Phosphorylation of RIG-I by Casein Kinase II Inhibits Its Antiviral Response[▽]

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Received 16 August 2010/Accepted 4 November 2010

RIG-I is an intracellular RNA virus sensor that mediates a signaling pathway that triggers the alpha/beta interferon (IFN- α / β) immune defenses. However, the mechanism for regulation of RIG-I activity remains largely unknown. Here we show that RIG-I activity is regulated by phosphorylation and dephosphorylation in its repressor domain (RD). Threonine at amino acid (aa) 770 and serine at aa 854 to 855 of RIG-I are phosphorylated by casein kinase II (CK2) in the resting state of the cell and dephosphorylated when cells are infected by RNA virus. Mutation at aa position 770 or 854 to 855 of RIG-I renders it constitutively active. Pharmacological inhibition of CK2 enhances virus-induced expression of IFN- β and suppresses virus proliferation, while inhibition of phosphatase reduces virus-induced expression of IFN- β . Overexpression of CK2 suppresses RIG-I-mediated signaling, while silencing of CK2 results in the increased suppression of virus proliferation. Our results reveal a novel mechanism of the regulation of RIG-I activity during RNA virus infection.

RIG-I is a recently identified RNA helicase containing two caspase activation and recruitment domains (CARDs). RNA binding and signaling by RIG-I are implicated in host defense against pathogens via stimulation of alpha/beta interferon (IFN- α / β) production and downstream transcription of various antiviral genes (26). In resting cells, RIG-I is maintained as a monomer in an autoinhibited state. Viral infection induces a conformational change in RIG-I that promotes self-association and CARD interactions with the VISA/IPS-1/Cardif/MAVS adaptor protein and triggers IFN regulatory factor 3- and NF- κ B-responsive gene expression (15). The internal repressor domain (RD) controls RIG-I multimerization and interaction with VISA/IPS-1/Cardif/MAVS. Deletion of the RD results in constitutive signaling to the IFN- β promoter, whereas RD expression alone prevents signaling and increases cellular permissiveness to hepatitis C virus (HCV) (16). Further study reveals that the C-terminal regulatory domain RD of RIG-I binds viral RNA in a 5'-triphosphate-dependent manner and activates the RIG-I ATPase by RNA-dependent dimerization. The RD contains a zinc-binding domain that is structurally related to the GDP/GTP exchange factors of Rab-like GTPases (2). However, regulation of RIG-I activity is underexplored, and the mechanism for its activity remains unknown.

Casein kinase II (CK2) is a highly conserved serine-threonine kinase that uses both ATP and GTP as phosphate donors. It is constitutively activated and ubiquitously expressed. It usually presents as a tetrameric holoenzyme complex of two cat-

alytic subunits (alpha and/or alpha') and two regulatory beta subunits, phosphorylates more than 300 substrates, and controls a wide range of processes, including the regulation of the cell cycle, apoptosis, transformation, and circadian rhythm (20). Recent evidences suggest a potentially important role for CK2 in the control of the inflammatory response (11, 13). However, whether CK2 is involved in viral infections has not been studied.

Reversible phosphorylation is an important regulatory mechanism for many biological processes in eukaryotic organisms, especially for signal pathways. The phosphorylation state of a protein is controlled dynamically by both protein kinases and phosphatases. Protein phosphorylation can lead to activation or inactivation of the substrate, depending on its nature. We here report that RIG-I activity is regulated by phosphorylation and dephosphorylation during viral infection.

MATERIALS AND METHODS

Plasmids. Flag- or hemagglutinin (HA)-tagged RIG-I, HA-CARD (amino acids [aa] 1 to 234), Flag- Δ CARD (aa 234 to 956), and HA-MDA5 were kindly provided by Hongbing Shu (Wuhan University, China), Flag-CK2 was a gift from David W. Litchfield (University of Western Ontario, Canada). The JHF1 plasmid used for the HCV replicon was a gift from Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (23).

