

# A Complex of Nuclear Pore Proteins Required for Pore Function

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**Abstract.** A family of proteins bearing novel *N*-acetylglucosamine residues has previously been found to be required to form functional nuclear pores. To begin to determine which of the proteins in this family are essential for pore function, antisera were raised to each of three members of the family, p62, p58, and p54. With these antisera, it was possible to deplete nuclear reconstitution extracts of the proteins and to test the depleted nuclei for nuclear transport. In the course of the experiments, it was found that the three proteins exist as a complex; antisera to any one, while specific on a protein blot, coimmunoprecipitated all three proteins. This complex of pore proteins is stable to 2 M

salt, 2 M urea, and the detergent Mega 10, indicating the presence of specific and tight protein-protein interactions. By gel filtration, the complex has a molecular mass of 550–600 kD. Nuclei containing pores depleted of the complex are found to be defective for nuclear transport; moreover, we observe a strict linear correlation between the amount of complex present in nuclei and the amount of nuclear transport of which those nuclei are capable. Thus, the p62-p58-p54 complex defines a group of proteins with strong protein-protein interactions that form a unit of pore structure essential for pore function.

**T**HE nuclear pore is a large and complex supramolecular assembly, estimated to be 120,000,000 D in mass (Reichelt et al., 1990). The pore is responsible for the regulated import of nuclear proteins and snRNPs (for reviews see Dingwall and Laskey, 1986; Newport and Forbes, 1987; Nigg, 1988; Hunt, 1989; Garcia-Bustos et al., 1991; Silver, 1991). In addition, the pore closely controls the export of mRNA, tRNA, and ribosomal subunits, as well as the export of certain proteins which shuttle between the nucleus and the cytoplasm, such as the glucocorticoid receptor (Picard et al., 1988; Borer et al., 1989; Mandell and Feldherr, 1990). Cryo-electron microscopy combined with Fourier averaging techniques have provided a detailed model of the nuclear pore (Unwin and Milligan, 1982; Stewart and Whytock, 1988; Akey, 1989; Reichelt et al., 1990). The most recent model suggests that the pore consists of three annuli or rings. The two outer rings contain eight subunits each and appear to be mirror images of one another. These rings are separated by a third ring composed of "spokes." The spokes surround a central granule, termed the transporter (Akey, 1989, 1990; Akey and Goldfarb, 1989). The central granule or "transporter" of the pore is postulated to serve as a diaphragm which opens to let macromolecules translocate through the pore. A more detailed but speculative model proposes that the transporter is in actuality a double iris; in this model, each iris is composed of eight arms that swing in to close the pore to large macromolecules, leaving a 90-Å channel for passive diffusion. When a nuclear protein binds to one or more of the arms of the iris, all arms of that iris swing out. The arms of the more internal iris then open and translocation into the nucleus occurs (Akey, 1990). In other studies, filamentous

structures have been observed to extend from the pores (Franke and Scheer, 1970; Maul, 1977; Fujitani et al., 1984; Carmo-Fonseca et al., 1987; Richardson et al., 1988; Allen and Douglas, 1989). The function of these filaments is unknown, but they could act as microtubule-like tracks leading to the pore.

Studies using labeled proteins and dextrans have shown the pore to have a resting channel size of 90–110 Å; this channel permits the free diffusion of molecules under ~40 kD, while excluding larger cytoplasmic proteins (Bonner, 1975; Paine et al., 1975; Paine and Horowitz, 1980; Peters et al., 1986; Peters, 1986; Lang et al., 1986; Lanford et al., 1986). For the entry of large nuclear proteins, the presence of one or more signal sequences on the protein is required to induce the opening of the pore (up to 280 Å; Feldherr et al., 1984; Dworetzky et al., 1988; Breeuwer and Goldfarb, 1990; see Dingwall and Laskey, 1986; Garcia-Bustos et al., 1991; Silver, 1991 for reviews). It has been possible to identify two separable steps in import, an ATP-independent binding of nuclear proteins to the pore, followed by ATP-dependent translocation through the pore (Newmeyer and Forbes, 1988; Richardson et al., 1988).

Much of the effort toward understanding nuclear import has been directed at identifying the proteins of the pore in the hope that the primary structure of these proteins will provide clues to the type of activities involved in nuclear transport, i.e., ATPases, cytoskeletal elements, kinases, et cetera. Because the pore is embedded in the nuclear lamina which is in turn connected to the nuclear matrix, it has proved refractory to isolation. In consequence, relatively few pore proteins have been identified. The recent finding of

spontaneous assembly of annulate lamellae in vitro (Dabauvalle et al., 1991) may allow purification of pores and remedy this. At present, however, the pore proteins that have been identified are gp210, NSP1, NUP1, a number of potential signal sequence receptors, and a family of novel glycoproteins. The protein gp210 is an abundant transmembrane glycoprotein thought to play a role in anchoring the pore complex to the nuclear envelope (Gerace et al., 1982; Filson et al., 1985; Wozniak et al., 1989; Graeber et al., 1990). Two pore proteins, NSP1 and NUP1, have recently been discovered in the yeast *Saccharomyces cerevisiae* (Hurt, 1988; Aris and Blobel, 1989; Nehrbass et al., 1990; Davis and Fink, 1990; Hurt, 1990). Both have been shown by genetic means to be essential; moreover, null mutants of NSP1 do not transport. A number of potential signal sequence receptors have also been identified either with chemical cross-linking techniques or by virtue of binding radiolabeled signal sequences on nitrocellulose blots (Yoneda et al., 1988; Adam et al., 1989; Silver et al., 1989; Lee and Melese, 1989; Li and Thomas, 1989; Yamasaki et al., 1989; Meier and Blobel, 1990). Such receptors might be predicted to reside at the pore or alternatively to be cytoplasmic proteins that shuttle nuclear proteins to the pore. Several of the potential signal sequence receptors have in fact been localized to the cytoplasm (Adam et al., 1989; Yamasaki et al., 1989). Indeed, a factor in the cytoplasm, the *N*-ethylmaleimide-sensitive nuclear import factor, NIF1, which is required for efficient import in an in vitro nuclear import system, may be such a shuttling receptor (Newmeyer and Forbes, 1990). *N*-Ethylmaleimide-sensitive cytosolic components have also been found to be required for nuclear transport in a permeabilized cell assay (Adam et al., 1990).

Of particular interest with respect to the mechanism of transport is a family of 8–10 proteins that have in common the possession of single *O*-linked *N*-acetylglucosamine residues (Davis and Blobel, 1986, 1987; Finlay et al., 1987; Hanover et al., 1987; Holt and Hart, 1986; Holt et al., 1987; Park et al., 1987; Schindler et al., 1987; Snow et al., 1987; see Hart et al., 1989, for review). This family of nuclear glycoproteins was identified both with mAbs raised against a rat nuclear lamina-pore complex fraction (Davis and Blobel, 1986, 1987; Hanover et al., 1987; Holt et al., 1987; Snow et al., 1987) and by their binding of <sup>125</sup>I-WGA on protein blots (Finlay et al., 1987). In rat nuclei, the family includes proteins of 45, 54, 58, 62, 100, 145, 180, 210, and 270 kD in mass (Snow et al., 1987). The most abundant of these is the pore protein, p62. The gene encoding p62 has been cloned and sequenced; however, to date, the sequence of p62 has failed to provide clues as to its role in the mechanism of import (D'Onofrio et al., 1988; Starr et al., 1990). A homologous family of glycoproteins has been identified in *Xenopus* eggs, the most abundant being 60, 97, and 200 kD in mass (Scheer et al., 1988; Featherstone et al., 1988; Finlay and Forbes, 1990). However, unlike the rat nuclear pore glycoproteins, which are isolated from nuclei, the *Xenopus* glycoproteins exist in a soluble pool in the unfertilized egg cytoplasm. This pool of proteins is used to construct the nuclear pores of nuclei that are formed during early development (Finlay and Forbes, 1990).

The importance of the family of glycoproteins to nuclear transport has been demonstrated by the finding that agents that bind to these glycoproteins block nuclear import. Spe-

cifically, the lectin WGA, when added to an in vitro nuclear transport assay or injected into cells, completely inhibits nuclear import (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988a; Wolff et al., 1988). WGA does not block the initial recognition and binding of a nuclear protein to the pore, but instead blocks the ATP-dependent translocation of that protein through the pore (Newmeyer and Forbes, 1988). Similarly, an mAb RL1, which binds to most of the glycoproteins by virtue of recognizing an *N*-acetylglucosamine-peptide epitope, when injected into *Xenopus* oocytes, inhibits import (Featherstone et al., 1988). Moreover, the injection of an anti-p62 mAb into cells (Dabauvalle et al., 1988b) or the addition of an anti-p62 polyclonal antibody to an in vitro transport assay also blocks transport (E. Meier and D. Forbes, unpublished results). In addition to blocking import, WGA also blocks the export of ribosomal precursors from the nucleus (Bataille et al., 1990). Thus, the data demonstrate that these glycoproteins are essential for both nuclear import and export.

To devise functional tests for the mechanism of action of individual pore proteins, we developed a system in which proteins can be selectively eliminated from the pore (Finlay and Forbes, 1990). For this, an in vitro nuclear reconstitution extract is used, which upon the addition of DNA or chromatin forms nuclei and thus nuclear pores (Lohka and Masui, 1983, 1984; Newport and Forbes, 1985; Blow and Laskey, 1986; Newmeyer et al., 1986a; Newport, 1987; Sheehan et al., 1988). The chromatin, when added to such an extract, decondenses, and binds membrane vesicles, which then flatten, fuse, and acquire pore complexes. The resulting nuclei exhibit high levels of nuclear import (Newmeyer et al., 1986a, b). The nuclear reconstitution extract can be depleted of the entire family of *Xenopus* *N*-acetylglucosamine-bearing proteins with WGA-Sepharose (Finlay and Forbes, 1990). Using such "WGA-depleted" extracts, we have shown that although the depleted extract can form nuclei containing nuclear pores, the pores that form are unable to transport. When the family of *Xenopus* glycoproteins is added back to a depleted extract (prior to nuclear formation), nuclei form that are fully capable of import. The related family of rat pore proteins also fully restores transport, indicating functional homology between the *Xenopus* and rat pore glycoproteins (Finlay and Forbes, 1990). From these experiments and others, it was concluded that the family of *N*-acetylglucosamine-bearing proteins is essential for pore function, but not for forming visible pore structures.

It has generally been assumed that all members of the 8–10 nuclear glycoproteins visualized with antisera such as RL1 are pore proteins. This assumption has arisen from the fact that the proteins all show an extraction profile taken to be typical of nuclear pore proteins, i.e., resistance to high salt and DNAase upon extraction of nuclei prepared in magnesium-containing buffers. A second indication that these glycoproteins are all pore proteins derives from the finding that mAbs that cross-react with the set of glycoproteins stain only the pore in immunoelectron microscopy (Davis and Blobel, 1986, 1987; Snow et al., 1987). However, neither of these pieces of data is conclusive. The extraction indicates only that the proteins are members of the residual pore, lamina, or nuclear matrix. Indeed, other types of nuclear proteins, such as transcription factors and chromatin proteins, have also been observed to have the novel *N*-acetylglucosamine modification

and may well be contained in such a matrix (Jackson and Tjian, 1988; Kelly and Hart, 1989). In reality, only p62 and p180 have been definitively shown to be pore proteins, through the use of specific antibodies and electron microscopy (Snow et al., 1987; Dabauvalle et al., 1988). Thus, it is now essential to the study of pore structure to determine not only whether the individual members of the glycoprotein family are pore proteins, but also to ask whether each protein in this family is required for nuclear import. In this report, we have raised polyclonal antisera to three members of the rat family of glycoproteins, p54, p58, and p62. We have used the antisera to construct nuclei depleted of these proteins. We find that all three antisera remove proteins essential for import. Moreover, we find that the three proteins exist in a large and stable complex. We conclude that all three are indeed pore proteins and that the complex defines a group of interacting proteins essential for pore function.

