wheat germ agglutinin (WGA) on binding to the envelope, permeabilized cells were first incubated with the indicated concentration of WGA diluted in import buffer for 15 min at 4°C. The coverslip was blotted to remove excess buffer and placed on a fresh drop of import buffer containing pH 7.4, NLS-receptor, APC-NLS and WGA at the indicated concentration. The samples were incubated for 5°C and processed for microscopy as described above.

Other Methods

Peptides used in this study were obtained from Multiple Peptide Systems (San Diego, CA), and had the following sequences: wild type SV-40 T antigen CGGGPPKKRRKVED, mutant CGGGPKKKKRRKVED. Proteins were analyzed by SDS-PAGE as described (Dreyfuss et al., 1984). Silver staining of gels was by the method of Rabilloud (Rabilloud et al., 1988).

Results

Purification of Nuclear Envelope Binding Factor

In an attempt to identify cytosolic factors required for protein import in digitonin permeabilized cells, bovine erythrocyte cytosol was fractionated by column chromatography. Column fractions were then assayed for the ability to stimulate nuclear accumulation of a fluorescent karyophilic protein. The fluorescent karyophile used was the phycobiliprotein allophycocyanin chemically coupled to a synthetic peptide containing the SV-40 large T antigen NLS, hereafter referred to as APC-NLS. This assay revealed column fractions that alone caused a distribution of APC-NLS at the surface of the nucleus as shown in Fig. 1. When focused on an equatorial section of the nucleus, the fluorescent signal was concentrated at the nuclear periphery, with some nucleolar binding observed in some experiments (see below). In many views the peripheral fluorescence was discontinuous, similar to the pattern of staining obtained with antibodies to the nucleoporin (Davis and Blobel, 1986; Snow et al., 1987)
or WGA (Finlay et al., 1987), suggesting that binding is at the nuclear pore. Focusing on the upper surface of the nucleus revealed a punctate pattern, again consistent with staining at the nuclear pore. Definitive identification of the envelope structures that are binding the APC-NLS will require electron microscopic analysis.

A protein that copurified with the envelope binding activity was purified from bovine erythrocytes (see Materials and Methods). After separation by hydroxylapatite chromatography and Mono Q, a high resolution strong anion exchanger, only a small number of fractions had greatly reduced envelope binding activity. Activity could be restored in these and several other fractions by the addition of purified NLS receptor (Adam and Gerace, 1991). Consequently, all fractions were assayed for stimulation of binding by the addition of purified NLS receptor. The purification procedure, described in detail in Materials and Methods, is similar to the purification of NLS receptor.

Fig. 2 shows a silver-stained gel of the purified protein and NLS receptor used in all of the experiments. The predominant protein in the active fraction by both silver and Coomassie blue staining is a 97-kD protein referred to hereafter as p97. A minor band at ~54 kD migrating slightly faster than the lower NLS receptor band was also seen in some preparations. Peptide cross-linking experiments and immunoblotting with polyclonal antibodies to the NLS receptor indicated that the protein was not receptor (data not shown). A typical purification from 1L of packed erythrocytes yields ~50–100 μg of protein. During purification, p97 eluted from anion exchange columns heterogeneously as did the envelope binding activity. This behavior was important for identification of the active species since p97 was the only protein that correlated with receptor-dependent envelope-binding activity. Tables estimating the recovery of activity

Figure 1. Binding to the nuclear envelope. The purified proteins were combined with APC-NLS as described in Materials and Methods. Focusing on an equatorial plane through the nucleus reveals a discontinuous peripheral nuclear stain. This pattern is more mottled/punctate in appearance when focusing on the upper surface of the nucleus. Bar, 20 μm.

Figure 2. SDS-PAGE of the purified components. 200 ng of each purified component were resolved on a SDS 12.5% polyacrylamide gel and stained with silver. The molecular weights of markers run in an adjacent lane are indicated. Lane 1 is purified NLS receptor. Lane 2 is purified p97.
Nuclear envelope binding is specific for a functional NLS. A 10–20-fold molar excess of wild type or mutant T antigen NLS peptide was added to each sample to compete for nuclear envelope binding of the APC-NLS. The fluorescence intensity of the nuclear envelope was determined by scanning photographic negatives in a laser scanning densitometer. Each bar represents averaged data from 30–40 nuclei.

are not presented due to the difficulty in obtaining meaningful values for activity in different fractions. Envelope binding in each column fraction depends upon the amount of receptor co-purifying with p97 as well as the ratio of the two proteins and the total amount of factors in the assay. Once receptor is separated from p97, activity is dependent upon an independently purified batch of receptor. No other envelope-binding activities, receptor dependent or independent, were observed in any other fractions throughout the purification.

