

# Macromolecular Interactions in the Nucleoporin p62 Complex of Rat Nuclear Pores: Binding of Nucleoporin p54 to the Rod Domain of p62

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**Abstract.** Nuclear pore complexes are constructed from a large number of different proteins, called collectively nucleoporins. One of these nucleoporins, p62, has an  $\alpha$ -helical coiled-coil COOH-terminal rod domain linked to an NH<sub>2</sub>-terminal domain that contains a series of degenerate pentapeptide repeats. In nuclear pores p62 forms a tight complex with at least two other proteins, p58 and p54, which can be extracted from isolated rat liver nuclei (Finlay, D. R., E. Meier, P. Bradley, J. Horecka, and D. J. Forbes. 1991. *J. Cell Biol.* 114:169–183). We have used a range of methods to demonstrate a strong binding between p62 and p54 in this complex and show that the rod domain of p62 appears to constitute the principal binding site for p54. Whole p62 and its rod domain expressed in *Escherichia coli* both bind strongly to p54 in

blot–overlay assays. Most of the epitopes on the p62 rod recognized by polyclonal antisera are masked in the complex, whereas epitopes on the NH<sub>2</sub>-terminal domain of p62 are still exposed, both in the isolated complex and also in nuclear pores stained in situ by immunofluorescence in isolated rat nuclei. Moreover, it has been possible to exchange recombinant p62 rod for some of the native p62 in complexes partially dissociated by 4 M urea. Overall these results suggest a key role for the p62 rod domain in maintaining the structural integrity of the complex and also suggest a molecular model for the complex. This model is consistent with data that indicate that the analogous coiled-coil region of yeast nucleoporin NSP1 may function in a similar way.

**T**HE transport of large macromolecules between cytoplasm and nucleus is facilitated by nuclear pore complexes (reviewed by Forbes, 1992; Gerace, 1992; Jarnik and Aebi, 1991; Panté and Aebi, 1993, 1994; Stewart, 1992). These are huge macromolecular assemblies that perforate the nuclear envelope and which have prominent eightfold rotational symmetry. The molecular mass of these complex macromolecular machines is of the order of 125,000,000 D (Reichelt et al., 1990) and they may contain as many as 100 different proteins. Nuclear localization sequences (NLSs)<sup>1</sup> target proteins to the nucleus (reviewed by Dingwall and Laskey, 1991) and nucleocytoplasmic transport appears to involve the interaction of NLSs with cytoplasmic receptors (Adam et al., 1989, 1990; Gerace, 1992). Transport takes place along the pore axis (Feldherr et al., 1984) and involves at least two steps (Newmeyer and Forbes,

1988; Richardson et al., 1988): an initial docking to the nuclear pore, which may involve interaction with pore glycoproteins (Gerace, 1992) and does not require metabolic energy, followed by translocation through the pore which requires ATP and is cold sensitive. However, the precise molecular mechanism of import has not yet been established. Export from the nucleus has not been characterized as to the same extent as import, but appears to involve an analogous pathway (reviewed by Izaurralde and Mattaj, 1992; Zapp, 1992).

An understanding of nucleocytoplasmic transport at the molecular level requires a knowledge of the protein and other components of nuclear pores; their structure, arrangement and interactions; and how these change to produce translocation. Recently considerable progress has been made in characterizing many of the protein components of both yeast and vertebrate nuclear pores (Grandi et al., 1993; Hurt, 1988; Kraemer et al., 1994; Loeb et al., 1993; Panté et al., 1994; Radu et al., 1993; Rout and Blobel, 1993; Starr et al., 1990; Sukegawa and Blobel, 1993; Wenté and Blobel, 1994; Wenté et al., 1992; Wimmer et al., 1992; Wozniak et al., 1989, 1994). A series of proteins, collectively called nucleoporins (Davis and Blobel, 1986), has been identified and is being extended rapidly. Many nucleoporins have dis-

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1. *Abbreviations used in this paper:* MBB, maleimido-butylryl-biotin; NLS, nuclear localization sequences; WGA, wheat germ agglutinin.

tinctive properties. Early work showed that several constituents of vertebrate nuclear pores were glycoproteins that had unusual O-linked *N*-acetyl-glucosamine residues that could bind to the lectin wheat germ agglutinin (WGA) and which were highly antigenic (Davis and Blobel, 1986, 1987; Featherstone et al., 1988; Snow et al., 1987). Moreover, many of the nucleoporins sequenced to date contain characteristic repeating amino acid sequence motifs, based on either a GLFG repeat or a degenerate pentapeptide repeat, xFxFx, in which the second and fourth residues are phenylalanine and the remainder are usually small, often hydrophilic residues, such as glycine, serine and threonine (reviewed by Dingwall, 1993; Forbes, 1992; Panté and Aebi, 1994; Stewart, 1992).

Nucleoporin p62 (Davis and Blobel, 1986, 1987) is a glycoprotein which appears to be involved in nucleocytoplasmic transport (Finlay et al., 1991). Because it contains a large number of O-linked *N*-acetyl glucosamine residues (Hanover et al., 1987; Starr et al., 1990; Starr and Hanover, 1990), p62 is readily identified on the basis of its ability to interact with WGA. Antibodies to p62 and WGA both inhibit transport (Dabauvalle et al., 1988; Finlay et al., 1987; Featherstone et al., 1988). Moreover, in systems based on reassembled *Xenopus* nuclei, transport is lost when the assembly mixture is depleted of glycoproteins including p62, but is restored when it is added back (Finlay et al., 1991). The p62 molecule (Fig. 1) appears to be constructed from an  $\alpha$ -helical coiled-coil rod domain, joined by a threonine-rich linker to an NH<sub>2</sub>-terminal domain containing xFxFx repeats (Buss et al., 1994; Carmo-Fonseca et al., 1991; Cordes et al., 1991; Starr et al., 1990). In nuclear pores, p62 appears to exist as a tight complex with at least two other proteins (Finlay et al., 1991; Kita et al., 1993) of molecular weights 58 (p58) and 54 kD (p54), and this complex can be extracted by detergents and high salt buffers. Although it clearly contains multiple copies of its constituent proteins, the precise size of the rat p62 complex is somewhat controversial. Gel filtration (Finlay et al., 1991) indicated a relative molecular weight of 500 kD, whereas sedimentation indicated a relative molecular weight of about half this value for both the rat (Kita et al., 1993) and *Xenopus* egg complex (Dabauvalle et al., 1990).

In this paper we have examined the molecular interactions within the p62 complex from rat liver nuclei and demonstrate a strong interaction between p54 and the  $\alpha$ -helical coiled-coil rod domain of p62. We propose a working model for the structure of the complex and discuss the possible similarities between the p62 complex and that formed in yeast with NSP1, which involves analogous binding of other nucleoporins to a coiled-coil rod domain. We have also developed methods that facilitate the exchange of recombinant p62 mutants for native p62 in the complex.

