

Nup180, a Novel Nuclear Pore Complex Protein Localizing to the Cytoplasmic Ring and Associated Fibrils

Norbert Wilken,* Ursula Kossner,* Jean-Luc Senécal,‡ Ulrich Scheer,* and Marie-Christine Dabauvalle*

*Department of Cell and Developmental Biology, Theodor-Boveri-Institute, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany; and ‡Division of Rheumatology, Notre-Dame Hospital, University of Montreal School of Medicine, Montréal, Quebec, Canada H2L4K8

Abstract. Using an autoimmune serum from a patient with overlap connective tissue disease we have identified by biochemical and immunocytochemical approaches an evolutionarily conserved nuclear pore complex (NPC) protein with an estimated molecular mass of 180 kD and an isoelectric point of ~ 6.2 which we have designated as nup180. Extraction of isolated nuclear envelopes with 2 M urea and chromatography of the solubilized proteins on WGA-Sephrose demonstrated that nup180 is a peripheral membrane protein and does not react with WGA. Affinity-purified antibodies yielded a punctate immunofluorescent pattern of the nuclear surface of mammalian cells and stained brightly the nuclear

envelope of cryosectioned *Xenopus* oocytes. Nuclei reconstituted in vitro in *Xenopus* egg extract were also stained in the characteristic punctate fashion. Immunogold EM localized nup180 exclusively to the cytoplasmic ring of NPCs and short fibers emanating therefrom into the cytoplasm. Antibodies to nup180 did not inhibit nuclear protein transport in vivo nor in vitro. Despite the apparent lack of involvement in NPC assembly or nucleocytoplasmic transport processes, the conservation of nup180 across species and its exclusive association with the NPC cytoplasmic ring suggests an important, though currently undefined function for this novel NPC protein.

NUCLEAR pore complexes (NPCs)¹ mediate the transmembrane exchange of macromolecules between the two major cell compartments, the nucleus and the cytoplasm. Knowledge of NPC structure, composition, and molecular organization is a necessary prerequisite to understanding the mechanisms of these highly selective and energy-requiring bidirectional transport processes (reviewed by Goldfarb and Michaud, 1991; Stochaj and Silver, 1992). NPCs are large and complex supramolecular assemblies with an outer diameter exceeding 0.1 μm and a total mass of 2×10^{-16} g or 120×10^6 D (Krohne et al., 1978; for a more detailed analysis and mass determination of various NPC substructures see Reichelt et al., 1990). Their overall structural features, in particular their striking eightfold rotational symmetry, are remarkably similar if not identical in all eukaryotic cells, thus attesting to the fundamental importance of NPCs in basic cellular activities (for reviews see Kessel, 1973; Franke and Scheer, 1974; Maul, 1977; Franke

et al., 1981; Scheer et al., 1988; Akey, 1992; Forbes, 1992; Stewart, 1992; Newmeyer, 1993).

By using various methods of sample preparation and EM analysis in combination with image analysis, considerable progress has recently been made in deciphering the three-dimensional architecture of NPCs (Unwin and Milligan, 1982; Akey, 1989, 1992; Reichelt et al., 1990; Jarnik and Aebi, 1991; Hinshaw et al., 1992; Stewart, 1992; Akey and Radermacher, 1993; Panté and Aebi, 1993). To a first approximation, NPCs are tripartite structures composed of a flat cylindrical body flanked at the top and bottom by two coplanar rings with octagonal symmetry, one attached to the cytoplasmic and one to the nucleoplasmic pore margin. Major components of the cylindrical body are a central plug or "transporter assembly" (Akey and Goldfarb, 1989) containing the functional pore channel through which active nucleocytoplasmic transport of macromolecules takes place (Feldherr et al., 1984; Dworetzky and Feldherr, 1988) and eight radially arranged spoke-like structures at the midplane of the NPC. The octagonal spoke assembly connects the central transporter to the nuclear membrane of the pore wall. Vertical supports link each of the eight spokes to the cytoplasmic and nucleoplasmic ring, thus creating a highly symmetrical NPC framework (Hinshaw et al., 1992; Akey and Radermacher, 1993). Eight granules ("annulus subunits";

Address all correspondence to M.-C. Dabauvalle, Biocenter, Zoology I, University of Würzburg, Am Hubland, D-97074 Würzburg, F.R.G.

1. *Abbreviations used in this paper:* GlcNAc, *N*-acetyl glucosamine; NPC, nuclear pore complex.

Franke and Scheer, 1970) are arranged in striking rotational symmetry on top of the cytoplasmic rings. Depending on the conditions used in nuclear envelope preparation, in particular in low-salt buffers without stabilizing divalent cations, these granules tend to unravel into short fibrils projecting from the cytoplasmic face of the NPCs (Franke and Scheer, 1974; Ris, 1991; Jarnik and Aebi, 1991). In addition, nucleoplasmic fibrils which, in amphibian oocytes, often constitute long cylindrical arrays (Franke and Scheer, 1970, 1974) or shorter basket-like filamentous assemblies (Ris, 1991; Jarnik and Aebi, 1991; Goldberg and Allen, 1992) terminate at the nucleoplasmic face of the NPCs.

Very little information is presently available on the biochemical nature of the various gross morphological components of the NPCs. Labeling studies with gold or ferritin-tagged WGA have shown that the O-linked *N*-acetylglucosamine (GlcNAc) modified nucleoporins (for review see Starr and Hanover, 1992) are localized preferentially to the central region of the NPC, i.e., to the transporter assembly with an overall diameter of ~40 nm (Finlay et al., 1987; Hanover et al., 1987; Scheer et al., 1988; Akey and Goldfarb, 1989). A similar localization was seen with mAbs against p62, the most abundant WGA-binding NPC glycoprotein (Dabauvalle et al., 1988a; Akey and Goldfarb, 1989). The topological disposition of the GlcNAc-containing nucleoporins suggests their having a role in nucleocytoplasmic traffic. In fact, protein import and RNA export are inhibited by WGA and mAbs recognizing p62, both in living cells after microinjection (Yoneda et al., 1987; Featherstone et al., 1988; Dabauvalle et al., 1988a,b; Benavente et al., 1989; Bataill  et al., 1990; Dargemont and K hn, 1992) and in cell-free systems (Finlay et al., 1987; Newmeyer and Forbes, 1988; Dabauvalle et al., 1990).