## Materials and Methods

### Preparation of Nuclear Reconstitution Extracts and Rat Liver Nuclei

Rat liver nuclei were isolated as previously described (Newmeyer et al., 1986b; Newport and Spann, 1987). Nuclear reconstitution extracts were prepared as in Finlay and Forbes (1990). Briefly, *Xenopus* eggs were de-jellied and washed in lysis buffer (10 mM Hepes [pH 7.4], 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 20 μg/ml cycloheximide, 5 μg/ml cytochalasin B, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The eggs were lysed by centrifugation in a Sorvall HB-5 rotor at 10,000 rpm for 10 min (Sorvall Div., Newton, CT). The low speed supernatant was removed and further centrifuged in a Beckman centrifuge (TL-100, Beckman Instruments, Palo Alto, CA) at 200,000 g for 1 h. The high-speed soluble fraction was then kept on ice for use in transport assays. The membrane fraction was collected, washed with 20 vol membrane wash buffer (MWB: 10 mM Hepes [pH 7.4], 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 1 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin), pelleted in a TL-100 centrifuge (Beckman Instruments) (15,000 rpm for 15 min), and resuspended in MWB to approximately 10 times the original concentration present in the crude low-speed supernatant. Sperm chromatin was prepared by demembrating sperm nuclei as described in Lohka and Masui (1983).

### Preparation of Rat WGA-binding Pore Proteins for Nuclear Reconstitution

Preparation of rat liver nuclear proteins for nuclear reconstitution was performed as previously described (Finlay and Forbes, 1990). Briefly, nuclei (6–8 × 10<sup>8</sup>) were washed once in reconstitution buffer (R buffer: 20 mM Hepes [pH 7.0], 100 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 0.5 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin), pelleted 45 seconds in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY), and resuspended in 500 μl of R buffer containing 2% Mega 10 detergent (Calbiochem-Behring Corp., La Jolla, CA). The nuclei were detergent-extracted at 4°C with tumbling for 30 min. The extracted nuclear pellet was removed by pelleting in an Eppendorf centrifuge for 45 s and discarded. The detergent extract containing the WGA-binding pore proteins was then incubated with WGA-Sepharose (E. Y. Laboratories, San Mateo, CA) for 45–60 min at 4°C with tumbling. The Sepharose was pelleted for 15 s in an Eppendorf centrifuge and the supernatant was discarded. The WGA-Sepharose containing bound nuclear glycoproteins was washed with 20 vol of R buffer, and the WGA-binding proteins eluted by incubation with 100 μl of high sugar buffer (HSB; 0.5 M N-acetylglucosamine [GlcNAc], 8 mM triacetylchitotriose [TCT], 20 mM Hepes [pH 7.0], 100 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 0.5 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin) for 20 min at 4°C with tumbling. The WGA-Sepharose was removed by centrifugation and the supernatant containing the eluted partially purified rat WGA-binding proteins was stored on ice until use.

## Polyclonal Antisera Production

For polyclonal antisera production, purification of rat liver nuclear WGA-binding proteins was scaled up as follows. Aliquots of rat liver nuclei were thawed and pooled to a final concentration of ~12.0 × 10<sup>9</sup> nuclei in 30 ml. The nuclei were then split into three tubes and centrifuged in a Sorvall HB-5 rotor at 5,000 rpm for 15 min at 4°C. The resulting nuclear pellets were then washed twice with 10 ml each of nuclei wash buffer (NWB; 10 mM Tris-HCl [pH 7.4], 250 mM sucrose, 1 mM DTT, 1 mM PMSF, 1 mM Pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin). The washed pellets were resuspended in 8 ml DNase buffer (10 mM Tris-HCl [pH 7.4], 250 mM sucrose, 2 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM PMSF, 1 mM Pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and DNase I (Worthington Biomedicals, Malvern, PA) was added to a final concentration of 5 μg/ml. The nuclei were DNase treated for 45 min at room temperature, then pooled, and kept on ice. The pooled nuclei were underlain with a 30% sucrose cushion in NWB and centrifuged in a swinging bucket rotor (SW28; Beckman Instruments) at 20,000 rpm, 4°C, for 15 min. The DNase pellet was resuspended in 3.45 μl 2% Mega-10 solution (PBS [pH 7.4], 2% Mega 10, 1 mM DTT, 1 mM PMSF, 1 mM Pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin) per 3 × 10<sup>6</sup> nuclei present in the original nuclear pool and extracted for 30 min at room temperature on a rotating platform. The extracted nuclei were centrifuged in a Sorvall HB-5 rotor at 5,000 rpm for 10 min at 10°C. The supernatant was decanted and saved on ice (S<sub>1</sub>) and the Mega 10 treatment was repeated once as above on the pellet (P<sub>1</sub>). After centrifugation the second supernatant (S<sub>2</sub>) was pooled with S<sub>1</sub>.

The pooled supernatants were applied to a 2-ml WGA-Sepharose column at a rate of 15 ml/h at room temperature with the flow-through collected on ice. The column was then washed with 10 column vol of column wash buffer (CWB: PBS, pH 7.4, 2% Triton X-100, 1 mM DTT, 1 mM PMSF, 1 mM Pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin) before elution. The proteins were eluted from the WGA-Sepharose with elution buffer (CWB plus 250 mM N-acetylglucosamine) and 200–300-μl fractions were collected. These fractions were assayed for the presence of WGA-binding proteins by electrophoresis of 2-μl aliquots on 10% polyacrylamide gels, transfer to polyvinylidene difluoride (PVDF; Immobilon, Millipore, Bedford, MA), and probing with <sup>125</sup>I-WGA.

The WGA-binding protein-containing fractions were pooled and electrophoresed in a single large lane on a 5–15% concave gradient polyacrylamide gel. The proteins were transferred to nitrocellulose using a Polyblot Electro-transfer semi-dry blotting apparatus (ABN). Two thin sections of the blot were probed with <sup>125</sup>I-WGA as markers for the bands of interest. The majority of the blot was stained with Fast green. Stained bands of 54, 58, and 62 kD were excised from the blot and the nitrocellulose solubilized in DMSO. Proteins prepared in this way were injected intramuscularly and subcutaneously into 5-lb female rabbits.

## Immunofluorescence Microscopy

For anti-p62 immunofluorescence on fixed rat cells, BRL cells were grown overnight on coverslips, rinsed in PBS, and fixed in a solution of PBS plus 3.7% formaldehyde for 5 min. The cells were washed briefly five times with PBS followed by three times with PBS containing 0.1% Triton X-100. A solution of PBS (130 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4), 0.1% Triton X-100, and 5% FCS (TBF) was placed on the cells for 10 min and then the desired antibody (diluted 1:50 in TBF) was incubated with the cells for 30 min. Cells were washed three times briefly with PBS, 5% FCS, followed by a 5-min wash. This sequence of washes was repeated three times before the secondary antibody, goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA), was added at a 1:50 dilution in PBS, 5% FCS. The cells were incubated 30 min in the dark, washed as after the primary antibody, and the DNA stained with a solution of PBS and 1 μg/ml bisbenzimidazole DNA dye (Hoechst 33258). The cells were further washed three times with PBS over a 15-min period, after which they were mounted in 3–5 μl of glycerol, sealed, and viewed with a Zeiss Photomicroscope III.

For immunofluorescence on unfixed cells (Adam et al., 1990), BRL cells grown on coverslips that were then incubated in 40 μg/ml digitonin in PBS for 5 min on ice. After a brief rinse in PBS, the coverslips were incubated in 0.2% Triton X-100 in 20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 1 mM EDTA, 2 mM DTT, 2 mM MgCl<sub>2</sub> for 6 min at room temperature. Cells were washed in PBS-0.2% gelatin, and probed with primary antisera (anti-p58, anti-54, or nonspecific antisera) at a 1:50 dilution in PBS-0.2% gelatin-1 mM trichitotriose for 5 min at room temperature. The coverslips were then rinsed briefly and treated with TRITC-conjugated goat

anti-rabbit IgG for 15 min at room temperature. The coverslips were rinsed again, and inverted onto slides in glycerol with the DNA dye Hoechst 33258 added.

### Depletion of WGA-binding Proteins from *Xenopus* Egg Extracts

WGA-Sepharose depletion of the high-speed *Xenopus* extract supernatant was performed as previously described in Finlay and Forbes (1990). In brief, the 200,000 g soluble fraction was incubated twice, consecutively, with 0.5 vol of packed washed WGA-Sepharose beads (E. Y. Laboratories, Inc.) for 45–60 min at 4°C with rocking. The Sepharose was pelleted by centrifugation for 15 s in an Eppendorf centrifuge, leaving a supernatant depleted of the WGA-binding proteins.

### Immunodepletion of the Rat Nuclear Pore Glycoproteins

1 ml of either anti-p54, -p58, or -p62 antisera was combined with 0.5 ml packed Protein A Sepharose and tumbled at room temperature for 1 h. The antibody-bound Sepharose was then centrifuged, the supernatant removed, and the Sepharose washed three times with 15 ml R buffer. The same procedure was performed with 1 ml rabbit whole serum (Cappel Laboratories) to yield a nonspecific Sepharose control. The coupled Sepharose preparations were stored in a 1:1 slurry of Sepharose:R buffer plus 0.01% azide at 4°C until needed.

For immunodepletion one volume of partially purified rat nuclear pore glycoproteins (RNPG) was combined with one volume of WGA-depleted *Xenopus* egg extract (to give RNPG mix) before the addition of the antibody-bound Sepharose. The presence of the depleted *Xenopus* extract served as an efficient way to decrease nonspecific binding to the Sepharose and had no adverse effect on the subsequent transport assay. In addition, to decrease nonspecific binding of proteins, the antibody-Sepharose was also washed with depleted *Xenopus* extract just prior to use. For immunodepletion of a given antigen, two serial immunoprecipitations were performed as follows: 1 vol of the RNPG mix was added to one-third vol of packed antibody-Sepharose, then tumbled at 4°C for 1.5 h, and centrifuged for 20 s in an Eppendorf centrifuge. The supernatant was decanted, a second one-sixth vol of antibody-Sepharose was added to the supernatant, and the immunoprecipitation repeated as described. After the final batch of Sepharose was pelleted from solution, the supernatant, consisting of the rat nuclear pore glycoprotein mix which had been depleted by the specific antisera, was used in transport assays as well as assayed by Western blotting for the depletion of the specific antigen. Quantitation of protein remaining in solution after immunoprecipitation was achieved by densitometric scanning of autoradiograms using an LKB Ultrascan XL Laser densitometer (LKB Instruments, Gaithersburg, MD).