Antibodies and oligonucleotides. Anti-Flag, anti-HA, anti-phosphoserine, and anti-phosphothreonine antibodies were obtained from Sigma-Aldrich, anti-CK2 α antibody was obtained from Chemicon, and anti-RIG-I antibody was obtained from Alexis Biochemical. Phosphospecific RIG-I antibodies were made by Shanghai Bio-Ferry Biotechnology Co., Ltd. The CK2 α inhibitor DMAT was obtained from Merck, and the phosphatase inhibitor OA (okadaic acid) was obtained from Alexis Biochemical. The following double-stranded oligonucleotides corresponding to the target sequences were purchased from Shanghai GenePharma Co., Ltd: CK2 α -siRNA1, CAAUUGUACCAGACGU UAA; CK2 α -siRNA2, GAUCCACGUUCAAUGAU; and CK2 α -siRNA3, GGAUUUCUUCAGUGCCAA. The negative-control sequence used was UUC UCCGAACGUGUCACGU. The antibodies against phosphopeptide were generated by Shanghai Bio-Ferry Biotechnology Co., Ltd, using synthetic peptides (see Fig. 6A).

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[▽] Published ahead of print on 10 November 2010.

Bioinformatics analysis of the phosphorylation sites at RIG-I. The bioinformatics analysis was performed based on EBI software that can be found at the EMBL-EBI website (<http://www.ebi.ac.uk/Tools/ppsearch/index.html>). The following regions are potential CK2 phosphorylation sites in RIG-I: 162 aa, SDKE; 698 aa, SVAD; 770 aa, TWDE; and 854 aa, SSFE.

In vivo phosphorylation assays. 293T cells were cultured in regular Dulbecco modified Eagle medium (DMEM) for 18 h. The medium was replaced with new DMEM without phosphate for 30 min. These cells were then cultured in phosphate-free DMEM (10% fetal bovine serum) with 0.5 mCi of [32 P]orthophosphate per dish for 8 h and treated with virus or IFN- β . Cells were harvested, and total protein was extracted. Endogenous RIG-I proteins were pulled down by immunoprecipitation with anti-RIG-I antibodies, followed by radioautography.

Kinase assay. His-RIG-I was expressed in BL21(DE3) cells and purified by a Ni-Sepharose 6 Fast Flow column. 293T cells (1×10^8) were seeded in 15-cm culture dishes treated 24 h later with or without Sendai virus for 8 h and then lysed in 3 ml of lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4 , 5 mM β -glycerophosphate at pH 7.5). The lysate was added to 10 μ l of the purified His-RIG-I with Ni beads and incubated for 4 h at 4°C. The beads were centrifuged for 5 min at 500 $\times$ g and washed three times in kinase buffer (25 mM Tris, 10 mM MgCl_2 , 5 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 2 mM dithiothreitol [DTT], 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF at pH 7.6). The beads were resuspended in 30 μ l of kinase buffer containing 100 μ M ATP incubated at 37°C for 30 min. A total of 6 μ l of sample buffer (300 mM Tris-Cl at pH 6.8, 600 mM DTT, 12% sodium dodecyl sulfate [SDS], 0.6% bromophenol blue, 60% glycerol) was added, and the samples were boiled for 5 min. The samples were then run on 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with antibody against phosphoserine and/or phosphothreonine.

In vitro kinase assay. A total of 0.5 μ g of recombinant glutathione S-transferase (GST)-CK2 α and 1 μ g of recombinant His-RIG-I were incubated in 30 μ l of kinase buffer containing 100 μ M unlabeled ATP and 5 μ Ci of [γ - 32 P]ATP and incubated at 37°C for 30 min. A total of 6 μ l of sample buffer (300 mM Tris-Cl at pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol) was added, and samples were boiled for 5 min. The samples were then run on 10% SDS-PAGE. The gel was exposed using X-ray film.

