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PIP2 epigenetically represses rRNA genes transcription interacting with PHF8

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ABSTRACT

Phosphoinositides are present in the plasma membrane, cytoplasm and inside the cell nucleus. Here we identify phosphatidylinositol-4,5-bisphosphate (PIP2) as a regulator of rRNA genes transcription at the epigenetic level. We show that PIP2 directly interacts with histone lysine demethylase PHF8 (PHD finger protein 8) and represses demethylation of H3K9me2 through this interaction. We identify the C-terminal K/R-rich motif as PIP2-binding site within PHF8, and address the function of this PIP2-PHF8 complex. PIP2-binding mutant of PHF8 has increased the activity of rDNA promoter (20%) and expression of pre-rRNA genes (47S-100%; 45S-66%). Furthermore, trypsin digestion reveals a potential conformational change of PHF8 upon PIP2 binding. These observations identify the function of nuclear PIP2, and suggest that PIP2 contributes to the fine-tuning of rDNA transcription.

1. Introduction

Phosphoinositides (PIs) are phosphorylated derivatives of phosphatidylinositol, a glycerol-based lipid. Phosphorylation of *myo*-inositol head group at every permutation of the 3-,4-,5-position yields seven isomers with independent biological functions. Phosphoinositides are known to act as signaling molecules involved in the regulation of membrane dynamics, cell architecture and motility, cell differentiation, proliferation and cell cycle progression, modulation of ion channels and transporters, or generation of second messengers [1,2]. PIs, as signaling molecules, are connected with direct dynamic regulation of nuclear receptors through which many of transcriptional effects can be mediated [3]. Not only PIs but also PIs-metabolizing enzymes such as Inositol polyphosphate multikinase (IMPK) also possess multiple functions. IMPK is able to act in transcription and epigenetic regulation through (i) interactions with steroidogenic factor 1 (SF1, nuclear receptor), (ii) generation of Ins(1,4,5,6)P4 thus increasing histone deacetylase activity, or (iii) interactions with transcription factors such as serum response factor (SRF), CREB-binding protein (CBP) and p53 [4]. Additionally, publications targeting the nuclear phosphoinositide-specific phospholipase C beta1 (PI-PLCbeta1), are showing its importance in processes such as myogenic, osteogenic or hematopoietic differentiation [5].

In the cell nucleus, the presence of almost all PIs, except PI(3,5)P2, has been documented [6–13]. To address PIs nuclear function, two high-throughput screens have been recently performed aiming to identify molecular interactions of PIs. More than 300 nuclear proteins were found and many of them contain lysine/arginine (K/R)-rich motif which mediates their interactions with the negatively charged phosphorylated *myo*-inositol head group of PIs [14,15]. These proteins are involved in processes such as cell division, cell signaling, and transcription, which indicate previously uncharacterized roles of nuclear PIs.

The most studied nuclear phosphoinositide is phosphatidylinositol-4,5-bisphosphate (PIP2) which localizes to nuclear speckles, small foci in the nucleoplasm, and to the nucleolus [6,7,11,16]. Nuclear PIP2 acts as a transcription activator interacting with the catalytic complex of both RNA polymerases I and II [11,16]. PIP2 regulates RNA polymerase II transcription probably by the direct interaction with histone H1 and histone H3 [17]. This interaction shields H1/H3 positive charges and thus interferes with their DNA binding ability, which leads to an altered chromatin structure. In addition, PIP2 negatively regulates RNA polymerase II transcription by binding to the myristoylated transcriptional co-repressor BASP1 [18]. It was shown that BASP1 requires PIP2 in order to recruit HDAC1. The presence of HDAC1 at chromatin leads to histone deacetylation which decreases promoter accessibility for

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transcription [18].

We have previously shown that PIP2 is in a complex with RNA polymerase I, and it binds directly to the upstream binding factor (UBF) and fibrillarin [19,16]. PIP2 binding provokes a conformational change of UBF and fibrillarin, and increases their affinities for DNA and for RNA. In addition, a PIP2 depletion by anti-PIP2 antibody from HeLa cells nuclear extract results in a decrease of transcription. The transcription activity can be restored by addition of exogenous PIP2 [16]. In conclusion, PIP2 increases the binding affinity of UBF and fibrillarin and thus facilitates not only RNA polymerase I transcription, but also subsequent rRNA processing.

Among proteins from the high-throughput screens, PHD finger protein 8 (PHF8) a histone lysine demethylase has been identified as a possible interacting partner of nuclear PIP2 [14,15]. The PHF8 demethylates mono- and dimethylated lysine 9 of histone H3 [20–22], dimethylated lysine 27 of histone H3 [22], and monomethylated lysine 20 of histone H4 [23]. As all these modifications are epigenetic repressive marks [24–26], PHF8 acts as a transcription activator. Possessing this ability, PHF8 is involved in the regulation of numerous physiological processes, such as craniofacial and brain development of zebrafish [23]. In contrast, the absence of PHF8 or its loss of function caused by mutation in the catalytic domain results in X-linked mental retardation and cleft lip/palate [27,28].

Besides RNA polymerase II transcription, PHF8 also facilitates rDNA transcription, which is executed by RNA polymerase I and its associated factors in the nucleolus. PHF8 regulates rDNA transcription *via* binding to H3K4me3 and subsequently demethylating H3K9me1/2. Moreover, PHF8 associates with the RNA polymerase I catalytic subunit as well as with the upstream binding factor (UBF) [21,22].

Current data suggest that PHF8 and PIP2 act at different stages of RNA polymerase I transcription however, there is no description of PIP2 as an epigenetic regulator. To find whether PIP2 indeed affects such events, we studied the role of the complex formed by PIP2 and PHF8. Here we demonstrate a direct interaction between PIP2 and PHF8, which affects the conformation of PHF8 and subsequently its ability to activate transcription of rRNA genes.