## Material and Methods

### Rat Liver Nuclei

Rat liver nuclei were prepared as described by (Blobel and Potter, 1966) with minor modifications. Instead of their TKM buffer, we used 80 mM KCl, 5 mM EDTA, 15 mM Pipes, pH 7.4, 15 mM NaCl (buffer A) containing 0.5 mM spermidine, 0.2 mM spermine, 1 mM DTT, 1  $\mu$ g/ml aprotinin, 1 mM PMSF, and 1  $\mu$ g/ml leupeptin (Finlay et al., 1991). Best results were obtained with livers from 3–4-wk-old weaners which were the generous gift

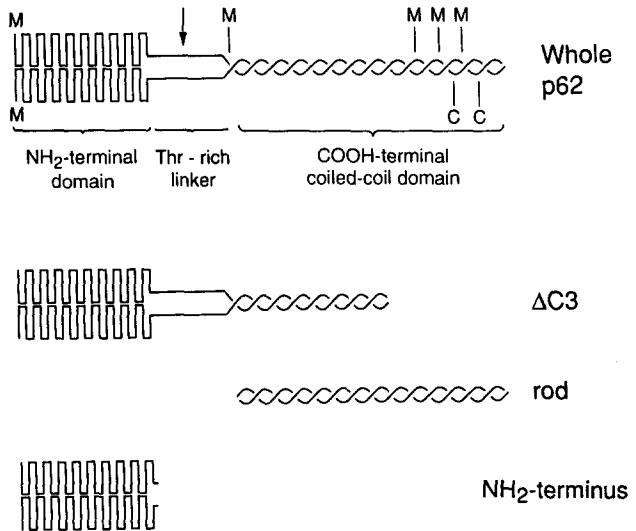
of several local laboratories. Livers were homogenized in a Polytron omnimixer (Camlab, Cambridge, UK) and nuclei isolated by sucrose density centrifugation (Blobel and Potter, 1966).

### Native p62 Complex

Native p62 complex was extracted from rat liver nuclei and purified essentially as described by Finlay et al. (1991). Nuclei ( $6 \times 10^9$ ) were pelleted in an Eppendorf centrifuge and extracted with 4 ml of reconstitution buffer (buffer R: 20 mM Hepes, pH 7.0, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 0.5 mM ATP, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin) containing 2% of the detergent Mega-10 (Sigma, Poole, UK) for 30 min at 4°C. The extract was centrifuged for 10 min in an Eppendorf centrifuge and the supernatant applied to a 1 ml WGA-agarose (Vector Laboratories, Burlingame, CA) column. The column was washed with  $\sim$ 40 ml buffer R containing 0.05% Triton X-100 and 1 M urea, and then the proteins bound to WGA were eluted with approximately 5 ml of 0.5 M *N*-acetyl-glucosamine in buffer R. This eluant was applied to a  $80 \times 1.5$  cm Sephacryl S-300 gel filtration column equilibrated with PBS containing 0.05% Triton X-100, 0.02% sodium azide, and 1 mM PMSF. The fractions containing the complex were identified by Western blotting with an anti-p62 polyclonal antibody and pooled. Native p62 complex was also prepared using high-salt extraction (Kita et al., 1993) and appeared indistinguishable to that prepared using detergent extraction.

### DNA Cloning and Plasmid Construction

Rat p62 cDNA (Starr et al., 1990) was cloned into expression vector pET3a (Studier et al., 1990) as described (Buss et al., 1994). We also constructed a series of deletion mutants corresponding to different domains of the p62 molecule (Fig. 1). Construct  $\Delta$ C3 was produced using the unique Sph I site, so that the expressed protein was truncated at His-428. This construct was cloned into the T-7 based vector pMW172 (modified from the original pET vectors of Studier et al., 1990 by Way et al., 1990) for expression. The rod domain construct was generated by using PCR (Bunnell and Kidd, 1989) to delete the first 990 bases so that translation was initiated at Met-331. This



**Figure 1.** Schematic 3 domain model for the structure of the whole p62 molecule and deletion constructs of p62. The schematic model is based on computer analysis of its sequence (Carmo-Fonseca et al., 1991; Cordes et al., 1991; Starr et al., 1990) and electron microscopy of expressed material (Buss et al., 1994). The NH<sub>2</sub>-terminal domain contains a degenerate xFxFx repeat motif that probably has a cross  $\beta$ -conformation. The COOH-terminal "rod" domain forms an  $\alpha$ -helical coiled-coil (Buss et al., 1994) and is joined to the NH<sub>2</sub>-terminal domain by a threonine-rich linker. The threonine-rich linker contains a thrombin site (arrow) that was used to produce the COOH-terminal fragment (Buss et al., 1994) and to immunize rabbits. The cysteine (C) and methionine (M) residues were used to generate labeled p62 molecules and fragments.

construct was expressed as a fusion protein containing a poly-histidine tag using the T-7-based vector pET15b (based on Studier et al., 1990, supplied by Novagen, Madison WI). The NH<sub>2</sub> terminus construct was also generated using PCR to introduce a STOP codon after Pro-194 and was expressed using pET3a. DNA sequencing was used to confirm the correctness of each construct.

### Expression and Purification of Recombinant Proteins

The expression and purification of p62 and the different deletion mutants was performed essentially as described (Buss et al., 1994). All constructs were expressed to high levels (of the order of 50–100 mg/l of culture) as inclusion bodies. After washing, the inclusion bodies were dissolved in 8 M urea, 25 mM Tris-HCl pH 8.0, 1 mM EGTA, 1 mM DTT (Nagai and Thøgersen, 1987), clarified by centrifugation at 30,000 g, and purified by ion exchange chromatography on DE-52 cellulose (Buss et al., 1994). Proteins were then renatured by removing the urea stepwise by dilution and dialysis as described (Buss et al., 1994). With the p62 rod fusion protein, the poly-histidine NH<sub>2</sub>-terminal peptide was removed by proteolysis with thrombin (Sigma) at a molar ratio of 1:1,000 at 37°C for 1 h. Fig. 2 shows SDS-PAGE of the purified proteins. All were essentially homogeneous and the relative molecular weight of all except the rod construct was close to that expected. Thus, the observed relative molecular weight for p62 was 55 kD (expected 58 kD); 45 kD for ΔC3 (expected 48 kD); 19 kD for the NH<sub>2</sub>-terminal domain (expected 21 kD) and 25 kD for the rod (expected 21 kD). The slightly higher relative molecular weight observed for the rod domain was not unexpected, as α-helical coiled-coil proteins often show aberrant mobility on SDS-PAGE (Sutoh et al., 1978; Stewart and Edwards, 1984). Circular dichroism (performed as described in Buss et al., 1994) indicated that the rod domain had the expected high α-helical content and shadowed rod preparations showed the expected rod-shaped molecules 30-nm long (data not shown). All constructs gave positive Western blots with a polyclonal anti-p62 antiserum (data not shown). The NH<sub>2</sub>-terminal construct had very low solubility at physiological pH (which was not unexpected because of its extremely low fraction of charged residues), but could be solubilized in 50 mM phosphate buffer, pH 6. When solubilized in this way, this construct was suitable for immunization but not for overlays and exchange studies.

### Labeling of Recombinant Proteins

Whole p62 and the p62 rod were labeled with biotin at the two cysteine residues near its COOH-terminus (see Fig. 1) using maleimido-butyryl-biotin (MBB; Calbiochem-Behring Corp., San Diego, CA). Rod (0.5 mg/ml) in 25 mM Tris-HCl, pH 8.0, 1 mM EGTA, 0.2 mM DTT was incubated 1 h at 4°C with 1 mM MBB. The reaction was stopped by adding 10 mM DTT and unreacted biotin removed by washing three to four times in a Centricon-30 microconcentrator (Amicon Corp., Beverly, MA).

Radioactively labeled rod was obtained by expression of the corresponding cDNA in *Escherichia coli* in the presence of [<sup>35</sup>S]methionine. An overnight culture of the p62 rod construct freshly transformed into BL21/DE3 cells (Studier et al., 1990) was diluted into 5 ml of M-9 medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.5% NaCl, 0.1% NH<sub>4</sub>Cl, 2 mM MgSO<sub>4</sub>, 0.2%

glucose, 0.1 mM CaCl<sub>2</sub>, pH 7.4) to an A<sub>600</sub> of 0.2. The cells were grown to an A<sub>600</sub> of 0.6 and induced by addition of 0.6 mM iso-propyl-thio-galactoside. After 15 min, 100 μCi of [<sup>35</sup>S]methionine was added and the cells grown for a further 30 min. The cells were then harvested and the pellet washed once with PBS before lysing the cells by freeze-thawing. The labeled p62 rod was present in inclusion bodies which were isolated by centrifugation and washed once with 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 M NaCl, 1% Na deoxycholate, 1% NP-40, and then twice with 20 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 2 mM DTT (Nagai and Thøgersen, 1987). The washed inclusion bodies were solubilized in 8 M urea, 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EGTA, and then the rod was renatured by removing the urea by diluting out with 25 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM DTT. This material was ~70–80% pure and was used without further purification.

