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Study of Human RIG-I Polymorphisms Identifies Two Variants with an Opposite Impact on the Antiviral Immune Response

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Abstract

Background: RIG-I is a pivotal receptor that detects numerous RNA and DNA viruses. Thus, its defectiveness may strongly impair the host antiviral immunity. Remarkably, very little information is available on RIG-I single-nucleotide polymorphisms (SNPs) presenting a functional impact on the host response.

Methodology/Principal Findings: Here, we studied all non-synonymous SNPs of RIG-I using biochemical and structural modeling approaches. We identified two important variants: (i) a frameshift mutation (P229fs) that generates a truncated, constitutively active receptor and (ii) a serine to isoleucine mutation (S183I), which drastically inhibits antiviral signaling and exerts a down-regulatory effect, due to unintended stable complexes of RIG-I with itself and with MAVS, a key downstream adapter protein.

Conclusions/Significance: Hence, this study characterized P229fs and S183I SNPs as major functional RIG-I variants and potential genetic determinants of viral susceptibility. This work also demonstrated that serine 183 is a residue that critically regulates RIG-I-induced antiviral signaling.

Introduction

Among all viral components that trigger the antiviral screen of the host, nucleic acids have been viewed as the most important [1]. In mammals, there are at least two receptor systems in place to detect such viral motifs and to further mount a type I interferon (IFN)-dependent antiviral immune response. The endosomal TLR3, 7, 8, and 9 interact with extracellular viral nucleic acids while the cytosolic helicases RIG-I and MDA-5 sense intracellular double-stranded (ds)RNA and/or 5’ triphosphate single-stranded RNA, two common byproducts of viral infection and replication [2,3,4,5].

Current knowledge posits RIG-I as a particularly critical surveillance molecule that detects numerous viruses such as the human pathogens influenza and hepatitis C (HCV) viruses [6,7]. RIG-I interacts with its ligands by means of its central ATP-binding helicase domain as well as its carboxyterminal regulatory domain (RD; see the schematic representation in Fig. 1A). For its amino-terminal tandem Caspase Recruitment Domains (CARDs), RIG-I homocomplexes relay a signal by binding MAVS (also known as IPS-1, CARDIF or VISA), an adapter protein that mediates CARD-dependent interactions with RIG-I. This signaling complex further activates the transcription factors NF-κB and interferon regulatory factor (IRF)-3 to ultimately upregulate the expression of pro-inflammatory and antiviral mediators and the subsequent induction of adaptive immune responses [2,3,4].

Importantly, the receptor function of RIG-I is non-redundant, as confirmed by knock-out studies [8]. Moreover, the Huh7.5 hepatocytic cell line is especially permissive to HCV as the result of an elaborate viral evasion strategy as well as to a defective RIG-I protein bearing a single mutation [9,10]. In that regard, unequivocal evidence shows that genetic mutations may be important determinants of increased susceptibility to viral diseases [11,12]. Among them, single-nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide is altered. There are more than 4 million SNPs in the human genome, 200,000 of which occur in coding regions, underlying the extent of genetic variability...
and its potential positive or negative effects on the host antimicrobial defense [13,14]. Interestingly, studies aiming to characterize RIG-I polymorphisms are scarce. Here, we characterized functional effects of two RIG-I SNPs that might help us to understand the basis of individual variations between normal and abnormal innate immune responses to viral pathogens as well as to better appreciate the molecular mechanism by which RIG-I is triggered by non-self RNA.

