O-linked Glycoproteins of the Nuclear Pore Complex Interact with a Cytosolic Factor Required for Nuclear Protein Import

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Abstract. Mediated import of proteins into the nucleus requires cytosolic factors and can be blocked by reagents that bind to O-linked glycoproteins of the nuclear pore complex. To investigate whether a cytosolic transport factor directly interacts with these glycoproteins, O-linked glycoproteins from rat liver nuclear envelopes were immobilized on Sepharose beads via wheat germ agglutinin or specific antibodies. When rabbit reticulocyte lysate (which provides cytosolic factors required for in vitro nuclear import) was incubated with the immobilized glycoproteins, the cytosol was found to be inactivated by up to 80% in its ability to support mediated protein import in permeabilized mammalian cells. Inactivation of the import capacity of cytosol, which was specifically attributable to the glycoproteins, involves stoichiometric interactions and is likely to involve binding and depletion of a required factor from the cytosol. This factor is distinct from an *N*-ethylmaleimide-sensitive receptor for nuclear localization sequences characterized recently since it is insensitive to *N*-ethylmaleimide. Cytosol inactivation is suggested to be caused by at least two proteins of the glycoprotein fraction, although substantial capacity for inactivation can be attributed to protein bound by the RL11 antibody, consisting predominantly of a 180-kD glycosylated polypeptide. Considered together, these experiments identify a novel cytosolic factor required for nuclear protein import that directly interacts with O-linked glycoproteins of the pore complex, and provide a specific assay for isolation of this component.

NUCLEAR pore complexes (NPCs)[1] are supramolecular assemblies of ~125,000 kD (Reichelt et al., 1990) that perforate the nuclear envelope and provide aqueous channels for exchange of both large and small molecules between the nucleus and cytoplasm (for reviews see Franke et al., 1981; Gerace and Burke, 1988). The NPC consists of a central "plug-spoke" assembly flanked by ~120-nm-diam peripheral rings which face the nucleoplasm and cytoplasm (Unwin and Milligan, 1982; Akey, 1989; Reichelt et al., 1990). Both the plug-spoke assembly and the two rings have pronounced eightfold radial symmetry with respect to an axis perpendicular to the nuclear envelope, suggesting that certain biochemical features of the NPC are repeated. The plug-spoke structure apparently provides the major permeability barrier across the NPC, and contains a central transport channel (Feldherr et al., 1984; Akey, 1989). The NPC acts as a molecular sieve with an effective diameter of 10 nm which allows passive diffusion of metabolites and small nonnuclear molecules (Paine et al., 1975; Peters, 1986). In contrast, import of karyophilic proteins (Goldfarb et al., 1986) and export of tRNA (Zasloff, 1983) and ribosomal subunits (Khanna-Gupta and Ware, 1989; Bataille et al., 1990) are mediated processes.

Mediated protein import has been the most thoroughly studied of nuclear translocation events (Gerace and Burke, 1988; Silver, 1991). This process is directed by short amino acid stretches in nuclear proteins called nuclear location sequences (NLSs). NLSs can direct nuclear import of large nonnuclear proteins when synthetic peptides containing these sequences are chemically coupled to the carriers or when chimeric genes encoding the targeting sequence are expressed in vivo (Kalderon et al., 1984; Goldfarb et al., 1986; Lanford et al., 1986). While NLSs do not conform to a strong consensus sequence, they usually contain a cluster of basic amino acids flanked by a glycine or proline residue as exemplified by the NLS of the SV-40 T antigen (Chelsky et al., 1989) or two short clusters of basic residues separated by a spacer as seen with the NLS of nucleoplasmin (Robbins et al., 1991).

In vivo and in vitro studies have shown that nuclear protein import requires ATP and is inhibited in the cold (Newmeyer et al., 1986; Richardson et al., 1988; Adam et al., 1990), and therefore is likely to be an active transport process. Interaction of particles containing NLSs with the NPC is thought to trigger enlargement of the pore channel to allow passage of particles up to ~30 nm in diameter, which is substantially

1. *Abbreviations used in this paper*: GlcNAc, *N*-acetylglucosamine; NEM, *N*-ethylmaleimide; NLS, nuclear location sequence; NPC, nuclear pore complex; PCG, NPC glycoprotein; WGA, wheat germ agglutinin.

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larger than the diffusional channel of the pore (Feldherr et al., 1984; Akey and Goldfarb, 1989). Although the transport steps that utilize ATP have not been defined, they are likely to include the process of channel opening.

Important insight into the biochemistry of mediated nuclear import has come from the use of cell-free systems to study this process. One such system involves use of cell-free extracts from *Xenopus* eggs to assemble nuclei from added DNA or to reseal isolated rat liver nuclei, which initially are broken open by the isolation process (Newmeyer et al., 1986). A fraction of the resulting nuclei are intact and able to concentrate NLS-containing ligands from the surrounding medium in a fashion that satisfies all available criteria for physiologically relevant nuclear import. A second system that reproduces physiological nuclear import involves the use of cultured mammalian cells permeabilized by treatment with digitonin, which perforates the plasma membrane but leaves the nucleus intact (Adam et al., 1990). The permeabilized cells, when supplemented with exogenous cytosol and ATP, efficiently concentrate ligands containing NLSs from the surrounding medium. One of the key points that has emerged from initial characterization of this system is that in vitro nuclear import absolutely requires cytosolic factors (Adam et al., 1990). Cytosolic factors were also shown to be required in the *Xenopus* egg nuclear assembly/import system (Newmeyer and Forbes, 1990).

The number of different cytosolic factors involved in nuclear import is unknown. In both the mammalian and *Xenopus* nuclear import systems, at least two cytosolic factors are sensitive to inactivation by the sulfhydryl alkylating reagent *N*-ethylmaleimide (NEM) (Adam et al., 1990; Newmeyer and Forbes, 1990). One NEM-sensitive factor has been recently purified from mammalian cytosol on the basis of its ability to specifically bind NLSs. This factor has been shown to constitute a functional transport receptor in vitro, since it can stimulate both nuclear import in the permeabilized cell assay, and transport in a system inactivated by NEM pretreatment (Adam and Gerace, 1991). Additional cytosolic factors have been directly implicated in the mammalian nuclear import system, since the purified transport receptor itself is insufficient to support nuclear import in the absence of added cytosol (Adam and Gerace, 1991).

Understanding the process of nuclear import will require biochemical characterization of the NPC as well as cytoplasmic transport factors. Up to now, only a handful of proteins present in the NPC have been identified. One of these is gp210, a transmembrane glycoprotein with asparagine-linked carbohydrate that is present at ~25 copies per NPC (Gerace et al., 1982; Wozniak et al., 1989; Greber et al., 1990). At least eight additional NPC polypeptides of 45–210 kD were identified by monoclonal antibodies directed against vertebrate NPCs (Davis and Blobel, 1987; Snow et al., 1987). These proteins, which vary in estimated abundance from two to eight copies per NPC, contain up to 10 or more mol of O-linked *N*-acetylglucosamine (GlcNAc) per mol of protein (Holt et al., 1987). These proteins also are recognized by wheat germ agglutinin (WGA; Finlay et al., 1987), a lectin that binds to proteins containing terminal GlcNAc. Some of the O-linked glycoproteins appear to contain both glycosylated and nonglycosylated subunits, since when nuclear envelopes are solubilized in buffers containing Triton X-100 and high salt, a number of major nonglycosylated polypeptides coimmunoprecipitate with the eight polypeptides bearing O-linked GlcNAc (Snow et al., 1987). At least three of the glycosylated polypeptides are present in a single complex that is stable after detergent solubilization of nuclear envelope (Finlay et al., 1991).