Specificity of Binding

In order to determine the specificity of the nuclear envelope binding, synthetic peptides containing either a wild type large T antigen sequence or a non-functional mutant sequence (see Materials and Methods), were used to competitively inhibit binding to the nuclear envelope. The standard binding reaction was assembled with 1 μg of peptide included as competitor before addition to the permeabilized cells. The results in Fig. 3 shows that the functional wild type sequence decreased binding by 75% while the mutant sequence reduced binding by only 21%. The wild type sequence did not completely abolish binding because the free peptide was present in only a 10–20-fold molar excess over the peptide conjugated to the APC. These results are in agreement with previous chemical cross-linking data (Adam et al., 1989).

ATP and Temperature Dependence

The association of NLS-containing proteins with the nuclear pore is independent of temperature or the energy state of the cell. The nuclear envelope binding seen with the purified proteins is also not ATP or temperature dependent (Fig. 4). Incubation with the purified binding factors at 0°C restricted accumulation of APC-NLS to the nuclear envelope. Addition of ATP to this incubation resulted in a small amount of internalization, evinced by the weak fluorescence of the nucleoli. When the binding reaction was carried out at 30°C
without added ATP, there was increased accumulation in the nuclear interior and the nuclear envelope fluorescence decreased slightly. If ATP was added at 30°C, intranuclear accumulation increased further, but was much less than would be observed with unfractionated cytosol. The weak translocation activity appeared to be associated with the permeabilized cells and was most obvious when a completely homologous system was used, i.e., bovine factors on bovine cells. When the purified bovine factors were used on normal rat kidney cells, this endogenous accumulation activity was much less pronounced (Hertzler, S., and S. Adam, unpublished results). In some experiments, the permeabilized cells accumulated small amounts of APC-NLS at the nuclear envelope in the absence of exogenous factors, but this was <5% of the signal observed in the presence of exogenous factors.

Receptor Dependence

Binding of nuclear proteins to the nuclear pore in the absence of translocation into the nucleus has been demonstrated in intact cells and in vitro (Newmeyer and Forbes, 1988; Richardson et al., 1988; Moore and Blobel, 1992). While it is likely that this binding is mediated by specific NLS-binding proteins, the evidence for this has been lacking. In earlier experiments, purified receptor did not exhibit nuclear transport activity in the absence of cytosol, nor was envelope binding observed in the absence of transport (Adam and Gerace, 1991). Results presented in Fig. 5 show that receptor alone did not lead to binding of the APC-NLS at the nuclear envelope. Purified p97 also did not lead to envelope binding alone. However, when the two were mixed, a dramatic increase in the amount of nuclear envelope binding was seen. The two factors were saturable with respect to each other and showed maximal binding when present in approximately equimolar concentrations (data not shown). Sequential addition of p97 and NLS receptor did not lead to envelope binding suggesting that both proteins must be present at the same time for binding to occur (data not shown).

NEM Sensitivity of Envelope Binding

NEM inhibits nuclear protein accumulation in cell-free and permeabilized cell transport assays (Newmeyer and Forbes, 1990; Adam et al., 1990). The purified NLS receptor or p97 were treated with NEM to identify the sensitive component in binding to the nuclear envelope (Fig. 6). When p97 was treated with 5 mM NEM and mixed with untreated receptor, binding to the envelope decreased by approximately 75%. However, if NLS receptor was treated with 5 mM NEM and mixed with untreated p97, a smaller decrease of only 27% was observed. NEM treatment of both receptor and p97 reduced binding to the same level as treatment of p97 alone.

Wheat Germ Agglutinin Inhibits Binding to the Envelope

WGA inhibits the accumulation of proteins within the nucleus, probably through interaction with the O-glycosylated nucleoporins (Finlay et al., 1987). When WGA was included with the purified factors in the envelope binding assay, a significant decrease in binding to the nuclear envelope was observed (Fig. 7). The effect of WGA is likely to be at the level of the nucleoporins at the pore complex, since neither the NLS receptor nor p97 bound immobilized WGA (data not shown). Inhibition was substantial at concentrations of

Figure 5. Binding requires both p97 and NLS receptor. Binding reactions containing only p97, NLS receptor or both together were carried out as described in the text. Envelope binding is only seen when both NLS receptor and p97 are present at the same time. Bar, 20 μm.
Figure 6. NEM inactivation of binding activity. NLS receptor and p97 were treated separately with NEM as described and combined with untreated p97 or NLS receptor, respectively. NEM treated NLS receptor and p97 were also combined. Each bar represents an average of 20–30 nuclei. p97 is more sensitive to NEM inactivation than the NLS receptor.

