Introduction

The nucleolus is a prominent structure within the cell nucleus which forms around the nucleolar organizing regions (NORs) in a cell cycle-dependent manner. The nucleolus is the cell ribosome factory and, in addition, it is also a multifunctional domain involved in a variety of processes and severe diseases including stress response, biogenesis of ribonucleoproteins, ribosomopathies and cancer (for reviews see refs. 1 and 2). It is therefore important to understand the function and regulation of this crucial cell compartment.

Throughout interphase, cells produce ribosomes with the maximal yield in G2 phase. The sequential stages of ribosome biogenesis are reflected in the general architecture of the nucleolus comprising three main sub-compartments, which have been well defined by electron microscopy: fibrillar centers (FC), dense fibrillar component (DFC), and granular component (GC). Transcription of ribosomal DNA (rDNA) by RNA polymerase I (Pol I) takes place mostly at the FC/DFC border. Pol I is a multi-polypeptide complex composed of constant subunits and temporarily associated factors. The main component of the Pol I machinery is the upstream binding factor (UBF). This architectural protein comprises six high mobility group (HMG) boxes enabling a single dimer of UBF to induce an almost 360° looping in 140 base-pairs of rDNA via multiple co-phased turns, forming the nucleo-protein structure referred to as the rRNA gene enhancesome.3-5 UBF binding to enhancer region of rDNA

UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity

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Abbreviations: NOR, nucleolar organizing region; FC, fibrillar center; DFC, dense fibrillar component; GC, granular component; rDNA, ribosomal DNA; Pol I, RNA polymerase I; UBF, upstream binding factor; HMG, high mobility group; PIC, pre-initiation complex; SL1, promoter selectivity factor; TBP, TATA-binding protein; TAF, TBP-associated factors; rRNA, ribosomal RNA; snrRNA, small nuclear RNA; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; AMD, actinomycin D; DRB, 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole; SIM, super-resolution structured illumination microscopy; HIF, high-pressure freezing; FS, freeze-substitution; IEM, immunoelectron microscopy; CTD, Pol II C-terminal domain; BH scale, basic and hydrophobic scale; U2OS cells, human osteosarcoma cells; HeLa cells, cervical carcinoma cells

To maintain growth and division, cells require a large-scale production of rRNAs which occurs in the nucleolus. Recently, we have shown the interaction of nucleolar phosphatidylinositol 4,5-bisphosphate (PIP2) with proteins involved in rRNA transcription and processing, namely RNA polymerase I (Pol I), UBF, and fibrillarin. Here we extend the study by investigating transcription-related localization of PIP2 in regards to transcription and processing complexes of Pol I. To achieve this, we used either physiological inhibition of transcription during mitosis or inhibition by treatment the cells with actinomycin D (AMD) or 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole (DRB). We show that PIP2 is associated with Pol I subunits and UBF in a transcription-independent manner. On the other hand, PIP2/fibrillarin colocalization is dependent on the production of rRNA. These results indicate that PIP2 is required not only during rRNA production and biogenesis, as we have shown before, but also plays a structural role as an anchor for the Pol I pre-initiation complex during the cell cycle. We suggest that throughout mitosis, PIP2 together with UBF is involved in forming and maintaining the core platform of the rDNA helix structure. Thus we introduce PIP2 as a novel component of the NOR complex, which is further engaged in the renewed rRNA synthesis upon exit from mitosis.
leads to the creation of open chromatin structure by displacing linker histone H1 and the assembly of pre-initiation complex (PIC) on the promoter. PIC formation involves concerted action of UBF and the promoter selectivity factor (SL1) consisting of TATA-binding protein (TBP) and Pol I-specific TBP-associated factors TAFI110, TAFI63, and TAFI48. UBF recruits SL1 via the interaction with TAFI48 and TBP, where TAFI3 provide highly sequence-specific promoter recognition.14-16 UBF also binds extensively across the transcribed regions of the rDNA and is involved in the early stages of rRNA processing.16 rRNA 2'-O-methyltransferase that localizes to the DFC region and is released into the cytoplasm resulting in Ca2+ mobilization, and inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to DAG remains bound to the membrane where it activates protein kinase C leading to a cellular response (for a review see ref. 22).

