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The Nucleolar Channel System of Human Endometrium Is Related to Endoplasmic Reticulum and R-Rings

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The nucleolar channel system (NCS) is a well-established ultrastructural hallmark of the postovulation endometrium. Its transient presence has been associated with human fertility. Nevertheless, the biogenesis, composition, and function of these intranuclear membrane cisternae are unknown. Membrane systems with a striking ultrastructural resemblance to the NCS, termed R-rings, are induced in nuclei of tissue culture cells by overexpression of the central repeat domain of the nucleolar protein Nopp140. Here we provide a first molecular characterization of the NCS and compare the biogenesis of these two enigmatic organelles. Like the R-rings, the NCS consists of endoplasmic reticulum harboring the marker glucose-6-phosphatase. R-ring formation initiates at the nuclear envelope, apparently by a calcium-mediated Nopp140-membrane interaction, as supported by the calcium-binding ability of Nopp140, the inhibition of R-ring formation by calcium chelators, and the concentration of Nopp140 and complexed calcium in R-rings. Although biogenesis of the NCS may initiate similarly, the reduced presence of complexed calcium and Nopp140 suggests the involvement of additional factors.

INTRODUCTION

For close to half a century, the nucleolar channel system (NCS) has been known as an ultrastructural hallmark of the postovulation human endometrium (Dubrauszky and Pohlmann, 1960; Clyman, 1963; Moricard and Moricard, 1964; Terzakis, 1965). It consists of several layers of tubular membrane cisternae in the nuclei of endometrial epithelial cells and is often associated with the nuclear envelope and nucleoli, hence the name (for a review see Spornitz, 1992). This organelle appears during a 3- to 4-d window in the midsecretory phase of the menstrual cycle when the endometrium is receptive to implantation of the fertilized egg (Clyman, 1963; Gordon, 1975). Oral contraceptives and intrauterine devices interfere with the timing and/or overall appearance of the NCS (Wynn, 1967; Feria-Velasco *et al.*, 1972; Azadian-Boulanger *et al.*, 1976; van Santen *et al.*, 1988; Dockery *et al.*, 1997). Several cases of unexplained primary infertility have been characterized by the absence or delayed development of NCSs as the only ultrastructural alteration in endometrial epithelial cells (Kohorn *et al.*, 1972; Gore and Gordon, 1974; Dockery *et al.*, 1996). This and other correlative evidence suggests a role for the NCS in the preparation of the uterine surface for embryo implantation and points to the NCS as a long sought after marker for human receptivity. Nevertheless, despite this tantalizing data and the detailed ultrastructural description of the NCS, it has resisted molecular characterization. Here we provide first insights as to the composition of this enigmatic organelle.

On the basis of their remarkable ultrastructural resemblance to the NCS, we previously characterized membrane systems, R-rings, that are induced in nuclei of tissue culture cells by overexpression of the nucleolar protein Nopp140 (Isaac *et al.*, 1998; Isaac *et al.*, 2001). Nopp140 functions as a chaperone for small nucleolar ribonucleoproteins (snoRNPs) in the nucleolus and Cajal bodies and has been implicated as a transcription factor (Meier and Blobel, 1992, 1994; Miau *et al.*, 1997; Isaac *et al.*, 1998; Chen *et al.*, 1999; Yang *et al.*, 2000; Chiu *et al.*, 2002; Wang *et al.*, 2002). Nevertheless, the presence of Nopp140 in R-rings suggests its physical involvement in their biogenesis. R-rings, like the NCS, consist of several layers of tubular membrane cisternae embedded in an electron-dense matrix and are often associated with nucleoli and the nuclear envelope. However, unlike the composition of the NCS, that of the R-rings is well defined. They consist of bona fide endoplasmic reticulum (ER), harboring integral membrane and luminal markers of both smooth and rough ER, but are devoid of nuclear envelope–specific structures, such as nuclear pore complexes and lamina. The electron-dense matrix of R-rings contains all markers tested from the dense fibrillar component and the fibrillar centers of nucleoli, in particular Nopp140, and its associated snoRNPs, but is devoid of proteins from the granular component, e.g., nucleolin and B23 (Isaac *et al.*, 2001). Despite this detailed molecular definition, however, it is unclear how the soluble Nopp140 can induce membrane systems in the normally membrane-free nucleus. We now demonstrate how Nopp140, aided by calcium, induces R-rings and explore if and how this biogenesis extends to the NCS of human endometrium.

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Abbreviations used: NCS, nucleolar channel system; ER, endoplasmic reticulum.

MATERIALS AND METHODS

Human Endometrial Biopsies

Endometrial biopsies were obtained by informed consent from normally cycling women in a protocol approved by the Institutional Review Board. Volunteer, reproductive aged women 18–38, who had a history of regular menstrual cycles and were free of any intrauterine lesions as assessed by hysterosonography before enrollment (Parsons and Lense, 1993), provided the endometrial biopsies for these studies. Biopsies were obtained at specified times within the menstrual cycle, based on previous cycle length (for follicular phase samples) and luteinizing hormone (LH) surge detection for postovulatory samples. A Pipelle catheter was inserted into the uterus and gently rotated as it was withdrawn under negative pressure. This method yielded intact tissue cylinders with preservation of tissue architecture. A portion of the tissue was fixed in 4% methanol-free paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and processed for paraffin embedding. A small amount of formaldehyde-fixed tissue was stored in 2% paraformaldehyde at 4°C for use in cryosectioning.

Cryosectioning and Immunostaining of Endometrial Tissue

Endometrial tissue was fixed in 4% paraformaldehyde in neutral phosphate-buffered saline. Cryosectioning was done by the method of Tokuyasu (Tokuyasu, 1973; Griffiths *et al.,* 1983b, 1984). Briefly, tissue was infiltrated after fixation using 2.3 M sucrose as a cryoprotectant. After mounting on cryopins and freezing in liquid nitrogen, 90-nm (ultrathin) cryosections were cut using a Leica UCT cryoultramicrotome and placed on nickel grids (Deerfield, IL). For immunostaining, sections were blocked in 1% powdered milk in phosphate-buffered saline and then stained by human Nopp140 antiserum RS8 at a dilution of 1:2 for 2 h followed by 10-nm gold-labeled secondary antibodies for 1 h. Human Nopp140 antibodies were raised in rabbits (Covance Research Products, Madison, WI) against bacterially expressed antigen (Isaac *et al.,* 2001). Sections were contrasted in 0.2% uranyl acetate and dried in a film of 2% methylcellulose before being observed by transmission electron microscopy.

