

Recombinant Nup153 Incorporates *in Vivo* into *Xenopus* Oocyte Nuclear Pore Complexes

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Nup153 is a molecular constituent of the nuclear basket of the nuclear pore complex (NPC) that plays a critical role in nuclear export of RNAs and proteins. In an effort to map this nucleoporin more precisely within the nuclear basket we have developed an experimental approach for localizing Nup153 expressed and incorporated *in vivo* into *Xenopus* oocyte NPCs. This approach involves the microinjection into the cytoplasm of *Xenopus* oocytes of *in vitro* synthesized mRNA from a vector encoding an epitope-tagged cDNA. Here we present results obtained by Western blots, fluorescence microscopy, and immuno-electron microscopy, which clearly document that the heterologous protein is properly expressed, targeted, and incorporated into preexisting *Xenopus* NPCs. This new approach for localizing nucleoporins within the structure of the NPC overcomes limitations of previous techniques and allows for greater specificity and resolution than have been possible with previous methods. © 2000 Academic Press

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INTRODUCTION

The nuclear pore complex (NPC) is a large and extremely elaborate supramolecular assembly that mediates the regulated exchange of molecules between the nucleus and the cytoplasm (Ohno *et al.*, 1998; Görlich and Kutay, 1999). Extensive high-resolution electron microscopy (EM) analysis of amphibian oocyte NPCs and, more recently, of yeast NPCs (Yang *et al.*, 1998; Fahrenkrog *et al.*, 1998) has laid the foundations of a consensus model of NPC's

three-dimensional architecture (Panté and Aebi, 1996a; Stoffler *et al.*, 1999). It consists of a massive central framework (with dimensions of ~120 nm × ~80 nm) embedded in the double membrane of the nuclear envelope (NE) and peripheral structures extending into both the cytoplasm and the nuclear interior. The central framework is formed by eight multidomain spokes connected at their distal ends, and on both their nuclear and cytoplasmic faces, by multisubunit rings (Hinshaw *et al.*, 1992). The whole assembly embraces a large central aqueous channel, of as yet ill-defined structure, that may accommodate particles with diameters up to 26 nm provided that they bear specific nuclear import/export signals (Dworetzky *et al.*, 1988; Feldherr *et al.*, 1984). Emanating from the cytoplasmic ring are eight short (about 100 nm) flexible filaments and extending from the nuclear ring are eight 50- to 100-nm-long filaments distally joined by a 30- to 50-nm-diameter ring forming a structure resembling a basket or fishtrap (Jarnik and Aebi, 1991; Ris, 1991). These peripheral structures provide an overall asymmetry to the NPC, which otherwise has a highly symmetric architecture, and contain docking sites for cargo going in and out of the nucleus (Richardson *et al.*, 1988; Panté and Aebi, 1996b).

With a mass of about 125 MDa (Reichelt *et al.*, 1990) it is thought that vertebrate NPCs are composed of at least 50–100 different protein subunits present in multiple copies (i.e., 8 or 16). To date, about 16 NPC proteins or nucleoporins (Nups for short) have been identified and molecularly characterized in vertebrates (Bastos *et al.*, 1995; Panté and Aebi, 1996b; Doye and Hurt, 1997). One of the best-studied vertebrate nucleoporins is Nup153, a large (153 kDa) O-linked glycoprotein that is a constituent of the nuclear basket structure. It can be released from the NPC of interphase cells as a homo-oligomer of approximately 1 MDa and remains as such during mitosis (Panté *et al.*, 1994; Bodoor *et*

