Essential Role of the N-terminal Domain in the Regulation of RIG-I ATPase Activity*

Received for publication, August 14, 2007, and in revised form, February 5, 2008. Published, JBC Papers in Press, February 11, 2008, DOI 10.1074/jbc.M706777200

Peter Gee, Pong Kian Chua, Jirair Gevorkyan, Klaus Klumpp, Isabel Najera, David C. Swinney, and Jerome Deval

From Roche Palo Alto LLC, Palo Alto, California 94304

Retinoic acid-inducible gene I (RIG-I) is a cytosolic receptor that recognizes viral RNA and activates the interferon-mediated innate antiviral response. To understand the mechanism of signal activation at the receptor level, we cloned, expressed, and purified human RIG-I containing the two caspase activation and recruitment domains (CARDs) followed by the C-terminal helicase domain. We found that recombinant RIG-I is a functional protein that interacts with double-stranded RNA with substantially higher affinity as compared with single-stranded RNA structures unless they contain a 5′-triphosphate group. Viral RNA binding to RIG-I stimulates the velocity of ATP hydrolysis by 33-fold, which at the cellular level translates into a 43-fold increase of interferon-β expression. In contrast, the isolated ATPase/helicase domain is constitutively activated while also retaining its RNA ligand binding properties. These results support the recent model by which RIG-I signaling is autoinhibited in the absence of RNA by intra-molecular interactions between the CARDs and the C terminus. Based on pH profile and metal ion dependence experiments, we propose that the active site of RIG-I cannot efficiently accommodate divalent cations under the RNA-free repressed conformation. Overall, these results show a direct correlation between RNA binding and ATPase enzymatic function leading to signal transduction and suggest that a tight control of ATPase activity by the CARDs prevents RIG-I signaling in the absence of viral RNA.

Pathogen recognition receptors constitute one of the first lines of human host cell defense against viral infections. Among them, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) helicases are specialized sensors of viral nucleic acid. RIG-I was shown to be involved in the recognition of Sendai virus, Hepatitis C virus (HCV), and vesicular stomatitis virus, whereas MDA-5 mediates the antiviral response to picornaviruses (1–3). Both RIG-I and MDA-5 are modular proteins of ~920 residues that contain an ATPase/helicase domain for RNA binding and two caspase activation and recruitment domains (CARDs) located in tandem at the N-terminal end of the protein (4). The CARDs, by way of homotypic interactions, are required for the signaling function between RIG-I and the newly discovered interferon-β promoter stimulator-1 (IPS-1), also called Cardif, MAVS, and VISA (for review see Ref. 5). The latter serves as an intermediate to the cascade leading to the activation of NF-κB and IRF-3/7, which results in the production of antiviral cytokines such as type-1 interferons (6–9). The receptor function of RIG-I is non-redundant and therefore essential to coordinate the signaling cascade leading to antiviral response (4), as confirmed by knock-out studies (10). Notably, the HuH7.5 cell line of hepatocytes is particularly permissive to HCV and vesicular stomatitis virus infection as the result of a defective RIG-I protein bearing a single mutation in its first CARD (11); the antiviral signaling pathway can be restored by overexpressing wild-type RIG-I (12). Viruses have found ways to avoid the RIG-I-mediated antiviral response. For instance, the NS3/4A protease of HCV cleaves IPS-1 to prevent its interaction with RIG-I (9, 13–15). Negative regulation of RIG-I signaling can also be mediated by cellular factors as a way to regulate uncontrolled production of interferon (IFN). Among them, LGP2 prevents the activation of RIG-I by sequestering its double-stranded RNA ligand (16), whereas RNF125 induces the ubiquitination of RIG-I (17).

The molecular mechanism of activation of RIG-I in response to RNA binding is still poorly understood. Although overexpression of the isolated CARDs induces a constitutive signaling independent from viral infection, mutagenesis experiments demonstrated that full-length RIG-I requires a functional ATPase to activate the response leading to IFN production (4). More recently, it has been proposed that RIG-I signaling is intrinsically inhibited by intramolecular interactions between the CARDs and the last 190 amino acids from the C terminus, named the repressor domain (18). According to this new model, latent RIG-I must undergo a conformational change and self-associate in order to activate IPS-1-mediated response. The present study was conducted to reconcile these independent observations and to establish a link between RNA binding, ATPase activity, and CARD-mediated signal transduction leading to IFN expression. Using enzyme kinetics combined with signal transduction assays, we discovered that the ATPase function of RIG-I is negatively regulated by the CARDs in the absence of RNA, which reveals the pivotal role of the ATPase/helicase domain in the modulation of RIG-I signaling.