Patients with systemic autoimmune diseases spontaneously produce antibodies against a variety of nuclear components (Tan, 1989, 1991). These autoantibodies are not only of diagnostic relevance, but also represent valuable immunological tools in molecular and cellular biology. High titers of autoantibodies against major protein components of the nuclear envelope such as nuclear lamins, the lamin B receptor, and the transmembrane NPC glycoprotein gp210 have been found in patients with liver and rheumatic autoimmune diseases (Sen cal and Raymond, 1992; reviewed by Worman and Courvalin, 1991). In addition, NPC-specific antibodies were identified in serum from a patient suffering from polymyositis (Dagenais et al., 1988a,b). These autoantibodies decorated the cytoplasmic side of NPCs and reacted in immunoblots with polypeptides of 200 and 130 kD. Based on their cross reactivity with myosin heavy chain of skeletal muscle, it was suggested that the 200-kD NPC protein might be a myosin-like protein (Dagenais et al., 1988b).

In the present study we have used the serum from a patient with an overlap connective tissue disease. This serum was selected because it produced a distinctly punctate fluorescence pattern of the surface of mammalian cell nuclei which is diagnostic for NPC-specific antibodies (e.g., Davis and Blobel, 1986; Sen cal and Raymond, 1991; Worman and Courvalin, 1991). Here we present evidence that the antigen, an evolutionarily highly conserved protein with an estimated molecular mass of 180 kD, forms part of the cytoplasmic ring of NPCs and associated fibers.

Materials and Methods

Biological Materials

Xenopus laevis were purchased from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). Cell lines derived from human (HeLa and HEP-2), hamster (BHK-21), rat (RVF-SM), and marsupial (PtK2) tissues were cultured in DME (GIBCO-BRL, Eggenstein, Germany) supplemented with 10% FCS (GIBCO-BRL) at 37°C in a 5% CO₂ incubator.

Antibodies

Autoimmune serum 7217 from patient DGP suffering from an overlap connective tissue disease was selected for further analysis by its distinct punctate fluorescence of the nuclear surface of HEP-2 cells. Monoclonal murine antibody S49H2 directed against *Xenopus* lamin L_{III} was a gift of Dr. G. Krohne (Theodor-Boveri-Institute, University of W rzburg). Antibodies against gp210 were raised in guinea pigs by immunization with a synthetic peptide coupled to keyhole limpet hemocyanin. The peptide comprised residues 1859–1873 of the predicted amino acid sequence of gp210 (Wozniak et al., 1989). The antibodies stained in immunoblots a 190-kD band of rat liver nuclei which also bound the lectin Con A (see Gerace et al., 1982). The serum (S71) was kindly provided by M. Marzini (Theodor-Boveri-Institute, University of W rzburg). mAb PII directed against nucleoporin p62 (Dabauvalle et al., 1988a) and mAb 72B9 directed against the nucleolar protein fibrillarin (Reimer et al., 1987) have been described.

Isolation of Nuclei and Nuclear Envelopes

Nuclei were isolated from cultured cells as described by Krohne et al. (1981) with some modifications. Cells grown in monolayer were washed with TKM buffer (10 mM Tris-HCl, pH 7.2, 70 mM KCl, 3 mM MgCl₂) containing 1 mM PMSF, then scrape harvested with a rubber policeman followed by centrifugation at 1,500 g for 5 min at 4°C. The cell pellet (~10⁷ cells) was resuspended in 10 ml of 10 mM Tris-HCl, pH 7.2, containing 1 mM PMSF, incubated for 10 min at 4°C and lysed in a glass-Teflon homogenizer. Nuclei were recovered by centrifugation at 1,000 g for 20 min at 4°C. Nuclear envelopes were prepared from isolated PtK2 nuclei as described by Gerace et al. (1984).

Nuclei were isolated from liver of young rats after the procedure of Krohne et al. (1978). Nuclei and nuclear envelopes from *Xenopus* oocytes were manually isolated as described (Krohne and Franke, 1983).

Extraction and WGA-Sepharose Affinity Chromatography of Nup180

Peripheral membrane proteins were extracted from nuclear envelopes derived from 2 × 10⁷ PtK2 nuclei with 2 M urea/1 mM EDTA essentially as described (Radu et al., 1993). The TCA-precipitated supernatant was solubilized by a 5-min incubation in SDS-buffer at 65°C, loaded onto a WGA-Sepharose 6MB column (Pharmacia, Uppsala, Sweden), eluted with appropriate sugars and fractionated (for details see Radu et al., 1993). Proteins of the flow-through and the eluates were precipitated with 10% TCA, washed with cold acetone, separated by SDS-PAGE on 12% acrylamide gels and analyzed by immunoblotting (see below).

Gel Electrophoresis and Immunoblot

Proteins were resolved by SDS-PAGE (Thomas and Kornberg, 1975) using 12% acrylamide. Two-dimensional gel electrophoresis with IEF in the first dimension was carried out according to O'Farrell (1975). For immunoblots, polypeptides were electrophoretically transferred from gels to nitrocellulose paper (Kyhse-Anderson, 1984). The membrane was blocked by overnight incubation with 10% nonfat dry milk in PBS at 4°C, then incubated for 2 h at 15°C with either serum 7217 diluted 1:100 with PBS, affinity-purified autoimmune antibodies at a concentration of 2 µg/ml or guinea pig antiserum against gp210 diluted 1:100. After several washes in PBS, appropriate secondary antibodies coupled to alkaline phosphatase (Dianova, Hamburg, Germany) were added at a 1:7,500 dilution in PBS and incubated for 1 h at room temperature. After several wash steps, bound antibodies were visualized enzymatically using NBT (p-nitroblue tetrazoliumchloride) and BCIP (5-bromo-4-chloro indolylphosphate, p-toluidine salt; Biomol, Hamburg, Germany).

Affinity Purification of Antibodies

The human 7217 autoantibodies were affinity-purified following the protocol of Cordes et al. (1991) with some modifications. In brief, nuclear proteins of HEp-2 or PtK2 cells were separated by one-dimensional SDS-PAGE and transferred to nitrocellulose. The filter was stained with Ponceau S and the strip containing the 180-kD band was excised, cut into small pieces and incubated overnight at 4°C in PBS containing 10% milk. Then the filter pieces were incubated with 60 μ l serum 7217 diluted in 4.5 ml PBS for 2 h at 15°C. After four wash steps of 10 min each in PBS, bound antibodies were eluted from the nitrocellulose by a 30 s incubation with 1 ml glycine buffer (10 mM glycine, pH 2.3, 0.5 M NaCl, 0.1 mg/ml BSA). The elution buffer was then immediately neutralized by adding 3 ml PBS and 1 ml of 0.1 M Na₂HPO₄. The elution procedure was repeated two times. Finally the antibodies were concentrated by centrifugation (Centricon 10; Amicon, Witten, Germany).