### Characterization of the 62-58-54 Complex

To test the stability of the complex, rat nuclear glycoproteins from  $3 \times 10^8$  liver nuclei were prepared as previously described (Finlay and Forbes, 1990). Briefly, the nuclei were extracted with 2% Mega 10 and the extracted proteins were bound to WGA-Sepharose. The Sepharose was washed and the rat nuclear pore glycoproteins were eluted in 120  $\mu$ l HSB. For immunoprecipitations, 100  $\mu$ l packed anti-p62-Sepharose was incubated with 100  $\mu$ l of depleted *Xenopus* egg extract for 30 min at room temperature. The Sepharose was pelleted 15 s in an Eppendorf centrifuge, the supernatant was discarded, and the Sepharose was incubated with 120  $\mu$ l of the above partially purified rat proteins for 3 h at room temperature. At the end of this incubation, the Sepharose was pelleted as before, the supernatant discarded, and the pellet washed with two aliquots of 10 ml PBS with 5  $\mu$ g/ml aprotinin and 5  $\mu$ g/ml leupeptin (PBS/I). The pelleted anti-p62 Sepharose was resuspended to 900  $\mu$ l total volume in PBS/I and split into nine equal aliquots for different treatment conditions (one being a control, untreated sample). The nine tubes were centrifuged to pellet the complex-bound Sepharose and the supernatants were discarded. Urea- (0, 2, 4, or 8 M final concentration) or NaCl-containing solutions (0.1, 0.5, 1, or 2 M final concentration) were added (100  $\mu$ l per aliquot) to determine the conditions that dissociate the complex. The antibody-Sepharose carrying bound complex was extracted for 45 min at room temperature and then centrifuged to pellet out the Sepharose. The supernatants were removed to new 1.5-ml Eppendorf tubes and 60  $\mu$ l of each was added to 20  $\mu$ l of 4 $\times$  SDS-PAGE sample buffer. The extracted pellets containing the anti-p62-Sepharose and the proteins remaining associated with the p62 protein were washed twice with PBS/I and boiled

in SDS-PAGE sample buffer before loading on a 10% polyacrylamide gel. The gel was transferred to PVDF and probed with a mixture of anti-p62, -p58, and -p54 antisera to identify all three proteins of the complex. The blot was then hybridized with  $^{125}$ I-protein A and exposed for autoradiography.

The size of the complex was determined by column chromatography on Sephacryl S-300 HR. For this, 0.5 ml of rat liver nuclei ( $3 \times 10^8$ /ml) were washed three times with 1 ml of R buffer, resuspended in 500  $\mu$ l 2% Mega 10 in R buffer, and incubated for 1 h at 4°C. The resulting extracted nuclei were pelleted for 1 min in a microfuge. The supernatant was added to 500  $\mu$ l of packed WGA-Sepharose beads (prewashed three times with 1 ml R buffer) and incubated for 1 h at 4°C. The beads were washed four times with 1 ml R buffer, before two sequential elutions of the WGA-binding proteins with 100  $\mu$ l of 0.5 M *N*-acetylglucosamine, 8 mM trichitriose in R buffer for 1 h at 4°C. The eluted rat proteins were applied to a Sephacryl S-300 HR column (1.5  $\times$  50 cm) equilibrated with 1 $\times$  PBS/0.05% Triton X-100/0.02% sodium azide. 0.5-ml fractions were collected; TCA and Triton X-100 were added to a final concentration of 20% TCA/0.2% Triton X-100. The fractions were incubated on ice 30–45 minutes and centrifuged in a microfuge for 15 min at 4°C. The supernatants were removed, 750  $\mu$ l acetone was added to the pellets that were incubated at ambient temperature for 15 minutes, and the pellets were re-centrifuged for 15 min at 4°C. After removal of the acetone, the pellets were dried on ice for 15 min, and resuspended in 80  $\mu$ l gel sample buffer plus BME. Samples were electrophoresed on 9% SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with the mAb 414 (Davis and Blobel, 1986, 1987) or all three antisera to p62, p58, and p54 to visualize the location of the three proteins. Marker proteins were similarly separated on either the same or parallel Sephacryl S-300 HR column, depending on the experiment. Multiple size determinations of the complex were performed.

To determine the composition and stoichiometry of proteins within the complex, WGA-binding proteins were prepared from rat liver nuclei ( $1.2 \times 10^9$ ) as described above. To immunoprecipitate the complex, 100  $\mu$ l of the eluted WGA-binding were combined with 200  $\mu$ l of 2 $\times$  IPB buffer (1 $\times$  IPB = 100 mM Tris, pH 7.4, 150 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM PMSF, 0.5% Mega 10, 10  $\mu$ g/ml aprotinin and leupeptin) and 50  $\mu$ l of Protein A-Sepharose that had previously been cross-linked to either whole rabbit serum (Cappel Laboratories) or to anti-p62 antisera with a DMP crosslinker (Pierce Chemical Co., Rockford, IL). After a 3-h incubation at room temperature, the antibody beads were pelleted and the supernatant removed to a new tube. The supernatants were then TCA precipitated and resuspended in gel sample buffer. The immunoprecipitates were washed 5 $\times$  in 500  $\mu$ l IPB, 2 $\times$  in R buffer, and resuspended in gel sample buffer. The pellets and supernatants were fractionated on 9% gels and stained with either Coomassie blue or a silver staining kit (Bio-Rad Laboratories, Richmond, CA).

### Depleted and Reconstituted Transport Assays

Depleted and reconstituted transport assays were performed as in Finlay and Forbes (1990) with the following modifications: The standard assay volume was increased to 40  $\mu$ l. FITC-Dextran (150 kD; 0.1 mg/ml final concentration) was added to samples that were to be assayed by video image analysis. The samples were then fixed with formaldehyde (3.7% final concentration) and stored at 4°C until analyzed. In brief, to assay the ability of the immunodepleted rat nuclear pore glycoprotein mix to reconstitute transport, 24  $\mu$ l of the depleted *Xenopus* extract containing the ATP-regenerating system (final concentration = 1–2.75 mM ATP, 9 mM creatine phosphate, 100 U/ $\mu$ l creatine kinase) were combined with 10  $\mu$ l of immunodepleted RNPG mix (partially purified rat nuclear pore glycoproteins and WGA-depleted *Xenopus* extract in a 1:1 ratio) or undepleted RNPG mix, 2  $\mu$ l of sperm chromatin (20,000/ $\mu$ l), and 4  $\mu$ l of a 10 $\times$  concentrated solution of washed membranes. Nuclei were allowed to form for 90 min, before addition of the transport substrate. The transport substrate, TRITC-HSA-wt (Newmeyer and Forbes, 1988; see also Goldfarb et al., 1986) used was a conjugate of human serum albumin (HSA) and a synthetic peptide containing the nuclear localization signal of the SV-40 large T-antigen (cys-thr-pro-pro-lys-lys-lys-arg-lys-val-COOH). Each molecule of HSA was conjugated to  $\sim$ 20 peptide molecules, on average, as determined by gel electrophoresis. After the TRITC-HSA-wt was added (1.25–2.5  $\mu$ g/ml), aliquots were removed at various times 20–80 min later, the dextran was added, and the samples were fixed as described above.

### Video Image Analysis

To compare the transport capability of different experimental samples, the nuclear accumulation of the transport substrate TRITC-HSA-wt was quantitated using video image analysis, previously described in Newmeyer and

Forbes (1990). Briefly, this system consists of a Dage MTI CCD-72 video camera mounted on a Nikon Optiphot fluorescence microscope with a Nikon 60 $\times$  plan-apochromat objective. Images were captured with an Imaging Technologies PC Vision Plus 512 frame-grabber board installed in an IBM AT-compatible computer, and displayed on a black-and-white video monitor. Optimas software (Bioscan, Inc., Edmonds, WA) was used to collect luminance and size data. Brightness and contrast were calibrated using a test pattern generated by the video camera. For our experiments all controls on the video camera were set to manual. The black level was set so that the background light level in the optimal system (i.e., in a specimen containing no fluorescent protein) was slightly above zero. The video camera gain was set at 10.0 (full scale): the brightest nuclei did not exceed the camera's range. Intact nuclei were selected at random by screening for exclusion of FITC-labeled 150 kD dextran. (Only undamaged nuclei have potential for nuclear protein accumulation, and it was therefore essential to assay only intact nuclei.) The perimeter of each nucleus was marked with a mouse, and the average luminance (fluorescence intensity) in that region was calculated. The perimeter of a nucleus with zero accumulation of TRITC-HSA-wt was marked by circling the excluding nucleus in the fluorescein channel. This average luminance (Integrated Grey Value, IGV) was then multiplied by the size of the nucleus to arrive at a value that represents the average total accumulated substrate for a given nucleus (Area  $\times$  IGV, or, ARIGV). For each sample, the ARIGV of 15–25 nuclei was averaged and the standard error calculated. Pixel luminances are expressed in arbitrary units in a range of 0 (completely black) to 255 (completely white), the values encodable by the eight-bit frame grabber.

### Gel Electrophoresis, Immunoblotting, and Radiolabeled Lectin Blotting

The proteins present in the nuclear or extract samples were fractionated on SDS-polyacrylamide gels (8, 9, or 10%) and transferred to nitrocellulose as described in Finlay et al. (1987). Pellets from immunoprecipitation using anti-p62, -p58, or -p54-Sepharose were washed in 40 vol R<sub>1</sub> buffer, resuspended in 50  $\mu$ l SDS-PAGE sample buffer, and incubated at 100°C for 3 min before electrophoresis and subsequent blotting onto PVDF. Immunoblotting with anti-p62, p58, or p54 antisera was performed by incubation of the blot with 5% nonfat dry milk, 0.1% Tween 20 in PBS for 1 h at room temperature. The blot was then incubated for 1 h with 15 ml of a 1:200 dilution of the antisera in 5% nonfat dry milk, 0.1% Tween 20, 0.25 M GlcNAc, in PBS. The blot was washed three times in PBS-Tween and twice with PBS alone, incubated for 1 h with <sup>125</sup>I-protein A (ICN: 0.033 mg/ml) at a concentration of 2–4  $\times$  10<sup>6</sup> cpm/15 ml, washed, and exposed for autoradiography. Probing with <sup>125</sup>I-WGA was done as described in Finlay and Forbes (1990). mAb 414 was the kind gift of L. Davis, T. Meier, and G. Blobel (Rockefeller University). It was used in a similar manner to those above at a 1:10,000 dilution, except that a secondary rabbit anti-mouse antibody (1:200; Cappel Laboratories) was added before the Protein A step.

### Results

Removal of the entire family of WGA-binding glycoproteins from a *Xenopus* nuclear reconstitution extract results in the formation of nuclei that contain visible pores at the electron microscopic level, but which are unable to import (Finlay and Forbes, 1990). These transport-defective nuclei can be rescued by addition of either the *Xenopus* glycoproteins or the rat nuclear glycoproteins (before nuclear formation). Thus, one or more of the rat proteins is functionally homologous with members of the *Xenopus* proteins. To determine which of the rat glycoprotein family are required for pore function, specific antibodies were raised against three individual glycoproteins, p62, p58, and p54.

