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

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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)[1] (Garcia-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 Laskey, 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 nucleoplasmin 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 (Rihs 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.

1. *Abbreviations used in this paper*: MDBK, Madin-Darby bovine kidney; NLS, nuclear localization sequence; TEA, triethanolamine; WGA, wheat germ agglutinin.

It is now clear that both pore complex and cytoplasmic proteins are required for nuclear transport. The requirement for nuclear pore complex components in the transport process has been demonstrated by genetic analysis in yeast (reviewed by Newmeyer, 1993), by antibody inhibition in higher eukaryotes (Featherstone et al., 1988), and by pore complex reconstitution studies (Finlay et al., 1991). Some control of transport is intrinsic to the pore complex since the permeability of the pore is altered in response to the growth and shape of the cell (Feldherr and Akin, 1993). Cell free or permeabilized cell systems that reproduce transport in vitro have directly demonstrated the requirement for cytoplasmic factors in nuclear protein transport (Newmeyer et al., 1986; Adam et al., 1990; Shi and Thomas, 1992; Moore and Blobel, 1992).

Protein transport into the nucleus can be divided into two identifiable steps: accumulation at the cytoplasmic face of the pore complex followed by translocation into the nuclear interior (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989). The first step is independent of ATP or temperature while the second requires ATP hydrolysis. In order to understand the mechanism of protein accumulation in the nucleus, we are purifying soluble cytosolic factors required for transport in permeabilized cells. We report here the purification of a protein that, in conjunction with the previously identified NLS receptor, reconstitutes the first step in protein transport, binding to the pore complex.

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

Laboratories, Rancho Dominguez, CA), nonessential amino acids, and penicillin/streptomycin. Cultures were maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Cells were removed from plastic dishes by trypsinization and replated on 18 × 18 mm glass coverslips 18–24 h before use.

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 culture media was aspirated from the cells and replaced with fresh media. 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 7.35, 110 mM potassium acetate, 2 mM magnesium acetate, 0.1 mM EGTA, and 2 mM DTT) and permeabilized with 50 μg/ml digitonin in import buffer at room temperature for 5 min. The coverslips were then incubated in import buffer on ice for 5 min. A standard reaction containing purified factors was comprised of 100 ng p97, 100 ng NLS receptor and 25–100 ng APC-NLS and import buffer to a final volume of 50 μl. In some experiments BSA was added to 20 mg/ml with no effect on binding. In experiments requiring ATP, an ATP-regenerating system was added containing 0.5 mM ATP, 10 mM creatine phosphate, and 20 μ/ml creatine phosphokinase. The coverslips were inverted over a 50-μl drop on a piece of parafilm inside a humidified chamber floating on an ice water bath. The incubation was carried out in the dark for 30 min and terminated by rinsing each coverslip briefly in import buffer. The cells were fixed in 2% formaldehyde in import buffer for 10 min at room temperature. The coverslips were mounted in import buffer and observed by epifluorescence with a Zeiss Axioskop microscope equipped with a 63× 1.25 NA objective. Photographs were taken with Kodak T-Max film. For quantitation, the image was focused on a plane roughly corresponding to a section through the center of the nucleus. Most of the nuclei in a particular field of view will be in the same focal plane. Quantitation of fluorescence intensity was determined by scanning photographic negatives with a scanning laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) as described (Adam and Gerace, 1991).

Purification of Binding Components

NLS receptor was purified from bovine erythrocytes as described (Adam and Gerace, 1991). Nuclear envelope binding activity was purified by a modification of the same method. One liter of packed erythrocytes (Biocell Laboratories) were lysed in 1.5 liters 5 mM Hepes-KOH (pH 7.4), 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM PMSF for 20 min at 4°C. The lysate was centrifuged at 10,000 *g* for 10 min to pellet any insoluble material. Ammonium sulfate was added slowly to the supernatants (30 g/100 ml) and allowed to mix for 1–3 h. The resulting precipitates were collected by centrifugation at 6,000 *g* for 20 min and washed once with 50% ammonium sulfate. The pellets were stored frozen at −20°C or immediately carried to the next step.