WGA as low as 10 μg/ml (~36% decrease) and increased to a maximum of 70–80% at 250 μg/ml WGA. Interestingly, higher concentrations of WGA did not decrease binding significantly below this level. Although the permeabilized cells were incubated with WGA before the cytosolic factors, the short incubation time may not have been sufficient to allow all of the available WGA-binding sites to become saturated. The inhibition of binding by WGA is specific as inhibition of binding is abolished by preincubation of the WGA with triacetylchitotriose (Fig. 7). Once the APC-NLS was bound at the nuclear envelope, it was not competed off by WGA or released during a 20-min incubation in import buffer (data not shown).

Figure 7. WGA inhibition of binding. Permeabilized cells were incubated with the indicated concentration of WGA for 15 min. The excess buffer was blotted off and the coverslips inverted over a fresh drop containing NLS receptor, p97 and WGA at the indicated concentration. Each point represents averaged data from ~40 nuclei. The open square represents the average nuclear envelope fluorescence when a 50-fold molar excess of triacetylchitotriose is mixed with 100 μg/ml WGA.

Discussion

Cytoplasmic Factors in Nuclear Protein Accumulation

The role of cytoplasmic factors in nuclear protein import is now well established. In microinjection experiments, Breeuwer and Goldfarb (1990) provided evidence for saturable cytoplasmic NLS-binding components that prevented the diffusion of small NLS-containing proteins into the nucleus in the absence of active transport. Direct evidence of the involvement of soluble cytoplasmic components in protein import has come from cell-free or permeabilized cell assays. Newmeyer and Forbes (1990) identified two factors that restore import or envelope binding to a Xenopus egg extract that had been inactivated by NEM. One factor, termed NIF-1, restored import activity to NEM-treated cytosol and was required for ATP-independent binding of proteins to the pore complex. The second factor, NIF-2, was also NEM sensitive and acted synergistically with NIF-1 to promote import.

Digitonin permeabilized cells have also been used to identify cytoplasmic factors required for import. Import in this system was sensitive to inactivation by NEM, but also required an NEM-insensitive cytosolic component (Adam et al., 1990; Adam and Gerace, 1991). Using this assay, Adam and Gerace (1991) identified specific NLS-binding proteins of 54/56 kD that stimulated import and comprised one of at least two NEM-sensitive cytosolic components. Using the same assay, Sterne-Marr et al. (1992) partially depleted import activity from cytosol using O-glycosylated nucleoporins immobilized on WGA-agarose beads. Moore and Blobel (1992) recently described a crude fractionation of Xenopus oocyte extracts that separated the envelope binding and translocation activities in permeabilized cells. The first fraction (fraction A) was required for NLS-mediated envelope binding, and the second fraction (fraction B) was required for the translocation of proteins to the nuclear interior. A similar assay developed in Drosophila melanogaster cultured cells did not require cytosolic factors for NLS binding to the permeabilized cells or the nuclear envelope, but required cytosol for import (Stochaj and Silver, 1992). Antibodies to a conserved 70-kD NLS-binding phosphoprotein inhibit the binding step (Stochaj et al., 1992; Stochaj and Silver, 1992).

The relationship of these many factors is unclear. NIF-1 and fraction A are both NEM-sensitive factors required for binding to the nuclear envelope, but fraction A is inactivated by ammonium sulfate while NIF-1 is not. In this respect fraction A is similar to NIF-2. It has been suggested that NIF-1 and fraction A contain specific NLS-binding proteins but this has not been demonstrated directly. The 54/56-kD NLS receptors from erythrocytes are the only NLS-binding proteins that have been shown to be directly involved in protein import (Adam and Gerace, 1991). The 97-kD protein described here, in conjunction with the NLS receptor, is sufficient to direct an NLS-containing protein to the nuclear envelope. The relationship of the purified bovine proteins to the Xenopus fractions is unknown. With further characterization, the Xenopus extracts will likely yield analogous proteins.