Rat liver nuclei were purified, treated with DNase, and extracted with the nonionic detergent Mega 10 to isolate *N*-acetylglucosaminylated nuclear proteins (Finlay and Forbes, 1990). The detergent extract was applied to a WGA-Sepharose column and the column was washed until no protein remained in the flowthrough. The WGA-binding proteins were eluted with *N*-acetylglucosamine (GlcNAc) and

electrophoresed on a gradient gel, followed by transfer to nitrocellulose. Upon staining the nitrocellulose with Fast green, protein bands of 62, 58, and 54 kD were found to be the most prominent along with a number of proteins of lesser abundance (Fig. 1). When a parallel blot was probed with <sup>125</sup>I-WGA, it was found that the 62-kD protein bound much <sup>125</sup>I-WGA, as expected. The 58-kD protein was detected as a faint WGA-labeled band, and the 54-kD band was rarely detectable with <sup>125</sup>I-WGA. This suggests that the 58- and 54-kD proteins are glycosylated to a much lesser extent than p62. Alternatively, because WGA prefers to bind clustered GlcNAc groups, it is also possible that the 54- and 58-kD proteins are glycosylated in a manner which is not efficiently recognized by WGA when the proteins are denatured on blots. The three protein bands, p62, p58, and p54, were excised from the nitrocellulose blot, solubilized in DMSO, and individually injected into rabbits. After multiple injections, polyclonal antisera were obtained specific for each protein (Fig. 2).

Because the injected proteins contain *N*-acetylglucosamine, a fraction of the antibodies generated react exclusively with the sugar epitope. This subset of antibodies can cross-react with a number of the nuclear pore glycoproteins. To eliminate this nonspecific crossreactivity, the competing sugar *N*-acetylglucosamine, which was found to block antibodies generated against the sugar epitopes, was included in all experiments.

When immunofluorescence was performed on fixed BRL cells in the presence of competing sugar, anti-p62 antisera stained the nuclear rim in a finely punctate manner (Fig. 3 a), consistent with a nuclear pore staining pattern (Davis and Blobel, 1986; Finlay et al., 1987; Snow et al., 1987). The

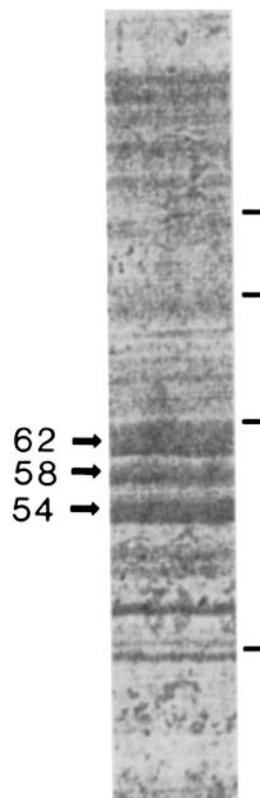
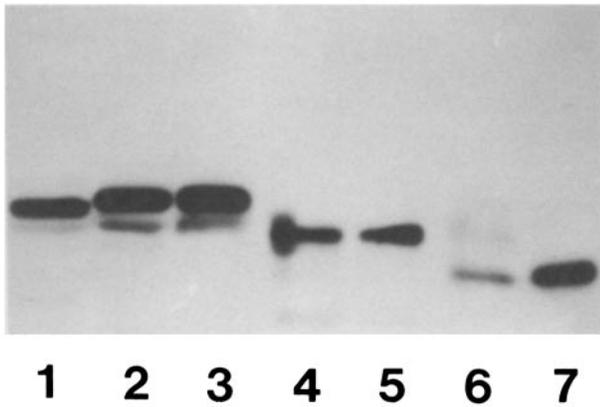


Figure 1. Purification of the WGA-binding proteins of rat nuclei. Large-scale purification of the rat nuclear pore glycoproteins was performed as described in Materials and Methods. The proteins were then electrophoresed on a 5–15% polyacrylamide gel, transferred to nitrocellulose, and stained with Fast green. The three most abundant proteins of 62, 58, and 54 kD are indicated. Bars indicate markers of 116, 97.4, 66, and 45 kD.

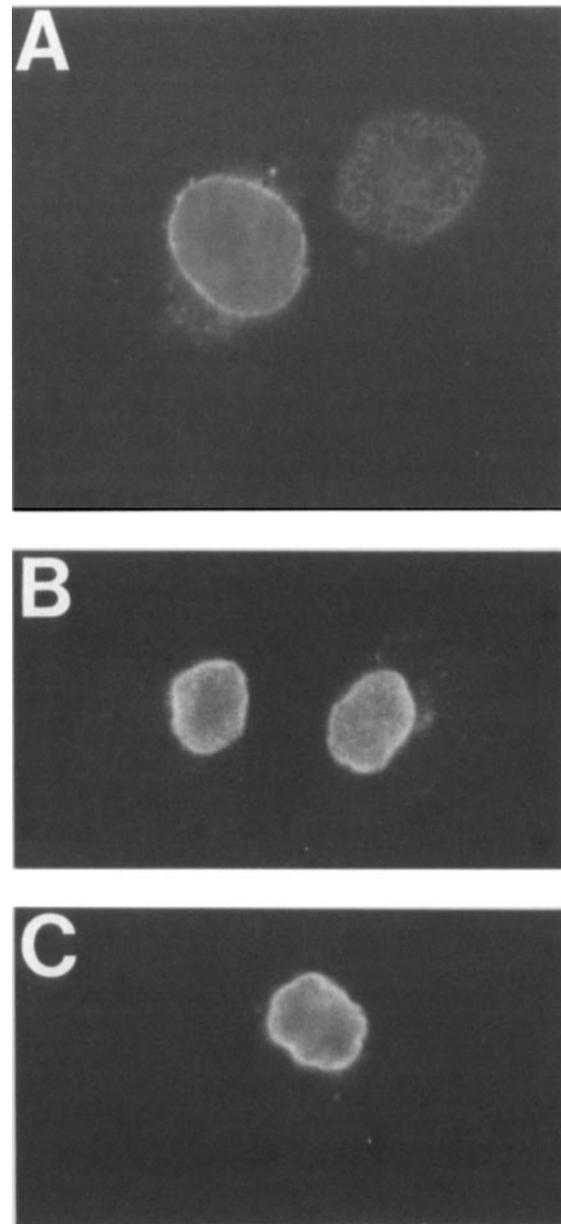


**Figure 2.** Polyclonal antisera recognize specific rat nuclear proteins. Specific polyclonal antisera generated versus the 62-kD protein (lanes 1-3), the 58-kD protein (lanes 4-5), or the 54-kD protein (lanes 6-7), were used to probe three separate strips of PVDF containing Mega 10 extracted rat nuclear proteins. The samples were: BRL cells ( $3.3 \times 10^6$ ; lane 1), rat liver nuclei ( $3 \times 10^6$ ; lanes 2, 4, and 6), and rat nuclear *N*-acetylglucosaminylated proteins (from  $6 \times 10^6$  nuclei; lanes 3, 5, and 7). The proteins were electrophoresed on a 10% polyacrylamide gel and transferred to PVDF. Groups of lanes were cut from the blot and probed with one of the three specific antisera in the presence of 0.25 M GlcNAc, followed by  $^{125}\text{I}$ -protein A, as described in Materials and Methods. The lower band seen in lanes 1-3 runs at  $\sim 60$  kD and may be a breakdown product of p62.

anti-p54 and anti-p58 antisera did not appear to stain fixed cells, but did show nuclear rim staining on unfixed BRL cells after mild Triton extraction (Fig. 3, *b* and *c*). (Polyclonal antisera to p62 and p54 were raised previously [Snow et al., 1987] that failed to stain fixed cells.) Our findings are consistent with the fact that anti-p54 and anti-p58 antisera have no effect on nuclear transport when added to a transport assay (Meier, E., and D. Forbes, unpublished results). We conclude that although only the anti-p62 antisera gives a punctate nuclear rim staining pattern on fixed and unfixed cells, all three of our antisera can recognize the individual protein to which they were raised on blots and in its native state. The data below support this conclusion.

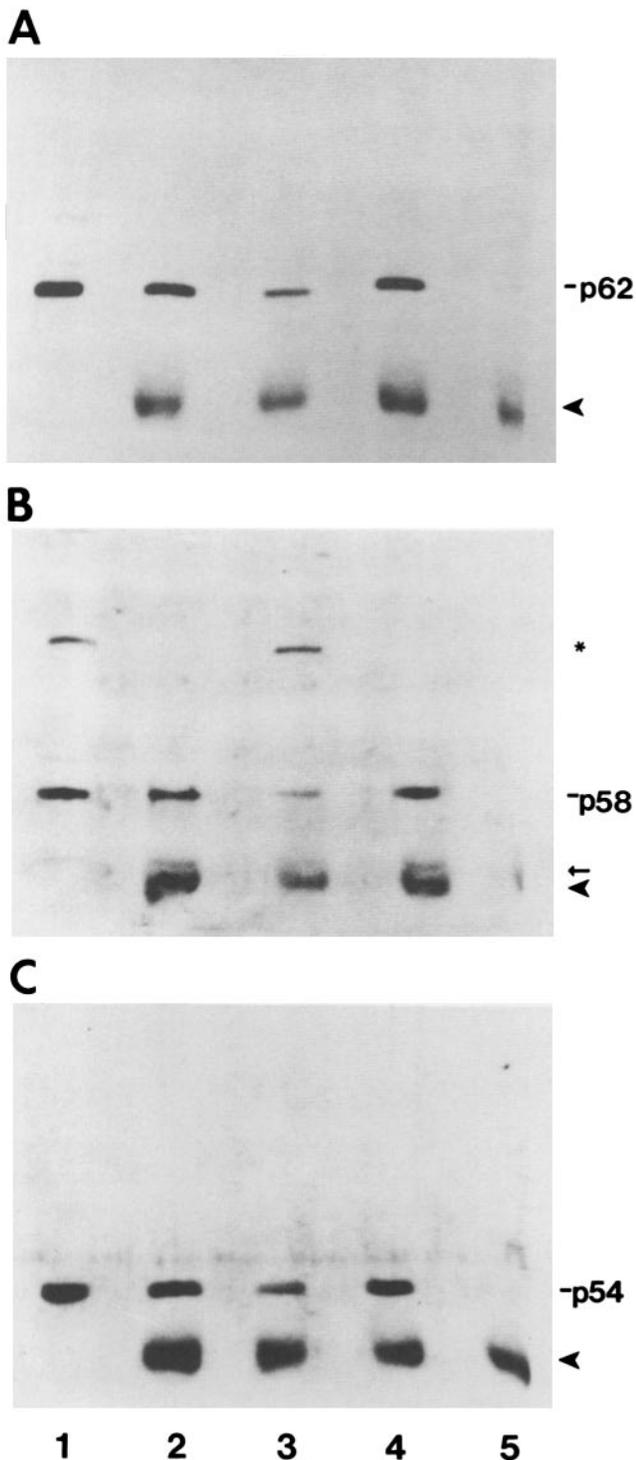
### Three Abundant Nuclear Pore Proteins Exist in a Complex

To immunodeplete a given pore protein from a nuclear reconstitution system using any of the above antisera, it was first necessary to determine whether the antisera were capable of immunoprecipitating their antigens. For this, each antisera was complexed to Sepharose beads and added to isolated rat WGA-binding glycoproteins (RNPG). When immunoprecipitation was performed in buffer compatible with subsequent nuclear formation (i.e., lacking detergent), a high nonspecific background of binding to the anti-p62 Sepharose resulted, as determined by probing with  $^{125}\text{I}$ -WGA (not shown). A similar bulk adsorption of proteins was seen when Sepharose coupled to a nonspecific antisera was used. To block non-specific binding sites on the Sepharose, *Xenopus* extract previously depleted of all WGA-binding proteins



