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Characterization of p92, Karyopherin β, Co-Purified with N-Acetylglucosamine-Bearing Nucleoporins from Rat Liver Nuclear Envelopes[1]

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A 92k protein (p92) was purified from the wheat germ agglutinin-Sepharose (WGA-Sepharose) bound fraction of a rat liver nuclear envelope salt-extract by DEAE-5PW and hydroxyapatite HPLCs. Partial amino acid sequence analysis of p92 revealed that it is karyopherin β, which was found recently in the cytosolic fraction. It was shown using anti-p92 antiserum that the protein is present in the nuclear envelope and cytosolic fractions, in almost the same amounts, but not in other subcellular fractions of rat liver. p92 bound to N-acetylglucosamine-bearing nucleoporins (GNPs) on WGA-Sepharose, but not directly to WGA. The amount of p92 found in the rat liver nuclear envelope fraction corresponded to about 10% of the nuclear pore complex in mass, and to as much as 140 mol of p92 per mol of nuclear pore complex. Hydrodynamic analysis of the purified p92 suggested that the molecule is present as a monomer and that it is a rod-shaped molecule. The interaction of p92 and GNPs seemed to be hydrophobic and ionic. Based on these results, the participation of nuclear envelope p92 in protein nuclear transport is discussed.

Key words: importin β, karyopherin β, NLS receptor, nuclear transport, PTAC97.

Nucleocytoplasmic transport takes place through nuclear pore complexes (NPC), which are macromolecular assemblies embedded in the double nuclear membrane. The nuclear import of karyophilic proteins, which contain the nuclear localization signal (NLS), is an active process. Using digitonin-permeabilized mammalian cells and a karyophilic protein, five cytosolic factors required for nuclear import have been characterized: karyopherin α/NLS receptor/importin α/PTAC58 (*1-5*), karyopherin β/p97/importin β/PTAC97 (*6-10*), GTPase Ran/TC4 (*11, 12*), Hsc70 (*13, 14*), and B-2/NTF2 (*15, 16*). It is known that karyopherin α associates with NLS-containing substrates and functions as an NLS-receptor. A proposed function of karyopherin β is that it associates with the karyopherin α-substrate complex in a 1:1 ratio and targets the complex to NPC (*6-10*). Functions have also been suggested for the other three factors.

Recently, analysis of the functions of NPC proteins, nucleoporins, has also progressed (for recent reviews, see Refs. *17* and *18*). There are several lines of evidence that a subgroup of nucleoporins is involved in protein nuclear import. This subgroup consists of nucleoporins with substitution (except, so far, in yeast) of Ser/Thr by single *N*-acetylglucosamine residues, making them reactive with wheat germ agglutinin (WGA). WGA has been shown to inhibit nuclear import (*19, 20*). Several of these *N*-acetylglucosamine-bearing nucleoporins (GNPs), *i.e.*, p62 (*21-23*), Nup153 (*24*), Nup214 (*25*), and Nup98 (*26*), were recently characterized at the molecular level. All these characterized molecules share numerous FXFG and/or GLFG motifs, and it was suggested that the domain containing these motifs serves as the binding site of karyophile-karyopherin complexes for nuclear transport in NPC (*26*). Therefore, it was expected that other GNP members and related proteins would also have important functions in the nuclear transport mechanism.

We recently purified several major GNPs from the WGA-bound fraction of rat liver nuclear envelopes, *i.e.*, p62 complex (*27*), p144 (*28*), p120 (*28*), p86 (*28*), and p39 (*29*), and characterized them. It was shown that all these purified GNPs are rod-shaped molecules (*28, 29*). p62 was purified as a tight complex with 60k and 54k GNPs (*27*), and it was suggested that p62 acts as a complex in the nuclear pore (*27, 30, 31*). During the purification of GNPs from the WGA-bound fraction of rat liver nuclear envelopes, we found a 92k protein bound to GNPs. Thus, in this study, we purified and characterized the GNP-related 92k protein, p92. Surprisingly, the protein was karyopherin β, which was found first in the cytosol fraction (*6, 7, 9, 32*). The results of immunoblotting of p92 suggested that the protein comprises as much as 10% of the total mass of the rat liver NPC. Based on the molecular characteristics and subcellular distribution of this protein, its function in

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Abbreviations: GlcNAc, *N*-acetylglucosamine; WGA-HRP, conjugate of wheat germ agglutinin and horseradish peroxidase; 2-ME, 2-mercaptoethanol; GNP, *N*-acetylglucosamine-bearing nucleoporin; NLS, nuclear localization signal; NPC, nuclear pore complex; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride; WGA, wheat germ agglutinin.

nuclear transport is discussed.

EXPERIMENTAL PROCEDURES

Buffers—Buffer A: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$, 500 mM NaCl, and 0.2 mM PMSF; buffer B: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$, 1 M NaCl, 1% Triton X-100 [polyoxyethylene (*10*) octylphenyl ether; Wako, Tokyo], and 0.2 mM PMSF; buffer C: 20 mM Tris-acetate buffer, pH 8.0, containing 0.2% Emulgen 109P [polyoxyethylene (*9*) lauryl ether; Kao, Tokyo], and 0.5% 2-ME (2-mercaptoethanol); buffer D: 10 mM sodium phosphate buffer, pH 6.8, containing 0.3 mM $CaCl_2$, 0.2% Emulgen 109P, and 0.5% 2-ME; buffer E: 500 mM sodium phosphate buffer, pH 6.8, containing 7.5 μM $CaCl_2$, 0.2% Emulgen 109P, and 0.5% 2-ME; buffer F: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$, 100 mM NaCl, and 0.2 mM PMSF; buffer G: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$ and 0.2 mM PMSF.

Preparation of Rat Liver Nuclear Envelopes and Their Salt Extract Fraction—Liver nuclei were isolated from fasting rats by the method of Blobel and Potter (*33*), except that all buffers were supplemented with 1 mM PMSF, 2 mM benzamidine, 10 μg/ml each of chymostatin and leupeptin, and 5 μg/ml each of antipain and pepstatin A. Nuclear envelopes and a nuclear envelope extract were prepared from the nuclei as previously reported (*27*). Briefly, the nuclei were treated with DNase I and RNase A, and the resulting nuclear envelopes were extracted with 10 mM Tris-HCl buffer, pH 7.4, at 4°C, containing 500 mM NaCl, 0.2 mM $MgSO_4$, 1% 2-ME, and protease inhibitors as above. The extract, which contained GNPs and their binding proteins, was designated as the "salt extract fraction" of nuclear envelopes. The amount of material derived from 1 A_{260} unit of isolated nuclei (approximately 3×10^6 nuclei) was designated as 1 U (*34*).