The second step in protein transport, translocation across the nuclear envelope, requires a distinct cytoplasmic factor.
that is not sensitive to NEM inactivation (Moore and Blobel, 1992). NLS receptor and p97 are physically separated from
an activity that causes rapid accumulation of the APC-NLS
in the nucleus when combined with NLS receptor and p97.
This activity is insensitive to NEM inactivation, as is frac-
tion B of Moore and Blobel (1992), and may represent the
erythrocyte equivalent of the Xenopus fraction (Hertzler, S.,
and S. Adam, unpublished). It is interesting to note that
NEM abolishes the ability of the NLS receptor to stimulate
transport (Adam and Gerace, 1991), yet does not dramati-
cally affect binding to the pore complex. This suggests that
the modified amino-acids in the receptor must be required
for a subsequent step in transport.

Mechanism of Binding to the Nuclear Pore

Nuclear protein import is undoubtedly composed of multiple
discrete steps that lead to accumulation of karyophiles within
the nucleus. One of the earliest steps in this pathway must
be recognition of an NLS by an NLS receptor in the
cytosol. In vitro peptide binding experiments suggest that
this is a very labile interaction although of rather high affinity
(Adam et al., 1989; unpublished results). At some point in
transport, the interaction of NLS receptor with the NLS
must be stabilized for efficient transport to occur. Perhaps
formation of a p97/receptor complex stabilizes NLS recep-
tor binding. Other fractions required for envelope binding in
Xenopus cytosol, NIF-1 and fraction A, exist as high molecu-
lar weight complexes (Moore and Blobel, 1992; New-
meyer, 1993). Neither the NLS receptor nor p97 can be
detected in a high molecular weight complex during puri-
fication. This may represent some fundamental difference
between Xenopus extracts and erythrocyte cytosol.

Interaction of the NLS receptor and p97 with the pore may
occur either individually or as a complex of the two proteins.
The results presented here indicate that receptor and p97
must be present at the same time for envelope binding. The
two proteins may form a stable complex, and it is this com-
plex that is recognized by the pore. Alternatively, a complex
may form with a third component provided by the pore,
stabilizing the interaction of receptor and p97. Binding of the
NLS to the free receptor or the receptor/p97 complex would
be possible in such a model. It is also possible that receptor
d and p97 do not interact directly. Association of the receptor
with the pore may be stabilized by binding of p97 to a differ-
ent site on the pore. Experiments are currently under way to
differentiate between these models. The fate of either the
NLS receptor or p97 after binding to the pore is unknown.
The presence of NLS receptor in the nucleus suggests that
some of the cytosolic transport components may shuttle be-
tween the cytoplasm and nucleus (Adam et al., 1989).

All of the results presented here were obtained with an
artificial karyophile consisting of a naturally fluorescent pro-
tein chemically coupled to a synthetic peptide representing
the SV40 large T antigen NLS. It should be noted that addi-
tional control mechanisms that affect the transport of authen-
tic karyophilic proteins are probably not operative in this as-
say. However, these experiments reveal the basic components
of the transport apparatus. Experiments with other NLSs of
the T antigen class as well as with sequences of bipartite
NLSs suggest that all NLSs can use these components for
pore binding and transport in permeabilized cells (Hertzler,
S., and S. Adam, manuscript in preparation). In this respect,
Michaud and Goldfarb (1993) have presented evidence that
both classes of NLS compete for a single receptor. It will be
interesting to see if p97 is a common factor for the import
of other karyophilic molecules such as snRNPs.

Inhibition of Binding by WGA

When microinjected into intact cells, WGA effectively
blocks the import of most karyophilic proteins, but does not
constrict the diffusion channel of the pore complex (Yoneda
et al., 1987; Dabauville et al., 1988). Cells injected with
WGA and a fluorescent karyophilic protein accumulate the
protein in the perinuclear region but not strongly with the nu-
clear envelope (Yoneda et al., 1987; Dabauville et al., 1988).
It has been suggested that WGA inhibits the translocation
step in transport, but does not affect binding to the pore
(Newmeyer and Forbes, 1988; Moore and Blobel, 1992).
The experiments presented here lead to a different interpre-
tation of import inhibition by WGA. Using purified proteins
to reconstitute binding to the envelope, there is a clear dose
response between the amount of WGA added and the amount
of NLS-mediated binding at the nuclear envelope. It is in-
teresting to note that WGA does not completely block bind-
ing at the envelope at the WGA concentrations used. This
suggests that at least some of the binding sites may be spa-
tially separated from the sugar residues of the nucleoporins.
An alternative explanation is that receptor bound at the pore
complex prior to permeabilization can release its bound
karyophile during the incubation allowing the fluorescent
protein to bind. The mechanism of WGA inhibition will re-
quire further experiments to determine the nature of the bind-
ing site for p97/receptor. The O-linked glycoproteins of
the pore complex may be involved in the active recognition
of cytoplasmic transport factors (Finlay et al., 1991; Sterne-
Marr et al., 1992). The results presented here provide fur-
ther evidence that the O-linked nucleoporins may represent
the docking site for the NLS receptor at the pore. The de-
crease in the rate of protein import by WGA may be due, in
part, to a reduced binding of transport factors at the cyto-
plasmic face of the pore.