Nuclear Assembly and Transport In Vitro

Extract was prepared from activated *Xenopus* eggs as described (Newport, 1987; Dabauvalle et al., 1991). Aliquots were frozen in liquid nitrogen and stored at -70°C. To initiate nuclear envelope assembly, demembrated *Xenopus* sperm nuclei prepared according to Blow and Laskey (1986) were added to the extract (~1000 nuclei/ μ l extract) together with an ATP-regenerating system. Aliquots were removed after an incubation time of 90 min at 22°C and processed for immunofluorescence microscopy. In some experiments, 5 μ l of affinity-purified anti-nup180 antibodies (2 mg/ml) or, as a control, purified mAb P11 directed against p62 (IgM, 4 mg/ml; Dabauvalle et al., 1990) were added to 50 μ l extract before incubation with sperm chromatin. Nuclear import was monitored by immunofluorescence microscopy using mAb 72B9 against the nucleolar protein fibrillarin (Bell et al., 1992).

Analysis of Nuclear Protein Import in *Xenopus* Oocytes

Microinjection experiments and analysis of the nucleocytoplasmic distribution of radioactively labeled nuclear proteins were performed as previously described (Dabauvalle et al., 1988a). Briefly, affinity-purified anti-nup180 antibodies (4 mg/ml) or, as control, nonimmune human IgGs at the same concentration were injected into the cytoplasm of *Xenopus* oocytes followed 3 h later by metabolically [³⁵S]methionine-labeled nuclear proteins. After an incubation time of 6 h, nuclei and cytoplasmic fractions were manually separated and analyzed by two-dimensional gel electrophoresis and autoradiography.

Immunofluorescence Microscopy

Small pieces of *Xenopus* ovary were shock frozen in isopentane cooled by liquid nitrogen. Frozen sections 5 μ m thick were air dried, incubated in acetone at -20°C for 10 min and air dried again. Cultured cells grown on coverslips were fixed for 10 min in -20°C methanol, transferred for 5 min in cold (-20°C) acetone and air dried. Nuclei assembled in egg extract were fixed and immobilized on microscope slides as described (Dabauvalle et al., 1991). The specimens were then incubated for 20 min at room temperature with the primary antibodies (serum 7217 diluted 1:50 in PBS or affinity-purified antibodies at a concentration of 2 μ g/ml). The lamin L_m-specific mAb S49H2 was used as 1:100 diluted ascites fluid. After several wash steps in PBS, appropriate Texas red-conjugated secondary antibodies (Dianova, Hamburg, Germany; diluted 1:150 in PBS) were added for another 20 min. Specimens were then counterstained for DNA with Hoechst 33258 (5 μ g/ml), washed in PBS, air dried from ethanol, and mounted in Mowiol (Hoechst, Frankfurt).

In some experiments cultured cells were rehydrated in PBS after methanol/acetone fixation for digestion with DNAase I (0.1 mg/ml in 10 mM Tris, pH 7.2, 2 mM MgCl₂) or pancreatic RNAase (0.1 mg/ml in 50 mM NaCl, 10 mM Tris, pH 7.2). After 10 min at room temperature the cells were washed for 5 min in PBS and processed for immunofluorescence microscopy as described above. Photographs were taken with a Zeiss Axiophot equipped with epifluorescence optics and the appropriate filter sets (Carl Zeiss Oberkochen, Germany).

Electron Microscopy

Procedures used for EM immunocytochemistry of isolated nuclear enve-

lopes from *Xenopus* oocytes have been described (Dabauvalle et al., 1988a). The manually isolated and unfixed nuclear envelopes were incubated with affinity-purified anti-nup180 at a concentration of 20 μ g/ml followed by anti-human IgG coupled to 10-nm gold particles (Amersham-Buchler, Braunschweig, Germany; diluted 1:10). Alternatively, incubation was performed with WGA coupled to 10-nm gold particles (diluted 1:10; Medac, Hamburg, Germany) for 45 min, followed by a PBS wash and fixation for EM. Immunogold-labeling with the p62-specific mAb P11 was done as described (Dabauvalle et al., 1988a). Cryostat sections (5 μ m) of ovaries from young *Xenopus* were fixed in acetone (5 min at -20°C), air dried and incubated with affinity-purified antibodies for 1 h. The tissue sections were then fixed with 2% formaldehyde in PBS (freshly prepared from paraformaldehyde) for 10 min. After thorough washing with PBS, anti-human IgG coupled to 10-nm gold particles was added for 3 h at room temperature. Specimens were then washed in PBS, postfixed, and processed for EM as described (Benavente et al., 1985). For double-immunolocalization experiments, antibodies against nup180 were applied together with mAb S49H2 (ascites fluid diluted 1:100 with PBS) followed by a mixture of human- and murine-specific secondary antibodies coupled to 12- and 5-nm gold particles, respectively.

Cultured rat (RVF-SM) cells grown on coverslips were fixed with 2% formaldehyde in PBS for 5 min. After several wash steps in PBS, the cells were permeabilized by treatment with 0.2% Triton X-100 in PBS for 5 min and washed again. The specimens were then incubated with affinity-purified antibodies followed by secondary gold-coupled antibodies and processed for EM as described above for the cryostat sections.

Results

Characterization of Autoimmune Serum 7217

The autoimmune serum 7217 from a patient suffering from overlap connective tissue disease was initially selected by immunofluorescence microscopy by its distinctly punctate nuclear surface staining of human HEp-2 cells. On immunoblots the serum reacted with several polypeptide bands of isolated nuclei from HEp-2, HeLa, BHK-21 and PtK2 cells including a prominent 180-kD component (not shown). To identify the antibodies that produced the characteristic punctate nuclear surface fluorescence, immunoglobulins were affinity-purified to different regions of nitrocellulose filters containing gel electrophoretically separated nuclear proteins from HEp-2 or PtK2 cells and probed separately by immunofluorescence microscopy. Only antibodies eluted from the 180-kD region yielded the fluorescence pattern indicative for NPCs. The other eluates were either negative or stained the whole nucleoplasm. Since the 180-kD protein turned out to be a component of the NPC (see below), we designate it nup (for nuclear pore complex protein or nucleoporin) 180 in accordance with Davis and Fink (1990). All results described below were obtained by using Western blot affinity-purified anti-nup180 antibodies.

