Nup155 Is a Novel Nuclear Pore Complex Protein That Contains Neither Repetitive Sequence Motifs Nor Reacts with WGA

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Abstract. We have molecularly cloned and sequenced a rat liver nuclear pore complex (NPC) protein of calculated molecular mass of 155 kD. Consistent with recently proposed nomenclature this protein is termed nucleoporin 155, or nup155. Unlike other nups that have so far been molecularly cloned and sequenced, nup155 does not contain repetitive sequence domains. It does not show similarity to the sequences of other proteins, including any nups, so far compiled in the data bases. Like other vertebrate nups which have been characterized nup155 possesses abundant (46 in total) consensus sites for various kinases. By immuno-electron microscopy, nup155 is associated with both the nucleoplasmic and the cytoplasmic aspect of the NPC and is therefore possibly a component of the symmetrically arranged NPC substructures. In mitotic cells, nup155 assumes a diffuse cytoplasmic distribution. Nup155 is among the ~30 proteins that were extracted from rat liver nuclear envelopes by 2.0 M urea/1.0 mM EDTA, separated from WGA-reactive proteins by WGA-Sepharose and further subfractionated by SDS–hydroxylapatite. These proteins are potential candidates for being nups.

THE nuclear pore complex (NPC)[1] is a large organelle (1, 22, 41, and for review see reference 29) with an estimated mass of 1.25×10^8 D (34). The collective term "nucleoporin" (11) (nup) has been introduced to refer to any of the hundred or more distinct proteins that are estimated to comprise the NPC. The nups are "peripheral" membrane proteins of the nuclear envelope membrane and are thus distinguished from integral proteins, such as gp210 (17, 45) that are specifically localized to the "pore membrane" domain of the nuclear envelope and that are likely to function in the anchoring of the NPC to the membrane.

Approximately a dozen of the nups have been identified using mAbs (10–13, 32, 36, 42). Most of these mAbs are polyspecific, reacting with more than one protein (11–13, 32, 36, 42) suggesting the presence of shared epitopes in these proteins. Moreover, the mAbs react with nups from mammals to yeast (3, 7, 12, 13, 36, 42) indicating that these epitopes are conserved. Indeed, molecular cloning and sequencing of several mAb-reactive proteins in yeast and vertebrates revealed the presence of highly repetitive and conserved sequence motifs that are part of these epitopes (7, 13, 31, 42). So far, five mAb reactive proteins have been cloned in yeast: NUP1 (13) and NSP1 (24, 31) share a repetitive nonapeptide motif and NUP49, NUP100, and NUP116 share a repetitive tetrapeptide motif (42). Only two nups have so far been molecularly cloned and sequenced in vertebrates: p62 (8, 37) and nup153 (38). Both of these proteins share a repetitive motif with yeast NSP1 and NUP1. The mAb-reactive nups of vertebrates, but apparently not those of yeast, also react with wheat germ agglutinin (11, 12, 19, 23, 32, 35) because of the modification of Ser or Thr by single O-linked N-acetylglucosamine residues (12, 19, 23, 32, 35, and for review see reference 21).

Although the precise number of copies of each of the dozen mAb- and WGA-reactive nups per NPC is not known it is likely that together these proteins may account for only ~10% of the total mass of NPCs. To begin characterization of the remaining nups that are not reactive with WGA we have attempted to develop methods to isolate and characterize them. Here we describe a procedure for the isolation of potential nups. Rat liver nuclear envelopes were extracted by 2 M urea and EDTA, the extracted proteins were fractionated by WGA-Sepharose chromatography and the proteins not reactive with WGA were subfractionated by SDS–hydroxylapatite chromatography and by SDS-PAGE. One of the proteins so isolated, with an estimated molecular mass of 140 kD (from the mobility in SDS-PAGE, and therefore referred to as p140) has been further characterized. Partial protein sequence analysis of p140, synthesis of a corresponding peptide, production of monospecific antibodies, and immunolocalization have shown p140 to be localized in the NPC and therefore to be a nup. Molecular cloning and sequencing revealed a protein with a calculated molecular mass of 155 kD. Consistent with a recently introduced nomenclature this protein is therefore termed nup155. Unlike the WGA- and mAb-reactive nups so far characterized, nup155 lacks repetitive sequence motifs. Homology searches have not revealed similarities to any proteins in the data banks.

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1. *Abbreviations used in this paper*: NE, nuclear envelope; NPC, nuclear pore complex; nup, nucleoporin.

Materials and Methods

Subfractionation of Peripheral Membrane Proteins of Rat Liver Nuclear Envelopes

Rat liver nuclei were isolated from 150–200 g Sprague Dawley rats as described by Blobel and Potter (5) and nuclear envelopes were obtained by two successive DNase and RNase digestions as described (14, 45).

For the extraction of peripheral membrane proteins, nuclear envelopes (NE) were incubated with 2 M urea, 1 mM EDTA, 20 mM Tris, pH 7.5, 1 mM DTT, and 0.1 mM PMSF for 15 min at 4°C at a concentration of 100 U of NE per ml of extraction buffer (1 U of NE is the amount of material derived from 1 A_{260} U of rat liver nuclei: ~3 × 10^6 nuclei; 1 g of rat liver yields ~20 A_{260} U of nuclei). The extracted proteins were separated from extracted NE by centrifugation at 20,000 g for 30 min at 4°C in a HB4 rotor (Sorvall Instruments, Newtown, CT). The supernatant was collected and the proteins precipitated with 10% TCA.

