Intranuclear endoplasmic reticulum induced by Nopp140 mimics the nucleolar channel system of human endometrium

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SUMMARY

Exogenous expression of the characteristic repeat domain of the nucleolar chaperone Nopp140 induces the formation of intranuclear structures, termed R-rings. Here, the R-rings are identified as extensive stacks of membrane cisternae in the otherwise membrane-free nucleus. They consist of bona fide endoplasmic reticulum (ER) containing integral membrane proteins of the smooth and rough ER. Although lacking nuclear pore complexes and lamina, the R-rings derive specifically from the inner nuclear membrane. These findings are consistent with the idea that all transmembrane proteins synthesized in the ER and the outer nuclear membrane can freely diffuse through the pore membrane domain into the inner membrane. Uniquely, the soluble transfected Nopp140 is directly involved in the generation of these membrane stacks as it localizes to the electron dense matrix in which they are embedded. The only well-documented example of intranuclear membrane proliferation is the nucleolar channel system of the postovulation human endometrium. The transient emergence of the nucleolar channel system correlates precisely with the readiness of the endometrium for the implantation of the fertilized egg. The nucleolar channel system exhibits an ultrastructure that is indistinguishable from R-rings, and nuclei of human endometrium harbor Nopp140 and ER marker containing structures. Therefore, the nucleolar channel system appears to be identical to the R-rings, suggesting a role for Nopp140 in human reproduction.

Key words: Endoplasmic reticulum, Nucleolar channel system, Human endometrium, Nucleolus, Nopp140

INTRODUCTION

The nucleus, unlike the cytoplasm, is completely membrane-free except for the enclosing double membrane of the nuclear envelope, which comprises an inner and an outer membrane. There is only one well-documented exception to this fact, the nucleolar channel system (NCS) of the human endometrium, also described as nucleolar canalicular or basket structure (Clyman, 1963; Dubrauszky and Pohlmann, 1960; Dubrauszky and Pohlmann, 1961; Moricard and Moricard, 1964; Terzakis, 1965). The NCS consists of intranuclear stacks of membrane cisternae embedded in an electron-dense matrix and often associated with the inner nuclear membrane and/or the nucleolus. It is one of the major ultrastructural hallmarks of the endometrial epithelium of the secretory phase, appearing transiently during days 16–24 of the menstrual cycle. These intranuclear membrane cisternae are strictly induced by progesterone (Kohorn et al., 1970; Kohorn et al., 1972; Pryse-Davies et al., 1979; Roberts et al., 1975). Moreover, there is a clear correlation between the emergence of the NCS and the readiness of the endometrium for the attachment of the blastocyst (Clyman, 1963). In birth control, oral contraceptives interfere with the formation of the NCS (Azadian-Boulanger et al., 1976; Feria-Velasco et al., 1972) and insertion of an intrauterine device prevents the implantation of the fertilized egg while causing premature induction of the NCS during the late proliferative phase (Wynn, 1967). Therefore, induction of the NCS in the endometrium at the right time of the menstrual cycle may be essential for human reproduction. In fact, the absence of the NCS from the secretory endometrium, as the only structural alteration, has been linked to infertility (Kohorn et al., 1972). Given the importance of the NCS, it is surprising that these intranuclear membrane cisternae have been characterized only morphologically and remain as enigmatic as 40 years ago when they were discovered (Dubrauszky and Pohlmann, 1960).

The nucleolus, the major membrane-free organelle of the nucleus, has been analyzed in depth as the site of ribosome biogenesis. We have characterized a nonribosomal, nucleolar and Cajal (coiled) body protein involved in this process, Nopp140 (Meier and Blobel, 1992; Meier and Blobel, 1994). Specifically, Nopp140 associates with small nucleolar ribonucleoprotein particles, required for ribosomal RNA modification and processing, and appears to function as a chaperone for their biogenesis and intranuclear transport (Isaac et al., 1998; Yang et al., 2000). The primary structure of Nopp140 reveals a unique amino and carboxy terminus separated by a long central repeat domain. The latter consists

of ten alternating repeats of acidic serine clusters and lysine-, alanine- and proline-rich basic stretches. Most of the serines in the acidic repeats are phosphorylated by casein kinase 2, rendering Nopp140 one of the most highly phosphorylated proteins in the cell with ~80 phosphates per molecule (Meier, 1996; Meier and Blobel, 1992). When expressed in bacteria (unphosphorylated) or in insect cells (phosphorylated), this repeat domain, termed NoppR, contributes to the solubility of Nopp140 owing to the high charge density (Meier, 1996) (data not shown). Exogenous expression of NoppR causes the formation of nucleoplasmic structures; we named them R-rings based on their induction by NoppR and their appearance, which is often ring-shaped by indirect immunofluorescence (Isaac et al., 1998). Endogenous Nopp140, together with all its associated nucleolar factors, is recruited to these R-rings and depleted from the remains of the nucleolus (Isaac et al., 1998).

Here we demonstrate that the R-rings consist of bona fide endoplasmic reticulum (ER) that invaginates from the inner nuclear membrane generating an intranuclear membrane network. These R-rings appear identical to the nucleolar channel system of the human endometrium, suggesting a role for Nopp140 in the uterine preparation for blastocyst attachment.

MATERIALS AND METHODS

Transfection and fluorescence

COS-1 cells, grown on cover slips, were transiently transfected with HA-tagged full-length Nopp140 or its ten repeats alone (HA-NoppR) to induce intranuclear R-rings exactly as previously described (Isaac et al., 1998). To determine the localization of a representative of the box H/ACA class of snoRNAs, the cells were transfected, alone or with HA-NoppR, with a plasmid containing snR36 inserted into the second intron of the human β-globin gene as previously described (pG/snR36) (Ganot et al., 1997).

The cells were fixed, permeabilized, and processed for double immunofluorescence as previously described (Isaac et al., 1998). The following primary antibodies were used at the dilutions indicated in parentheses: anti-Nopp140 serum (RH10 at 1:1000) (Meier, 1996); anti-HA ascites fluid (12CA5 at 1:200) (Wilson et al., 1984); anti-LAP2 ascites fluid (#34 at 1:1000; kindly provided by Sui Huang and David Spector); anti-nucleoporin p62 ascites fluid (414 at 1:5000) (Davis and Blobel, 1986); anti-lamin B serum (1:1000) (Chaudhary and Courvalin, 1993); anti-calnexin serum (1:1000) (Kim et al., 1996); anti-Sec61 (1:200) and anti-TRAM serum (1:50) (Nicchitta et al., 1995); anti-SRP receptor serum (1:200; kindly provided by Chris Nicchitta); anti-TRAPα serum (formerly anti-SSRa at 1:200) (Nicchitta et al., 1991); anti-BiP serum (1:50; kindly provided by Chris Nicchitta); anti-protein disulfide isomerase (PDI) serum (1:100) (Terada et al., 1995); anti-HMG-CoA reductase serum (1:50) (Roitelman et al., 1992); anti-cytochrome P450b, c and j sera (1:100; kindly provided by Frank Gonzalez); anti-inositol 1,4,5-trisphosphate (IP_3) receptor serum (1:50) (Moschella and Marks, 1993). Secondary antibodies were rhodamine-labeled goat anti-rabbit IgGs and fluorescein-labeled goat anti-mouse IgGs from Boehringer Mannheim Corp. (Indianapolis, IN, at 1:200). The cells were labeled with the lipophilic dye $DiOC_6$ (Molecular Probes, Inc., Eugene, OR) according to Terasaki et al. (Terasaki et al., 1984). The fluorescence was observed and the images analyzed exactly as described previously (Isaac et al., 1998).