**Figure 3.** Immunofluorescent staining of BRL cells with anti-p62, anti-p58, and anti-p54 antisera. (A) Rat BRL cells were fixed with formaldehyde, permeabilized with Triton X-100, and probed with primary antisera, followed by addition of rhodamine-conjugated goat anti-rabbit secondary IgG. (A) Cells stained with anti-p62 antisera at different planes of focus. Two nuclei can be observed to be stained. In the lower cell, the level of focus is at the surface of the nucleus where a punctate stain is obvious, while in the upper cell the focus is at the equator of the nucleus and a rim stain is observed. (B) Unfixed BRL cells stained with anti-p58 antisera, as described in Materials and Methods. (C) Unfixed BRL cells stained with anti-p54 antisera. In both B and C, a nuclear rim stain was observed, although this stain only rarely had a punctate appearance.

was added to the rat pore glycoproteins (RNPG mix). The depleted *Xenopus* extract inhibited nonspecific binding and in addition was compatible with subsequent nuclear reconstitution and transport assays. Pellets resulting from such immunoprecipitations with each of the specific antisera were



**Figure 4.** p62, p58, and p54 coprecipitate in a complex. A mixture of rat nuclear pore glycoproteins and WGA-depleted *Xenopus* extract in a ratio of 1:1 (RNPG mix; 150  $\mu$ l) was split into five aliquots. Four were immunoprecipitated twice for 1.5 h with one of the antisera-Sepharose conjugates listed below. One aliquot was not immunoprecipitated and served as a control (RNPG mix). The pellets of each immunoprecipitate were washed, further split into three samples, and electrophoresed on three separate polyacrylamide gels. The lanes are as follows: RNPG mix (lane 1; 10  $\mu$ l), anti-p62-Sepharose pellet (lane 2), anti-p58-Sepharose pellet (lane 3), anti-p54-Sepharose pellet (lane 4), and a nonspecific Sepharose pellet (lane 5). The three gels were transferred to PVDF and probed with one

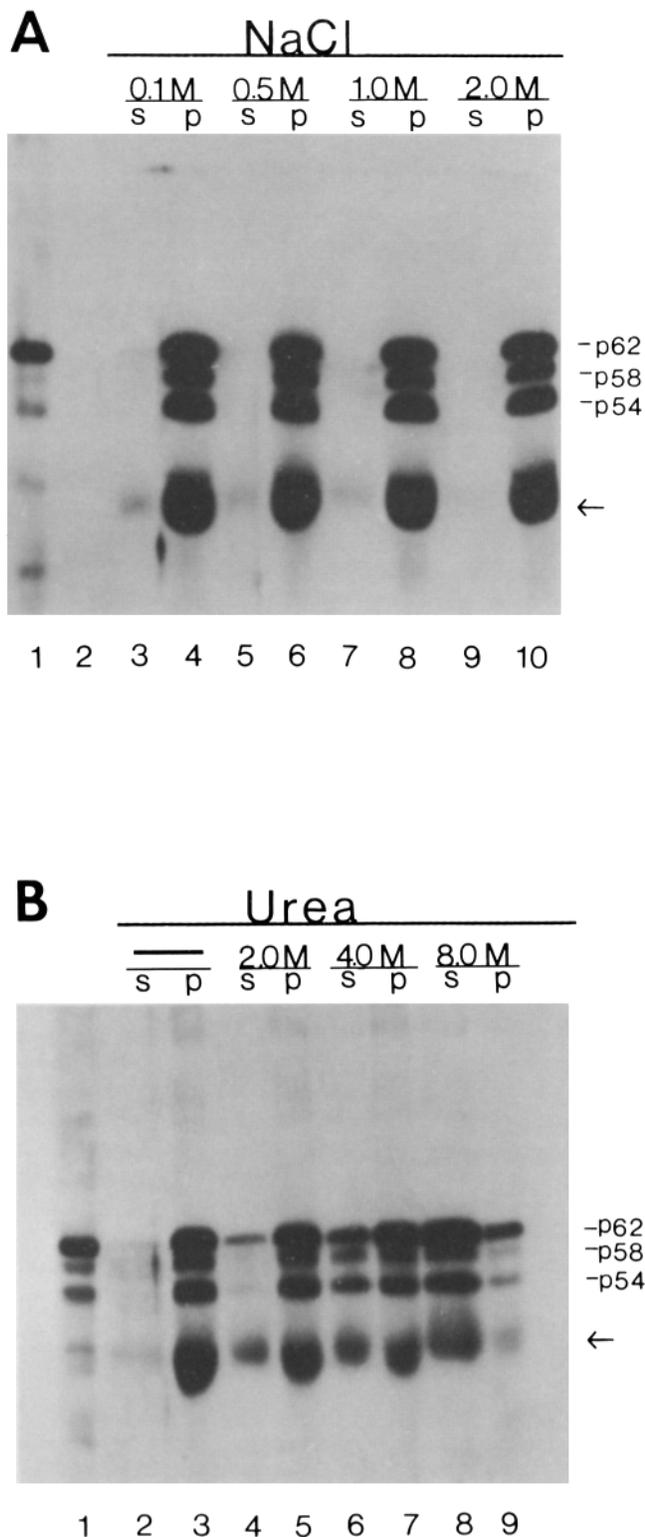
applied to polyacrylamide gels and probed with the respective antisera. Each antisera was found to be capable of specifically immunoprecipitating its antigen from a mixture of rat nuclear glycoproteins (anti-p62, Fig. 4 a, lane 2; anti-p58, Fig. 4 b, lane 3; anti-p54, Fig. 4 c, lane 4).

To determine whether any of the three proteins to which our antisera were raised interact with one another, we asked whether the antigens coprecipitate. The total rat glycoprotein mixture was immunoprecipitated with anti-p62 antisera, for example. The resulting pellet was split and electrophoresed on three separate gels. Western transfers of the gels were probed with anti-p62, anti-p58, or anti-p54 antisera (Fig. 4, a-c, respectively). Surprisingly, we found that each of the specific antibodies immunoprecipitated all three proteins. Nonspecific antisera did not significantly precipitate any of the proteins (Fig. 4, a-c, lane 5). These results indicate that p62, p58, and p54 exist in a complex when extracted from rat liver nuclei with the mild detergent Mega 10.

To maintain the rat pore proteins in as functionally active a state as possible (for later pore reconstitution), fairly gentle conditions were used for the extraction and immunoprecipitation described above. With these relatively mild conditions, it was possible that the apparent complex might result from weak nonspecific interactions between the three proteins. To examine the stability of the complex, an anti-p62 immunoprecipitate of the rat WGA-binding proteins was extracted with increasing concentrations of salt or urea. The results indicate that the complex is held together by strong molecular interactions between the three proteins. No disruption of the complex was observed at salt concentrations of up to 2 M NaCl (Fig. 5 a) or urea concentrations of 2 M (Fig. 5 b). The antibody itself, however, began to dissociate from the Protein A-Sepharose at  $\sim$ 4 M urea, as indicated by the presence of the IgG heavy chain in the supernatant (see arrow in Fig. 5 b), and the antibody with bound complex completely dissociated at 8 M urea. It is worth noting that the complex that remains associated with the Protein A-Sepharose pellet at 4 M urea (Fig. 5 b, lane 7) appears to maintain the original ratio of p62:p58:p54, suggesting that the complex does not yet dissociate at this urea concentration. These results argue that the three proteins form a tightly associated protein complex which can be extracted intact from existing pores.

To determine the size of the complex, an aliquot of the isolated rat WGA-binding proteins was applied to a Sephacryl HR300 gel filtration column. Fractions were collected, the

of the following antisera at a 1:200 dilution: (A) anti-p62 antisera, (B) anti-p58 antisera, (C) anti-p54 antisera. Note that each lane, which is the immunoprecipitate of only one antisera, contains all three proteins when probed with the different antisera. (The protein in lane 5 in blot B did not transfer well, however there is very little if any p58 immunoprecipitated in this nonspecific control pellet, as expected. The absence of the complex can also be seen in lanes 5, blots A and C, where the other two-thirds of the nonspecific antisera pellet lack any significant amount of the p54 or p62 proteins.) The arrowhead indicates the IgG heavy chain. The asterisk indicates a 50-kD protein (see text). The \* in B indicates a *Xenopus* 124-kD protein with which the p58 antisera cross-reacts. This 124-kD protein is, however, only present in the sample immunoprecipitated by the anti-p58-Sepharose and thus is not a part of the complex immunoprecipitated by the three antisera.



**Figure 5.** Salt and urea extraction of the 62-58-54 complex. Partially purified rat nuclear pore glycoproteins, extracted from  $3 \times 10^8$  nuclei, were immunoprecipitated with anti-p62-Sepharose. The washed pellets were incubated at room temperature with 100  $\mu$ l of either NaCl- (*A*) or urea- (*B*) containing solutions (concentrations as indicated in figure). After 45 min, the Sepharose was pelleted and aliquots of the supernatants (*S*) and pellets (*P*) were subjected to SDS-PAGE on 9% gels. The proteins were transferred to nitrocellulose and incubated with anti-p62, p58, and p54 anti-

proteins electrophoresed on a polyacrylamide gel, transferred to nitrocellulose, and probed with an mAb, 414, which reacts with numerous members of the pore glycoprotein family including p62 and p58 (Fig. 6 *a*; Davis and Blobel, 1987), or probed with all three of our antisera (Fig. 6 *b*). The results demonstrate that p62, p58, and p54 elute from the column in identical fractions (Fig. 6, *a* and *b*). The complex fractionates with a molecular weight of 550–600 kD, migrating between the  $\beta$ -galactosidase (464 kD) and thyroglobulin (672 kD) markers. This discrete size argues again that the complex is a distinct structure, and not a random or heterogeneous aggregate.

It should be noted that a protein of  $\sim 50$  kD is recognized by the anti-p58-antisera and is coprecipitated by all the antibodies. In the initial blots of the rat nuclear pore glycoproteins the 50 kD band was not visible (Fig. 2, lanes 4–5), but is visible in all three immunoprecipitates probed with anti-p58 antisera (*small arrow*, Fig. 4 *b*). We think it is most likely a breakdown product of the 58 kD protein which accumulates during the course of immunoprecipitation.