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et al., 1999). The Nup153 molecule exhibits a tripartite structure consisting of similarly sized N- and C-terminal domains (~600 residues each) flanking a central zinc finger domain. Nup153 is one of the two nucleoporins (the other being Nup358, a constituent of the cytoplasmic filaments (Yokoyama *et al.*, 1995)) known to contain a series of zinc fingers (McMorrow *et al.*, 1994). This zinc finger region represents a Ran binding domain (Nakielny *et al.*, 1999) and is also able to bind DNA *in vitro* (Sukegawa and Blobel, 1993). All the targeting and assembly information of Nup153 is contributed by its N-terminal domain (Bastos *et al.*, 1996; Enarson *et al.*, 1998), which contains an M9-like nuclear localization signal (Nakielny *et al.*, 1999). Consistent with this, Nup153 has been recently identified as a novel cargo for transportin (Nakielny *et al.*, 1999). The C-terminal domain contains the majority (26 of 33) of the FXFG repeats, found in many nucleoporins, as well as the binding sites for importin α/β (Shah *et al.*, 1998). Overexpression of this region in mammalian cells leads to a dramatic accumulation of poly(A)⁺ RNA inside the nucleus, possibly by titrating out an essential factor for mRNA export (Bastos *et al.*, 1996). A role for Nup153 in RNA export has been confirmed by antibody microinjection studies. When antibodies against Nup153 are injected into *Xenopus* oocytes, a block in the export of three classes of RNA (mRNA, snRNA, and 5S rRNA) occurs. These antibodies also affect NES-mediated protein export (Ullman *et al.*, 1999).

The interaction between Nup153 and transport factors and the role of this NPC protein in different transport pathways are consistent with its location within the NPC. Indeed, Nup153 is the only nucleoporin thus far identified that is situated at the distal ring of the nuclear basket, a structure that may represent a docking site for cargo in transit through the NPC. Interestingly, it is an epitope contained within the C-terminal domain that has been mapped on the terminal ring (Panté *et al.*, 1994). However, the disposition of Nup153 within the nuclear basket structure, i.e., whether the Nup153 oligomer actually comprises the entire distal ring, for example, or whether it is also part of the basket forming filaments, is not yet known.

In this study, we have employed a novel approach to start to address these questions based on the high-resolution localization of Nup153 assembled *in vivo* into *Xenopus* oocyte NPCs. This approach consists of expressing human Nup153 in oocytes by microinjection of *in vitro* synthesized mRNA from a vector encoding an epitope-tagged cDNA followed by immunogold labeling of the expressed protein using anti-tag antibodies. Here, we present the results obtained on conventional cross sections of *Xenopus* nuclear envelopes where we show the correct target-

ing and incorporation at the nucleoplasmic face of the NPC of the full-length Nup153. Further work based on this method and employing different mutants and EM techniques (quick freezing/freezing-drying/rotary metal shadowing, which by stereo pairs yields a 3D view of the nuclear basket) will allow us to map different domains of the molecule within the basket structure. This new approach may also permit the precise localization of other nucleoporins and will overcome limitations of standard immuno-electron microscopy (immuno-EM) resulting from high cross-reactivity of many anti-nucleoporin antibodies caused by the presence of highly antigenic repetitive sequence motifs within many nucleoporins.

MATERIALS AND METHODS

Plasmid construction and mRNA synthesis. The pGEM 3Z-based vector containing a myc and GFP tags (pGEM-myc/GFP) was obtained from Lorenza Lanini and Frank McKeon (Harvard Medical School). The polylinker region of this vector was modified by ligating a double-stranded synthetic oligonucleotide adaptor as previously described (Bastos *et al.*, 1996) between the unique *Xho*I and *Cl*aI sites. Full-length human Nup153 cloned in the expression vector pCMV-HA (Bastos *et al.*, 1996) was subcloned downstream of the myc/GFP tags of the modified pGEM-myc/GFP vector using the *Xho*I (in-frame with the tags) and *Bgl*II sites contained in the adaptor. To produce mRNA encoding myc/GFP-Nup153, this construct was linearized with *Nhe*I and transcribed with T7 RNA polymerase using the mCAP mRNA capping kit (Stratagene, La Jolla, CA) following the manufacturer's protocol.