### Electrophoresis, Immunoblotting, and Overlay

Electrophoresis of proteins was carried out using 7.5–17.5% acrylamide gradient SDS-gels as described by Matsudaira and Burgess (1978). The separated proteins were transferred electrophoretically to nitrocellulose membranes with the help of a semi-dry blotter (Kyhse-Anderson, 1984). The blotted proteins were visualized with 0.2% Ponceau-S in 10% acetic acid. After blocking with 5% non-fat dry milk, the blots were incubated with polyclonal rabbit antisera (diluted 1:1,000) followed by goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma). Immunodetection was carried out using BCIP/NBT as substrate as described in Harlow and Lane (1988) with the modification of dissolving the BCIP in 50% aqueous DMF instead of 100%.

For most overlay experiments, we used p62/p58/p54 complex that had been partially purified using WGA-affinity column chromatography, although some experiments were also performed using the purified complex obtained by gel filtration. After SDS-PAGE, the samples were blotted onto nitrocellulose membranes as described above and blocked with 5% non-fat milk. The nitrocellulose membrane containing the blotted proteins was then incubated for 1–2 h with recombinant rod that had been labeled with either biotin (5 μg/ml) or [<sup>35</sup>S]methionine (1 μg/ml) diluted in 5% non-fat milk in PBS (Billingsley et al., 1990). After three 10 min washes with PBS containing 0.1% Tween 20, biotin-labeled proteins were visualized with anti-biotin coupled to alkaline phosphatase (1:2,000) (Sigma). With p62 rod labeled with [<sup>35</sup>S]methionine, blots were washed the same way, and then exposed to X-ray film overnight or visualized using a Molecular Dynamics (Sunnyvale, CA) Phosphorimager.

### Antibodies

Polyclonal antibodies against p62, the rod and the NH<sub>2</sub> terminus were raised in rabbits. Antibodies against the rod domain were generated by immunization with the purified thrombin fragment that contains mainly the rod domain of the p62 molecule (Buss et al., 1994). Whole p62 and the NH<sub>2</sub> terminus were expressed in *E. coli* and purified as described. Because of its low solubility at pH 7, the NH<sub>2</sub>-terminal construct was dissolved in 50 mM phosphate buffer, pH 6. An initial injection (250 μg, intradermally) was given as an emulsion in complete Freund's Adjuvant, with subsequent boosts twice every 4 wk in incomplete adjuvant. Serum was obtained from venous blood and tested for specific activity against p62 or specific regions of the molecule on immunoblots as shown in Fig. 3. All antisera reacted with whole p62, but fragments only reacted with the domain against which they had been raised and not with the others. In addition to reacting on Western blots, these antisera also recognized non-denatured p62 and the appropriate fragments in dot blots (see Fig. 9 A) and ELISA assays (not shown). In contrast to many antibodies raised against native glycosylated proteins (e.g., Snow et al., 1987), all three antisera which we raised against material expressed in *E. coli* recognized only the p62 band in Western blots of whole nuclei (Fig. 4) and did not stain any other bands. Anti-whole p62 (not shown) and anti-NH<sub>2</sub> terminus (see Fig. 10) gave rim staining of nuclei by indirect immunofluorescence, but anti-rod gave only weak staining of nuclei (see Fig. 10). Affinity-purified NH<sub>2</sub> terminus antibodies were produced using ΔC3 (which had much more favourable solubility properties than the NH<sub>2</sub>-terminal construct) coupled to CNBr-Sepharose (Pharmacia, Uppsala, Sweden), whereas for affinity-purified rod antibodies, we used a recombinant p62 rod column. Affinity-purified antibodies were eluted using 100 mM glycine, pH 2.5, and used at dilutions of 1–10 μg/ml for indirect immunofluorescence.

Polyclonal antisera raised against rat p58 and p54 which have been characterized extensively (Finlay et al., 1991) were the generous gift of Dr. Douglas Forbes (University of California at San Diego, San Diego, CA).

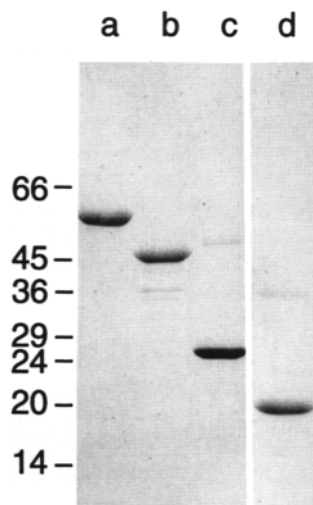
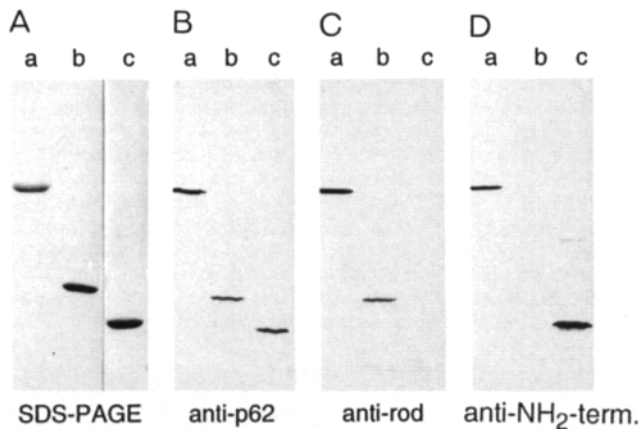


Figure 2. SDS-PAGE showing recombinant p62 and the different recombinant p62 fragments after purification. (a) whole p62; (b) ΔC3; (c) rod; (d) NH<sub>2</sub>-terminal domain.



**Figure 3.** Western blots characterizing the specificity of the polyclonal antibodies raised against different regions of p62. Lane *a* is whole p62; lane *b* is rod; and lane *c* is the NH<sub>2</sub>-terminal domain. (A) SDS-PAGE; (B) blot using anti-p62; (C) is using anti-rod and (D) is anti-NH<sub>2</sub>-terminal domain.

### Relative Antibody Avidity

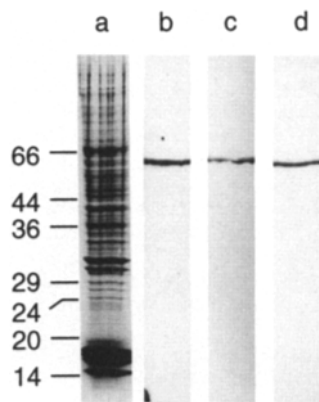
We compared the avidity of different p62 antisera for recombinant p62 or p62 in the complex by using dot blots on nitrocellulose. Serial dilutions of recombinant p62 or the purified p62 complex were dotted in 1  $\mu$ l samples onto nitrocellulose. They were then blocked in 5% non-fat milk and incubated for 1 h in 1:1,000 antiserum. After washing, the blots were probed with <sup>125</sup>I-protein A. After washing and drying, the blots were exposed to film to identify the position of each sample. Individual dots were then cut from the filter and counted for 1 min in a Wallac 1261 Multigamma gamma counter (Pharmacia, Turku, Finland).