Electron Microscopy

For ultrastructural analysis, transfected tissue culture cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, and dehydrated through a graded series of ethanol, and the monolayer was scraped up and embedded in Epon resin. Ultrathin sections (90 nm) were cut on a Reichert Ultracut E microtome (Vienna, Austria), stained with uranyl acetate followed by lead citrate and viewed on a JEOL 100CXII transmission electron microscope (Peabody, MA) at 80 kV. Endometrial tissue was fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and processed as above. For electron spectroscopic imaging, specimens were prepared without using heavy metals such as osmium tetroxide and embedded in the resin Quetol 651 (Electron Microscopy Sciences). Electron spectroscopic imaging was performed as previously described (Dellaire *et al.,* 2004) by using a Tecnai 20 transmission electron microscope (FEI, Eindhoven, The Netherlands) equipped with an electron imaging spectrometer (Gatan, Pleasanton, CA).

Glucose-6-Phosphatase Assay

The histochemical localization of glucose-6-phosphatase was adapted from published protocols (Wachstein and Meisel, 1956; Griffiths *et al.,* 1983a). Briefly, 2-mm pieces of secretory endometrial tissue were fixed in 0.5% glutaraldehyde in 100 mM PIPES buffer, pH 7.0, containing 5% sucrose. After fixation, tissues were placed in an incubating medium made by dissolving 0.19 g of glucose-6-phosphate in 10 ml of 80 mM Tris-maleate buffer, pH 6.5, and slowly adding 80 μl of a 12% lead nitrate solution. After incubation for 4–12 h at room temperature, tissues were washed in Tris-maleate buffer, dehydrated, and processed for Epon resin embedding for transmission electron microscopy. The assay was done identically on monolayers of COS-1 cells that were transfected with the Nopp140 repeat domain to induce R-ring formation, with an incubation time of 2 h at room temperature.

Tissue Culture, Transfection, and Calcium Overlay

COS-1 cells were cultured in high-glucose DMEM (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen). U2OS cells were cultured in low-glucose DMEM containing 10% FBS. Cells were grown on 12-mm coverslips and transiently transfected using FuGene 6 (Roche, Indianapolis, IN) for 30 h according to manufacturer's protocol. The following constructs were used: HA-hNopp140 (pNK59, full-length human Nopp140 [Isaac *et al.,* 2001] was cloned into pSVM [Isaac *et al.,* 1998]); mGFP-Sec61γ (Snapp *et al.,* 2003); and HA-hNoppΔN (pNK53, human Nopp140 lacking its 59 amino terminal residues was amplified and cloned into pSVM). For BAPTA-AM treatment, cells were treated with 10 μM BAPTA-AM (Sigma, 10 mM stock solution in DMSO) for 30 h. For calcium overlay assays, proteins were separated by standard SDS-PAGE, transferred to nitrocellulose membranes, and probed with ^{45}Ca as described (Maruyama *et al.,* 1984). After exposure for autoradiography, the membranes were stained with amido black. The calcium-binding protein TRAP (formerly SSR) was a kind gift from G. Migliaccio (Instituto di Ricerce Biologia Moleculare, Rome, Italy) (Migliaccio *et al.,* 1992).

Immunofluorescence and Antibodies

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-HA ascites fluid (12CA5 at 1:200; Wilson *et al.,* 1984) and anti-calnexin polyclonal serum (SPA860 at 1:200; StressGen, San Diego, CA). Imaging was performed at room temperature using a 60×/1.4 NA planapo objective on an inverted microscope (1× 81; Olympus, Melville, NY) containing automatic excitation and emission filter wheels connected to an air-cooled charge-coupled device camera (Sensicam QE; Roper Scientific, Tucson, AZ) run by IPLab Spectrum software (Scanalytics, Billerica, MA). Images were processed for contrast and brightness using Photoshop CS2 (Adobe, San Jose, CA). When indicated, images were deconvolved using Hazebuster software (VayTek, Fairfield, IA) quick deconvolution method.

RESULTS

The NCS is ER related

To characterize NCSs, we collected biopsies of human endometrium during the secretory phase of the menstrual cycle and fixed and processed them for transmission and cryoelectron microscopy. A low-magnification transmission electron micrograph of an endometrial biopsy from day 18 of the menstrual cycle reveals the ultrastructural hallmarks of the postovulatory epithelial cells, namely subnuclear vacuoles with glycogen deposits (Figure 1A, single asterisks), giant mitochondria (mito), and NCSs (boxed). Three NCSs are visible in this section consistent with previous studies suggesting that cells containing these enigmatic membrane cisternae appear to cluster (More and McSeveney, 1980). Interestingly, the apical plasma membranes of some of the epithelial cells have lost their microvilli and are bulging into the lumen, reminiscent of pinopodes (Figure 1A, double asterisks). Pinopodes have been proposed as ultrastructural markers for receptivity and have been observed near or at the site of blastocyst attachment (Martel, 1981; Nikas *et al.,* 1995; Bentin-Ley *et al.,* 1999; Nikas, 1999a,b). Therefore, NCSs appear at the right time and place to play a role in endometrial receptivity, although it should be noted that recently pinopodes have been questioned as markers for the implantation window (Petersen *et al.,* 2005; Quinn *et al.,* 2007).