For fluorescent in situ hybridization of endogenous U14 and transfected snR36 snoRNAs, the protocol of Samarsky et al. (Samarsky et al., 1998) was followed. To detect U14, an aminomodified DNA oligonucleotide (Samarsky et al., 1998) was synthesized and labeled with Cy3 as previously described (Kislauskis et al., 1993). Analogously, to visualize snR36, the DNA oligonucleotide 5′-T*ACGATACCGT*GAGATAAAAAT*AGCGATATCCT*CGTACTCAAAGAAGT*TC-3′ was synthesized, where T* represents the aminomodified thymidine.

Electron microscopy

For ultrastructural analysis, NoppR-transfected COS-1 cells were trypsin detached, pelleted, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington, VT). Ultrathin sections (90 nm) were cut on a Reichert Ultracut E microtome, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

For immunogold labeling, the cell pellet was fixed for 5 minutes with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer and embedded in LR White resin. Ultrathin sections were incubated with anti-HA ascites fluid (12CA5 at 1:200) (Wilson et al., 1984) or affinity-purified anti-Nopp140 rabbit IgGs (RF11 at 1 μg/ml) (Meier and Blobel, 1992) in 1% powdered milk in phosphate-buffered saline. Secondary antibodies were goat anti-mouse or anti-rabbit IgMs conjugated to 15 nm gold (EY Laboratories, San Mateo, CA).

Paraffin-embedded endometrial tissue

Tissue samples of endometrium in the secretory phase were collected from healthy women and paraffin-embedded in a previous study (Daiter et al., 1992). Sections (5 μm) were cut on a Leica RM2165 microtome, deparaffinized and rehydrated through a graded ethanol series. Antigen retrieval was performed essentially as described (Shi et al., 1991) except that the sections were boiled for 10 minutes in 0.01 M sodium citrate (pH 6.0). Subsequently, the sections were blocked with 1% powdered milk, 0.5% Tween-20 in phosphate-buffered saline, incubated with anti-calnexin rabbit serum (at 1:1000) (Kim et al., 1996) or affinity-purified anti-Nopp140 rabbit IgGs (RE10 at 0.3 μg/ml) (Meier and Blobel, 1992) and antinucleolin mouse ascites fluid (7G2 at 1:2000) (Pinol-Roma, 1999) or anti-BiP monoclonal mouse IgGs (anti-KDEL at 5 μg/ml; StressGen Biotechnologies Corp., Victoria, Canada). Secondary antibodies were as described for indirect immunofluorescence above. For ultrastructural analysis, the sections were deparaffinized, rehydrated, and fixed with 4% glutaraldehyde.

To improve the reactivity of the anti-rat Nopp140 antibodies with the human homolog, rat Nopp140 antiserum was affinity purified over a column of immobilized recombinant human Nopp140. For this purpose, the full-length human Nopp140 cDNA was generated by PCR amplification using as template the gene KIAA0035 (kindly obtained from the Kazusa DNA Research Institute, Chiba, Japan) (Nomura et al., 1994). Human Nopp140 was expressed in bacteria as described for the rat protein but in soluble form and purified from whole bacterial lysate over a hydroxylapatite (Bio-Gel HTP, Bio-Rad) column like its rat homolog (Meier, 1996). The recombinant protein was coupled to an Affi-Gel 10 agarose support (Bio-Rad) and used to affinity purify RE10h IgGs from rat Nopp140 antiserum RE10. The RE10h IgGs (at 0.4 μg/ml) were used specifically to produce the indirect immunofluorescence data in Fig. 6C.

RESULTS

R-rings are membranous structures

We previously described the induction of R-rings by partial Nopp140 constructs (Isaac et al., 1998) (Fig. 1c) and show here