Several experiments support the possibility that the O-linked glycoproteins play an important role in mediated nuclear import, although the evidence for this is not conclusive. Mediated nuclear import is inhibited by monoclonal antibodies recognizing these glycoproteins (Dabauville et al., 1988*a*; Featherstone et al., 1988) as well as by WGA (Finlay et al., 1987; Yoneda et al., 1987; Dabauville et al., 1988*b*; Adam et al., 1990). The antibodies and WGA could act directly by inhibiting a hypothetical transport function of the glycoproteins, or alternatively, could act indirectly by sterically inhibiting other components of the NPC. When the O-linked glycoproteins are depleted from a cell-free *Xenopus* extract that mediates nuclear assembly, the NPCs that are assembled from this depleted extract are deficient in nuclear protein import (Finlay and Forbes, 1990). This indicates that either the O-linked glycoproteins themselves are involved in nuclear import, or alternatively, that these proteins are needed for assembly of other proteins in the NPC that have an essential transport role. Two yeast genes related to the mammalian glycoproteins, NUP1 (Davis and Fink, 1990) and NSP1 (Nehrbass et al., 1990), are essential for cell growth, although the direct relevance of these genes to NPC assembly and/or function is unknown. Considered together, these experiments argue that further analysis is required to determine whether the O-linked glycoproteins themselves have a direct role in nuclear import.

In this study we have used a biochemical approach to investigate this question. Using a nuclear import system consisting of digitonin-permeabilized cells supplemented with rabbit reticulocyte lysate as a source of cytosol, we found that preincubation of cytosol with the immobilized O-linked glycoproteins substantially reduces the ability of the cytosol to support nuclear import. This effect is highly specific and is likely to be due to depletion of an essential transport factor from cytosol by interaction with the glycoproteins. This transport factor is insensitive to inactivation by NEM and is distinct from the NLS receptor previously described. These results constitute strong evidence that O-linked glycoproteins themselves have a direct role in nuclear protein import. The experiments also provide a specific assay for isolation of a cytosolic factor that interacts with these glycoproteins, and promise to further our understanding of the process of nuclear protein import.

Materials and Methods

Assay and Quantitation of Nuclear Import in Vitro

Normal rat kidney cells were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 atmosphere. 12–48 h before use in the import assay, cells were passaged onto 18 × 18-mm glass coverslips (Thomas Scientific, Swedesboro, NJ). Cells were permeabilized with digitonin as previously described (Adam et al., 1990) except that transport buffer was: 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 20 mM HEPES, pH 7.4, 2 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF. Rabbit reticulocyte lysate (Promega Corp., Madison, WI) was used as exogenous cytosol in the transport assay, and before use was dialyzed against

transport buffer using a collodion membrane (25,000 mol wt cut-off) apparatus (Schleicher & Schuell, Inc., Keene, NH). Collodion dialysis allowed rapid and efficient buffer exchange with ~20% dilution of the extract (dialyzed extracts contained ~80 mg/ml protein). In a typical import assay, cytosol accounted for 50% of a 40-μl reaction volume. Allophycocyanin conjugated to four to eight peptides containing the SV-40 large T antigen nuclear localization sequence was prepared as described by Adam et al. (1990) and used at a final concentration of 100 nM in the import assay. The ATP regenerating system was as described and the reaction mixture was brought to 40 μl with transport buffer. Coverslips containing washed permeabilized cells were inverted over 35 μl of this reaction mixture and incubated as described (Adam et al., 1990). After the transport reaction, coverslips were washed twice with transport buffer and fixed for 10 min at 4°C in transport buffer containing 4% paraformaldehyde. Coverslips were again washed in transport buffer, mounted on microscope slides, and sealed with nail polish. In this study, we used washed and formaldehyde-fixed samples to quantitate the level of nuclear import in permeabilized cells. This procedure offered technical advantages compared with our previous procedure involving unfixed samples mounted directly in the medium used for nuclear import (Adam et al., 1990), since fixed cells remained better attached to coverslips during image analysis and could be stored overnight at 4°C before analysis. For qualitative visualization, nuclear transport was observed by epifluorescence with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) using a 63× objective. Nuclei were photographed with Kodak Tmax film.

An image analyzer, the ACAS (anchored cell analysis and sorting) 470 Interactive Laser Cytometer (Meridian Instruments Inc., Okemos, MI) equipped with a rhodamine filter, was used to quantitate nuclear fluorescence due to import of the fluorescent substrate. For each sample, 10 fields (containing a total of ~300-1,000 permeabilized cells) were randomly selected and scanned. The photomultiplier was set to a value of 40-45, while the scan strength ranged between 20 and 25. All other settings were default values. The fluorescence associated with each nucleus was assigned a numerical value representing average fluorescence per pixel in that nucleus in arbitrary units. For simplicity, we have expressed nuclear fluorescence as average fluorescence of all nuclei in the sample.

Immobilization of NPC Glycoproteins with WGA-Sepharose

Rat liver nuclear envelopes were isolated as previously described (Gerace et al., 1982) and frozen in liquid nitrogen. 1 U of nuclear envelopes is derived from 3×10^6 rat liver nuclei and a 1 U/ml solution of nuclei yields an A_{260} of 1. Nuclear envelopes were resuspended at 100 U/ml in 2% octyl-β-D-glucopyranoside (Calbiochem Corp., La Jolla, CA), 500 mM NaCl, 250 mM sucrose, 0.25 mM $MgCl_2$, 20 mM HEPES, pH 7.5, 2 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF (OG buffer), and NPC glycoproteins (hereafter designated PCGs) were solubilized by incubation for 90 min at 4°C with end-over-end mixing. Insoluble material was removed by centrifugation at 44,000 rpm for 15 min in a 70.1 Ti rotor (Beckman Instruments, Carlsbad, CA) and the supernatant fraction was adsorbed for 3 h at 4°C to WGA-Sepharose (Sigma Chemical Co., St. Louis, MO) that had been prewashed in OG buffer, yielding WGA-PCG-Sepharose beads. Preparation of this matrix involved 8 U of nuclear envelopes per microliter of packed WGA-Sepharose beads. To analyze the specificity of the WGA-PCG-Sepharose matrix, 20 mM GlcNAc was included as competitor during incubation of WGA-Sepharose with nuclear envelopes (to yield WGA-GlcNAc-Sepharose) and/or WGA-Sepharose was incubated with OG buffer in the absence of solubilized nuclear envelopes. WGA-PCG-Sepharose beads, WGA-GlcNAc-Sepharose beads, and WGA-Sepharose beads were washed once with OG buffer and three times with transport buffer before use.