WGA-Affinity Chromatography of the Salt Extract Fraction—The salt extract fraction (300 ml, 10,000 U) was dialyzed against buffer A and then applied at 0.33 ml/min to a WGA-Sepharose (Sigma Chem., St. Louis, USA) column (5 ml) equilibrated with buffer A. The flow-through fraction containing proteins that did not bind to the column was designated as the "GNP-depleted salt extract fraction." Proteins that bound to the column were eluted with buffer B, after washing with 5 bed-volumes of buffer A. The GNP-depleted salt extract fraction was reapplied to the column and eluted with buffer B. This chromatography was repeated five times. The fractions thus eluted with buffer B were combined and designated as the "GNP-bound fraction" of the nuclear envelope salt extract. A GNP-fraction was prepared by eluting with a 0.5 M GlcNAc solution from the WGA-Sepharose after washing with buffer B as previously described (*27*).

Purification of p92—The GNP-bound fraction of the nuclear envelope salt extract was dialyzed against 20 mM Tris-acetate buffer, pH 8.0, containing 0.5% 2-ME and 0.2 mM PMSF, applied to a TSK gel DEAE-5PW column (0.75×7.5 cm; Tosoh, Tokyo) equilibrated with buffer C, and then eluted with a gradient of 0 to 1,000 mM sodium acetate in buffer C. The flow rate was 0.5 ml/min. p92 was eluted with about 400 mM sodium acetate (Fig. 2A). Then, the fractions containing p92 were combined and applied to a hydroxyapatite HPLC column (0.76×10 cm, Hydroxyapatite-MP; Cica-Merck, Tokyo) equilibrated with buffer D. The column was developed with a phosphate buffer gradient formed from buffers D and E at 0.5 ml/min and room temperature. p92 was eluted with about 100 mM sodium phosphate (Fig. 2B).

Amino Acid Sequence Analysis—To obtain the internal peptides of p92 by proteolytic digestion, the GNP-bound fraction was subjected to SDS-PAGE. After Coomassie Blue staining, the gel band containing p92 was excised. After digestion of p92 in gel slices with lysylendopeptidase, the resultant peptides were isolated by reversed-phase HPLC on a silica-based C8 column (4.6×250 mm, Capcell Pak C8 Column; Shiseido, Tokyo). The sequences of the isolated peptides were determined with a Protein Sequencer 470A (Applied Biosystems). The two internal amino acid sequences thus obtained were compared with the sequence of rat karyopherin β in the GenBank database, using Genetic Information Processing Software (Genetyx-Mac).

Gel Chromatography and Density Gradient Centrifugation—The Stokes' radius of p92 was determined on a Superdex 200 HR 10/30 column (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 0.5 M NaCl, 0.1% Triton X-100, and 0.5% 2-ME at 4°C. The Stokes' radii of the standard proteins used to calibrate the column were as follows: thyroglobulin, 8.6 nm; catalase, 5.23 nm; bovine serum albumin, 3.62 nm; and ovalbumin, 2.83 nm. The sedimentation coefficient of p92 was determined by glycerol density gradient centrifugation. A 0.5 ml aliquot of purified p92 was loaded onto a 4 ml 10–40% glycerol gradient in 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and 0.2% Emulgen 109P, centrifuged at 140,000×*g* for 18 h, fractionated, and then analyzed by SDS-PAGE. The sedimentation coefficients of the standard proteins used were as follows: β-amylase, 8.9 S; alcohol dehydrogenase, 7.6 S; ovalbumin, 3.7 S; and carbonic anhydrase, 3.1 S.

Preparation of Anti-p92 Antiserum—Antiserum was raised by immunizing female rabbits with p92. The GNP-bound fraction of the rat liver nuclear envelope salt extract was subjected to SDS-PAGE, and then the gel containing the protein band corresponding to p92 was excised, and the gel piece was used for the immunization of rabbits.

SDS-PAGE and Western Blotting—Proteins were separated by 8% SDS-PAGE, and visualized by silver staining according to the methods of Laemmli (*35*) and Morrissey (*36*), respectively. Immunoblotting (*37*) and WGA blotting (*28*) were carried out as previously described.

Preparation of Rat Liver Subcellular Fractions—The preparation of nuclei, nuclear envelopes, and the salt extract and insoluble fractions of nuclear envelopes was described above. A nucleoplasm fraction was obtained as the supernatant on DNase I treatment of nuclei as described above.

Mitochondrial, microsomal, and cytosol fractions were prepared from the postnuclear supernatant by ultracentrifugation as reported previously (*37*). A part of the cytosol fraction was stored in 50% glycerol supplemented with 0.2 mM PMSF at −20°C until use for GNP-Sepharose affinity chromatography.

Preparation of GNP-Sepharose and p92-Bound GNP-Sepharose—The salt extract fraction of rat liver nuclear

envelopes (240 ml, 8,000 U) was dialyzed against buffer A and then applied to a WGA-Sepharose (4.5 ml) column equilibrated with buffer A at 0.33 ml/min. The column was washed with 5 bed-volumes of buffer A. The resin thus obtained was designated as "p92-bound GNP-Sepharose" because it retained p92 and GNPs on WGA-Sepharose beads. The resin was further washed with 10 bed-volumes of buffer B and 5 bed-volumes of buffer A, successively, to remove p92 from the resin. This WGA-Sepharose retained GNPs free of p92, and was designated as "GNP-Sepharose."

Binding of p92 with GNP-Sepharose—The GNP-depleted salt extract fraction (12 ml, 400 U) was applied to a GNP-Sepharose column (0.1 ml) equilibrated with buffer A at 2.5 ml/h. Proteins that bound to the column were eluted with buffer B after washing with 30 column-volumes of buffer A. The eluted fraction which contained p92 was analyzed by SDS-PAGE. In a control experiment, WGA-Sepharose was used instead of GNP-Sepharose.

