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Nup358, a Cytoplasmically Exposed Nucleoporin with Peptide Repeats, Ran-GTP Binding Sites, Zinc Fingers, a Cyclophilin A Homologous Domain, and a Leucine-rich Region*

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The Ras-related nuclear protein, Ran, has been implicated in nuclear transport. By screening a HeLa cell λ expression library with Ran-GTP and sequencing overlapping cDNA clones, we have obtained the derived primary structure of a protein with a calculated molecular mass of 358 kDa. Using antibodies raised against an expressed segment of this protein, we obtained punctate nuclear surface staining by immunofluorescence microscopy that is characteristic for nucleoporins. Electron microscopy of immunogold-decorated rat liver nuclear envelopes sublocalized the 358-kDa protein at (or near) the tip of the cytoplasmic fibers of the nuclear pore complex (NPC). In agreement with current convention, this protein was therefore termed Nup358 (for nucleoporin of 358 kDa). Nup358 contains a leucine-rich region, four potential Ran binding sites (*i.e.* Ran binding protein 1 homologous domains) flanked by nucleoporin-characteristic FXFG or FG repeats, eight zinc finger motifs, and a C-terminal cyclophilin A homologous domain. Consistent with the location of Nup358 at the cytoplasmic fibers of the NPC, we found decoration with Ran-gold at only the cytoplasmic side of the NPC. Thus, Nup358 is the first nucleoporin shown to contain binding sites for two of three soluble nuclear transport factors so far isolated, namely karyopherin and Ran-GTP.

Bidirectional traffic of macromolecules (proteins, ribonucleoproteins, and deoxyribonucleoproteins) into and out of the nucleus proceeds through the nuclear pore complex (NPC).[1] The NPC consists of a central opening of ~40 nm in diameter surrounded by a scaffold that is attached to the pore membrane. Fibers emanate from the NPC for at least 50 nm into the cytoplasm, and a nuclear basket extends into the nucleoplasm (for review, see Ref. 1). A number of NPC proteins (collectively termed nucleoporins) have now been molecularly characterized. Of particular interest is a subgroup of nucleoporins that contain domains with short peptide repeats (for review, see Ref. 1). These peptide repeat-containing nucleoporins have been shown to function as docking sites in transport factor-mediated protein import (2), and, for one of these nucleoporins, Nup98, the docking site has indeed been mapped to its peptide repeat-containing domain (3). These data provide a direct biochemical link between nucleoporins and soluble transport factors.

The requirement for soluble transport factors for protein import into nuclei has been demonstrated in an *in vitro* system (4). This system consisted of digitonin-permeabilized cells that retained import-competent nuclei with intact nuclear envelopes (NEs) but that had lost most of their soluble cytosolic proteins. Protein import into these nuclei depended on exogenously added cytosol. Subfractionation of the cytosol yielded two fractions (A and B) with distinct activities (5). Fraction A activity recognized a nuclear localization sequence (NLS)-containing transport substrate and docked it at the NPC, whereas fraction B activity mediated translocation into the nucleus. The active component of fraction A has been purified and shown to consist of a stoichiometric complex of two proteins referred to as karyopherin α and β (2, 6–8). Karyopherin α (synonymous with NLS receptor or importin) recognizes the NLS, whereas karyopherin β functions as an adapter that links the karyopherin α-NLS substrate complex to the repeat-containing nucleoporins (2, 3, 8). The active components of the B fraction are the small GTPase Ran (9, 10) and a 10-kDa Ran interactive protein (11). A Ran-GTP/GDP cycle has been proposed to mediate docking and release of the karyopherin-substrate complex along an array of repeat-containing nucleoporins extending from the tips of the cytoplasmic fibers to the nucleoplasmic basket, resulting in guided diffusion across the NPC (3, 12).