Immobilization of PCGs Using Sepharose-coupled Monoclonal Antibodies

Rat liver nuclear envelope-specific monoclonal antibodies RL2, RL11, and RL20 were coupled to Sepharose and used to adsorb nuclear envelope antigens. The preparation of these antibodies has been described (Snow et al., 1987). Monoclonal antibodies were purified from ascites fluid by binding to a protein G-Sepharose matrix (Pharmacia LKB Biotechnologies, Piscataway, NJ) and eluting with 50 mM glycine, pH 3. The eluate was dialyzed extensively against 100 mM Na-bicarbonate, pH 8.8, and coupled to Sepharose beads using cyanogen bromide-activated Sepharose 4B at a ratio of 10 mg antibody/ml Sepharose beads. Antibody beads were stored in PBS containing 10 mM sodium azide at 4°C. Before use, antibody beads were washed three times in OG buffer.

400 U of nuclear envelopes solubilized in OG buffer at 100 U/ml were mixed overnight at 4°C with 100 μl of antibody beads containing RL2, RL11, or RL20. The unbound fraction was removed and subsequently incubated with 50 μl packed WGA-Sepharose for 3 h at 4°C. The resulting WGA-Sepharose beads contained all O-linked NPC glycoproteins except the RL2, RL11, or RL20 antigens and are designated RL2-depleted WGA, RL11-depleted WGA, or RL20-depleted WGA, respectively. Immobilized glycoprotein resins were equilibrated in transport buffer before use in inactivation reactions.

Inactivation of Cytosolic Transport Factors

In a standard cytosolic inactivation reaction, 100 μl of dialyzed reticulocyte lysate was mixed with 50 μl packed WGA-PCG-Sepharose beads (or 100 μl monoclonal antibody-Sepharose beads) containing 400 U nuclear envelope protein (1.2×10^7 nuclear equivalents/μl cytosol) for 30 min at 4°C. As a control, cytosol was incubated with the same volume of WGA-Sepharose beads in a parallel reaction. The beads were removed by microcentrifugation for 2 s and the resulting supernatant fraction was either assayed immediately for nuclear transport competence or frozen in liquid nitrogen and stored at −70°C for assay at a later time.

To determine the level of inactivation resulting from incubation of cytosol with various levels of nuclear envelope protein, PCGs were coupled at a ratio of 12 U of nuclear envelopes/μl of packed WGA-Sepharose beads. Increasing volumes of WGA-PCG-Sepharose beads (0.83-25 μl) were mixed with enough WGA-Sepharose to bring the total bead volume to 25 μl, and beads were then incubated with 50 μl of dialyzed reticulocyte lysate. After 30 min of incubation at 4°C, beads were pelleted and the unbound cytosol was processed as described above.

A two-stage reaction was carried out to determine whether inactivation of cytosol by nuclear pore glycoproteins in a catalytic or stoichiometric event. In the first stage, a constant amount of WGA-PCG-Sepharose (200 U of nuclear envelopes absorbed to 25 μl WGA-Sepharose) was preincubated with increasing amounts of dialyzed reticulocyte lysate (25-300 μl) for 30 min at 4°C. After removal of unbound cytosol, preincubated beads were incubated in the second step with 50 μl fresh cytosol under standard inactivation conditions. The ability of cytosol-preincubated beads to inactivate fresh cytosol was then assayed by determining the transport competence of the unbound cytosol after the second incubation.

Stimulation of Depleted Cytosolic Extracts

The standard condition for analyzing the ability of various extracts to stimulate the import competence of inactivated cytosolic extracts was as follows: 15 μl of the supernatant fractions resulting from incubation of cytosol with WGA-Sepharose (mock-inactivated) or WGA-PCG-Sepharose (PCG-inactivated) were supplemented with 5 μl of untreated cytosol (dialyzed reticulocyte lysate), transport buffer, mock-inactivated cytosol, or PCG-inactivated cytosol in a 40-μl import reaction. In the experiment in Fig. 5, the ability of 5 μl of NEM-treated cytosol to stimulate transport competence of 15 μl of PCG-inactivated or mock-inactivated cytosol was tested in an analogous fashion. NEM-treated cytosol was prepared as described by Adam et al. (1990). Nuclear import was measured as described above.

Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis and immunoblotting with RL1 or RL2 antibodies were carried out as described (Snow et al., 1987).

Results

Inactivation of Cytosol by Preincubation with Immobilized PCGs

In this study we have investigated the possibility that the process of nuclear protein import involves an interaction between a specific cytosolic factor and O-linked glycoproteins of the NPC (designated PCGs). Our assay involved use of a cell-free nuclear import system consisting of digitonin-permeabilized cells supplemented with exogenous cytosol, which is absolutely required to obtain transport in the per-