Upon entry into mitosis, the ribosome production stops in prophase and the nucleus is then disassembled in a sequential manner. The repression of Pol I transcription is connected with the ordered release of the processing complexes from rDNA transcription machinery. At the end of prophase, upon chromosome condensation and breakdown of nuclear envelope, the interphase nuclear architecture is not detectable, but the components of rDNA transcription machinery and pre-rRNA processing machinery remain partially assembled and are kept at different sites throughout mitosis. rDNA, that was actively transcribed in the preceding interphase, remains associated with the Pol I subunits, UBF, SL1 subunits and transcription termination factor TTF1 and resides in the mitotic NORs.20 The proteins and small nucleolar RNA (snoRNAs) of the processing complexes, ribosomal proteins as well as the pre-rRNA molecules remain assembled and stored in either the nucleoli or fibrillar regions, although Pol I transcription is preserved.29 We show that PIP2 is associated with Pol I subunits and UBF in a transcription-independent manner. On the other hand, PIP2/fibrillarin colocalization is dependent on the production of rRNA. These results indicate that PIP2 is required not only during rRNA production and biogenesis, as we have shown before, but also plays a structural role as an anchor for the Pol I pre-initiation complex during the cell cycle. We suggest that throughout mitosis PIP2 together with UBF is involved in forming and maintaining the core platform of the rDNA helix structure. Thus we introduce PIP2 as a novel component of the NOR complex, which is further engaged in the renewed rRNA synthesis upon exit from mitosis.

Results

PIP2 association with Pol I complex is transcription-independent

We showed recently that PIP2 participates in formation of Pol I transcription foci in interphase nucleus.27 Nevertheless, the dependence of PIP2 localization to the sites of Pol I transcription factory on the transcriptional status of the cell is not known. We chose mitotic cells with physiologically blocked transcription and used an immunoprecipitation assay to detect the components of Pol I transcription machinery still associated with PIP2. We confirmed the detection reliability by monitoring NOR integrity with immunofluorescent microscopy taking the samples during cell fractionation. We specifically detected UBF and two subunits of Pol I: PAF53 and RPA116, in the same complex with PIP2 (Fig. 1). Based on the association of the components of Pol I pre-initiation complex with PIP2 in mitotic cells, we asked whether this association is retained in NORs during all mitotic stages. To test this, we used super-resolution structured illumination microscopy (SIM) combined with multi-immunolabeling approach as well as high-pressure freezing and freeze-substitution (HPF/FS) combined with immunoelectron microscopy (IEM). We showed previously that HPF/FS preserves the near-native state of nuclear compartments and molecular complexes.30 We
observed regular colocalization patterns of PIP2 and UBF during different mitotic phases in which transcription is physiologically inhibited. UBF serves as a marker of mitotic NORs due to its persistent presence on extended fibers of rDNA during the entire cell cycle. SIM showed colocalization between PIP2 and UBF in NORs throughout prophase and metaphase, continuing in “daughter” NORs in anaphase and telophase (Fig. 2). HPP/F5 IEM confirmed this colocalization of PIP2 with UBF in clearly distinguishable NORs, characterized by less compacted structure as compared with the bulk of condensed chromatin, from prophase to telophase (Fig. 3).

Low concentration of AMD was used to investigate the effect of chemically provoked inhibition of Pol I transcription on PIP2 colocalization with Pol I and UBF. In transcriptionally active interphase nuclei, we showed by confocal microscopy that PIP2 colocalizes with Pol I and UBF, as seen by signal intensity profiles (Fig. 4A). In agreement with this, IEM also showed PIP2 localization in a close proximity to Pol I and UBF in the FC region of nuclei, which is the counterpart of mitotic NORs, where the inactive components of Pol I transcription machinery accumulate during interphase (Fig. 5A). Pol I inhibition by AMD treatment results in nuclear segregation and formation of a central body associated with caps. Immuno- fluorescence microscopy showed that PIP2 still colocalized with Pol I and UBF even though Pol I transcription was blocked (Fig. 4B). IEM further demonstrated intermingling clusters of PIP2 with Pol I and UBF in the light part of the caps as seen in Figure 5B.