For immunoblots, nuclear proteins from various mammalian cell lines were separated by one-dimensional SDS-PAGE and transferred to nitrocellulose. Affinity-purified anti-nup180 antibodies reacted in all samples selectively with a 180-kD band, occasionally in form of a double band (Fig. 1, lanes 1'-4'). Manual subfractionation of *Xenopus* oocyte nuclei revealed that the 180 kD protein remained associated with the isolated nuclear envelopes (Fig. 1, lane 5'). In addition to nup180, the antibodies recognized a 260-kD polypeptide of nuclear envelopes isolated from *Xenopus* oocytes (Fig. 1, lane 5'). The electrophoretic mobility of nup180 was consistently higher than that of myosin heavy chain run as molecular weight standard (Fig. 1). It is worth mentioning that nup180 was highly susceptible to degrada-

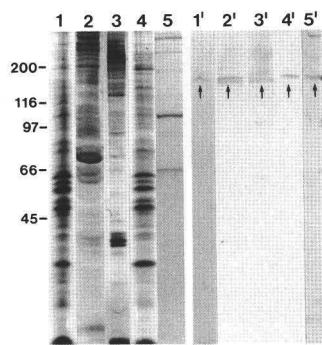


Figure 1. Immunoblots with affinity-purified anti-nup180 antibodies. Nuclear and nuclear envelope proteins were separated by SDS-PAGE and stained with Coomassie blue (lanes 1-5). Total proteins of 1×10^6 nuclei from HEP-2 (lane 1), HeLa (lane 2), BHK-21 (lane 3), and PtK2 (lane 4) cells and of 200 manually isolated nuclear envelopes from *Xenopus* oocytes (lane 5) were analyzed. Proteins from

a gel run in parallel were electrophoretically transferred to nitrocellulose and probed with affinity-purified anti-nup180 antibodies (lanes 1'-5'). In all samples the antibodies react with a 180-kD band (arrows), sometimes in form of a double band (lane 2'). An additional high molecular weight polypeptide of ~ 260 kD appears in the *Xenopus* nuclear envelope fraction (lane 5'). Positions of the molecular mass markers are indicated (in kD; from top to bottom myosin heavy chain, β -galactosidase, phosphorylase a, BSA, ovalbumin). Note that nup180 migrates faster than myosin heavy chain.

tion, even during storage of isolated nuclei at -20°C . The most prominent degradation product was a 74-kD polypeptide.

To determine the isoelectric point of nup180 we separated nuclear proteins of PtK2 cells by two-dimensional gel electrophoresis using IEF in the first dimension, followed by immunoblotting. Under these conditions nup180 displayed an isoelectric point which spread between pH 6.2 and pH 6.6 (not shown). Since nup180 was detectable neither in Coomassie blue nor silver stained gels it obviously represents only a minor component of total nuclear proteins.

To clarify whether nup180 is a peripheral or integral membrane protein, and whether or not it belongs to the GlcNAc-bearing nucleoporin family, we extracted nuclear envelopes of PtK2 cells with 2M urea/1 mM EDTA, i.e., conditions known to solubilize nucleoporins (Radu et al., 1993). The solubilized proteins were then fractionated by WGA-Sepharose chromatography and analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 2, nup180 was extractable from the nuclear envelopes under these conditions, but did not bind to WGA (Fig. 2 a, lane 1). In control experiments, p62 was detected in the WGA-binding fraction whereas gp210, a transmembrane glycoprotein associated with NPCs, was not extractable and remained in the nuclear envelope pellet (data not shown; see also Radu et al., 1993).

Some human autoimmune sera have been reported to contain antibodies directed against gp210 (Worman and Courvalin, 1991). In SDS-PAGE this protein has an apparent molecular mass of 190 kD (Gerace et al., 1982). To compare nup180 with gp210, proteins of isolated rat liver nuclei were separated by one-dimensional SDS-PAGE, blotted and probed with antibodies against nup180 and gp210. As illustrated in Fig. 2 b, nup180 clearly differed from gp210 by its higher mobility in SDS-PAGE. Furthermore, nup180 did not bind the lectin Con A in contrast to gp210 (data not shown). From these experiments we conclude that nup180 is a peripheral nuclear membrane protein, is not a member of the

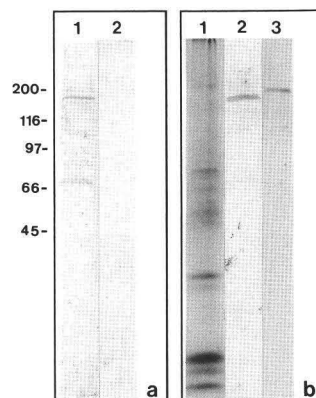


Figure 2. Biochemical characterization of nup180. Peripheral membrane proteins were extracted from isolated PtK2 nuclear envelopes by 2 M urea and fractionated by WGA-Sepharose affinity chromatography (a). Proteins that did not bind to WGA (flow-through, lane 1) and bound proteins (lane 2) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to nup180. Nup180 appears exclusively in the flow-through

fraction (lane 1, the lower reactive band of 74 kD represents the major degradation product of nup180). (b) Proteins of rat liver nuclei were separated by SDS-PAGE and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose and probed with affinity-purified anti-nup180 (lane 2) or guinea pig antiserum to gp210 (lane 3). Note that nup180 runs faster than gp210, which has a mobility corresponding to 190 kD. Position of the molecular mass markers are indicated in kD.

WGA-binding nucleoporin family, and is different from gp210.

Purified anti-nup180 antibodies did not react in immunoblots with rabbit myosin heavy chain, even when 10 μg purified myosin was applied to a single gel lane. This result together with the above mentioned finding that nup180 migrated faster in SDS-PAGE than myosin heavy chain (Fig. 1) speaks against a relationship between both proteins.

Light Microscopic Immunolocalization

When human HEP-2 cells were labeled with affinity-purified antibodies to nup180 for immunofluorescence microscopy, optical sections of the nuclei revealed a prominent nuclear "rim" staining (Fig. 3 a'). When focused on the top surface of nuclei, a punctate pattern characteristic for NPC proteins could be discerned as illustrated for rat kangaroo PtK2 cells (Fig. 3 b'). Essentially the same fluorescent pattern was seen with other mammalian cell lines such as HeLa and BHK. During mitosis, fluorescence was no longer restricted to the nuclear periphery but became dispersed throughout the cell with no detectable enrichment at the chromosome surfaces (Fig. 3 a', arrows). Analogous changes of the fluorescence pattern during mitosis have been observed with antibodies against GlcNAc-modified nucleoporins (e.g., Davis and Blobel, 1986; Park et al., 1987; Snow et al., 1987; Benavente et al., 1989), a nucleoporin lacking GlcNAc residues (nup155; Radu et al., 1993) and the integral membrane glycoprotein gp210 (Gerace et al., 1982). After treatment of cells with DNAase or RNAase before incubation with anti-nup180, the fluorescence was essentially unaltered.

Fluorescent dot-like structures were also seen in the cytoplasm of HEP-2 cells, though in variable amounts from cell to cell (Fig. 3 a'). By using immunogold EM we have recently obtained evidence that these fluorescent cytoplasmic bodies represent annulate lamellae (Ewald, A., J. L. Senécal, U. Scheer, and M.-C. Dabauvalle, manuscript in preparation).