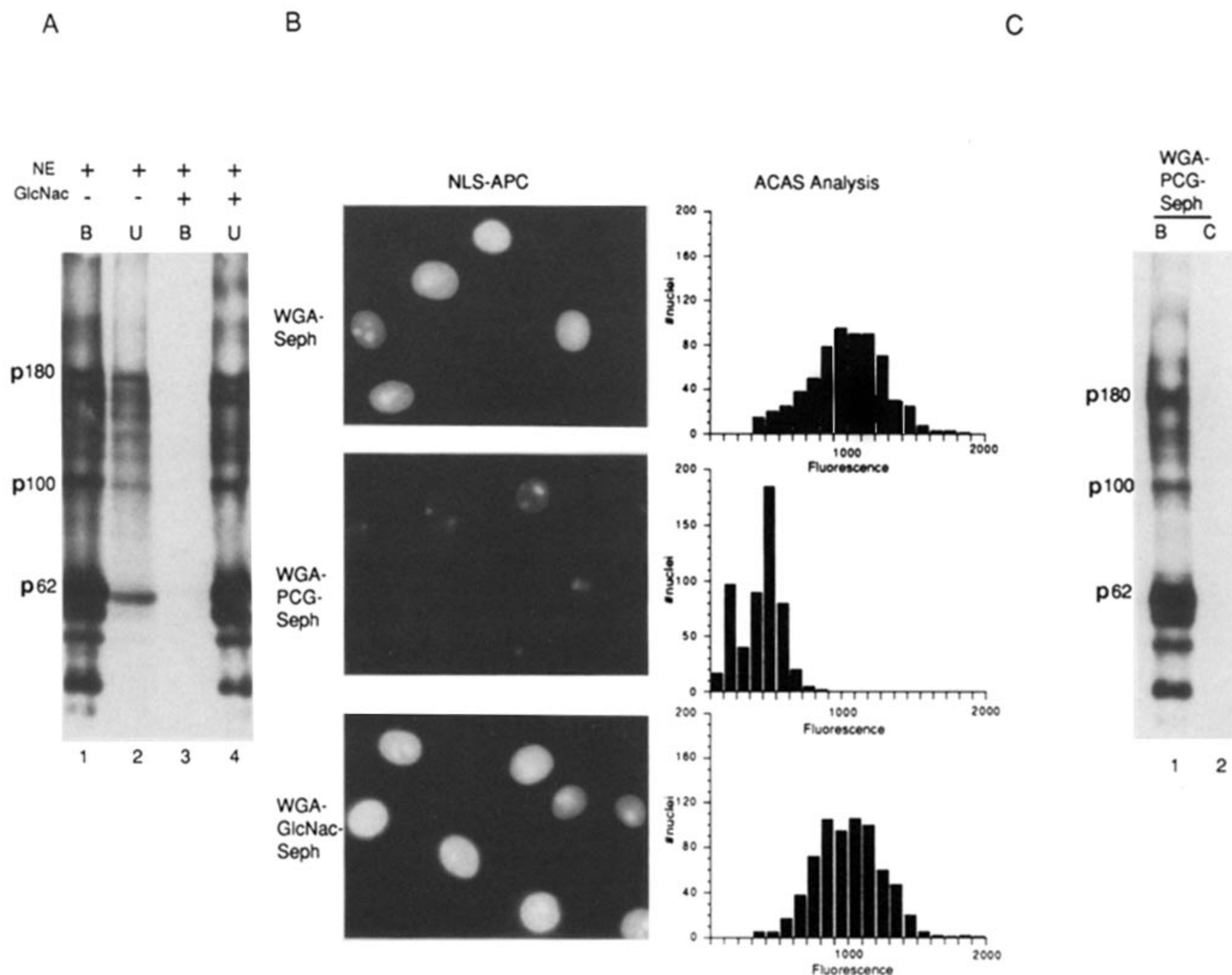

Figure 1. Preincubation of cytosol with immobilized PCGs decreases its transport capacity. (*A*) WGA-Sepharose beads were incubated with solubilized nuclear envelopes in the absence (lanes *1* and *2*) or presence (lanes *3* and *4*) of 20 mM GlcNAc. The beads were removed by centrifugation. O-linked glycoproteins associated with the beads (lanes *1* and *3*) or unbound nuclear envelope proteins (lanes 2 and *4*) were separated on 10% SDS-PAGE gels, transferred to nitrocellulose, and analyzed by immunoblotting with the RL2 monoclonal antibody. *B*, associated with WGA-Sepharose beads; *U*, unbound; *NE*, solubilized nuclear envelopes. (*B*) WGA-Sepharose, WGA-PCG-Sepharose, or WGA-GlcNAc-Sepharose beads were incubated with rabbit reticulocyte lysate, the beads were pelleted, and the unbound lysates were used to assay nuclear import of NLS-bearing allophycocyanin in permeabilized normal rat kidney cells (*left panel*). Transport was quantitated using the ACAS 470 image analyzer as described in Materials and Methods. For each sample, nuclei from 10 randomly selected fields were scanned and analyzed. The histograms display the number of nuclei from all 10 scans which had attained the various fluorescence concentrations in arbitrary units. (*C*) After incubation of immobilized PCGs with cytosol, both the beads (lane *1*) and the unbound cytosol (lane *2*) were analyzed for the presence of O-linked glycoproteins by immunoblotting as described in *A*. *B*, associated with WGA-Sepharose beads after cytosol incubation; *C*, cytosol resulting from incubation with immobilized NPC proteins.

meabilized cells (Adam et al., 1990). We reasoned that if an essential cytosolic factor associates with PCGs during the process of nuclear import, preincubation of cytosol with an affinity matrix containing isolated PCGs may deplete this factor, resulting in "inactivated" cytosol that is deficient in its capacity to support transport in the permeabilized cells.

For many of the experiments in this study, inactivation of cytosol was carried out using PCGs derived from rat liver nuclear envelopes and immobilized on WGA-Sepharose beads after solubilization in a buffer containing octylglucoside and high salt. The characterization of this affinity matrix is described in Fig. 1 *A*. Incubation of solubilized rat liver nuclear envelopes with WGA-Sepharose resulted in binding of most of the total PCG mass to the matrix (Fig. 1 *A*, lanes *1* and 2), as determined by immunoblotting of bound (*B*) and unbound (*U*) fractions with RL2, a monoclonal antibody that binds to eight discrete PCGs in rat liver nuclear envelopes (Snow et al., 1987). As expected, almost all immunoreactive material remained unbound when nuclear envelopes were incubated with WGA-Sepharose in the presence of excess competing sugar, GlcNAc (Fig. 1, *A*, lanes *3* and *4*).

Three matrices were used for initial characterization of cytosol inactivation: WGA-Sepharose, PCGs bound to WGA-Sepharose (WGA-PCG-Sepharose), and a WGA matrix resulting from incubation with nuclear envelopes in the presence of excess GlcNAc (WGA-GlcNAc-Sepharose). Each

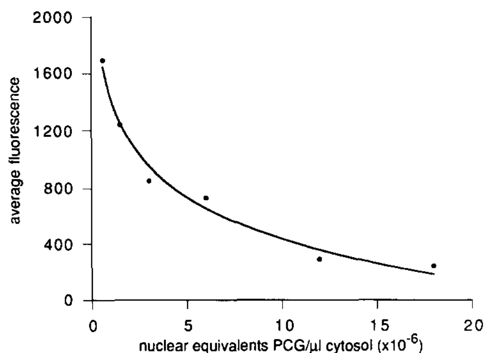

Figure 2. Dependence of cytosol inhibition on the concentration of PCGs. 50 μl of cytosol were incubated with increasing amounts of immobilized PCGs (from 0.6 to 18 $\times$ 10^6 nuclear equivalents of NPC glycoproteins per microliter of cytosol) before being assayed for the ability to direct transport. The average fluorescence of nuclei in each sample was quantitated by ACAS analysis and plotted as a function of PCG concentration. The level of fluorescence obtained from cytosol incubated with WGA-Sepharose alone was identical to that obtained from incubation of cytosol with 0.6 $\times$ 10^6 nuclear equivalents of PCGs.

matrix was incubated with rabbit reticulocyte lysate, a cytosolic fraction that is capable of efficiently directing nuclear transport in permeabilized mammalian cells (Adam et al., 1990). The resulting cytosol was assayed for its ability to support nuclear import of a large fluorescent protein (allophycocyanin) conjugated with synthetic peptides containing the nuclear location sequence of the SV-40 T antigen. As shown in Fig. 1 *B*, preincubation of cytosol with WGA-PCG-Sepharose decreased the transport activity of the cytosol to ~30% of the control level, while preincubation with WGA-Sepharose or WGA-GlcNAc-Sepharose had no effect. Therefore, substantial inactivation of cytosol can be specifically achieved by preincubation with WGA-PCG-Sepharose.

The transport inhibition achieved by WGA-PCG-Sepharose does not result from release of WGA from the beads into the cytosol where it could subsequently bind PCGs and inhibit transport, since incubation of cytosol with WGA-Sepharose lacking PCGs did not affect subsequent nuclear import. This experiment also indicates that there are no WGA-binding proteins present in the cytosol fraction which are required for nuclear import with this system. One other possible explanation for the decrease in the transport capacity of cytosol incubated with WGA-PCG-Sepharose is that PCGs are released from the beads into the cytosol where they might themselves inhibit transport. This is not the case, since the RL2 antigens remained exclusively associated with the beads and were not found in the unbound cytosol fraction after incubation of reticulocyte lysate with WGA-PCG-Sepharose (Fig. 1 *C*, lanes *1* and *2*).