Oocyte microinjection. Mature (stage 6) oocytes were surgically removed from female *Xenopus laevis* as described previously (Jarnik and Aebi, 1991) and stored in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 0.82 MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mM Hepes, pH 7.5. Oocytes were defolliculated by treatment with 5 mg/ml collagenase (Sigma, St. Louis, MO) in calcium-free MBS for 3 h. Oocytes were then washed with MBS and used for microinjection within the next 2 days. Fifty nanoliters of a solution containing 2 μ g/ μ l mRNA encoding myc/GFP-Nup153 was microinjected into the cytoplasm of *Xenopus* oocytes. Injected oocytes were incubated at room temperature for 24 h. Oocytes were then prepared for immunoblotting, immunofluorescence microscopy, or immuno-EM as described below.

Immunoblotting. After microinjection with mRNA encoding myc/GFP-Nup153 and incubation at room temperature, the nuclear and cytoplasmic fractions of the oocytes were prepared by manual dissection in low-salt buffer (LSB: 1 mM KCl, 0.5 mM MgCl₂, 10 mM Hepes, pH 7.5) containing CLAP as a protease inhibitor (Bastos *et al.*, 1996) at 4°C. CLAP was added during all of the subsequent steps. Proteins from the cytoplasmic fraction were prepared by acetone precipitation according to the procedure described by Ullman *et al.* (1999). For the nuclear fractions, nuclei were directly resuspended in SDS sample buffer and incubated at 96°C for 10 min. Extracts from five nuclei and cytosolic extracts from 1.5 oocytes were loaded per lane on 8% SDS-PAGE, and proteins were separated using standard methods. For immunoblotting, proteins were transferred onto nitrocellulose membranes (BA85; Schleicher and Schuell Inc., Keene, NH) using a wet blotting apparatus (Hoefer Scientific Instruments Inc., San Francisco, CA). Blots were blocked, labeled with primary antibodies, and then developed with peroxidase-conjugated secondary antibodies (goat anti-mouse, Pierce Chemical Co., Rockford, IL) and ECL

Plus (Amersham Pharmacia Biotech) according to the manual. The monoclonal antibody QE5 at a dilution of 1:200 (Panté *et al.*, 1994) or an anti-GFP monoclonal antibody at a dilution of 1:1000 (Roche Diagnostics) was used as primary antibody.

Fluorescence microscopy. Nuclei from oocytes microinjected with mRNA encoding myc/GFP-Nup153 were manually dissected in LSB and cleaned of yolk and other materials by up and down suction with a 5- μ l pipette in LSB. The isolated nuclei were then placed on coverslips in LSB and viewed and photographed directly with a Zeiss Axioplan 2 microscope equipped with a 40 \times objective lens using Openlab software (Improvision, Coventry) for digital image processing.

Immuno-electron microscopy. For immuno-electron microscopy, 8-nm-diameter gold particles were prepared and an anti-myc monoclonal antibody (9E10) was directly conjugated to the colloidal gold particles as described by Panté *et al.* (1994). After cytoplasmic injection with mRNA encoding myc/GFP-Nup153 and incubation at room temperature for 24 h, nuclei were manually dissected in LSB. The isolated nuclei were then extracted with 0.1% Triton X-100 for 30 s before they were incubated with colloidal gold-tagged anti-myc antibody for 1 h. Next, the labeled nuclei were washed three times in LSB, fixed in 2% glutaraldehyde for 30 min, post fixed in 1% OsO₄ for 1 h, dehydrated, and embedded in Epon resin (Fluka, Buchs, Switzerland). Thin sections (50 nm) were cut on a Reichert Ultracut microtome (Reichert-Jung Optische Werke, Vienna, Austria) using a diamond knife. The sections were collected on collodion/carbon-coated copper grids and stained with 6% uranyl acetate for 1 h and 2% lead citrate for 2 min. Electron micrographs were recorded with a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Tokyo, Japan) operated at an acceleration voltage of 100 kV.