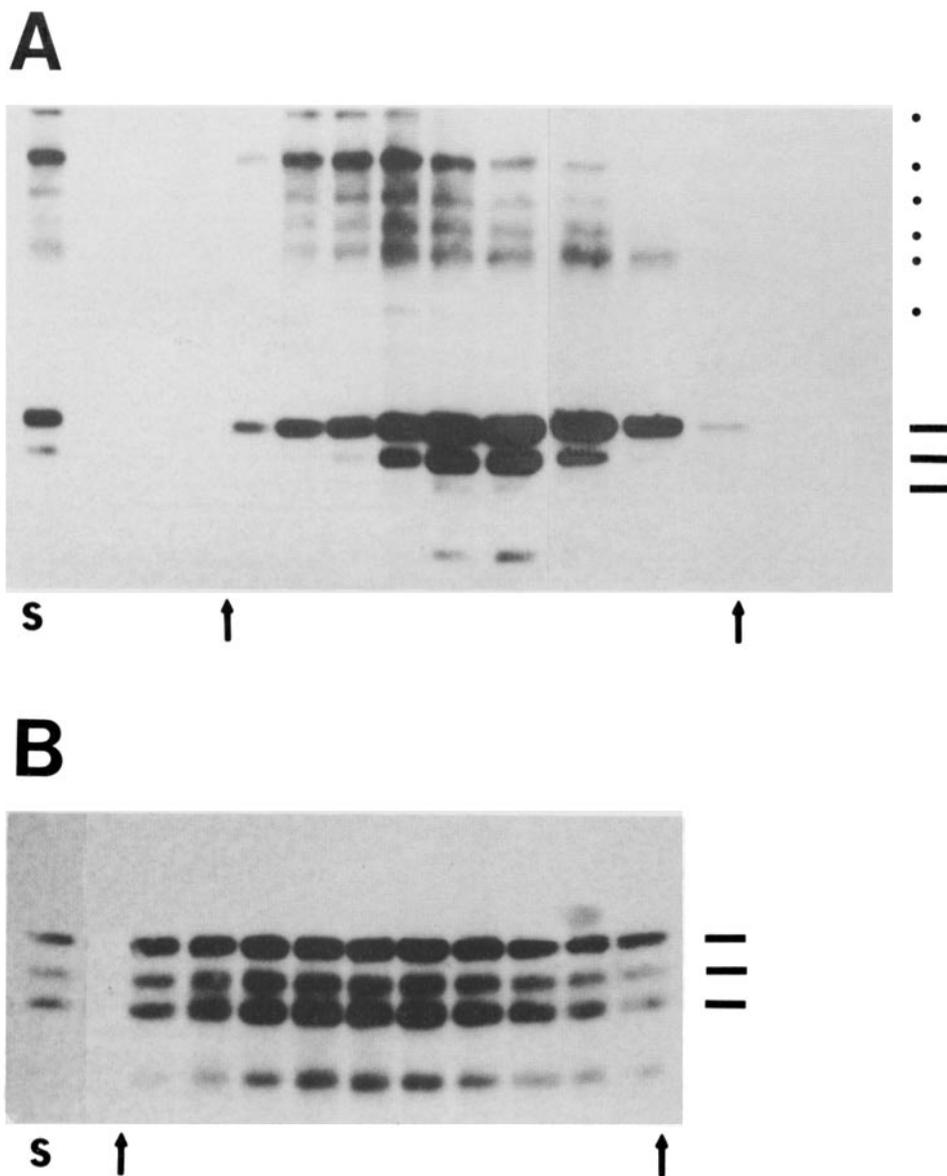
We found that *N*-acetylglucosamine-bearing proteins of 94, 130, 143, 157, 180, and 270 kD were not in the complex. The 180- and 270-kD pore glycoproteins migrated faster than the 550–600-kD complex (*top dots*, Fig. 6 *a*). Proteins of 94, 130, 143, and 157 also migrated slightly faster than the rat p62-p58-p54 complex (Fig. 6 *a*). Moreover, the anti-p62 antisera does not significantly immunodeplete these higher molecular weight bands, indicating they are not quantitatively included in the complex (see Figs. 8 and 9 below). Interestingly, the relative mobility of the 180–270 and the 94–130–143–157 proteins indicate that they too are present in complexes of large size relative to their molecular weight. It remains to be determined whether these latter high molecular weight entities are due to oligomerization of the individual proteins or to heterologous complexes of the different proteins.

To determine more rigorously the composition of the complex, isolated rat WGA-binding proteins were immunoprecipitated using Sepharose beads to which anti-p62 antisera was cross-linked. The resulting immunoprecipitate was fractionated on a polyacrylamide gel and stained with Coomassie blue (lanes 2 and 6, Fig. 7). In four separate immunoprecipitations, p62, p58, and p54 were the only proteins observed reproducibly in large amounts. The ratio appeared to be  $\sim 4:1:4$  (522 kD). This is an estimate based on a staining which is sensitive to amino acid composition and may not be entirely accurate. Other proteins which stain poorly may also be present. (Silver staining showed the same three proteins, but was greatly biased towards p62 which appears to stain to a much greater extent than p54 and p58 with this technique.) We conclude that, although other proteins may be present in lesser amounts, the complex contains primarily p62, p58, and p54.

#### ***Nuclei Deficient in the p62-58-54 Complex Are Deficient for Transport***

To ask whether nuclei depleted of complex are capable of nu-

sera, simultaneously. Lane 1 contains an aliquot of untreated immunoprecipitate. The arrow indicates the IgG heavy chain, which also binds  $^{125}$ I-protein A. Note that the 62-58-54 complex appears stable to 2 M NaCl or 2 M urea extraction.



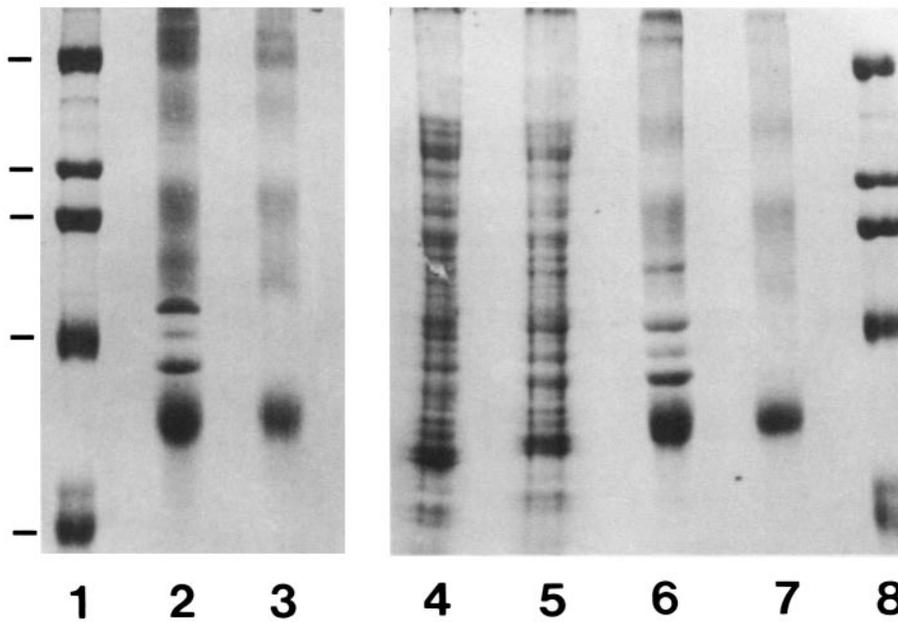
**Figure 6.** Gel filtration determination of the complex size. (A) Partially purified rat WGA-binding glycoproteins were applied to a Sephacryl S-300 HR column and fractions were collected, concentrated, and electrophoresed on a 9% SDS-polyacrylamide gel. After transfer to PVDF membrane, the fractions were probed with mAb414. The same column was used to run the protein size markers, thyroglobulin,  $\beta$ -galactosidase, catalase, bovine serum albumin,  $\alpha$ -amylase, ovalbumin, and soybean trypsin inhibitor. Fractions 13-29, demarcated by the arrows, include the peak of the eluted complex. (Only the odd numbered fractions were electrophoresed on this gel.) The 62-58-54 complex peaked at approximately fraction 22, giving a size of 550-600 kD. For reference, thyroglobulin (672 kD) peaked in fraction 20, whereas galactosidase (464 kD) peaked in fraction 26. A similar size determination was obtained when the protein size markers were fractionated simultaneously with the rat glycoproteins. Lane S contains  $\sim$ 1% of the starting material. The bars mark p62, p58, and p54. The dots mark proteins of  $\sim$ 270, 180, 157, 143, 130, and 94 kD. (B) Partially purified rat WGA-binding glycoproteins were applied to a Sephacryl S-300 HR column as in A with the following changes: Smaller fractions ( $\sim$ 0.5 ml) were collected for finer resolution, and a blot of the transferred protein fractions was probed with all 3 antisera (anti-p62, anti-p58, and anti-p54). The bulk of

the complex eluted in fractions 38-47, again demarcated with arrows. The complex peaked in fractions 42-43 as determined by densitometry of the autoradiograph. For reference, thyroglobulin peaked in fractions 39-40 and  $\beta$ -galactosidase peaked in fraction 47. Again, lane S contains  $\sim$ 1% of the starting material. The bars mark the 62, 58, and 54 kD proteins.

clear import, the depletion-reconstitution system previously described was used. Anti-p62 antisera was coupled to Protein A Sepharose and used to immunodeplete a mixture of partially purified rat nuclear pore glycoproteins. To determine the amount of complex removed under these conditions, the immunodepleted supernatants were electrophoresed on polyacrylamide gels, transferred to nitrocellulose, and probed with  $^{125}$ I-WGA. Densitometric scans of the resulting autoradiograms indicated that two serial immunodepletions using anti-p62-Sepharose removed up to 90% of the p62 protein complex present, depending on the experiment (compare lanes 1 and 3, Fig. 8 a). Nonspecific antisera when coupled to Sepharose removed  $<$ 10-15% of p62 (mock depletion). Because the supernatants were probed with  $^{125}$ I-WGA, it was possible to visualize not only the amount of p62 removed, but also the high molecular weight rat glycoproteins (Fig. 8

b, lanes 3-6). We found that there was no significant difference in the high molecular weight bands when the sample was immunodepleted with either the nonspecific antisera-Sepharose or anti-p62-Sepharose. Thus, immunoprecipitation using anti-p62-Sepharose leads to a large specific reduction in the p62 protein complex without a corresponding decrease in the higher molecular weight WGA-binding proteins.

To test the effect on nuclear import of depleting p62 and its complexed proteins, the immunodepleted mixture of rat pore proteins (Fig. 8 b, lane 4) was added to a nuclear reconstitution extract from which the equivalent *Xenopus* WGA-binding proteins had been removed (Fig. 8 b, lane 1). Chromatin and membranes were added and nuclei were allowed to form. Once formed, the nuclei containing p62 complex-depleted pores were assayed for nuclear transport by the ad-



**Figure 7.** Coomassie blue staining of the immunoprecipitated complex. Rat WGA-binding proteins from  $6 \times 10^8$  nuclei were immunoprecipitated in two different experiments, one shown in lanes 2–3 and a second shown in lanes 4–7. The pellets were immunoprecipitated with either anti-p62 (lanes 2 and 6) or nonspecific antisera (lanes 3 and 7). The supernatants from the immunoprecipitated pellets shown in lanes 6 and 7 were concentrated by TCA precipitation; half was loaded in lanes 4 (anti-p62 supernatant) and 5 (whole rabbit antisera supernatant). (It should be noted that the WGA-binding proteins used in this experiment were impure and contained many non-WGA-binding proteins.) The two proteins other than p62, p58, and p54 seen in lane 6 were present in relatively high amounts in this experiment, but not in other immunoprecipitations. The gel was stained with Coomassie blue. The ratio of the three proteins varied somewhat from experiment to experiment. Molecular weight markers of 205, 116, 97, 66, and 45 kD are shown in lanes 1 and 8.

dition of a rhodamine-labeled transport substrate (TRITC-HSA-wt), consisting of multiple copies of the SV40 T antigen signal sequence covalently coupled to HSA. At various times after substrate addition, the nuclei were fixed, observed briefly in the fluorescence microscope, and analyzed by video imaging which allows the level of accumulated fluorescent transport substrate to be quantitatively measured in individual nuclei (Newmeyer and Forbes, 1990). An average of accumulated fluorescence per nucleus could then be obtained for each sample to determine the relative efficiency of transport.

A comparison of transport in p62 complex-depleted nuclei, mock-depleted nuclei, and control nuclei is shown in Table I. (Control nuclei are those reconstituted with the complete set of rat nuclear glycoproteins present.) In a typical experiment, the p62 complex-depleted nuclei accumulated only 11% of the amount of substrate imported by control nuclei. Nuclei formed with nonspecifically depleted rat glycoproteins transported at 85% the control level. Thus, in this experiment the removal of p62 and its complexed pore proteins reduced the ability of depleted nuclei to import by eightfold.