To examine whether the inactivation of cytosol by WGA-PCG-Sepharose was due to binding and depletion of a soluble transport factor, we eluted WGA-PCG-Sepharose matrix with a number of conditions, including low pH and high salt buffers, in an attempt to recover an activity that would complement the PCG-inactivated cytosol. We were unsuccessful in recovering activity with any of the conditions we tested (data not shown). Therefore, we have carried out a more detailed analysis of the characteristics of cytosol inactivation to obtain evidence for the presence of a factor that interacts with the PCGs.

Titration of Cytosol Inactivation by PCGs

To determine the concentration dependence for inactivation of cytosol by PCGs, increasing amounts of WGA-PCG-Sepharose beads were incubated with a constant volume of cytosol, and the cytosol was subsequently assayed for its transport activity (Fig. 2). This analysis showed that a maximum of ~80% inhibition could be achieved with our conditions. At maximal inactivation, 1.2 $\times$ 10^7 nuclear equivalents of PCGs were required to inhibit the transport activity of 1 μl of cytosol (Fig. 2). This PCG/cytosol ratio is used for most of the inactivation experiments described in this study. In practice, the actual amount of cytosol inactivation we achieved with this PCG/cytosol ratio varied from experiment to experiment. This variation may be due to different levels of transport activity found in different lots of cytosol, or to the presence of a low concentration of certain of the required cytosolic factors in the permeabilized cell fraction as suggested by previous studies (Adam and Gerace, 1991). Inactivation of cytosol appears very specific since only ~2 μM of total PCGs are necessary to achieve this inhibitory effect. Furthermore, 11 μg of PCGs are sufficient to inactivate or deplete most of the factor in 4 mg of cytosolic protein.

Inactivation of Cytosol Is Likely to Involve Stoichiometric Interactions with the PCGs

WGA-PCG-Sepharose could cause a decrease in the transport activity of cytosol either by enzymatically inactivating a required cytosolic factor or by binding and depleting the cytosolic factor. The former possibility would involve a catalytic event, while the latter possibility would involve stoichiometric interactions. A modifying enzyme should be in-

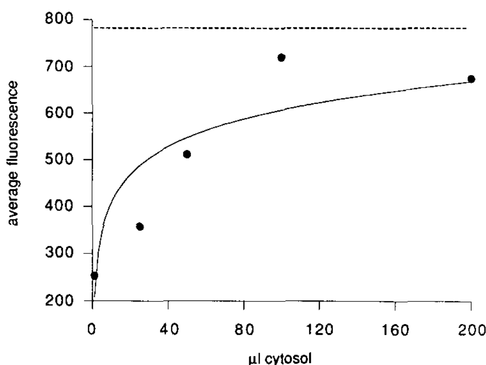

Figure 3. PCGs preincubated with cytosol lose their inactivation capacity. Immobilized PCGs (6 $\times$ 10^8 nuclear equivalents) were preincubated with various amounts of cytosol from 0 to 200 μl. Cytosol was removed and the immobilized PCGs were then incubated with a second aliquot (50 μl) of fresh cytosol. Cytosol from the second incubation was assayed in an import reaction to indicate whether the preincubated PCGs retained their ability to inactivate cytosol. The import directed by the second incubation cytosol is plotted as a function of the volume of cytosol during the first incubation. The level of transport given by cytosol that was mock-inactivated by incubation with WGA-Sepharose in this experiment is indicated by the dotted line.

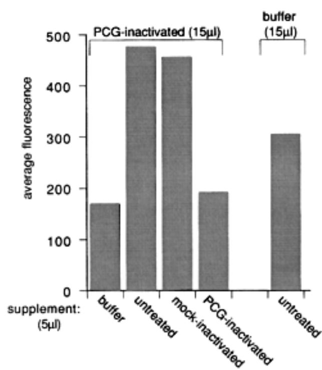

Figure 4. Stimulation of inactivated cytosol with untreated cytosol. PCG-inactivated cytosols (15 μl) were supplemented with 5 μl untreated or mock-inactivated (i.e., incubated with WGA-Sepharose lacking PCG) cytosol in 40 μl transport assays as described in Materials and Methods. As negative controls, inactivated cytosols were supplemented with 5 μl buffer or PCG-inactivated cytosol. 15 μl buffer was supplemented with 5 μl untreated cytosol to determine the contribution of small volumes of these extracts to the import reaction. The control for maximal activity in this experiment consisted of 20μl mock-inactivated cytosol, which gave a fluorescence value of 893.

sensitive to prior contact with its substrate, while a binding protein would lose its activity as it became saturated with ligand. To distinguish between these latter possibilities, samples with a constant amount of WGA-PCG-Sepharose beads (6×10^8 nuclear equivalents of PCGs) were preincubated with increasing amounts of cytosol (0–200 μl). The first aliquots of cytosol were removed and the beads were then incubated with a single volume of fresh cytosol under standard "inactivation" conditions. The ability of cytosol-preincubated beads to subsequently inactivate the fresh cytosol was determined by measuring the transport activity of cytosol from the second incubation (Fig. 3). As the volume of cytosol and hence the ratio of cytosol/PCGs in the preincubation was increased, the ability of WGA-PCG-Sepharose beads to inactivate a second aliquot of cytosol was progressively lost. At ~200 μl cytosol, the beads no longer retained the ability to significantly inactivate additional cytosol (Fig. 3). Proteolysis of NPC proteins by cytosolic proteases cannot account for the loss of "inactivation" ability of WGA-PCG-Sepharose, because proteolysis of the PCGs during the course of incubation with cytosol was undetectable (e.g., compare Fig. 1 *A*, lane *1* with Fig. 1 *C*, lane *1*).

In summary, incubating cytosol with increasing amounts of WGA-PCG-Sepharose progressively inactivates the transport capacity of cytosol (Fig. 2). In a reciprocal relationship, incubating WGA-PCG-Sepharose with increasing amounts of cytosol progressively abolishes the capacity of these beads to inactivate a second lot of cytosol (Fig. 3). These observations indicate that inactivation of cytosol is likely to occur by stoichiometric interaction of a cytosolic factor with PCGs rather than by catalytic modification of the transport factor by PCGs leading to its inactivation.

Inactivation of Cytosol Does Not Occur by the Generation of a Stoichiometric Inhibitor

Two mechanisms are compatible with the finding that inactivation of cytosol by PCGs involves a stoichiometric interaction between a factor in the cytosol and PCG fractions. First, as indicated above, immobilized PCGs may bind and deplete a required cytosolic factor. Alternatively, mixing cytosol and PCGs may result in the generation of a stoichiometric inhibitor of the import process, such as a modified form of a cytosolic transport factor that can interfere with the activity of the unmodified factor. To distinguish these two possibilities, we investigated whether the transport activity of cytosol inactivated with a moderate "excess" of PCGs could be stimulated by the addition of a small amount of untreated cytosol (Fig. 4). If a stoichiometric inhibitor of nuclear import was generated by preincubation of cytosol with PCGs, then "excess" inhibitor generated by PCGs in the preincubation should still be effective in inhibiting most of the activity of a small volume of untreated cytosol. In other words, the activity of the cytosol mixture should reflect the ratio of PCG/(inactivated cytosol + untreated cytosol) derived from the separate components assembled in the final transport assay. On the other hand, if the PCGs depleted an active factor from cytosol during the preincubation, then the untreated cytosol could stimulate transport in a roughly additive fashion with the activity of the inactivated cytosol.

