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Research Article

Interaction between the inner nuclear membrane lamin B receptor and the heterochromatic methyl binding protein, MeCP2

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ABSTRACT

The nuclear membrane has an important role for the dynamic regulation of the genome, besides the well-established cytoskeletal function. The nuclear lamina is emerging as an important player in the organization of the position and functional state of interphase chromosomes. Epigenetic modifications such as DNA methylation and histone modifications are required for genome reprogramming during development, tissue-specific gene expression and global gene silencing. The Methyl-CpG binding protein MeCP2 binds methyl-CpG dinucleotides in the mammalian genome and functions as a transcriptional repressor *in vivo* by interacting with Sin3A, thereby recruiting histone deacetylases (HDAC). MeCP2 also mediates the formation of higher-order chromatin structures contributing to determine the architectural organization of the nucleus. In this paper, we show that MeCP2 interacts *in vitro* and *in vivo* with the inner nuclear membrane protein LBR and that the unstructured aminoacidic sequence linking the MBD and TRD domains of MeCP2 is responsible for this association. The formation of an LBR–MeCP2 protein complex might help providing a molecular explanation to the distribution of part of the heterochromatin at the nuclear periphery linked to inner membrane.

Introduction

In the last few years, the structural and functional organization of the nucleus has become subject of intensive studies and the general perspective now assigns to the nuclear membrane an important role for the dynamic regulation of the genome, besides the well established cytoskeletal functions [1]. The nuclear lamina is emerging as an important player in the organization of the position and functional state of interphase chromosomes.

The control of the expression of eukaryotic genes can be viewed as a complex regulatory mechanism that includes three hierarchical levels: DNA sequence, chromatin structure and nuclear organization [2,3]. At the chromatin level, a complex network of both epigenetic modifications and specific protein regulators allows a tight regulation of the underlying DNA sequence. In mammals, specifically methylated forms of histone H3, deacetylated histone H4, and DNA methylation characterize heterochromatin. Both methylated lysine 9 of histone H3 and methylated CpG dinucleotides are binding sites for chromatin modifiers such as the heterochromatic protein 1 (HP1) and the methyl CpG binding protein MeCP2, respectively [4]. This last protein, characterized by a methyl binding domain (MBD) and a transcription repression domain (TRD), can repress transcription by recruiting silencing complexes and histone deacetylases, thereby stabilizing and consolidating the heterochromatic state of DNA [5,6]. Both HP1 and MeCP2 have been shown to be particularly concentrated at

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pericentromeric heterochromatin where they are involved in the control of gene expression and play a key structural role [7,8].

Yeast [9], Drosophila [10], and mammals [11] have provided strong evidences for a role of nuclear topology in the control of gene transcription and, in most cells, a large part of transcriptionally silent heterochromatin localizes near the nuclear periphery [2]. This chromatin compartmentalization suggests interactions between chromatin components and proteins of the nuclear envelope. Lamins of the nuclear membrane seem to be important for the attachment of chromatin to the nuclear envelope and LBR is the best example of a chromatin- and lamin-binding membrane protein [1]. It is an integral membrane protein, essential during human development [12] and plays an active role in positioning the heterochromatin at the nuclear periphery. Whereas the C-terminal portion of the protein is embedded within the nuclear membrane through its eight transmembrane segments, the highly charged N-terminal region faces the nucleoplasm where it is engaged in the interaction with several nuclear proteins, DNA and chromatin [13–15]. Significantly, LBR has been shown to bind also HP1 [16], suggesting a direct link between heterochromatin and a nuclear membrane protein. *In vivo*, the interaction of LBR with chromatin is mediated by the formation of a tight complex consisting of LBR itself together with the core histones H3 and H4 and HP1 [17].

We reasoned that MeCP2, being a key protein for the transcriptional regulation and massively present in heterochromatin, might interact with LBR at the periphery of the nucleus, as it has been seen for other important determinants involved in gene silencing. The experiments performed in this article were undertaken to verify this hypothesis.