The ammonium sulfate pellets were dissolved in 400 ml cold Hepes/EDTA buffer (5 mM Hepes, pH 7.4, 1 mM EDTA) with 2 mM DTT and 1 mM PMSF. The solution was centrifuged at 15,000 *g* for 30 min to pellet any insoluble material and the supernatants applied to a 7 × 5 cm column of phenyl Sepharose CL4-B (Pharmacia Fine Chemicals, Piscataway, NJ) at a flow rate of approximately 150 ml/h. The column was washed with 750 ml of the Hepes/EDTA buffer with 2 mM DTT and eluted with 300 ml 80% ethylene glycol in Hepes/EDTA buffer with 2 mM DTT.

The eluant was diluted twofold with 20 mM Triethanolamine (TEA), pH 8.0, 100 mM NaCl, 2 mM EDTA and 2 mM DTT, and loaded on a 2.6 cm × 14 cm DEAE Sepharose Fast Flow (Pharmacia Fine Chemicals) column at 2 ml/min. The column was washed to baseline absorbance with 20 mM TEA, pH 8.0, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.01% Brij 35, and eluted stepwise first with three column volumes of 200 mM NaCl followed by 1 M NaCl. The 1 M NaCl eluant was immediately loaded on a 1.6 cm × 5 cm hydroxylapatite column at 0.5 ml/min. The column was washed with 1 mM sodium phosphate, pH 7.0, 1 mM DTT to baseline absorbance. The proteins were eluted with a 50-ml linear gradient of 1–150 mM sodium phosphate, 1 mM DTT. The fractions containing envelope binding activity (30–90 mM phosphate) were pooled and loaded on a Mono Q HR5/5 column (Pharmacia Fine Chemicals) equilibrated in 20 mM Hepes, pH 7.0, 100 mM sodium acetate, 0.1 mM EDTA, 0.01% Brij 35, and 2 mM DTT. The column was washed with the same buffer to baseline absorbance and eluted with a linear gradient of 0.4–1.2 M sodium acetate in the same buffer.

At this point in the purification, envelope binding became entirely dependent upon the addition of purified receptor. The fractions containing envelope binding activity (0.56 M–0.06 M acetate) were precipitated by addition of ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation at 10,000 *g* for 10 min and resuspended in 300 μl of import buffer. The sample was applied to a Superose 12 HR10/30 gel filtration column (Pharmacia Fine Chemicals) equilibrated in 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 MonoQ HR5/2 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 collodion 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 were mixed 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 cover slip was blotted to remove excess buffer and placed on a fresh drop of import buffer containing p97, NLS-receptor, APC-NLS and WGA at the indicated concentration. The samples were incubated at 4°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 CGYGPKKKRKVED, mutant CGYGPKNKRKVED. 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 nucleoporins (Davis and Blobel, 1986; Snow et al., 1987)