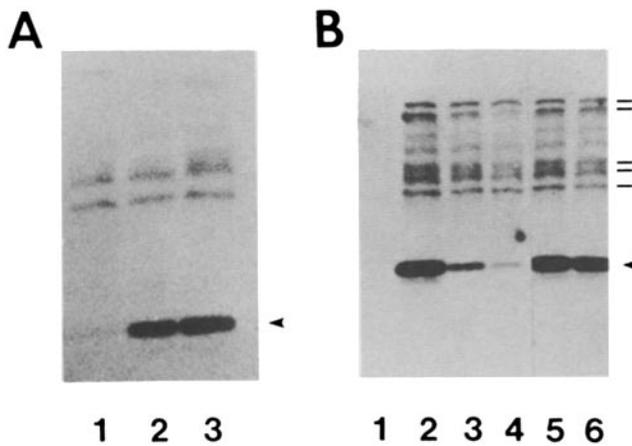
To test the effect of depletion by anti-p54 or -p58 antisera, each antisera was bound to Protein A Sepharose and used to immunodeplete the rat nuclear pore glycoproteins in the presence of WGA-depleted *Xenopus* extract. The immunodepleted supernatants were electrophoresed on polyacrylamide gels, transferred to nitrocellulose, and probed with the corresponding antibody followed by  $^{125}\text{I}$ -Protein A. Anti-p58-Sepharose removed  $\leq 50\%$  of the 58-kD protein (not shown). Since the anti-p58 antisera also cross-reacts with a 124-kD *Xenopus* protein (Fig. 4 b), this protein may compete with the rat p58 for antibody binding, reducing the efficiency of p58 immunoprecipitation. Immunodepletion with anti-p54-Sepharose, on the other hand, removed much of p54 and its associated proteins. Immunodepletion of the complex by anti-p54 and subsequent nuclear formation resulted in a drop in nu-

clear transport to one third the mock-depleted control (Table I). Thus, these data support the anti-p62 immunodepletion finding that removal of the complex greatly reduces nuclear transport.

#### **Amount of Nuclear Transport Is Directly Related to the Amount of Complex Present**

In the above experiment, nuclei depleted with anti-p62-Sepharose showed a much greater reduction in transport than p54-depleted nuclei (Table I). If, as our data suggest, the complex is necessary for forming functional nuclear pores, then the amount of the complex present at the time of nuclear formation would be a limiting factor in the ability of the resulting nuclei to transport. If true, the differences in transport observed would be due to the varying amounts of complex removed by different antisera. To test this, an aliquot of rat nuclear pore glycoproteins mix was split into five identical parts that were then immunodepleted by anti-p54, -p58, -p62, or nonspecific antibody-Sepharose, or by no antisera at all. The proteins remaining after immunodepletion were added to a larger aliquot of WGA-depleted *Xenopus* extract. Chromatin and membranes were added and nuclei allowed to form. The transport substrate was added and the nuclei analyzed 40 min later by video imaging to measure nuclear transport. The amount of p62-58-54 complex remaining in each immunodepleted extract was measured by gel electrophoresis, transfer to nitrocellulose, probing with  $^{125}\text{I}$ -WGA, and densitometric scanning of the p62 band (Fig. 9 a, lanes 4–8). Total nuclear import was then plotted versus the amount of p62 that remained, taking this as a measure of the amount of complex remaining (Fig. 9 b and Table II).

As predicted, we found that the different antisera did indeed remove differing amounts of p62 and its associated proteins under the immunodepletion conditions. More importantly, the lower the amount of the p62-58-54 complex that



**Figure 8.** Immunodepletion of the 62-kD rat nuclear pore glycoprotein. (A) Partially purified rat nuclear pore proteins were added to a depleted *Xenopus* extract in a 1:1 ratio (RNPG mix). The depleted *Xenopus* extract was added to block nonspecific binding to the Sepharose. The immunodepleted supernatants are shown after a 3-h immunoprecipitation with anti-p62 antisera-conjugated Sepharose (lane 1; 10  $\mu$ l) or nonspecific antisera-conjugated-Sepharose (lane 2; 10  $\mu$ l). 10  $\mu$ l of starting material are shown in lane 3. (The fractions were electrophoresed, blotted, and probed with  $^{125}$ I-WGA to visualize p62, indicated by an arrowhead, and additional WGA-binding nuclear proteins.) Immunoprecipitation with anti-p62 specifically depleted the p62 protein (arrowhead; lane 1) compared to immunoprecipitation with a nonspecific control antibody (lane 2). (B) To determine the proportions of WGA-binding proteins present in transport assays when testing the effect of the p62 protein immunodepletion on transport, the following transport assay components were electrophoresed, blotted, and probed with  $^{125}$ I-WGA: WGA-Sepharose-depleted *Xenopus* extract (lane 1; 11.5  $\mu$ l), RNPG mix (lane 2; 5  $\mu$ l), RNPG mix immunoprecipitated once for 1.5 h with either anti-p62-Sepharose (lane 3; 5  $\mu$ l) or twice for 1.5 h with anti-p62-Sepharose (lane 4; 5  $\mu$ l); RNPG mix immunoprecipitated once with nonspecific control antisera-Sepharose (lane 5; 5  $\mu$ l), or twice with nonspecific control Sepharose (lane 6; 5  $\mu$ l). (5  $\mu$ l of the various immunodepleted supernatants were added back to 11.5  $\mu$ l of the depleted *Xenopus* extract to assay for transport in the nuclei formed.) The arrowhead denotes p62. The bars denote proteins of 230, 210, 143, 130, and 118 kD.

remained in the nuclear transport assay, the less the amount of transport substrate that was accumulated by the nuclei (Fig. 9 b). In this experiment, immunodepletion with anti-p62-Sepharose left 21% of the complex in the assay; such con-

**Table I. Immunodepletion of p62 or p54 Reduces Nuclear Transport**

Antisera used for immunodepletion	Total accumulated RITC-ss-HSA	% Transport
None	16,076 $\pm$ 3,056 ( $n = 15$ )	100
Anti-p62	1,712 $\pm$ 563 ( $n = 15$ )	11
Nonspecific antisera	13,630 $\pm$ 3,405 ( $n = 15$ )	85
Anti-p54	4,592 $\pm$ 933 ( $n = 14$ )	29

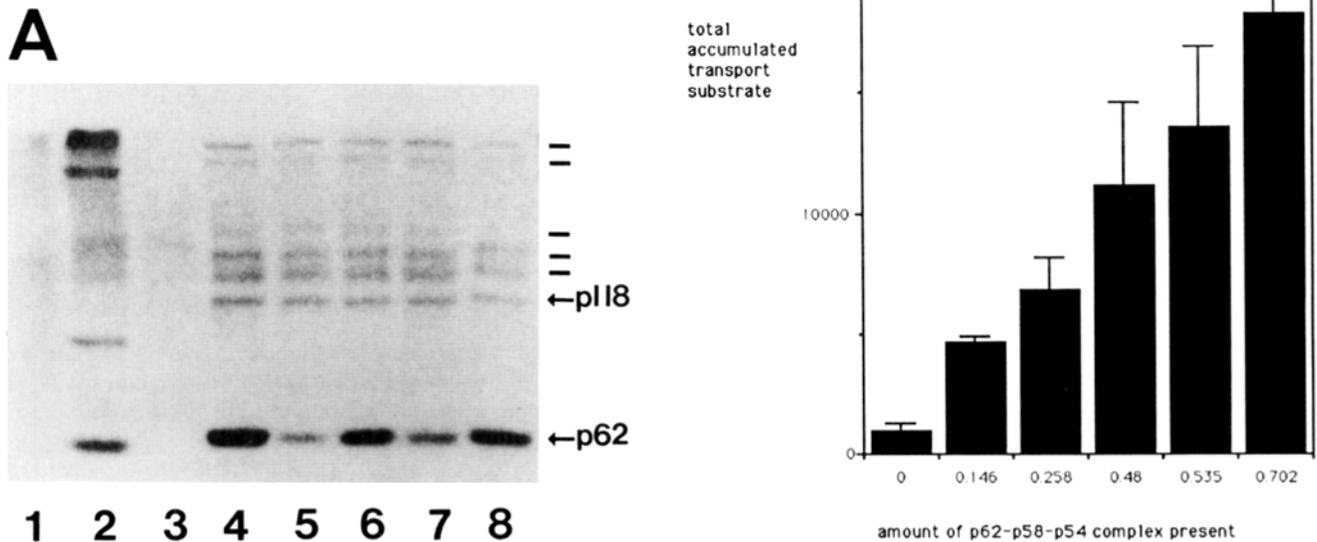
Samples of partially purified rat proteins mixed in a 1:1 ratio with depleted *Xenopus* extract were immunodepleted with the indicated antisera-Sepharose. 10  $\mu$ l of the immunodepleted supernatants were then added to 30  $\mu$ l of WGA-depleted *Xenopus* extract, chromatin, and membranes. The resulting nuclei were assayed for their ability to transport. Total nuclear transport substrate accumulation was measured using video imaging. The numbers represent values for the total accumulated substrate for  $n$  nuclei  $\pm$  the SEM, as described in Materials and Methods.

ditions supported 25% of the control level of transport. Anti-p54-Sepharose immunodepletion left 37% of the complex in the assay and showed 37% the control level of transport. Immunodepletion with anti-p58-Sepharose, which has the lowest affinity for the complex, left 68% of the complex and transported at 61% of the control level. Monitoring the amount of a representative higher molecular weight WGA-binding protein (p118; arrow in Fig. 9 a) showed that there was no correlation between the amount of this protein or any other high molecular weight glycoprotein present and the transport rate of the immunoprecipitated samples (Table II). This indicates that the drop in transport is not due to a general lowering in concentration of all rat nuclear pore glycoproteins by immunoprecipitation, but instead to the removal of the p62-p58-p54 complex. We conclude that there is a strict linear correlation between the amount of p62-p58-p54 complex available during nuclear formation and the ability of the nuclei formed to import nuclear proteins.

## Discussion

The recently identified family of novel pore glycoproteins has been estimated to comprise  $\sim$ 2-3% of the protein of rat nuclear pores (Snow et al., 1987). The glycoproteins are conserved among different organisms and a similar family of proteins has been identified in *Xenopus* eggs. When the entire *Xenopus* glycoprotein family is removed from a nuclear reconstitution extract, the nuclei formed are defective both in the binding and translocation steps of nuclear import (Finlay and Forbes, 1990). Rat pore glycoproteins can reconstitute binding and transport when substituted for the missing *Xenopus* proteins. Based on these observations, it is clear that some or all of the members of this protein family are critical for proper pore function. To obtain specific functional and structural information for individual members of the pore glycoprotein family, we have raised polyclonal antibodies specific to each of three abundant members of the rat family, p62, p58, and p54.