Using indirect immunofluorescence, we established that R-rings consist of ER containing all tested marker proteins of rough and smooth ER (Issac *et al*., 2001). As ultrastructural approaches are the only methods to unequivocally identify NCSs, we used a histochemical method to identify the ER marker enzyme glucose-6-phosphatase in R-rings and in our NCS-positive endometrial biopsies (Figure 2). In this method, concomitant incubation with glucose-6-phosphate and lead nitrate leads to lead-phosphate precipitates that are detectable by electron microscopy at sites where glucose-6-phosphatase liberates phosphate (Wachstein and Meisel, 1956; Griffiths *et al.,* 1983a). As expected, in tissue culture cells transfected with the Nopp140 repeat domain for R-ring induction, lead phosphate precipitates specifically in the cytoplasmic ER, the contiguous nuclear envelope (NE), and in R-rings (Figure 2A). A higher magnification micrograph reveals all precipitates in the lumen of these organelles consistent with the intraluminal active site of this membrane-bound enzyme (van Schaftingen and Gerin, 2002; Figure 2B). Similarly, in endometrial biopsies, lead phosphate precipitates in the cytoplasmic ER, the nuclear envelope, and, importantly, the NCS (Figure 2C). Note the specificity of the labeling of only the ER but not mitochondria (mito), which are surrounded by a single layer of ER, a hallmark of

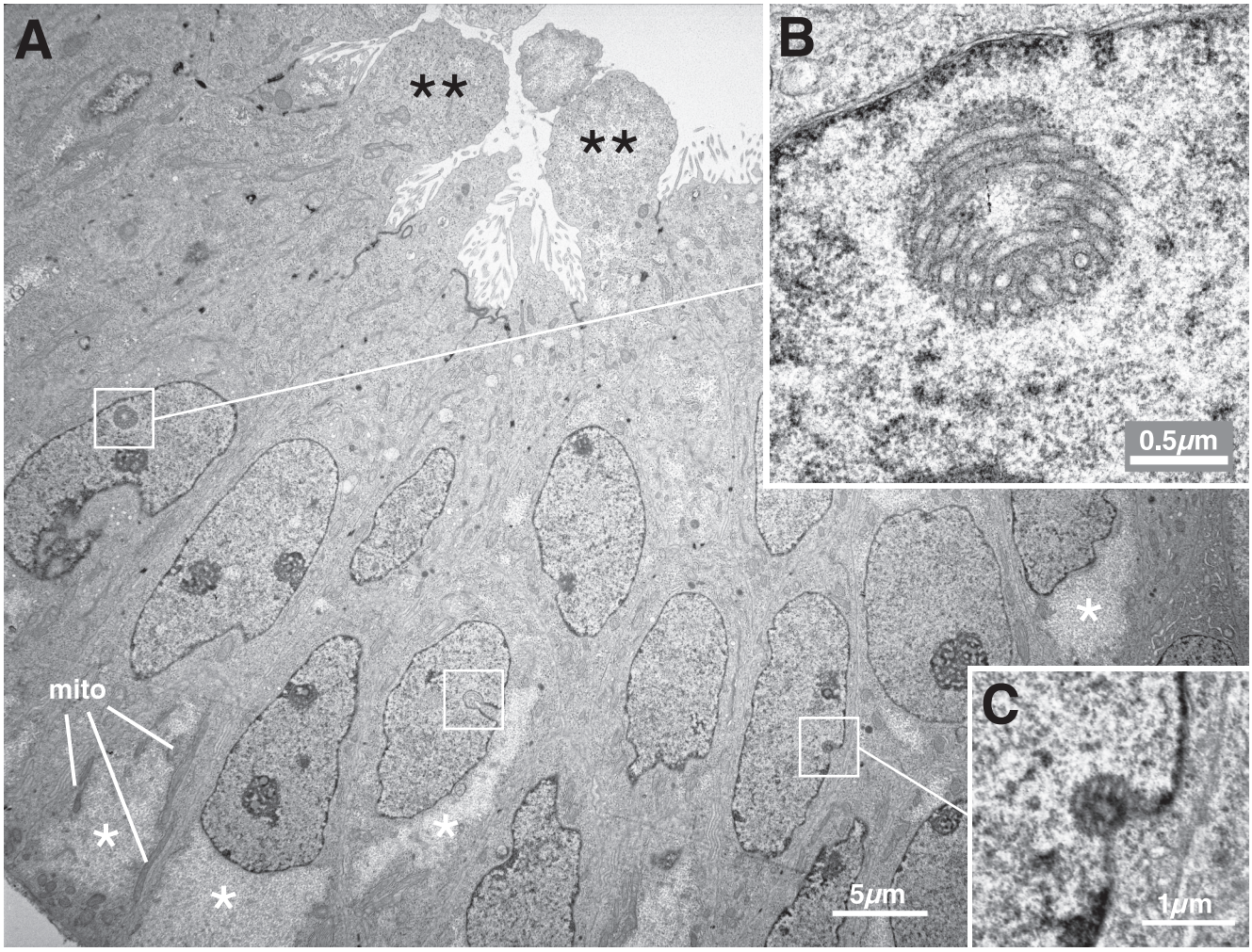

Figure 1. Transmission electron micrograph of a human endometrial gland from a biopsy of cycle day 18. (A) Low magnification illustrates the hallmarks of the epithelial cells from this stage of the cycle, subnuclear vacuoles (single asterisks), pinopodes (double asterisks), giant mitochondria (mito), and NCSs (boxed). (B) and (C) Higher magnifications of the respective NCSs.

the epithelial cells of this stage of the cycle (Verma, 1983). The presence of lead phosphate precipitates, and therefore glucose-6-phosphatase, in the lumen of the NCS cisternae identifies this organelle as ER derived (Figure 2D). Significantly, glucose-6-phosphatase is the first unequivocally identified molecular component of the NCS.