Assay of the Mode of Binding of p92 with GNPs—The GNP-depleted salt extract fraction (36 ml, 1,200 U) was dialyzed against buffer F, then applied to a GNP-Sepharose column (0.8 ml) equilibrated with buffer F at 2.4 ml/h, and the resin was washed with 10 bed-volumes of buffer F. The p92-bound GNP-Sepharose thus obtained was taken out of the column and suspended in buffer F. An aliquot containing 30 μl of the resin was incubated in an assay medium for 1.5 h with occasional vortex-mixing. The suspension was centrifuged and the supernatant was subjected to SDS-PAGE. The amount of p92 released was determined by densitometry after silver-staining of the gel.

Separation of p92 from the Cytosol Fraction on a GNP-Sepharose Column—A rat liver cytosol fraction stored as a 50% glycerol solution (2.5 ml) at −20°C was dialyzed against buffer A and then applied to a GNP-Sepharose column (0.2 ml) equilibrated with buffer A at 0.6 ml/h. Proteins that bound to the column were eluted with buffer B after washing with 10 bed-volumes of buffer A.

Preparation of p92-Bound GNP-Sepharose with Cytosolic p92—A rat liver cytosol fraction (1.3 ml) was dialyzed against buffer F and then applied at 0.5 ml/h to a GNP-Sepharose column (0.8 ml) equilibrated with buffer F. After washing with 10 bed-volumes of buffer G, the resin was taken out and suspended in buffer G.

Protein Determination—Protein concentrations were determined by the Lowry method (*38*).

RESULTS

Purification of p92 from Rat Liver Nuclear Envelopes—Recently, we purified GNPs from the WGA-Sepharose bound fraction of rat liver nuclear envelopes (*27-29*). During the WGA-Sepharose affinity chromatography, we found that several proteins seemed to be non-glycosylated ones, though the proteins bound to the resin, because they could be eluted from the WGA-Sepharose with 1% Triton X-100 and 1 M NaCl in the absence of GlcNAc. Among these proteins, a 92k species was observed reproducibly in most preparations. We speculated that this protein is a subunit of a GNP on NPC, or the protein interacts with GNPs transiently in cells. We decided to purify and characterize the 92k protein.

Nuclear envelopes prepared from rat liver nuclei by treatment with DNase and RNase (Fig. 1, lane 1) were extracted with 500 mM NaCl (Fig. 1, lane 2). The salt extract fraction was applied to a WGA-Sepharose column, and proteins that bound to the column *via* GNPs were eluted with a buffer containing 1% Triton X-100 and 1 M NaCl. The flow-through fraction (referred to as the "GNP-depleted salt extract") was reapplied to the column and eluted in the same manner. These procedures were repeated five times and the eluted fractions thus obtained were combined (Fig. 1, lane 3: GNP-bound fraction). Repetition of these procedures was important for recovering most of the 92k protein in the salt extract. The 92k protein was the major component of the fraction (Fig. 1, lane 3). WGA-bound proteins were eluted successively with a buffer containing 0.5 M GlcNAc (Fig. 1, lane 4: WGA-bound fraction). They included GlcNAc-bearing nucleoporins purified previously: 62k, 60k, and 54k, derived from the p62 complex (*27*), 144k (*28*), and 39k nucleoporins (*29*). The 92k protein (p92) in the GNP-bound fraction was further purified by DEAE-5PW and hydroxyapatite HPLCs (Fig. 2). A typical purification and yield of p92 are summarized in Table I.

Partial Amino Acid Sequence of p92—We analyzed the partial amino acid sequence of p92 to obtain structural information on the protein. p92 was digested with lysylendopeptidase and the resulting peptides were separated by

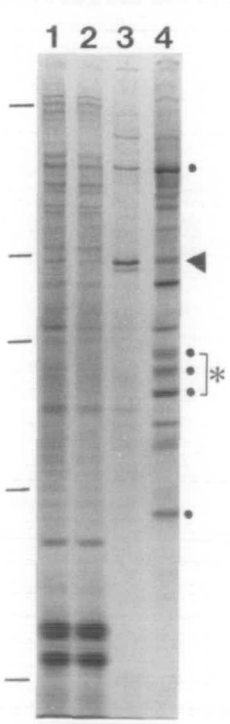

Fig. 1. **WGA-affinity chromatography of the salt extract fraction of rat liver nuclear envelopes.** A salt extract fraction of nuclear envelopes was applied to a WGA-Sepharose column. Then, the column was eluted with buffers containing 1% Triton X-100 and 1 M NaCl (GNP-bound fraction), and 0.5 M GlcNAc (WGA-bound fraction), successively. These fractions were electrophoresed and then stained with silver. Lanes: 1, nuclear envelopes, 0.02 U; 2, salt extract fraction, 0.02 U; 3, GNP-bound fraction, 0.3 U; 4, WGA-bound fraction, 2 U. The bars at the left of the gel indicate the positions of marker proteins of 210, 97.4, 66.3, 43, and 28.7 kDa, from top to bottom. An arrowhead shows the position of p92. Dots on the right of lane 4 indicate GlcNAc-nucleoporins purified previously.