2. Materials and methods

2.1. Cell cultures and transfections

Human cervical carcinoma (HELA), osteosarcoma (U2OS), and human embryonic kidney 293 (HEK 293) cells were grown in D-MEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 humidified atmosphere. Suspension HELA cells were kept in S-MEM supplemented with 5% FBS at 37 °C in 5% CO_2 humidified atmosphere. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stable cell lines were prepared by Lipofectamine 2000 transfection and selected in D-MEM with 10% FBS with addition of geneticin (G418) in final concentration 0.5 mg/ml.

2.2. Constructs

PHF8 was cloned from human cDNA into pET-42(a)$^+$ (Novagen) and used for protein purification. For mammalian expression, PHF8 was inserted into pCMV-Tag4B (Strategene). Truncated forms of PHF8 as followed 1-360 AA, 361-674 AA, 675-1024 AA were inserted into pET-42(a)$^+$ and used for protein purification. PIP2 binding mutant of PHF8 with mutated 282-836 AA (KSRPKKKK motif mutated to ASRPAGAA) was prepared by Q5 site direct mutagenesis kit (New England Biolabs) in pET-42(a)$^+$ (Novagen) and pCMV-Tag4B (Strategene). Human rDNA promoter for luciferase assay was prepared as described previously [29] and cloned into pGL4.10 (Promega). The primers used in this study are summarized in Sup. Table 1.

2.3. Antibodies

Following primary antibodies were used in this study: anti-PIP2 mouse monoclonal IgM antibody (Echelon Biosciences Inc., clone 2C11, Z-A045), anti-PHF8 rabbit polyclonal (Abcam, ab36068 and kind gift from Ingrid Grummt, GCRC Heidelberg, Germany); anti-H3 goat polyclonal IgG (Abcam, ab12079), anti-H3K9me2 rabbit polyclonal IgG antibody (Abcam, ab32521); anti-H3K9me1 rabbit polyclonal IgG antibody (Abcam, ab9045), anti-H3K27me2 rabbit polyclonal IgG (Abcam, ab24684), anti-H3K36me2 rabbit polyclonal (Abcam, ab9049), anti-H3K9 acetyl rabbit polyclonal (Abcam, ab10812), anti-H4K20me1 rabbit polyclonal (Abcam, ab9051), anti-Flag mouse monoclonal IgG (Stratagene, clone M2, 200471), anti-GST mouse monoclonal (Abcam, ab92), anti-GAPDH mouse monoclonal (Acris, clone 6G5), anti-RPA194 (Santa Cruz, sc-28714), control mouse anti-IgG (Abcam, ab81032), control mouse anti-IgM (Abcam, ab18401), control rabbit anti-IgG (Abcam, ab46540).

Following secondary antibodies were used in this study: goat anti-mouse IgM (μ-chain specific) antibody conjugated with Alexa Fluor 555 (Invitrogen, A21426), goat anti-rabbit IgG (H + L) antibody conjugated with Alexa Fluor 488 (Invitrogen, A11034), IRDye 680 donkey anti-mouse IgG (H + L) antibody (LI-COR Biosciences, 926-68072), IRDye 800 donkey anti-mouse IgG (H + L) antibody (LI-COR Biosciences, 926-32212), IRDye 800 donkey anti-rabbit IgG (H + L) antibody (LI-COR Biosciences, 925-32213), IRDye 680 donkey anti-rabbit IgG (H + L) antibody (LI-COR, Biosciences, 926-68073), IRDye 800 Goat anti-Mouse IgM (μ chain specific) antibody (LI-COR Biosciences 926-32280), IRDye 800 donkey-anti Goat IgG (H + L) antibody (LI-COR Biosciences, 926-32214), IRDye 680 donkey-anti Goat IgG (H + L) antibody (LI-COR Biosciences, 926-68074).

2.4. Expression and purification of recombinant proteins

Construct of truncated forms of PHF8-GST and PIP2 binding mutant of PHF8-GST in pET-42(a)$^+$ plasmid vector (Novagen) were transformed and expressed in *E. coli* BL21(DE3). Transformed cells were incubated for approximately 4 h at 37 °C until OD = 0.6. Expression was then induced by 0.5 mM IPTG for 2 h at 30 °C. Samples were lysed by sonication in buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton-X, complete protease inhibitors (Roche)) and purified using GST-agarose beads according to manufacturer's protocol (Sigma). SDS-PAGE electrophoresis was used to check expression and purity.

2.5. Indirect immunofluorescence for super-resolution fluorescence microscopy

U2OS cells seeded on glass coverslips (18 × 18 mm) were fixed and permeabilized with 90% ice-cold methanol in MeS buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$) for 5 min at 4 °C. Coverslips were further blocked with 4% BSA in PBS for 20 min at room temperature. After three washes with PBS, coverslips were incubated with the particular primary antibody diluted in PBS overnight at 4 °C in a wet chamber. After incubation with primary antibody, coverslips were washed with PBS-T (PBS supplemented with 0.05% Tween20). Subsequently, coverslips were incubated with corresponding secondary antibody for 1 h at room temperature in a wet chamber. After three washes in PBS-T, coverslips were mounted in Vectashield antifade medium with DAPI (Vector laboratories). Images were acquired using microscope ECLIPSE Ti-E equipped with Andor iXon3 897 EMCCD camera and objective CFI SR Apochromat TIRF 100 ×/1.49 oil (Nikon). Software NIS-Elements AR 4.20.01 and NIS Elements AR 4.30 was used for capturing and analysis of the images. Pearson's correlation coefficient was measured from 10 region of interest of five cells nuclei as is expressed as mean ± standard deviation.