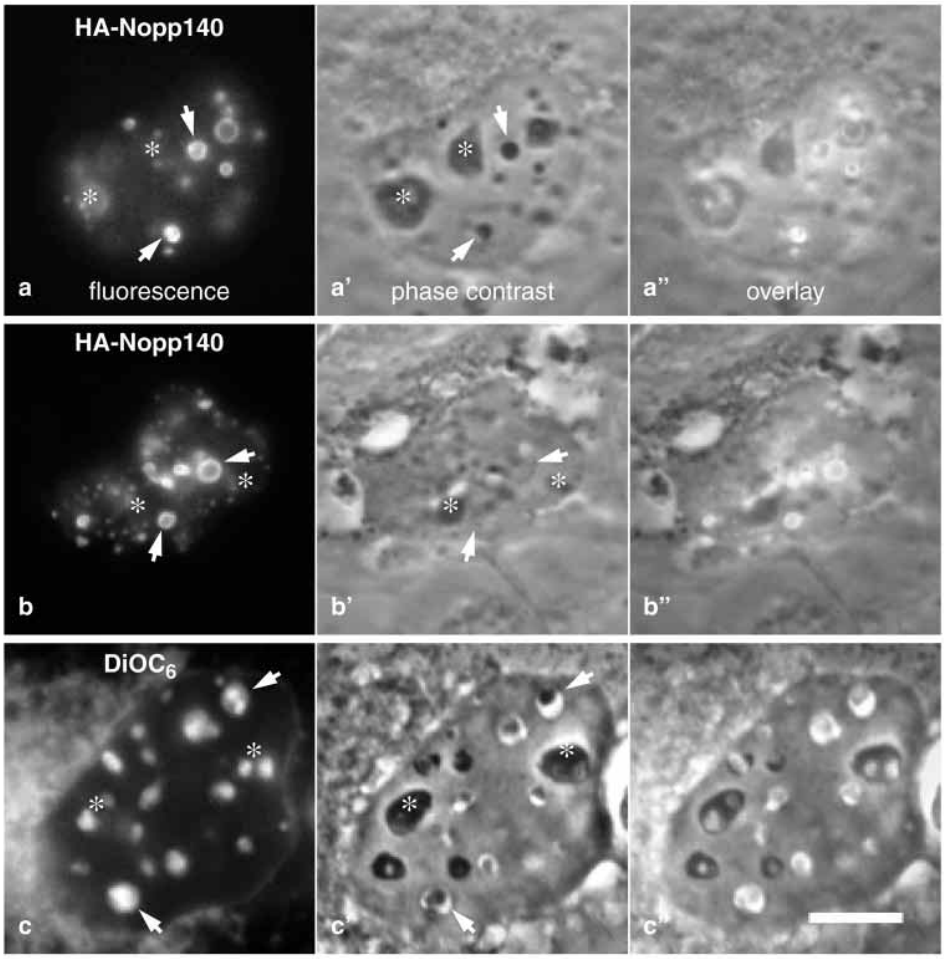


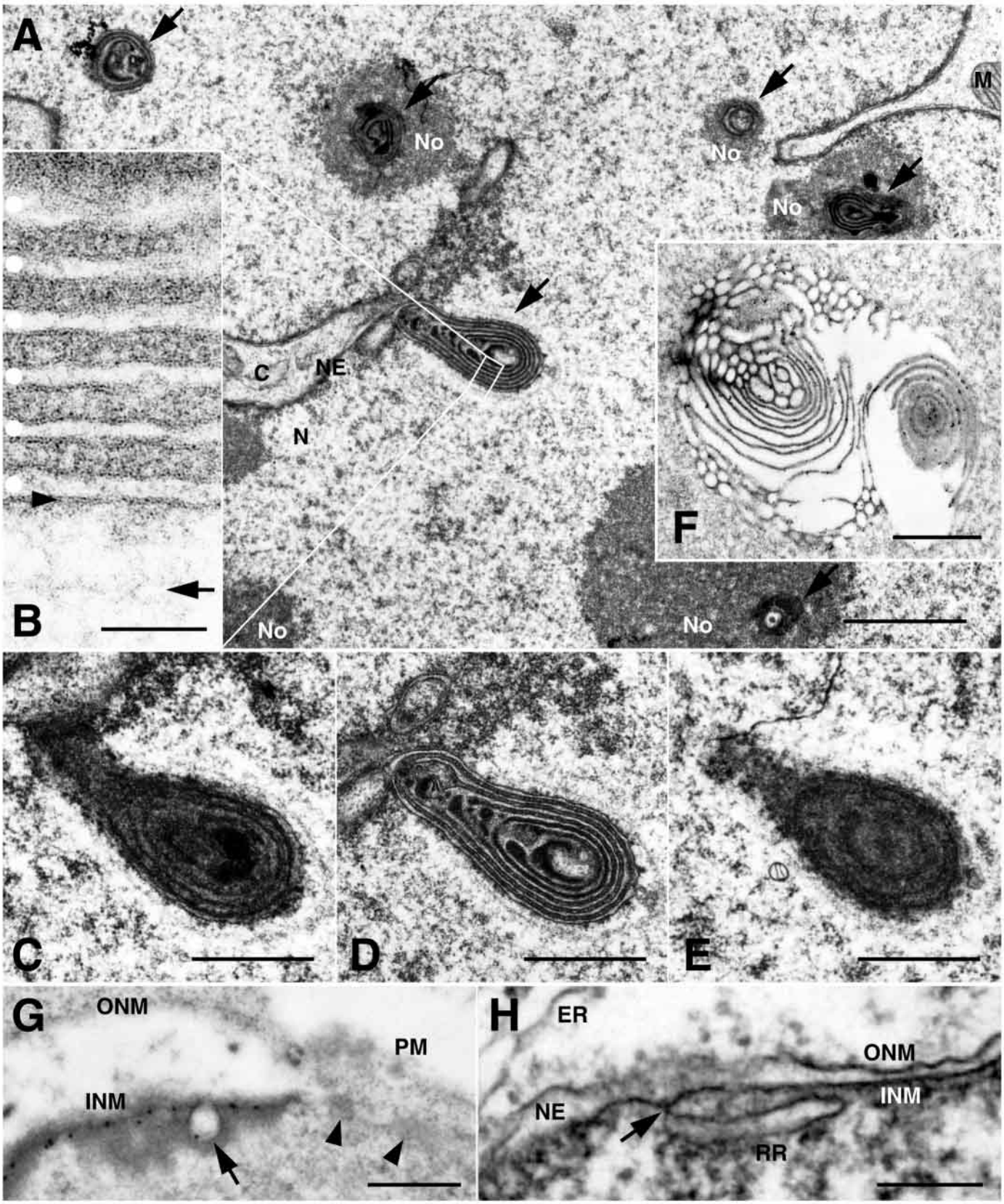
Fig. 1. R-rings are membranous structures. Fluorescence of COS cells transfected with the HA-tagged full-length Nopp140 (HA-Nopp140) (a and b) or the Nopp140 repeat domain (c). The cells were stained for the transfected protein with HA-antibodies (a,b) or for membranes with the lipophilic dye, $DiOC_6$ (c). Note the varied appearance of the R-rings in different cells by indirect immunofluorescence (a,b) and by phase contrast microscopy (a'-c'). Two R-rings are pointed out in each panel (arrows). Note that the phase dense particles of the R-rings (arrows in a' and c') are distinct from nucleoli (asterisks). An overlay of the fluorescence with the phase contrast images shows the location of the R-rings in the nucleoplasm and in the nucleoli (a"-c"). Bar, 10 μm.

that they were also induced by the exogenous expression of full-length Nopp140 (Fig. 1a,b). Additionally, we determined that the shortest construct that was expressed to a detectable degree and that formed R-rings mapped to the first four repeats of Nopp140, residues 60-241. R-ring induction was specific for the repeat domain of Nopp140 as its amino or carboxy terminus alone (Isaac et al., 1998) or the closely related repeats of another nucleolar protein, treacle, failed to induce similar structures (Isaac et al., 2000). Likewise, exogenous expression of other nucleolar proteins or small nucleolar RNAs that were recruited to the R-rings (Table 1), such as NAP57, GAR1, fibrillarin, UBF and snR36 did not lead to R-ring formation (data not shown) (Chen and Huang, 2001; Heiss et al., 1999; Pogacic et al., 2000; Snaar et al., 2000). Finally, the appearance of R-rings was not limited to COS-1 cells but was also observed in HeLa cells when human Nopp140 was exogenously expressed (Chen et al., 1999). Therefore, R-ring production is specific for the repeat domain of Nopp140 but not limited by the cell type. Moreover, R-ring induction occurs during interphase as NoppR-transfected cells lose viability (Isaac et al., 1998), arrest RNA polymerase I transcription and have not been observed undergoing mitosis or in pairs characteristic for postmitotic cells (data not shown).

The appearance of the R-rings by phase contrast and indirect immunofluorescence microscopy varied from cell to cell (Fig. 1, arrows). Often they occurred as free rings in the nucleoplasm (Fig. 1a,b, arrows), which appeared as dense particles (a'), as barely visible structures (b'), or as lucent rings around a dense core (c') by phase contrast microscopy. R-rings were frequently observed within nucleoli (Fig. 1a,c; asterisks) and many nuclei contained multiple small foci of emerging R-rings (Fig. 1b). The accumulation of many nucleolar and Cajal body factors in the R-rings (Table 1, top half) (Isaac et al., 1998) suggested their possible aggregation into an antibody impermeable Nopp140-based complex. Surface labeling of such a complex could result in the ring-shaped Nopp140 pattern observed by indirect immunofluorescence (Fig. 1a,b; arrows). Alternatively, such a pattern could indicate a vesicular and membranous structure. To test the latter possibility, we stained NoppR-transfected cells with the lipophilic dye, $DiOC_6$. Indeed, this vital membrane dye clearly labeled the R-rings in addition to the ER and the nuclear envelope (Fig. 1c, compare to c' and c"). Surprisingly, therefore, the exogenous expression of the soluble Nopp140 induced the proliferation of membranes in the otherwise membrane-free nucleus of the cell.

R-rings consist of intranuclear stacks of membrane cisternae

To investigate the morphology of the R-rings, we studied thin sections of HA-NoppR-transfected cells by transmission electron microscopy. The nuclei of these cells contained various numbers of multilamellar membranous structures, apparently the R-rings (Fig. 2A, arrows). As expected from our indirect immunofluorescence analysis, these structures often associated with nucleoli and the nuclear envelope or formed apparently independent structures in the nucleoplasm (Fig. 2A, arrows). Higher magnification of an R-ring revealed the two dark lines characteristic for a lipid bilayer (Fig. 2B, arrowhead) and identified these structures as stacks of membrane cisternae (Fig. 2B). Their lumen, like that of the ER and the nuclear envelope, exhibited a constant distance between the two



membranes (Fig. 2B, white dots). Each cisternae was separated from the next by a layer of electron dense material of even thickness of approximately 30 nm (Fig. 2B). The number of layers varied from a few to over 30. The stacks were often arranged in concentric spheres that formed structures like onion leaves rather than concentric tubes. This was especially