EXPERIMENTAL PROCEDURES

RNA Methods—Short RNAs were either transcribed from DNA oligonucleotides by using the T7 MEGASHortscript (Ambion) as recommended by the manufacturer or chemically...
synthesized (IDT). If used in double-stranded context, synthetic ssRNA (5'-GAGUCAGCAGCUUGUAAAAGA-3') was annealed to reverse ssRNA (3'-CUCAGUGUCAGUACAUUUUCUUU-5'). Triphosphorylated ssRNA was obtained in vitro from the DNA oligonucleotide TR10P1-FOR (5'-AATTATA-TACGACCTAGTACAGTACGACGTTGTAAAGAA-3') annealed with the reverse complement strand. Radiolabeling of in vitro transcribed RNA was performed with T4 RNA ligase 1 (New England Biolabs) and [32P]pCp (GE Healthcare), while T4 PNK (Invitrogen) was used for 5'-end labeling of synthetic RNA in the presence of [32P]ATP (GE Healthcare). All enzymes were subsequently heat-inactivated, and radiolabeled material was further purified with G-25 columns (GE Healthcare). The cIRES RNA was synthesized with the T7 Megascript kit (Ambion) as previously described (19). Poly(U) and poly(I/C) were purchased from GE Healthcare.

**Protein Expression and Purification**—The wild-type cDNA clone encoding human RIG-I (GenBank™ accession number AF038963) was purchased from Origene Technologies Inc. (Rockville, MD). Full-length and different truncated versions of the gene were subcloned to a modified pET11 vector containing 10 histidine codons in-frame at the 5'-end of the cloning site. K270A and D372A mutants were generated by site-directed mutagenesis with QuikChange II (Stratagene), and sequence changes were controlled on both strands. Recombinant RIG-I was generated in *Escherichia coli* expression system (BL21DE3), with induction started with 0.25 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 25 °C after a 3-h cold shock at 4 °C. Cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 10% glycerol, 500 mM NaCl, 0.2% Nonidet P-40, and 10 mM imidazole), sonicated, and clarified by centrifugation at 13,000 × g for 30 min. The supernatant was incubated with Ni2+/agarose affinity chromatography (MP Biomedicals, LLC) and finally dialyzed overnight against storage buffer (30 mM HEPES, pH 7.4, 40% glycerol, 300 mM NaCl, 0.13% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA). Protein samples were quantified, aliquoted, and stored at −80 °C.

**ATPase Activity**—Unless indicated, all ATPase assays were performed in the helicase buffer (25 mM Tris, pH 7.4, 3 mM dithiothreitol) in the presence of 2 mM ATP, 3 mM MgCl2. We used either the 25 nM RIG-I domain or 225 nM full-length RIG-I in a 20-μL reaction at 37 °C for various times, typically ranging from 5 to 90 min. Reaction samples were stopped by rapid dilution (20-fold) in acidic malachite green solution (Bioassay Systems) supplemented with 10 mM EDTA and incubated for 15 min, and the absorbance was determined at 650 nm. All kinetic data were analyzed using the software PRISM (GraphPad Software Inc.). At saturating RNA concentrations, activation coefficients were calculated under Michaelis-Menten conditions as follows: $K_m(R) = \alpha K_m(R)$, and $k_{cat}(R) = \beta k_{cat}(R)$.

**RNA Binding**—Purified RIG-I or RIG-Ih was incubated at different concentrations with radiolabeled RNA (4 nM) for 15 min at 22 °C in helicase buffer before adding the Novex high density buffer (Invitrogen). Complexes were resolved on a 6% acrylamide Tris borate-EDTA non-denaturing gel (Invitrogen) after a 30-min migration at 22 °C, visualized, and quantified by phosphorimaging. The best fit of titration curves were generated with a cooperative binding model using the following sigmoidal equation, as previously described (20), $Y = Y_g + Y_{max}(R^n/R^n + K_{diss}^{-1/2})$, where $Y$ is the fraction of bound RNA, $Y_g$ is the background signal, $Y_{max}$ is the maximum fraction of bound RNA, $R$ is the concentration of RIG-I monomer, $K_{diss}$ is the dissociation constant, and $N$ is the Hill coefficient.

**Cell Lines and Cell Culture**—All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with Glutamax™, 100 mg/ml sodium pyruvate, and 5% (v/v) fetal bovine serum. HSG Neo L1+ cell line (Apath LLC, St. Louis, MO) are stably transfected with a genotype 1a HCV subgenomic replicon and are a derivative of the Huh7.5 cell line. The replicon was cured from the HSG Neo L1+ cell line, resulting in the cell line HSG7.5. To confirm the presence of the T551 substitution in RIG-I, cDNA was generated from the HSG7.5 cells. The RIG-I coding region was amplified using the following primer pairs, (S)5'-GTCCGGCCCTCAT-TTCCTCGGAAAATC-3', (AS)5'-GGTACAAGGATCC-ATGATTATACCCACTATGTTTG-3', and sequenced using ABI 3730xl DNA Analyzer.