To separate proteins that bind to WGA from those that do not, precipitated proteins were solubilized in 2% SDS (wt/vol), 20 mM Tris, pH 7.5, and 20 mM DTT. After incubation at 65°C for 20 min the sample was diluted with the appropriate reagents to 100 U of extract per ml of 0.5% Triton X-100 (vol/vol), 0.1% SDS, 200 mM NaCl, 20 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF and then loaded onto a WGA-Sepharose column which had been pretreated overnight at 4°C with 0.5% Tween 20, 150 mM NaCl, 20 mM Tris, pH 7.5, to block nonspecific sites. Generally, 1 ml of WGA-Sepharose was used per ~2,000 U of extract. Loading was performed batch-wise at 4°C for period of 4–5 h. The slurry was then poured into a column and the "flow through" collected. The gel was then washed with 20 vol of 0.5% Triton X-100, 0.1% SDS, 200 mM NaCl, 20 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and the WGA-binding proteins eluted at room temperature with 4 vol of a wash buffer containing 0.5 M N-acetylglucosamine (Aldrich, Milwaukee, WI) and 0.38 mM N,N′,N″-triacetylchitotriose (Sigma, St. Louis, MO).

For further subfractionation, the proteins in the flow through of the WGA-Sepharose were precipitated with 10% TCA, washed with acetone, solubilized in 2% SDS, 100 mM sodium phosphate buffer, pH 6.8, 100 mM DTT, and then subjected to SDS-hydroxylapatite chromatography as previously described (9) except that a linear gradient of 0.1 to 0.5 M sodium phosphate buffer, pH 6.8, containing 0.1% SDS and 1 mM DTT was used for elution.

Fractions from the eluate of the hydroxylapatite column containing polypeptides of interest were pooled. Proteins were precipitated with 10% TCA and then solubilized in SDS-sample buffer, incubated for 20 min at 65°C, and then separated by SDS-PAGE on a 6% acrylamide gel. Polypeptides were electrophoretically transferred to nitrocellulose and visualized with 1% Ponceau red in 1% acetic acid. A protein with an apparent molecular mass of 140 kD and therefore referred to as p140 (see arrowhead in Fig. 2) was excised and subjected to partial NH2-terminal sequence analysis or cleaved with endopeptidase Lys-C as described (15) and several of the internal peptides subjected to NH2-terminal sequence analysis.

In comparative extraction procedures, 2.0 M urea/1.0 mM EDTA was replaced by 7.0 M urea/1.0 mM EDTA or 500 mM NaCl/5.0 mM MgCl₂. In the case of the 7.0 M urea/1.0 mM EDTA, extraction was performed at 20°C, and then the samples were centrifuged at 356,000 g for 10 min at 20°C.

Anti-peptide Antibodies

A peptide was synthesized on the basis of peptide sequence obtained from p140 (amino acid residues 951–970, see Fig. 8). An NH2-terminal cysteine was introduced for coupling purposes. The peptide was linked to keyhole limpet hemocyanin (Calbiochem-Novabiochem Corp., La Jolla, CA) using 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride (Pierce, Rockford, IL) (30) and the conjugate used to elicit antibodies in rabbits. The antibodies were affinity purified using the same synthetic peptide coupled to SulfoLink gel (Pierce) via its NH2-terminal cysteine (28). The bound antibody was eluted by 0.1 M glycine buffer, pH 2.5 (20).

Immunoblot Analysis

Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (40). Blots to be incubated with antibodies were washed two times in distilled water, once in 40% 2-propanol for 5 min, and then extensively with water. The blots were blocked for 3 h at room temperature with 0.1% gelatin, 0.1% Tween 20, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.075 mM PMSF, and probed for 3 h at room temperature with primary anti-
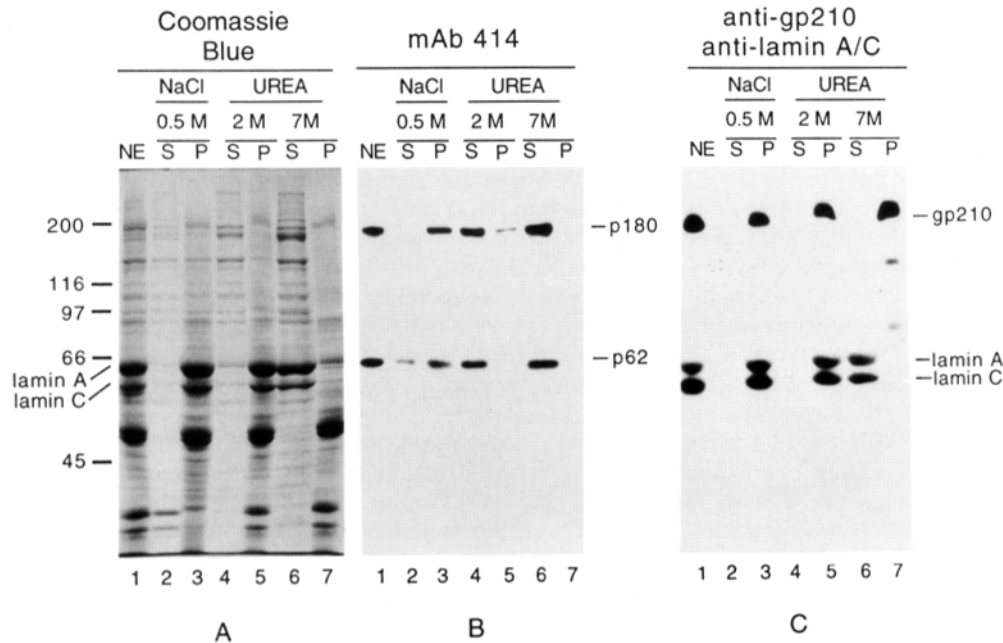

Figure 1. Differential extraction of peripheral membrane proteins by treatment of NE with high salt or urea/EDTA. Nuclear envelopes were incubated either with 500 mM NaCl/5.0 mM MgCl₂, 2.0 M urea/1.0 mM EDTA, or 7.0 M urea/1.0 mM EDTA and centrifuged to separate the extracted proteins in the supernatant (*S*) and the extracted nuclear envelopes in the pellet (*P*). The proteins were analyzed by SDS-PAGE and either Coomassie blue staining (*A*) or immunoblotting with mAb 414 (*B*) reacting with the two nucleoporins p62 and p180 or with antibodies against gp210 and lamins A/C (*C*). The amount of protein in each lane is derived from 2.0 OD U of nuclear envelopes. Numbers on the left indicate the position of the molecular mass markers in kilodaltons. The additional bands in *C*, lane 7, are likely degradation products of gp210.