### Immunofluorescence

For indirect immunofluorescence, isolated rat nuclei ( $2 \times 10^7$ ) were fixed in 4% paraformaldehyde in PBS for 5 min then centrifuged through a 30% sucrose cushion onto poly-L-lysine coated coverslips. The nuclei were then refixed on the coverslips with 4% paraformaldehyde and incubated with the required affinity-purified primary antibodies (0.1 mg/ml) at dilutions between 1:10 and 1:100 for 30 min at 37°C followed by fluorescein-labeled goat anti-rabbit IgG (1:50; Vector Laboratories) for 30 min. After each step, the nuclei were washed several times with PBS. Specimens were mounted on slides containing mowiol (Hoechst-AG, Frankfurt, Germany) and analysed and photographed in a Bio-Rad MRC-600 confocal microscope.

### Protein Exchange in the p62 Complex

The native p62 complex obtained from the WGA column was mixed with a twofold excess of recombinant p62 rod and dialyzed against 4 M urea in



**Figure 4.** Specificity of antisera raised against expressed p62. (Lane *a*) SDS-PAGE of whole nuclei (lanes *b-d*) Western blots of whole nuclei using anti-whole p62 (lane *b*), anti-rod (lane *c*), and anti-NH<sub>2</sub> terminus (lane *d*). Each antiserum reacted with only one band which corresponded to p62.

PBS containing 1 mM DTT and 1 mM EGTA. The urea was removed by dialysis against PBS containing 1 mM DTT and 1 mM EGTA. The mixture was then applied to a  $80 \times 1.5$  cm Sephacryl S-300 column and separated as described above for the purification of the native complex.

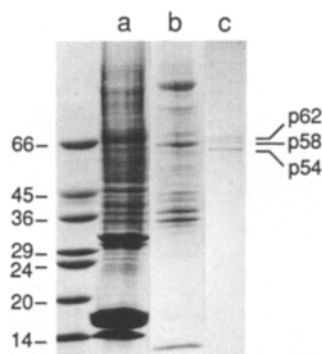
### Protein Chemical Techniques

Protein concentration was determined by A<sub>280</sub> using the following absorption coefficients for a concentration of 1 mg/ml calculated from the amino acid composition: p62, 0.39; rod, 1.06;  $\Delta$ C3, 0.32.

## Results

### Purification and Characterization of p62 Complex from Rat Liver Nuclei

In rat liver nuclei p62 forms a tight complex with two other nuclear pore proteins p58 and p54 (Finlay et al., 1991; Kita et al., 1993). We isolated this complex from rat liver nuclei using both detergent (Finlay et al., 1991) and high salt (Kita et al., 1993) methods, followed by affinity chromatography using WGA-agarose and gel filtration on Sephacryl S-300. The components of the p62 complex were present in such small quantities that they could not be detected in Coomassie-stained SDS-PAGE of whole nuclei (Fig. 5 *a*) and were barely visible after WGA affinity chromatography (Fig. 5 *b*). The material obtained by WGA-affinity chromatography still contained considerable quantities of contaminants (Fig. 5 *b*). However, because of the large size of the complex, gel filtration gave quite pure material that showed three major bands on SDS-PAGE (Fig. 5 *c*). In our SDS-PAGE system, p58 migrated very close to p62, and it was sometimes difficult to separate these two bands completely on Coomassie-stained gels. Kita et al. (1993) observed a similar migration pattern, with the p62 and p58 bands closely spaced, so that the apparent molecular weight of p58 was closer to 60 kD. In both our material and that prepared by Kita et al. (1993), the p54 band was well separated from the p62/p58 doublet. We confirmed that we had prepared the same material as Finlay et al. (1991) by probing the purified complex with WGA, a polyclonal antibody raised against recombinant p62, and the two polyclonal antisera against p58 and p54 used by Finlay et al. (1991), which were the gener-



**Figure 5.** Preparation and purification of native p62 complex from rat liver nuclei. (Lane *a*) shows SDS-PAGE of whole rat liver nuclei ( $2 \times 10^6$  nuclei). (Lane *b*) pooled fractions eluted from the WGA-affinity column. The concentration of the components of the p62 complex was still so low that they could barely be detected by Coomassie staining. The strong band at  $\sim 66$  kD was a contaminant and not p62. The intensity of this band

varied between preparations as can be seen by comparing this lane with Fig. 7, lane *a*; (lane *c*) shows purified complex after gel filtration over Sephacryl S-300 which shows three clear bands corresponding to p62, p58, and p54. The p54 band is clearly stained more intensely than the other two.

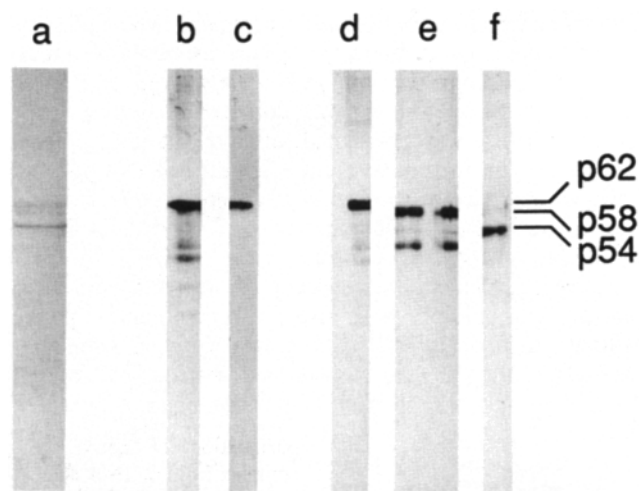
ous gift of Dr. Douglas Forbes (University of California at San Diego). All three antibodies gave distinctive bands on immunoblots (Fig. 6) and so confirmed that the material we had prepared contained the same three proteins observed by Finlay et al. (1991). There was also a weak and variable band at about 50 kD, which cross-reacted with the p58 antibody. This band was also observed by Finlay et al. (1991) and was characterized in more detail by Kita et al. (1993) who used two-dimensional electrophoresis and peptide mapping to show that this 50-kD band was probably a breakdown product of p58.

In our Coomassie-stained SDS-PAGE of the complex, the p54 band was always markedly more intense than either the p62 or p58 bands (Fig. 5, lane c, Fig. 6 a, and Fig. 7 j). Densitometry of these gels indicated that the p54 band was usually very close to twice as intense as either p58 or p62, which were present in roughly equal amounts. Although Coomassie staining does not necessarily reflect protein concentration precisely, Kita et al. (1993) observed a similar molar ratio of p62/p58/p54 near to 1:1:2 using silver-stained SDS-PAGE. Finlay et al. (1991) estimated the molar ratio as 4:1:4. It may be that the lower quantity of p58 seen by Finlay et al. (1991) resulted from partial proteolysis of p58 (see Kita et al., 1993). The p62 complex we prepared had a relative molecular weight of 400–500 kD estimated by gel filtration, which was in good agreement with that determined by Finlay et al. (1991). As p62 forms a coiled-coil dimer (Buss et al.,

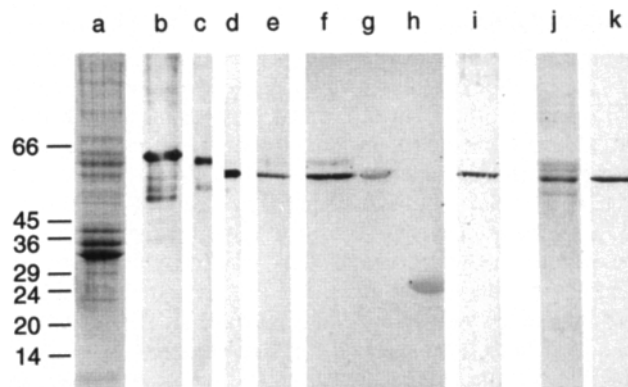
1994), it seems likely that the stoichiometry of p62/p58/p54 in the complex is 2:2:4.