Results

Genetic variability profile of human RIG-I

Information collected on 04/2009 from NCBI SNP database indicates that at least 342 SNPs are present in the human RIG-I gene. Among them, 14 are situated within coding sequences but only 7 result in amino acids substitutions, i.e. R7C, S144F, S183I, T260P, I406T, D580E, F789L (Fig. 1A). An additional SNP corresponds to a frameshift at nucleotide position 845 of RIG-I mRNA (accession number NM_014314), which results in a frameshift (fs) and in a truncated RIG-I protein. This mutant is herein defined as P229fs as it includes the first 229 residues (instead of 925 residues in the WT RIG-I protein) followed by 4 unintended residues (i.e. FRSV; Fig. 1B) and thus, does not contain the helicase and the RD domains. As illustrated in Fig. 1A, RIG-I SNPs map to the different domains of the protein. Missense SNPs differentially affect RIG-I-mediated innate immune signaling

Elucidating the functional role of non-synonymous SNPs in RIG-I may enhance our understanding of viral pathogenesis and host defense mechanisms as well as to contribute to a more detailed knowledge in structure-function relationship of RIG-I. To this effect, plasmids containing the eight SNPs were generated by site-directed PCR mutagenesis. We first observed that R7C, S144F, S183I, T260P, I406To rF 789L mutants was similar to that induced by WT RIG-I and lower in cells expressing the R7C, S144F, S183I, T260P, I406To rF 789L mutants (Fig. 1C). The frequency values provided indicate that at least 342 SNPs are present in the human RIG-I gene. Among them, 14 are situated within coding sequences but only 7 result in amino acids substitutions, i.e. R7C, S144F, S183I, T260P, I406T, D580E, F789L (Fig. 1A). An additional SNP corresponds to a frameshift at nucleotide position 845 of RIG-I mRNA (accession number NM_014314), which results in a frameshift (fs) and in a truncated RIG-I protein. This mutant is herein defined as P229fs as it includes the first 229 residues (instead of 925 residues in the WT RIG-I protein) followed by 4 unintended residues (i.e. FRSV; Fig. 1B) and thus, does not contain the helicase and the RD domains. As illustrated in Fig. 1A, RIG-I SNPs map to the different domains of the protein. Missense SNPs differentially affect RIG-I-mediated innate immune signaling

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To determine whether non-synonymous SNPs can alter RIG-I-induced antiviral and/or pro-inflammatory signaling pathways, we used a functional cell-based assay to evaluate RIG-I-dependent promoter or an NF-kB promoter (Fig. 2C) and the molecular weight of all RIG-I proteins was similar, with the exception of P229fs RIG-I which resulted in a truncated protein with a size comparable to the 2CARD module (Fig. 2A).

Figure 1. Genetic variability profile of human RIG-I. (A) Schematic representation of major domains of RIG-I (adapted from [7,17,34]). RIG-I non-synonymous SNPs described in NCBI SNP database are indicated as R7C (rs10813831), S144F (rs55789327), S183I (rs11795404), T260P (rs35527044), I406T (rs951618), D580E (rs17217280) and F789L (rs35253851). (B) Alignment of protein sequence of RIG-I SNPs from Homo sapiens (NM_014314), Pan troglodytes (XM_001266662), Macaca mulatta (NM_001042668) and Rattus norvegicus (NM_01106645). (C) Frequency of RIG-I SNPs alleles. This latter information was collected from NCBI SNP database and refers to the sum of SNP containing alleles in both human to platypus, using ClustalW software. Amino acids in blue and red correspond to conserved and non-conserved residues, respectively. (C) Frequency of RIG-I SNPs alleles. This latter information was collected from NCBI SNP database and refers to the sum of SNP containing alleles in both homozygous and heterozygous individuals for a given SNP. ND: not determined.

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and/or HEK 293T cells (black bars) were co-transfected with a three is shown. in (vector-transfected cells (activity of triplicate samples minus basal activity measured in empty expressed as the mean 6 SNPs RIG-I (filled bars) (luciferase-reporter plasmid and a vector encoding WT (empty bars) or