RESULTS

We have set up an experimental approach for performing high-resolution localization studies of

Nup153 incorporated *in vivo* into *Xenopus* oocyte NPCs. This approach consists of expressing the protein in oocytes by microinjecting *in vitro* synthesized mRNA from a vector encoding an epitope-tagged cDNA and localizing the expressed protein with specific antibodies against the tag. For this purpose we cloned a human Nup153 cDNA downstream of the myc and GFP tags into a suitable vector containing the T7 promoter. To evaluate whether the tagged Nup153 is expressed in *Xenopus* oocytes we first performed immunoblots. As documented in Fig. 1A, when an anti-GFP antibody was used to immunoblot cytosolic and nuclear extracts from oocytes microinjected with mRNA of tagged Nup153 a band with an apparent molecular mass of 180 kDa appeared in the cytosolic fraction within 2 h postinjection. This band, which corresponds to the tagged Nup153 expressed within the *Xenopus* oocytes, was not present in the nuclear fraction at this time. The amount of tagged protein in the cytoplasmic fraction increased over time, reaching a maximum at about 12 h after microinjection of the mRNA. Beyond this time cytoplasmic recombinant Nup153 started to decrease concomitantly with the appearance of increasing amounts of recombinant Nup153 in the nuclear fractions (Fig. 1A). We also used the monoclonal QE5 antibody, which recognizes three nucleoporins (CAN/Nup214, Nup153, and p62; Panté *et al.*, 1994), to immunoblot extracts from *Xenopus* oocytes expressing epitope-tagged Nup153.

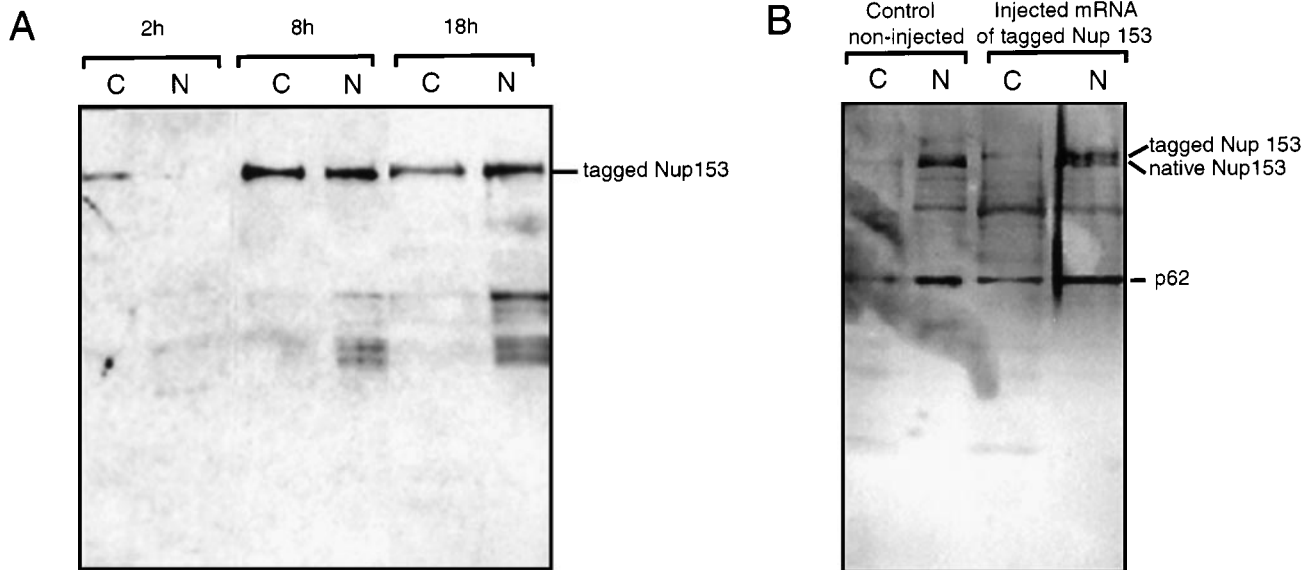


FIG. 1. Recombinant Nup153 is expressed in *Xenopus* oocytes. Western blots of cytosolic (C) and nuclear (N) extracts from oocytes microinjected with myc/GFP-Nup153 mRNA probed with an anti-GFP antibody (A) or with monoclonal QE5 antibody that recognizes the nucleoporins CAN/Nup214, Nup153, and p62. CAN/Nup214 is not revealed due to the low affinity of QE5 for this protein on Western blots (B). Proteins from each subcellular fraction were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GFP or QE5 antibodies. For (A) samples were prepared 2, 8, and 18 h after microinjection. For (B) samples were prepared 24 h after microinjection.