Materials and methods

Plasmid constructs

pFLAG-MAC-LBR vector contains the LBR N-terminal domain (aa 1–208) cloned into the HindIII–XmaI sites of the pFLAG-MAC vector (Sigma). pGST-hMeCP2 (aa 1–486) was obtained by inserting the PCR amplified hMeCP2 cDNA into the BamHI site of pGEX-4T-1 vector (Pharmacia Biotech). pSG-FLAG-hMeCP2 (1–486 aa), pSG-FLAG-NtMBD (aa 1–162), pSG-FLAG-MBD-linker (aa 78–201), pSG-FLAG-linker-TRD (aa 202–311) plasmids were obtained by inserting the PCR fragments into the BamHI site downstream of the N-terminal FLAG tag in pSG5 (Stratagene).

hMeCP2 Δlinker mutation was introduced with Quick Change XL Site-Directed Mutagenesis Kit (Stratagene). The reaction was performed on pEGFPC1-hMeCP2 plasmid with the forward primer 5′-actgggagagggagcccctccgccacgtcagagggtgtgcag-3′ and the reverse primer 5′-ctgcacaccctctgacgtggcggaggggctccctctcccagt-3′; the AflII–XmaI cassette was then swapped into pSG FLAG-hMeCP2 construct obtaining pSG FLAG-hMeCP2 Δlinker plasmid.

pEYFP-N1 plasmid was purchased from Clontech. This plasmid contains the yellow fluorescent protein cDNA under the control of the CMV promoter. Mammalian expression vectors pEYFP YN and pEYFP YC were obtained by cloning the NheI–EcoRI fragments encoding for aa 1–154 and aa 155–238 of yellow fluorescent protein into the corresponding sites of pEGFP C1 and pEGFP C2 plasmids, respectively. cDNAs encoding hMeCP2 and hLBR isoforms were excised as BamHI fragments from pEGFP C1 and pEGFP C2 expression vectors, respectively, and cloned into the corresponding sites of BiFC expression plasmids (see text for reference).

Inserts encoding SV40 Large T antigen (aa 87–708) and mouse p53 (aa 72–390) were excised as BamHI and EcoRI–BamHI fragments, respectively, from plasmids pTD1 and pVA3 (Clontech Laboratories, Inc.) and cloned into pEYFP YN and pEYFP YC plasmids.

All constructs were verified by sequencing.

Cells culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% V/V fetal bovine serum and antibiotics. About 10^5 cells were seeded on coverslips and transiently transfected with 1 μg of the indicated vectors using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. For bimolecular fluorescence complementation assay, cells were co-transfected with a total of 1 μg of pEYFP YN and pEYFP YC expression plasmids using Lipofectamine 2000 (Invitrogen). Fluorescence was determined after 24 h. Images were acquired with Zeiss LSM410 laser scanning confocal microscope using the 63× objective lens.

Antibodies

The rabbit anti-LBR anti-serum (Covance Research Products Inc.) was raised against recombinant h-LBR amino acids 1–203, whereas the rabbit anti-MeCP2 anti-serum (Covance Research Products Inc.) was raised against a recombinant portion of the h-MeCP2 (residues 171–486). Recombinant hMeCP2 (aa 1–486) and hLBR (aa 1–208) were expressed in *E. coli* ER2566, induced with 0.5 mM IPTG at 30 °C for 5 h. Extracts were prepared by sonicating the bacteria in lysis buffer (750 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.1% V/V Triton X-100, protease inhibitor mixture (Sigma P4380). Following centrifugation at 6000 ×*g* for 10 min, the cleared lysate was applied to chitin-agarose (New England Biolabs Inc.) pre-equilibrated with lysis buffer. The column was washed with 20 volumes of lysis buffer and the fusion proteins were cleaved on the column over night with the lysis buffer containing 50 mM DTT. Eluted fractions containing the bulk of MeCP2 or LBR were pooled and the recombinant purified proteins were dialyzed against phosphate-buffered saline. Anti p53 (IC12) monoclonal antibodies were purchased from Cell Signaling and the anti-LAMIN B (C-20) sc-6216 antibodies were purchased from Santa Cruz Biotechnology.

Immunofluorescence microscopy

HeLa cells were washed with 1× PBS buffer, fixed with 4% V/V paraformaldehyde, permeabilized with 0.1% V/V Triton X-100 (room temperature, 10 min) and blocked using 1× PBS with 2% V/V FBS. In order to detect the expression of pSG-FLAG recombinant constructs and endogenous LBR, cells were incubated with anti-FLAG M2 mouse monoclonal antibody (1:1000 dilution, SIGMA) or with anti-LBR polyclonal antibody (1:3000 dilution) for 60 min. The coverslips were washed and incubated with secondary antibody conjugated to Cy3 fluorophores anti-mouse (1:500 dilution, Jackson Immunoresearch) and Alexa Fluor 488 F(ab′)2 fragment anti-rabbit (1:500 dilution, Molecular Probes) for 60 min. Cells were washed twice and incubated with Hoechst