Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PACT is required for MDA5-mediated immunoresponses triggered by *Cardiovirus* infection via interaction with LGP2

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ARTICLE INFO

Article history:
Received 18 September 2017
Accepted 10 October 2017
Available online xxx

Keywords:
LGP2
PACT
MDA5
Type-I interferon
RIG-I-like receptors
Picornavirus
Cardiovirus

ABSTRACT

Laboratory of genetics and physiology 2 (LGP2) and melanoma differentiation-associated gene 5 (MDA5) cooperatively detect viral RNA in the cytoplasm of *Cardiovirus*-infected cells and activate innate immune responses. Here, we evaluated whether the double-stranded RNA-binding protein PACT plays a role in this anti-viral response to further elucidate the mechanism. Immunoprecipitation experiments demonstrated that PACT interacts with LGP2 and that this interaction is enhanced by encephalomyocarditis virus (EMCV) infection. *In vitro* interaction analyses using purified recombinant proteins confirmed that the single-stranded Theiler's murine encephalitis virus genome enhanced the interaction between LGP2 and PACT. Small interfering RNA knockdown experiments further indicated that PACT is required for *Cardiovirus*-triggered interferon responses. To support this functional interaction with LGP2, overexpressed PACT was shown to enhance EMCV-triggered interferon promoter activity only when LGP2 and MDA5 were co-expressed but not when MDA5 is expressed alone. Together, our findings indicate a possible role of PACT in regulating the *Cardiovirus*-triggered immune responses mediated by MDA5 and LGP2, which opens the door to novel therapeutic strategies in interferon-related autoimmune diseases and cancer.

1. Introduction

Anti-viral innate immune recognition is mediated by pattern recognition receptors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), including RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [1–3]. These receptors can detect accumulating viral RNAs during viral infection and initiate appropriate antiviral signaling responses and type-I interferon (IFN) production to achieve anti-viral effects. The RLRs belong to the superfamily 2 helicases, which are characterized by a DExD/H-box helicase domain and a C-terminal RNA-binding domain. The RNA-dependent ATP hydrolysis activity of RIG-I and MDA5 is suggested to be important for discriminating non-self RNA from self-RNA [4–6]. Accordingly, genetic mutations that lead to malfunction of the ATP hydrolysis activity in RIG-I and MDA5 are implicated in autoimmune diseases such as Aicardi-Goutieres syndrome and Singleton Merten syndrome, respectively [7–10]. The binding of viral RNA to RIG-I or MDA5 results in ATP hydrolysis-dependent structural reorganization to cause displacement of the caspase activation and recruitment domains (CARDs) for transducing the signal to the downstream mitochondrial adaptor, mitochondrial antiviral signaling (MAVS) [11–15]. In contrast to RIG-I and MDA5, LGP2 cannot initiate downstream signaling since it lacks CARDs, although LGP2 is considered to participate in the sensing of viral RNAs together with RIG-I and MDA5 [16–19]. The various RLRs play different functional roles in the specific recognition of viral RNA species. RIG-I is responsible for the recognition of viral RNAs that possess 5′ tri- or di-phosphorylated ends within a double-stranded region [20–22], including influenza virus and Sendai virus (SeV). MDA5 detects long double-stranded RNAs (dsRNAs) and higher-order RNA structures, and is responsible for the recognition of RNAs from the genus *Picornavirus*, including encephalomyocarditis virus (EMCV) and Theiler's murine encephalitis virus (TMEV). Several studies have demonstrated that lack of LGP2 results in greater sensitivity to MDA5-responsive viruses compared to RIG-I-responsive viruses [17,18]. MDA5 has also been suggested to show the lowest RNA binding affinity among the RLR proteins [23], and several co-factors for MDA5 have been identified. A recent study revealed that LGP2 increases the initial rate of the RNA–MDA5

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https://doi.org/10.1016/j.bbrc.2017.10.048
0006-291X/

interaction and regulates MDA5 filament formation, which triggers a greater signaling response than observed by MDA5 alone [24]. We recently identified the TAR-RNA-binding protein (TRBP) as an additional factor involved in MDA5/LGP2 signaling triggered by *Cardiovirus* infection; TRBP increased EMCV-mediated IFN responses via functional interaction with MDA5/LGP2 but not with MDA5 alone [25]. TRBP has been reported as a cellular factor utilized for human immunodeficiency virus 1 replication [26] and is also known as an essential component of the RNAi machinery [27,28].

To further elucidate this mechanism, in the present study, we used a similar immunoprecipitation experiment to focus on the role of other components of the RNAi machinery [29,30], the dsRNA-binding protein PACT and Dicer, in the functional interaction with RLRs to mediate *Cardiovirus*-triggered IFN responses.

We further knocked down PACT and Dicer expression in mouse L929 cells to evaluate the role of PACT and Dicer in the induction of the *Cardiovirus*-induced type-I IFN response. Together, we expect these results to shed new light on the mechanism of non-self RNA vs. self-RNA recognition to highlight new therapeutic targets.