To prove that this effect is Pol I–specific and not caused by the side inhibition of Pol II transcription, we used DRB as an inhibitor of the phosphorylation of Pol II C-terminal domain (CTD), blocking its activity. DRB-induced Pol II inhibition causes alterations in the nucleolar architecture and results in “beads on a string” morphology. Despite the fact that there are prominent changes in the structure of nucleolar subdomains, rRNA genes remain transcriptionally active. Upon Pol II transcription inhibition, nucleolar beads were assembled and PIP2 colocalization with Pol I and UBF persisted as documented in the corresponding signal intensity profiles (Fig. 4C). In agreement, IEM demonstrated PIP2 localization in very close vicinity to Pol I and UBF in DRB-treated cells (Fig. 5C).

Taken together, these results show that the association of PIP2 with the components of Pol I pre-initiation complex is not dependent on active transcription, and it is maintained throughout the cell cycle.

PIP2 colocalization with fibrillarin in nuclei is transcription-dependent

Fibrillarin methylates pre-rRNAs and its recruitment to the DFC region of the nucleolus is dependent on active transcription. We investigated the effect of transcription inhibition on the localization of PIP2 and fibrillarin in the DFC region of nuclei. PIP2 and fibrillarin colocalize in actively transcribing cells (Fig. 4A). IP2/fibrillarin clusters can be readily seen in the DFC region by IEM (Fig. 5A). However, upon inhibition of Pol I transcription, IP2/fibrillarin colocalization is lost as seen in the signal intensity profile (Fig. 4B). Fibrillarin is concentrated in the dense part of the caps, and in most cases does not colocalize with PIP2 after AMD treatment (Fig. 5B). Upon Pol II inhibition by DRB treatment, PIP2 colocalization with fibrillarin is restricted to a limited area in the nucleolus due to the segregation of DFC and FC regions in newly formed nucleolar beads (Fig. 4C). In accordance, we clearly distinguished DFC region, where PIP2 intermingled with fibrillarin, and FC region, where only PIP2 was localized (Fig. 5C).

These results indicate that PIP2/fibrillarin colocalization is dependent on active transcription.

Discussion

It has been known for many years that the nucleus retains significant amounts of lipids, including phospholipids, even after experimental removal of the nuclear membrane. Due to the lack of membranous structures inside the nucleus, it has been suggested that other hydrophobic molecules maintain a particular environment for phospholipids to be localized in the nucleus (for reviews see refs. 38 and 39). The nucleolus is not separated from the nucleoplasm by a membrane yet it has a unique structure and composition of the nucleolus has been intensively studied, and the mechanisms of their formation and maintenance remain unclear (for review see ref. 41). Here we studied the dependence of subnucleolar localization of PIP2 and the proteins involved in pre-rRNA synthesis and processing on the transcriptional activity of the cells.