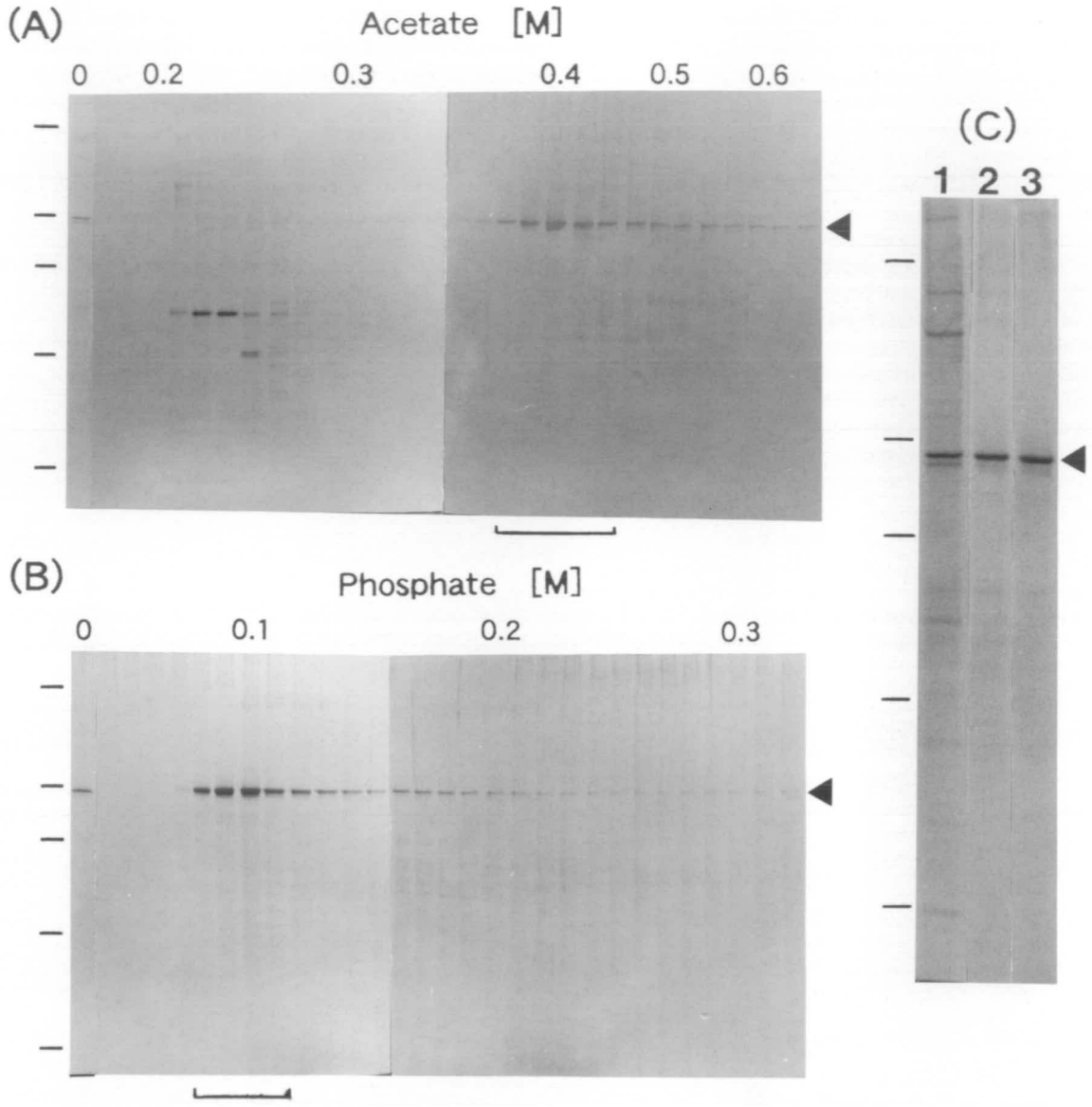

Fig. 2. **Purification of p92 by DEAE-5PW and hydroxyapatite HPLCs.** The GNP-bound fraction, 1,000 U, was applied to a DEAE-5PW column and then eluted with a sodium acetate gradient (A). The fractions containing p92 shown by a bar were combined and then applied to a hydroxyapatite column. The column was eluted with a phosphate buffer gradient (B). Fractions containing the purified p92, shown by a bar, were collected. In both chromatographies, the eluate was collected in 0.5 ml fractions, and 20 μl (A) or 40 μl (B) of each fraction was analyzed by SDS-PAGE. Lanes 0 in (A) and (B) are the applied sample, 0.3 U (A) and 1.6 U (B), respectively. The acetate (A) and phosphate (B) concentrations in gradient programs are indicated above the figures. Samples after each purification step were electrophoresed and stained with silver (C). Lane 1, GNP bound fraction, 0.3 U; lane 2, p92 purified by DEAE-5PW, 2 U; and lane 3, p92 purified by hydroxyapatite, 3 U. Arrowheads indicate the positions of p92. The bars at the left of the gels indicate the positions of the same marker proteins as in Fig. 1.

reversed-phase HPLC, then the two major peptides were sequenced. Surprisingly, the amino acid sequences of these peptides completely matched two regions of the amino acid sequence of rat karyopherin β (*6*), as shown in Table II. We examined whether these two amino acid sequences contain a known amino acid sequence motif or not, but no such motif was found. So, the probability that these two proteins are different was simply estimated to be less than $1/20^{19}$. From the results as to partial amino acid sequences, molecular weight, GNP-binding activity (see below), and subcellular distribution, we concluded that p92 is karyopherin β. p92 is referred to hereafter as karyopherin β.

TABLE I. **Purification of p92 from rat liver nuclear envelopes.** The protein was purified from 10,000 U of nuclei derived from 240 g of rat liver. The amounts of p92 in nuclear envelopes and the salt extract fraction were estimated by densitometry on immunoblots probed with anti-p92 antiserum.

	Protein amount (mg)	Purification (-fold)	Yield (%)
Nuclear envelope	1,248	1	100
Salt extract	897	0.95	69
GNP-Sepharose	6.3	75	38
DEAE-5PW	0.44	200	7
Hydroxyapatite	0.12	420	4

Characterization of the Purified Karyopherin β—As described above, karyopherin β (p92) was eluted from a WGA-Sepharose affinity column with Triton X-100/NaCl in the absence of GlcNAc, although the protein was initially retained on the column. Such behavior of the protein suggested that it is not a GlcNAc-bearing protein. To confirm this, WGA-HRP was applied to a blot of purified karyopherin β. As shown in Fig. 3D, lane 2, the purified karyopherin β did not react with WGA-HRP, while several nuclear envelope proteins did so strongly (Fig. 3D, lane 1). These results show that karyopherin β has no GlcNAc residue. Thus, the protein seemed to bind to WGA-Sepharose indirectly *via* GNPs.

The sedimentation coefficient and the Stokes' radius of karyopherin β in the native form were determined by glycerol density gradient centrifugation and gel filtration, respectively, to analyze the hydrodynamic properties of the protein. The native molecular mass was calculated from the S-value and the Stokes' radius. The value was almost the same as that obtained on SDS-PAGE (Table III), which suggests that the karyopherin β molecule exists as a monomer under the examined conditions. The axial ratio of the molecule estimated from the frictional ratio using the table of Schachman (*39*) was 26. This value suggests that the shape of this molecule deviates from a globular form, and it may be rod-shaped.