As shown in Fig. 1B, QE5 labeled both the native and the heterologous tagged Nup153. As the recombinant Nup153 increased in the nuclear fraction, the intensity of the band representing the native nucleoporin decreased. This is an indication that there is an exchange of the native Nup153 with the recombinant Nup153. Note that in both gels the antibodies labeled proteolytic products of Nup153. Such proteolysis has been reported previously (Nakielny *et al.*, 1999). Thus the use of two different types of antibody demonstrates that the tagged Nup153 is well expressed in *Xenopus* oocytes and accumulated in the nuclear fraction.

The GFP tag also allowed us to check the expression of the protein by fluorescence microscopy of manually isolated *Xenopus* oocyte nuclei. As shown in Fig. 2, the signal was concentrated at the nuclear envelope, indicating that in *Xenopus* oocytes the protein is not only expressed but also properly

targeted to the nuclear envelope. In order to determine the location of the tagged protein on an ultrastructural level, we then carried out preembedding immuno-electron microscopy on isolated *Xenopus* oocyte nuclei expressing tagged Nup153. To allow the antibody to reach the nuclear side of the nuclear envelope, nuclei were briefly treated with Triton X-100 before being labeled with an anti-myc antibody conjugated with 8-nm gold particles. As documented in Fig. 3, we found that the anti-myc antibody significantly labeled the nuclear face of the NPCs of oocytes that expressed myc-tagged Nup153. The gold particles were predominantly detected at a distance of 50 to 100 nm away from the central plane of the NPC. These positions correspond to the distal part of the nuclear baskets (see Fig. 3, arrowheads). This finding confirms our previous immuno-localization data (Panté *et al.*, 1994). In addition, a significant number of gold particles were located near or at