**Cell Stimulation Assays**—Cells were transfected with 0.5 μg of His-tagged wild-type, K270A, or D372A mutant RIG-I (or empty vector as a control) cloned into pDEST26 (Invitrogen) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Cell culture medium was replaced 4 h post-
transfection. Twenty-four hours post-transfection, cells were transfected with in vitro transcribed 5′-phosphorylated cIRES RNA or lipid (mock), and 24 h later total cellular RNA was extracted. Total RNA was isolated using versaGene 96 RNA purification kit (Gentra Systems, Inc.) according to the manufacturer’s instructions. Reverse transcription was carried out in a 10-µl reaction using 2 µl of total cellular RNA (Taqman Reverse transcriptase reagents; Applied Biosystems). To quantitate the level of IFN-β and RIG-I gene expression, 4 µl of cDNA (diluted 1:4 in H₂O) was amplified in a 20-µl reaction consisting of Taqman Universal PCR mix (Applied Biosystems) with the following sets of primers and probe (6 µM each) (fluorogenic probe labeled with FAM, IFN-β: 5′-CAAGCCTCCATTCATTGCAGCAG-3′; forward primer, 5′-CAGGAACCTTGCTCAGAAGC-3′; reverse primer, 5′-TCATCTCCTTGAGGCTAGA-3′; fluorogenic probe labeled with FAM, RPS-9: 5′-ACCCCGGAGACCTTTCGC-3′; forward primer, 5′-GACTGTTGTTTGTCCCAA-3′; reverse primer, 5′-GCTTGGTGTCGAGACTGAGATT-3′) using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems). The human RIG-I primer and probe set was obtained from Applied Biosystems. Gene expression levels were normalized to endogenous gene RPS-9. Changes in gene expression after transfection with wild-type or mutant RIG-I in the presence of cIRES RNA were expressed as a ratio to the levels observed in vector-only transfected cells, set arbitrarily as 1.

RESULTS

The ATPase Activity of Latent RIG-I Is Autoinhibited—We expressed and purified the full-length human RIG-I containing the two CARDs responsible for signal transduction and the helicase/ATPase domain serving as a RNA ligand receptor (Fig. 1A). The ATPase activity of RIG-I was monitored by following the steady-state formation of inorganic phosphate (Pi) over time with increasing concentrations of substrate. The same reaction was also conducted in the presence of RNA mimicking a virus-induced stimulus. The partially double-stranded cIRES sequence was chosen for this purpose because it contains the hairpin loops within the 5′-untranslated region of the HCV genome. Under these conditions, we observed that the catalytic efficiency (kcat/Km) of the reaction increased from 15.8 to 223 min⁻¹ when 3P-ssRNA (Fig. 1B). This RNA-induced activation was essentially caused by a 33-fold higher kcat (7.6 to 253 min⁻¹) with without major changes in ATP binding. We next investigated how various RNA structures can influence the activation state of RIG-I. The basal ATPase activity of RIG-I was also increased in the presence of homopolymeric poly(U/C) but remained unchanged with ssRNA poly(U), suggesting that the ATPase domain is activated after binding to double-stranded RNA (dsRNA) or secondary structures of ssRNA in hairpin loops (Fig. 1C). We also assessed the selectivity of NTP usage. In the absence of RNA, RIG-I showed little difference in catalysis between nucleotides (Fig. 1D). In comparison, the nucleoprotein complex had a marked preference for the adenine base, as judged by the 30- and 29-fold increase in catalysis of ATP and dATP, respectively. Together, these results show that purified RIG-lisafunctionalRNAreceptorcoupledwithinRNAstructure-dependent ATPase. In the absence of ligand, the ATPase activity of RIG-I is autoinhibited.

Efficiency of RNA Binding Modulates RIG-I Activation—We decided to use short RNA sequences to establish a mechanistic link between ligand binding and ATPase activity. We characterized the RNA binding properties of RIG-I by developing an electromobility shift assay (EMSA). Increasing amounts of protein were incubated with radiolabeled short synthetic RNA for 15 min at room temperature, and the resulting complexes were submitted to a native PAGE. Under these conditions, RIG-I was able to bind to dsRNA (K1/2 = 26.4 ± 1.3 nM) significantly
FIGURE 3. Functional assignment and characterization of RIG-I helicase domain. A, ClustalW sequence alignment of RIG-I and LGP2 around the N terminus of the helicase domain. Three constructs of the helicase domain were generated to evaluate the role of the linker region around position 218. The boxes at positions 270 and 372 represent the conserved residues subject to site-directed mutagenesis. B, hydrophobic cluster analysis of the linker sequence with a predicted disordered region devoid of hydrophobic clusters underlined in blue (40). Stars represent prolines, and diamonds are for glycines. Open and dotted boxes are for threonines and serines, respectively. Hydrophobic residues are encircled in clusters and represent regions likely to be structured. C, SDS-PAGE analysis by Coomassie stain of purified proteins. M, molecular weight marker; 239, RIG_239 (RIG-Ih); 218, RIG_218; 201, RIG_201; 1, RIG-I_1 (RIG-I). D, ATP concentration-dependence curve of the velocity of Pi formation in the absence of RNA ligand. The catalytic efficiency was calculated as the ratio of $k_{cat}/K_m$: 552 mM$^{-1}$ min$^{-1}$ for RIG_201, 861 mM$^{-1}$ min$^{-1}$ for RIG_218, and 589 mM$^{-1}$ min$^{-1}$ for RIG_239. All values are the mean ± S.D. of at least three experiments.
Activation of RIG-I