Subcellular Distribution of Karyopherin β—To confirm the localization of karyopherin β in subcellular fractions by

TABLE II. **Amino acid sequences of two peptides obtained on lysylendopeptidase digestion of p92, and their alignment with the corresponding sequences of rat karyopherin β.** Two internal amino acid sequences of p92 determined after lysylendopeptidase digestion were compared with those of rat karyopherin β. Vertical lines between the two sequences connect identical amino acid residues.

p92	TVSPDRLELEAAQ	TLATWA
	||||||||||||||	||||||
Rat karyopherin β	10TVSPDRLELEAAQ	859TLATWA

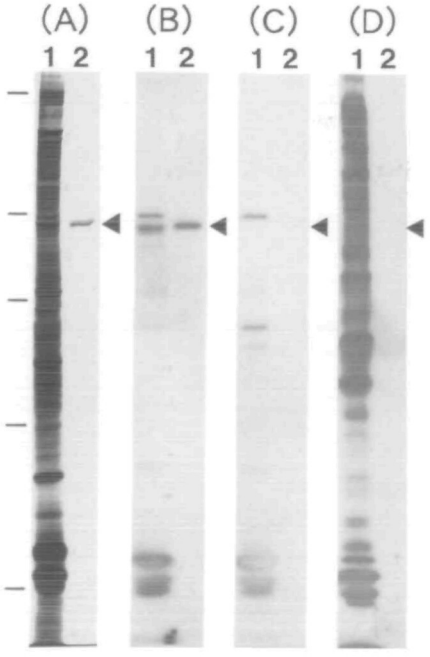

Fig. 3. **Specificity of the anti-karyopherin β antiserum.** Rat liver nuclear envelopes (0.2 U, lane 1) and purified karyopherin β (5 U, lane 2) were separated by SDS-PAGE and then stained with silver (A). The same samples as those obtained on electrophoresis were transferred to PVDF filters and probed with anti-karyopherin β antiserum (B), preimmune serum (C), or WGA-HRP (D). Arrowheads indicate the positions of karyopherin β. The bars at the left of the gels indicate the positions of the same marker proteins as in Fig. 1.

TABLE III. **Physicochemical properties of the native form of karyopherin β.**

Molecular[a] mass (kDa)	Sedimentation coefficient (S)	Stokes' radius (nm)	Native molecular mass[b] (kDa)	Frictional ratio (f/f_0)	Axial ratio
92	3.5	6.9	102	2.2	26

[a]Estimated by SDS-PAGE. [b]Estimated from the sedimentation coefficient and Stokes' radius.

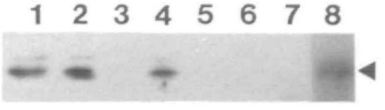

Fig. 4. **Subcellular distribution of karyopherin β.** Rat liver subcellular fractions (0.2 U) were separated by SDS-PAGE, transferred to PVDF filters, and then probed with anti-karyopherin β antiserum. Lanes: 1, nuclei; 2, nuclear envelopes; 3, nucleoplasm; 4, the salt extract fraction of nuclear envelopes; 5, salt-extracted nuclear envelopes; 6, mitochondria; 7, microsomes; and 8, cytosol. An arrowhead indicates the position of karyopherin β. The diffuse band in lane 8 is attributed to the large amount of protein in the lane.

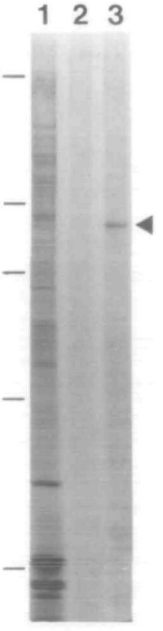

Fig. 5. **Binding of karyopherin β with GNPs.** The GNP-depleted salt extract fraction of rat liver nuclear envelopes (lane 1) was applied to a WGA-Sepharose (control) or GNP-Sepharose column, and then the proteins bound to the column were eluted with 1% Triton X-100 and 1 M NaCl (lane 2 or 3), respectively. The fractions eluted from these columns were subjected to SDS-PAGE and stained with silver. The bars at the left of the gel indicate the positions of the same marker proteins as in Fig. 1.

Western blot analysis, antiserum was raised by the immunization of rabbits with karyopherin β. As shown in Fig. 3, the antiserum reacted with the purified karyopherin β (Fig. 3B, lane 2) and a 92k protein in the nuclear envelopes (Fig. 3B, lane 1). The position of the karyopherin β band in the nuclear envelope fraction (Fig. 3B, lane 1) was a little lower than that of the purified molecule (Fig. 3B, lane 2) in this figure because there was a heavy protein band just above it. Bands other than that of karyopherin β, seen in the lane for nuclear envelopes stained with the antiserum (Fig. 3B, lane 1), were also detected with the preimmune serum (Fig. 3C, lane 1). Therefore, these bands should represent non-specific staining with the rabbit serum. We examined the subcellular localization of karyopherin β using this antiserum. Subcellular fractions prepared from rat liver were analyzed by immunoblotting (Fig. 4). Karyopherin β was detected in the fractions of nuclei, nuclear envelopes, and salt extract of nuclear envelopes, but not in the nucleoplasm fraction. These results suggested that, among the subnuclear fractions, karyopherin β is located in the nuclear envelope. Then, other subcellular fractions were also examined with the antiserum, it being found that karyopherin β is present in the cytosol fraction, but not in the mitochondrial or microsomal fraction. These results indicate that the protein is present not only in the nuclear envelope fraction, but also in the cytosol fraction. The amounts of karyopherin β in the subcellular fractions were estimated by densitometry of Western blots using a calibration curve for known amounts of karyopherin β in the same blot. These results showed that the ratio of the amounts of karyopherin β in the nuclear envelope and cytosol fractions is about 1 : 1.1. Moreover, the amount of karyopherin β in rat liver nuclei was estimated to be roughly 5.6×10^5 molecules per nucleus.

Mode of Binding of Karyopherin β and GNPs—The native form of karyopherin β was purified recently by other groups from a *Xenopus* egg extract (*9*) and bovine erythrocytes (*7*). In contrast with our case, the protein was purified from soluble fractions in both cases. Moreover, the interaction of the protein and GNPs was little studied. Therefore, we characterized the mode of binding of GNPs and karyopherin β.

To confirm the binding of karyopherin β to GNPs, but not to WGA, a GNP-depleted nuclear envelope extract was applied to columns of WGA-Sepharose and GNP-bearing WGA-Sepharose (GNP-Sepharose), and then the bound proteins were analyzed by SDS-PAGE. As can be seen in Fig. 5, karyopherin β did not bind to WGA-Sepharose in the absence of GNPs (Fig. 5, lane 2), though it did bind to GNP-Sepharose (Fig. 5, lane 3). These results show that karyopherin β binds specifically to GNPs on WGA-Sepharose beads, and also that immobilized GNPs on WGA-Sepharose can be used for analysis of the binding of karyopherin β and GNPs.