Fig. 2. The R-rings are multilamellar intranuclear membrane cisternae. Transmission electron microscopy for ultrastructural analysis (A-E and H) and postembedding immunodetection (F,G). (A) Low-power overview of a NoppR-transfected COS cell. Several R-rings (arrows) are detectable in the nucleoplasm (N), the nucleoli (No) and at the nuclear envelope (NE). A mitochondrion (M) can be seen in the cytoplasm (C). Bar, 2 μm. (B) High-power magnification of the framed section of the central R-ring in (A). Note the two electron-dense lines characteristic for biological membranes (arrowhead) that separate the individual lumina (white dots) of the cisternae from the electron-dense material between the membranes, and the regular structure separating them from the nucleoplasm (arrow). Bar, 0.1 μm. (C-E) Close-up of a serial section through the central R-ring in (A). Note the rounding off on either side (C,E) of the central section (D). Bars, 1 μm. (F) R-ring immunogold labeled with anti-HA antibodies for the transfected HA-NoppR. Note the specific labeling of the electron-dense matrix between the membrane cisternae, which were often disrupted during preparation because they presented the point of least resistance during sectioning. Bar, 1 μm. (G) Close-up of the nuclear envelope, where the outer (ONM) and inner nuclear membrane (INM) were separated during sectioning on the left side and held together by two nuclear pore complexes (arrowheads) on the right side. The section was immunogold labeled with anti-Nopp140 antibodies, which only recognize the transfected NoppR but not the endogenous Nopp140. Note the gold decoration of specifically the inner membrane and of a bud (arrow) forming a putative R-ring. The dark material attached at the inner membrane is a nucleolus based on the comparable appearance of other nucleoli in the same section (not shown). In this section, the nucleus is in close proximity with the plasma membrane (PM). Bar, 0.5 μm. (H) High magnification of a bleb off the inner nuclear membrane (INM, arrow) forming a putative R-ring (RR). A section of endoplasmic reticulum (ER) coming off the outer membrane (ONM) of the nuclear envelope (NE) is visible in the cytoplasm. Bar, 0.2 μm.

evident in serial sections (Fig. 2C-E), which revealed the opposing surfaces (C and E) of an R-ring (D). The membrane sheets frequently fused into a multitude of tubules that resulted in a honeycomb-type pattern in cross-sections (Fig. 2F). It should be noted that the morphology of the R-rings varied dramatically from cell to cell and that we only depict a small sample (compare Fig. 2A,F and Fig. 3A).

To demonstrate that these membrane stacks corresponded to the R-rings observed by immunofluorescence, we embedded the cells in LR White resin and stained the thin sections with immunogold for the transfected HA-NoppR. In these preparations, the membranes were poorly preserved and mostly the electron-dense material between the membrane stacks was visible. Indeed, antibodies to the HA-epitope specifically labeled the electron-dense material between and surrounding the membranes, confirming their identity as R-rings (Fig. 2F). The direct association of the transfected NoppR with the membranes suggested that it was physically involved in the formation of the R-rings. This was particularly well illustrated in an example where HA-NoppR, detected with Nopp140 antibodies, lined the inner nuclear membrane and a putative R-ring in the process of budding from the inner membrane (Fig. 2G, arrow). On the basis of the colocalization with the transfected NoppR of all the nucleolar antigens listed in Table 1, the electron-dense Nopp140 matrix consisted of all these factors from the fibrillar parts of the nucleolus. The ring-shaped appearance of the R-rings by indirect immunofluorescence was likely due to the lack of penetration of the antibodies through the dense layers of this Nopp140 matrix.

The membrane stacks exerted various effects on their immediate surroundings. If they occurred in the nucleoplasm, the outermost membrane was surrounded by a halo or coat-like regular structure about 100 nm away, which blended in another 100 nm or so into the nucleoplasm (Fig. 2B, arrow and Fig. 2D). The intranucleolar membrane stacks, however, integrated seamlessly into the nucleoli (Fig. 2A). The nucleolus itself lost its characteristic tripartite appearance and formed an amorphous mass consisting mainly of granular component (Fig. 2A). This was in agreement with the loss of all the factors from the fibrillar parts of the nucleolus, including the snoRNAs, but the retention of nucleolin and B23/NO38 of the granular component (Table 1) (Isaac et al., 1998). Because preribosomes appear to make up the bulk of the granular component of the nucleolus, the retention of nucleolin and B23/NO38 was consistent with their documented association with these particles (Hügle et al., 1985; Pinol-Roma, 1999).

NoppR-transfected cells frequently exhibited invaginations of specifically the inner membrane of the nuclear envelope. For example, where the inner and outer nuclear membranes were separated during sectioning, transfected NoppR was observed lining the inner membrane including a bud (Fig. 2G, arrow). Additionally, invaginations were frequently noted attaching to the inner membrane but leaving the outer membrane unperturbed (Fig. 2H, arrow). These buds and invaginations likely represented R-rings at early stages of development, as they were not observed in untransfected cells. Therefore, these data are consistent with our findings that R-rings are specifically derived from the inner nuclear membrane (see

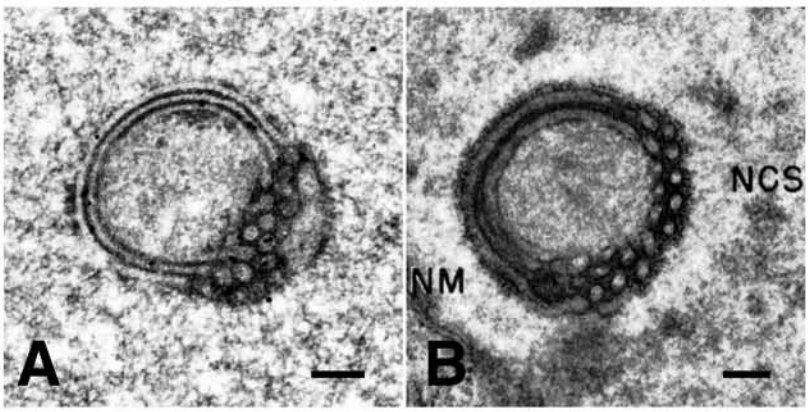


Fig. 3. The ultrastructure of the R-rings is indistinguishable from that of the nucleolar channel system. (A) Immunoelectron micrograph of an R-ring induced by NoppR in COS cells and labeled with Nopp140 antibodies as in Fig. 2G. (B) Electron micrograph of a nucleolar channel system (NCS) induced by progesterone in a human endometrial cell in the secretory phase. Reproduced from *The Journal of Cell Biology* (Terzakis, 1965) by copyright permission of The Rockefeller University Press. A section of the nuclear envelope (nuclear membrane, NM) is visible. Note the granular matrix that embeds both tubular membrane systems and the halo-like appearance of their surrounding nucleoplasm. Bars, 0.2 μm.