A

![Graph](image)

B

![Graph](image)

C

![Graph](image)

FIGURE 4. Effect of RNA binding on RIG-Ih ATPase activity. A, effect of polynucleotide binding on the rate of ATPase activity compared to poly(U), poly(I/C), and cIRES. All values are the mean ± S.D. of at least three experiments. B, EMSA of synthetic ssRNA and dsRNA (4 nM) incubated at 22 °C in the presence of increasing concentrations of RIG-I: lanes 0–7, 0, 1.25, 2.5, 5, 10, 20, 40, 80 nM. Lane C is used in the right panel (dsRNA) as a migration size control containing ssRNA with no enzyme. The arrow indicates the position of the wells. Curve fitting yielded a $K_{1/2}$ of 16.7 ± 0.7 nM and a Hill coefficient of 2.4 for dsRNA. Dotted lines represent unshifted data set when bound product was <50% of input RNA. C, RNA-free NTPase activity of RIG-Ih in the presence of ATP, GTP, CTP, UTP, and DATP. Experiments were performed similarly as A, using saturating NTP concentrations. All values are the mean ± S.D. of at least three experiments.

better than to ssRNA (<50% of input RNA bound at 320 nM RIG-I) (Fig. 2, A and B). The formation of high molecular weight complexes involving more than one protein/molecule of dsRNA was consistent with a cooperative binding model yielding a Hill coefficient $n = 3.5$. We also evaluated the contribution of the triphosphate moiety to ssRNA binding, as it was recently reported to play an important role in RIG-I activation (1, 3). A 21-mer oligonucleotide transcribed in vitro by T7 RNA polymerase was incubated with the helicase prior to electrophoresis. As a result, the presence of the 5′-triphosphate group on ssRNA increased the stability of the nucleoprotein complex ($K_{1/2} = 131 ± 17.8$ nM, Fig. 2B) as compared with ssRNA. Overall, we discovered that RIG-I displays a selective profile of polynucleotide binding with a preference for RNA over DNA as follows: dsRNA > 3P-ssRNA > ssRNA > ssDNA = dsDNA. This binding property is consistent with the differentiated stimulation profile for these short oligonucleotides on ATPase activity, with a maximum increase of ~40-fold observed with dsRNA (Fig. 2C). It also confirms previous independent observations suggesting that RIG-I does not functionally interact with DNA (4, 12).

The Truncated RIG-Ih Domain Is Constitutively Activated—We assigned the boundaries of the helicase domain of RIG-I by aligning its amino acid sequence against the one of LGP2 sharing 30% identity (Fig. 3A). The linker region is highly acidic and predicted to be disordered around position 230 (Fig. 3B); it could therefore contribute to the functional integrity of RIG-I and/or the regulation of its signaling. To test these possibilities, we decided to express and purify three constructs of the helicase domain containing different parts of the linker region (Fig. 3C). All three truncated enzymes displayed similar catalytic efficiency between 552 and 861 min$^{-1}$ (Fig. 3D). We concluded that the linker does not regulate the enzymatic function of the helicase domain and decided to use the RIG_239 construct exclusively throughout the rest of this study, referred to as RIG-Ih.

We also looked at the effect of RNA on ATPase activity. To our surprise, none of the polynucleotides tested was able to stimulate the activity of the isolated helicase domain (Fig. 4A). To ensure that this defect in regulation was not caused by an impaired RNA binding function, we measured the formation of the nucleoprotein complex by EMSA. The isolated RIG-Ih domain showed the same selective RNA binding properties as the full-length enzyme (Fig. 4B). Additionally, the rates and selectivity profile of nucleotide accommodation in the absence of RNA were similar to the ones of the activated full-length RIG-I (Fig. 4C). Collectively, these results provide convincing evidence that the helicase domain is constitutively activated in the absence of the CARDs.

