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Generation of mice deficient in RNA-binding motif protein 3 (RBM3) and characterization of its role in innate immune responses and cell growth

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ABSTRACT

The activation of innate immune responses is critical to host defense against microbial infections, wherein nucleic acid-sensing pattern recognition receptors recognize DNA or RNA from viruses or bacteria and activate downstream signaling pathways. In a search for new DNA-sensing molecules that regulate innate immune responses, we identified RNA-binding motif protein 3 (RBM3), whose role has been implicated in the regulation of cell growth. In this study, we generated *Rbm3*-deficient (*Rbm3*$^{-/-}$) mice to study the role of RBM3 in immune responses and cell growth. Despite evidence for its interaction with immunogenic DNA in a cell, no overt phenotypic abnormalities were found in cells from *Rbm3*$^{-/-}$ mice for the DNA-mediated induction of cytokine genes. Interestingly, however, *Rbm3*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed poorer proliferation rates as compared to control MEFs. Further cell cycle analysis revealed that *Rbm3*$^{-/-}$ MEFs have markedly increased number of G2-phase cells, suggesting a hitherto unknown role of RBM3 in the G2-phase control. Thus, these mutant mice and cells may provide new tools with which to study the mechanisms underlying the regulation of cell cycle and oncogenesis.

1. Introduction

Nucleic acids from viruses or bacteria potently activate immune responses through nucleic acid-sensing pattern recognition receptors (PRRs), namely, membrane-bound Toll-like receptors (TLRs) such as TLR3, TLR7 and TLR9, and cytosolic receptors, which include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), DNA-dependent activator of IRFs (DAI) [1–4]. The hallmark of the activation of these receptors is the induction of genes encoding type-I IFN and proinflammatory cytokine gene expression [1–4]; however, the detailed signaling pathways and mechanisms of gene activation following activation of these receptors still remain elusive. Currently, the DNA-sensing system remains less well known than the RNA-sensing system, perhaps suggesting a more complicated system, and there is evidence for an as yet unknown cytosolic DNA sensor(s) that activates the signaling pathway for proinflammatory cytokine genes [3–6]. It has also been known that TLR9 (and also TLR7) needs to translocate from the endoplasmic reticulum to endosomes/lysosomes upon stimulation [7–11], a process which requires Unc-93 homolog B1 (UNC93B1) is involved in the translocation [7,9,11]. How the trafficking signal is activated and regulated still remains to be clarified, and it is therefore possible that another DNA-sensing molecule participates in the regulation of the entire TLR9 signaling process.

To gain new insights into the complexity of the DNA-sensing mechanisms in the cell, we searched for immunogenic DNA-binding proteins and identified RNA-binding motif protein 3 (RBM3). RBM3 was originally described as a nuclear protein with one RNA recognition motif [12]. The *Rbm3* gene is located on chromosome X of mice (also Xp11.23 in humans) and is ubiquitously expressed in a variety of cell types but is expressed at relatively high levels in cancer cell lines [13,14]. Because of its potential role in oncogenesis, RBM3 has been studied in relation to cell growth activity and viability. It has been reported that the suppression of RBM3 expression by siRNA knockdown in cancer cell lines results in an inhibition of cell proliferation and increases susceptibility to anti-cancer drugs, whereas the overexpression of RBM3

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promotes cell proliferation [14,15]. Thus, although these observations are intriguing, the physiological role of RBM3 in cell growth control still remains unclear. To study the role of RBM3 in immunity and cell growth, we newly generated *Rbm3*-deficient (*Rbm3*$^{-/-}$) mice. Our results indicate that RBM3 is not involved in the regulation of nucleic acid-mediated cytokine gene induction, but it does play a critical role in cell cycle regulation. We discuss our findings in terms of the utility of these mutant mice for the analysis of innate immune signaling, cell cycle, and oncogenesis.

2. Materials and methods

2.1. Generation of Rbm3-deficient mice

Genomic DNA containing *Rbm3* gene was amplified by polymerase chain reaction (PCR) from 129/Sv genome DNA. An *Rbm3*-targeting construct that replaces exon 3–6 with a phosphoglycerate kinase promoter-driven neomycin-resistant gene was transfected into E14–1 ES cells. Homologous recombinants were injected into C57BL/6 blastocysts. The resulting chimera mice were intercrossed heterozygous F_1 progenies to obtain *Rbm3*$^{-/-}$ mice as described previously [16]. C57BL/6J mice were purchased from CLEA Japan.

2.2. Screening for CpG-B ODN-binding protein

The screening of CpG-B DNA-binding proteins was performed by ZOEGENE Corporation (Japan) with the cell-free protein display (CFPD) method that is developed on the basis of the technology linking between mRNA and their translated protein through puromycin linker [17]. For this screening, mRNA purified from bone marrow cells cultured with the Flt3-ligand was used. CpG-B DNA-binding proteins were enriched using a column filled with CpG-B DNA-conjugated beads. The binding proteins were assigned by reading the sequence of complementary DNA (cDNA) from mRNA attached to the protein.