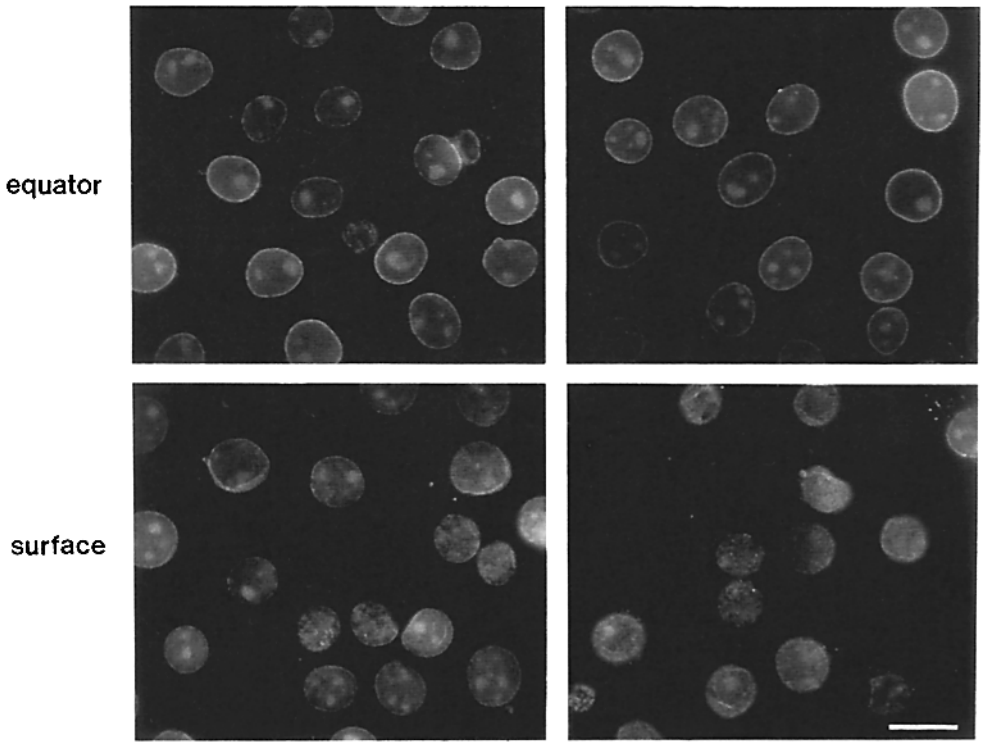

Figure 1. Binding to the nuclear envelope. The purified proteins were combined with APC-NLS as described in Materials and Methods. Focusing on an equatorial plane through the nucleus reveals a discontinuous peripheral nuclear stain. This pattern is more mottled/punctate in appearance when focusing on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

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

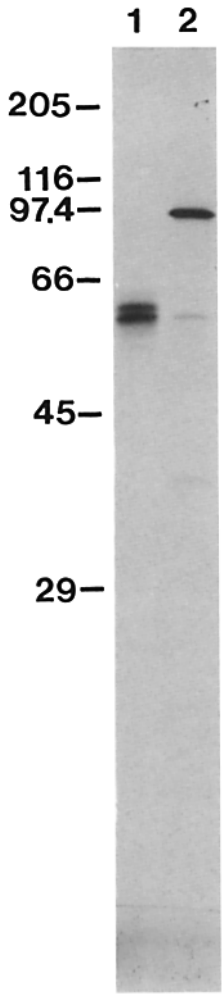

Figure 2. SDS-PAGE of the purified components. 200 ng of each purified component were resolved on a SDS 12.5% polyacrylamide gel and stained with silver. The molecular weights of markers run in an adjacent lane are indicated. Lane *1* is purified NLS receptor. Lane *2* is purified p97.

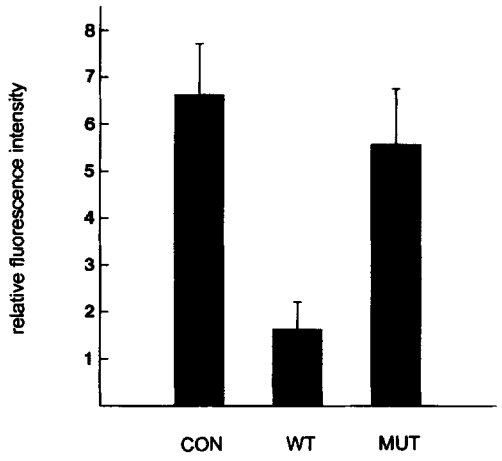

Figure 3. Nuclear envelope binding is specific for a functional NLS. A 10–20-fold molar excess of wild type or mutant T antigen NLS peptide was added to each sample to compete for nuclear envelope binding of the APC-NLS. The fluorescence intensity of the nuclear envelope was determined by scanning photographic 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