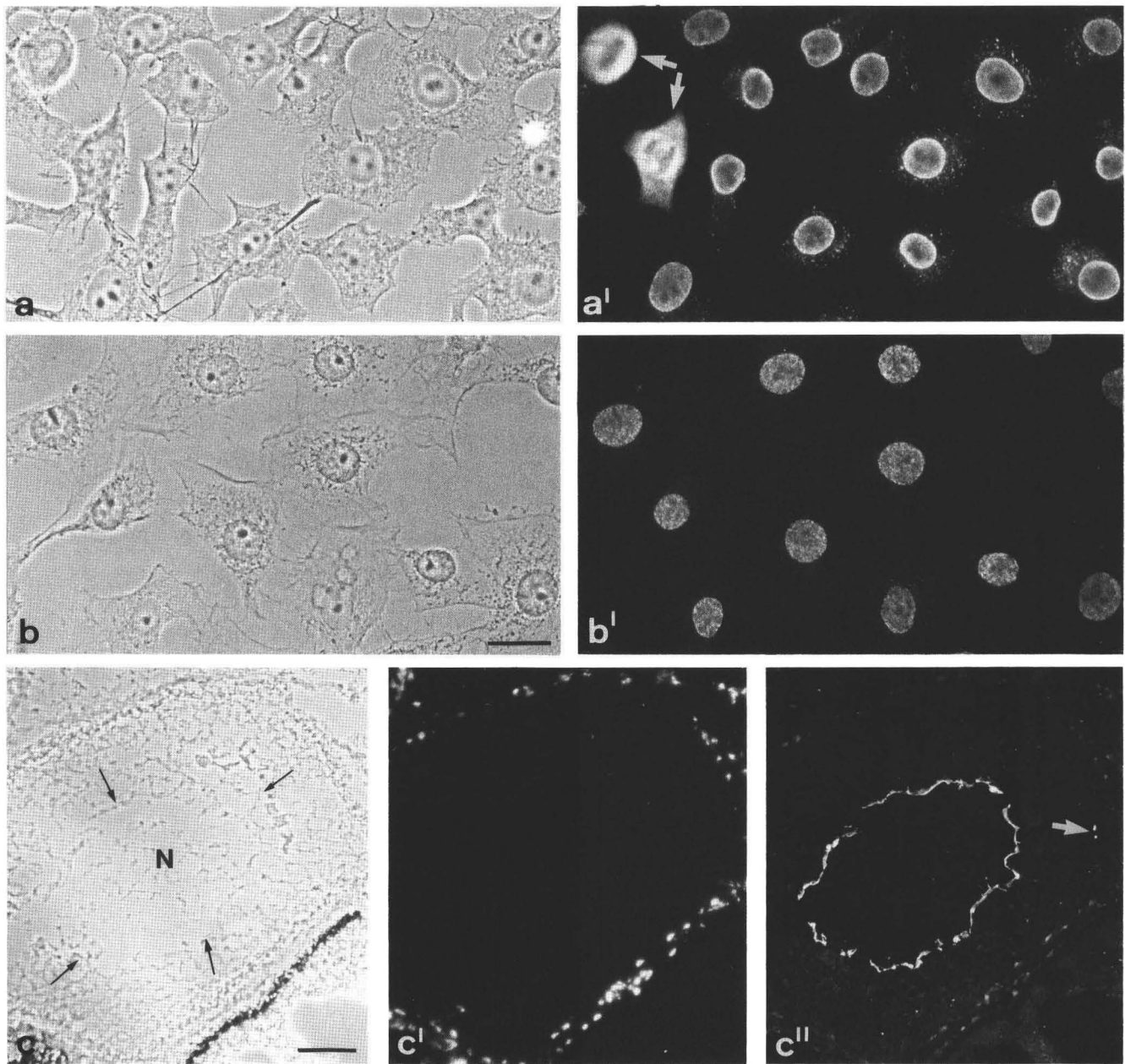


Figure 3. Localization of nup180 in cultured human HEP-2 cells (*a*), rat kangaroo PtK2 cells (*b*), and on a frozen section of a *Xenopus* oocyte (*c*) by immunofluorescence microscopy. The corresponding phase contrast images are shown (*a*–*c*). Depending on the focal plane, nuclei reveal either a prominent “rim” staining (*a*) or the characteristic punctate surface fluorescence (*b*). The fluorescent dots seen in the cytoplasm of HEP-2 cells most likely reflect the presence of annulate lamellae. During mitosis, nup180 is dispersed throughout the cytoplasm (*a*). A previtellogenic *Xenopus* oocyte with its surrounding follicle cell layer is shown in phase contrast (*c*), the position of the nuclear envelope is indicated by arrows) and after DNA staining with Hoechst (*c'*). Hoechst fluorescence reveals the nuclei of the follicle cells. Anti-nup180 antibodies stain strongly the nuclear envelope of the oocyte and, though weaker, the nuclear periphery of the follicle cells (*c''*). Some fluorescent dots, probably reflecting annulate lamellae, are observed in the oocyte cytoplasm (arrow in *c''*). *N*, nucleus. Bars, (*a* and *b*) 20 μm ; (*c*) 50 μm .

Immunofluorescence microscopy on frozen sections through *Xenopus* ovary showed strong and specific staining of the nuclear envelope of oocytes (Fig. 3 *c''*). The fluorescence pattern was not punctate but rather uniform, most likely due to the high pore density in this material (a continuous labeling of the nuclear periphery of *Xenopus* oocytes has also been observed with antibodies against p62; Dabauvalle

et al., 1988a). In addition to the nuclear envelope staining, some fluorescent dot-like structures occurred in the cytoplasm of the oocytes (Fig. 3 *c''*, arrow), probably reflecting the presence of annulate lamellae. Comparable cytoplasmic fluorescent dots have also been observed in *Xenopus* oocytes after staining with antibodies to p62 (Dabauvalle et al., 1991).