Detection of RIG-I phosphorylation. FLAG-RIG-I was transfected into 293T cells and lysed 24 h later in 1 ml of lysis buffer. A total of 40 μ l of protein A/G plus agarose beads was added to the lysates, and samples were incubated for 4 h at 4°C. The beads were centrifuged at 500 $\times$ g for 5 min, washed three times in lysis buffer, and resuspended in 50 μ l of sample buffer. The samples were run on 10% SDS-PAGE gels, transferred onto a PVDF membrane, and immunoblotted with antibodies against RIG-I and phosphoserine and/or phosphothreonine.

Site-directed mutagenesis of RIG-I. The Muta-Direct kit (SBS Bio, Beijing, China) was used for the preparation of the mutants of the RIG-I gene. The manufacturer's instructions were followed, using primers containing the desired mutations. For the RIG-I S162A mutant, the sense primer used was 5'-TGGAATGCCTTCTCAGAGCAGACAAGGAAAAGTGG-3', and the antisense primer used was 5'-CCAGTTTCTCTGTCTGCTCTGAGAAGGCATTC-3'. For the RIG-I S698A mutant, the sense primer used was 5'-ATTCTGATTGCCACGCGAGTTGCTGATGAAGG-3', and the antisense primer used was 5'-CCTTCATCAGCAACTGCGGTGGCAATCAGAAT-3'. For the RIG-I T770I mutant, the sense primer used was 5'-ATTTTACGCCTTCAGATATGGGACGAAGCAGTA-3', and the antisense primer used was 5'-TACTGCTTCGTCCTCATATCTGAAGGCGTAAAT-3'. For the RIG-I S854G mutant, the sense primer used was 5'-CCCAAGCCAAAGCAGTTTGAGGT TTTGAAAAAGAGCAAAG-3', and the antisense primer used was 5'-CTTTGCTCTTTTTCAAAACCTGCAAACCTGCTTGGCTTGGG-3'.

Luciferase reporter gene assays. HEK293T cells were seeded in 24-well plates at a density of 1×10^5 cells per milliliter. The next day, the cells were transfected with 100 ng of the IFN- β or interferon-stimulated response element (ISRE)-luciferase reporter constructs along with different genes, as indicated in the figures. After 24 h, the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured by using a luminometer. The data were expressed as mean fold stimulations relative to the control levels. Each experiment was performed at least three times, and all statistic data are represented as the means $\pm$ standard deviations (SD).

Coimmunoprecipitation and immunoblot analysis. For transient transfection and coimmunoprecipitation experiments, 293T cells (1×10^6) were transfected with the various genes for 24 h. Transfected cells were lysed in 1 ml of lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF at pH 7.5). For each immunoprecipitation,

total cell extract was incubated with antibody and 20 μ l of a 1:1 slurry of protein A/G PLUS-Agarose (Santa Cruz) at 4°C for 4 h. The beads were then washed three times with 0.5 ml of lysis buffer. The precipitates were separated by SDS-PAGE and transferred to a PVDF membrane for the immunoblot assay. For endogenous coimmunoprecipitation experiments, cells (2×10^8) were lysed in 3 ml of lysis buffer, and a 1.5-ml aliquot of lysate was incubated with specific or control antibody and 20 μ l of a 1:1 slurry of agarose beads. The following procedures were performed as described above.

GST pulldown assay. A total of 10 μ g of recombinant GST-CK2 α was incubated with 5 μ l of glutathione-Sepharose 4B in 1 ml of 0.1 M Tris-Cl and 1 mM PMSF at pH 8.0 at 4°C for 4 h. The beads were centrifuged at 500 $\times$ g for 5 min and washed three times in phosphate-buffered saline (PBS). The beads were resuspended in 1 ml of 0.1 M Tris-HCl, pH 8.0, containing 1 mM PMSF, 10 μ g of bovine serum albumin (BSA), and 2 μ g of His-tagged fragments of RIG-I as indicated and incubated at 4°C for 4 h. The beads were centrifuged at 500 $\times$ g for 5 min and washed three times in PBS to remove nonspecific binding. A total of 30 μ l of 1 $\times$ sample buffer was added, and samples were boiled for 5 min. The samples were then run on 15% SDS-PAGE gel and stained with Coomassie blue after electrophoresis.