R-Ring Formation

To explore possible mechanisms of NCS biogenesis, we turned to our tissue culture model of NCS induction, R-rings (Isaac *et al.*, 2001). These membrane cisternae are generated in nuclei of tissue culture cells by exogenous expression of the repeat domain of Nopp140. Additionally, full-length rat Nopp140 induces intranuclear ring-shaped structures that are membranous based on their staining with the lipophilic dye $DiOC_6$ at the light microscopic level (Isaac *et al.*, 2001). It came therefore as a surprise, when these structures proved to be mostly electron-dense aggregates devoid of any discernable membranes at the ultrastructural level (not shown, but see Figure 3B, asterisks). Because the NCS is human specific and absent from all other species tested, we asked if full-length human Nopp140 would behave differently. Full-length human Nopp140, like its rat counterpart, accumulated in intranuclear ring-shaped structures when overexpressed (Figure 3A, I). Some of these contained the ER integral membrane marker calnexin (Figure 3A, II). Indeed, on an ultrastructural level, some of these structures are bona fide R-rings adjacent to or engulfed by nucleoli (Figure 3B, arrow), whereas some appear as electron-dense aggregates like those induced by rat Nopp140 (Figure 3B, asterisks). Identical results were obtained with the human Nopp140 alpha and beta isoforms, which differ by the inclusion of a 10-amino acid exon in the repeat region of Nopp140 alpha (Pai and Yeh, 1996). Therefore, full-length human Nopp140 induces R-rings consisting of intranuclear membrane cisternae reminiscent of the human-specific endometrial NCSs.

How are membrane cisternae generated in the usually membrane free nucleus? Two ways come to mind: de novo and through growth of preexisting membrane systems. The latter is the most prevalent form of cellular membrane induction. Indeed, previous evidence suggested that R-rings are derived from the inner membrane of the nuclear envelope (Isaac *et al.*, 2001). Therefore we investigated more directly if and how the soluble Nopp140 causes the formation of R-rings from the inner nuclear membrane. In addition to forming R-rings, transfected Nopp140 frequently

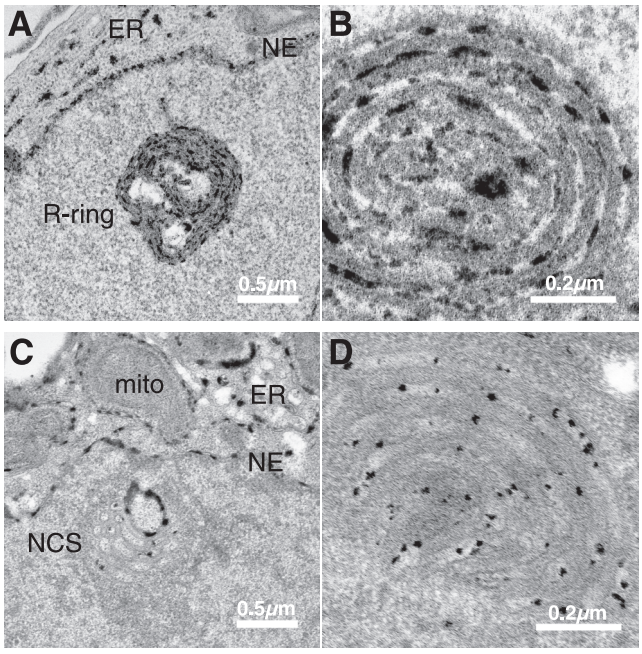

Figure 2. Transmission electron micrographs of histochemical labeling of glucose-6-phosphatase activity in R-rings (A and B) and in NCSs (C and D). (A) Black lead-phosphate precipitates in the ER, the nuclear envelope (NE), and R-rings of Nopp140 repeat domain transfected COS-1 cells after fixation and incubation with glucose-6-phosphate and lead nitrate. (B) Higher magnification of an R-ring identifies the precipitates in the lumen of R-ring cisternae. (C) As in A but on an endometrial biopsy from the secretory phase. Note the specific labeling of the NCS and the ER, but not the mitochondria (mito), which are surrounded by a single layer of ER in these cells. (D) Higher magnification of an NCS demonstrating the precipitates in the lumen of the NCS cisternae.

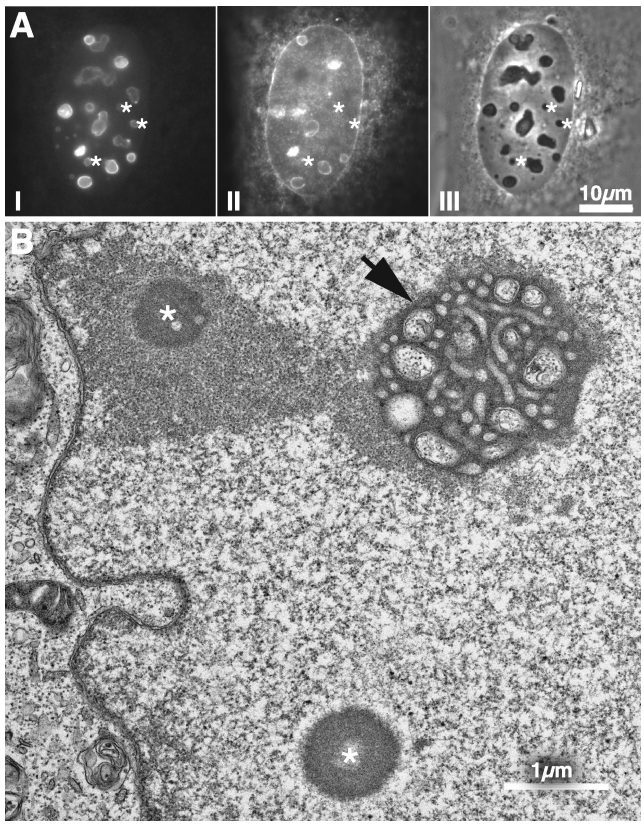

Figure 3. Induction of R-rings and intranuclear aggregates by the transfection of HA-tagged, full-length human Nopp140 into U2OS cells. (A) Double immunofluorescence of a transfected cell stained for the HA-epitope (I) and the integral ER membrane protein calnexin (II); and a phase-contrast image of the same cell (III). Note that some HA-, i.e., Nopp140-positive structures are devoid of calnexin (some are marked by asterisks). (B) Transmission electron micrograph of a transfected cell with an R-ring (arrow) and intranuclear aggregates that are apparently membrane free (asterisks).