ATPase Activity Is Important for cIRES-mediated Activation of IFN-β—Walker A and B motifs in helicases are a signature of nucleotide binding. We decided to investigate by mutagenesis the contribution of each of these conserved sequences to the ATPase activity of RIG-I (Fig. 3A). The resulting proteins containing a mutation in Walker A (K270A) or in Walker B (D372A) could still bind to RNA but displayed reduced enzymatic activity compared with wild-type RIG-I under RNA activation conditions (Fig. 5A). The same results were observed with the truncated proteins (data not shown), which highlights the importance of each of the two conserved residues for ATPase activity. To explore the biological relevance of our findings at the cellular level, we measured IFN-β gene expression in hepatocytes as a surrogate for RIG-I activation. Transfection of cIRES RNA induced a 340-fold increase in IFN-β gene expression in Huh7 cells (Fig. 5B). The specific induction of IFN-β was also confirmed by Taqman analysis of IFN-inducible gene IFI-6 expression levels that showed induction only in cells that
We present the first detailed enzymatic characterization of human recombinant RIG-I and show that the CARDs play an essential role in the regulation of the ATPase domain. Both full-length enzyme and the isolated helicase domain are functional proteins that can be purified after being expressed in E. coli. Our experiments using these two forms show that RIG-I prefers ATP over the other ribonucleotides. This selective activity is largely conserved among SF2 helicases (27, 28) and can be inhibited by mutating the Lys-270 and Asp-372 within the conserved Walker A and B motifs. Importantly, our results show that recombinant RIG-I is also a selective RNA-binding protein. Using a protein shift assay (EMSA), we determined the equilibrium binding constants for different types of small RNAs in the nucleophilic attack only in the RNA-stimulated reaction. Consistently, divalent metal ion-chelating agents, such as EDTA, do not alter the basal rate of formation of $P_i$ by RIG-I, which excludes the possibility of contaminating metal ions (Fig. 6C). The reaction is specific to RIG-I as the rate remains sensitive to D372A mutation. This is confirmed by the fact that other agents, such as NaCl, can equally disrupt the basal ATPase activity of RIG-I and RIG-Ih domain (Fig. 6D). We also looked at the effect of pH on catalysis. It has been previously shown with other helicases that acidic conditions decrease the binding affinity of the enzyme to magnesium (24), resulting in a general loss of activity (25, 26). In our experiments, the ATPase activity of the isolated RIG-Ih domain was consistently sensitive to low pH in all tested conditions and peaked between pH 7.0 and 7.5 (Fig. 6E). On the other hand, the basal activity of full-length RIG-I was not affected by pH ranging from 5.5 to 8.0 and only became sensitive to acidic conditions once bound to RNA (Fig. 6F). This suggests that the level of protonation of polar residues from the active site might affect the binding of magnesium to RIG-I, which, in turn, selectively regulates the catalytic rate of the RNA-stimulated reaction but does not participate in the basal ATPase reaction. A likely interpretation of this observation is that RNA binding induces a conformational change of the active site of RIG-I in order to place the catalytic residues in a position similar to the ones of the intrinsically activated helicase domain.

**DISCUSSION**

The Basal ATPase Activity of RIG-I Is Independent of Magnesium—Most helicases require metal ions to coordinate the $\beta$ and $\gamma$ phosphates of ATP before the nucleophilic attack by a water molecule. Nevertheless, there are a few reports of related enzymes with magnesium-independent ATPase activity (21–23). Our ATPase reactions were routinely conducted with 3 mM magnesium. This concentration allows RIG-Ih to achieve efficient catalysis whether or not it is bound to RNA (Fig. 6A). However, we discovered that full-length RIG-I does not require magnesium for its basal ATPase activity (Fig. 6B). Instead, the metal ion participates in the nucleophilic attack only in the RNA-stimulated reaction. Consistently, divalent metal ion-chelating agents, such as EDTA, do not alter the basal rate of formation of $P_i$ by RIG-I, which excludes the possibility of contaminating metal ions (Fig. 6C). The reaction is specific to RIG-I as the rate remains sensitive to D372A mutation. This is confirmed by the fact that other agents, such as NaCl, can equally disrupt the basal ATPase activity of RIG-I and RIG-Ih domain (Fig. 6D). We also looked at the effect of pH on catalysis. It has been previously shown with other helicases that acidic conditions decrease the binding affinity of the enzyme to magnesium (24), resulting in a general loss of activity (25, 26). In our experiments, the ATPase activity of the isolated RIG-Ih domain was consistently sensitive to low pH in all tested conditions and peaked between pH 7.0 and 7.5 (Fig. 6E). On the other hand, the basal activity of full-length RIG-I was not affected by pH ranging from 5.5 to 8.0 and only became sensitive to acidic conditions once bound to RNA (Fig. 6F). This suggests that the level of protonation of polar residues from the active site might affect the binding of magnesium to RIG-I, which, in turn, selectively regulates the catalytic rate of the RNA-stimulated reaction but does not participate in the basal ATPase reaction. A likely interpretation of this observation is that RNA binding induces a conformational change of the active site of RIG-I in order to place the catalytic residues in a position similar to the ones of the intrinsically activated helicase domain.