RNA isolation and real-time PCR. Total RNA was isolated from 293T cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA (2 μ g) was reverse transcribed using SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) to generate first-strand cDNA. Real-time PCR experiments were performed using a Stratagene Mx3005P real-time thermocycler (Stratagene, La Jolla, CA). SYBR Premix Ex Taq (TaKaRa Bio, Inc.) was used for all reactions with the following specific primers: CK2 α , 5'-C GAGTTGCTTCCCGATAC-3' and 5'-GCTGACAAGGTGCTGATT-3'; actin, 5'-CATGTACGTTGCTATCCAGGC-3' and 5'-CTCCTTAATGTCACGCAC GAT-3'; IFN- β , 5'-TGCTCTCCTGTGTGCTCTCTCC-3' and 5'-CATCTCAT AGATGGTCAATGCGG-3'; HCV, 5'-ATGGCGTTAGTATGAGTGTGTC-3' and 5'-GGCATTTGAGCGGGTGTATC-3'.

RNA interference (RNAi) experiments. Double-stranded oligonucleotides corresponding to the target sequences were purchased from Shanghai GenePharma Co., Ltd. The following sequences were targeted for human CK2 α cDNA: CK2 α -siRNA1, CAUUGUACCAGACGUUAA; CK2 α -siRNA2, GAUCCACGUU UCAAUGAUA; CK2 α -siRNA3, GGGAAUUCUUCAGUGCCAA. The negative-control sequence used was UUCUCCGAACGUGUCACGU.

Multimerization assay. Non-SDS-polyacrylamide gel (7%) was prerun for 30 min at 40 mA, with 25 mM Tris-HCl and 192 mM glycine, pH 8.4, with and without 0.2% deoxycholate (DOC) in the cathode and anode chambers. Samples in the native sample buffer (62.5 mM Tris-Cl, pH 6.8; 15% glycerol) were applied to the gel and electrophoresed at 25 mA for 80 min. Immunoblotting was performed with antibody, as indicated after electrophoresis.

Mass spectrometry. A total of 0.5 μ g of recombinant GST-CK2 α and 1 μ g of the recombinant His-RIG-I RD was incubated in 30 μ l of kinase buffer containing 100 μ M unlabeled ATP and incubated at 37°C for 30 min. The sample was then run on 10% SDS-PAGE. The band containing the His-RIG-I RD was isolated from the gel and digested with trypsin in gel at 37°C for 18 h. The sample was then subjected to mass spectrometry (24).

Virus infection and assay. 293 cells (1×10^5) were transfected with RIG-I or RIG/CK2 for 24 or 36 h prior to vesicular stomatitis virus (VSV) infection (multiplicity of infection [MOI] of 10.0). At 1 h after infection, cells were washed with PBS three times, and then medium was added. The supernatants were harvested 24 h after being washed. The supernatants were diluted from 1:106 and used to infect confluent BHK21 cells cultured on 24-well plates. At 1 h postinfection, supernatant was removed, and 3% methylcellulose was overlaid. The overlay was removed at 3 days postinfection. Cells were then fixed with 4% formaldehyde for 1 h and stained with 0.2% crystal violet in 20% methanol. The number of plaques were counted, averaged, and multiplied by the dilution factor to determine the viral titer as the number of PFU/ml. HCV replicon RNA was transcribed and purified from the respective linearized plasmids, this purified RNA was transfected into Huh7 cell, and 72 h after transfection, culture medium was collected and concentrated using an Amicon Ultra-15 filter (Millipore). The experiment was performed according to the literature (23). Each experiment was performed at least three times. These data were statistically analyzed by the two-tailed Student test, with a *P* value of less than 0.05 considered statistically significant.