Perhaps the discrepancy between the results of WGA inhibi-
tion experiments presented here and earlier reports relates
to fundamental differences between egg/oocyte extracts and
mammalian somatic cell cytosolic factors, or to the differ-
ence between isolated nuclei in egg extracts and the nuclei
in permeabilized cells. Akey and Goldfarb (1989) have sug-
gested that import involves at least three distinct steps: bind-
ing to structures peripherally associated with the pore, dock-
ing over the center of the pore and translocation to the
nuclear interior. Additionally, Richardson and co-workers
(1988) have shown that gold particles arrested at the cyto-
plasmic side of the pore appear to aggregate on filaments ex-
tending into the cytoplasm. Filaments extending from the
cytoplasmic ring of the pore can be visualized in thin sections
and scanning electron micrographs (Ris, 1991; Jarnik and
Aebi, 1991; Goldberg and Allen, 1992). It is possible that
the cytoplasmic filaments are not well preserved in isolated
nuclei, but are retained in digitonin permeabilized cells. If
the WGA-sensitive binding sites are present on these fila-
ments, they might not be observed on isolated nuclei in
Xenopus extracts. However, this argues that the filaments
are not obligatory participants in the transport process, and that
in their absence, other WGA-insensitive binding sites remain and transport can still occur.

Other Factors in Nuclear Protein Import

Two recent reports have implicated hsc70 in the import of nuclear proteins. Import activity can be depleted from cytosol used in the permeabilized cell assay with ATP-agarose (Shi and Thomas, 1992). The activity could be reconstituted with proteins eluted from the ATP-agarose, or with bacterially expressed hsp70 and hsc70. In another study, Imamoto et al. (1992) isolated a 69-kD protein by nucleoplasmin NLS affinity chromatography. This protein was recognized by an antibody that inhibits transport when microinjected into cells (Yoneda et al., 1988). Protein sequence analysis identified the 69-kD protein as hsc70. Two hsp70 cognate proteins shuttle between the nucleus and cytoplasm in Xenopus oocytes (Mandell and Feldherr, 1990), and hsp70/hsc70 colocalize to the nucleus with certain karyophilic proteins, suggesting a possible transport function for these proteins (Koskinen et al., 1991; Henriksson et al., 1992; Okuno et al., 1993). Neither the 54/56-kD NLS receptor nor p97 are recognized by antibodies specific for hsp70, hsc70, or hsp90. Purified NLS receptor and p97 do not bind ATP agarose under the conditions used by Shi and Thomas (1992), and their envelope binding function does not require ATP. We conclude that if hsc70 is involved in protein import, it is likely to be at a step after binding to the pore.

During the preparation of this manuscript, two groups reported the involvement of the GTP-binding protein Ran/TC4 in protein transport (Moore and Blobel, 1993; Melchior et al., 1993). In our hands, with erythrocyte cytosols, non-hydrolyzable GTP analogs do not inhibit transport or binding, nor does GTP stimulate transport. However, preliminary experiments with brain cytosol demonstrate a strong inhibition of import by the non-hydrolyzable analogs (Hertzler, S., and S. Adam, unpublished). Given that we are able to reconstitute the binding step in vitro with two purified proteins, it seems unlikely that GTP or a GTP-binding protein are required for this step. The requirement for GTP-binding proteins in protein transport would provide an efficient integration of protein import with RNA export and other nuclear functions.

The authors would like to thank Neil Chi, Shannon Hertzler, and Patrick Hamblin for critical reading of the manuscript.

This work was supported by grants from the Cancer Research Foundation, grant GM47866-01 from the National Institutes of Health, and grant JFRA-433 from the American Cancer Society.

Received for publication 10 December 1993 and in revised form 10 February 1994.

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