accumulates in patches at the nuclear envelope suggestive of R-ring initiation at the nuclear periphery (Figure 4A). To monitor if R-rings indeed bud from the nuclear envelope, we performed live imaging experiments on cells whose ER was labeled by transient transfection of an integral ER membrane marker tagged with monomeric green fluorescent protein (GFP), mGFP-Sec61γ. When these cells were concomitantly transfected with the Nopp140 repeat domain to induce R-rings, membranous extensions developed from the nuclear envelope resulting in a globular ER structure inside the nucleus (Figure 4B). Over time these structures grew (Figure 4B, compare insets in II and III). These R-rings often remained attached to the nuclear envelope through a stalk (Figure 4B, arrows). In fact, similar stalks coming off the nuclear envelope with attached R-rings were identified in electron micrographs of cells transfected with full-length human Nopp140 (Figure 4C). These findings, supported by previously published data (Isaac *et al.*, 2001), suggest that R-rings are a direct derivative of the inner nuclear membrane and that Nopp140, through its presence, plays a physical role in their initiation.

A Role for Calcium in R-ring Biogenesis

The repeat domain of Nopp140, which is responsible for R-ring formation (Isaac *et al.*, 1998), is a highly charged domain consisting of alternating positively and negatively charged repeats. Positively charged stretches of amino acids can bind directly to the head groups of phospholipids as in the case of myosin and other proteins with polybasic clusters (Doberstein and Pollard, 1992; Reizes *et al.*, 1994; Areas *et al.*, 1998; Heo *et al.*, 2006). Alternatively or in addition, negatively charged amino acids, such as in the highly phosphorylated Nopp140 repeats, can interact with phospholipids via calcium bridges as in the case of synaptogamin I, PKCβ, and dextran sulfate (Huster and Arnold, 1998; Zhang *et al.*, 1998; Huster *et al.*, 1999; Nalefski *et al.*, 2001). Because the lumen of the ER and the nuclear envelope is highly enriched in calcium and because R-rings contain calcium channels, such as the IP_3 receptor (Isaac *et al.*, 2001), we tested if Nopp140 is a calcium-binding protein by using a blot overlay technique (Maruyama *et al.*, 1984). For this purpose, we prepared Western blots from supernatants and pellets of immunoprecipitations with Nopp140 peptide antibodies in the presence and absence of free competing Nopp140 peptide, using low salt rat liver nuclear extracts (Figure 5A). Under these conditions, Nopp140 is precipitated and partially removed from the supernatant only in the absence of competing peptide (Figure 5A, lanes 1/2 and 6/7) but not in its presence (lanes 3/4 and 8/9). For visualization of calcium-binding proteins, the nitrocellulose membranes were first probed with ^{45}Ca followed by autoradiography (Figure 5A, lanes 1–5) and subsequently for general visualization of proteins with amido black (lanes 6–10). The major calcium-binding protein in these extracts is a band of 140 kDa (Figure 5A, lane 3) that is identified as Nopp140 (arrowhead) by its precipita-

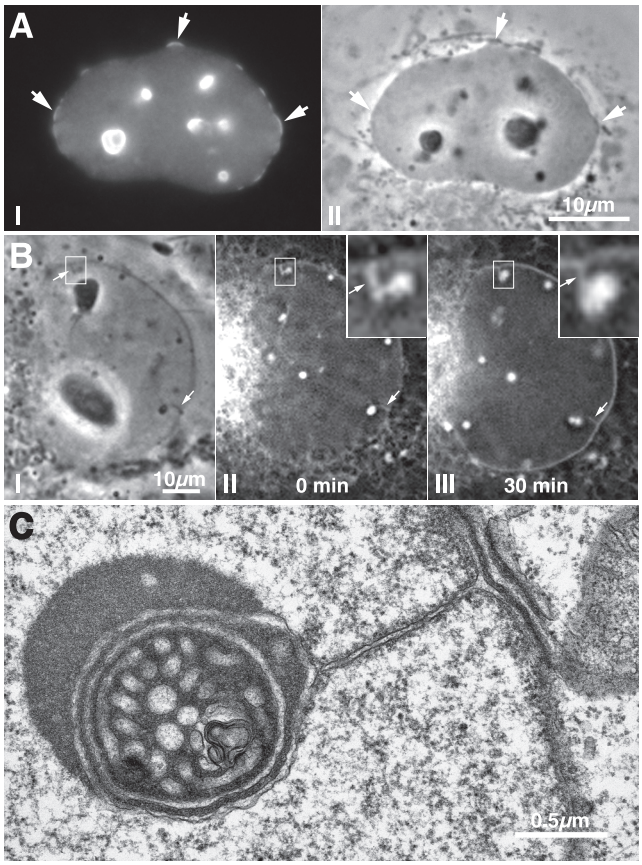

Figure 4. Initiation of R-ring formation at the nuclear envelope. (A) HA immunofluorescence of a COS-1 cell transfected with human Nopp140 lacking its amino terminus, which only induces R-rings but no aggregates, (I) and the corresponding phase contrast image (II). Note the Nopp140 accumulation at the nuclear envelope (arrows) in addition to the intranuclear R-rings. (B) R-ring formation in live cells visualized by cotransfection of the Nopp140 repeat domain and the GFP-tagged ER integral membrane marker, Sec61γ. GFP fluorescence of the same cell observed 30 min apart (II and III, deconvolved images) and phase contrast image at time zero (I). Note the stalklike connections to the nuclear envelope of some of the intranuclear membrane structures (arrows). Insets document the growth of an R-ring over time. (C) Transmission electron micrograph of an example of an R-ring attached to the inner membrane of the nuclear envelope through a stalk in a Nopp140-transfected cell.

tion (lane 2) and partial removal from the supernatant (lane 1) in the absence but not presence of competing peptide (lanes 3 and 4, respectively). In addition to Nopp140, only one or two minor, lower molecular weight calcium-binding proteins are detected in the extract (Figure 5A, lane 3, asterisk). Purified translocon-associated protein (TRAP) was included as a positive control on the blots, because only the alpha but not the beta form binds calcium (Wada *et al.*, 1991; Hartmann *et al.*, 1993; Figure 5A, compare lanes 5 and 10). To test if phosphorylation of Nopp140 was required for its calcium binding, the calcium overlay assay was performed on phosphorylated Nopp140 purified from rat liver and unphosphorylated recombinant Nopp140. Indeed, phosphorylated, but not unphosphorylated, Nopp140 bound calcium avidly (Figure 5B). We conclude that Nopp140 binds calcium in a phosphorylation-dependent manner.