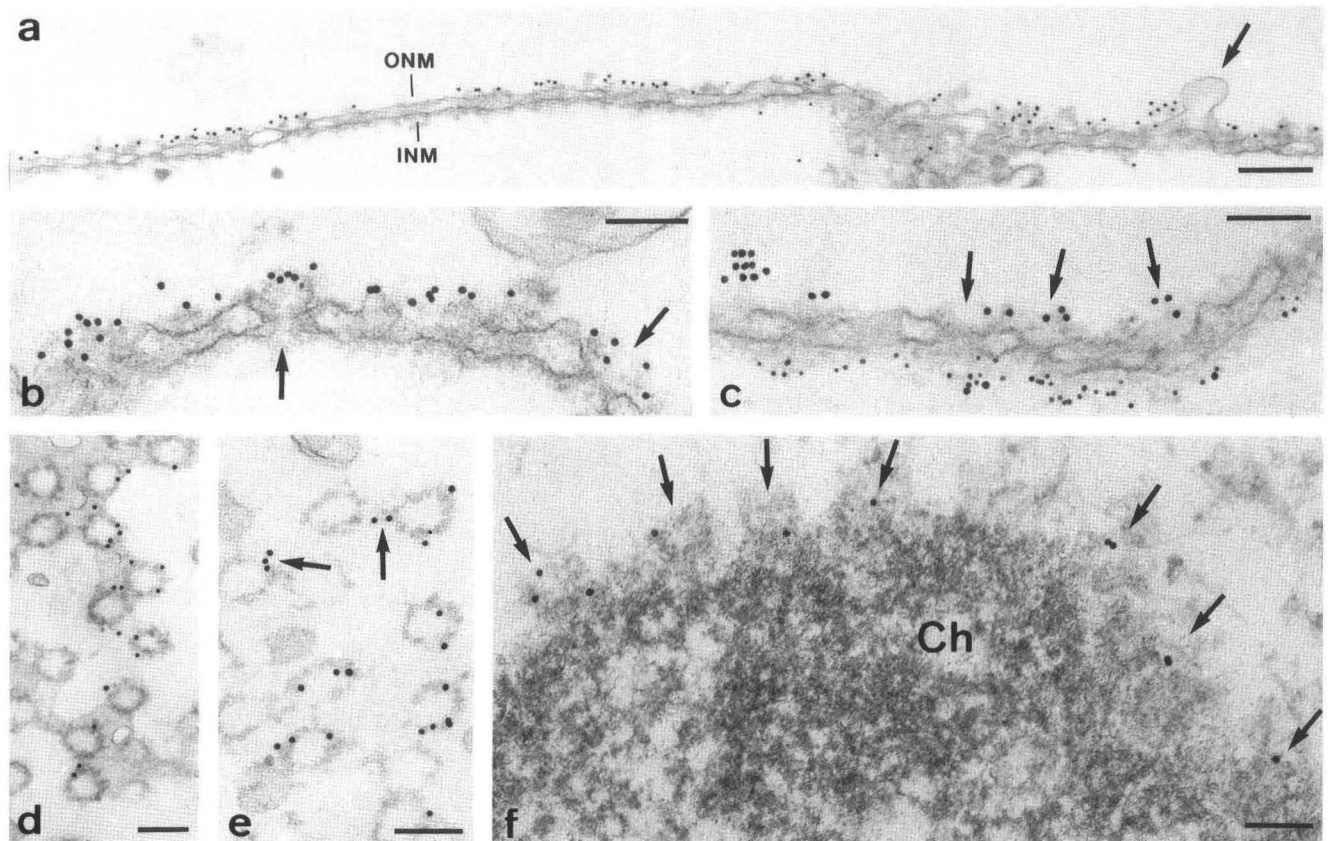


Figure 4. Preembedding immunogold EM localization of nup180. Cryosections of *Xenopus* oocytes (*a-c*), manually isolated nuclear envelopes from *Xenopus* oocytes (*d* and *e*) and Triton-permeabilized rat RVF-SM cells (*f*) were incubated with affinity-purified anti-nup180 followed by secondary gold-conjugated antibodies. The outer (cytoplasmic) face of NPCs is selectively labeled as seen in transversely sectioned nuclear envelopes (*a-c*). The outer nuclear membrane (*ONM*) often forms blebs (*arrow* in *a*; *INM*, inner nuclear membrane). Gold particles decorate the cytoplasmic NPC rings or short fibrils projecting therefrom (*b* and *c*, some NPCs are denoted by *arrows*). The nucleoplasmic side of the nuclear envelope is identified in *c* by double label immunogold EM using a mAb against *Xenopus* lamin L_{III} (small gold; the larger gold particles reveal the distribution of anti-nup180). Tangential sections reveal labeling of cytoplasmic pore rings (*d* and *e*) and associated fibers (*arrows* in *e*). Gold particles also decorate the cytoplasmic face of NPCs in rat cells (*f*, NPCs are denoted by *arrows*). *Ch*, chromatin. Bars, 0.1 μ m.

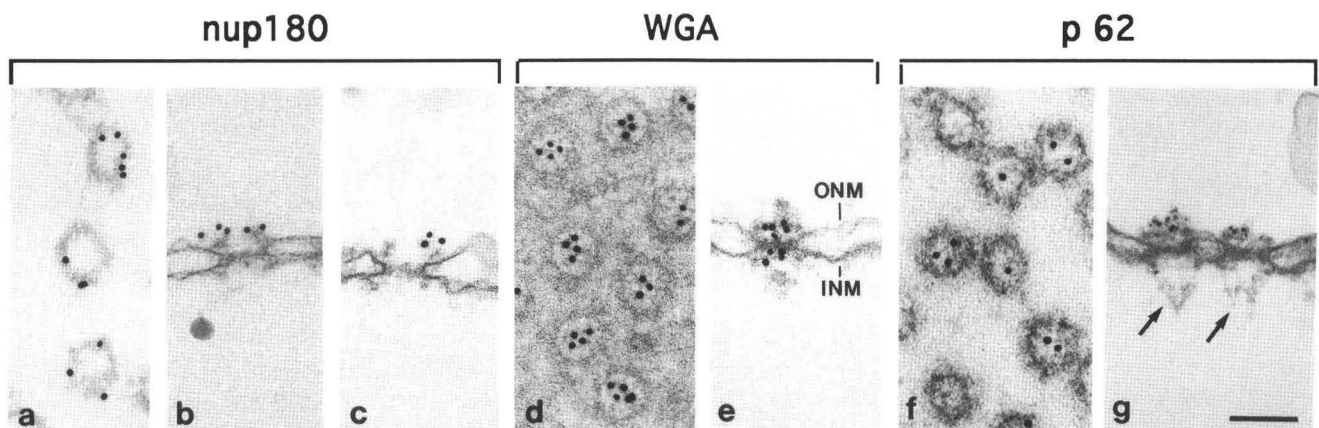


Figure 5. Comparison of NPC labeling with anti-nup180 (*a-c*), gold-conjugated WGA (*d* and *e*) and anti-p62 (*f* and *g*). Nuclear envelopes were isolated manually from *Xenopus* oocytes, incubated with the antibodies or WGA and processed for EM. Shown are representative examples of transversely and tangentially sectioned nuclear envelopes. The cytoplasmic side of all cross-sectioned nuclear envelopes faces the top of the figure. Fibrils projecting from the nucleoplasmic NPC ring are indicated (*g*, *arrows*). Bar, 0.1 μ m.