### p62 Interacts with p54

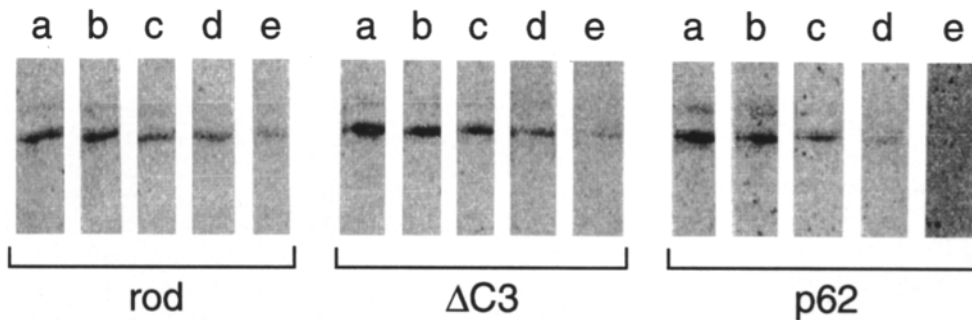
To examine molecular interactions in the p62 complex, we developed a blot-overlay assay. Partially purified p62 complex from rat livers was blotted onto nitrocellulose and probed with recombinant p62 or rod labeled with either biotin or [<sup>35</sup>S]methionine. As shown in Fig. 7, the position of p62 (lane b), p58 (lane c), and p54 (lane d) on the nitrocellulose was determined using Western blots. In the blot overlay whole p62 and its rod domain gave the same result except that the binding of p62 was generally weaker, which might be due to the molecule's unfavourable solubility under the conditions used (see Buss et al., 1994). Therefore we concentrated on using the p62 rod domain for binding studies. The strongest binding was to p54, with weaker binding to p62. Although there were a large number of other proteins present in the crude complex used (as could be seen by SDS-PAGE, Fig. 7 a), no other binding was observed. The binding of the probe to p62 or rod on the nitrocellulose might be due to dimerization of coiled-coils or aggregation of the rod, which can be induced by salt (Habeler, T., F. Buss, and M. Stewart, unpublished results). We also probed purified p62 complex (Fig. 7, j and k) to confirm that the rod was binding to p54 and not to a contaminant in the crude complex preparation.



**Figure 6.** Immuno-characterization of native p62 complex purified by gel filtration (see Fig. 5, lane c). (a) SDS-PAGE; (lane b) and (lane d) Western blots using anti-p62; (lane c) WGA-biotin; (lane e) anti-p58; (lane f) anti-p54. Because the p62 and p58 bands ran so closely together, we cut the lanes down their middle to ensure they were aligned precisely. Therefore, lane d contains the left-hand half of a lane and lane e contains the right-hand half, together with the left-hand half of the next lane (whose right-hand half is in lane f). In this way we could be certain that anti-p62 and anti-p58 were reacting with different bands. The pattern of reactivity parallels that reported by Finlay et al. (1991). We also found a variable quantity of a lower molecular weight band (~50 kD) that crossreacted with anti-p58 and which was probably a proteolytic fragment. The reaction between p62 and WGA was consistent with this protein's high content of O-linked N-acetyl glucosamine residues (Starr et al., 1990).



**Figure 7.** Blot-overlay using biotin-labeled whole p62 and p62 rod. (Lane a) shows Coomassie-stained SDS-PAGE of proteins eluted from the WGA column. The positions of p62 (lane b), p58 (lane c), and p54 (lane d) were established by Western blotting (see Fig. 6). Lanes e–h and k are overlays in which nitrocellulose blots were probed with biotinylated p62 rod instead of antibodies, lanes e, f, and i are the WGA-column eluate shown in (lane a). Clearly the p62 rod is binding strongly to p54 and, with light loading, appears to be the only interaction present. However, higher loadings (lane f) of the crude complex also show weaker binding of the biotinylated rod to whole native p62. Lane g shows binding of biotinylated rod to recombinant p62, which, because it is not glycosylated, runs at close to 54 kD (see Buss et al., 1994). (Lane h) Binding of biotinylated rod to the 24-kD recombinant rod, illustrating that the binding to p62 probably results from rod-rod interactions. Lane i shows an overlay using whole p62 instead of rod. Again, binding is primarily to the p54 band. We also performed the overlays with pure p62 complex to confirm that the binding was indeed to p54. Lane j is a Coomassie-stained SDS-PAGE of purified complex and lane k is an overlay of this material using biotinylated rod.



**Figure 8.** Competition of the binding to p54 of  $^{35}\text{S}$ -labeled p62 rod by a range of different p62 constructs. A typical autoradiogram of an overlay experiment in which radioactively-labeled rod was displaced by progressively higher concentrations of unlabeled rod,  $\Delta\text{C3}$  or whole p62. The molar ratios of labeled rod to unlabeled protein are 1:1 (lanes a); 1:10 (lanes b); 1:50 (lanes c); 1:100 (lanes d); and 1:200 (lanes e).

We also attempted to compare the relative affinities of different regions of the p62 molecule for p54 in a competition assay in which  $^{35}\text{S}$ -labeled rod was mixed with increasing quantities of unlabeled whole p62, rod or  $\Delta\text{C3}$  and then used to probe p54 on nitrocellulose (Fig. 8). We could obtain a measure of the relative binding strengths by observing how much labeled rod was displaced. We had to use substoichiometric quantities of  $^{35}\text{S}$ -labeled p62 rod (relative to p54) to avoid background problems on the overlay, and so rather high concentrations of the unlabeled proteins were required to show significant competition. Although the results obtained in this way did not give a precise measure of absolute binding affinities, they were quite reliable in indicating relative affinities of the different fragments. Whole p62 was slightly more effective in replacing labeled rod from p54 than was rod itself or  $\Delta\text{C3}$ . Unfortunately the low solubility of the  $\text{NH}_2$ -terminal fragment prevented our testing whether it was able to compete to any extent with the p62 rod domain for p54. The  $\Delta\text{C3}$  construct, from which roughly half of the COOH-terminal rod domain had been deleted (Fig. 1), appeared to be as effective as the rod construct which contained the whole  $\alpha$ -helical domain. This result implied that p54 binding site was not only restricted to the extreme COOH terminus of the rod. Interestingly, Buss et al. (1994), found that the COOH terminus of the p62 rod domain was important in determining the aggregation and solubility behaviour of the molecule. Taken together, our present data indicate that, although minor contributions from other parts of the molecule cannot be excluded, the p62 rod domain contains the major binding site for p54 and that this binding site is not located exclusively at its very COOH terminus, which previous work has shown is important for aggregation.