Using these protein-specific antibodies, we have found that antisera against any one protein coimmunoprecipitates the other two. This demonstrates that the three proteins interact with one another to form a high molecular weight multimeric complex. Since p62 is one of the few members of the glycoprotein family shown conclusively to be a pore protein, its association in a complex with p54 and p58 indicates that they are also authentic nuclear pore proteins. Although poorly glycosylated, they are among the most prominent bands observed after our WGA-Sepharose purification of nuclear glycoproteins. We now think that their retention on WGA-Sepharose is indirect and results from the binding of the heavily glycosylated p62 member of the complex to the WGA Sepharose. Using gel filtration, we have found that the complex has a molecular weight of  $\sim$ 550-600 kD. The complex is resistant to dissociation by low concentrations of the detergent Mega 10, 2 M urea, or 2 M NaCl, indicating that the association of the proteins is mediated by specific and tight interactions. By immunodepletion, we have demonstrated that the efficiency of transport of proteins into nuclei is directly dependent on the initial concentration of this complex within a nuclear reconstitution extract. Together our results demonstrate that the 3 glycoproteins, p62, p58, and p54, physically



**Figure 9.** The amount of p62 and its associated proteins correlates directly with nuclear import. Transport assays were performed as described in Materials and Methods by adding either total rat nuclear pore glycoproteins (RNP mix), immunodepleted RNP mix, or high sugar buffer to a WGA-depleted *Xenopus* nuclear reconstitution extract. After nuclear formation, TRITC-labeled nuclear transport substrate was added. Aliquots were taken 40 min later, fixed with formaldehyde, and assayed with video imaging. (A) The amount of the 62–58–54 complex remaining after immunodepletion with anti-p62, -p58, -p54, or nonspecific antisera Sepharose is indicated by the amount of p62 present in lanes 5–8.  $^{125}\text{I}$ -WGA was used to probe a nitrocellulose blot of the transport assay components which had been electrophoresed on an 8% polyacrylamide gel. ( $^{125}\text{I}$ -WGA allows observation of the upper molecular weight WGA-binding proteins, as well as the 62-kD component of the 62–58–54 complex. Although the 58- and 54-kD proteins are present, they do not appear to bind WGA well and therefore are not visible on this blot.) The lanes are as follows: 10 $\times$  *Xenopus* membranes in MWB (2  $\mu\text{l}$ ; lane 1), undepleted *Xenopus* extract (17  $\mu\text{l}$ ; lane 2), WGA-depleted *Xenopus* extract (12  $\mu\text{l}$ ; lane 3), RNP mix (50% partially purified RNPGs and 50% WGA-depleted *Xenopus* extract) (5  $\mu\text{l}$ ; lane 4). Lanes 5–8 contain: 5  $\mu\text{l}$  RNP mix immunodepleted with anti-p62-Sepharose (lane 5), anti-p58-Sepharose (lane 6), anti-p54-Sepharose (lane 7), or nonspecific-antisera-Sepharose (lane 8). 5  $\mu\text{l}$  of the various RNP mix immunodepleted supernatants were added to 11.5  $\mu\text{l}$  of the depleted *Xenopus* extract for nuclear reconstitution. Lane 1 represents the amount of membranes added to this 18  $\mu\text{l}$  of extract. Note that the different antisera have differing efficiencies of p62 removal. Arrows denote p62 and p118, the latter being a representative high molecular weight rat WGA-binding glycoprotein present in WGA blots. The bars indicate proteins of  $\sim$ 230, 210, 157, 143, and 130 kD. (B) The total accumulation of transport substrate under conditions in Fig. 9a where differing amounts of complex were present is plotted. Total accumulation of transport substrate is indicated in arbitrary units (ARIGVs) derived from video imaging, as explained in Materials and Methods. Densitometric scanning of the blot in A resulted in a relative measure of p62 present per sample. The values noted are the integrated area under the absorption curve of p62 measured by densitometry of each band in Fig. 9a.

interact with one another to form a complex which is essential for nuclear import.

With respect to the organization of the complex, it appears from immunoprecipitation and Coomassie blue staining that the bulk of the complex is composed of the three proteins that we have studied. Further work will be required to ascertain whether other proteins are included. Determining the stoichiometry of the different proteins within the complex is dependent on an equally sensitive staining of each protein by the dye chosen; we already know that such equal staining is not the case with silver staining. Fast green stain of the proteins transferred to nitrocellulose gives a stoichiometry of  $\sim$ 2:1:2, while Coomassie blue staining of protein gels gives a stoichiometry of  $\sim$ 4:1:4 (or often even less p58). If the complex consists of four subunits of p62, one subunit of p58, and four

of p54, the molecular weight would be  $\sim$ 522 kD. This calculated stoichiometry approximates the gel filtration value of 550–600 kD, but cannot be said to be more than an estimate at present and will be the focus of future work.

It is worth asking what features p62, p58, and p54 share, both structurally and functionally. From earlier work, when solubilized as monomers by 2% Triton-high salt treatment of nuclear envelopes, the three proteins were found to be among the proteins immunoadsorbed by a monoclonal antibody RL2. This antibody recognizes protein-sugar epitopes and stains the nuclear pore (Snow et al., 1987; Holt et al., 1987). Thus, each must share a common protein-sugar epitope. Our antibodies do not appear to recognize this epitope (see Fig. 2). By comparison of radioiodinated tryptic peptides, Snow et al. (1987) determined that p62 and p54 show

**Table II. Comparison of Transport to the Amount of p62-complex Present**

Antisera used for immunodepletion	% p118 present	% Complex remaining	% Transport
None	100	100	100
Anti-p62	81	21	25
Anti-p58	70	68	61
Anti-p54	82	37	37
Nonspecific antisera	77	76	74

The data from Fig. 9 are presented here in tabular form. Samples of partially purified rat proteins added in a 1:1 ratio with depleted *Xenopus* extract were immunodepleted with the indicated antisera-Sepharose. 10  $\mu$ l of the immunodepleted supernatants were then added to 30  $\mu$ l of depleted *Xenopus* extract, chromatin, and membranes. The resulting nuclei were assayed for their ability to transport. Total substrate accumulation was measured using video imaging. The amount of complex present was measured by densitometric scanning of the p62 band in Fig. 9 a. Linear regression analysis performed on the individual samples resulted in a positive correlation of 0.99, with 1.0 being the most positive correlation possible. The amount of p118 present was measured by densitometric scanning of Fig. 9 a to provide a measure of nonspecific protein removal.

distinctly different tryptic patterns, but that p62 may share some partial homology with p58. Again, if there are common epitopes between p62 and p58, our polyclonal antisera do not recognize such epitopes, as the antisera are specific for their respective proteins on Western blots. Together these observations support the conclusion that p62, p58, and p54 are structurally distinct proteins. With respect to function, the association of the three proteins with one another in the nuclear pore suggests that they act together during a common step in nuclear transport.

Initially the possibility existed that the rat pore protein complex was artificially formed during detergent extraction of rat liver nuclei or, alternately, during immunoprecipitation. However, treatment of the complex with 2 M salt or 2 M urea did not disassemble the complex, arguing against a random aggregate and indicating that the proteins are tightly and specifically associated with one another. The complex is not induced by immunoprecipitation, since an identical complex was observed with gel filtration. In a separate gel filtration study, we found that there is also a 600-kD complex containing three similarly sized WGA-binding pore proteins present in soluble form in *Xenopus* eggs (Meier, E., and D. Forbes, in preparation). Because neither detergent extraction nor immunoprecipitation was carried out on the *Xenopus* complex, it is likely that this complex is analogous to the rat complex and represents the mitotic state of p62, p58, and p54 in *Xenopus* eggs. Thus, there is evidence that the complex exists not only within the pore itself, but is maintained in a stable state after the pore is disassembled at mitosis. The complex then appears to represent a fundamental unit of nuclear pore structure. It should be noted that a completely different complex of *Xenopus* proteins of 254 kD has been observed by Dabauvalle et al. (1990), which they find necessary for nuclear pore formation; a somewhat multispecific antibody that removes this complex and other proteins from a crude nuclear reconstitution extract results in defective nuclei. We do not know how this complex relates to the larger ones that we observe.

In studies that were directed at observing the rate of incorporation of newly synthesized p62 into nuclear pores, Davis and Blobel (1986) found that in tissue culture cells p62 exists

as a monomer for up to 12 h after synthesis. This evidence suggests that assembly of the complex does not occur immediately after synthesis, but may take place only at the time of pore formation (S phase). Alternatively, p62 may be made in large excess relative to the amount of p54 and p58 synthesized in the tissue culture cells used in their study. Clearly it will be of interest to determine when and in what manner newly synthesized pore proteins initially become incorporated into the stable complex that we observe.

If present in only one copy per pore, the 550–600 kD complex would represent 0.5% of the pore protein ( $6 \times 10^5$  D/1.2  $\times 10^8$  D). Snow et al. (1987), however, have estimated that each pore contains eight copies of p62. Based on this observation, if all p62 is part of a complex, and if there are four copies of p62 per complex, then there would be two such complexes per pore. Alternatively, given the eightfold symmetry of each half of the pore, one might expect there to be either 8 or 16 complexes per pore. It is possible that this complex represents a significant fraction of the mass of an intact pore; careful measurements will be required to assess this possibility. It will also be interesting to ask in future experiments whether, when the complex is depleted, additional pore proteins fail to assemble onto the pore.

In our gel filtration studies, we observed that other members of the glycoprotein family migrate at equally large but distinct molecular weights, indicating their presence in other complexes. Such proteins are present in much lesser amounts in our isolated rat mixture, but this may be due to their poor extractability from the pore with the mild detergent Mega 10 during purification. The presence of additional complexes suggests that the pore is assembled from a group of modular parts. Indeed, the complexes seen here combined may represent a substantial fraction of the total pore mass (10–20%).

By immunodepletion, we find a direct correlation between the amount of the p62-p58-p54 complex present and the rate of nuclear import. This correlation indicates that the amount of complex that a given nucleus is able to incorporate during formation is directly related to its ability to transport. One possibility is that each pore requires a given number of such complexes in order to be "on." The observed transport rate difference would then be due to the total number of "on" pores present. It is equally possible that a single complex allows a pore to be "on," but that the more complexes that are present, the faster the rate of transport for that particular pore. Currently it is not possible to distinguish between the two models, but the complex appears essential for nuclear import.

Interestingly, Akey and Goldfarb (1989) have found that when WGA-gold is added to isolated nuclear envelopes it binds to the central granule of the pore, a region which they have termed the "transporter." The central granule or transporter has been estimated to have a mass of 12,000,000 D (Reichelt et al., 1990) and has been further hypothesized to consist of 16 arms, arranged in two irises of eight arms each (Akey, 1990). If this model is correct, each arm would be calculated to be 750 kD in mass ( $1.2 \times 10^7$  kD/16), a value not inconsistent with the size of the 600-kD complex we observe. One might hypothesize then that the p62-58-54 complex we observe represents all or part of such an arm. Immunoelectron microscopy with p62-specific antisera such as described here will be required to test this possibility.

In summary, we have used specific polyclonal antisera, in

conjunction with a depletion-reconstitution system, to demonstrate the necessity of the p62-p58-p54 pore proteins to nuclear import. We have shown that the proteins exist in a protein complex in rat nuclear pores. Identification of such a complex is of interest for three reasons. First, it defines proteins that must closely interact with one another within the pore; with such knowledge the hierarchy of interactions which create the structure of the pore can be derived. Second, such complexes, when found in mitotic systems such as the *Xenopus* egg, can be used to define the way in which the pore is disassembled and reassembled each cell cycle. Third, it may now be possible to ask more specifically what role the complex plays during specific stages of nuclear transport. For example, are these proteins necessary for the initial binding step of a nuclear protein to the pore or only for translocation? With the recent discovery of potential signal sequence receptor proteins, it will also be of interest to explore the relationship of the complex to these receptors.

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