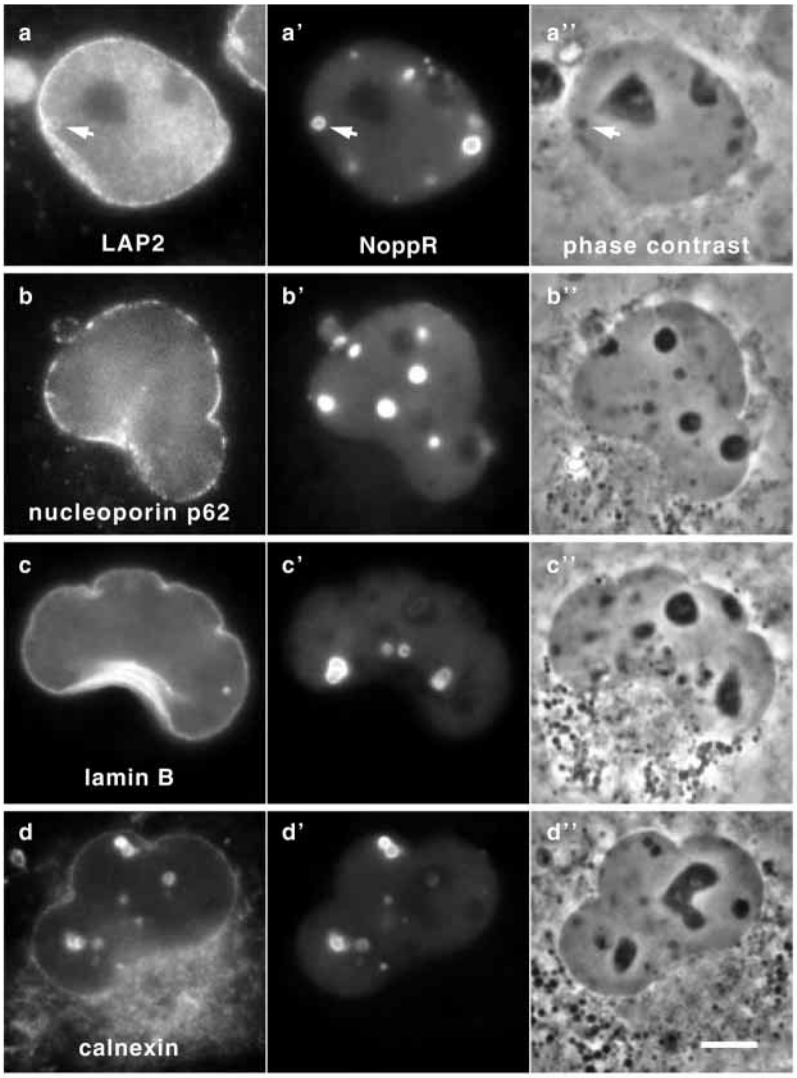


Fig. 4. R-rings are distinct from the nuclear envelope but contain an ER-specific membrane protein. Double immunofluorescence of HA-NoppR-transfected COS cells stained for the inner nuclear membrane protein, LAP2 (a), the nuclear pore complex protein, p62 (b), the nuclear lamina protein, lamin B (c) and the ER transmembrane protein, calnexin (d). To identify the R-rings, the cells were double labeled with HA-antibodies for the transfected HA-NoppR (a'-d') and imaged by phase contrast (a"-d"). One R-ring is pointed out (arrow). Bar, 10 μm.

Discussion) (Fig. 7). Nevertheless, multilamellar R-rings were rarely observed connected to the nuclear envelope, suggesting that they may become detached with increase in size and that they might twist off from the nuclear envelope as documented for the NCS (More and McSeveney, 1980). In summary, exogenous expression of the Nopp140 repeat domain caused the induction of intranuclear stacks of membrane cisternae.

The R-rings appear identical to the nucleolar channel system

The unexpected discovery of intranuclear membrane cisternae made us wonder whether similar observations had previously been reported. To our knowledge, the only well-documented occurrence of intranuclear membrane cisternae is the NCS of the human endometrium. The NCS forms an intricate channel system originating from the inner nuclear membrane and often associating with nucleoli (Terzakis, 1965). To test whether the NCS was related to the R-rings, we compared the published ultrastructure of the NCS to that of R-rings. Like the R-rings, the NCS frequently forms multilamellar and tubular membrane cisternae in the nucleus (Fig. 2) (Clyman, 1963; Dubrauszky and Pohlmann, 1961; More and McSeveney, 1980; Terzakis, 1965). The most striking example of similarity between the two nuclear membrane systems is depicted in Fig. 3. It compares two transmission electron micrographs taken 35 years apart and shows an R-ring in a COS cell nucleus immunogold labeled for Nopp140 (Fig. 3A) apposed to a reproduction of an NCS in a human endometrial cell (Fig. 3B) (Terzakis, 1965). The membrane lamellae and tubules of the R-rings and the NCS look identical and the granules of the electron-dense matrix in which they are embedded are of the same size. In both cases, the nucleoplasmic inclusion in the center has a slightly different appearance from that surrounding these structures. Finally, both structures are surrounded by a lighter halo and appear to be tethered to the rest of the nucleoplasm. Taken together, the R-rings and the NCS are morphologically indistinguishable.

R-rings are distinct from the nuclear envelope and consist of ER

Because both the R-rings and the NCS appear to be structurally derived from the inner nuclear membrane, we investigated whether they were nuclear envelope related. Employing indirect immunofluorescence in NoppR-transfected cells, we probed for the presence in R-rings of an integral membrane protein of the inner nuclear membrane, the lamin associated protein, LAP2 (Foisner and Gerace, 1993; Furukawa et al., 1995). Surprisingly, LAP2 failed to accumulate in the R-rings (Fig. 4a) identified by double immunofluorescence for the transfected HA-NoppR (Fig. 4a'-d') and by phase contrast imaging (Fig. 4a"-d"). Only on rare occasions, minimal amounts of LAP2 were detected in R-rings (Fig. 4a, arrow). Additionally, the R-rings were devoid of nuclear pore complexes or nuclear lamina as documented by the absence of the respective marker proteins, p62 (Fig. 4b) (Davis and Blobel, 1986) and lamin B (Fig. 4c) (Gerace and Blobel, 1980). Antibodies to these proteins stained the nuclear envelope but never the R-rings (Fig. 4b,c). The unaffected localization of the lamina in NoppR-transfected cells explained the absence of LAP2 in the R-rings because LAP2 remained tethered to the lamina in the nuclear envelope (Ellenberg et al., 1997; Foisner and Gerace, 1993; Furukawa et al., 1995). Finally, no nuclear pore complexes were detected in any of the electron micrographs of the R-rings and the NCS (Fig. 2; Fig. 3) (Terzakis, 1965). Taken together, these data demonstrated that the R-rings are distinct from the nuclear envelope.

To examine whether any integral membrane proteins were part of the R-rings, we tested for the presence of calnexin, an ER chaperone (Wada et al., 1991). Unlike LAP2, calnexin