We found that PIP2 is not localized in the GC region, where the assembly and maturation of pre-ribosome particles take place, but it forms clusters in the FC and DFC regions responsible for transcription and processing of rRNA. Recently, we have demonstrated that PIP2 promotes Pol I transcription and directs UBF binding to a more selective site on the rDNA promoter. Further, we showed the alteration in fibrillarin binding to
rRNA upon interaction with PIP2. Since UBF and fibrillarin are reported to have roles in the formation of nucleoli, these results suggest a role for PIP2 as well in nucleoli formation. Here we investigated if PIP2 is still present in the sites of Pol I transcription factory following inhibition of transcription. The effect of transcription inhibition was tested in native conditions during mitosis as well as using the treatment with AMD and DRB, which alter nucleolar architecture significantly. Indeed, we found that upon either physiological or chemically induced inhibition of Pol I transcription, PIP2 maintains its association with the components of Pol I pre-initiation complex but not with fibrillarin. These data reinforce the view that PIP2 interacts with fibrillarin only upon active pre-rRNA transcription while its binding to Pol I complex is not dependent on the synthesis of rRNA. It is known that the transcription factors are shuttling between the NORs and the cytoplasm even during mitosis, and this trafficking depends on the mitotic stage. In spite of this, UBF owing to its DNA-binding capacity is indispensable associated with non-condensed fibers of rDNA during the cell cycle. According to the modified basic and hydrophobic (BH) scale developed by Brzeska et al., UBF sequence includes potential lipid-binding sites. Three of them are located in the HMG box 1 and the forth is positioned in the HMG box 4. Interestingly, all four sites are distributed inside the DNA-binding sites. We suggest that PIP2 associated with UBF is engaged in the formation of the core part of rDNA helix structure thus maintaining the open chromatin state of NORs independent of the Pol I transcription. During mitosis, certain Pol I transcription factors are phosphorylated by the Cdk1-cyclin B kinase, which is a prerequisite for establishing and retaining the rDNA transcription in the repressed state. To switch on the rRNA synthetic activity at the exit from mitosis, UBF has to lose the mitosis-specific inhibitory phosphorylations and be activated by phosphorylation at Ser 484 by G1-specific Cdk4/cyclin E&A kinase. Our data suggest that the interaction between PIP2 and UBF is not governed by these general phosphorylation-dephosphorylation mechanisms, but it is preserved throughout the cell cycle, indicating a structural role for PIP2 in the formation and maintenance of nucleolar architecture. It has been shown that Pol I subunit PAF53 contacts UBF directly, and both PAF53 and RPA116 subunits remain associated with Pol I transcription machinery independent of the rRNA synthesis. By BH scale, two potential lipid-binding sites are positioned in PAF 53 sequence and one potential site is located in RPA 116 sequence. In accordance with these data, we showed that PAF53 and RPA116 retain the binding with UBF and PIP2 in the absence of Pol I transcription supporting the notion of PIP2 involvement in the core complex of NORs. It is known that in yeast cells mutations impairing Pol I elongation also impair the cleavage of precursor rRNA, suggesting a connection between production and processing of rRNA. Our data that PIP2 interacts with fibrillarin only upon active pre-rRNA transcription, support the notion that coordination of

![Figure 2. PIP2 retains its colocalization with UBF during mitosis as demonstrated by SIM. Multi-immunolabeling followed by super-resolution structured illumination microscopy showed that PIP2 colocalizes with UBF in NORs, which are the mitotic counterparts of the interphase FCs, in prophase and metaphase. This colocalization pattern persists in anaphase and telophase in "daughter" NORs. Scale bar: 5 μm.](image-url)
Pol I transcription and pre-rRNA processing factors is mediated by the production of rRNA.

In summary, we showed the interaction of PIP2 with the indispensable components of Pol I pre-initiation complex regardless of Pol I transcription suggesting the role for PIP2 in the formation and maintenance of the core platform of rDNA helix structure. Thus, we identified for the first time a lipid component of the nucleo-protein NOR complex. Obviously, proving this idea will require further work on the alterations in NOR architecture, nucleolar structure, and ribosomal gene transcription upon depletion of nucleolar PIP2 in situ.

Material and Methods

Cell culture and inhibitors
Human osteosarcoma (U2OS) cells and cervical carcinoma (HeLa) cells were kept in DMEM with 10% fetal calf serum in 5% CO₂/air, 37 °C, humidified atmosphere. Cells were treated with AMD (0.02 µg/ml) for 2 h or with DRB (50 µg/ml) for 1 h.