15 μl inactivated cytosol was supplemented with 5 μl of either buffer, untreated cytosol, mock-inactivated cytosol, or inactivated cytosol in a 40-μl transport assay. Supplementation of PCG-inactivated cytosol with an untreated cytosol or a cytosol that had been mock-inactivated by preincubation with WGA-Sepharose beads both stimulated transport ~2.5-fold (Fig. 4), while supplementation of the cytosol with PCG-inactivated cytosol only minimally stimulated transport compared with the addition of buffer alone. The PCG/cytosol ratio in the sample of 15 μl inactivated cytosol supplemented with 5 μl inactivated cytosol was 1.2×10^7 and gave 22% of the control transport activity (see Fig. 4 legend), while the PCG/cytosol ratio in the sample containing 15 μl inactivated cytosol supplemented with 5 μl untreated cytosol was 0.9×10^7 (because only 15 μl cytosol was exposed to PCGs) and gave 51% of the control activity. If a stoichiometric inhibitor were responsible for cytosol inactivation, much less stimulation would be expected. Based on Fig. 2, this model would predict an activity ~30% of the control. However, this experiment actually yielded a much higher level of transport activity, similar to the sum of activity of 15 μl of PCG-inactivated cytosol when assayed alone plus the activity of 5 μl of untreated cytosol when assayed alone (Fig. 4). Since fresh cytosol can stimulate the transport activity of PCG-inactivated cytosol in an additive fashion

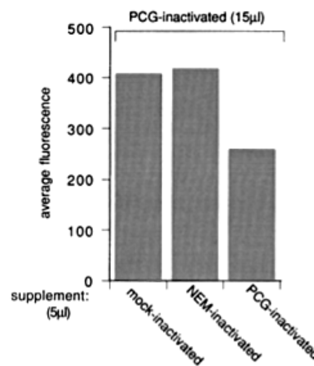

Figure 5. NEM-treated cytosol complements the transport capacity of PCG-inactivated cytosol. 15 μl PCG-inactivated cytosol or 15 μl buffer was supplemented with 5 μl mock-inactivated, NEM-inactivated, or PCG-inactivated cytosol in a 40-μl transport reaction. In this experiment 20 μl of mock NEM-treated cytosol gave a transport value of 571, while cytosol that had been mock-inactivated by incubation with WGA-Sepharose beads had a transport value of 857. Hence, dialysis of cytosol for NEM inactivation appears to lead to nonspecific reduction in the transport capacity of cytosol.

when cytosol is inactivated with an "excess" of PCGs, this argues that a stoichiometric inhibitor of transport is not produced during PCG incubation.

The Cytosolic Factor Inactivated by PCGs Is Insensitive to NEM

Since treatment of cytosol with NEM dramatically decreases the import competence of the resulting extract, we asked whether the cytosolic factor inactivated by WGA-PCG-Sepharose is sensitive to inactivation by NEM. To examine this question, we determined whether NEM-treated cytosol could stimulate transport in a PCG-inactivated cytosol. PCG-inactivated cytosols (15 μl) were supplemented with 5 μl of either inactivated, mock NEM-treated, or NEM-treated cytosol in a 40 μl total sample size, and samples were assayed for transport activity (Fig. 5). Including 5 μl of NEM-treated cytosol in a 40 μl final volume gave no detectable nuclear transport above background (equivalent to a fluorescence value of essentially 0). However, supplementing PCG-inactivated cytosol with NEM-treated cytosol stimulated transport as much as did mock NEM-inactivated cytosol (Fig. 5). In contrast, supplementing PCG-inactivated cytosol with PCG-inactivated cytosol under similar conditions gave no significant stimulation (see Fig. 4). This indicates that the factor inactivated by WGA-PCG-Sepharose, unlike other cytosolic factors implicated in nuclear transport, is not NEM sensitive. Therefore, the factor that interacts with PCGs is distinct from the NEM-sensitive 54/56-kD NLS receptor.

It should be noted that the addition of NEM-treated or mock NEM-treated cytosol to PCG-inactivated cytosols in this experiment did not yield the 2.5-fold stimulation of transport observed in Fig. 4. The preparation of cytosol for NEM or mock NEM treatment, which involved an extended period of dialysis (Adam et al., 1990), resulted in a loss of transport activity which was independent of the chemical modification (see Fig. 5 legend). Thus, NEM- or mock NEM-treated cytosols would not be expected to stimulate PCG-inactivated extracts as much as an untreated cytosol.

We previously demonstrated that pretreatment of permeabilized cells with NEM inhibited subsequent protein import into the nuclei of those cells in the presence of transport-competent (non-NEM-treated) cytosol (Adam et al., 1990). It is conceivable that PCGs themselves are a component of the permeabilized cell fraction that is sensitive to NEM, and that NEM treatment interferes with the interactions of these proteins with cytosolic factors. We therefore determined whether NEM treatment of PCGs had any affect on the ability of WGA-PCG-Sepharose to inactivate cytosol. WGA-PCG-Sepharose beads were treated with NEM (or mock treated) at the concentration required to inhibit transport in permeabilized cells (1 mM), and the beads were subsequently incubated with cytosol, which was then assayed for transport activity. NEM-treated WGA-PCG beads were fully capable of rendering cytosol inactive for transport, compared with WGA-PCG Sepharose that was mock treated with NEM (data not shown).

Inactivation of Cytosol Can Be Ascribed to at Least Two Polypeptides in the PCG Fraction

To begin an analysis of which polypeptide in the PCG fraction is responsible for inactivating the cytosol, we fractionated the group of glycoproteins using the monoclonal antibodies RL2, RL11, and RL20. RL2 recognizes an epitope containing O-linked GlcNAc that is present on all eight nuclear envelope polypeptides bearing this sugar. After solubilization of nuclear envelopes in nonionic detergent/high salt conditions similar to those used in this study, RL2 immunoprecipitates the eight polypeptides containing O-linked GlcNAc, as well as several major nonglycosylated polypeptides that are tightly associated with the glycosylated polypeptides and therefore are apparently subunits of the glycoproteins (Snow et al., 1987). RL11, in contrast, recognizes almost exclusively a 180-kD glycosylated polypeptide as seen by immunoprecipitation analysis (Snow et al., 1987). The control antibody RL20 recognizes gp210, an integral membrane protein of the NPC protein containing N-linked carbohydrate that is not expected to interact with cytosolic transport factors since most of the protein is located in the lumen of the nuclear envelope (Greber et al., 1990). Monoclonal antibodies coupled to Sepharose were used to immunoadsorb PCGs from solubilized nuclear envelopes, and PCGs that remained unbound to the antibody beads were subsequently adsorbed to WGA-Sepharose. The resulting antibody and WGA-Sepharose matrices were then assayed for their ability to inactivate cytosol, to obtain a qualitative description of the inactivation capacity of different matrices.