RESULTS

Virus infection induces dephosphorylation of RIG-I. We hypothesized that the activity of RIG-I during viral infections is

regulated by phosphorylation and dephosphorylation. To test this, bacterially expressed His-tagged RIG-I was incubated with cellular extracts from cells treated with or without Sendai virus. His-RIG-I was then pulled down with Ni beads and suspended in a buffer containing 100 μ M ATP. We show that RIG-I was highly phosphorylated at both serine and threonine after incubation with resting cells, while in contrast, RIG-I was dephosphorylated after exposure to cells infected by virus (Fig. 1A). These results indicate that RIG-I is phosphorylated in resting cells and dephosphorylated in virus-infected cells. To address whether endogenous RIG-I is regulated in this way, cells were treated by Sendai virus and phosphorylation measured at different time points. Endogenous RIG-I was isolated by immunoprecipitation followed by Western blot analysis of serine and threonine phosphorylation. In resting cells, RIG-I was phosphorylated at serine and threonine and both amino acids were dephosphorylated in virus-infected cells (Fig. 1B). We further show that the activation of pRIG-I/p-Ser/p-Thr occurs in a time-dependent way, with complete dephosphorylation at 6 h postinfection. In cells infected by virus, a decrease of the RIG-I phosphorylation signal was shown to coincide with an increase of IFN- β expression (Fig. 1C), suggesting that virus infection induces dephosphorylation of RIG-I, which leads to the activation of its signaling and results in the expression of IFN- β . Dephosphorylation of RIG-I is a specific response to RNA virus, as no dephosphorylation was observed after infection of the cells with a DNA virus (Fig. 1D). To further identify the dephosphorylation of RIG-I upon RNA virus infection, cells were cultured in the presence of [32 P]orthophosphate, followed by the treatment with Sendai virus, adenovirus (AdV), and IFN- β for 8 h. As shown in Fig. 1E, dephosphorylation of RIG-I was observed only in cells treated with RNA virus and not with AdV and IFN- β .

CK2 inhibits RIG-I signaling and suppresses RIG-I-mediated antiviral response. The protein kinase CK2 is a universal kinase that phosphorylates proteins at Ser/Thr. Bioinformation analysis reveals that RIG-I contains several potential CK2 phosphorylated sites, with most of them located in its RD region. Since the RD of RIG-I has been considered a regulatory domain (2, 15), we therefore addressed whether CK2 is involved in the regulation of RIG-I activity. The CK2 wild type and its mutant, a dominant negative form, CK2 α K68M (22), were cotransfected with RIG-I and analyzed by a reporter assay. We show that wild-type CK2 inhibits RIG-I-mediated activation of ISRE and IFN- β reporters, whereas the CK2 mutant was not effective (Fig. 2A). CK2 did not inhibit VISA/MAVS/IPS-1/Cardif-mediated ISRE activation, nor did CK2 inhibit TRIF-mediated ISRE activation (Fig. 2A). MDA5 is homologous to RIG-I and mediates antiviral signaling similar to that mediated by RIG-I. Our study shows that CK2 did not affect MDA5-mediated activation of ISRE (Fig. 2A), suggesting that CK2 specifically affects RIG-I signaling at the level of RIG-I and not its downstream molecules and signaling pathways. Next, we tested whether CK2 inhibited Sendai virus-induced IFN- β gene expression. 293T cells were transfected with CK2 α/β or CK2 α K68M/ β , followed by virus infection. As shown in Fig. 2B, overexpression of CK2 α/β resulted in a dose-dependent decrease of virus-induced IFN- β , and CK2 α K68M/ β seemed to enhance virus-induced expression of IFN- β .