Figure 2. RIG-I-mediated constitutive innate immune signaling, but not expression level, is differentially affected by RIG-I SNPs. Expression of wild-type (WT) and non-synonymous SNPs RIG-I as assessed by western-blot using BEAS-2B cells (A) and flow cytometry (B) using an anti-Flag antibody and BEAS-2B and/or HEK 293T cells 42 h post-transfection. RIG-I SNP proteins are expressed at the same level as WT RIG-I with the exception of P229fs in BEAS-2B cells. (C–E) BEAS-2B (grey bars) and/or HEK 293T cells (black bars) were co-transfected with a lβ-galactosidase reporter plasmid and either a NF-kB- or IFN-β-luciferase-reporter plasmid and a vector encoding WT (empty bars) or SNPs RIG-I (filled bars) (C–E) or WT 2CARD (E) or a control plasmid. Data were collected 42 h (C) or 24 h (D–E) post-transfection and are expressed as the mean ± SD of RLU normalized to β-galactosidase activity of triplicate samples minus basal activity measured in empty vector-transfected cells (C–D). One representative experiment out of three is shown. // in (C) means that this condition was not tested. (E) Stimulated HEK 293T cells as shown in panels (D) were assessed for IL-8 and RANTES release by ELISA. Data are mean ± SD of triplicate samples and are representative of three independent experiments. doi:10.1371/journal.pone.0007582.g002

triggered by the 2CARD module. This finding is particularly important as it suggests that individuals carrying such mutation may constitutively produce exaggerated amounts of immune mediators.

By contrast, no constitutive IFN-β expression was triggered by the S183I RIG-I mutant (n = 3, p<0.0001; Fig. 2C). More importantly, S183I SNP uniquely inhibited IRF-3 (not shown), IFN-β and NF-kB reporter activities elicited by the viral mimic poly(C) in agreement with previous studies that have shown that poly(C) is a potent RIG-I stimulus ([16,17,18,19]; Fig. 3A; n = 3 p<0.0001). To confirm the pathophysiological relevance of the above findings, we sought to address the responsiveness of the mutant proteins to viral infection. We clearly demonstrated that S183I mutation had a deleterious effect on RIG-I antiviral activity as it drastically reduced IFN-β and NF-kB-mediated responses triggered by intact, replicative Sendai or influenza A viruses (Fig. 3B and 3C). Noteworthy, while R7C SNP slightly inhibited RIG-I signaling triggered by Sendai virus stimulation, D90E inhibited RIG-I signaling in response to dsRNA and IAV, but not to Sendai virus infection (n = 3, p≤0.0003). Nevertheless, as S183I SNP uniquely resulted in the strongest inhibition of RIG-I-dependent signaling induced by all stimuli, we decided to focus the rest of our study on this specific mutation. Thus, the clear loss-of-function effect of S183I RIG-I SNP was confirmed by measuring the secretion of IL-8 (n = 3 p<0.0001; Fig. 3D) and RANTES (not illustrated) in the supernatants of stimulated HEK 293T cells. This result well extends Shigemoto et al.’s findings using RIG-I-deficient murine embryonic fibroblasts [20]. Finally, specificity controls are

Figure 3. SNPs differentially modulate RIG-I-mediated immune signaling in response to a viral dsRNA mimetic as well as to influenza A and Sendai viruses. HEK 293T cells (A–B) and BEAS-2B cells (C) were co-transfected with WT (open bars) or non-synonymous SNP RIG-I (black bars) expression vectors and IFN-β- or NF-kB-dependent luciferase reporter plasmids. 24 h later, cells were challenged for 18 h by poly(I:C) (p(I:C), 1 μg/well) (A) or infected with Sendai virus (SeV, 2 HAU/well) (B) or influenza A virus (IAV, MOI = 1) (C). Data are expressed as in Fig. 2c and are representative of three independent experiments. // in (B) means that this condition was not tested. (D) The stimulated or infected cells as shown in panels (A–B) were subsequently assessed for IL-8 release by ELISA. Data are mean ± SD of triplicate samples and are representative of three independent experiments. IL-8 was undetectable in supernatants of non-stimulated transfected cells. (E) S183I SNP does not alter RIG-I-independent signaling. BEAS-2B and HEK 293T cells were co-transfected with WT or S183I RIG-I vectors or empty vector (control) and a NF-kB-dependent luciferase reporter plasmid. 24 h later cells were stimulated with PMA (100 nM) or TNF-α (20 ng/ml). doi:10.1371/journal.pone.0007582.g003
provided to make sure the alteration of cell signaling by S183I variant is specific to the RIG-I-dependent pathway. Thus, NF-κB signaling in HEK 293T or BEAS2B cells triggered by two non-viral stimuli (i.e. the cytokine TNFα and the potent PKC signaling activator PMA) was not down-modulated by S183I RIG-I, in comparison with WT RIG-I (Fig. 3E). Altogether, these data stressed the critical role of S183 residue in mediating RIG-I-induced innate immune signaling.