body diluted in the same solution. The affinity-purified anti–peptide anti-bodies were diluted to 0.6 μg/ml and the rabbit antisera against gp210 (43) and lamins A and C (6) were diluted 1:1,000. The source of the mAb414 (11) was a hybridoma culture medium and was used at a 1:20 dilution. The mAb414-incubated blots were washed 3 × 15 min and incubated with affinity-purified rabbit anti–mouse IgG at 2 μg/ul (Organon Teknika, West Chester, PA). All blots were then washed 3 × 15 min with blocking solution and incubated for 1 h in ^{125}I-protein A (New England Nuclear, Boston, MA) in blocking solution. After this incubation blots were washed 4 × 15 min with the blocking solution and exposed to X-ray film for autoradiogra-phy at −80°C.

Immunofluorescence

Monolayer cultures of BALB/c 3T3 or BRL cells grown on glass coverslips were washed with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl), fixed, and permeabilized with cold methanol for 6 min at −20°C, rinsed twice with TBS containing 0.1% Tween 20 (TBST), and blocked for 1 h at room temperature with 2% nonfat dry milk (Carnation Co., Los Angeles, CA) in TBST (TBSTM). The cells were then incubated for 30 min at room tem-perature with the affinity-purified anti–peptide p140 antibodies (3 μg/ml in TBSTM), washed five times with TBSTM, and then incubated for 15 min at room temperature with fluorescein-labeled goat anti–rabbit IgG (20 μg/ml) (Cappel Organon Teknika, Durham, NC). Where indicated DNA staining Hoechst 33258 dye was added to the second antibody solution at a concentration of 0.5 μg/ml. Finally, the cells were washed six times in TBSTM, once in TBST containing 0.1% BSA and mounted in 90% (vol/vol) glycerol containing 1 mg/ml p-phenylenediamine, 10 mM Tris-HCl, pH 8.0 (25). Specimens were examined using an Axiophot Microscope (Carl Zeiss, Oberkochen, Germany) and the images were recorded on film (TMAX-400; Eastman Kodak Co., Rochester, NY).

Immunoelectron Microscopy

3T3 cells grown as monolayers were detached with trypsin, washed in DME, and pelleted by centrifugation. The cell pellet was fixed with 2% paraformaldehyde-0.05% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, at 4°C for 30 min. The pellet was then dehydrated in 70% ethanol and embedded in L. R. White (Electron Microscopy Sciences, Fort Washington, PA). Sections were cut on a Reichert-Jung Ultracut E ultramicrotome (Mi-croscopic Optical Consulting Inc., Valley Cottage, NY) and placed on Formvar carbon–coated nickel grids. The grids were blocked with 1% BSA in PBS, probed with affinity-purified rabbit anti–peptide antibodies (3 μg/ml) and binding detected with goat anti–rabbit IgG bound to 10 nm gold particles (Amersham Life Sciences, Arlington Heights, IL). The grid was stained with uranyl acetate and viewed on an electron microscope (100 CX; JEOL USA, Peabody, MA) operated at 80 kV.

For cryo-sectioning an aliquot of cells fixed as above was incubated for 30 min with 2.3 M sucrose in PBS at room temperature and then frozen in liquid nitrogen. Ultrathin frozen sections were prepared according to Tokuyasu (39). Rainbow-colored sections were cut on a cryo-ultramicro-tome (FC-4E; Reichert Jung S. A., Paris) and collected on Formvar carbon–coated nickel grids. Grids were blocked and probed as described for the L. R. White sections. After binding of the goat anti–rabbit bound to 10 nm gold, the grids were washed and stained according to Griffiths et al. (18).

Synthesis of a p140-specific cDNA Probe

The exact cDNA sequence for a portion of p140 was determined using the PCR procedure described by Lee et al. (27) as follows. On the basis of a 25 amino acid peptide sequence (amino acids 443–467; see Fig. 8), two par-tially degenerate oligonucleotide primers were synthesized corresponding to the sense sequence of amino acid residues 443–449 (sense primer) and the antisense sequence of amino acid residues 462–467 (antisense primer). As a template we used first-strand cDNA synthesized from size-selected poly A$^+$ RNA of NlS1 cells using random primers (45). The in vitro am-plification was performed using Taq polymerase (Perkin-Elmer Corp., Nor-walk, CT) in a 0.1-ml reaction containing 0.1 μg of cDNA and 6 pmoles of each of the degenerate sense and antisense primer mixtures. The sample was exposed to 25 cycles of denaturation (94°C for 1 min), annealing (42°C for 2 min), and polymerization (72°C for 1 min). The reaction products were analyzed on a 4% low-melting agarose gel (NuSieve GTG; FMC Bio-Products, Rockland, MD). A band of 72 bp was excised, phenol extracted, ethanol precipitated, and then subjected to a second round of amplification under the same conditions. The DNA product was analyzed on an agarose
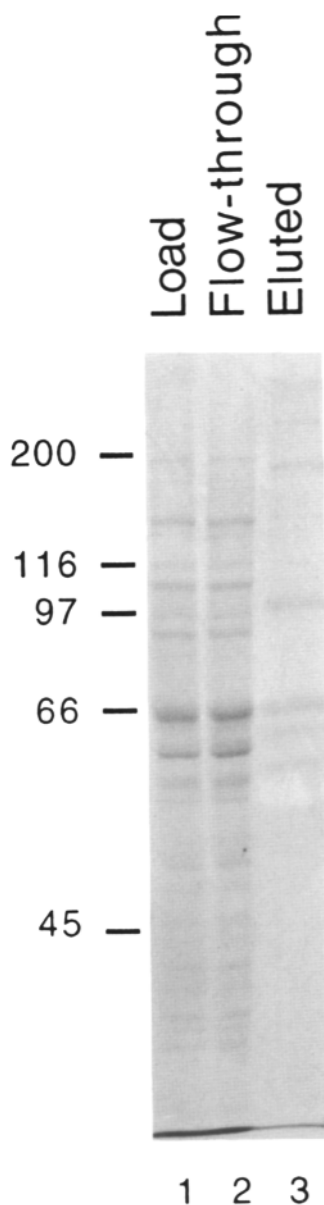