To test if R-ring formation itself was calcium dependent, we used a membrane-permeable intracellular calcium chelator, BAPTA-AM, on COS-1 cells transfected with an HA-tagged human Nopp140 construct lacking the N terminus. This construct induces R-rings without inducing the protein aggregations that the full-length protein also induces, making it more suitable for this experiment. BAPTA-AM was added immediately after transfection, whereas an equivalent amount of the solvent, DMSO, was used as a control. Cells were fixed 30 h later and analyzed for R-ring formation by immunostaining with anti-HA antibodies. Results were expressed as the percentage of transfected cells (detected by nuclear HA staining) with observable R-rings and showed that treatment with the calcium chelator BAPTA-AM reduced R-ring formation by one third (Figure 5C). Taken together, these data support the initiation of R-ring formation by a calcium-mediated Nopp140-membrane interaction.

Complexed Calcium in the Matrix of R-Rings and NCSs

Calcium should be complexed between the phosphate groups of Nopp140 and those of the phospholipids in the membrane if it is involved in membrane invagination in this manner. Such enriched calcium phosphate complexes, unlike the soluble calcium stores within the ER, can be detected using electron spectroscopic imaging. This electron microscopic technique is based on the principle that each element in a sample absorbs characteristic amounts of energy from the incident electron beam. The loss of energy of the incident electrons can be analyzed by an electron spectrometer, which is also capable of forming an image with electrons of a particular energy loss. Thereby, the spectrometer produces an element-specific image (Dellaire *et al.*, 2004). For this

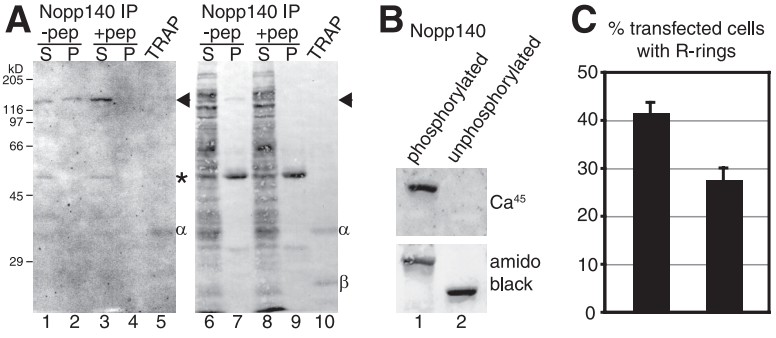

Figure 5. A role for calcium in R-ring formation. (A) Nopp140 is a calcium-binding protein. Autoradiogram of a nitrocellulose membrane overlaid with ^{45}Ca (lanes 1–5) and subsequently stained with amido black (lanes 6–10). Nopp140 was immunoprecipitated from low-salt nuclear extracts with peptide antibodies (lanes 2, 4, 7, and 9) in the absence (lanes 1, 2, 6, and 7) or presence of competing peptide (lanes 3, 4, 8, and 9). Half of each supernatant after precipitation was analyzed (lanes 1, 3, 6, and 8). Purified translocon associated protein (TRAP) was loaded as a control (lanes 5 and 10). (B) Calcium overlay (top panel) and amido black stain (bottom panel) as in A of purified phosphorylated (lane 1) and unphosphorylated recombinant Nopp140 (lane 2). Note the difference in mobility due to the difference of ~80 phosphates between the two Nopp140 forms. (C) Effect of the membrane permeable calcium chelator BAPTA-AM on R-ring formation. Cells were transfected with Nopp140 lacking its amino terminus as in Figure 4A and treated with 10 μM BAPTA-AM. The transfected cells containing R-rings were counted 30 h later. The average of 11 independent transfections with SE is depicted as the percentage of cells with R-rings. Approximately 200 transfected cells were counted in each case; $p < 0.001$.

purpose, cells transfected with the repeat domain of Nopp140 for R-ring induction were embedded in Quetol resin, and ultrathin unstained sections were observed using an electron microscope equipped with an electron spectrometer capable of electron spectroscopic imaging. In the resulting images, R-rings were most highly enriched in complexed calcium when compared with any surrounding structure, e.g., heterochromatin or the nuclear envelope (Figure 6A, I). However, phosphorus was more enriched in heterochromatin than in R-rings, validating the specificity of calcium imaging (Figure 6A, II). When viewed at higher magnification, complexed calcium was enriched in the electron-dense matrix between the membrane tubules of the R-rings (Figure 6A, III) where Nopp140 is concentrated (Isaac *et al.*, 2001). Similarly, phosphorus was enriched at the same location (Figure 6A, IV). This was not surprising given the accumulation of the highly phosphorylated Nopp140 and its associated small nucleolar ribonucleoproteins (Isaac *et al.*, 2001). In summary, these data support a calcium-mediated Nopp140-membrane association.

