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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Keywords: RIG-I; virus; interferon;

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- κ B, Nuclear factor- κ B; SeV, Sendai virus; poly (I:C),

polyinosinic:polycytidylic acid;

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[1,2,3,4]. 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].

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, [12,13,14,15]). 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- κ B, 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 μ g) was mixed with 1 ml DMEM and 5 μ l polyethylenimine Max (Polysciences, Warrington, PA, USA), and then incubated for 15 minutes. 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 μ l: 1 μ g purified recombinant RIG-I protein, 100 ng RNA, 20 mM Tris-HCl pH 8.0, 1.5 mM MgCl₂, 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 μ g) were mixed with the indicated RNAs (0.1 μ g) in a 20 μ l reaction mixture (20 mM Tris-HCl pH 8.0, 1.5 mM MgCl₂, 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.

2.9 Luciferase reporter assay.

Huh7.5 or Huh7 cells were seeded in a 12-well plate (5×10^4 cells/well/0.5 ml medium).

Then, 2 µg p-125 Luc IFN-β luciferase reporter plasmid, 20 ng pRL-tk, and 4 µg

expression plasmid for RIG-I wt or mutants were mixed with 150 µl Opti-MEM and 9

µl Fugene 6 (Roche), and incubated for 15 minutes. The mixture was dispensed into 3

wells (50 µl/well). After 20 h of incubation, cells in each well were split into two

wells for virus infection. After cells had attached to the dish, one well was mock

treated and the other well was infected with SeV (moi 1) for 20 h. Cells were

subjected to a Dual-Luciferase (R) Reporter Assay System (Promega, Madison, WI,

USA). Luciferase activity was normalized using Renilla luciferase activity (pRL-tk)

as a reference. The values show relative luciferase activity as fold induction normalized

by the value of unstimulated cells transfected with empty vector (control) for each experiment.

2.10 Trypsin digestion.

The reaction mixture (10 μ l) contained 0.5 picomoles of GST-RIG-I or GST-RIG-I mutants in elution buffer 2. Five nanograms of trypsin (TPCK-treated) was added to the mixture and incubated at 37 °C for 20 min. The reaction was terminated by adding SDS loading buffer (Nacalai Tesque, Kyoto, Japan) (10 μ l) and boiling for 1 min. The digestion products were analyzed by SDS-PAGE (5-20 % acrylamide gradient) followed by silver staining or Western blotting using monoclonal ANTI-FLAG M2 antibody.

3. Results

3.1. Delimitation of RIG-I repressor domain

Previously, the region of RIG-I encompassing a.a. 735-925 was mapped as RD [9]. Deletion of a.a.735-925 results in constitutive active RIG-I. Moreover, RD is sufficient to repress virus-induced activation of endogenous RIG-I when a.a.735-925 alone is over expressed . a.a.735-925 encompasses the entire CTD (a.a. 802-925), which was defined as a structural domain by a protease digestion experiment and contains the RNA-binding basic cleft [10], and a linker (a.a. 747-801) connecting the helicase domain and the CTD (Fig. 1A). To delimit the functional RD further, we prepared detailed deletion mutants of RIG-I and examined their stimulatory activity on IFN- β luciferase reporter (Fig. 1A, B). Without viral stimulus, wt RIG-I exhibited no stimulatory activity. Consistent with the previous report [9], RIG-I1-734 exhibited marked activation of the reporter. Interestingly, RIG-I1-801, which lacks the entire CTD, exhibited little activity; however, partial and complete deletion of the linker (1-770 and 1-746, respectively) exhibited enhanced reporter activity (Fig. 1B), suggesting that the linker is the minimal repression domain. We generated a GST

fusion construct, GST-RIG-I 747-801, in which the linker was connected with GST. Cells expressing GST as a control showed elevated IFN- β promoter activity upon SeV infection which selectively activates RIG-I [20]. (Fig. 1C); however, over-expression of GST-RIG-I747-801 suppressed reporter gene activation upon viral stimulus, suggesting that the newly defined repression domain is capable of acting in *trans*, the feature described for a.a. 735-925. The secondary structure prediction program (Materials and methods) suggested that a.a.747-801 is mostly composed of α -helix (Fig. 1D). Alignment of the primary sequence corresponding to linkers of LGP and RIG-I from different species revealed that several amino acids are highly conserved (Fig. 1E); notably, ϕ XX ϕ Q motif (in which ϕ is I, L or V) is conserved at a.a. 765 and 780 (human RIG-I numbering). We constructed mutants in which each of the motif was disrupted: mutant 1 (ILQ/765, 768, 769/AAA) and mutant 2 (IIQ/780, 783, 784/AAA), and analyzed their basal activity. Interestingly, both mutant 1 and 2 promoted reporter gene expression without viral infection (Fig. 1B), consistent with the hypothesis that the linker structure is critical for repression function.

3.2. ATP binding site of RIG-I is required for virus-induced activation of IFN- β promoter but not for constitutive activity of the linker mutants.