2.3. Pull-down assay

Pull-down assay was performed as described previously [2]. Briefly, 300 μg of Dynabea ds M-280 Streptavidin (Invitrogen) and 60 pmol of 5′-biotinylated CpG-B DNA were mixed and incubated for 15 min at room temperature. After three washes with a buffer [0.5 M Tris–HCl (pH 7.5), 2.5 M NaCl, and 0.5 M EDTA], the Dynabeads-CpG-B DNA complex was equilibrated and resuspended in a lysis buffer [50 mM Tris–HCl (pH8.0), 300 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM APMSF]. Whole cell lysate (90 μg) was mixed with 30 μl of the complex and incubated for 30 min at room temperature. Pulled-down samples were washed with the buffer three times and subjected to immunoblot analysis.

2.4. Fluorescence microscopy

RAW 264.7 cells and mouse embryonic fibroblasts (MEFs) (1×10^5 cells) were plated on a 15-mm micro cover glass (Matsunami Glass). RAW264.7 cells expressing YFP-tagged RBM3 were analyzed using an Olympus FV-1000 confocal microscope (Olympus). LysoTracker Red was purchased from Invitrogen. MEFs were fixed with PBS containing 4% paraformaldehyde, permealized with 0.2% Triton X, and then stained with antibodies. Primary antibodies for anti-mouse RBM3 (Abgent) and anti-phospho-Histone H3 at Ser10 (H3-pS10) (Millopore) were used. Secondary antibodies for FITC-conjugated donkey anti-mouse IgG antibody and Cy3-conjugated goat anti-rabbit IgG were purchased from Invitrogen. Images were captured using an IL-X71 Applied Precision Deltavision microscope (Olympus) and processed with DeltaVision SoftWorx software (Applied Precision).

2.5. RNA analysis

Total RNA isolation and cDNA synthesis were performed as described previously [2]. mRNA was purified from total RNA using Oligotex-dT30 (TaKaRa). Quantitative real-time PCR (qRT-PCR) analysis was carried out using LightCycler480 and the SYBRGreen system (Roche). The primer sequences for GAPDH, IL-6, IFN-α4, IFN-β, and TNF-α have been described [2]. The following primers for RBM3 were used: 5′-CCTTCACAAACCCAGAGCAT-3′ (sense) and 5′-TAGACCGCCCATACCCATA-3′ (anti-sense). All data are presented as relative expression units after normalization to GAPDH expression level. Additional information is in Supplementary Materials and Methods.

3. Results

3.1. Identification of RBM3 as an immunogenic DNA-associated protein

We first sought to identify proteins involved in immunogenic DNA recognition systems and performed biochemical screening by the cell-free protein display (CFPD) method [17] using purified mRNAs from dendritic cells (DCs) differentiated from bone marrow cells cultured with the Flt3-ligand (Supplementary Fig. S1A). In this screening, we identified RBM3 as the most prominent protein that binds to CpG-B DNA, a TLR9 agonist (Supplementary Fig. S1B). Previously, RBM3 was studied in the context of oncogenesis but not immune regulation [13,14,18]. We further studied the interaction of RBM3 with CpG-B DNA by co-precipitation assay. Protein lysates from HEK293T cells transiently expressing FLAG-tagged RBM3 was subjected to pull-down assay with biotin-labeled CpG-B DNA and streptavidin-conjugated magnetic beads. As shown in Fig. 1A, RBM3 was precipitated with the DNA and this precipitation was inhibited by an excess amount of nonconjugated CpG-B DNA. Thus, these findings indicate that RBM3 indeed binds to immunogenic CpG-B DNA *in vitro*.

It has been reported that RBM3 mainly localizes to the nucleus when cell is at rest, but otherwise shuttles between the cytoplasm and the nucleus [14]. Since CpG-B DNA localizes in the lysosomal compartment in the cytoplasm [8,10], we next examined whether the cellular localization of RBM3 is regulated following CpG-B DNA stimulation. To test this, we stimulated RAW264.7 cells with CpG-B DNA and measured RBM3 protein levels in the nuclear and cytoplasmic fractions. Interestingly, the RBM3 protein level in the cytoplasmic fraction increased after CpG-B DNA stimulation in a time-course-dependent manner, while that in the nuclear fraction decreased (Fig. 1B). Notably, the accumulation of RBM3 in the cytoplasmic fraction was not induced when the cells were stimulated by the TLR4 agonist lipopolysaccharide (LPS), suggesting that the recruitment of RBM3 into the cytoplasm specifically occurs upon CpG-B DNA stimulation (Fig. 1C). We also examined the co-localization of RBM3 with CpG-B DNA by fluorescence microscopy. YFP-tagged RBM3 co-localized with rhodamine-labeled CpG-B DNA in RAW264.7 cells (Fig. 1D) and also merged with LysoTracker, a lysosomes marker (Fig. 1E), suggesting that RBM3 binds to CpG-B DNA in lysosomes, where TLR9 signaling occurs [8,10]. Collectively, these observations suggest the potential involvement of RBM3 in the CpG-B DNA-TLR9 signaling process.