New Phenomenon

Poly(C)-binding protein 2 positively regulates interferon downstream signaling

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Host innate immunity protection against pathogen infection is universal in diverse species of invertebrates and vertebrates. Type I interferon (IFN-I) is induced by pathogen infection and secreted from the pathogen-infected host cells, and then binds to IFN-I receptors (IFNAR1 and IFNAR2) on the cell surface. This binding leads to the activation of the receptor-associated Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). Then the activated kinases phosphorylate the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2). Afterwards, the activated STAT1/2 complex combines with IFN-regulatory factor 9 (IRF9) to form a complex that binds to the IFN-stimulated response elements on cellular DNA, which leads to the expression of the multiple IFN-stimulated genes (ISGs) [1,2]. Due to the importance of JAK-STAT pathway to antiviral immunity, down-regulation of the IFN-I receptors will result in a disastrous damage of an IFN-I antiviral response [3]. Therefore, maintaining IFNAR1 protein stability is a matter of cardinal importance for the enhancement of the antiviral efficacy of IFN-I.

In our previous study, we reported that RBM47 is an interferon-inducible RNA-binding protein that plays an essential role in enhancing host IFN downstream signaling [3]. Mechanistically, RBM47 binds to the 3′UTR of IFNAR1 mRNA, increases mRNA stability, and retards the degradation of IFNAR1. In order to elucidate the mechanisms of action of RBM47 on innate antiviral immunity, immunoprecipitation (IP) and mass spectrometry (MS) assays were performed to identify proteins that interact with RBM47.

Another RBP called poly(C)-binding protein 2 (PCBP2) was identified to be associated with RBM47. PCBP2 belongs to a class of proteins that bind with poly(C) sequences in both RNA and DNA, and is involved in host cell mRNA stability, translational regulation of cellular mRNAs and cellular antiviral responses [4]. PCBP2 functions as a negative regulator of IFN-β activation by mediating the degradation of MAVS [5,6].

In the present study, we found that when 293T cells were stimulated with RIG-III or poly(I:C), PCBP2 inhibited the activation of IFN-β promoter (Supplementary Figure S1A), which is consistent with the result of You et al. [5]. Furthermore, when 293T cells were infected with Sendai virus (SeV), PCBP2 inhibited the mRNA level of IFN-β (Supplementary Figure S1B) but not ISGs, such as IFIT1 and Cig5 (Supplementary Figure S1C). However, once PCBP2 was co-transfected with RBM47, the mRNA levels of IFIT1 and Cig5 were increased obviously (Supplementary Figure S1C), although the mRNA level of IFN-β was not changed (Supplementary Figure S1B). These data suggest that PCBP2 may play important roles in IFN downstream signaling.

In addition, PCBP2 was reported to interact with porcine reproductive and respiratory syndrome virus (PRRSV) Nsp1β and support viral replication [7,8]. Therefore, it will be interesting to investigate the relationship between PCBP2 and RBM47 on innate antiviral activity. Here, Co-IP assay was performed to confirm the interaction between PCBP2 and RBM47. RBM47-Flag plasmid was transfected into 293T cells with or without plasmid PCBP2-Myc for 24 h, and then cells were harvested to perform IP assay using anti-Flag antibody or anti-Myc antibody. The IP of PCBP2 with anti-Myc antibody successfully co-precipitated RBM47-Flag (Figure 1A). Likewise, the reciprocal test using anti-Flag antibody also immunoprecipitated PCBP2 with anti-RBM47 antibody (Figure 1A). To further confirm the interaction between PCBP2 and RBM47, lysate from 293T cells was divided into three parts which were incubated with control IgG, anti-PCBP2 antibody and anti-RBM47 antibody respectively. The results showed that endogenous PCBP2 and RBM47 interact with each other (Figure 1B). Furthermore, GST-pulldown assay was performed using recombinant fusion protein GST-PCBP2 and purified RBM47-Flag, which also confirmed that GST-conjugated PCBP2 could successfully pulldown RBM47 in the cell lysate (Figure 1C). To further validate the interaction between PCBP2 and RBM47, the cellular distributions of these two proteins were detected by confocal microscopy. HeLa cells were transfected with the plasmid RBM47-Flag and/or PCBP2-Myc for 24 h. The cells were then stimulated with IFN-α for another 6 h. Confocal micros-copy results showed that when co-expressed with RBM47 in HeLa cells,
PCBP2 was redistributed from a predominantly nuclear localization to punctuate sites colocalizing with RBM47 in the cytoplasm (Figure 1D). Once the cells were stimulated with IFN-α, PCBP2 re-localized to the nucleus. However, most part of the PCBP2 protein remained in the cytoplasm and still co-localized with RBM47 (Figure 1D).