In overlay assays, Ran-GTP (but not Ran-GDP) has been shown to bind specifically to a number of cellular proteins (13, 14). One of these proteins, termed RanBP1 (for Ran Binding Protein), has previously been characterized (13, 15). Here we report the molecular characterization and cellular localization of a second Ran binding protein. Its molecular mass of 358 kDa was calculated from its cDNA-derived primary structure. Immunoelectron microscopy localized the protein to the NPC, specifically to its cytoplasmically exposed fibers. We therefore termed this protein Nup358. In addition to four RanBP1 homologous domains, Nup358 contains eight zinc finger motifs of the Cys_2-Cys_2 type, several FXFG and FG repeats, a leucine-rich region, and a C-terminal cyclophilin A-like domain. Electron microscopy (EM) of isolated NEs that were incubated with Ran coupled to gold showed decoration of only the cytoplasmic side of the NPC, consistent with the localization of Nup358.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L41840.

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[1] The abbreviations used are: NPC, nuclear pore complex; Ran, Ras-related nuclear protein; RanBP, Ran binding protein; Nup, nucleoporin; NE, nuclear envelope; NLS, nuclear localization sequence; EM, electron microscopy; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Molecular Cloning—Recombinant Ran was prepared and loaded with [α-^{32}P]GTP as described (13). A HeLa cell cDNA library was made in λEXlox vector (Novagen) as described (16) and was screened with Ran-[α-^{32}P]GTP (13). Of 1.2 million plaques screened, 69 positive clones were identified. Among them, 32 were RanBP1 (13) and 28 were Nup358. Clones 7-1, 13-2, 7-4, 11-2, 14-1, 23-2, and 6-2, spanning most of Nup358, were characterized. To obtain sequences further to the 5′ end, the same library was screened by DNA hybridization with a probe of about 380 base pairs from the 5′ end of the clone 7-4. Nine positive clones were identified, among which one clone, 5A, contained sequences extending further to the 5′ terminus. 5′ End sequences were also obtained through two rounds of PCR amplification of the library with two pairs of nested primers. The upstream nested primers annealed to the vector directly upstream of the cloning site, and downstream nested primers annealed to sequences of clone 5A, corresponding to amino acids 93–100 and 84–92. The amplified PCR fragment was cloned into TA cloning vector (Invitrogen) and sequenced. Standard molecular biology techniques were used for all analyses (17).

Production of a Recombinant Protein and Antibodies and Immunological Analyses—A DNA fragment encoding amino acids 2550–2837 was obtained by PCR using clone 6-2 as a template and was inserted into *Escherichia coli* expression vector pQE-30 (Qiagen). Recombinant protein (called C-288) was induced by isopropyl-1-thio-β-D-galactopyranoside and was purified using a Nickel-NTA agarose column according to the manufacturer (Qiagen). Antibodies were produced by immunizing mice with the purified recombinant protein C-288 (18). The resulting anti-Nup358 antiserum was used at a dilution of 1:200 for immunoblotting (18) and immunofluorescence microscopy (19).

Microsequencing of the Rat Homolog of Nup358—NEs from rat livers were extracted with 2 M urea and 1 mM EDTA as described previously (20). The proteins were separated by SDS-PAGE (3.75–7.50% acrylamide) and transferred to polyvinylidene difluoride membrane. The 350-kDa band was cut out, digested with the endoproteinase Glu-C (Sigma), and peptides were separated and sequenced as described (21).

Electron Microscopy—Rat liver NEs were prepared as described previously (22), with slight modifications.[2] For immunogold localization of Nup358, isolated NEs were fixed for 15 min in 2.5% formaldehyde in STM (10% sucrose, 20 mM triethanolamine-HCl (pH 7.5), 0.1 mM $MgCl_2$) and pelleted at 2,000 × *g* for 5 min onto 35-mm plastic dishes. Subsequent antibody incubations and washes were performed in the dishes with buffer A (1% bovine serum albumin, 68 mM NaCl, 13 mM KCl, 15 mM KH_2PO_4, 40 mM Na_2HPO_4, 0.5 mM phenylmethylsulfonyl fluoride). Attached NEs were washed three times, samples were incubated with anti-Nup358 polyclonal mouse serum diluted 1:400, and binding was detected with goat anti-mouse IgG conjugated to 10 nm gold (Amersham Inc.) and diluted 1:50. Processing for thin sectioning and EM were performed as described previously (23).