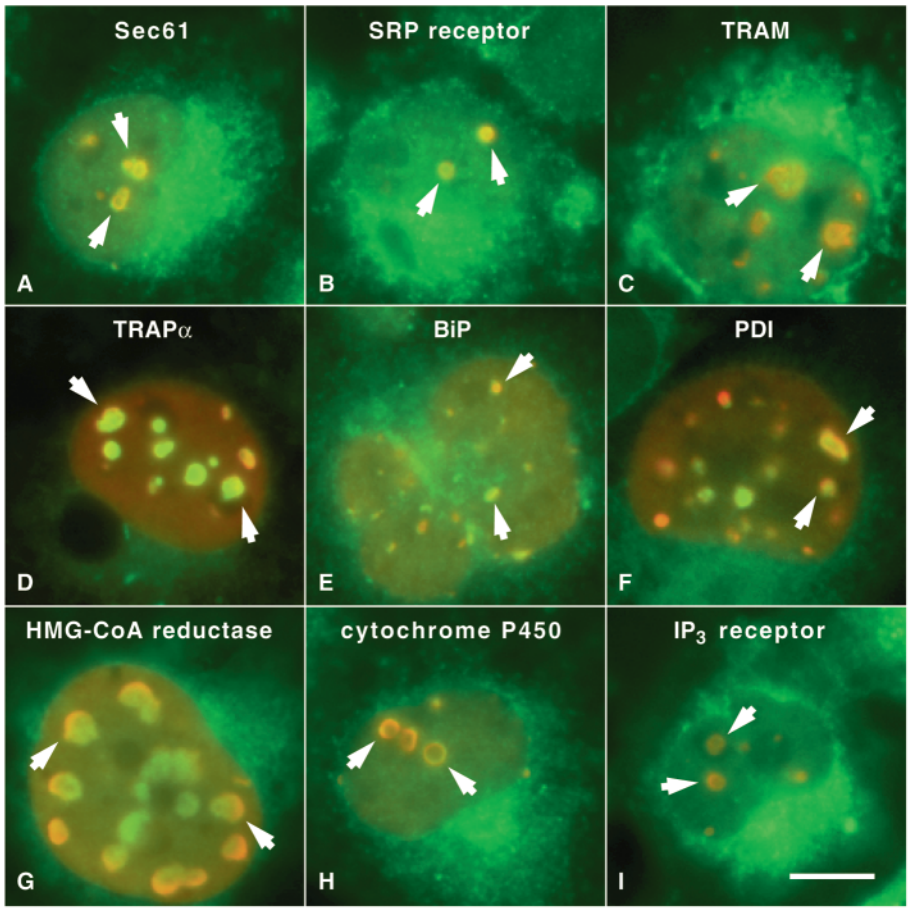


Fig. 5. R-rings consist of intranuclear ER. Merged images of double immunofluorescence of HA-NoppR-transfected COS cells stained for HA-NoppR (red) and the ER marker proteins (green), Sec61 (A), SRP receptor (B), TRAM (C), TRAPα (D), BiP (E) PDI (F), HMG-CoA reductase (G), cytochrome P450 (H) and the IP_3 receptor (I). Note the overlap in yellow and orange identifying the R-rings (two are pointed out by arrows in each panel). Bar, 10 μm.

accumulated in every single R-ring (Fig. 4d) identified by the transfected HA-NoppR (Fig. 4d'). In fact, calnexin concentrated to an even higher degree in the R-rings than in the ER and in the nuclear envelope (Fig. 4d). Because calnexin is a specific ER resident, our results suggested that the R-rings represented a proliferation of ER in the nucleoplasm. To confirm this possibility, we tested for the presence of additional ER membrane proteins in R-rings. Specifically, we stained NoppR-transfected cells by indirect immunofluorescence with antibodies to the protein translocation machinery associated proteins Sec61, SRP receptor, TRAM and TRAPα (Johnson and van Waes, 1999). All these integral membrane proteins (green) localized, in addition to the ER and the nuclear envelope, to the R-rings (Fig. 5A-D, yellow and orange, arrows; Table 1). As in the previous experiments (Fig. 1; Fig. 4), the R-rings were identified by double immunofluorescence for the transfected HA-NoppR (red) and by phase contrast microscopy (not shown). Even the lumenal ER proteins, BiP and PDI accumulated in the R-rings demonstrating that the lumen of the R-rings is similar to that of cytoplasmic ER (Fig. 5E,F; Table 1). Extensive proliferation of ER in the cytoplasm has been observed by the overexpression of integral ER membrane proteins, such as HMG-CoA reductase, cytochrome P450 and IP_3 receptor (Chin et al., 1982; Schunck et al., 1991; Takei et al., 1994). We tested to see if these smooth-ER-associated antigens also accumulated in our intranuclear membranes. Indeed, HMG-CoA reductase, three isoforms of cytochrome P450, and the IP_3 receptor were all present in the R-rings (Fig. 5G-I, arrows; Table 1). Thus, the nuclear R-rings contained marker proteins of the rough and smooth ER indicating that they were equivalent to cytoplasmic ER.

The nucleolar channel system is biochemically related to the R-rings

To test whether the NCS was also biochemically equivalent to the R-rings, we stained paraffin sections of human endometrium from the secretory phase of the menstrual cycle for calnexin, BiP and Nopp140. In double-immunofluorescence experiments, calnexin antibodies recognized, in addition to the cytoplasmic ER, an intranuclear structure in epithelial cells (Fig. 6A, arrow in top panel). This structure was closely apposed to the nucleolus identified by staining with nucleolin antibodies (Fig. 6A, middle panel). The merged image of the two immunostains showed that the intranuclear structure was separate from but adjacent to the nucleolus (Fig. 6A, bottom panel). The presence of the integral ER membrane protein calnexin in this structure suggested that it was membranous, ER derived and, given its occurrence in endometrial epithelial cells of the secretory phase of the menstrual cycle, an NCS. To investigate whether Nopp140 was present in similar structures, sections from the same sample were stained with Nopp140 and nucleolin antibodies. Indeed, in some nuclei Nopp140 (red) was located, in addition to the nucleolus, in extranucleolar foci (Fig. 6B, arrows in top panel). These structures were devoid of nucleolin (Fig. 6B, green, middle panel) and were particularly visible if the immunofluorescence images of Nopp140 and nucleolin were merged (arrows, bottom panel). These structures were unlikely to be Cajal bodies because they were too large and because they were present in only a few nuclei that usually occurred in clusters; both arecharacteristics of the NCS (More and McSeveney, 1980) but not of Cajal bodies. Unfortunately, these endometrial sections were particularly refractory to staining with antibodies to any protein of the dense fibrillar component of the nucleolus (e.g. Nopp140 and fibrillarin; note the poor labeling in Fig. 6B,C) or of the related Cajal bodies (p80 coilin, not shown). Therefore, to investigate a direct nuclear colocalization between an ER marker and Nopp140 in these cells, we affinity purified the rat Nopp140 antibodies against recombinant human Nopp140. Although this improved the situation only marginally (Fig. 6C), it allowed these antibodies

Table 1. Summary of molecules tested for their presence in R-rings

	Molecule	Description/function	Subcellular location	In R-rings
Nucleus	Nopp140	snoRNP chaperone	DFC, CB, nucleoplasm	+*
	NAP57/dyskerin	Core components of box	DFC, CB	+*
	GAR1	H/ACA snoRNPs		+§
	snR36 snoRNA‡			+§
	Fibrillarin	Core components of box	DFC, CB	+*
	U14 snoRNA	C/D snoRNPs		+§
	RNA polymerase I	Transcription of	FC	+*
	UBF	ribosomal RNA		+*
	Nucleolin	Ribosome biogenesis	GC¶, nucleoplasm	–*
	B23/NO38			–*
	p80 coilin	CB marker	CB, nucleoplasm	+*
	Sm antigens	Core components of spliceosomal snRNPs	Nucleoplasm	–*
Nuclear envelope	LAP2	Lamin-associated protein	Inner nuclear membrane (integral)	±§
	p62	Nucleoporin	Nuclear pore complex	–§
	Lamin B	Lamin	Nuclear lamina	–§
Cytoplasm (ER)	Calnexin	Chaperone	RER membrane (integral)	+§
	Sec61	Translocon and	RER membrane (integral)	+§
	TRAM	associated proteins		+§
	SRP receptor			+§
	TRAPα			+§
	BiP	Soluble chaperones	RER lumen	+§
	PDI			+§
	Cytochrome $P450_b$	ER-associated enzymes	SER membrane (integral)	+§
	Cytochrome $P450_c$			+§
	Cytochrome $P450_j$			+§
	HMG-CoA reductase			+§
	IP_3 receptor			+§
Membranes	$DiOC_6$	Lipophilic dye	All membranes	+§

Abbreviations: CB, Cajal body; DFC, dense fibrillar component of the nucleolus; FC, fibrillar center of the nucleolus; GC, granular component of the nucleolus; RER, rough ER; SER, smooth ER.

*Isaac et al., 1998.

‡Exogenously expressed yeast box H/ACA snoRNA.