These data demonstrate that PCBP2 interacts with RBM47 directly. RBM47 is known to increase mRNA stability and retard the degradation of IFNAR1 for immune regulation during virus infection [3]. The association of PCBP2 with RBM47 prompted us to examine whether PCBP2 regulates the protein stability of IFNAR1 as well. RBM47-Flag was transfected into 293T cells with or without PCBP2-Myc. IP was performed using anti-Flag antibody, or anti-PCBP2 antibody, respectively, and then IP assay was performed. The expression of PCBP2-GST was induced by IPTG (1 mM) in Escherichia coli BL21(DE3). Then, the recombinant fusion proteins were incubated with GST affinity agarose separately. Thereafter, 293T cell lysate with RBM47-Flag ectopic expression was incubated with the agarose for 2 h at room temperature, and the immunoprecipitated proteins were analyzed by western blot analysis. WCE, whole-cell extract. (D) Confocal microscopy of PCBP2-Myc (green) and RBM47-Flag (red). RBM47-Flag or PCBP2-Myc was transfected into HeLa cells individually or together for 48 h, then the cells were treated with or without IFN-α for 6 h before fixation. The nucleus was stained with DAPI. Scale bar = 5 μm. (E,F) PCBP2-Myc and RBM47-Flag were solely or together transfected into 293T cells for 24 h. The cells were then harvested to test the mRNA level (E) or protein expression (F) of IFNAR1. RT-PCR results are presented as the relative levels to that of β-actin. (G) RIP assay was performed to test the interaction between IFNAR1 mRNA and PCBP2-Flag together with or without RBM47-Flag. The data shown are representative of three independent experiments. Data are presented as the mean ± SEM. NS, non-significant. **P < 0.01, ***P < 0.001.

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Figure 1. PCBP2 interacts with RBM47 and synergistically stabilizes IFNAR1 mRNA  (A) Co-IP staining. Left: Co-IP of lysate from 293T cells expressing PCBP2-Myc, with or without RBM47-Flag. IP was performed using anti-Flag antibody. Right: Co-IP of lysate from 293T cells transfected with RBM47-Flag, with or without PCBP2-Myc. IP was performed using anti-Myc antibody. (B) The endogeneous interaction between RBM47 and PCBP2. 293T cell lysate was incubated with IgG, anti-PCBP2 antibody, or anti-RBM47 antibody, respectively, and then IP assay was performed. (C) GST-pulldown was performed to test the association between PCBP2 and RBM47. (D) Confocal microscopy of PCBP2-Myc (green) and RBM47-Flag (red). RBM47-Flag or PCBP2-Myc was transfected into HeLa cells individually or together for 48 h, then the cells were treated with or without IFN-α for 6 h before fixation. The nucleus was stained with DAPI. Scale bar = 5 μm. (E,F) PCBP2-Myc and RBM47-Flag were solely or together transfected into 293T cells for 24 h. The cells were then harvested to test the mRNA level (E) or protein expression (F) of IFNAR1. RT-PCR results are presented as the relative levels to that of β-actin. (G) RIP assay was performed to test the interaction between IFNAR1 mRNA and PCBP2-Flag together with or without RBM47-Flag. The data shown are representative of three independent experiments. Data are presented as the mean ± SEM. NS, non-significant. **P < 0.01, ***P < 0.001.
proteins and IFNAR1 mRNA using an RNA-Binding Protein Immunoprecipitation Kit (Medical & Biological Laboratories, Tokyo, Japan). Finally, we performed qRT-PCR on the RBM47- or PCBP2-associated RNA mixture absorbed by the Protein A/G PLUS-Agarose. As shown in Figure 1G, IFNAR1 mRNA was not precipitated by PCBP2-Flag alone. However, IFNAR1 mRNA was precipitated by RBM47-Flag, and this effect was enhanced by co-expression of the two proteins. As a control, IFNAR2 mRNA was not pulled down by any of the proteins in the same assays (Figure 1G). These results indicate that PCBP2 does not influence IFNAR1 function but does enhance the modulatory activity of RBM47 on IFNAR1.