Plasmids and production of recombinant proteins
GST-tagged PLCδ1PH (1–140) was received from Dr Hitoshi Yagisawa. Recombinant PLCδ1PH domain is a commonly used PIP2 probe due to its high affinity to the head domain of PIP2 molecule. For GST-tagged PLCδ1PH, the purification was performed on glutathione-agarose column (G4510, Sigma Aldrich) which had been equilibrated with BC100 (20 mM Tris pH 8.0, 0.1 mM EDTA, 20% glycerol, 100 mM NaCl). After washes with BC100, 0.1% NP40, 1 mM DTT, and complete protease inhibitors cocktail (5056489001, Roche Diagnostics GmbH) proteins were eluted with 50 mM Tris-HCL, pH 8.0 having 0.1g of reduced L-Glutathione (G4251, Sigma Aldrich).

Cell fractionation and immunoprecipitation
For obtaining chromosomal fraction enriched with NORs, adherent HeLa cells were treated with nocodazole (20 ng/ml) for 6 h. Mitotic cells were harvested by shaking-off, lysed in the buffer (110 mM CH₃COOK, 10 mM HEPES, pH 7.5, 2 mM MgCl₂, 0.5% Brij98, complete protease inhibitors cocktail) and homogenized with a G22 needle. The resultant extract was centrifuged at 600 g for 5 min at 4 °C. The pellet was resuspended in the IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1% NP-40, complete protease inhibitors cocktail), sonicated and treated with Benzonase (CNB70664, Millipore; 4000 units) for 1 h at 4 °C. After centrifugation at 16100 g for 20 min at 4 °C, supernatant was used in the
immunoprecipitation assays. Samples were incubated with 2 µg of either anti-PIP2 mouse monoclonal IgM antibody (ZA045, Echelon Biosciences Inc) or mouse monoclonal IgM-isotype control (ab18401, Abcam) overnight at 4 °C, and then with 50 µl of protein L-agarose beads (20520, Thermo Scientific Pierce) for 2.5 h at 4 °C. Immunoprecipitates were thoroughly washed with the IP buffer, boiled in Laemmli sample buffer for 10 min, and separated by SDS-PAGE for western blot detection. NOR integrity was monitored with double immunolabeling of UBF and PIP2 or Pol I and PIP2 followed by confocal microscopy taking the samples during cell fractionation.

Immunoblot analysis
Proteins transferred to nitrocellulose membrane (Pall 66485, Pall Corporation) were blocked with 5% non-fat milk in PBS for 1 h and incubated with the appropriate specific antibodies diluted in 1% BSA in PBST overnight at 4 °C. Immunoblotting signals were detected with the appropriate IRDye donkey anti-rabbit or IRDye goat anti-rabbit antibodies and analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences).

Light microscopy
Images were taken with either a confocal microscope (Leica TCS SP5 AOB5 TANDEM) with 100 × (NA 1.4) oil immersion objective lens or a superresolution structured illumination microscope (ELYRA PS.1, Carl Zeiss; Andor iXon3 885 EMCCD camera, pixel size 8 × 8 µm) with Plan-Apochromat 63×/1.4 Oil DIC M27 oil immersion objective lens using the parameters as follows: number of SIM rotations = 5; SIM grating periods varied according to the excitation wavelength from 34.0 µm to 42.0 µm.

Immunoelectron microscopy
Interphase HeLa cells were fixed in 3% formaldehyde plus 0.1% glutaraldehyde and embedded into LR White resin by standard procedure.66 Mitotic HeLa cells were high-pressure frozen, freeze-substituted and embedded into LR White resin according to a previously published protocol.66 Thin sections (70 nm) were examined in a FEI Morgagni 268 transmission electron microscope at 80 kV and in a Tecnai G2 20 LaB6 electron microscope (FEI) at 200 kV. The images were captured with Mega View III CCD camera (pixel size 6.45 × 6.45 µm) and with Gatan Model 894 UltraScan 1000 camera (pixel size 14 × 14 µm). Multiple sections of at least three independent immunogold labeling experiments were analyzed. Adobe Photoshop CS3 Version 10.0 was used to identify the geometrical centers of 6 nm gold nanoparticles and then cover co-centrically with red dots to facilitate the visualization of these small nanoparticles in images.