**DISCUSSION**

The Basal ATPase Activity of RIG-I Is Independent of Magnesium—Most helicases require metal ions to coordinate the $\beta$ and $\gamma$ phosphates of ATP before the nucleophilic attack by a water molecule. Nevertheless, there are a few reports of related enzymes with magnesium-independent ATPase activity (21–23). Our ATPase reactions were routinely conducted with 3 mM magnesium. This concentration allows RIG-Ih to achieve efficient catalysis whether or not it is bound to RNA (Fig. 6A). However, we discovered that full-length RIG-I does not require magnesium for its basal ATPase activity (Fig. 6B). Instead, the metal ion participates in the nucleophilic attack only in the RNA-stimulated reaction. Consistently, divalent metal ion-chelating agents, such as EDTA, do not alter the basal rate of formation of $P_i$ by RIG-I, which excludes the possibility of contaminating metal ions (Fig. 6C). The reaction is specific to RIG-I as the rate remains sensitive to D372A mutation. This is confirmed by the fact that other agents, such as NaCl, can equally disrupt the basal ATPase activity of RIG-I and RIG-Ih domain (Fig. 6D). We also looked at the effect of pH on catalysis. It has been previously shown with other helicases that acidic conditions decrease the binding affinity of the enzyme to magnesium (24), resulting in a general loss of activity (25, 26). In our experiments, the ATPase activity of the isolated RIG-Ih domain was consistently sensitive to low pH in all tested conditions and peaked between pH 7.0 and 7.5 (Fig. 6E). On the other hand, the basal activity of full-length RIG-I was not affected by pH ranging from 5.5 to 8.0 and only became sensitive to acidic conditions once bound to RNA (Fig. 6F). This suggests that the level of protonation of polar residues from the active site might affect the binding of magnesium to RIG-I, which, in turn, selectively regulates the catalytic rate of the RNA-stimulated reaction but does not participate in the basal ATPase reaction. A likely interpretation of this observation is that RNA binding induces a conformational change of the active site of RIG-I in order to place the catalytic residues in a position similar to the ones of the intrinsically activated helicase domain.

**DISCUSSION**

The Basal ATPase Activity of RIG-I Is Independent of Magnesium—Most helicases require metal ions to coordinate the $\beta$ and $\gamma$ phosphates of ATP before the nucleophilic attack by a water molecule. Nevertheless, there are a few reports of related enzymes with magnesium-independent ATPase activity (21–23). Our ATPase reactions were routinely conducted with 3 mM magnesium. This concentration allows RIG-Ih to achieve efficient catalysis whether or not it is bound to RNA (Fig. 6A). However, we discovered that full-length RIG-I does not require magnesium for its basal ATPase activity (Fig. 6B). Instead, the metal ion participates in the nucleophilic attack only in the RNA-stimulated reaction. Consistently, divalent metal ion-chelating agents, such as EDTA, do not alter the basal rate of formation of $P_i$ by RIG-I, which excludes the possibility of contaminating metal ions (Fig. 6C). The reaction is specific to RIG-I as the rate remains sensitive to D372A mutation. This is confirmed by the fact that other agents, such as NaCl, can equally disrupt the basal ATPase activity of RIG-I and RIG-Ih domain (Fig. 6D). We also looked at the effect of pH on catalysis. It has been previously shown with other helicases that acidic conditions decrease the binding affinity of the enzyme to magnesium (24), resulting in a general loss of activity (25, 26). In our experiments, the ATPase activity of the isolated RIG-Ih domain was consistently sensitive to low pH in all tested conditions and peaked between pH 7.0 and 7.5 (Fig. 6E). On the other hand, the basal activity of full-length RIG-I was not affected by pH ranging from 5.5 to 8.0 and only became sensitive to acidic conditions once bound to RNA (Fig. 6F). This suggests that the level of protonation of polar residues from the active site might affect the binding of magnesium to RIG-I, which, in turn, selectively regulates the catalytic rate of the RNA-stimulated reaction but does not participate in the basal ATPase reaction. A likely interpretation of this observation is that RNA binding induces a conformational change of the active site of RIG-I in order to place the catalytic residues in a position similar to the ones of the intrinsically activated helicase domain.

**DISCUSSION**
 Activation of RIG-I

FIGURE 6. Effects of MgCl₂ and pH on RIG-I basal and stimulated activity. A, dependence of ATPase activity of the RIG-Ih domain on MgCl₂ concentration. Values were normalized and expressed in % activity based on maximum rates of Pi formation measured independently with and without cIRES RNA. B, same experiment as in A, with increasing concentrations of MgCl₂ applied to RIG-I. C, full-length RIG-I (225 nM) was incubated with 3 mM MgCl₂ with or without 10 mM EDTA in the presence of 2 mM ATP, and the formation of Pi over time was followed. The same reaction was conducted for up to 135 min with full-length RIG-I containing the D372A mutation. Reactions were quenched by dilution of the samples into the malachite green reagent. D, effect of NaCl on the ATPase activity of full-length RIG-I and RIG-Ih domain. Values were normalized to the maximum rate obtained in the absence of NaCl and expressed in % activity. E, dependence of ATPase activity of RIG-Ih domain on pH. F, same experiment as in E, with full-length RIG-I. All values are the mean ± S.D. of at least three experiments.