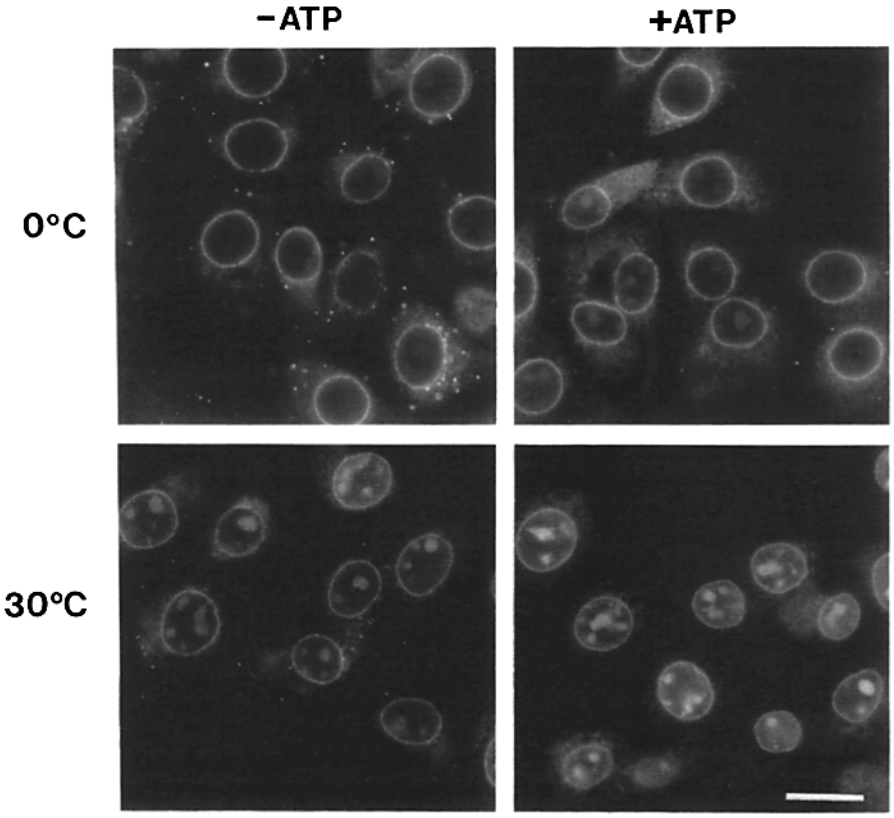

Figure 4. ATP and temperature dependence of binding. Each binding reaction was carried out at the indicated temperature with or without ATP and an ATP-regenerating system. Bar, 20 μm.

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

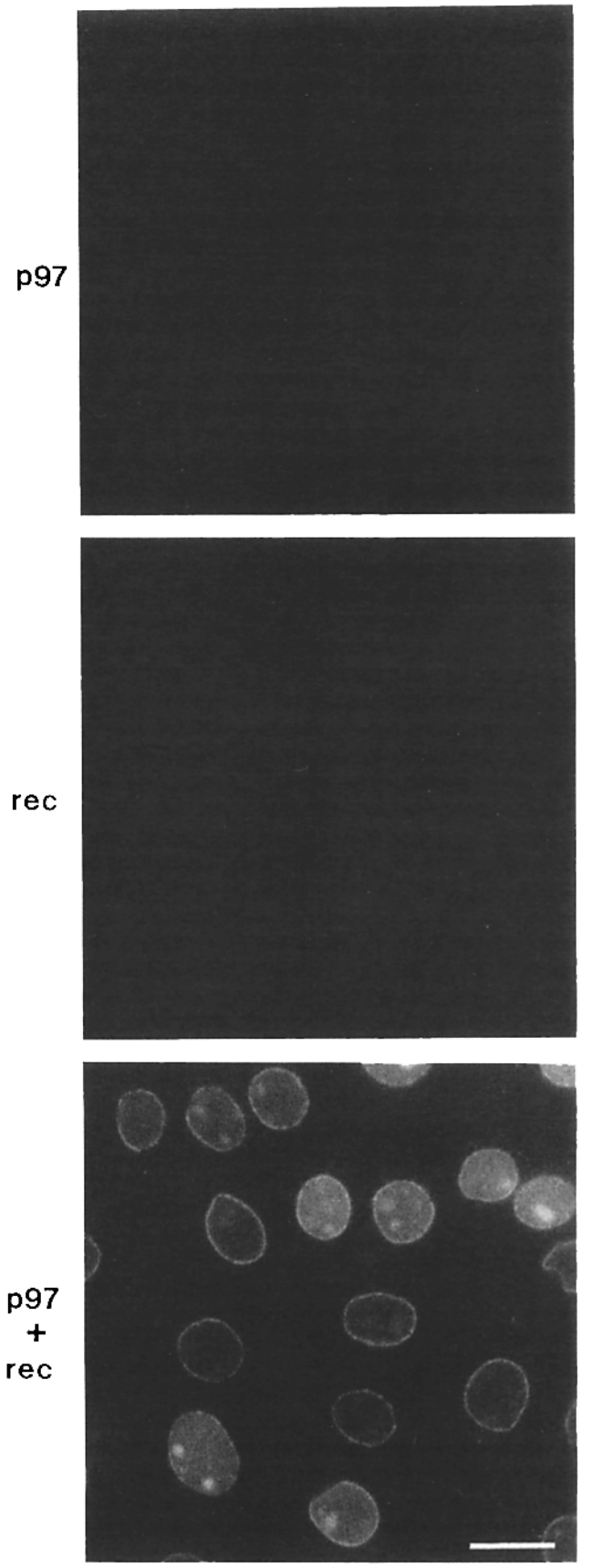

Figure 5. Binding requires both p97 and NLS receptor. Binding reactions containing only p97, NLS receptor or both together were carried out as described in the text. Envelope binding is only seen when both NLS receptor and p97 are present at the same time. Bar, 20 μm.