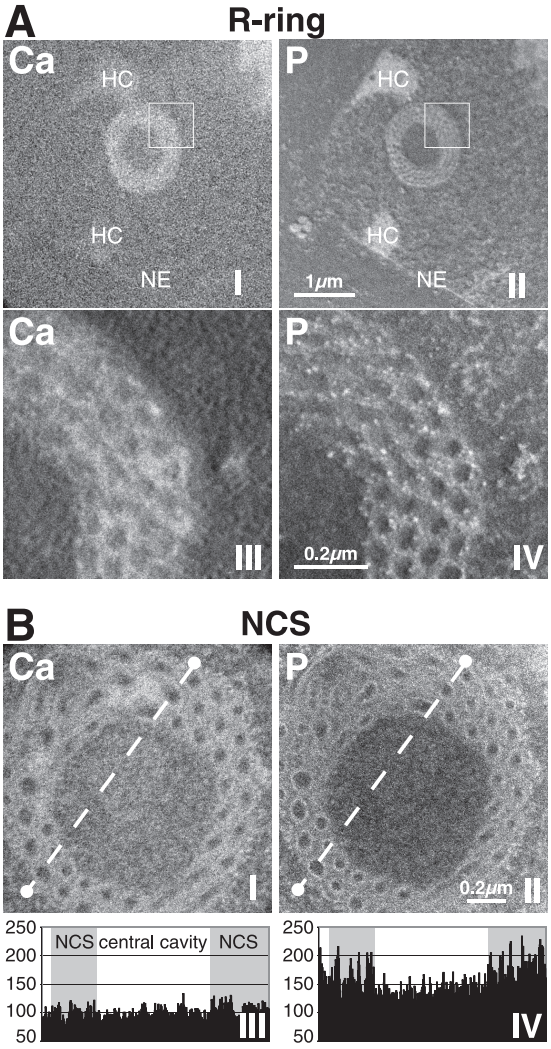

Figure 6. Electron spectroscopic imaging reveals enrichment of complexed calcium between the membrane cisternae of R-rings and NCS. (A) Electron spectroscopic micrographs of complexed calcium (I) and phosphorus (II) of a cell containing an R-ring. The nuclear envelope (NE) and heterochromatin (HC) are indicated. III and IV show enlarged images of the boxed sections of the R-ring in I and II, respectively. Note the enrichment of complexed calcium and phosphorus in the matrix but not the membrane cisternae of the R-ring. (B) The same as A but of an NCS in a human endometrial biopsy. III and IV represent histograms of the pixel intensities (based on the originally collected data) along the dashed line between the two dots in I and II, respectively. Note how calcium is enriched to a much lesser extent in the NCS (gray background) relative to the central NCS cavity and surrounding chromatin (white background) compared with phosphorus.

To test if the matrix of the NCS was similarly enriched in complexed calcium, endometrial biopsies of secretory human endometrium were fixed, embedded, and analyzed by electron spectroscopic imaging. As in the case of R-rings, complexed calcium was found in the electron-dense matrix between the membrane cisternae, although less enriched relative to neighboring structures, e.g., the central opening (Figure 6B, I and III). However, phosphorus was equally concentrated in the matrix of the NCS as in that of R-rings (Figure 6B, II and IV). Line scans through the NCS (Figure 6B, I and II, dashed line) measuring relative calcium and phosphorus pixel intensities revealed differences in the distribution of the two elements, arguing against erroneous calcium signal based on mass-density (Figure 6B, III and IV). Additionally, the line scan confirmed the only minor accumulation of calcium signal in the matrix of the NCS relative to the central cavity (Figure 6B, III). Therefore, formation of the NCS, like that of the R-rings, could be initiated by a calcium-mediated Nopp140-membrane interaction.

Nopp140 in the NCS

Using immunogold labeling on LR White–embedded tissue culture cells, we previously demonstrated the abundance of Nopp140 in the electron-dense matrix of the R-rings (Isaac *et al.*, 2001). However, immunogold labeling of endometrial tissue sections proved more challenging. Only the combination of mild fixation conditions (4% paraformaldehyde) and ultrathin cryosectioning preserved the Nopp140 epitope sufficiently for detection by immunogold labeling. Although Nopp140 was readily detected in the dense fibrillar component of nucleoli (Figure 7, A and B), the NCS was only labeled to a minor extent (Figure 7, A and C) or not at all (Figure 7B). Therefore, despite their striking ultrastructural resemblance, the NCS differs from the R-rings in molecular composition. In particular, its content of complexed calcium and Nopp140 is lower, indicating that NCS induction, unlike that of R-rings, is not a mere consequence of increased Nopp140 expression but that additional factors are involved in NCS biogenesis.

DISCUSSION

During the receptive phase of the menstrual cycle, the nuclei of epithelial cells of human endometrium develop multilayered tubular membrane cisternae, NCSs (Clyman, 1963; Terzakis, 1965; Spornitz, 1992). We now identify glucose-6-phosphatase as a first molecular marker for these unusual organelles, suggesting that they are ER derived. This finding adds to a number of unique features of these organelles, e.g., they are membrane tubules in the otherwise membrane-free nucleus, they are human-specific and apparently absent even from baboon endometrium (MacLennan *et al.*, 1971), they are transient structures appearing for only 3 to 4 d, their appearance correlates with the receptive phase of the menstrual cycle, and now they appear to represent intranuclear ER. Despite all these intriguing characteristics, the function of the NCS remains obscure. Nevertheless, the newly gained

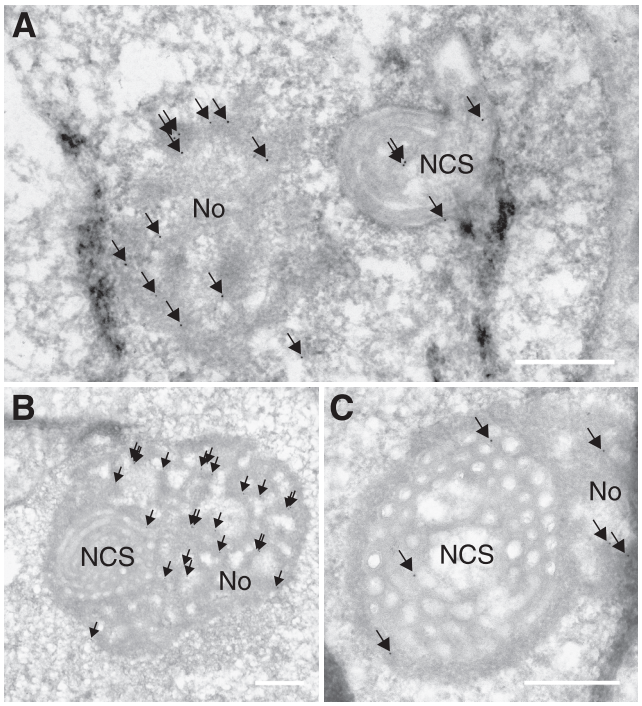

Figure 7. Immunoelectron micrographs with Nopp140 antibodies on ultrathin cryosections. (A–C) Nopp140 is enriched in the dense fibrillar component of nucleoli (No) but only moderately present in NCSs (A and C) or not at all (B). All gold particles are pointed out by arrows. Bars, 0.5 μm.