Antibodies
Primary antibodies: anti-PIP2 mouse monoclonal IgM antibody (ab11039, Abcam; 4 µg/ml for SIM, 16 µg/ml for confocal microscopy, 2 µg/µl for immunoblotting), anti-GST rabbit polyclonal antibody (gift from Dr I. Shevelev; 5 µg/ml), anti-UBF rabbit polyclonal antibody (sc-9131, Santa Cruz Biotechnology; 0.4 µg/ml for immunoblotting), anti-UBF antibody from human autoimmune serum (gift from Dr Renate Voit; 1:1200 for SIM, anti-UBF rabbit polyclonal antibody (HPA006385, Sigma Aldrich; 2.6 µg/ml for IEM), anti-UBF mouse monoclonal IgG1 antibody (sc313125, Santa Cruz Biotechnology; 2 µg/ml for confocal microscopy), anti-PAF53 rabbit polyclonal antibody (gift from Prof Ingrid Grummt; 1:1000 for immunoblotting), anti-PAF53 mouse monoclonal IgG1 antibody (611413, BD Transduction Laboratories; 2.5 µg/ml for IEM), anti-PAF116 rabbit polyclonal antibody (gift from Prof Ingrid Grummt; 1:1000 for immunoblotting).
for confocal microscopy), anti-fibrillarin rabbit monoclonal IgG antibody (2639, Cell Signaling Technology Inc.; 0.3 µg/ml for IEM), anti-fibrillarin mouse monoclonal IgG1 antibody (ab4566, Abcam; 1:100 for confocal microscopy). Secondary antibodies: IRDye 680RD donkey anti-rabbit IgG (H+L) (926-68073, LI-COR Biosciences; 1:10000), IRDye 800CW goat anti-rabbit IgG (H+L) (926-32211, LI-COR Biosciences; 1:10000), goat anti-mouse IgM conjugated with Alexa 555 (A21426, Invitrogen; 10 µg/ml for SIM), donkey anti-mouse IgM conjugated with Cy3 (715-165-140, Jackson ImmunoResearch; 10 µg/ml for confocal microscopy), donkey anti-mouse IgG conjugated with Alexa 488 (A21202, Invitrogen; 5 µg/ml for confocal microscopy), goat anti-rabbit IgG conjugated with Alexa 647 (A21245, Invitrogen; 5 µg/ml for confocal microscopy), goat anti-human IgG conjugated with Alexa 488 (A11013, Invitrogen; 5 µg/ml for SIM), goat anti-mouse IgM (µ-chain specific) antibody coupled with either 6 nm (115-195-075) or 12 nm (111-205-144) colloidal gold particles, goat anti-mouse IgG (H+L) antibody coupled with 6 nm colloidal gold particles (115-195-068), goat anti-rabbit IgG (H+L) antibody coupled with either 6 nm (111-195-144) or 12 nm (111-205-144) colloidal gold particles (Jackson ImmunoResearch Laboratories Inc); all gold-conjugated secondary antibodies were diluted 1:30.

Figure 5. PIP2 colocalization with Pol I and UBF is not influenced by transcription inhibition while PIP2 colocalization with fibrillarin is disrupted by transcription inhibition as shown by IEM. (A) IEM results show that PIP2 is in close proximity to Pol I in the nucleolus and colocalizes with UBF in the FC and with fibrillarin in DFC regions, respectively. (B) In AMD inhibited cells, PIP2 localizes to the light part of the caps together with Pol I and UBF while fibrillarin localizes mainly to the denser part of the caps after the inhibition of Pol I transcription. (C) Upon DRB treatment, PIP2 colocalizes with Pol I and UBF in the inner space of FCs as well as on the border between FC and DFC regions, while in the DFC, PIP2 and fibrillarin are arranged in a necklace-like manner. N, nucleus; NL, nucleolus; FC, fibrillar center; DFC, dense fibrillar component. Scale bar: 200 nm.
Disclosure of Potential Conflicts of Interest
There are no conflicts of either financial or personal interest.

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