#### ***p62 Rod Epitopes Are Masked in the Isolated Complex and in Nuclear Pores***

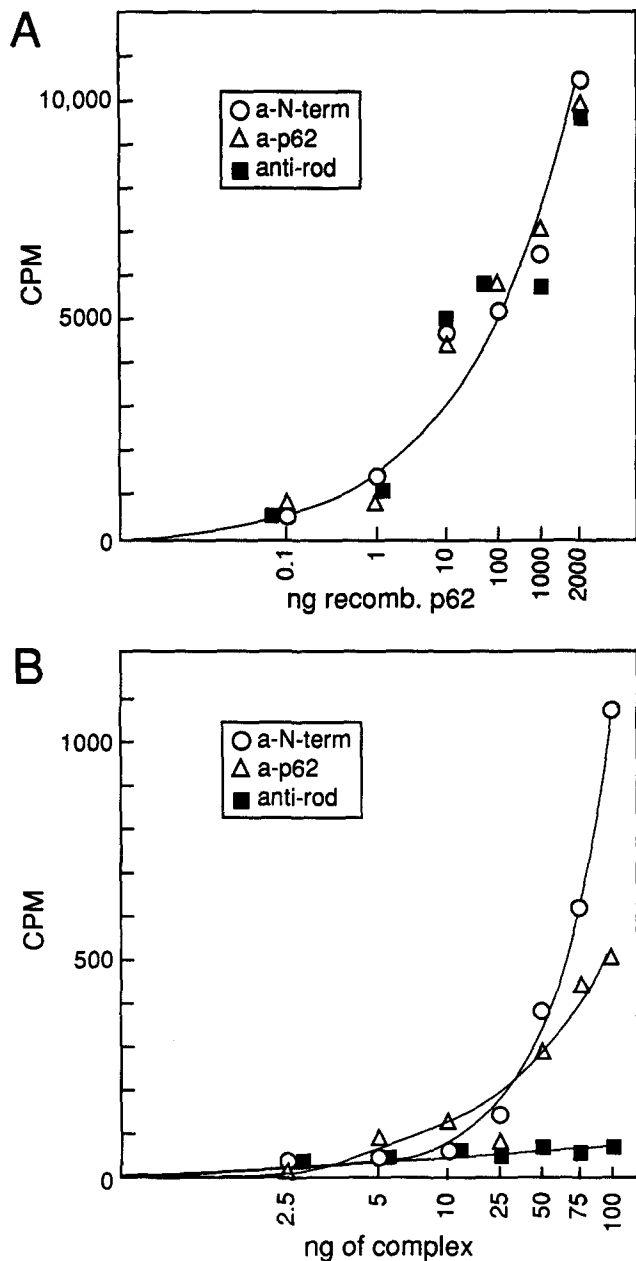
We used polyclonal antibodies raised against the  $\text{NH}_2$  terminus, the rod, or whole p62 to examine the accessibility of these different domains in the isolated native complex or in nuclear pores in intact nuclei. The antibodies we prepared against whole p62, the rod domain and the  $\text{NH}_2$ -terminal domain all had similar affinity for recombinant p62. As these were all polyclonal sera, one cannot obtain a binding constant for each, but one can easily determine their relative avidities by probing serial dilutions of antigen. Fig. 9 A shows the amounts of each antibody that bound to dot blots of serial dilutions of recombinant p62 and over a very wide range the three antibodies were almost indistinguishable.

Thus, each of the antibody preparations had comparable avidity for p62 under these conditions. However, the behavior of dot blots of serial dilutions of the native p62 complex was quite different and the affinity-purified serum raised against the  $\text{NH}_2$ -terminal domain showed a much higher avidity than that raised against the rod domain. The avidity of the  $\text{NH}_2$ -terminal antibody for the complex was roughly comparable to that observed for recombinant p62. Thus, 100 ng of complex (which contains roughly 25 ng of p62) gave 1074 CPM, whereas 100 ng of recombinant protein gave 5,077 cpm. However, the signal obtained from the complex using the affinity-purified anti-rod serum was at least a factor of 10 less than that obtained with the anti- $\text{NH}_2$ -terminal serum and was difficult to distinguish from the background. The signal from the polyclonal serum against whole p62 was intermediate between these two values, as would be anticipated if it recognized epitopes on both rod and  $\text{NH}_2$ -terminal domains. The striking decrease in avidity of the affinity-purified rod polyclonal antibody in the complex compared with the native molecule indicated that p62 rod epitopes had become masked in the complex, which would be consistent with p54 binding to this part of p62 as indicated by the blot overlay experiments.

Indirect immunofluorescence of whole nuclei indicated that p62 rod epitopes were also masked in nuclear pores. Affinity-purified anti- $\text{NH}_2$  terminus polyclonal antibodies gave very strong nuclear rim staining, even at a dilution of 1:100 (1  $\mu\text{g}/\text{ml}$ ), and showed the punctate pattern typical of nuclear pore proteins (Fig. 10). However, affinity-purified anti-rod only gave very weak staining, even at 1:10 dilution (10  $\mu\text{g}/\text{ml}$ ). The relative avidity of both antibodies was comparable on dot blots (Fig. 9 A) and on Western blots of crude complex that had been fixed after transfer to nitrocellulose using the same conditions used for immunofluorescence (data not shown). Western blots of whole nuclei (Fig. 4) indicated that both antisera recognized only p62, and so the higher staining with the anti- $\text{NH}_2$  terminus polyclonal was unlikely to be due to its crossreacting with other nuclear pore proteins containing analogous repeating motifs. Therefore our immunofluorescence data were also consistent with a decreased avidity of the rod antiserum for p62 in the complex and indicated that p62 rod epitopes were also being masked in nuclear pores.

#### ***Exchange of Rod and p62 in Native Complex***

To assess the binding of recombinant rod to p54 in solution we examined the extent to which p62 rod could be exchanged



**Figure 9.** Masking of some p62 epitopes in the native complex. Serial dilutions of recombinant p62 (A) or whole native p62 complex purified by gel filtration (B) were dotted onto nitrocellulose and then probed with anti-p62 (triangles), anti-NH<sub>2</sub> terminus (circles), or anti-rod (squares). The amount of each antibody bound was then determined using <sup>125</sup>I-labeled protein A. A Shows that over a considerable range of dilutions all three antibodies had comparable avidity for the expressed p62. However, as shown in B, the binding of anti-rod was very much reduced in the native complex while that of anti-p62 was intermediate between that of anti-NH<sub>2</sub> terminus and anti-rod. These data indicate that some of the epitopes that were exposed on the rod domain of recombinant p62 were masked in the whole complex.

for the p62 within the native complex. Because the cohesive forces within the complex were too great for exchange to be observed in benign buffers (not shown), we used urea to partially dissociate the complex. As shown in Fig. 11 A, both 4 M (lane b) and 6 M urea (lane c) dissociated the complex,

but only the material dissociated with 4 M urea was able to reconstitute to a significant extent when the urea was removed. Thus, native material and material renatured from 4 M urea could be pelleted at 100,000 g (Fig. 11 A, lane d), and still migrated as an ~400-kD complex on gel filtration (Fig. 11 B). By contrast, most of the material that had been treated with 6 M urea remained in solution after the urea was removed (Fig. 11 A, lane e) and gel filtration showed that the complex was dissociated by 6 M urea. We therefore decided to use 4 M urea in our exchange experiments.