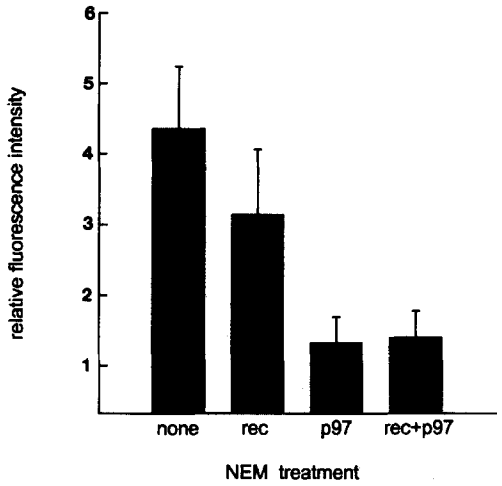

Figure 6. NEM inactivation of binding activity. NLS receptor and p97 were treated separately with NEM as described and combined with untreated p97 or NLS receptor, respectively. NEM treated NLS receptor and p97 were also combined. Each bar represents an average of 20–30 nuclei. p97 is more sensitive 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).

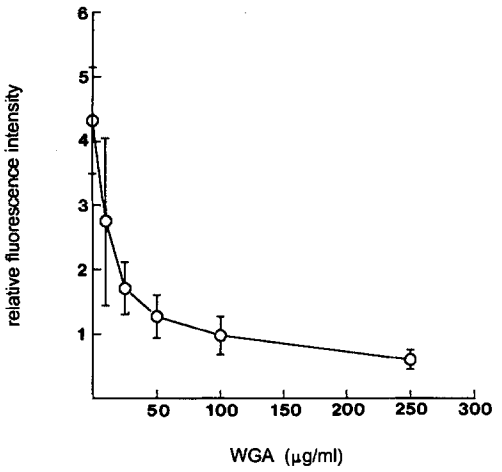

Figure 7. WGA inhibition of binding. Permeabilized cells were incubated with the indicated concentration of WGA for 15 min. The excess buffer was blotted off and the coverslips inverted over a fresh drop containing NLS receptor, p97 and WGA at the indicated concentration. Each point represents averaged 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., 1991; 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 fraction 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 dramatically 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 cytoplasm. 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 receptor binding. Other fractions required for envelope binding in Xenopus cytosol, NIF-1 and fraction A, exist as high molecular weight complexes (Moore and Blobel, 1992; Newmeyer, 1993). Neither the NLS receptor nor p97 can be detected in a high molecular weight complex during purification. 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 complex 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 and p97 do not interact directly. Association of the receptor with the pore may be stabilized by binding of p97 to a different 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 between 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 protein chemically coupled to a synthetic peptide representing the SV40 large T antigen NLS. It should be noted that additional control mechanisms that affect the transport of authentic karyophilic proteins are probably not operative in this assay. 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 nuclear 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 interpretation 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 interesting to note that WGA does not completely block binding at the envelope at the WGA concentrations used. This suggests that at least some of the binding sites may be spatially 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 require further experiments to determine the nature of the binding 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 further evidence that the O-linked nucleoporins may represent the docking site for the NLS receptor at the pore. The decrease in the rate of protein import by WGA may be due, in part, to a reduced binding of transport factors at the cytoplasmic face of the pore.

Perhaps the discrepancy between the results of WGA inhibition experiments presented here and earlier reports relates to fundamental differences between egg/oocyte extracts and mammalian somatic cell cytosolic factors, or to the difference between isolated nuclei in egg extracts and the nuclei in permeabilized cells. Akey and Goldfarb (1989) have suggested that import involves at least three distinct steps: binding to structures peripherally associated with the pore, docking 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 cytoplasmic side of the pore appear to aggregate on filaments extending 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 filaments, 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 the GTP-binding protein Ran/TC4 in protein transport (Moore and Blobel, 1993; Melchior et al., 1993). In our bands, 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.

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