2. Materials and methods

2.1. Plasmids

The construction of FLAG-tagged LGP2, RIG-I, MDA5, His-tagged LGP2, HA-tagged LGP2, and TRBP plasmids is described elsewhere [16,25]. FLAG-tagged Dicer was a generous gift from Dr. V. N. Kim (Seoul National University). The pCKFLAG-PACT plasmid was a generous gift from Dr. J. Marques (Universidade Federal de Minas Gerais). The open reading frame of PACT was polymerase chain reaction (PCR)-amplified with restriction enzyme sites and ligated to multiple cloning site of pCold I vector (Takara).

2.2. Recombinant proteins

For recombinant RLR protein purification, HEK293T cells (ATCC; CRL-3216) were transfected with FLAG-tagged LGP2, RIG-I, or MDA5 plasmids. Cell lysates were incubated with anti-FLAG M2 affinity gel (Sigma) and elution was achieved by the addition of 3 × FLAG peptide (Sigma). PACT protein was expressed in *Escherichia coli* BL21 (Thermo) tagged with a His_6 epitope in the pCold I vector according to the manufacturer's instructions (Takara-Clontech). The expressed protein was purified using cOmplete His-Tag purification resin (Roche).

2.3. Interaction analysis with purified recombinant proteins

FLAG-tagged LGP2, FLAG-MDA5, or FLAG-RIG-I (150 ng each) and 300 ng of His-PACT were incubated in the presence or absence of RNAs (100–200 ng) in the cell extract buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.2 mM EGTA, 2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM $MgCl_2$] for 15 h at 4 °C. In certain experiments, the RNAs were pre-incubated with Benzonase (9 U; Sigma-Aldrich) for 2 h at 4 °C before the addition of PACT and RLR as pre-treatment, or Benzonase (1–9 U) was added to the RLR/RNA/PACT complex and incubated for 2 h at 4 °C. After incubation, 10 μl of M2 FLAG-beads was added to purify the protein/RNA complex. The complex was analyzed by western blotting using anti-PACT (Abcam) and anti-FLAG (Sigma) antibodies.

2.4. Immunoprecipitation assay for overexpressed proteins

HEK293T cells were transfected with a total of 5 μg of each plasmid using Lipofectamine 3000. Twenty-four hours after transfection, the cells were lysed with whole cell extract buffer [25], and anti-FLAG M2 beads (Sigma-Aldrich) were added to the lysate. After incubation for 2 h, the beads were washed with the whole cell extract buffer and then eluted with sodium dodecyl sulfate sample buffer followed by western blot analysis using the anti-FLAG and PACT antibodies.

2.5. Virus infection

HEK293T, L929 (ATCC; CCL-1), Vero cells (ATCC; CRL-1587), and BHK cells (ATCC; CCL-10) were cultured and infected with TMEV (DA strain), SeV Z strain, and EMCV (ATCC, VR-129B) as described previously [25]. Viral infection was performed with a multiplicity of infection of 1–10 in Dulbecco's modified Eagle medium (DMEM) supplemented with 1–2% fetal calf serum for 2 h, and then the medium was subsequently replaced with complete medium.

2.6. RNAs and transfection

ON-TARGETplus Non-targeting siRNA#1, SMARTpool ON-TARGETplus mouse Prkra (PACT) siRNAs and mouse Dicer1 siRNAs (Supplementary Table I) were purchased from Dharmacon-GE Lifescience. siRNA knockdown in L929 cells was performed as previously described [25].

Phi6 lambda phage dsRNA was purchased from Thermo Scientific. TMEV genomic RNA was purified from the media and cell lysate of BHK cells infected with TMEV. In brief, BHK cells were infected with TMEV and grown until the cytopathic effect was complete. The lysate was then subjected to three freeze-thaw cycles, cleared with a 0.45-μm filter, and centrifuged at 25,000 rpm for 6 h in an SW28 rotor at 4 °C. The virus pellet was suspended in TRIzol reagent (Thermo) and RNA was isolated according to the manufacturer's instructions.

2.7. Plaque formation assay

For the plaque formation assay, BHK or Vero cells were infected with serial 10-fold dilutions of the virus in media. Infected cells were overlaid with media containing 0.9% agarose. At 24–72 h after infection, the cells were stained with 0.2% crystal violet containing 20% ethanol.

2.8. RNA analysis and cytokine assay

Quantitative RNA analysis was performed as described previously [25]. Culture media from infected cells or control cells were analyzed for cytokine production using the mouse IFNβ enzyme-linked immunosorbent assay kit (PBL Assay Science).

2.9. Reporter gene assays

HEK293T cells were transfected with the p3xFLAG-CMV10 empty vector or p3xFLAG-LGP2 plasmid (20 ng), together with pEFBOS-FLAG-MDA5 (20 ng) and pcKFLAG-PACT (20–200 ng) along with IFNβ-110luc (125 ng) and pRLnull (25 ng) (Promega) using Lipofectamine 3000. The Dual-Luciferase Assay System (Promega) was used for luciferase assays as described previously [25].

3. Results

3.1. LGP2 co-precipitates with PACT and Dicer in mammalian cells

We have previously demonstrated that LGP2 associates with an RNAi-regulatory factor TRBP [25]. In order to assess whether LGP2

Please cite this article in press as: M. Miyamoto, A. Komuro, PACT is required for MDA5-mediated immunoresponses triggered by *Cardiovirus* infection via interaction with LGP2, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.10.048