Ran binding to isolated NEs was detected using recombinant Ran (13) conjugated, as described by the manufacturer (Amersham Inc.), directly to 10 nm gold. A standard binding reaction consisted of: 20 μl of isolated NEs (derived from 3 × 10^6 nuclei) suspended in STM plus 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin A, and aprotinin, 14 μl of buffer B (20 mM Hepes (pH 7.3), 110 mM potassium acetate, 2 mM $Mg(OAc)_2$, 1 mM EGTA), 2 μl of 20 mM GTP in buffer B, 2 μl of 20 mg/ml bovine serum albumin in buffer B, and 2 μl of Ran-gold in buffer B. Reactions were incubated for 15 min at room temperature and terminated by the addition of 40 volumes of reaction buffer (50% STM, 50% buffer B) plus 2.5% glutaraldehyde. After 30 min on ice, samples were spun onto plastic dishes and processed as above for thin sectioning and EM. To detect the binding of Ran to the surface of intact isolated NEs, fixed samples were pelleted at 2,000 × *g*, washed once with distilled water, and, after resuspending in 10 μl of distilled water, bound to glow-discharged Formvar carbon-coated copper grids. Samples were viewed by EM after staining with 2% uranyl acetate.

RESULTS

We have probed a λ expression library of HeLa cell cDNAs with Ran-GTP. Sequencing of overlapping cDNA clones yielded the deduced primary structure for a protein with a calculated molecular mass of 358 kDa (Fig. 1, *A* and *B*). Because this protein was localized to the NPC (see below), it was named Nup358. Consistent with the ability of Nup358 to bind Ran-GTP, it possesses four sites that showed similarity to RanBP1 (Fig. 1*C*). Nup358 also contains repeats of FG and FXFG that are among the signature motifs of peptide repeat-containing nucleoporins. In addition, Nup358 has eight similar zinc finger motifs of the Cys_2-Cys_2 type (Fig. 1*D*) as well as a C-terminal cyclophilin A-like domain and an N-terminal leucine-rich region (Fig. 1*B*).

A 288-amino-acid segment corresponding to residues 2550–2837 of Nup358 (Fig. 1*A*) was expressed in *E. coli*, purified (using its His tag), and injected into mice for antibody production. Probing SDS-PAGE separated proteins of rat liver NEs with the antibodies yielded decoration primarily of one protein of ~350 kDa (Fig. 2, *lane 4*). There were also weaker reactions with bands below and above the 200-kDa marker. These bands are likely to be degradation products of Nup358 since they are not consistently seen, although they could also represent distinct cross-reactive proteins. A polypeptide of similar mobility as the antibody-reactive 350-kDa band can be seen among the Amido Black-stained polypeptides of the rat liver NEs (Fig. 2, *lane 1*). As expected, a protein of similar mobility was also one of the major Ran-GTP-reactive polypeptides (Fig. 2, *lane 3*) and reacted with monoclonal antibody 414 (Fig. 2, *lane 2*), most likely because of the presence of FXFG repeats. To determine whether this rat NE protein is the homolog of human Nup358, we did partial amino acid sequencing of the 350-kDa band. We found that the obtained peptide sequences of this rat protein indeed matched the corresponding cDNA-deduced amino acid sequence of human Nup358 (Fig. 3).

Immunofluorescence microscopy of paraformaldehyde-fixed HeLa cells showed the nucleoporin-characteristic punctate staining when focusing on the nuclear surface (Fig. 4) and occasional punctate staining in the cytoplasm.

To sublocalize Nup358 within the NPC, immunogold EM was done using isolated rat liver NEs. Nup358 appears to be associated with the tips of the cytoplasmic fibers emanating from the NPC (Fig. 5, *top*). Quantitative analysis of the distribution of gold particles (n = 87) showed them to peak at a mean distance of 59 nm from the midplane of the NE (data not shown). These data suggest that Nup358 is located at or near the tip of the cytoplasmically exposed NPC fibers.

As Nup358 binds Ran-GTP, NPCs in nuclear envelopes could be expected to bind to it as well. Using Ran directly coupled to 10 nm gold, we found gold decoration of NPCs, exclusively on their cytoplasmic side (Fig. 5, *middle*), consistent with the localization of Nup358. Quantitative analysis (n = 65) showed Ran-gold to peak at a mean distance of 46 nm from the midplane of the NE (data not shown).