information may provide a reason for its existence. Thus, the ER in the nucleus may provide membrane surface in addition to the nuclear envelope for signaling from the nucleus to the ER and beyond. Or, these intranuclear membranes might be sequestering inhibitory molecules from the cell surface, thus facilitating implantation. Alternatively, these secretory cells are simply overproducing ER membranes (although this is not evident in the cytoplasm) that for some reason end up in the nucleus. It is also possible that the functionally important part of these structures is their electron-dense matrix, which we now show to contain some Nopp140 and complexed calcium. In that scenario, the membranes would merely provide a framework for the matrix. Which ever it is, the intriguing correlation of their appearance with the readiness of the endometrial surface for blastocyst attachment warrants their further scrutiny.

The Nopp140-induced R-rings, with their striking ultrastructural resemblance to the NCS, provided a model system for the generation of intranuclear and nucleolus-associated membrane systems. Five lines of evidence suggest that R-ring biogenesis is initiated at the inner nuclear membrane by a calcium-mediated Nopp140-membrane interaction. First, R-rings constitute simple invaginations of the inner nuclear membrane but not of the entire nuclear envelope (Isaac *et al.*, 2001). Second, transfected Nopp140 often appears in patches at the nuclear envelope (Figure 4; Isaac *et al.*, 2001). Third, Nopp140 is the most prominent calcium-binding protein in nuclear extracts (Figure 5A). A kinase and/or phosphatase may regulate calcium binding as only the phosphorylated form binds calcium (Figure 5B). In the case of the kinase it is likely casein kinase II, which is responsible for the extreme Nopp140 phosphorylation (Meier and Blobel, 1992; Meier, 1996; Li *et al.*, 1997). Unfortunately, we have been unable to directly test this possibility because none of the available inhibitors of casein kinase II and other kinases yielded dephosphorylated Nopp140 in cell culture (data not shown). Fourth, complexed calcium is enriched between the membrane tubules where Nopp140 is concentrated (Figure 6A; Isaac *et al.*, 2001). Finally and perhaps most importantly, calcium chelation interferes with R-ring formation consistent with a direct role for calcium in this process (Figure 5C). The fact that the inhibition by calcium chelation is only partial could have several explanations. For example, it is possible that calcium is not the only mediator for a Nopp140 membrane interaction. Thus, the positively charged stretches of the Nopp140 repeats could interact directly with the negatively charged head groups of the phospholipids as in the case of myosin and other proteins with polybasic clusters (Doberstein and Pollard, 1992; Reizes *et al.*, 1994; Areas *et al.*, 1998; Heo *et al.*, 2006). Of course, lowering the calcium concentration of a cell also could have many indirect effects eventually resulting in reduced R-ring formation. However, during the limited time of BAPTA-AM exposure, we did not observe any general impact on cell viability or morphology of the cells. The simplest explanation for the only partial inhibition is that the cellular calcium concentration was lowered insufficiently, but higher concentrations of BAPTA-AM could not be used because they did have adverse effects on cell viability (data not shown).

The important question, of course, was how related the biogenesis of the R-rings was to that of the NCS. Despite the restricted experimental approaches possible with human endometrial biopsies, we succeeded in determining the presence of complexed calcium and Nopp140 in the NCS. Although they are both present in the electron-dense matrix, they are less enriched than in R-rings, particularly Nopp140, which is extremely concentrated in R-rings, but barely detectable in NCSs by immunoelectron microscopy. Therefore, it does not appear that NCS formation is a mere consequence of Nopp140 overexpression in the endometrium but that additional factors are involved. Such factors are likely specific to the human endometrial epithelial cells, as Nopp140 is a ubiquitously expressed protein. Indeed, transfection of Nopp140 into a human endometrial adenocarcinoma cell line, Ishikawa (Nishida *et al.*, 1985) caused R-ring formation in much shorter time after transfection compared with transfection into nonendometrial cell lines (N. Kittur and U. T. Meier, unpublished observation). This suggests that endometrial epithelial cells may be specifically primed for NCS induction.

The surprising finding that full-length human but not rat Nopp140 induces R-rings may constitute an interesting parallel between the presence of NCSs in human but not rodent endometrium. Thus, despite their high sequence conservation in their amino and carboxyl termini, the repeat domain responsible for R-ring induction exhibits the largest sequence differences between human and rat Nopp140 (Meier and Blobel, 1992; Pai *et al.*, 1995; Meier, 1996). Therefore, the difference in Nopp140 could be directly responsible for the species differences in NCS formation. It was further surprising that nuclear structures induced by full-length rat Nopp140 stained with the lipophilic dye $DiOC_6$ (Isaac *et al.*, 2001), although, as judged by transmission electron microscopy, they were devoid of membranes (Figure 3B). It is thus possible that although lipids are present in these aggregates, they have not formed into visible membrane bilayers (compare R-rings and aggregates in Figure 3B). Unusually therefore, if these lipids could spontaneously form bilayers, R-rings could also form de novo.

Overall, our results suggest that despite their remarkable ultrastructural resemblance and their common derivation from the ER, NCSs differ from R-rings in their composition and biogenesis. Nevertheless, the true relationship between these two unusual organelles will not be known until technical advances allow a more complete picture of NCS composition to emerge, e.g., by detection and labeling at the light microscopic level.

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