Figure 2. Separation of pe-ripheral membrane proteins extracted from nuclear enve-lopes by 2.0 M urea/1.0 mM EDTA into those that bind and those that do not bind to WGA–Sepharose. Extract (*Load*) was incubated with WGA–Sepharose. Equivalent aliquots of the load and the flow-through fraction and five times more of the material eluted specifically by sugars were analyzed by SDS-PAGE (8% acrylamide) and Coomas-sie blue staining. Numbers on the left indicate molecular mass in kilodaltons.

gel and the major 72-bp fragment was isolated, subcloned into pBluescript II SK (Stratagene, La Jolla, CA), and sequenced with Sequenase (United States Biochemical Corp., Cleveland, OH).

Screening of the cDNA Library

A λZAP cDNA library produced from rat NlS1 cells mRNA (45) was screened with a 41-mer oligonucleotide complementary to the sense strand of the subcloned PCR product. The oligonucleotide was labeled with γ[^{32}P]ATP (New England Nuclear) using polynucleotide kinase (New En-gland Biolabs, Beverly, MA), and used to screen 1 million recombinants. Phage lifts were performed as described in reference 4. Filters were pre-hybridized at 65°C for 1–3 h in a solution containing 50% formamide, 5× Denhardt's solution, 0.2% SDS, 50 mM NaP$_i$, pH 7.7, 900 mM NaCl, 5 mM EDTA, and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 24 h using ∼3 × 10^5 cpm of labeled oligonu-cleotide/ml hybridization buffer (identical to prehybridization buffer except 250 μg/ml tRNA is used instead of denatured salmon sperm DNA). Filters were washed three times at room temperature and twice at 45°C in 1× SSC, 0.1% SDS. Positive plaques were purified. pBluescript SK(−)–containing cDNA inserts were rescued from plaque-purified clones. Plasmid DNA was sequenced with Sequenase. To obtain a full-length sequence, screening was repeated using 50-mer oligonucleotide probes synthesized on the basis of
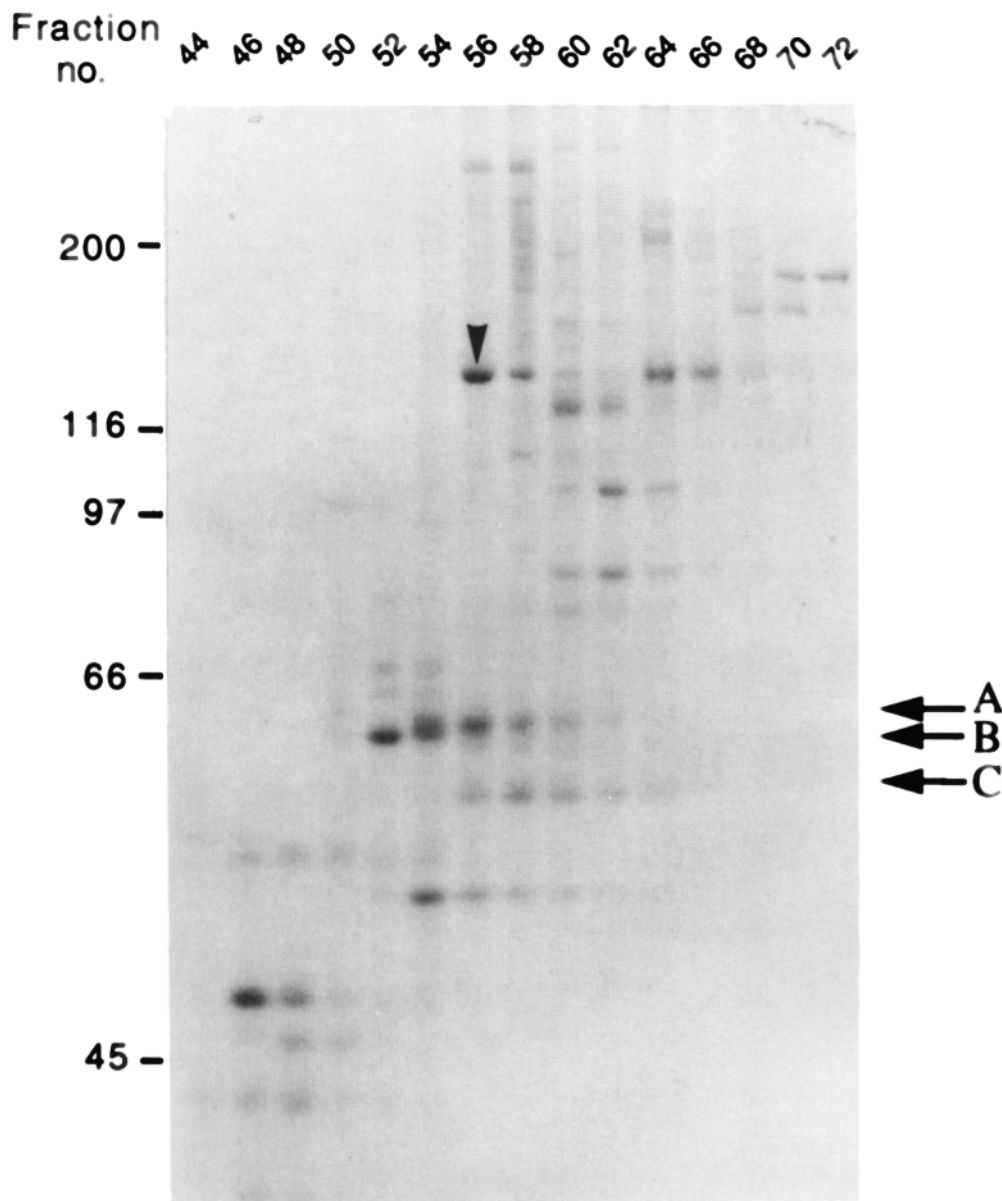
Figure 3. SDS-hydroxylapatite chromatography of peripheral nuclear membrane proteins that did not bind to WGA-Sepharose. Proteins extracted from nuclear envelopes by 2.0 M urea/1.0 mM EDTA and not bound to WGA-Sepharose were further subfractionated by SDS-hydroxylapatite chromatography. Every other fraction, beginning with No. 44 was analyzed by SDS-PAGE (8% acrylamide) and polypeptides visualized by Coomassie blue staining. Arrowhead indicates p140 and arrows to the right indicate positions of lamins A, B, and C. Numbers on the left indicate molecular mass in kilodaltons.