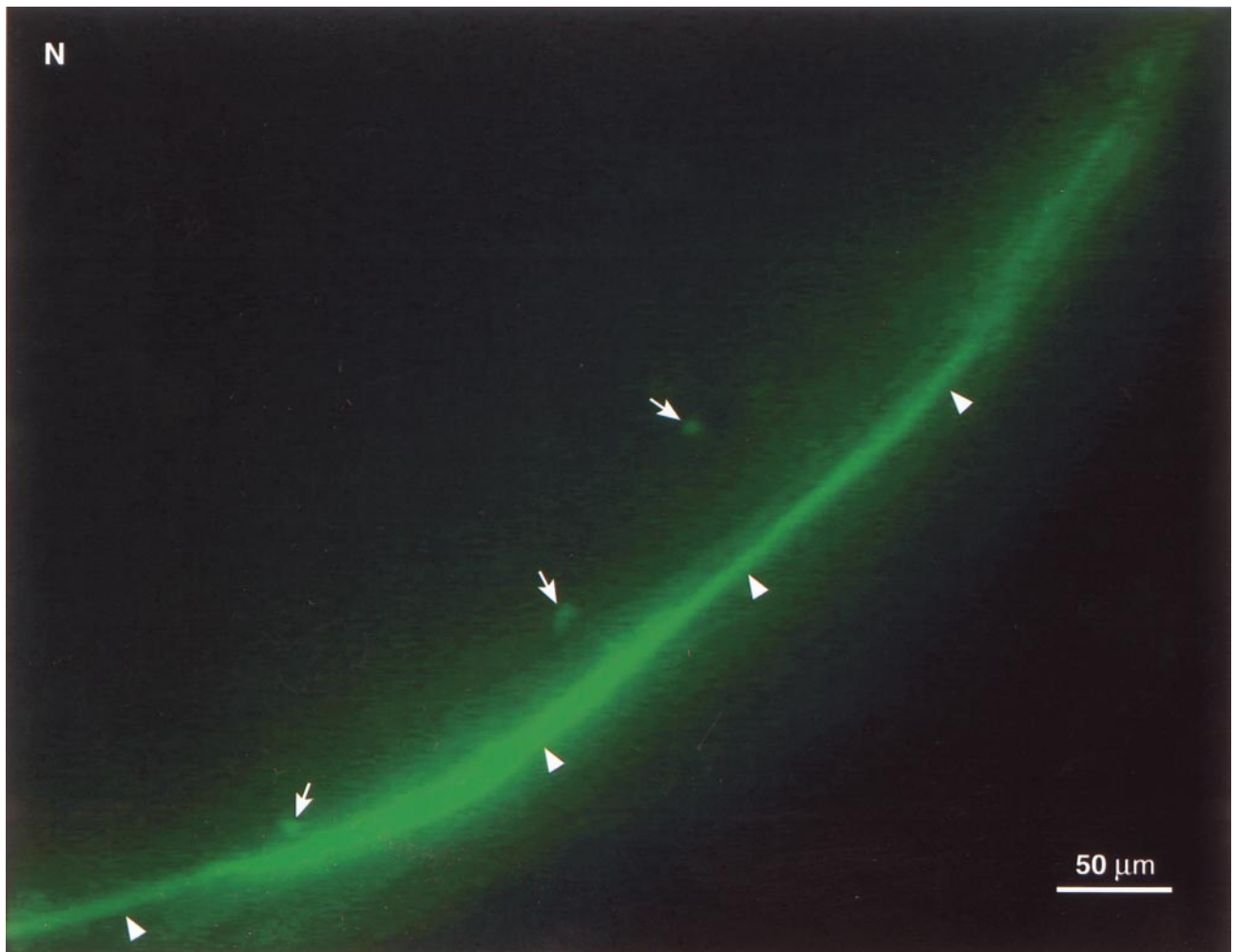


FIG. 2. GFP-tagged Nup153 is targeted to the nuclear envelope of *Xenopus* oocytes. Fluorescence micrograph of a nucleus isolated from a *Xenopus* oocyte microinjected with mRNA encoding myc/GFP-Nup153, viewed directly under the microscope (see Materials and Methods). Arrowheads indicate the nuclear rim staining, and arrows point to clusters of labeled NPCs. The nuclear side (N) is indicated.