Good agreement with new information showing that RIG-I can also discriminate against RNAs that do not contain a free triphosphate on their 5'-end (1, 3) that provides a mechanistic basis for the distinction between self and non-self RNA. On the other hand, RIG-I specifically recognizes small double-stranded structures. From a structural perspective, it is interesting to note that the affinity of RIG-I for dsRNA is ~6-fold higher than for 3P-ssRNA, probably because of the limited number of protein contacts in the latter case.

Our study also demonstrated that RNA binding leads to a conformational change that stimulates the ATPase activity of RIG-I in vitro, with a preference for dsRNA over 3P-ssRNA (Fig. 2C). Consistently, it is known that many double-stranded siRNAs can trigger the induction of IFN expression (29, 30).

One recent study showed that RIG-I itself recognizes 21-mer dsRNAs that do not contain any overhang (31), which is precisely the type of RNA structure used in our biochemical experiments. Unfortunately, our dsRNA was not able to activate IFN expression in transfected cells (data not shown), perhaps due to a lower sensitivity of induction under our conditions. In agreement with other groups, we observed that the long double-stranded poly(I/C) is also a strong activator of RIG-I ATPase activity (4, 12, 32). In contrast, two knock-out studies reported that poly(I/C) instead activates MDA-5 in vivo, which might reflect differences in response between human and mouse cells (2, 33). We found a good correlation between activation of the ATPase activity and induction of IFN expression when an HCV-derived RNA (cIRES) was used as a ligand. This viral sequence of partially dsRNA containing a triphosphate group increased the basal rate of Pi formation by RIG-I up to 33-fold (Fig. 7) without severely changing Kₘ for ATP (α = 2.3). The enzymatic activation translated into a 43-fold increase of IFN expression in hepatocytes stimulated with cIRES (Fig. 5). In summary, our study reveals that “double-strandedness” is, in vitro, a strong molecular marker of activation for purified RIG-I. However, the molecular features for specific binding of RNA to RIG-I in vivo probably involve a combination of dsRNA motifs, the triphosphate group, and/or stem loop structures such as the ones present in HCV RNA.

To our surprise, bound RNA failed to stimulate the ATPase activity of the isolated RIG-Ih domain (Fig. 4A). Although it is difficult to completely rule out that the truncated ATPase/helicase lost its structural integrity due to an artificial N-terminal boundary, we showed that the interdomain connection between the CARDs and the ATPase is dispensable for the enzymatic activity and RNA binding (Figs. 3D and 4B). Consistently, our bioinformatics analysis predicted that RIG-I is a modular protein with two functional domains linked by a disordered region. Therefore, we interpreted this lack of ligand-induced regulation of enzymatic activity as an intrinsic activation of the helicase domain in the absence of the CARDs. Indeed, the basal activity and the nucleotide selectivity of the helicase domain alone are comparable with the activity of full-length RIG-I after RNA stimulation (Figs. 1D and 4C). This result is particularly interesting for two reasons. First, it makes the link between two earlier findings that show (i) that ATPase activity is essential for the signaling function of RIG-I (4), and (ii) that the C terminus part of RIG-I represses interactions with
Likewise, the formation of the RIG-I-nucleotide complex about three turnovers of ATP hydrolysis per unwinding step. One example is given by HCV NS3 helicase, which requires catalytic changes needed to separate strands of nucleic acid. 

Mutational changes needed to separate strands of nucleic acid. 

The intramolecular interaction between the CARDs (C) and the repressor domain (r) of the helicase domain (green) maintain RIG-I in an inactive conformation (R, black star). After RNA ligand (L) binding, RIG-I undergoes a first conformational change (R’) that activates the ATPase function at the catalytic step (β = 33) by reorganizing the active site (yellow star). RNA might also serve as a scaffold for self-association of RIG-I (n). In a second conformational and/or oligomerization step (R”), hydrolysis of ATP is believed to generate the energy required to facilitate the interaction between RIG-I and IPS-1. 