RIG-I 2CARD module carrying the S183I SNP is unable to trigger signal transduction

Next, we investigated the mechanism by which S183I SNP results in inhibition of RIG-I antiviral immune response. First, we found that this was neither due to an alteration at a very early step of RIG-I signaling, i.e. the ligand-binding capacity (Fig. 4A) nor to a RIG-I cellular mislocalization (not illustrated). In a very recent report, Fujita’s laboratory also demonstrated that the inhibitory phenotype of S183I RIG-I was neither due to a failure of ubiquitination [20]; a post-translational process essential for RIG-I activity [21]. Next, we took advantage of the fact that the isolated tandem WT 2CARD elicits a vigorous and spontaneous induction of downstream signaling [7] to examine whether S183I mutation could also inhibit this constitutive cell response. As shown in Fig. 4B, contrary to WT 2CARD, the S183I 2CARD could not induce IFN-β and NF-κB activities in HEK 293T (n = 3, p<0.0001) and BEAS-2B cells (not shown). This loss-of-function effect was confirmed by measuring the secretion of endogenous mediators in supernatants of HEK 293T cells (n = 5, p<0.0001; Fig. 4C).

RIG-I isoleucine 183 residue closes off RIG-I homodimers and RIG-I/MAVS complexes

CARD domains mediate homotypic or heterotypic interactions

**Figure 4. Analysis of the loss-of-function mechanism of S183I SNP: evidence for an inhibition of the constitutive signal transduction triggered by 2CARD RIG-I. (A) S183I does not affect dsRNA binding activity of RIG-I as assessed by a pull-down of Flag-tagged WT and S183I RIG-I proteins using poly(C)-coated agarose beads, 42 h after transfection of HEK 293T cells. (B, C) S183I strongly inhibits RIG-1 2CARD-induced IFN-β-dependent antiviral and NF-κB-dependent pro-inflammatory signaling as demonstrated by luciferase reporter assays (B) or by measuring RANTES and IL-8 release by ELISA in HEK 293T cells (C) co-transfected for 42 h with WT or S183I 2CARD or empty expression vector and luciferase reporter plasmids. Data are mean ± SD of triplicate samples and are representative of three independent experiments. doi:10.1371/journal.pone.0007582.g004**

S183I SNP exerts a down-modulatory effect

Phenotypes of several heritable disorders are linked to missense mutations in single alleles. In some cases, the mutant protein exhibits a regulatory effect whereby heterozygous co-expression of mutant and WT gene has a deleterious consequence, relatively to the case in which two WT alleles are expressed [25,26]. Such a down-regulatory effect usually involves homomeric or heteromeric proteins. In regard to the ability of S183I SNP to impair antiviral
signaling through an increase of RIG-I homocomplexes and RIG-I/MAVS heterocomplexes, it might be speculated that in a heterozygous host, the mutant protein would interfere with the function of the normal protein being produced from the WT allele. To test this hypothesis, we titrated WT RIG-I with increasing amounts of S183I RIG-I in mock treated-HEK 293T and in HEK 293T cells activated by the viral mimetic poly(I:C) or infected by Sendai virus (Fig. 6, panels A–C). As a single example, IFN-β response was reduced by 50% in cells co-transfected with an equimolar concentration of WT RIG-I and S183I RIG-I expressing vectors and further activated by these stimuli (n = 3, p < 0.0001). We also observed that this S183I 2CARD mutant reduced IFN-β activity of WT 2CARD by 50% when transfected at a 1:1 ratio and up to 70% at a fourfold excess of transfected plasmid DNA (n = 3, p < 0.0001, Fig. 6D). Interestingly, the negative impact of S183I SNP was less potent when considering...
RIG-I-mediated NF-κB activation triggered by poly(I:C) or Sendai virus, consistently with the primary role of RIG-I in type I-IFN-inducing antiviral signaling pathways [2,3,4,27].