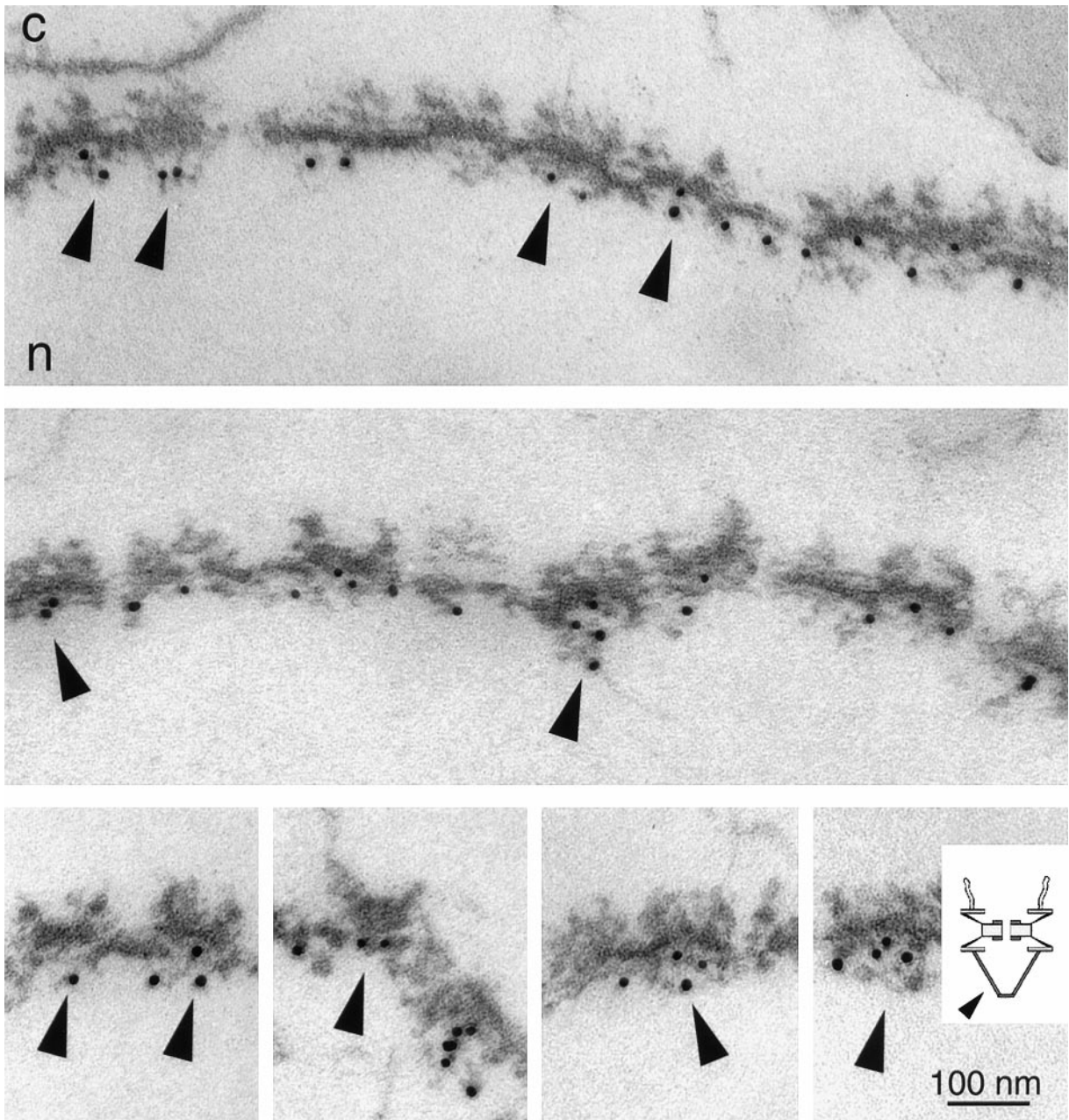


FIG. 3. Recombinant Nup153 associates with *Xenopus* NPCs. Cross sections of nuclear envelopes and selected examples of gold-labeled oocyte NPCs from Triton X-100 treated nuclei isolated from *Xenopus* oocytes that expressed myc/GFP-Nup153. Nuclei were incubated with an anti-myc antibody conjugated directly to 8-nm-diameter colloidal gold particles prior to embedding and thin-sectioning. The cross sections revealed that the anti-myc antibody labeled the distal part of the nuclear baskets (arrowheads). The cytoplasmic (c) and nuclear (n) faces of the nuclear envelope are indicated. The inset at the bottom (right) represents a schematic diagram of the NPC where the small arrowhead marks the nuclear basket. Scale bar; 100 nm.

the nuclear exit of the central gated channel. At the moment we cannot rule out the possibility that this labeling is due to recombinant Nup153 being in transit or perhaps due to the nuclear baskets that might have collapsed during sample preparation. In addition, this localization of recombinant Nup153 might also be due to overexpression of the fusion

protein. Taken together, the results of immunoblot analysis, fluorescence microscopy, and immunoelectron microscopy of *Xenopus* oocytes microinjected with an mRNA encoding epitope-tagged Nup153 demonstrate that the recombinant protein is properly expressed and correctly targeted and incorporated into *Xenopus* oocyte NPCs.