Striking ring-like surface decorations of NPCs can be seen after isolated rat liver NEs were reacted with Ran-gold, deposited on a grid, and slightly stained with uranyl acetate (Fig. 5, *bottom*).

DISCUSSION

Nup358 is the largest nucleoporin that has so far been molecularly characterized. It has previously been detected in urea extracts of NEs as a potential nucleoporin that reacted with monoclonal antibody 414 but not with wheat germ agglutinin (2). Most importantly, in an overlay assay in which NE proteins separated by SDS-PAGE were probed with NLS-containing substrate in the presence of karyopherin, Nup358 (previously referred to as p270 (2)) was found to be one of several docking site nucleoporins for karyopherin-mediated binding of an import substrate (2). The repeat-containing domains of the nucleoporins have been proposed to form an array of multiple docking sites that extend from the cytoplasmic to the nucleoplasmic ends of the NPC along which the transport substrate would be moved in a factor-mediated fashion by guided diffusion (3, 12). That overlay assay result provided direct biochem-

[2] M. J. Matunis and G. Blobel, manuscript in preparation.

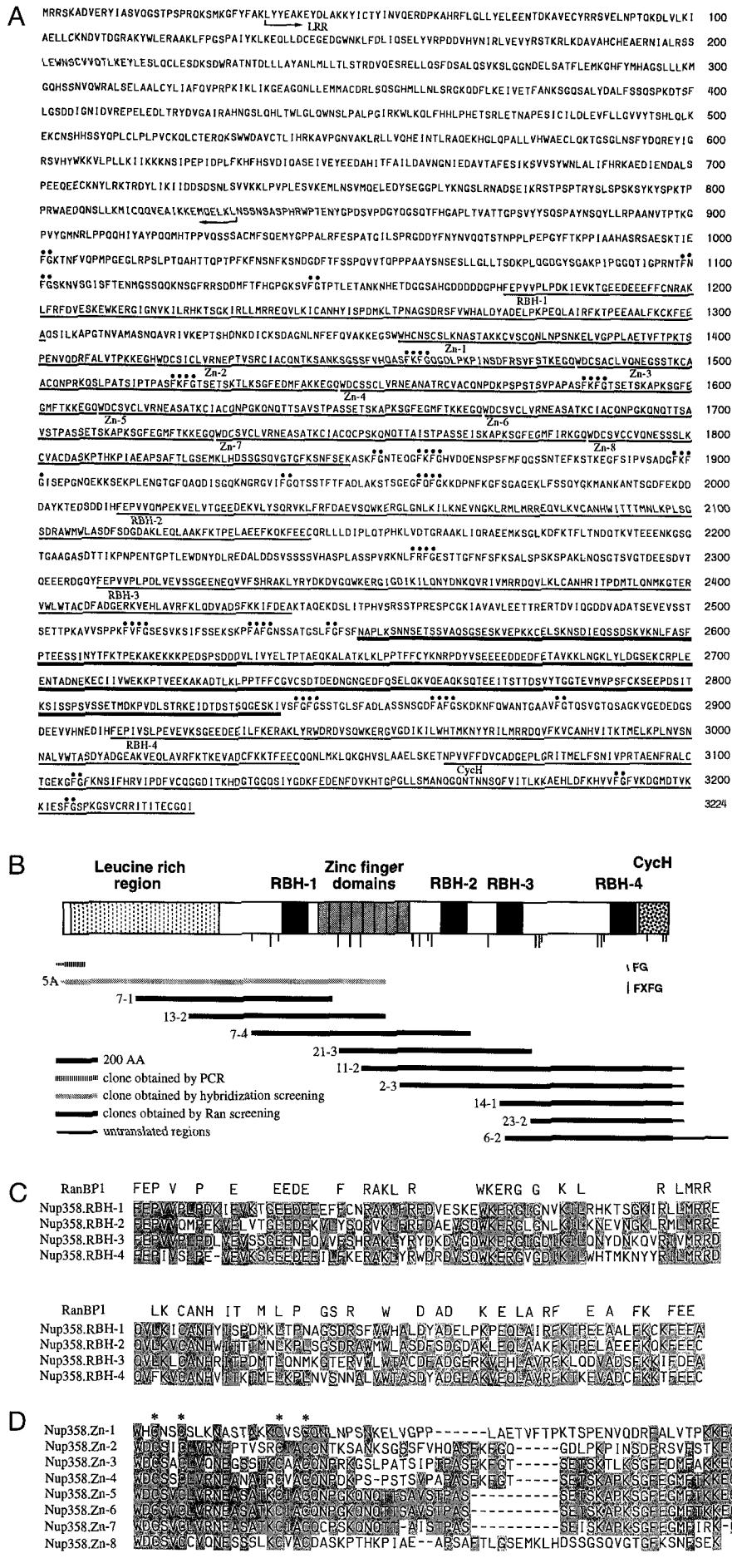

FIG. 1. **Sequence and analysis of human Nup358.** *A*, amino acid sequence of human Nup358 deduced from the cDNA sequence. Amino acid residues are *numbered* on the *right*. The *region between the two arrows* indicates a leucine-rich region (*LRR*), *regions with thin underlines* indicate four RanBP1 homologous domains (*RBH*), eight zinc finger domains (*Zn*), and a cyclophilin A homologous domain (*CycH*), the *region with a thick underline* indicates the segment of the protein used to raise antibodies; *residues with overhead dots* represent FG or FXFG repeats. *B*, a schematic representation of human Nup358 domains and cloning strategy. Human Nup358 domains detailed in *A* are shown here schematically with *boxes*. *Vertical lines* indicate locations of FG or FXFG repeats. *Horizontal bars* below the boxes indicate cDNA clones obtained by Ran screening, DNA hybridization screening, or PCR amplification. *C*, an alignment of four RanBP1 homologous domains (*RBH*) of Nup358. Identical residues are shown in *shaded areas*. Residues of RanBP1 (13) identical with those found in at least three of the Nup358 RBHs are shown at the *top*. *D*, an alignment of eight zinc finger domains (*Zn*) of Nup358. Identical residues are shown in *shaded areas*. *Overhead asterisks* indicate conserved cysteines.