FIG. 1. Dephosphorylation of RIG-I during virus infection. (A) A kinase assay was performed on purified His-RIG-I and lysates from 293T cells treated with or without Sendai virus, as described in Materials and Methods. (B) Endogenous RIG-I was immunoprecipitated from 293T cells treated with or without Sendai virus for 8 h in the presence of RIG-I antibody and immunoblotted with antiphosphoserine, antiphosphothreonine, and anti-RIG-I. (C, top) 293T cells were treated with SeV for the indicated periods of time, and endogenous RIG-I was immunoprecipitated and immunoblotted with an antiphosphoserine/antiphosphothreonine mixture, anti-RIG-I antibody, and antiactin antibody. (Bottom) Total cellular RNA was extracted and analyzed by real-time PCR for the expression of IFN- β . (D) Endogenous RIG-I was immunoprecipitated from 293T cells treated with or without Sendai virus (SeV) or adenovirus (AdV) for 8 h in the presence of RIG-I antibody and immunoblotted with an antiphosphoserine/antiphosphothreonine mixture and anti-RIG-I antibody. (E) The 293T cells cultured in the presence of [32 P]orthophosphate, as described in Materials and Methods, were stimulated with SeV, AdV, or IFN- β (1,000 IU/ml) for 8 h. Total protein was extracted after treatments. RIG-I was immunoprecipitated with the antibody against RIG-I. The phosphorylation state of RIG-I was viewed by radioautography. Expression of RIG-I and actin was monitored by immunoblotting with antibody against RIG-I and actin, respectively. CT, control; *, result from radioautography.

FIG. 2. CK2 α inhibits RIG-I signaling. (A) 293T cells were transfected with different luciferase constructs or expression plasmids containing the genes indicated. Luciferase assays were performed 18 h after transfection. Values are shown as the means $\pm$ SD from one representative experiment. The results were obtained from three independent experiments. Rel. Luc. Acti., relative luciferase activity. (B) The indicated amounts of CK2 α / β or the CK2 α K68M/ β mutant were transfected into 293T cells and HUVEC for 16 h, respectively. Cells were treated with SeV or AdV for 8 h. Total RNA was extracted and analyzed by real-time PCR for the expression of IFN- β . (C) 293T cells (top) and HUVEC (bottom) were transfected with CK2 α K68M/ β or empty plasmids and then pretreated with increasing concentrations of CK2 inhibitor DMAT for 2 h, followed by SeV or AdV infection for 8 h. Total RNA was extracted and analyzed by real-time PCR for the expression of IFN- β . (D) 293T cells (1×10^5) were transfected with CK2 α (2 μ g) and CK2 β (2 μ g) expression plasmids together with an empty control plasmid (2 μ g) or with CK2 α (2 μ g) and CK2 β (2 μ g) expression plasmids with a RIG-I (2 μ g) expression plasmid. At 24 h after transfection, cells were infected with VSV (MOI of 10), and supernatants were harvested at 24 h postinfection. Supernatants were analyzed for VSV production using standard plaque assays. Plaques were counted, and titers were calculated as the number of PFU/ml. (E) 293T cells were pretreated with the indicated concentrations of CK2 inhibitor DMAT for 2 h, followed by the infection of VSV (MOI of 10) for 24 h. Supernatants were analyzed for VSV production using standard plaque assays. (F, left) 293T cells (1×10^5) were transfected with CK2 α -RNAi (2 μ g/ml) double-strand oligonucleotides and infected with VSV at 36 h after transfection. Supernatants were analyzed for VSV production using standard plaque assays. (Right) Result from Western blotting with specific anti-CK2 α and antiactin antibodies for normalization. All statistic data are represented as the means $\pm$ SD. The results were obtained from three independent experiments.