the extreme 5' and 3' ends of the known sequence. Screening was performed as above except that the formamide concentration was adjusted to 45% and the temperature of the last two washes was increased to 60°C. This strategy was repeated through one more round of screening to deduce the entire amino acid sequence.

Subsequently three full-length cDNAs were isolated from a BRL cell cDNA library in lambda gt10 (generously provided by Dr. Jun Sukegawa, Howard Hughes Medical Institute, The Rockefeller University). This library was screened as above with a 54-mer oligonucleotide complementary to the 5' most end of the overlapping, partial cDNA: it confirmed and extended the sequence obtained from the overlapping partial cDNA clones.

Sequence Analysis

The deduced amino acid sequence was analyzed on a Macintosh computer using the programs Protean 1.08 from DNASTAR Inc. (Madison, WI), DNA Strider 1.1, and MACPROT from Macintosh. The deduced amino acid sequence was compared with the sequences in the GenBank and EMBL data bases by the FASTA program of Pearson and Lipman (33).

Results

Extractions and Subfractionation of Potential Nups

Rat liver nuclear envelopes were prepared from isolated nuclei (5) by two successive DNase and RNase digestions (14, 45). Various conditions for the extraction of peripheral membrane proteins were then used. The extracted peripheral membrane proteins (supernatant, *S*) were separated from the membranes (pellet, *P*), by centrifugation for 15 min at 89,000 *g*. The extent of extraction was monitored by SDS-PAGE, Coomassie blue staining and immunoblot analysis with various antibodies (Fig. 1). Under the mildest extraction condition, 500 mM NaCl/5 mM MgCl₂, a considerable number of proteins were already extracted (Fig. 1 *A*, lanes *2* and *3*). One of these proteins is the partially extracted p62, a nup that reacts with mAb414 (11) (Fig. 1 *B*, lanes *2*
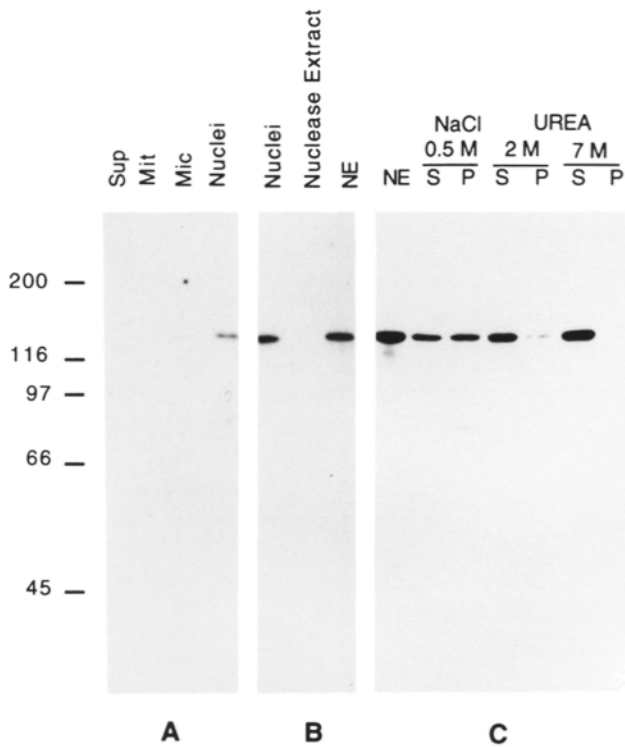
Figure 4. Immunoblots with affinity-purified anti-peptide (p140) antibodies of SDS-PAGE separated proteins of cellular fractions (A), subfractions of purified rat liver nuclei (B) and subfractions of nuclear envelopes (NE) (C). (A) a rat liver homogenate was fractionated by differential centrifugation at 800 g for 10 min at 4°C to yield a crude pellet of nuclei; the 800 g supernatant was then centrifuged at 12,000 g for 15 min to yield a pellet of mitochondria (Mit). The resulting supernatant was centrifuged at 128,000 g for 15 min to yield a pellet of microsomes (Mic), and a postmicrosomal supernatant (Sup). Approximately equal amounts of total protein are present in each lane. (B) Purified rat liver nuclei (Nuclei) were incubated consecutively with DNase and RNase to yield a "nuclease extract" and nuclear envelopes (NE). The amount of protein in each lane corresponds to 0.4 OD U of nuclei. (C) Nuclear envelope (NE) extracts identical to those shown in B. Numbers on the left indicate molecular mass in kilodaltons.

and 3). However lamins A and C are not extracted (Fig. 1 C, compare lanes 2 and 3). Neither is gp210 (Fig. 1 C, lanes 2 and 3) an integral membrane protein that is used here as a marker for complete sedimentation of membranes. Under harsher extraction conditions, using 2.0 M urea and 1 mM EDTA, more proteins appear to be extracted and those that were already partially extracted at 500 mM NaCl/5 mM MgCl₂ are more completely extracted (Fig. 1 A, compare lanes 2–5). Two of the mAb414 reactive nups, p62 and p180, were completely extracted (Fig. 2 B, lanes 4 and 5). Only small amounts of the lamins were extracted under these conditions (visible in Fig. 1 A, lane 4 and in a longer exposure of the immunoblot [data not shown] of Fig. 1 C, lane 4). As expected, gp210 was not extracted (Fig. 1 C, lanes 4 and 5). Finally, under the harshest conditions, 7.0 M urea and 1 mM EDTA, extraction of peripheral membrane proteins, including the lamins, appeared to be complete (Fig. 1, compare lanes 6 and 7 of A, B, and C).