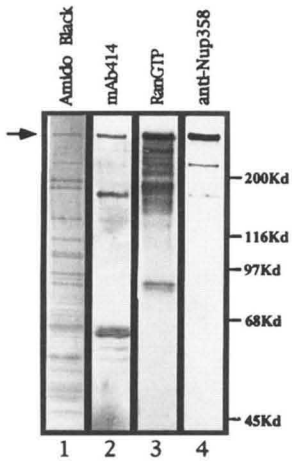

FIG. 2. **Characterization of the rat homolog of Nup358.** Proteins of rat liver nuclear envelopes were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and stained with Amido Black (*lane 1*) or probed with 414 monoclonal antibody (*lane 2*), Ran-[α-^{32}P]GTP as described (13) (*lane 3*), or anti-Nup358 antibody (*lane 4*). *Arrow* indicates Nup358.

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          1302          1311     1959      1965
Nup358    QSILKAPGTN             FQFGKKE
          |·|||| |||             |||||||·
rhNup358  QNILKALGTN             FQFGKKD

          2143          2152     2995          3007
Nup358    QRLLLDIPLQ             PLNVSNNALVWTA
          | |||||||||            ||| |||||||||
rhNup358  QLLLLDIPLQ             PLNFSNNALVWTA
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FIG. 3. **Comparison of partial amino acid sequences of the rat homolog of Nup358 (*rhNup358*) with human Nup358.** The *numbers* indicate the position of human Nup358 residues. *Vertical lines* indicate identical amino acids; *dots* indicate similar amino acids.

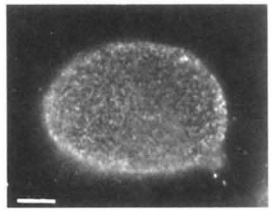

FIG. 4. **Localization of Nup358 by immunofluorescence microscopy.** HeLa cells were grown on coverslips, fixed with paraformaldehyde, solubilized with Triton X-100, and probed with anti-Nup358 antibodies (1:200 dilution) and fluorescein-labeled goat anti-mouse IgG. Focusing on the nuclear surface yielded a punctate staining pattern that is typical for nucleoporins. *Bar* equals 5 μm.

ical evidence of a link between a subgroup of nucleoporins (functioning as a stationary phase) and one of the transport factors, karyopherin, functioning as a mobile phase. The data here show that Nup358 can interact with a second transport factor, namely Ran-GTP. A Ran-GTP/GDP cycle has been proposed to affect the interaction between the stationary and the mobile phase (3, 12).