FIG. 3. CK2α phosphorylates RIG-I. (A) Interactions of endogenous CK2α and RIG-I. Cell lysate from 293T cells was immunoprecipitated with anti-RIG-I antibody or control IgG (mlgG). The immunoprecipitates were analyzed by immunoblotting (IB) with anti-CK2α. The expression levels of the endogenous proteins were analyzed by immunoblotting with anti-RIG-I and anti-CK2α antibodies. Co-IP, coimmunoprecipitation. (B, left) 293 cells (2×10^6) were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with anti-HA antibody after transfection. The immunoprecipitates were then analyzed by anti-HA and anti-Flag antibody. Expression of the ectopic genes was analyzed by immunoblotting with anti-HA and anti-Flag antibodies. (Right) 293 cells were transfected with the indicated plasmids, and cell lysates were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were analyzed by anti-HA and anti-Flag antibodies. Expression of the ectopic genes was analyzed by immunoblotting with anti-HA and anti-Flag antibodies. (C) Different His-tagged fragments of RIG-I were incubated with GST-tagged CK2α containing glutathione-Sepharose 4B beads. The complex was spun down after incubation and subjected to SDS-PAGE as indicated as in Materials and Methods. (D) RIG-I interacts with CK2α through its RD *in vivo*. (Top) Schematic presentation of the domain structure of RIG-I. (Middle) 293T cells were transfected with the indicated plasmids containing genes and different fragments of RIG-I. Total

(Fig. 2B). It might result from the fact that the mutant competes with the action of the wild type of CK2. CK2-suppressed virus-induced expression of IFN- β is RNA virus specific, because CK2 did not affect the cellular response to the DNA virus. Pretreatment of 293T cells and human umbilical vein endothelial cells (HUVEC) with the CK2 inhibitor DMAT, followed by virus infection, led to a dose-dependent increase of IFN- β expression (Fig. 2C). These observations suggest that CK2 inhibits RIG-I signaling. To investigate the downstream consequences, we next tested whether CK2 affects the RIG-I-mediated antiviral response. A virus plaque assay for VSV was used to evaluate the effect of CK2 on virus proliferation. We show that overexpression of CK2 reduced the endogenous cellular antiviral response as well as the RIG-I-mediated antiviral response (Fig. 2D). Pretreatment of cells with a CK2 inhibitor led to the enhancement of the endogenous antiviral response as well as the RIG-I-mediated antiviral response (Fig. 2E). To confirm the role of CK2 in virus proliferation, we knocked down CK2 in cells using small interfering RNA (siRNA). As shown in Fig. 2F, two specific siRNAs were able to effectively silence endogenous CK2. The suppression of virus proliferation was significantly enhanced in CK2-silenced cells with or without overexpressed RIG-I. These data demonstrate that CK2 promotes virus proliferation by the inhibition of RIG-I signaling.

CK2 phosphorylates RIG-I. To investigate if RIG-I is a substrate of CK2, we first tested the interaction of CK2 and RIG-I. Coimmunoprecipitation experiments showed that endogenous CK2 and RIG-I interact with each other (Fig. 3A). Similar results were found for ectopic CK2 and RIG-I (Fig. 3B). Structurally, the N terminus of RIG-I has two CARDs, while the C terminus contains a helicase and RD region (2, 15). To determine which region interacts with CK2, we expressed the His-tagged CARD, helicase domain, and RD of RIG-I in bacteria and incubated these with bacterially expressed GST-CK2. As showed in Fig. 3C, only the RD, and not the CARD and helicase domain, interacted with CK2. RIG-I or its fragments with CK2 were then cotransfected into cells, and coimmunoprecipitation confirmed the interaction between the RD and CK2 (Fig. 3D). To determine if RIG-I is phosphorylated by CK2, Flag-tagged CK2 and HA-tagged RIG-I were cotransfected at different concentrations. Ectopic RIG-I was then pulled down with HA antibody and immunoblotted with antiphosphorylated Ser/Thr. Cotransfection of CK2 dose dependently increased the phosphorylation of RIG-I (Fig. 3E), while pretreatment of the cells with the CK2 inhibitor DMAT decreased RIG-I phosphorylation (Fig. 3F). The phosphorylation of RIG-I by CK2 *in vitro* was also inhibited in the presence of

DMAT (Fig. 3G). These data suggest that RIG-I is the substrate of CK2 in resting cells.