As shown in Figure 2A, mRNA of IFNAR1, rather than IFNAR2, was significantly increased after IFN-α stimulation when PCBP2-Myc and RBM47-Flag were co-expressed. Western blot analysis also confirmed that PCBP2 enhanced the effect of RBM47 on IFNAR1 stabilization and the subsequent phosphorylation of STAT1/2 (Figure 2B). Furthermore, PCBP2-Myc and/or RBM47-Flag, ISRE-Luc, and pRL-TK (internal control) were co-transfected into 293T cells for 24 h. Then the cells were treated with or without IFN-α stimulation for another 6 h. Cells were harvested and the DLR assays

**Figure 2. PCBP2 positively regulates JAK-STAT signaling and potentiates the antiviral activity of RBM47** (A, B) PCBP2-Myc and RBM47-Flag were solely or together transfected into 293T cells for 24 h. Then cells were stimulated with PBS or IFN-α for 6 h. RT-PCR was performed to test mRNA levels of IFNAR1, IFNAR2 (A). Western blot analysis of IFNAR1, phosphorylation of STAT1 and STAT2 (B). (C) ISRE-Luc was transfected into 293T cells together with or without PCBP2-Myc or RBM47-Flag. After 24 h, cells were stimulated with PBS or IFN-α for another 6 h. DLR assays were performed to test the promoter activity of ISRE. (D) qRT-PCR analysis of the expressions of IFIT1 and Cig5 in 293T cells treated with or without IFN-α. (E) mRNA level of IFNAR1 was tested. RBM47-Flag was transfected into WT or PCBP2-knockout 293T cells for 24 h. The cells were then infected with VSV for another 8 h. (F) mRNA level and viral titers of VSV were measured. The cells were treated as in 2E. The titers of VSV in cell-free supernatants were determined by a median tissue culture infective dose (TCID50) assay. Cultured supernatants containing VSV viruses were serially diluted with DMEM and then placed on the monolayer of Vero cells in 96-well plates. (G) Model of PCBP2 on IFNAR1 signaling. PCBP2 cooperates with RBM47 to stabilize IFNAR1 mRNA, and amplifies JAK-STAT signaling. RT-PCR results are presented as the relative levels to that of β-actin. The data shown are representative of 3 independent experiments. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
were performed using a luciferase assay kit (Vazyme, Nanjing, China). DLR assays also demonstrated that PCPB2-Myc further enhanced IFN-α-induced ISRE promoter activity in the presence of ectopic expression of RBM47-Flag (Figure 2C). The mRNA levels of IFI15 and Cig5 were further upregulated by co-expression of PCPB2-Myc and RBM47-Flag with IFN-α stimulation (Figure 2D). Overall, PCPB2 promotes RBM47-mediated enhancement of JAK-STAT signaling.

To investigate the effect of PCBP2 on virus replication, we constructed a PCBP2-knockout 293T cell line using the CRISPR-Cas9 technique. 293T cells were firstly transfected with CRISPR/Cas9-PCBP2 plasmid, and then selected with puromycin (2 μg/mL) for at least three weeks. PCBP2−/− cell line was derived from a single cell clone and was confirmed by western blot analysis. As shown in Figure 2E, the effect of RBM47 on IFNAR1 mRNA stability was significantly decreased in PCBP2-knockout cells. Furthermore, the inhibitory effect was only 50% in PCBP2-knockout cells (Figure 2F). In addition, the viral titers from the supernatant also confirmed the effect (Figure 2F). This confirms that PCBP2 promotes the antiviral activity of RBM47. Thus, we speculated that PCBP2 enhances the RBM47-mediated host antiviral response by potentiating the effect of RBM47 to stabilize IFNAR1 mRNA (Figure 2G).

Previous studies also supported that PCBP2 inhibits VSV replication [9] and enhances the inhibitory effect of IFN-α to HCV [10]. To verify the broad-spectrum antiviral role of PCPB2, dengue virus (DENV) was also selected to confirm the antiviral effect of PCBP2 (Supplementary Figure S2). The results are in line with our current findings that PCPB2 has a positive role in IFN downstream signaling. Taken together, we conclude that PCPB2 plays multiple roles in IFN innate immunity. It not only has an inhibitory effect on IFN-β production, but also stabilizes IFNAR1 mRNA via binding to RBM47. These could be shrewd strategies for the host to combat the virus after IFN attenuation in the late stage of virus infection.

**Supplementary Data**

Supplementary data is available at *Acta Biochimica et Biphysica Sinica* online.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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