By utilizing GNP-Sepharose, the mode of binding of karyopherin β and GNPs was examined (Fig. 6). Karyopherin β in the GNP-depleted salt extract of nuclear envelopes bound to the resin. Then, the release of karyopherin β from the resin due to various reagents was examined. A detergent in a high-ionic-strength medium,

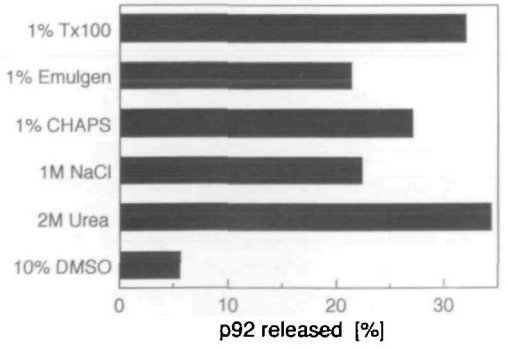

Fig. 6. **Binding of karyopherin β and GNPs.** The GNP-depleted salt extract fraction was dialyzed against 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM $CaCl_2$ and 100 mM NaCl, and then applied to a GNP-Sepharose column. An aliquot of the karyopherin β-bound GNP-Sepharose thus obtained was put into a microtube and then one of the assay media indicated in the figure was added. The suspensions were incubated for 1.5 h, and the released karyopherin β was estimated by SDS-PAGE followed by silver staining. The amount of the protein released is represented as the relative amount (%) to that released with 1% Triton X-100 and 1 M NaCl, because most karyopherin β was released by this treatment. Emulgen, Emulgen 109P; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethyl sulfoxide.

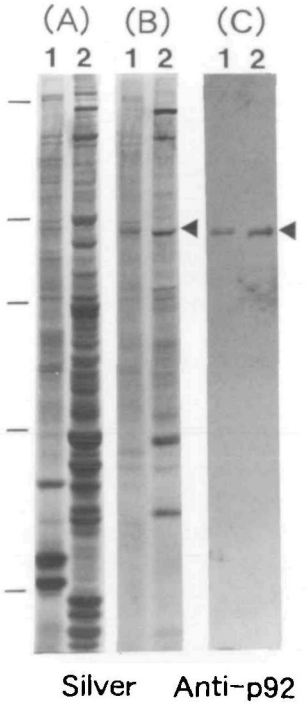

Fig. 7. **Separation of karyopherin β from the nuclear envelope and cytosol fractions on a GNP-Sepharose column.** The rat liver cytosol fraction was dialyzed against 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$ and 500 mM NaCl. The dialyzed cytosol fraction (lane A2) and the GNP-depleted salt extract fraction of rat liver nuclear envelopes (lane A1: control) were applied separately to a GNP-Sepharose column, and then the bound proteins were eluted with 1% Triton X-100 and 1 M NaCl (lanes B2 and B1, respectively). The applied samples (A) and bound fractions (B) were analyzed by SDS-PAGE and stained with silver. The same fractions as in (B) were also separated by SDS-PAGE, transferred to PVDF filters, and then probed with anti-karyopherin β antiserum (C). Arrowheads indicate the positions of karyopherin β. The bars at the left of the gels indicate the positions of the same marker proteins as in Fig. 1.

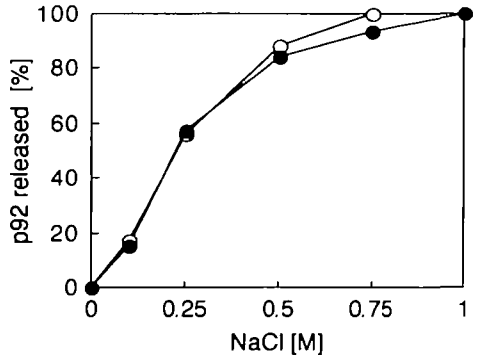

Fig. 8. **Release of karyopherin β from karyopherin β-bound GNP-Sepharose prepared from the nuclear envelope and cytosol fractions.** The cytosol and GNP-depleted nuclear envelope salt-extract fractions were dialyzed against 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$ and 100 mM NaCl, and then applied to a GNP-Sepharose column, separately. The karyopherin β-bound GNP-Sepharose thus prepared was taken from the column and aliquots (30 μl) were put into microfuge tubes, then a 1% Triton X-100 solution (150 μl) supplemented with 0, 0.1, 0.25, 0.5, 0.75, or 1 M NaCl solution was added to the tubes. The suspensions were incubated for 1.5 h and then centrifuged. The supernatants were analyzed by SDS-PAGE. The amounts of released karyopherin β with 1% Triton X-100 and various concentrations of NaCl (●, nuclear envelope karyopherin β; ○, cytosolic karyopherin β) were determined by densitometry, and are shown as relative values to that with 1% Triton X-100-1 M NaCl.

such as 1% Triton X-100 and 1 M NaCl, dissociated most karyopherin β from GNPs. However, detergents such as 1% Triton X-100, 1% Emulgen 109P, and 1% Chaps in a low-ionic-strength medium could only dissociate 20-30% of karyopherin β from GNPs. A high-ionic-strength medium in the absence of a detergent could also dissociate karyopherin β from GNPs, but only to the extent of about 20%. These data suggested that both ionic and hydrophobic interactions are involved in the binding of karyopherin β and GNPs. A denaturing reagent, 2 M urea, also partially dissociated karyopherin β, but 10% DMSO had little effect on the binding.

Comparison of Karyopherins β in Nuclear Envelope and Cytosolic Fractions—We applied our GNP-Sepharose method to a cytosol fraction to obtain partially purified cytosolic karyopherin β for comparison with the nuclear envelope karyopherin β (Fig. 7). The rat liver cytosol fraction that bound to the GNP-Sepharose contained a 92k protein (Fig. 7B, lane 2), which migrated to the same position as nuclear envelope karyopherin β on SDS-PAGE (Fig. 7B, lane 1). The 92k protein was confirmed to be karyopherin β by the use of anti-karyopherin β antiserum (Fig. 7C). It was thus shown that the GNP-Sepharose method is applicable to the purification of cytosolic karyopherin β.