Immunoblotting with RL2 was used to analyze the proteins adsorbed to monoclonal antibody beads and WGA-Sepharose (Fig. 6 *A*). As expected, RL2 beads adsorbed the major mass of the PCGs, although some p62 and a major fraction of p100 was not bound by RL2 beads and was subsequently adsorbed to WGA-Sepharose. RL11 beads immunoadsorbed >95% of p180 (and its apparent degradation products migrating between ~150 and 180 kD; see Snow et al., 1987) as determined by quantitative immunoblotting. Almost all of the remaining PCGs were not bound to RL11, and were subsequently adsorbed to WGA-Sepharose. As expected, RL20 did not adsorb any of the PCGs, and all of these proteins were subsequently adsorbed to WGA-Sepharose.

The fraction of PCGs that bound to RL2 beads efficiently inactivated the transport capacity of cytosol (62% inactivation relative to the RL20 control), while a smaller amount of inactivation capacity remained in the RL2-depleted fraction that subsequently bound to WGA (Fig. 6 *B*). As expected, no inactivation was obtained by RL20-Sepharose beads compared with Sepharose beads lacking bound antibodies (Fig. 6 *B* and data not shown). Rather, all inactivation activity was obtained with the RL20-depleted fraction adsorbed to WGA, which is identical in composition to PCGs directly bound to WGA. The degree of cytosol inactivation by PCGs bound to RL2-Sepharose slightly exceeded the inactivation obtained with PCGs bound to WGA-Sepharose, possibly due to steric effects.

Surprisingly, very efficient inactivation was achieved with RL11-Sepharose, which inactivated cytosol nearly as well (58% inactivation) as RL2-Sepharose (62% inactivation). Since RL11 immunoadsorbs predominantly a 180-kD band (Snow et al., 1987), these results suggest that the 180-kD glycosylated polypeptide is probably involved in the inactivation of a requisite cytosolic factor, although the possibility cannot be eliminated that a minor component tightly bound to p180 is responsible for this effect. At the same time, sub-

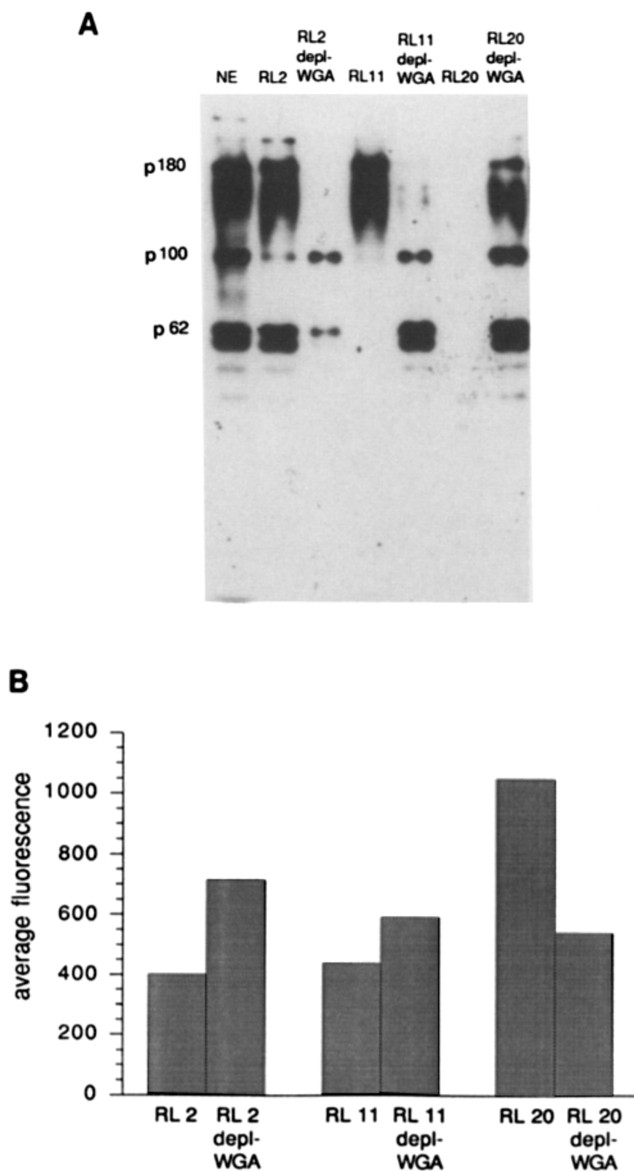

Figure 6. The inactivation capacity of the PCG fraction can be attributed to at least two different proteins. (*A*) O-linked glycoproteins from solubilized nuclear envelopes were immunoadsorbed to Sepharose beads containing immobilized monoclonal antibodies RL2, RL11, and RL20 (designated RL2, RL11, and RL20). O-linked glycoproteins not bound by the antibodies were subsequently adsorbed to WGA-Sepharose (designated RL2 depl-WGA, RL11 depl-WGA, and RL20 depl-WGA). PCGs bound to Sepharose via antibodies or WGA were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting with RL1 antibody. *NE*, total solubilized nuclear envelopes. (*B*) The six resins described above were incubated with reticulocyte lysate and the resulting cytosols were assayed for import activity.

stantial inactivation capacity remained in the RL11-depleted PCG fraction that bound to WGA and was virtually devoid of p180 (45% inactivation). This indicates that the capacity for cytosol inactivation also resides in other proteins in the PCG fraction. This is further supported by the presence of some inactivation capacity in the RL2-depleted fraction adsorbed to WGA, which is virtually devoid of p180.

We obtained very similar results to those presented in Fig. 6 when solubilization of nuclear envelopes was accomplished by a buffer containing 2% Triton and 0.5 M NaCl instead of 2% octylglucoside and 0.5 M NaCl (data not shown). Since the profile of polypeptides immunoadsorbed by RL11 and RL2 after solubilization of nuclear envelopes in 2% Triton X-100 and 0.5 M NaCl (Snow et al., 1987) was identical to that obtained with considerably higher salt (2% Triton and 1.5 M NaCl; data not shown), 2% Triton and 0.5 M NaCl clearly provide a stringent immunoadsorption condition. Considering these results, it is very unlikely that inactivation of cytosol by the PCG fraction is due to proteins loosely or adventitiously associated with the PCGs, but rather must be due to intrinsic subunits of the PCGs.

In summary, these data clearly indicate that the ability of the PCG fraction to inactivate cytosol for transport resides in at least two different components of the PCG fraction, including a fraction highly enriched in the p180 glycosylated polypeptide. However, completely definitive assignment of depletion activity probably will require analysis of proteins expressed from cDNA clones.

Discussion

Mediated nuclear import of proteins requires multiple cytosolic factors (Adam et al., 1990; Newmeyer and Forbes, 1990). One of these is a 54/56-kD NLS binding protein, which recently has been shown to represent an NEM-sensitive receptor for nuclear protein import with a permeabilized cell assay (Adam and Gerace, 1991). However, the purified NLS receptor alone in the absence of cytosol is insufficient to support nuclear import in permeabilized cells, indicating that additional cytosolic components are involved in this process. These additional factors are suggested to include at least one other NEM-sensitive factor and at least one NEM-insensitive factor (Adam and Gerace, 1991; see also Newmeyer and Forbes, 1990). We have described an assay that identifies at least one of these additional cytosolic transport factors, based on a specific interaction of this factor with O-linked glycoproteins of the NPC.