The current autoinhibition model for the control of RIG-I signaling is reminiscent of the regulation of another nucleic acid sensor, the RNA-dependent protein kinase (PKR). In this case, PKR exists predominantly in a latent (or repressed) form (13). RNA binding induces a conformational change that relieves the autoinhibition; however, the validity of this mechanism is still being debated (35). Others have recently reported that dimerization of PKR is required for signal transduction (36). Similarly, multimerization of RIG-I is likely to play an important role in activation of the signaling functions (18). We observed in our EMSA the formation of high molecular weight complexes involving more than one protein/molecule of RNA. These results certainly argue in favor of an oligomerization of RIG-I after RNA binding, although preliminary cross-linking experiments suggest that unliganded RIG-I can also form dimers (data not shown). Also, the titration curves for RNA binding do not fit properly with a single hyperbolic function but would instead involve a cooperative binding model. Interestingly, cooperativity seems to be more pronounced with full-length RIG-I than with the isolated helicase domain as judged by the Hill coefficient and the formation of slowly migrating complexes, suggesting a participation of the CARDs in protein-protein interactions (Figs. 2A and 4B). The direct role of these conserved domains in the autoregulation of unrelated proteins such as Apaf-1 and NOD-1 has already been described (37–39), and it would not be surprising if the level of oligomerization of RIG-I also controls its capacity to interact with IPS-1. Again, identifying which residues are involved in interdomain communication and multimerization of RIG-I will help to define the molecular mechanisms that govern the multiple interconnected functions of this protein, i.e. ligand binding, ATPase activity, and signal transduction. In conclusion, we provide the first detailed enzymatic characterization of RIG-I and report that RIG-I ATPase activity is autoinhibited by the CARDs. We also show that particular structures of RNA can release this inhibition and potentiate signal transduction to downstream effectors. The stability of the RIG-I-RNA complex maximizes the activation of the ATPase function, which makes RIG-I a sensitive sensor of non-self RNA inside infected cells.

Activation of RIG-I

**FIGURE 7. Model for the activation of RIG-I signaling.** The intramolecular interaction between the CARDs (C) and the repressor domain (r) of the helicase domain (green) maintain RIG-I in an inactive conformation (R, black star). After RNA ligand (L) binding, RIG-I undergoes a first conformational change (R’) that activates the ATPase function at the catalytic step (β = 33) by reorganizing the active site (yellow star). RNA might also serve as a scaffold for self-association of RIG-I (n). In a second conformational and/or oligomerization step (R”), hydrolysis of ATP is believed to generate the energy required to facilitate the interaction between RIG-I and IPS-1. 

The intramolecular interaction between the CARDs (C) and the repressor domain (r) of the helicase domain (green) maintain RIG-I in an inactive conformation (R, black star). After RNA ligand (L) binding, RIG-I undergoes a first conformational change (R’) that activates the ATPase function at the catalytic step (β = 33) by reorganizing the active site (yellow star). RNA might also serve as a scaffold for self-association of RIG-I (n). In a second conformational and/or oligomerization step (R”), hydrolysis of ATP is believed to generate the energy required to facilitate the interaction between RIG-I and IPS-1.

The intramolecular interaction between the CARDs (C) and the repressor domain (r) of the helicase domain (green) maintain RIG-I in an inactive conformation (R, black star). After RNA ligand (L) binding, RIG-I undergoes a first conformational change (R’) that activates the ATPase function at the catalytic step (β = 33) by reorganizing the active site (yellow star). RNA might also serve as a scaffold for self-association of RIG-I (n). In a second conformational and/or oligomerization step (R”), hydrolysis of ATP is believed to generate the energy required to facilitate the interaction between RIG-I and IPS-1.
Activation of RIG-I

REFERENCES

1. Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006) Science 314, 994–997

2. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsu, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimoto, T., Koh, C. S., Reis e Sousa, C., Matsuura, Y., Fujita, T., and Akira, S. (2006) Nature 441, 101–105

3. Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006) Science 314, 997–1001

4. Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., and Akira, S. (2005) Immunity 23, 727–740

5. Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Barten-schlager, R., and Tschopp, J. (2005) Nature 437, 1167–1172

6. Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006) Science 314, 994–997

7. Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z., and Shu, H. B. (2005) J. Virol. 80, 335–345

8. Pan, G., O'Rourke, K., and Dixit, V. M. (1998) J. Biol. Chem. 273, 27415–27419

9. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., and Nunez, G. (1999) J. Biol. Chem. 274, 11474–11486

10. Klumpp, K., Leveque, V., Le Pogam, S., Ma, H., Jiang, W. R., Kang, H., Granycome, C., Singer, M., Laxton, C., Hang, J. Q., Sarma, K., Smith, D. B., Heindl, D., Hobbs, C. J., Merrett, J. H., Symons, J., Cammack, N., Martin, J. A., Devos, R., and Najera, I. (2006) J. Biol. Chem. 281, 3793–3799

11. Marques, J. T., Devosse, T., Wang, D., Zamanii-Daryoush, M., Serbinoiswi, P., Hartmann, R., Fujita, T., Behlke, M. A., and Williams, B. R. (2006) Nat. Biotechnol. 24, 559–565

12. Loz, M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M. A., Garcia-Sastre, A., Katze, M. G., and Gale, M. Jr. (2008) J. Virol. 82, 335–345

13. Gaboriaud, C., Bissery, V., Benchetrit, T., and Biron, P. (1987) FEBS Lett. 224, 149–155