DISCUSSION

To better understand the functional role of individual nucleoporins in molecular detail it is necessary not only to know how they interact with transport factors and other nucleoporins but also to map their location within the 3D architecture of the NPC. This information has been scarce due to the limited availability of suitable antibodies, cross-reactivity problems, and limited EM sample preparation protocols. Only a handful of nucleoporins have been immunolocalized at the EM level within the NPC. One of these is Nup153, which is the only NPC protein that has been found so far on the terminal ring of the nuclear basket (Panté *et al.*, 1994). This finding is based on EM immuno-cytochemical observations on both thin-sectioned/embedded and freeze-dried/rotary metal-shadowed *Xenopus* nuclear envelopes using antibodies that recognize epitopes contained in the C-terminal half of the protein. Therefore, strictly speaking, we know only that the corresponding Nup153 epitope resides in the distal ring. In fact, we do not know whether Nup153 forms only part of the distal ring or is also a constituent of the structure of the nuclear basket. In order to map Nup153 more precisely within the NPC we have developed an alternative approach to the more conventional EM localization of nucleoporins. This approach involves the expression of a tagged Nup153 in *Xenopus* oocytes combined with the use of anti-tag antibodies to immunogold-label the recombinant nucleoporin. The results presented here clearly indicate that tagged Nup153, when expressed in *Xenopus* oocytes after mRNA injection, is exclusively targeted to the nuclear face of the NPC, in agreement with our previous EM immuno-cytochemical observations (Panté *et al.*, 1994). Furthermore, these data show that human Nup153 properly associates with the *Xenopus* oocyte NPCs. This is a remarkable finding considering that there is little or no pore biogenesis in these oocytes. Obviously, the finding raises the question about the mechanisms by which the expressed protein is correctly targeted to and associated with the NPCs. One interpretation of these results is that there are additional binding sites available on the nuclear basket for extra copies of this protein. For example, the tagged Nup153 may interact and bind to another nuclear basket component or it may associate with endogenous Nup153. Since Nup153 can be released from the NPC as a homo-oligomer of about 1 MDa (Panté *et al.*, 1994), the capacity of the *Xenopus* NPCs to bind and assemble the expressed protein could be explained by the ability of this nucleoporin to oligomerize. Another possible explanation is that the endogenous Nup153 is being displaced and exchanged for the recombinant protein. While the mechanism by which

the recombinant Nup153 associates with NPCs remains to be further elucidated, it is evident from the data presented that this approach may be employed to localize at least certain nucleoporins within the NPC. Moreover, it has the advantage of overcoming the limitations set by the standard immuno-EM involving anti-nucleoporin antibodies due to their frequent cross-reactivity, which has generated conflicting results in the field.

From the EM preparations (cross sections) presented in this study it is not possible to unambiguously identify distinct substructures of the NPC and therefore to localize expressed Nup153 more precisely. That Nup153 is at least partly involved in forming the distal ring of the nuclear basket has been supported by the recent finding that bacterially expressed GST-fused Nup153 forms round particles with a diameter of about 30 nm. In fact, in the presence of calcium the Nup153 particles appear donut-like in the EM, whereas in the absence of calcium they appear disk-like (Burke and Aebi, unpublished results). This remarkable finding indicates that the basic framework of the distal ring is a Nup153 oligomer (probably an octamer; Panté *et al.*, 1994) that acts as a calcium-sensitive "iris-diaphragm" (Stoffler *et al.*, 1999). Although in some cases gold particles appeared to decorate the filaments of the nuclear baskets, we cannot conclude that Nup153 forms part of the filaments. In order to establish a more precise topological mapping of Nup153 the use of an EM technique (such as the quick freezing/freezing-drying/rotary metal shadowing technique) that provides a 3D view of the nuclear basket is required. Further work based on such high-resolution EM techniques and employing deletion constructs of Nup153 should allow us to map more precisely different domains of the molecule within the basket structure. Ultimately, such analysis combined with other structural studies will provide more detailed structural information on the 3D molecular organization and the functional dynamics of the nuclear basket.

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