Discussion

The efforts conducted by international consortiums – such as the HapMap Project and Perlegen – to identify and characterize the levels of polymorphic variation in humans has yielded an ever-growing list of SNPs [28]. These include variation located in genes involved in innate immunity, which may account for individual differences in the response to pathogens. For instance, mutations in TLR2, TLR4, TLR5 and IRAK4 have all been associated with increased risk to develop infectious diseases [13,29,30]. In regard to genes encoding CARD-containing proteins, mutations in the peptidoglycan receptors NOD1 and NOD2 have been associated with several inflammatory disorders, including Crohn’s disease, Blau syndrome and asthma [23]. A non-synonymous SNP in MDA-5 was also reported to show an association with type I diabetes [31]. Remarkably, no human disease has yet been linked to RIG-I. Nonetheless, the defective response of a human hepatoma cell line, Kupffer cell line, found permissive to HCV replication, was due to a single mutation (T55I) [9,21,32]. Here, we characterized functional effects of RIG-I SNPs that might help us to understand the basis of individual variations between normal and abnormal innate immune responses to viral pathogens as well as to better appreciate the molecular mechanism by which RIG-I is triggered by non-self RNA.

Among the eight RIG-I SNPs reported in NCBI SNP database, we characterized two distinct functional SNPs which strongly alter RIG-I-mediated signaling. First, we identified P229S as a SNP which results in a truncated constitutively active RIG-I. This finding is particularly important as it suggests that individuals carrying such mutation may constitutively produce exaggerated amounts of antiviral and pro-inflammatory mediators. Conversely, in agreement with a very recent study from T. Fujita’s laboratory [20], we characterized the loss-of-function S183I SNP. Interestingly, this natural mutation allowed us to further demonstrate the importance of S183I in the transient complex formation that is required for proper RIG-I signaling. Thus, our findings strongly support the hypothesis that regulation of RIG-I/RIG-I and RIG-I/MAVS association/dissociation constitutes a major checkpoint of this antiviral signaling pathway.

CARDs-containing proteins are members of a large group of the ‘death domain superfamily’, which also include the DD (death domain) subfamily and the DED (death effector domain) subfamily. All these domains have a six-helical bundle (H1–H6) structural fold and mediate homotypic interactions within each domain subfamily [22,33,34]. At the amino acid level, the CARD#2 domain of RIG-I differs from other CARDs (see alignment in Fig. S1), whose molecular structures have been previously determined (e.g. MAVS, Apaf-1, RAIDD, Nod1 [22]). We predicted the CARD#2 three-dimensional structural implications of the S183I SNP. In our model, serine 183 maps to helix H6 (cf. Ref. [34]). In molecular dynamics studies, which allowed to assess protein flexibility, H6 with S183I appeared quite flexible and moved perpendicular to the remaining helices in contrast to H6 with I183 which seemed more rigid. More pertinent, our findings suggest that the replacement of a hydrophilic serine by a hydrophobic isoleucine may alter the flexibility as well as the surface architecture of the CARD#2 domain, in particular the exposure of hydrophobic areas. These changes may enhance and/or stabilize hydrophobic interactions in H6, critical for CARD-CARD interactions between RIG-I per se as well as with MAVS. This hypothesis is consistent with studies that have established that hydrophobic residues put constraints on the relative orientations of protein helices [35]; this process being critical for CARD-CARD complex structures [22,34,36]. While additional structural studies outside the scope of this work will be necessary to fully uncover the structural impact of the S183I mutation, our in silico analysis points to a potential impact on the basis of helical packing in the RIG-I CARD#2 domain.