Because the ATP binding site is indispensable for virus-induced activation of RIG-I [5], we examined whether the constitutive active RIG-I mutants described in Fig. 1B are similarly dependent on ATP binding. We introduced K270A mutation into each of the deletions, mutant 1 and 2 and examined their activity (Fig. 1F). Interestingly, KA mutation did not change the trans-activation potential of the constitutive active mutants. As expected, K270A mutation totally abolished SeV-induced activation of IFN- β promoter by wt RIG-I (Fig. 1G). On the other hand, although mutant 1 and 2 are constitutively active, their activation level was not enhanced by SeV infection in K270 or K270A context. To further confirm whether the constitutive active mutants activate the reporter gene through the activation of IRF-3, we examined IRF-3 dimerization in cells over-expressing wt and mutants (Fig. 2). IRF-3 dimer formation was observed in cells over-expressing constitutively active mutants 1-770, 1-746 and 1-734 (Fig. 2A), and the level of IRF-3 dimer was roughly proportional to the reporter assay (Fig. 1B).

Likewise, mutant 1 and 2 induced IRF-3 dimer (Fig. 2B).

3.3. Mutation 1 and 2 within the linker did not affect RNA binding activity of RIG-I

Next, the RNA binding activity of RIG-I mutant 1 and 2 was examined using 25 bp dsRNA (p25/25), 5' tri-phosphate-containing RNA (5'pppGG25), and poly (I:C) (Fig. 3A, B). Wild-type RIG-I, and mutant 1 and 2 exhibited binding to these RNA. Mutant 1 and 2 bound to 5' pppGG25 and poly (I:C) with slightly increased efficiency (in repeated experiments, data not shown). These results suggest that mutation within the linker did not cause overall structural distortion and at least preserved the function of RNA recognition.

3.4. Mutation 1 and 2 within the linker disconnected dsRNA binding and ATPase activity of RIG-I

Wild-type RIG-I, and mutant 1 and 2 were produced as GST fusion, purified and

subjected to the ATPase assay (Materials and methods). Although wild-type RIG-I exhibited very low ATPase activity, it was markedly enhanced by 5' pppGG25 or poly(I:C) (Fig. 3C). In contrast, ATPase activity of mutant 1 and 2 was not increased even in the presence of ligands. As both mutant 1 and 2 bind dsRNA similarly to the wild-type (Fig. 3 A, B), these mutants are incapable of connecting the dsRNA binding signal to the activation of ATPase. These results suggest that mutation 1 and 2 result in altered conformation of RIG-I.

3.5. Conformational change of RIG-I induced by mutation 1 and 2

To investigate the conformational change of RIG-I, we subjected wild-type RIG-I and mutants to limited trypsin digestion and analyzed the digested product by SDS-PAGE (Fig. 3D). Trypsin digestion of wild-type RIG-I generated an intermediate RIG-I fragment of 90 kD after 5 min of digestion. This fragment persisted at least until 20 min of digestion, suggesting that it is relatively resistant to this protease. Western blotting using anti-Flag antibody, whose epitope is attached at the amino terminus of the

RIG-I construct, revealed that the 90 kD fragment contains the intact Flag epitope attached to the N-terminus of RIG-I (Fig. 3E). Notably, the result suggests that the CARD was protected from digestion. From the calculated molecular mass of the fragment, we mapped the cleavage site around amino acid 800 of RIG-I and GST was cleaved off. In sharp contrast, neither mutant 1 and 2 exhibited a trypsin-resistant fragment within this range of digestion, strongly suggesting that the conformation of mutant 1 and 2, particularly the complex of CARD and helicase domain, is much less resistant to trypsin digestion.

4. Discussion

In the present work, we demonstrated that the linker, which connects the helicase domain and CTD, is the minimal domain for the repression of constitutive activity of RIG-I. Prediction of the secondary structure suggested that the linker is mostly composed of α -helix. Furthermore, comparison of the primary sequences of the linkers of RIG-I and LGP2 proteins revealed conserved amino acid residues. Mutagenesis of these key amino acids resulted in impaired repression function. We hypothesized a model, described in Fig. 4. Wild-type RIG-I conforms a closed structure so CARD is masked (Fig. 4A); thus, the basal activity of wild-type RIG-I is minimized. Mutation of the linker disrupts the closed conformation and results in the release of CARD. The protease digestion of wild-type and mutant RIG-I strongly suggests a conformational change by linker mutation (Fig. 4B). Interestingly, once such a conformational change is allowed by mutation, ATPase activity is dispensable for signaling (Fig. 1F). This is consistent with the hypothesis that RIG-I CARD is essential for signaling but its availability is strictly regulated by the overall

conformation of full-length RIG-I. Our protease digestion experiment demonstrates for the first time that two distinct conformations, repressed (wild-type RIG-I) and de-repressed (mutant RIG-I) exist and that the linker of 55 amino acids is responsible for autorepression.

