Biochemical and Biophysical Research Communications 415 (2011) 75–81

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

55 Amino acid linker between helicase and carboxyl terminal domains of RIG-I functions as a critical repression domain and determines inter-domain conformation

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ARTICLE INFO

Article history:
Received 21 September 2011
Available online 12 October 2011

Keywords:
RIG-I
Virus
Interferon

ABSTRACT

In virus-infected cells, viral RNA with non-self structural pattern is recognized by DExD/Hbox RNA helicase, RIG-I. Once RIG-I senses viral RNA, it triggers a signaling cascade, resulting in the activation of genes including type I interferon, which activates antiviral responses. Overexpression of N-terminal caspase activation and recruitment domain (CARD) is sufficient to activate signaling; however basal activity of full-length RIG-I is undetectable. The repressor domain (RD), initially identified as a.a. 735–925, is responsible for diminished basal activity; therefore, it is suggested that RIG-I is under auto-repression in uninfected cells and the repression is reversed upon its encounter with viral RNA. In this report, we further delimited RD to a.a. 747–801, which corresponds to a linker connecting the helicase and the C-terminal domain (CTD). Alanine substitutions of the conserved residues in the linker conferred constitutive activity to full-length RIG-I. We found that the constitutive active mutants do not exhibit ATPase activity, suggesting that ATPase is required for de-repression but not signaling itself. Furthermore, trypsin digestion of recombinant RIG-I revealed that the wild-type, but not linker mutant conforms to the trypsin-resistant structure, containing CARD and helicase domain. The result strongly suggests that the linker is responsible for maintaining RIG-I in a "closed" structure to minimize unwanted production of interferon in uninfected cells. These findings shed light on the structural regulation of RIG-I function.

1. Introduction

Innate immune responses are initiated upon detection of microorganisms such as viruses and bacteria. With no exception, viruses replicate inside the host cells; therefore, detection of viral components within infected cells is the critical step for triggering primary immune responses [1–4]. Viral RNA with a non-self structural signature is sensed by RIG-I-Like Receptor (RLR), which includes RIG-I, MDA5 and LGP2 [5,6]. Sensing viral RNA triggers a cascade of signaling events, leading to the activation of genes, including those encoding type-I interferon (IFN) and proinflammatory cytokines, which in turn participate in the antiviral response.

RIG-I is activated by double-stranded (ds)RNA and its detection is markedly enhanced if tri-phosphate moiety is present at the 5′ end of the RNA [7,8]; therefore, RIG-I acts as a specific sensor of viral dsRNA with 5′-ppp. RIG-I is composed of three functional domains: N-terminal caspase activation and recruitment domain (CARD, a.a. 1–190), RNA helicase domain (a.a. 218–746) and C-terminal domain (CTD, a.a. 802–925). Initial analyses revealed that CARD is essential and sufficient for signaling: over-expression of CARD alone is sufficient for signaling and RIG-I devoid of CARD acts as a dominant inhibitor of signaling. The observation that full-length RIG-I exhibits significantly low basal signaling activity compared to CARD alone led to the hypothesis of auto-repression, in which repressor domain (RD) masks CARD. Deletion mapping revealed that a.a. 735–925 acts as RD [9]. A similar region of LGP2 (a.a. 489–543) but not that of MDA5 exhibits repression function [9].

Abbreviations: RLR, RIG-I-like receptor; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; LGP2, laboratory of genetics and physiology 2; IFN, interferon; dsRNA, double-stranded RNA; CARD, caspase activation and recruitment domain; CTD, C-terminal domain; IPS-1, IFN-β promoter stimulator 1; IRF-3, interferon regulatory factor-3; NF-κB, nuclear factor-κB; SeV, sendai virus; poly (I:C), polyinosinic:polycytidylic acid.

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doi:10.1016/j.bbrc.2011.10.015

The repression of full-length RIG-I can be liberated upon its encounter with viral RNA. Thus, the de-repression mechanism is critical for the function of RIG-I for acting as a sensor and a switch for signaling. CTD binds to dsRNA with its basic cleft [10]; thus, its involvement in RNA recognition is suggested. It is presumed that the helicase domain also participates in viral RNA recognition, since full-length RIG-I exhibits higher RNA binding affinity [10,11]. Binding of dsRNA induces ATPase activity of RIG-I in vitro. ATPase-deficient RIG-I acts as a dominant inhibitor [5], suggesting that ATP binding and/or its hydrolysis is a critical step in de-repression. RIG-I is a functional RNA helicase, which consumes ATP for dsRNA unwinding [10]; however, dsRNA substrates resistant to RIG-I helicase activity, but not those susceptible, induce IFN production, suggesting that dsRNA unwinding is not critical for signaling. Therefore it is hypothesized that ATPase activity of RIG-I induces a conformational change, resulting in unmasking CARD. Once CARD is liberated, RIG-I may undergo oligomeric complex formation [9]. The oligomer also recruits another CARD-containing adaptor, IPS-1 (IFN-β promoter stimulator 1) (also known as MAVS, VISA and Cardif, [12–15]). IPS-1 is a unique signaling adaptor, expressed on the outer membrane of mitochondria, and its specific localization is critical for its function [13]. IPS-1 transmits a signal through TRAF proteins, resulting in transcription factors IRF-3, IRF-7 and NF-κB, which are responsible for the activation of IFN and cytokine genes [16].