To determine whether all the karyopherin β in the cytosol fraction has the ability to bind to GNPs or not, a large excess of GNP-Sepharose was used, and the bound karyopherin β was compared with that in the starting cytosol fraction by Western blotting analysis. The amount of karyopherin β bound to the GNP-Sepharose was almost the same as that of all karyopherin β in the cytosol fraction (data not shown). These results show that the majority of karyopherin β in the cytosol fraction has the ability to bind to GNPs.

The purified cytosolic and nuclear envelope karyopherin β preparations were compared with each other as to their binding with GNP-Sepharose (Fig. 8). The two karyopherin β preparations showed very similar dissociation profiles, depending on NaCl concentration in the presence of 1% Triton X-100. These results suggested that karyopherin β in both fractions exhibits a similar mode of binding to GNPs.

DISCUSSION

The native form of p97 (karyopherin β) was purified first by Adam and Adam from bovine erythrocytes (*7*). They used conventional procedures comprising ammonium sulfate precipitation followed by as many as six chromatographies (*7*). Recently, Görlich *et al.* purified importin β (karyopherin β) from a *Xenopus* egg extract on a special affinity column, an anti-importin α immunoabsorbent, and a Superdex 200 column (*9*). We described a purification method for native karyopherin β from rat liver nuclear envelopes involving three steps of chromatography. GNP-affinity chromatography gave about 80-fold purification of karyopherin β in a single step and was very effective for the purification. This method should be applicable to the purification of karyopherin β not only from rat liver nuclear envelopes, but also from the cytosolic fraction (Fig. 7) and other animal tissues.

The subcellular localization of karyopherin β in cultured cells was studied by indirect immunofluorescence microscopy using specific antibodies by other groups, it being shown that this protein is distributed in the cytoplasmic and nuclear envelope regions (*8, 40*). We studied the distribution of karyopherin β in rat liver cells by cell fractionation followed by SDS-PAGE and immunoblotting, and obtained results consistent with those of the above groups (Fig. 4). We also clarified that karyopherin β is absent in the nucleoplasmic, mitochondrial, and microsomal fractions (Fig. 4). Moreover, densitometric analysis of immunoblots revealed that the nuclear envelope fraction contains almost the same amount of karyopherin β as that in the cytosol fraction. Within the nuclear envelope, karyopherin β is probably localized on NPC, because the protein binds strongly with GNPs (Figs. 5, 6, and 7). The punctate staining of nuclear envelopes of cultured cells with the anti-karyopherin β antibody on immunofluorescence microscopy (*8*), and the location of colloidal gold particles coated with anti-karyopherin β on the nuclear pores on immunoelectron microscopy (*41*) support the above idea.

The amount of karyopherin β was estimated to be 5.6×10^5 molecules per nucleus from the results in Fig. 4. This value corresponds to as many as about 140 copies of karyopherin β per nuclear pore and about 10% of NPC in mass, when the number of pore complexes per rat liver nucleus and the molecular mass of NPC are taken as 4,000 (*42*) and 124 MDa (*43*), respectively. On the other hand, the amount of total GNP per NPC of rat liver nucleus can be estimated as follows. The yield of GNP was calculated to be 0.36×10^{-6} g/U nucleus, the average of our eight independent experiments (*27, 28*). This amount corresponds to 3×10^{-17} g/NPC, when 1 U$=3\times10^6$ nuclei (*34*) and 4,000 nuclear pores/rat liver nucleus (*42*) are postulated. The mass of one NPC was calculated to be 20×10^{-17} g from the

reported molecular mass of NPC, 124 MDa (*43*). These values mean that GNPs extracted from rat liver nuclear envelopes correspond to about 15% of the total mass of NPC. When we assume an average molecular weight of GNPs of 1.0×10^5, the above value corresponds to 190 GNP molecules/NPC. These results suggested that the number of karyopherin β molecules/NPC, 140, is close to that of GNP molecules/NPC, 190. In other words, this means that most GNPs in NPCs of rat liver nucleus are occupied by karyopherin β, if most karyopherin β molecules are in the bound state with GNPs.

It was shown by physicochemical analysis that the purified karyopherin β is present as a monomer in 0.5 M NaCl containing 0.2% Emulgen 109P (Table III). The axial ratio of the molecule was calculated to be 26 from the sedimentation coefficient and Stokes' radius. This value means that the molecule is not globular but, most likely, rod-like in shape. Enenkel *et al.* mentioned in their paper that purified Kap95p, which is a yeast homologue of karyopherin β expressed in *Escherichia coli*, behaved on molecular sieving chromatography as a monomeric globular protein (*44*). The expected molecular shapes of our rat liver natural karyopherin β and *yeast* Kap95p expressed in *E. coli* are thus different from each other. This difference may imply that their molecular shapes change depending on solvent conditions, unknown posttranslational modification (*8*), or species.

Chi *et al.* examined the extractability of karyopherin β from rat liver nuclear envelopes, and showed that the protein is extracted most effectively with a combination of a detergent and high salt (*8*). However, their data do not allow discrimination of the binding of karyopherin β to GNPs, from that of GNPs to the nuclear pore complex framework. We determined the binding of rat liver karyopherin β and GNPs directly using GNP-Sepharose, and showed that some detergents and salts could partially dissociate the protein from GNPs when they were used separately (Fig. 6). However, the use of a combination of a salt and a detergent caused complete dissociation of the protein. These results suggested that both ionic and hydrophobic interactions can be attributed to the binding of karyopherin β to GNPs (Figs. 6 and 8).

The presence of a large amount of karyopherin β on NPC, which can cover most GNP molecules, raised a new question regarding the mechanism of nuclear protein transport. Where does the karyophile-karyopherin α complex associate with karyopherin β during nuclear transport, in the cytoplasmic region or on the nuclear pore complex? Imamoto *et al.* reported that the PTAC58-PTAC97 complex (karyopherins α and β complex) did not accumulate at the nuclear rim in the absence of a karyophile, but did so in the presence of a karyophile in digitonin-permeabilized cultured cells (*10*). Their data suggest that the association of karyopherin β and the karyophile-karyopherin α complex occurs in the cytoplasmic region. If so, the affinity of the karyopherin β-karyopherin α-karyophile complex for GNPs is probably higher than that of free karyopherin β, so the complex formed in the cytoplasmic region is exchanged with karyopherin β on GNPs and then binds to NPC. However, more studies are necessary to clarify the site at which karyopherin β associates with the karyopherin α-karyophile complex during nuclear transport in rat liver.