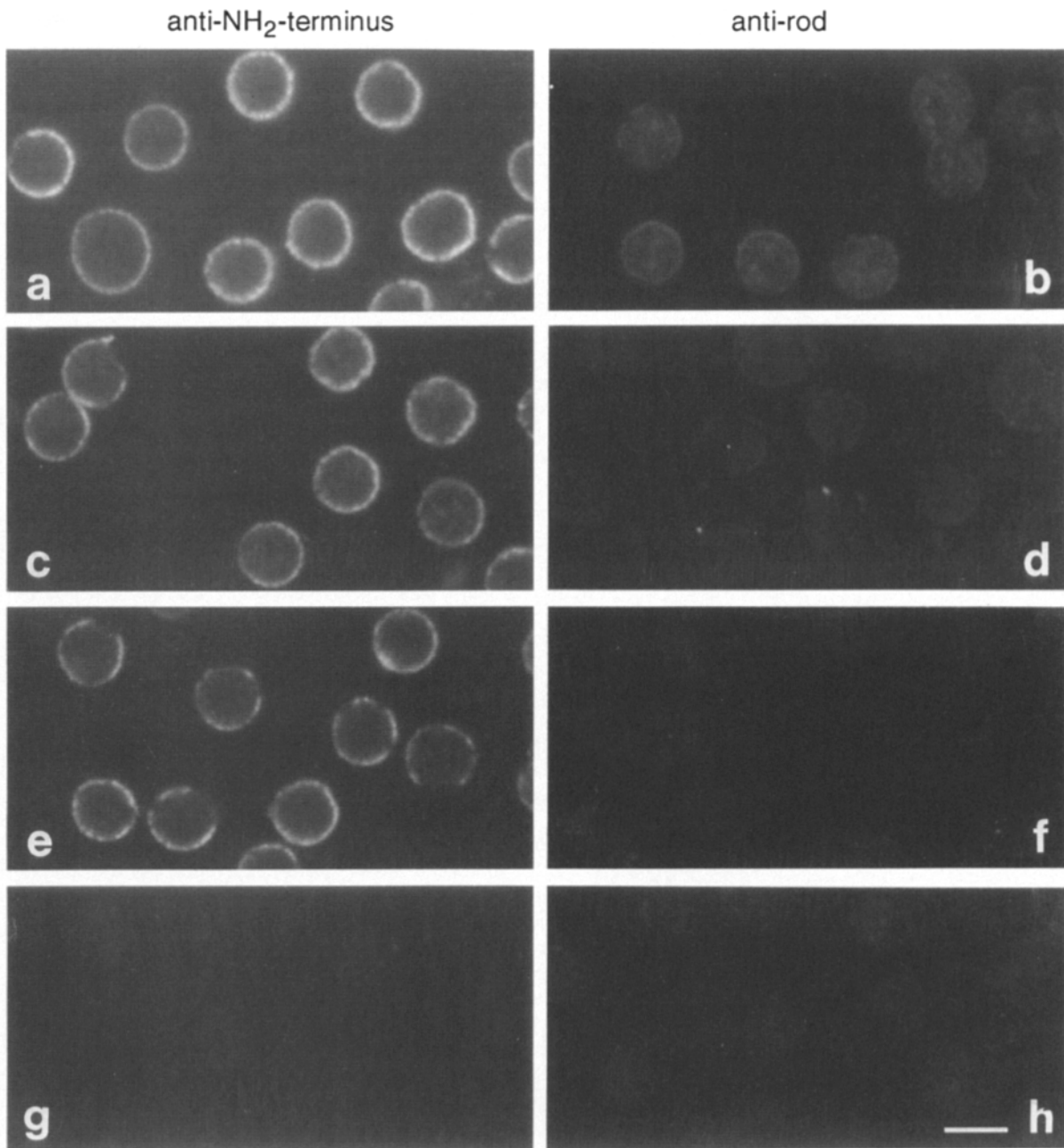
We assessed whether exchange had occurred by using gel filtration to separate the complex from unbound rod and p62 in the material obtained after the removal of urea. We used a twofold excess of rod. Under these conditions, most of the rod eluted at 75–85 ml, near BSA (66 kD), but there was always a portion of the added rod that migrated with the native complex at 55–62 ml near apoferritin (440 kD), which we could identify unequivocally from its high Mr and the presence of whole p62 (Fig. 11 B). Although exchange was never complete, the fact that even a partial exchange could be effected indicated that recombinant rod was able to substitute for whole p62 in the complex. Unfortunately, we could not perform this experiment with whole recombinant p62 because of its unfavourable solubility properties (Buss et al., 1994) and because it aggregated strongly under the conditions used for gel filtration. When the complex and rod were mixed in the absence of urea they gave separate peaks on gel filtration with the rod migrating at 75–85 ml and the complex at 55–62 ml.

## Discussion

An understanding of the function of nuclear pore complexes at the molecular level requires an inventory of the constituents of the pores, together with a knowledge of their structure and interactions with other pore components and how these change to facilitate nucleocytoplasmic transport. To complement extensive studies that are identifying and characterizing both yeast and vertebrate nucleoporins (Grandi et al., 1993; Hurt, 1988; Kraemer et al., 1994; Loeb et al., 1993; Panté et al., 1994; Radu et al., 1993; Rout and Blobel, 1993; Starr et al., 1990; Sukegawa and Blobel, 1993; Wentz and Blobel, 1994; Wentz et al., 1992; Wimmer et al., 1992; Wozniak et al., 1989, 1994), we have started to characterize the molecular interactions within the macromolecular complex formed by nucleoporin p62 in rat liver nuclei, both to understand the molecular architecture of this complex and also to facilitate the introduction of p62 molecules modified by directed mutagenesis so that we can define structure–function relationships. We have used a range of complementary methods to demonstrate a strong interaction between p54 and the  $\alpha$ -helical coiled–coil rod domain of p62 both in vitro and in intact nuclear pores. These data give clues to the molecular architecture of the p62 complex and suggest some analogies with yeast nucleoporin NSPI. Furthermore, characterization of the conditions required for exchange of recombinant p62 mutants for the native material in the rat liver p62 complex should facilitate structure–function investigations of the different domains of the p62 molecule.

### p54 Binds to the Rod Domain of p62

Our data from blot overlays (Figs. 7 and 8) and epitope masking (Figs. 9 and 10) both indicated that there was a

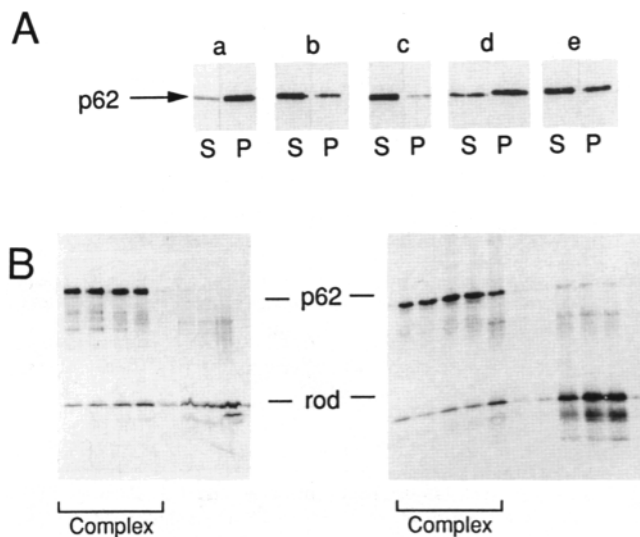


**Figure 10.** Indirect immunofluorescence showing the affinity for nuclear pores in isolated nuclei of antibodies to different portions of the p62 molecule. Affinity-purified antibodies (0.1 mg/ml) were used at 1:10; 1:50, and 1:100. Even at the highest dilution anti-NH<sub>2</sub> terminus antibodies (*a*, *c*, and *e*) showed the punctate nuclear rim staining typical of nuclear pore proteins whereas anti-rod (*b*, *d*, and *f*) showed only very weak staining even at 1:10 dilution. As both antisera had comparable avidity for recombinant p62 (Fig. 9 *A*) and bound only to p62 in Western blots of whole nuclei (Fig. 4), these results indicate that some p62 rod epitopes were masked when the molecule is present in intact nuclear pores and confirms the result obtained with isolated p62 complex (Fig. 9). *g* and *h* show staining of isolated nuclei with a 1:10 dilution of preimmune serum for anti-NH<sub>2</sub> terminus and anti-rod, respectively. Bar, 10  $\mu$ m.

strong interaction between p62 and p54, and that this interaction involved the binding of p54 to the  $\alpha$ -helical coiled-coil rod domain of p62. Both the strength and specificity of the p62-p54 interaction on blot overlays appeared to be roughly equivalent to that seen with antibodies in our Western blots and even in comparatively crude preparations (Fig. 7, lane *a*) the applied p62 or p62 rod bound only to p54 and,

less weakly, to any p62 or rod present on the nitrocellulose. We have previously demonstrated that p62 molecules have a marked tendency to self-associate and that this interaction involves regions at the ends of the rod domain (Buss et al., 1994). Consequently, some interaction between the applied p62 and any p62 construct on the nitrocellulose that contained the rod domain was not unexpected. However, the in-