Repression of RIG-I in uninfected cells is crucial for tight regulation of the host immune system and prevents unwanted production of IFN under ordinary conditions; however, the precise mechanism of auto-repression is not known and there is no evidence for the existence of "active" and "inactive" conformations. In the current study, we further delimited RD to the 55 amino acid linker between the helicase domain and CTD. Mutant RIG-I containing amino acid substitutions within the linker conferred constitutive activity. Furthermore, trypsin digestion of wild-type and mutant RIG-I revealed a distinct conformation, suggesting that the mutation inactivated RD and induced an "open" conformation. These findings shed light on the role of RD in RIG-I regulation.

2. Materials and methods

2.1. Cell culture

HEK293T, Huh7.5 and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

2.2. Plasmid construction

All RIG-I full-length mutant constructs used in this study were generated with Prime Star (R) HS DNA Polymerase (TAKARA, Shiga, Japan) or the KOD-Plus Mutagenesis Kit (TOYOBO LIFE SCIENCE, Osaka, Japan), using primers containing the desired mutation, and were sequenced using an ABI 3130xl automatic DNA sequencer to verify the presence of the mutation.

2.3. Protein sequence alignment

Multiple protein sequence alignment was performed using the ClustalX program [17]. DSC, MLRC, and PHD methods were used to predict the protein secondary structure of the linker sequences on the NPS@ (Network Protein Sequence Analysis) server [18].

2.4. Protein purification

The cDNA encoding fusion protein, consisting of Flag-tag and human RIG-I or mutants, was inserted into pAcGHLT-B vector (BD Biosciences, CA, USA), which has GST and His-tag, between NcoI and SmaI sites. To obtain recombinant baculoviruses, SF9 insect cells were co-transfected with the expression plasmid and BD Baculo Gold Linearized Baculovirus DNA (BD Biosciences) according to the manufacturer's protocol. Recombinant virus was recovered from the culture supernatant. The recombinant RIG-I proteins were expressed in Sf9 or High five insect cells (2×10^7 cells/150 mm dish) by infection with recombinant baculovirus (moi 10) for 4 days. The cells were lysed in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.5 mM DTT, 1% Triton X-100) with protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged at 15,000 rpm for 20 min. The supernatants were mixed with Ni-NTA super-flow (QIAGEN, Hilden, Germany) and the beads were washed with binding buffer (20 mM imidazole, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.5 mM DTT). Proteins were eluted with elution buffer 1 (500 mM imidazole, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.5 mM DTT). The eluted protein was further purified with glutathione Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Bound proteins were washed with wash buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.5 mM DTT) and eluted with elution buffer 2 (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 20 mM glutathione).

2.5. Immunoblot analysis

Huh7.5 cells were seeded in a 6 cm dish (5×10^5 cells/dish/2 ml medium). Before transfection, culture medium was replaced with serum-free DMEM. Expression plasmid for RIG-I wt or mutants (2 μg) was mixed with 1 ml DMEM and 5 μl polyethylenimine Max (Polysciences, Warrington, PA, USA), and then incubated for 15 min. The mixture was added to the culture. After incubation for 1 h, FBS was added to a final concentration 10%. After harvest, whole cell lysate was prepared with NP40 lysis buffer and subjected to Native PAGE or SDS PAGE as described previously [6,19].

2.6. ATPase assay

Reaction mixture (25 μl: 1 μg purified recombinant RIG-I protein, 100 ng RNA, 20 mM Tris–HCl pH 8.0, 1.5 mM $MgCl_2$, 1.5 mM DTT, 20 units Protector RNase Inhibitor, 1 mM ATP) was incubated at 37 °C for 30 min. The product, inorganic phosphate, was quantified using BioMol Green (Enzo, Farmingdale, NY, USA).

2.7. RNA

25 bp dsRNA was prepared by annealing chemically synthesized complementary RNA (p25/25) as described previously [10]. Then, 5′-triphosphate-containing RNA was synthesized using a DNA template and T7 RNA polymerase as described previously [10] as 5′pppGG25, presumably containing copy-back 3′end. Poly (I:C) pull down assay was performed as described previously [5].

2.8. RNA binding assay

Purified GST-fused RIG-I proteins (5 μg) were mixed with the indicated RNAs (0.1 μg) in a 20 μl reaction mixture (20 mM Tris–HCl pH 8.0, 1.5 mM $MgCl_2$, 1.5 mM DTT) and incubated at 37 °C for 15 min. The mixture was resolved by 15% native PAGE. The gel was stained with EtBr to visualize free RNA and protein/RNA complex.