Collectively, on the basis of the data presented here, we consider that serine 183 residue plays a central role in the molecular ordering that leads to RIG-I-mediated NF-κB and IRF-3 activation pathways. Nevertheless, one can wonder how S183I SNP inhibits RIG-I-induced signaling pathways despite its enhancing effect on RIG-I complexes formation. Based on our biochemical assays and structural modeling showing that this mutation does affect hydrophobicity and flexibility of the CARD#2 domain of RIG-I but does not influence its ligand binding activity, we hypothesize that S183I rather induces an abortive conformation of RIG-I, rendering it incapable of downstream signaling. Concerning the inhibitory effect of S183I on RIG-I/MAVS-dependent signal transduction, a recent study clearly supports the concept that MAVS association with RIG-I is not per se sufficient for inducing immune gene expression [37]. Thus, a splicing form of MAVS called MAVS 1a, which shares little sequence similarity with WT MAVS but still contains CARD domain as well as a TRAF-binding motif, can interact strongly with RIG-I but cannot trigger cell signaling. Therefore, like S183I, expression of MAVS 1a interferes with the formation of
productivity of RIG-I/MAVS signaling complexes, which likely contributes to its inhibitory outcome.

Elucidating the functional role of RIG-I SNPs may enhance our understanding of the pathogenesis of viral infections, to ultimately decrease morbidity and mortality through improved risk assessment and early administration of prophylactic therapies [13,29].

Clinical studies assessing S183I SNP frequency in control healthy individuals and patients infected with viruses will certainly clarify the contribution of RIG-I variation to the pathogenesis of viral diseases. Likewise, investigating the clinical relevance of the potent immunostimulatory PRRs ds SNP may be particularly interesting in patients with autoimmune diseases where cytokines play a pivotal pathogenic role. Among them, evidence linking IFN-α/β with the pathogenesis of lupus and insulin-dependent diabetes mellitus in humans are the most convincing [30].

Meanwhile, our study demonstrates that serine 183 is a pivotal residue involved in communication between CARD modules of RIG-I themselves as well as with MAVS and emphasizes the complexity of molecular events that governs RIG-I-induced antiviral signaling.

Materials and Methods

Viruses and reagents

Influenza/A/Scotland/20/74 (H3N2) virus was prepared as previously described [39]. Sendai virus (Cantell strain, ATCC VR-907 Paramyxovirus 1) was a kind gift of E. Meurs (Institut Pasteur, Paris, France). The viral dsRNA mimetic polyinosinic:polycytidylic acid (poly(I:C)) and phosphor 12-mristate 13-acetate (PMA) were from Sigma. Human recombinant TNFα was purchased from Peprotech.

Phylogenetic analysis of RIG-I SNPs

RIG-I SNPs were as described in NCBI’s SNP database (cf. http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId = 23586).

Plasmids construction and site-directed mutagenesis

The pEFBOS(+)−Flag-RIG-I (amino acids 2–925) or 2CARD (amino acids 2–229) vectors were previously described [17] and pcDNA3-Flag-MAVS and pcI-V5-WT 2CARD plasmids were a kind gift of Dr. Z. Chen and Dr. E. Meurs, respectively. SNPs containing plasmids were made using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene). Sequences of oligonucleotides used for mutagenesis are indicated in Table S1. An Site-Directed Mutagenesis kit (Stratagene). Sequences of oligonucleotides used for mutagenesis are indicated in Table S1. An Site-Directed Mutagenesis kit (Stratagene). Sequences of oligonucleotides used for mutagenesis are indicated in Table S1.

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a final elongation step at 68°C for 7 min. After PCR, template digestion by DpnI restriction enzyme and transformation of bacteria were performed according to manufacturer’s instructions. We thank Dr. Z. Chen (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. E. Meurs (Institut Pasteur, Paris, France) for kindly providing the pcDNA3-Flag-MAVS and pCI-V5-2CARDWT plasmids, respectively. Dr. J. Hiscott (McGill University, Montreal, Canada) generously provided the IFN-β promoter and IRF-3 luciferase reporter plasmid. We thank Dr. A. Sakuntabhai (Institut Pasteur) for his interest in our study.

Author Contributions
Conceived and designed the experiments: JP MST. Performed the experiments: JP AB AK BS ANW. Analyzed the data: JP AB AK GC. Wrote the paper: JP AK ANW MST.

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