Because of the minimal extraction of lamins and the apparently complete extraction of the mAb414-reactive nups at 2.0

M urea/1 mM EDTA, we settled for this condition and used this extract as starting material for further subfractionation of nups by WGA-Sepharose affinity chromatography (Fig. 2). As expected, most of the proteins did not bind to WGA-Sepharose and appeared in the flow-through (Fig. 2, lane 2). Only a distinct set of proteins bound and was specifically eluted with sugars (Fig. 2, lane 3).

The proteins that did not bind to WGA-Sepharose were precipitated, dissolved in an SDS-containing buffer, fractionated by SDS-hydroxylapatite chromatography and separated by subsequent SDS-PAGE of the fractions (Fig. 3). More than 30 proteins, among them lamins A, B, and C, were separated by these procedures. Most of these proteins are likely candidates for being nups. One of the more abundant of these proteins (see arrowhead in fraction No. 56 in Fig. 3), termed p140 on the basis of its estimated molecular mass of 140 kD on SDS-polyacrylamide gels, has been chosen for further characterization.

p140 Is a Nup

We obtained several partial amino acid sequences from p140, both from the NH₂ terminus and several endoproteolytic fragments. A peptide corresponding to an internal region was synthesized and antibodies were elicited in rabbits. The p140 specific antibodies were then used to determine the intracellular location of p140.

A rat liver homogenate was subfractionated by differential centrifugation into nuclei, mitochondria (Mit), microsomes (Mic), and a postmicrosomal supernatant (Sup). Proteins in these fractions were separated by SDS-PAGE, transferred to nitrocellulose and analyzed for reactivity with anti-p140 antibodies. p140 was found exclusively in the nuclear fraction (Fig. 4 A).

Next, purified rat liver nuclei were subfractionated by two successive DNase and RNase digestions. The material released by nuclease digestion was separated from the NE by centrifugation. The proteins were again analyzed by SDS-PAGE and subsequent immunoblotting. p140 was found exclusively in the nuclear envelope (Fig. 4 B).

To further sublocalize p140, nuclear envelopes were extracted as in Fig. 1 and the extracted and nonextracted proteins analyzed by SDS-PAGE and subsequent immunoblotting (Fig. 4 C). About 50% of p140 was extracted by 500 mM NaCl/5 mM MgCl₂, whereas most or all of p140 was extracted with 2 M urea/1 mM EDTA or 7 M urea/1 mM EDTA, respectively. Thus by all these fractionation criteria p140 behaves like a peripheral membrane protein of the nuclear envelope, consistent with it being a nup.

The association of p140 with the nuclear envelope was further examined by immunofluorescence microscopy using cultured buffalo rat liver (Bethesda Research Laboratories, Gaithersburg, MD) cells that had been fixed and permeabilized with methanol. Cells were probed with affinity-purified anti-peptide antibodies and binding visualized with fluorescein-labeled goat anti-rabbit IgG. The antibodies reveal a finely punctate nuclear surface staining (Fig. 5 B) or punctate nuclear rim staining (Fig. 5 A) that is characteristic for nups (11, 36) and pore membrane proteins as such gp210 (10, 44).

In mitosis, p140 assumes a diffuse cytoplasmic distribution (Fig. 6, b–d) that reverses back to a punctate nuclear
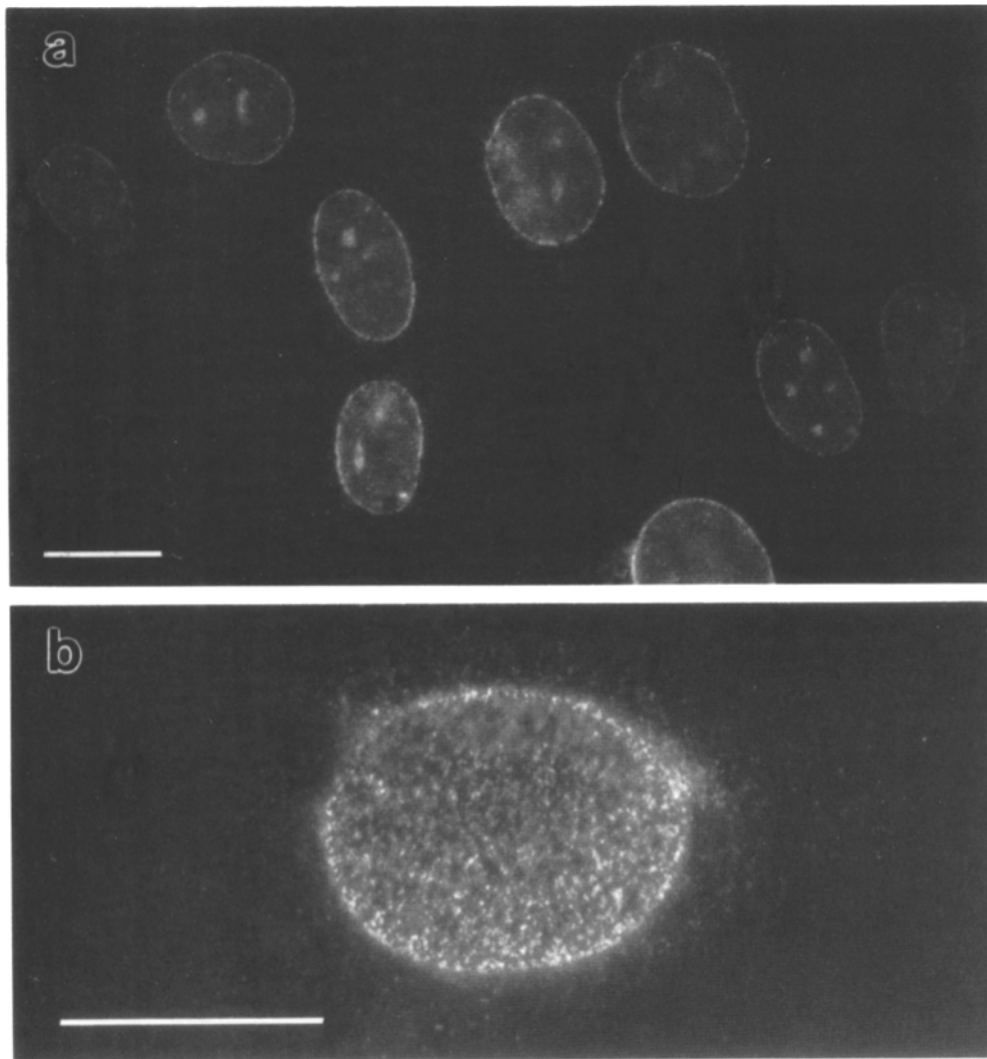
Figure 5. Localization of p140 by indirect immunofluorescence microscopy. Methanol fixed and permeabilized 3T3 cells were incubated with affinity-purified anti-peptide (p140) antibodies and then with fluorescein-conjugated goat anti-rabbit IgG. The fluorescent signal appears as (*a*) discontinuous fluorescent ring if the image is focused on the nuclear rim and (*b*) punctate if the image is focused on the surface of the nuclei. Both patterns are consistent with the presence of the antigen in the nuclear pore complexes. Bars, 20 μm.