The autorepression state can be reversed by viral infection through a multiple step mechanism: i) recognition of non-self RNA, ii) binding and hydrolysis of ATP, iii) conformational change and iv) ubiquitination. The non-self RNA patterns, such as dsRNA and 5'tri-phosphate are recognized by the CTD and helicase domain [10]. RNA binding induces ATPase activity of RIG-I (Fig. 3C), leading to the conformational change (Fig. 4C). In addition, there are several reports that ubiquitination plays an important role in RIG-I activation, although the proposed models are not consistent [21,22,23]. It is therefore important to determine the repressed and de-repressed conformation of full-length RIG-I at atomic resolution. Such structures will explain how the linker region regulates the overall conformation of the RIG-I molecule.

Figure legends

Figure 1. RIG-I 747-801 is the minimal repression domain

(A) Domain structure of the RIG-I constructs used in this study. The amino acid region encoded by each construct is shown on the left. Mutations are indicated by arrows.

(B, C) Activation of IFN- β promoter by RIG-I wt and mutants. Huh7.5 (B) or Huh7 cells (C) were transfected with indicated expression vectors, IFN- β luciferase reporter and pRL-tk as the internal control. In C, 52 h after transfection, cells were infected with Sendai virus (SeV) for 20 h. 24 h (B) or 72 h (C) after transfection, the cells were harvested, and cell lysates were subjected to dual luciferase assay. The values (Y axis) show relative luciferase activity as fold induction normalized by the value of unstimulated cells transfected with empty vector (control) for each experiment. Data represent the mean $\pm$ standard deviations (SD) (n=3).

(D) Predicted secondary structure of the linker of RIG-I.

(E) Multiple sequence alignment of the linker of RIG-I and LGP2 from different species.

The sequences were aligned using the ClustalX program. Invariant residues are marked

with an asterisk. Highly conserved residues are marked with a colon. Similar residues are marked with a dot. Residues targeted for mutation are indicated at the top and were substituted with alanine, indicated as A.

(F, G) The effect of ATP binding site mutation on the activity of RIG-I and mutants.

Huh7.5 cells were transfected with the indicated expression vectors in the presence of the luciferase reporter and pRL-tk, as in (B). KA: K270A mutation, which disrupts ATP binding motif. 24 h (F) or 72 h (G) after transfection, the cells were harvested. Constitutive activity (F) or SeV induced activity (G) was determined.

Figure 2. Linker region of RIG-I regulates the activation of IRF-3

Dimerization of IRF-3 induced by RIG-I mutants. Huh7.5 cells were transfected with empty vector (control), expression vector for Flag-tagged RIG-I wt or indicated mutants. 36 h (A) or 16 h (B) after transfection, cell lysates were subjected to Native PAGE and immunoblot analysis with anti-IRF-3 antibody. IRF-3 monomer and dimer are indicated by arrows.

Figure 3. Characterization of RIG-I mutant protein

(A) RNA-binding activity of RIG-I wt and mutants. Recombinant full-length RIG-I was incubated with p25/25 (left) or 5'pppGG25 (right) and separated by gel electrophoresis (Materials and methods). Arrows denote the position of unbound RNA (lower) or RNA-RIG-I complex (upper).

(B) Poly (I:C) pull-down of RIG-I proteins. Extract from HEK293T cells transfected with empty vector (control), Flag-tagged RIG-I wt or mutant vectors was mixed with poly (I:C)-agarose beads, and subjected to pull-down assay. RIG-I proteins in the input material (lower panel) and recovered from pull-down (upper panel) are shown.

(C) ATPase activity of RIG-I. ATPase activity of the recombinant GST-RIG-I proteins was determined as described in Materials and methods. Data are the mean $\pm$ SD (n=2). Similar data were obtained from a separate experiment.

(D and E) Protease digestion of RIG-I. Recombinant GST-RIG-I proteins were

digested with trypsin at 37 °C for the indicated times, resolved by SDS-PAGE and visualized by silver staining (D). Similarly digested RIG-I (for 20 min) was analyzed by immunoblotting with anti-Flag (E).

Figure 4. Model for basal repression and de-repression of RIG-I

Schematic model of RIG-I suppression by its linker region and de-repression by viral RNA is shown. Tandem CARDs and domains encoding the helicase, linker and CTD are shown. The linker repressed RIG-I signaling by masking CARDs in the absence of viral RNA (A). Trypsin digestion partially digested RIG-I, resulting in digestion-resistant fragment consisting of CARD and helicase domain. Linker mutation changes the linker structure, resulting in the open conformation, in which CARD is exposed (B). Mutant 1 and 2 were highly sensitive to trypsin digestion and no resistant fragment was observed. Binding of viral RNA to RIG-I induces ATPase activity, leading to its conformational change, resulting in unmasking of CARD (C).

Acknowledgements

We thank Pro. S. Yonehara for valuable discussion. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan, the Ministry of Health, Labor and Welfare, the PRESTO Japan Science and Technology Agency, the Uehara Memorial Foundation, Takeda Science Foundation, and Nippon Boehringer Ingelheim.

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FIG.1

FIG.2 A

B

FIG.3

FIG.4