Our immunoelectronmicroscopic sublocalization data indicate that Nup358 is located at or near the tips of the cytoplasmic fibers that extend from the NPC into the cytoplasm. Thus, Nup358 could function as a port of entry into a multiple docking site pathway across the NPC (and/or as a port of exit in nuclear export).

The functions of the other domains of Nup358 remain to be elucidated. The leucine-rich region at the N-terminal region could function in protein-protein interaction (24). Likewise, the zinc finger domain could function in protein-protein, protein-RNA, or protein-DNA interaction. Nup358 is the second nucleoporin with zinc finger domains that has so far been identified. The other, Nup153, contains four zinc finger domains (also of

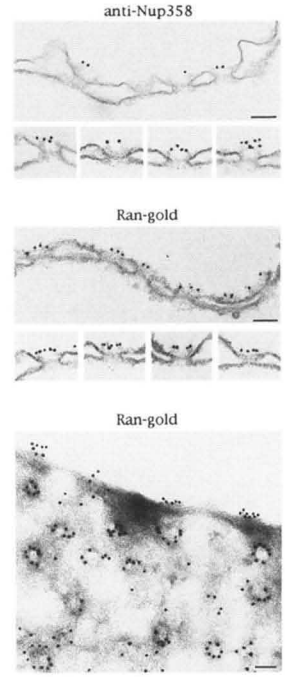

FIG. 5. **Nup358 and Ran binding sites localize to the cytoplasmic face of the NPC.** *Top panel*, isolated rat liver NEs were fixed and incubated with anti-Nup358 antibodies followed by gold-conjugated goat anti-mouse IgG. Samples were processed for thin sectioning and observation by EM as described under "Materials and Methods." *Middle panel*, isolated rat liver NEs were incubated with Ran-gold conjugate, fixed, and processed for thin sectioning and observation by EM as described under "Materials and Methods." Shown in the *top* and *middle panels* are views along a single NE, as well as four individual NPCs depicting typical patterns of gold labeling. In each case, the cytoplasmic face of the NE (as determined by double labeling with an anti-lamin B antibody (data not shown)) is oriented toward the top of the figure, and gold was localized exclusively to the fibers associated with the cytoplasmic face of the NPC. *Bottom panel*, isolated rat liver NEs were incubated with Ran-gold conjugate, fixed, and deposited onto EM grids. Samples were stained with 2% uranyl acetate and observed by EM. Up to eight gold particles can be seen forming rings around individual NPCs. *Bars* equal 0.1 μm.

the Cys_2-Cys_2 type), is localized at the nucleoplasmic side of the NPC (25–27), and has been shown to bind DNA in a zinc-dependent fashion (25). The cyclophilin A homologous domain at the C terminus could function as a peptidyl-prolyl *cis-trans* isomerase or a chaperone (28).

The striking decoration of isolated NEs by Ran-coupled gold at only the cytoplasmic side of NPC is consistent with the localization of Nup358. Quantitative analysis indicates that Ran bound to the NPC at a distance of 46 nm from the midplane of the NE and that the α-Nup358 antibodies bound at a distance of 59 nm. Given the size of Nup358, the 13 nm difference between binding sites is also consistent with the binding of antibody and Ran to the same protein. Nevertheless, these data do not prove that the Ran-gold is in fact binding to Nup358, as there could be other Ran-binding nucleoporins. Furthermore, whether the bound Ran is in a GTP-bound form will require further experiments with Ran mutants and GTP analogs. A definitive identification of the observed Ran binding sites (and other potential Ran binding sites not detected under these conditions) at the NPC is the focus of current investigation.

The availability of a nucleoporin with both karyopherin and Ran binding sites should now allow a more detailed biochemical analysis of how a GTP/GDP cycle may affect karyopherin-mediated docking of transport substrate.

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