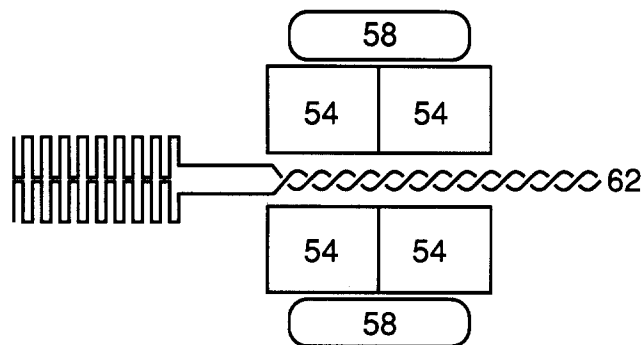




**Figure 11.** Exchange of p62 in the native p62 complex. *A* shows the effect of urea on the dissociation and reassociation of the native p62 complex. In each case, the material was centrifuged for 30 min at 100,000 *g* and the concentration of p62 in pellet (*P*) and supernatant (*S*) assessed by Western blotting with anti-p62. With native complex (lane *a*) almost all of the p62 was pelleted under these conditions; in 4 M urea (lane *b*) the majority was in the supernatant and, in 6 M urea (lane *c*), almost all of the p62 was in the supernatant. When the complex was reformed by removing the urea by dialysis, the material treated with 4 M urea (lane *d*) behaved like the native material, with most of the p62 sedimenting, whereas with the material treated with 6 M urea (lane *e*), most of the p62 still remained in the supernatant. We therefore used 4 M urea to partially dissociate the complex and monitor the extent native p62 could be exchanged by p62 rod. We assessed the degree of exchange by incubating the complex and p62 rod in 4 M urea, renaturing and then applying to a Sephacryl S-300 gel filtration column to see if any of the rod would co-migrate with the reconstituted complex. *B* shows fractions of the gel filtration columns from two different runs probed on Western blots using anti-p62. The intact complex eluted at 55–62 ml from this column, close to apoferritin (*M<sub>r</sub>* 440,000) which eluted at 56 ml. Rod, on the other hand, eluted at 75–80 ml, near to BSA (*M<sub>r</sub>* 66,000) which eluted at 72 ml. In each run in which rod and complex were mixed in 4 M urea and renatured, some of the p62 rod ran with the complex (indicated by the presence of whole p62) and eluted at 55–65 ml, indicating that partial exchange of rod for whole p62 complex had taken place.

teraction between p54 and the p62 rod domain was clearly very much stronger and, at low loadings, appeared to be the only interaction present. Even at high loadings, the only other interaction observed was the p62 self-association and, even in quite crude preparations, no binding of p62 to any other component was observed. Further evidence for the specificity of the p54–p62 rod interaction was obtained from the displacement experiments.

The masking of p62 rod epitopes observed in both the extracted complex (Fig. 9) and in intact nuclei (Fig. 10) was also consistent with the binding of p54 primarily to the p62 rod domain. On both dot blots and by indirect immunofluorescence, the binding of rod antibodies to p62 in the complex was strongly inhibited, although these same antibodies bound to recombinant p62 molecules with affinities comparable to those observed with antibodies to whole p62



**Figure 12.** Highly schematic illustration of a possible arrangement of the different protein components in the native p62 complex consistent with the molar ratios of 1:1:2 for p62, p58, and p54 inferred from SDS-PAGE and our evidence for the binding of p54 to the p62 rod. The position of p58 in the complex is not known, although the accessibility of the p62 NH<sub>2</sub>-terminal epitopes would indicate that p58 was probably attached primarily to either the p62 rod or to p54.

or the NH<sub>2</sub>-terminal domain (Fig. 9 *A*). By contrast, antibodies to the NH<sub>2</sub>-terminal domain bound with roughly equal affinity to both recombinant p62 and p62 in the complex, indicating that their epitopes were not masked to a significant extent in the complex. Similarly, the binding of antibodies to whole p62 (which recognized epitopes on both the rod and NH<sub>2</sub>-terminal domains) was reduced, but not eliminated, in the complex compared with p62 molecules. Although, of course, the p62 in the complex was glycosylated whereas the recombinant material was not, glycosylated native p62 from the complex reacted well with rod antibodies on Western blots (Fig. 6, *b* and *d*). Furthermore, since the antibodies were raised against recombinant p62, they would not be directed against any of the sugar epitopes, which are located primarily in the NH<sub>2</sub>-terminal domain and threonine-rich linker (Cordes and Krohne, 1993; Starr and Hanover, 1990). The weak immunofluorescence signal obtained on whole nuclei with p62 rod antibodies compared with that obtained with antibodies against the NH<sub>2</sub>-terminal domain indicated that the rod epitopes were masked in whole pores as well as in the extracted complex.

Although of course we cannot exclude the possibility that the NH<sub>2</sub>-terminal domain and threonine-rich linker make some contribution to the p54–p62 interaction, both our blot-overlay and epitope masking results indicated that the p62 rod domain contained the major binding site for p54.

### Structural Model for the p62 Complex

Our results suggest a potential model for the p62 complex which contains two polypeptide chains each of p62 and p58 together with four p54 chains (Fig. 12). This stoichiometry would be consistent with our SDS-PAGE results which indicated that p62 and p58 were present in roughly equimolar quantities whereas p54 was present in about twice the abundance of either p58 or p62. Two p62 chains would be consistent with its rod domain forming of an  $\alpha$ -helical coiled-coil as suggested by both sequence analysis (Carmo-Fonseca et al., 1991; Cordes et al., 1991; Starr et al., 1990) and experimental data (Buss et al., 1994). A macromolecular complex with this composition would have a predicted molecular

mass of ~456 kD, which would be consistent with estimates for the rat complex of the order of 500 kD (Finlay et al., 1991). The p54 binding site is probably primarily in the rod domain. It is possible that p54 dimerizes, as there is no obvious sequence repeat in the p62 rod that might be associated with duplicated p54 binding sites. The position of p58 in the complex is still unclear. Our data gave no indication of an interaction between p58 and p62, but such an interaction may not have been preserved under the conditions used for our blot overlays. The observation that the NH<sub>2</sub>-terminal epitopes were still available might indicate that p58 was not interacting extensively with this region of p62 and so it is probably more likely that either p58 interacts with p54, or with the p62 rod, or indeed with both. Since antibodies against both p54 and p58 can immunoprecipitate the complex (Finlay et al., 1991), at least part of both proteins must be exposed. Clearly further work will be necessary to clarify the molecular interactions involving p58. However, our data offer clear and direct evidence for a strong and specific interaction between p54 and the p62 rod domain.

Although quite large, the p62 complex probably constitutes only a small fraction of the mass of nuclear pores. Thus, if eight copies were present per pore (Snow et al., 1987), they would only account for a mass of about 4,000 kD compared with 125,000 kD for the whole pore complex (Reichelt et al., 1990). Although several groups have used immuno-electron microscopy to localize p62, a clear consensus has not yet emerged (reviewed by Panté and Aebi, 1994; Panté et al., 1994). The epitope masking we observed in the p62 complex may help explain the difficulties that have been encountered in localizing this material in nuclear pores.

### Analogies to Yeast Nucleoporin NSP1

Yeast nucleoporin NSP1 (Hurt, 1988; Nehrbass et al., 1990) is in some ways analogous to vertebrate p62. The NSP1 sequence indicates that the molecule has an NH<sub>2</sub>-terminal domain containing 19 xFx<sub>2</sub> repeats and a COOH-terminal  $\alpha$ -helical coiled-coil rod domain. Interestingly NSP1 null mutants can be rescued with constructs corresponding to only the rod domain of the molecule (Nehrbass et al., 1990). Moreover, NSP1 also binds a number of proteins strongly and the COOH-terminal rod domain appears to be the principal site on the molecule involved in these interactions (Grandi et al., 1993; Wimmer et al., 1992). It may be, therefore, that both p62 and NSP1 share a number of organizational similarities and their coiled-coil domain may represent a common way in which analogous complexes are formed in yeast and vertebrates. Since the NH<sub>2</sub>-terminal domain containing the phenylalanine-rich pentapeptide repeats is present in a range of different nucleoporins, it may have considerable redundancy within p62 and NSP1, whereas the rod domain may serve a vital function in organizing the different components into a macromolecular complex.

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