§Present study.

¶Nucleolin is also present in the DFC.

to detect Nopp140 (red) in nuclear structures (arrow) besides nucleoli (arrowheads, compare to phase contrast panel). Double labeling with antibodies against BiP also stained this putative NCS in addition to the ER (Fig. 6C, arrow in green panel). This was particularly evident when the Nopp140 and BiP panels were overlaid (Fig. 6C, arrow in merge panel). Phase contrast imaging (Fig. 6C, bottom panel) revealed the relatedness of this putative NCS (arrow) to R-rings by displaying a lucent area around a dense core as observed for R-rings (Fig. 1c'). To further assure that these tissue sections from staged human endometrium contained NCSs, they were deparaffinized, postfixed, epon embedded and sectioned for electron microscopy. Indeed, electron micrographs revealed NCSs in some of the epithelial cell nuclei (Fig. 6D). The NCS was often adjacent to a nucleolus (Fig. 6D), suggesting that the structures detected by calnexin, BiP and Nopp140 immunofluorescence in these sections (Fig. 6A-C) represented bona fide NCSs. We conclude that the NCS and the R-rings are not only structurally related but also both contain Nopp140 and ER markers.

DISCUSSION

We report that the overexpression of Nopp140 or of its hallmark repeat domain induces the unique proliferation of ER cisternae in the nucleus. These multilamellar structures, termed R-rings, have an apparent counterpart in human endometrial cells, the NCS. Our findings have fundamental implications for membrane proliferation, nuclear envelope topology and the physiology of the human endometrium. The striking likeness of the R-rings and the NCS in ultrastructure (Fig. 3) and in the protein components calnexin, BiP and Nopp140 (Fig. 6) strongly suggests that the NCS, like the R-rings, consists of bona fide ER whose formation is triggered by Nopp140. Thus, Nopp140 may play a direct role in the preparation of the endometrium for the implantation of the fertilized egg and thereby be essential for human reproduction.

The induction of ER proliferation by the overexpression of integral membrane proteins has been well documented. Thus, the overexpression of HMG-CoA reductase, IP_3 receptor, cytochrome P450, cytochrome b_5, lamin B receptor, 180 kDa ribosome receptor and aldehyde dehydrogenase, among others, leads to the accumulation of various forms of tightly packed ER stacks in the cytoplasm (Chin et al., 1982; Schunck et al., 1991; Smith and Blobel, 1994; Takei et al., 1994; Vergères et al., 1993; Wanker et al., 1995; Wright et al., 1988; Yamamoto et al., 1996). These membranes usually originate from the outer nuclear membrane and often remain associated with it. However, no soluble protein before has caused such an effect on membrane proliferation and, particularly, not within the nucleus. The most closely related case is the overexpression of

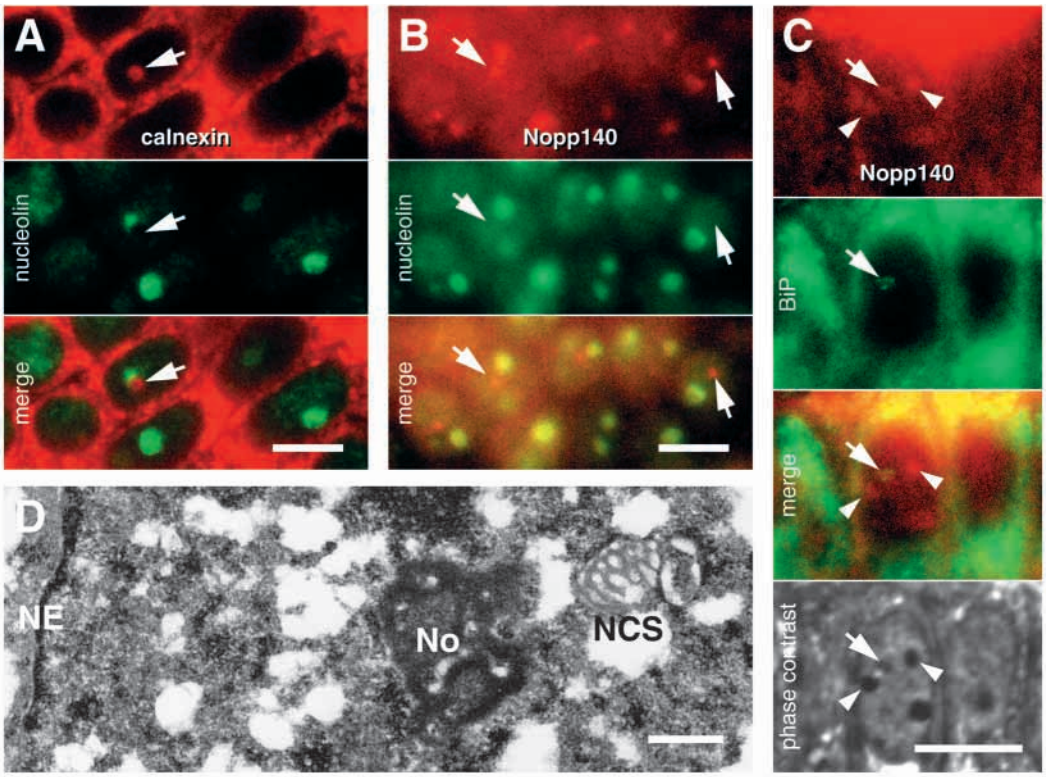


Fig. 6. The NCS, like the R-rings, contains calnexin, BiP and Nopp140. (A–C) Paraffin sections of human endometrium in the secretory phase, stained by double immunofluorescence for the ER transmembrane marker calnexin (A) and for Nopp140 (B and C, red panels). (A) and (B) were double labeled for the nucleolus with nucleolin and (C) for ER with BiP antibodies (green panels). The two images in each were merged. Some putative NCSs are indicated by arrows and two nucleoli are pointed out by arrowheads in (C). Bars, 10 μm. (D) Transmission electron micrograph of a deparaffinized section as in (A-C). The nucleolus (No), an adjacent NCS and the nuclear envelope (NE) are labeled. Note, the white holes in the nucleoplasm reflect the poor preservation of the paraffin sections. Bar, 0.5 μm.

the nuclear pore complex protein Nup153, which causes the formation of intranuclear membrane arrays (Bastos et al., 1996). However, the Nup153 induced structures are clearly distinct from R-rings because they contain lamins and nuclear pore complexes and because they are mostly apposed to the nuclear envelope. Therefore, they may be more closely related to tubular invaginations of the nuclear envelope (Fricker et al., 1997). Similarly, membrane induction caused by the overexpression of Nup53 in yeast suggests a role for this nucleoporin in nuclear envelope biogenesis unrelated to R-rings (Marelli et al., 2001).