Our detection assay involves immobilizing the group of PCGs from rat liver nuclear envelopes on Sepharose beads using WGA or specific antibodies. After incubating these matrices with transport-competent cytosol, the resulting cytosol is analyzed for its ability to support NLS-mediated nuclear import in permeabilized mammalian cells. Preincubation of cytosol with PCGs reduced the transport activity of cytosol up to a maximum of ~80%. This effect is specific for the PCGs, since when cytosol was incubated with either WGA-Sepharose alone or WGA-Sepharose that had been incubated with solubilized nuclear envelopes in the presence of excess GlcNAc to block PCG binding, transport was equivalent to import directed by untreated cytosol. Neither the release of WGA nor PCGs into the resulting cytosol can account for the observed inhibition of transport activity. Furthermore, inactivation of cytosol by PCGs is independent of the matrix used for immobilization since the pore glycoproteins coupled via antibodies to Sepharose inhibited transport as efficiently as proteins coupled through WGA-Sepharose.

While we could only achieve a maximum of ~80% inhibition of transport when cytosol was incubated with saturating amounts of PCGs, this result may indicate the presence of

some cytosolic factors in the permeabilized cell fraction used for the transport assay that can partially complement the deficiency in the cytosol fraction. Consistent with this possibility, the NLS receptor has been shown to be present in the permeabilized cell fraction as well as in cytosol (Adam et al., 1990). Alternatively, a second less active nuclear import pathway that is independent of this factor may exist.

Immobilized PCGs are likely to act stoichiometrically in the inactivation of the cytosolic transport factor(s). This was indicated by the observation that the capacity of PCGs to inactivate cytosol was abolished by preincubation with a sufficiently large volume of cytosol. This neutralization of the inactivation capacity of PCGs would not be expected if PCGs acted enzymatically to inactivate cytosol, but would be expected if the PCGs became saturated with the specific nuclear import factor that they deplete from cytosol.

Cytosol that had been inactivated by preincubation with saturating amounts of PCGs could have its activity restored in an additive fashion by a small amount of untreated cytosol. This argues that a stoichiometric inhibitor of transport is not created by preincubation with PCGs. Since inactivation of cytosol is specific and stoichiometric and apparently does not involve creation of an inhibitor, our data strongly support the notion that immobilized PCGs bind and deplete a factor required for nuclear import from cytosol. While we have been unsuccessful in eluting an activity from PCG affinity matrices that will complement PCG-inactivated cytosol, this may reflect the lability of this putative bound factor upon exposure to the elution conditions that we have explored.

The cytosolic factor that interacts with PCGs is distinct from the 54/56-kD NLS receptor that has been previously characterized. The factor that interacts with PCGs is NEM insensitive, since the activity of a PCG-inactivated cytosol can be complemented with an NEM-treated cytosol. In contrast, the NLS receptor is completely inactivated by pretreatment with NEM (Adam and Gerace, 1991).

In principle, inactivation of the transport capacity of cytosol by PCGs could be accomplished by either one or multiple polypeptides in the PCG fraction. These active components could either be glycosylated subunits of PCGs, or nonglycosylated subunits that remain tightly associated with the latter under our nonionic detergent/high salt solubilization conditions (Snow et al., 1987). Subfractionation of the O-linked glycoproteins with monoclonal antibody matrices suggested that at least two separable components in the PCG fraction are responsible for inactivating cytosol for transport. Substantial inactivation capacity was associated with protein bound to the RL11 antibody matrix, which consisted predominantly of a 180-kD glycosylated polypeptide (Snow et al., 1987). Hence, it is most likely that the glycosylated p180 is one of the components involved in cytosol inactivation. At the same time, PCG fractions almost totally depleted of p180 with the RL11 monoclonal antibody also had substantial ability to inactivate cytosol, suggesting that at least one additional polypeptide must be involved as well. This could be glycosylated like p180, or alternatively, could be a nonglycosylated subunit of a PCG.

Our experiments do not allow quantitative comparison of the inactivation capacity of p180 relative to other PCGs, since the inactivation ability of a single glycoprotein (e.g., p180) may differ when it is immobilized and oriented by different affinity matrices. Furthermore, the contribution of different components in the PCG fraction to cytosol inactivation would relate to their molar abundance (which apparently is not equal in nuclear envelopes; Snow et al., 1987) as well as to their affinity for a cytosolic factor.

Immunoelectron microscopic studies indicate that the RL11 epitope is located on the nucleoplasmic side of NPCs (Snow et al., 1987). However, this is not inconsistent with the indication that this protein interacts with a cytosolic factor involved in nuclear import. It is possible that p180 spans the NPC, that some events of nuclear import occur on the nucleoplasmic surface of the NPC, or that the factor that interacts with p180 functions in both import and export of macromolecules from the nucleus.

While at least two proteins in the PCG fraction have the capacity to inactivate cytosol for nuclear protein import, all proteins with inactivating capacity may interact with a single cytosolic factor. A number of structural similarities have been described among the different mammalian PCGs and their yeast homologues that could account for this possibility. First, the mammalian proteins are known to contain multiple copies of O-linked GlcNAc (Holt et al., 1987). Second, p62 of rat (Starr et al., 1990) and the NSP1 protein of budding yeast (Nehrbass et al., 1990) contain an ~200-residue stretch containing mostly heptad repeats that is favored to form a coiled-coil alpha helix. Finally, p62 of rat and both NSP1 and NUP1 of yeast contain multiple copies of a degenerate pentapeptide repeat that is favored to form beta sheet secondary structure (Davis and Fink, 1990; Nehrbass et al., 1990; Starr and Hanover, 1990, 1991). Whether any of the remaining rat PCGs contain heptad repeats or pentapeptide repeats is presently uncertain for lack of their sequences. In addition to the possibility that a single cytosolic factor is inactivated by different PCGs, it also is conceivable that multiple cytosolic factors are inactivated by different proteins in the PCG fraction. Resolving this question will require analysis of the inactivation assay using highly purified individual glycoproteins.

While the 54/56 NLS receptor is not the cytosolic target of inactivation by immobilized PCGs, it is possible that during the process of nuclear import, the interaction of the cytosolic factor(s) with PCGs in some fashion involves this receptor. At present there is no evidence to indicate whether or not the NLS receptor itself interacts with PCGs during the process of nuclear import. However, most of the NLS receptor pool in cells is not associated with NPCs (Adam et al., 1989; Yamasaki et al., 1989), so if this interaction were to occur, it must be very transient. Supporting this notion, no proteins in extracts of rat cells that become photocrosslinked to a radioactive NLS (including a 55-kD band that presumably represents the NLS receptor) are detectably associated with WGA binding proteins (Yamasaki et al., 1989).

In conclusion, we have shown that immobilized PCGs from rat liver nuclear envelope specifically interact with a cytosolic factor involved in nuclear protein import. This interaction probably causes depletion of this factor from cytosol and the resulting loss of its transport activity when assayed in vitro with permeabilized cells. Complementation of this inactivated cytosol will provide a facile assay for isolation of this factor. Furthermore, molecular analysis of this component and specific characterization of its binding site on specific PCGs should yield important insight into the functions of PCGs and the process of nuclear protein import.

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