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REFERENCES

1. Moroianu, J., Blobel, G., and Radu, A. (1995) Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA* **92**, 2008-2011
2. Adam, S.A. and Gerace, L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* **66**, 837-847
3. Weis, K., Mattaj, I.W., and Lamond, A.I. (1995) Identification of hSRP1α as a functional receptor for nuclear localization sequences. *Science* **268**, 1049-1053
4. Görlich, D., Prehn, S., Laskey, R.A., and Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* **79**, 767-778
5. Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995) *In vivo* evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. *EMBO J.* **14**, 3617-3626
6. Radu, A., Blobel, G., and Moore, M.S. (1995) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**, 1769-1773
7. Adam, E.J.H. and Adam, S.A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* **125**, 547-555
8. Chi, N.C., Adam, E.J.H., and Adam, S.A. (1995) Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* **130**, 265-274
9. Görlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R.A., Hartman, E., and Prehn, S. (1995) Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* **5**, 383-392
10. Imamoto, N., Shimamoto, T., Kose, S., Takao, T., Tachibana, T., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995) The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile. *FEBS Lett.* **368**, 415-419
11. Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* **123**, 1649-1659
12. Moore, M.S. and Blobel, G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* **365**, 661-663
13. Imamoto, N., Matsuoka, Y., Kurihara, T., Kohno, K., Miyagi, M., Sakiyama, F., Okada, Y., Tsunasawa, S., and Yoneda, Y. (1992) Antibodies against 70-kDa heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* **119**, 1047-1061
14. Shi, Y. and Thomas, J.O. (1992) The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* **12**, 2186-2192
15. Moore, M.S. and Blobel, G. (1994) Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA* **91**, 10212-10216
16. Paschal, B.M. and Gerace, L. (1995) Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* **129**, 925-937
17. Davis, L.I. (1995) The nuclear pore complex. *Annu. Rev. Biochem.* **64**, 865-896
18. Simos, G. and Hurt, E.C. (1995) Nucleocytoplasmic transport: Factors and mechanisms. *FEBS Lett.* **369**, 107-112
19. Finlay, D.R., Newmeyer, D.D., Price, T.M., and Forbes, D.J. (1987) Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* **104**, 189-200
20. Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M., and Uchida,

T. (1987) Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* **173**, 586-595
21. Starr, C.M., D'Onofrio, M., Park, M.K., and Hanover, J.A. (1990) Primary sequence and heterologous expression of nuclear pore glycoprotein p62. *J. Cell Biol.* **110**, 1861-1871
22. Carmo-Fonseca, M., Kern, H., and Hurt, E.C. (1991) Human nucleoporin p62 and the essential yeast nuclear pore protein NSP1 show sequence homology and a similar domain organization. *Eur. J. Cell Biol.* **55**, 17-30
23. Cordes, V., Waizenegger, I., and Krohne, G. (1991) Nuclear pore complex glycoprotein p62 of *Xenopus laevis* and mouse: cDNA cloning and identification of its glycosylated region. *Eur. J. Cell Biol.* **55**, 31-47
24. Sukegawa, J. and Blobel, G. (1993) A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell* **72**, 29-38
25. Kraemer, D., Wozniak, R.W., Blobel, G., and Radu, A. (1994) The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl. Acad. Sci. USA* **91**, 1519-1523
26. Radu, A., Moore, M.S., and Blobel, G. (1995) The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell* **81**, 215-222
27. Kita, K., Omata, S., and Horigome, T. (1993) Purification and characterization of a nuclear pore glycoprotein complex containing p62. *J. Biochem.* **113**, 377-382
28. Saito, M., Kita, K., Sekiya, K., Omata, S., and Horigome, T. (1995) Purification and molecular shape of a 144 kDa protein bearing *N*-acetylglucosamine residues from rat liver nuclear envelopes. *J. Biochem.* **117**, 47-53
29. Imai, N., Saito, M., Denta, K., Ichimura, T., Omata, S., and Horigome, T. (1995) Purification of 39 kDa and 50 kDa putative nucleoporins from rat liver nuclear envelopes. *Biochem. (Life Sci. Adv.)* **14**, 59-66
30. Finlay, D.R., Meier, E., Bradley, P., Horecka, J., and Forbes, D.J. (1991) A complex of nuclear pore proteins required for pore function. *J. Cell Biol.* **114**, 169-183
31. Guan, T., Müller, S., Klier, G., Pantē, N., Blevitt, J.M., Haner, M., Paschal, B., Aebi, U., and Gerace, L. (1995) Structural analysis of the p62 complex, an assembly of *O*-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. *Mol. Biol. Cell* **6**, 1591-1603
32. Imamoto, N., Tachibana, T., Matsubae, M., and Yoneda, Y. (1995) A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* **270**, 8559-8565
33. Blobel, G. and Potter, V.R. (1966) Nuclei from rat liver: Isolation method that combines purity with high yield. *Science* **154**, 1662-1665
34. Davis, L.I. and Blobel, G. (1986) Identification and characterization of a nuclear pore complex protein. *Cell* **45**, 699-709
35. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
36. Morrissey, J.H. (1981) Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**, 307-310
37. Kawahire, S., Tachibana, T., Umemoto, M., Yoneda, Y., Imai, N., Saito, M., Ichimura, T., Omata, S., and Horigome, T. (1996) Subcellular distribution and phosphorylation of the nuclear localization signal binding protein, NBP60. *Exp. Cell Res.* **222**, 385-394
38. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
39. Schachman, H.K. (1959) *Ultracentrifugation in Biochemistry*, p. 239, Table IX, Academic Press, New York and London
40. Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995) Mammalian karyopherin $\alpha_1\beta$ and $\alpha_2\beta$ heterodimers: α_1 or α_2 subunits binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**, 6532-6536
41. Görlich, D., Vogel, F., Mills, A.D., Hartmann, E., and Laskey, R.A. (1995) Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**, 246-248
42. Maul, G.G. (1977) The nuclear and the cytoplasmic pore complex: Structure, dynamics, distribution and evolution. *Int. Rev. Cytol. Suppl.* **6**, 75-186
43. Reichelt, R., Holzenburg, A., Buhle, E.L., Jr., Jarnik, M., Engel, A., and Aebi, U. (1990) Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. *J. Cell Biol.* **110**, 883-894
44. Enenkel, C., Blobel, G., and Rexach, M. (1995) Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.* **270**, 16499-16502