Electron Microscopic Immunolocalization

To localize nup180 at the ultrastructural level, we used preembedding immunoelectron microscopy. Manually isolated nuclear envelopes of *Xenopus* oocytes or, alternatively, frozen sections (5 μm thick) of previtellogenic *Xenopus* oocytes were incubated with affinity-purified anti-nup180 followed by 10-nm gold-conjugated anti-human IgG. Strikingly, the gold particles exclusively decorated the cytoplasmic side of transversely sectioned nuclear envelopes, and were concentrated at the NPCs (Fig. 4, *a* and *b*). At higher magnification the gold particles were seen to decorate the cytoplasmic ring of the NPCs as well as short fibrils projecting therefrom (Figs. 4 *b* and 5, *b* and *c*).

To unequivocally identify the topology of the nuclear envelopes, we performed double immunogold labeling using anti-nup180 and a mAb directed against a protein of the nuclear lamina. The result clearly demonstrated that nup180 was located at the cytoplasmic side of the NPCs (Fig. 4 *c*) and, further, that the nucleoplasmic face of the nuclear envelope was accessible to antibodies under the incubation conditions. The topology of the nuclear envelope could also be inferred from the tendency of the outer nuclear membrane to locally disintegrate by bleb formation, probably due to the absence of a stabilizing lamina layer (Fig. 4 *a*, arrow). In sections glancing the outer surface of nuclear envelopes, the association of gold particles with the cytoplasmic ring of the NPCs was especially evident (Figs. 4, *d* and *e* and 5 *a*). Not infrequently, short fibrils radiating from the cytoplasmic NPC ring were also labeled (Fig. 4 *e*, arrows). It is interesting to note that the amount of labeling was generally lower when isolated oocyte nuclear envelopes were used as opposed to frozen sections of oocytes. This may indicate that the antigen is partially lost from the NPCs during the isolation procedure due to connections with cytoplasmic structures.

When cultured rat cells were labeled with anti-nup180, gold particles were also found exclusively at the cytoplasmic face of the NPCs (Fig. 4 *f*).

The distribution of nup180 within the substructures of the NPC clearly differed from that of the GlcNAc-modified nucleoporins. Fig. 5 presents a panel of representative NPCs in transverse and tangential views after labeling with anti-nup180, WGA and anti-p62 (mAb PII). With anti-nup180, immunogold decoration was found exclusively on the cytoplasmic ring of the NPCs or short fibers projecting into the cytoplasm (Fig. 5, *a-c*). In contrast, WGA-gold labeled preferentially the central pore region, i.e., the site of the transporter assembly (Fig. 5, *d* and *e*; for a quantitative evaluation of WGA binding sites, see Akey and Goldfarb, 1989). The most abundant member of the GlcNAc-bearing nucleoporins, p62, localized close to the central pore axis, with some preference for the cytoplasmic side (Fig. 5, *f* and *g*).

Antibodies to Nup180 Do Not Interfere with Nuclear Import

Recently we have shown that injection of the p62-specific mAb PII into the cytoplasm of *Xenopus* oocytes effectively inhibited nuclear protein import (Dabauvalle et al., 1988a; Benavente et al., 1989). In striking contrast, nuclear protein

transport was not affected after injection of anti-nup180 antibodies (data not shown).

Extract prepared from activated *Xenopus* eggs is capable of reconstituting nuclear envelopes around exogenously added chromatin or DNA. The resulting nuclei contain a large number of NPCs which are morphologically indistinguishable from those of normal nuclei, and are functionally active since they transport karyophilic proteins in a signal sequence and ATP-dependent manner (reviewed in Lohka, 1988; Laskey and Leno, 1990; Forbes, 1992). When probed

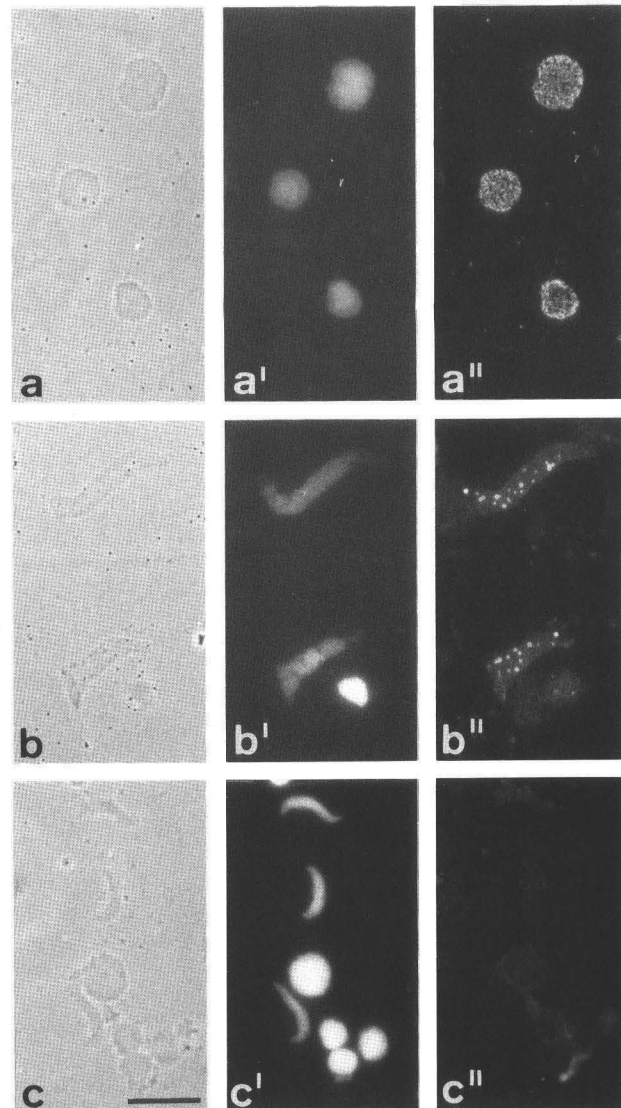


Figure 6. Nuclei assembled from sperm chromatin in *Xenopus* egg extract show a punctate surface fluorescence after reaction with anti-nup180 (*a'*). When the nuclei are allowed to form in the presence of affinity-purified anti-nup180 antibodies, nuclear protein uptake is not inhibited. This is shown by immunofluorescence of the newly assembled nuclei with antibodies to the nucleolar protein fibrillarin which stain numerous intranuclear dot-like structures (*b'*; see Bell et al., 1992). In contrast, in the presence of antibodies to p62 the in vitro nuclei are unable to take up proteins and are thus devoid of the fluorescent dots (*c'*). The corresponding phase contrast images (*a-c*) and Hoechst fluorescence (*a'-c'*) are shown. Bar, 20 μm .

with anti-nup180, the *in vitro* reconstituted nuclei revealed a distinctly punctate fluorescence pattern on their outer surfaces (Fig. 6 *a''*), indicating that nup180 is a component of the newly formed NPCs.