surface localization at the end of mitosis (Fig. 6 *e*). This is consistent with the reversible disassembly of the NPC during mitosis. Similar results were obtained previously with the mAb-reactive nups (11, 36, and for review see reference 16). In addition sucrose gradient sedimentation analysis of mitotic HeLa cell extracts show that p140 sediments at ∼6 S (data not shown) suggesting its disassembly into a monomer.

Definitive evidence for the location of p140 in the NPC was obtained by immunoelectron microscopy using aldehyde fixed BRL cells and immunolabeling, either of frozen thin sections (Fig. 7, *b–d*) or of sections obtained after embedding (Fig. 7 *a*). The immunogold particles clearly labeled the NPCs (Fig. 7) with particles visible on the nucleoplasmic and cytoplasmic sides.

cDNA Sequence and Deduced Primary Structure of p140

We used amino acid sequence information derived from an internal position of p140 and PCR to synthesize an exact probe for screening rat liver cDNA libraries. Several partial
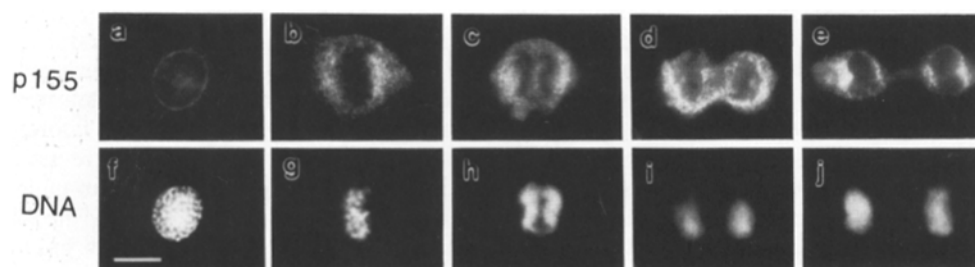
Figure 6. Distribution of p140 in mitotic BRL cells. Methanol-fixed and permeabilized cells were incubated with affinity-purified anti-peptide (p140) antibodies and then with fluorescein-conjugated second antibodies containing the DNA-staining dye Hoechst 33258. The upper row of images (*a–e*) presents the localization of p140, while the lower row of images (*f–j*) shows the distribution of DNA in the corresponding cells. (*a*) Interphase; (*b*) metaphase; (*c*) anaphase; (*d* and *e*) telophase. All pictures are taken at the same magnification. Bar, 20 μm.

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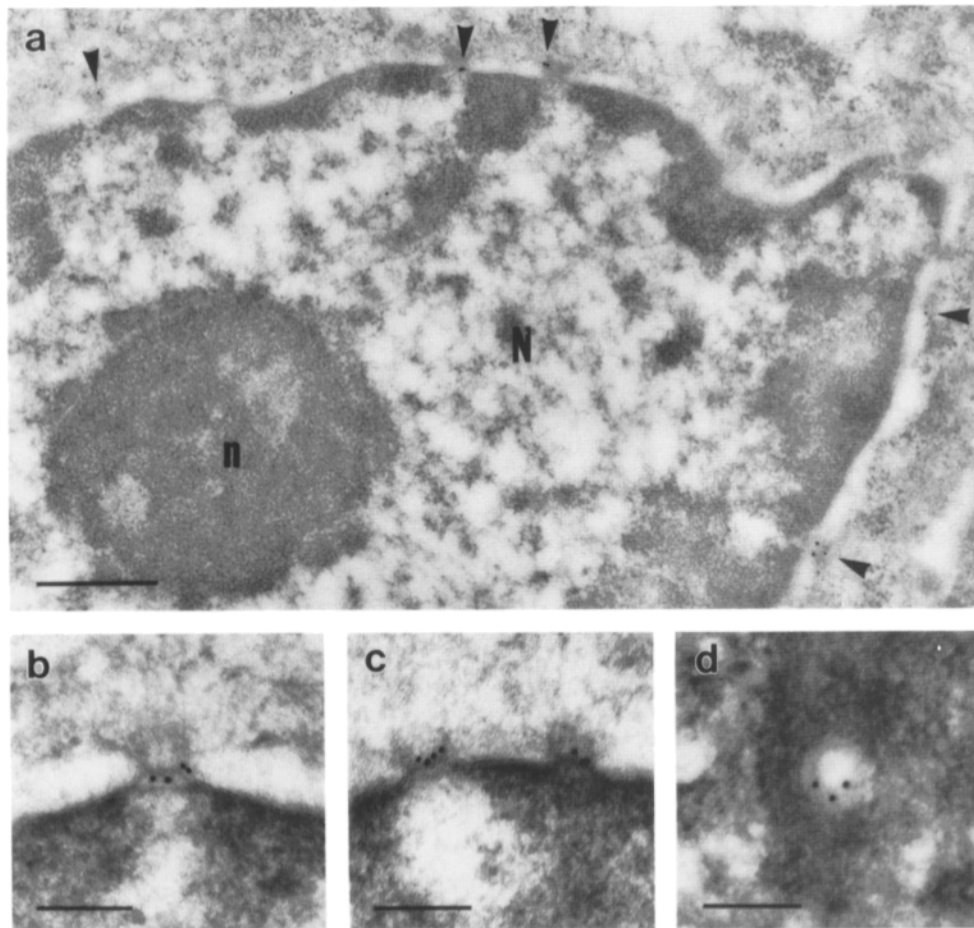
Figure 7. Localization of p140 by immunoelectron microscopy. 3T3 cells were fixed with 2% paraformaldehyde–0.05% glutaraldehyde and either embedded in L. R. White (*a*) or infused with sucrose and frozen (*b–d*). The L. R. White or the frozen sections were incubated with affinity-purified anti–peptide (p140) antibodies followed by goat anti-rabbit IgG linked to 10 nm gold particles. (*a*) Arrowheads point to gold-labeled nuclear pore complexes; *N*, nucleus; *n*, nucleolus. (*b–d*) Transverse (*b*), oblique (*c*), and tangential (*d*) sections, respectively, through the nuclear membrane. Bar: (*a*) 100 nm; (*b–d*) 20 nm.