How can ER cisternae form in the nucleoplasm? Other than de novo, there are only two possible models for the biogenesis of the R-rings. They are schematically represented in Fig. 7. In model A, the R-rings could result from the invagination of the entire nuclear envelope, i.e. the inner and outer membranes (Fig. 7A). In model B, the invagination of only the inner membrane could form the R-rings (Fig. 7B). In both cases, the invaginations are directly triggered and eventually accompanied by the transfected Nopp140 and all the nucleolar factors that are recruited to that location (see below) (Table 1), forming an electron dense Nopp140 matrix (Fig. 7, gray sprinkled layer). Therefore, the two models can easily be distinguished by the repeat unit of ER cisternae and Nopp140 matrix in close-ups of R-ring cross-sections (Fig. 7, boxed section). In model A, two layers of Nopp140 matrices would be separated by two apposed ER cisternae that may additionally be separated by a layer of cytoplasm (Fig. 7A). In model B, every single ER cisternae is separated by a layer of Nopp140 matrix (Fig. 7B). On the basis of these two models, the high magnification of a section through an R-ring demonstrates that they must originate exclusively from the inner nuclear membrane (compare Fig. 2B with Fig. 7B). This is further supported by the direct visualization of the budding of putative R-rings from the inner membrane in NoppR-transfected cells (Fig. 2G,H). Additionally, model A is unlikely because it predicts the presence of nuclear pore complexes and lamina as seen in other examples of nuclear envelope invaginations (Fricker et al., 1997). R-rings, however, are clearly devoid of pore complexes and lamina (Fig. 4b,c). Moreover, it is well documented that the NCS, which we show here to be equivalent to R-rings, originates specifically from the inner membrane of the nuclear envelope (Armstrong et al., 1973; More and McSeveney, 1980; Terzakis, 1965). Finally, in a recent study, intranuclear membrane cisternae in Schwann cells, likely induced by the overexpression of HMG-CoA reductase, also appear as invaginations of the inner nuclear membrane (Berciano et al., 2000). Therefore, the R-rings/NCS form a contiguous membrane network with the inner nuclear membrane and, through the pore membrane domain, with the outer nuclear membrane and with the smooth and rough ER (Fig. 7B).

Unlike the inner nuclear membrane and the smooth and rough ER, however, the R-rings/NCS are not endowed with a

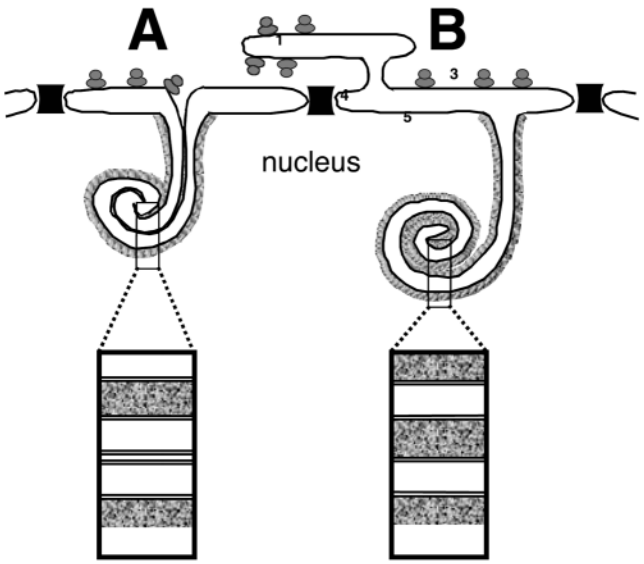


Fig. 7. Models of R-ring/NCS biogenesis from the nuclear envelope. (A) R-rings/NCS form by the invagination of the entire nuclear envelope, the outer and inner nuclear membranes, lined by an electron-dense Nopp140 matrix (gray sprinkled layer). (B) R-rings/NCS form by the exclusive invagination of the inner nuclear membrane. Integral membrane proteins of the rough (1) and the smooth ER (2) and synthesized on the outer nuclear membrane (3) can laterally diffuse through the pore membrane domain (4) and the inner nuclear membrane (5) into the R-rings. The boxed regions show how a close-up of the repeat patterns of Nopp140 matrix and intranuclear membrane cisternae would look for each model.

specific subset of transmembrane proteins but rather with a mixture of smooth and rough ER proteins (Table 1). Consequently, integral membrane proteins synthesized in the ER or the outer nuclear membrane can laterally diffuse through the pore membrane domain and the inner membrane into the R-rings/NCS. Thus, all the integral membrane proteins of the outer nuclear membrane and the ER have free access to the inner nuclear membrane as originally observed for viral glycoproteins (Bergmann and Singer, 1983; Torrisi and Bonatti, 1985; Torrisi et al., 1987). Furthermore, all these ER proteins can laterally diffuse into the R-rings, whereas the inner nuclear membrane proteins are retained in their proper location by multimerization or by binding to the nuclear lamina and/or chromatin (Smith and Blobel, 1993; Soullam and Worman, 1993; Soullam and Worman, 1995). This observation is consistent with a diffusion/retention mechanism for targeting of inner membrane proteins (Ellenberg et al., 1997) and with our results of the R-rings lacking lamins and the lamina-associated LAP2 (Fig. 2a,c).

How does a soluble protein induce ER proliferation? Is this effect direct or indirect? In the latter case, one could imagine that Nopp140 stimulates transcription of an integral membrane protein, which could lead to membrane proliferation as outlined above. Although Nopp140 can exhibit transcriptional activation activity in certain systems (Miau et al., 1997), this scenario fails to explain why these membranes are formed in the nucleus. By contrast, the embedding of the R-rings in a matrix, to which the transfected Nopp140 localizes exclusively, strongly favors a direct involvement of Nopp140 in R-ring formation. This is further supported by the direct localization of the transfected NoppR to a bud of the inner nuclear membrane (Fig. 2G, arrow) that likely represents an emerging R-ring. Therefore, we propose a direct and physical role for Nopp140 in R-ring/NCS biogenesis similar to that of coat proteins in vesicle formation (Scales et al., 2000). For example, the lysine-rich regions of the repeat domain could directly interact with the negatively charged head groups of the phospholipids as in the case of myosin (Areas et al., 1998; Doberstein and Pollard, 1992; Reizes et al., 1994). Alternatively, or in addition, the highly phosphorylated acidic serine stretches could form a similar interaction via divalent cations, such as calcium, as documented for the interaction of dextran sulfate with phospholipid bilayers (Huster and Arnold, 1998; Huster et al., 1999). The latter model is attractive because Nopp140 indeed binds calcium in a phosphorylation-dependent manner (U.T.M., unpublished) and because of the presence of calcium channels in the R-rings (Fig. 5I). The physical interaction of Nopp140 with the inner nuclear membrane may also provide the molecular mechanism underlying nuclear envelope attachment of nucleoli observed in most cells (Bourgeois and Hubert, 1988).

The protein composition of specific membranes is usually a testimony of their function, i.e. the rough ER in protein synthesis, the smooth ER in enzymatic activities and the inner nuclear membrane in nuclear structure and organization. No such specialization of the R-rings/NCS can be determined because of their complex composition of both smooth and rough ER proteins. We speculate that the R-rings/NCS lack a mechanism to specifically retain transmembrane proteins so that all accumulate nonselectively. Nevertheless, it is likely that only one or few of these proteins directly pertain to the function of the R-rings/NCS. This could occur in two ways; first, by a direct function of that particular protein/enzyme in the intranuclear membranes or second, by siphoning the protein/enzyme into the R-rings thereby depleting its function from the ER. Our model system of R-ring/NCS induction will now help to dissect which of these proteins is important in this context.

A possible role for the NCS may be derived from the following facts. The peak of NCS induction, six days postovulation, correlates exactly with the time of blastocyst attachment to the endometrium. Moreover, only ~5% of all the epithelial cells contain NCSs and these cells are clustered throughout the endometrium (More and McSeveney, 1980; Ryder et al., 1995). Therefore, it is tempting to speculate that the blastocyst for its endometrial attachment selects these clusters of epithelial cells containing NCSs. Thus, there may be a specific signaling event required between the epithelial NCS and the trophoblast of the blastocyst to ensure proper attachment. Our model R-ring/NCS tissue culture cell system will allow us to test if these cells exhibit specific secretory functions or a specific response to extracellular stimuli. Ultimately, these studies will contribute to our understanding of how these intranuclear membranes may be crucial for blastocyst attachment.

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