As antibodies to p62 have been shown to inhibit active transport of karyophilic proteins into nuclei assembled in *Xenopus* egg extract (Dabauvalle et al., 1990), we have analyzed the effect of antibodies to nup180 on nuclear transport with this experimental system. After addition of affinity purified anti-nup180 antibodies to the extract, nuclei formed normally from sperm chromatin and were perfectly capable of accumulating karyophilic proteins as demonstrated by the emergence of numerous fibrillar-containing aggregates (Fig. 6 *b''*; see also Bell et al., 1992). When NPC-mediated transport was inhibited by addition of anti-p62 (mAb P11) to the extract, the dense aggregates did not form (Fig. 6 *c''*). In conclusion, antibodies to nup180 did not interfere with nuclear protein transport, neither *in vivo* nor *in vitro*.

Discussion

In the present study we have identified an evolutionarily conserved NPC protein which we have designated nup180 (for nuclear pore complex protein or nucleoporin) with a molecular mass of 180 kD as estimated from its mobility in SDS-PAGE and an isoelectric point at a pH of ~ 6.2 . On the basis of its extractability from isolated nuclear envelopes with 2 M urea/1 mM EDTA we classify nup180 as a peripheral membrane protein (Radu et al., 1993). Nup180 is not reactive with WGA and hence does not belong to the family of GlcNAc-containing pore proteins. Another nucleoporin lacking GlcNAc sugar moieties has recently been described in rat liver (nup155) with an apparent molecular mass of 140 kD as determined by SDS-PAGE, which localizes to the nucleoplasmic and cytoplasmic sides of the NPCs (Radu et al., 1993).

By immunogold EM performed on isolated nuclear envelopes and cryosections of *Xenopus* oocytes, human autoimmune antibodies against nup180 resulted in a striking asymmetric labeling of NPCs. Gold particles occurred almost exclusively at the cytoplasmic face of the NPCs and specifically decorated the cytoplasmic rings and the short fibrils projecting from the rings into the cytoplasm. The topological disposition of nup180 was clearly different from that of the WGA-binding nucleoporins (a comparison of the NPC labeling patterns with anti-nup180, WGA, and anti-p62 is shown in Fig. 5). Thus, gold-tagged WGA did not label the cytoplasmic rings, but rather the central regions of the NPCs where nucleocytoplasmic transport processes are known to take place (for a detailed mapping of the WGA binding sites see Akey and Goldfarb, 1989). A similar labeling pattern was obtained with mAb P11 against p62, the predominant member of the WGA-binding nucleoporin family.

Nup 180 is distinct from other previously identified NPC proteins. Among the GlcNAc-modified nucleoporins is a protein with an apparent molecular mass of 180 kD (p180; Snow et al., 1987). Using a monospecific mAb (antibody RL11), these authors detected p180 exclusively at the nucleoplasmic side of the NPC. With the help of another mAb, termed mAb 322, Sukegawa and Blobel (1993) have identified a WGA-binding NPC protein which appears to be identical with p180. Just as for p180, it is also located on the

nucleoplasmic side of the NPC (Sukegawa and Blobel, 1993). This protein (now termed nup153) has been sequenced and found to contain zinc finger motifs characteristic for DNA-binding proteins. Taken together, the absence of WGA reactivity, and the localization to the NPC cytoplasmic ring and associated fibers clearly distinguishes nup180 from p180/nup153.

Nup180 is also distinct from a transmembrane NPC glycoprotein of an apparent molecular mass of 190 kD on SDS-polyacrylamide gels (Gerace et al., 1982) that, based on its predicted molecular mass, was renamed gp210 (Wozniak et al., 1989). First, nup180 is solubilized from nuclear membranes with 2 M urea whereas gp210 resists such extraction conditions; second, nup180 migrates faster on SDS-polyacrylamide gels than gp210; third, nup180 does not react with Con A unlike gp210; and fourth, the topology of nup180 in the NPC differs from that of gp210 (Gerace et al., 1982; Greber et al., 1990).

Nup180 is also distinct from myosin heavy chain. Apart from the clearly different gel electrophoretic mobilities of both proteins, antibodies to nup180 react neither in immunoblot nor immunocytochemical analyses with myosin. Further, the symmetrical labeling of NPCs with anti-myosin antibodies (Berrios et al., 1991) clearly differs from the asymmetric pattern obtained in the present study with the nup180 antibodies.

From EM studies it is well known that short fibrils extend from the cytoplasmic NPC ring and/or the eight attached annulus granules into the cytoplasm (Kessel, 1973; Franke and Scheer, 1974; Maul, 1977; Scheer et al., 1988; Ris, 1991; Jarnik and Aebi, 1991; Forbes, 1992; Panté and Aebi, 1993). We found immunogold decoration on these fibers as well as the cytoplasmic ring proper, indicating that nup180 is part of both structures. Since it has been shown that the cytoplasmic rings and the fibers projecting from them bind karyophilic proteins before their translocation through the NPC (Richardson et al., 1988; Newmeyer and Forbes, 1988) we assessed the effect of anti-nup180 antibodies on nuclear protein transport. Microinjection of the antibodies into the cytoplasm of *Xenopus* oocytes as well as their addition to a cell-free system did not affect nuclear protein transport to any noticeable extent. Similarly, injection of the antibodies into the cytoplasm or nucleus of *Xenopus* oocytes did not inhibit RNA export (A. Jarmolowski; European Molecular Biology Laboratory, Heidelberg, unpublished observations). Thus, at the moment we have no positive evidence that nup180 is directly involved in nucleocytoplasmic transport processes as this is the case for the WGA-binding nucleoporins (for references see introduction).

A number of electron microscopic studies have suggested connections of cytoplasmic intermediate filaments to the nuclear envelope (for reviews see French et al., 1989; Carmo-Fonseca and David-Ferreira, 1990; Hansen and Ingber, 1992). It is tempting to speculate that nup180 may be involved in establishing a structural link between the nucleoplasmic face of the NPCs and the cell cytoskeleton. In fact, short 5-nm-thick filaments which morphologically resemble the fibrils emanating from the cytoplasmic NPC ring have been described to link bundles of intermediate filaments to NPCs (Carmo-Fonseca et al., 1987). It is conceivable that NPCs not only provide the gateways for nucleocytoplasmic exchange of macromolecules, but also provide a physical

connection between the genome via the DNA-binding nucleoporin nup153 (Sukegawa and Blobel, 1993) and the cytoskeleton via the cytoplasmically exposed NPC protein nup180 described in the present study.

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