and full-length clones were obtained and sequenced. They established a 4,321-bp contiguous sequence which is consistent with a 4.4-kb mRNA species identified by RNA blotting (data not shown). There is a 75-bp 5'-untranslated region followed by an ATG initiation codon (nucleotide +1) that is flanked by conserved bases found at eukaryotic translational start sites (26). Translational initiation at this position was confirmed by NH₂-terminal protein sequence analysis of p140. The opening reading frame continues for 4,170 bp to a TAA termination codon and is followed by a short 3'-untranslated region containing a potential poly-A addition site (position 4,213) and a poly-A tail.

The cDNA codes for a protein composed of 1,390 amino acid residues with a calculated molecular mass of 155,020 D. Its identity is confirmed by protein sequence data obtained from the NH₂ terminus and six endoproteolytic peptide fragments (see underlines in Fig. 8). Analysis of its primary and predicted secondary structure reveals no striking characteristics with the possible exception of a proline rich region between amino acid residues 584–654. The protein contains numerous consensus sites for various kinases (30 sites for casein kinase II, 16 sites for protein kinase C, and one site each for tyrosine kinase and cAMP-dependent kinase). Consistent with its peripheral membrane association, p140 contains no hydrophobic segments of sufficient length for integration into the membrane. Finally, the primary structure of p140 appears to be unique and unrelated to any sequences present in the available data bases including previ-

ously sequenced rat and yeast nucleoporins. As p140 is a nup and consistent with a recently introduced nomenclature that incorporates the calculated molecular mass of unmodified primary structure as a means to classify nups, we propose to term p140 as nup155.

Discussion

We have identified and molecularly cloned and sequenced a novel rat liver NPC protein of calculated molecular mass of 155 kD, termed nup155. This nup differs from the others hitherto molecularly cloned and sequenced nups in that it contains neither repetitive sequence motifs nor reacts with WGA.

Nup155 is one of a group of about 30 abundant peripheral membrane proteins (Fig. 2) that do not react with WGA. These proteins were extracted from isolated rat liver nuclear envelopes by 2.0 M urea/1.0 mM EDTA, and then separated from WGA-reactive proteins using WGA–Sepharose and further subfractionated by SDS–hydroxylapatite chromatography. Extraction with 2.0 M urea/1.0 mM EDTA was chosen because it allowed us to produce an extract enriched in nups and largely separated from lamins (Figs. 1 and 4). Most of nup155, the WGA-binding nups, and probably most nups are extracted under these conditions. Although it remains to be investigated how many of the proteins in this fraction (Fig. 2) are nups, it is likely that many of them are. Characterization of another of these proteins by similar methods has, in

Figure 8. cDNA and deduced amino acid sequence of nup-155 (p140). Nucleotides are numbered on the left; the co-ordinate +1 was assigned to the first nucleotide of the reading frame. Amino acids, represented by one-letter codes, are numbered on the right. The heavy lines mark the initiation codon, the termination codon and the poly A addition sites. Stretches of amino acid sequence obtained by peptide sequencing are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z21780.

fact, shown that it is a nup as well (Radu, A., G. Blobel, and R. W. Wozniak, unpublished results). As complete extraction of nup155 and the WGA-binding nups occurred at 7.0 M urea/1.0 mM EDTA and, although 7.0 M urea/1.0 mM EDTA extracts the lamins (Fig. 2), it may, for further work, serve as a more quantitative extraction condition that yields a more complete recovery of all classes of nups particularly as the lamins are likely to be readily separable from most of the potential nups (Fig. 2). It is interesting to note that about half of nup155 is extracted by 500 mM NaCl/5 mM MgCl₂, a condition that has previously been used in the preparation of a "nuclear pore complex lamina" fraction (14). It is likely that nup155 is not the only nup that is partially extracted under these conditions, judging from the considerable number of peripheral nuclear envelope proteins, all of them potential

nups, that were extracted under these conditions (Fig. 1, lane 24).

Nup155 was found by immunoelectron microscopy to be localized on the NPC (Fig. 7). A noteworthy feature of p155 is the presence of 48 consensus sites for various protein kinases (nup155 is in fact a phosphoprotein) (Courvalin, J. C., A. Radu, G. Blobel, and R. Wozniak, unpublished data). Regulated phosphorylation and dephosphorylation of these sites may be important for the function of nup155 and may play a role in the reversible disassembly of the NPC during mitosis.

8

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