Dephosphorylation of threonine at aa 770 and serine at aa 854 to 855 results in the activation of RIG-I. Based on bioinformation analysis, there are several potential CK2 sites in RIG-I, one site at aa position 162 of the N-terminal region and three in the RD region, including sites at aa 698, aa 770, and aa 854 to 855 (see Materials and Methods). To determine which sites are the targets of CK2, we mutated these sites at either serine or threonine. The activity for these mutants was evaluated by a reporter assay. We show that the protein with the mutation of serine at aa 162 (aa162 mutant) led to the reduction of RIG-I activity (Fig. 4A). On the contrary, all mutations in the RD region led to the enhancement of ISRE activation, except for the aa698 mutant. The activity of these two mutants was similar to that of its active form, the CARD. Forced expression of the mutation at aa 770 and aa 854 to 855 in cells increase the expression of IFN- β , whereas the aa162 mutant led to the inhibition of IFN- β expression and the aa698 mutant did not show an obvious effect (Fig. 4B). We next addressed whether these mutation sites were the phosphorylation sites of CK2. We show that the RD, but not the CARD and helicase domain, is phosphorylated by CK2 when these fragments are incubated with CK2 by the *in vitro* kinase assay (Fig. 4C). Incubation of the aa698, aa770, and aa854 to 855 mutants with CK2 demonstrated the phosphorylation of the aa698 mutant, while the aa770 and aa854 to 855 mutants showed reduced phosphorylation signals under the same condition (Fig. 4D). The aa770 and aa854 to 855 mutants also were not phosphorylated when transfected into cells (Fig. 4E, panels 2 and 3), while the aa698 mutant remained phosphorylated after transfection (Fig. 4E, panel 1). These data demonstrate that dephosphorylation at aa sites 770 and 854 to 855 is associated with RIG-I activation. To confirm this finding, cells were pretreated with the general phosphatase inhibitor OA (okadaic acid), followed by virus infection. We show that inhibition of phosphatase dose dependently decreased virus-induced IFN- β in both 293T cells and HUVEC (Fig. 4F). We next performed mass spectrography to determine the phosphorylation sites by CK2. The purified RIG-I RD was phosphorylated by CK2 *in vitro*, followed by mass spectrometry analysis. The results revealed 2 phosphorylation sites, including threonine 770 and serine 854 to 855 (Fig. 4G).

Amino acids 770 and 854 to 855 are essential for the intermolecular interaction and multimerization of RIG-I. It has been previously suggested that the RD mediates conformational constraints that, through internal interactions, mask the CARDs. During viral infection, RIG-I is activated following

proteins were extracted 18 h after transfection and were immunoprecipitated with anti-CK2 α antibody. The immunoprecipitates were analyzed by immunoblotting with anti-Flag and anti-CK2 α antibodies. (Bottom) Expression of the ectopic genes was analyzed by immunoblotting with anti-CK2 α and anti-Flag antibodies. IgG L, IgG light chain; WT, wild type. (E) 293T cells were transfected with HA-RIG-I and increasing amounts of the Flag-CK2 α plasmid for 24 h. The RIG-I protein was immunoprecipitated using anti-HA antibody and immunoblotted with an antiphosphoserine/antiphosphothreonine mixture and anti-HA antibody. (F) 293T cells were pretreated with the indicated concentrations of CK2 inhibitor DMAT for 2 h, followed by the transfection of the HA-RIG-I plasmid for 24 h. RIG-I protein was immunoprecipitated using anti-HA antibody and immunoblotted with an antiphosphoserine/antiphosphothreonine mixture and anti-HA antibody. (G) Bacterially expressed RIG-I was incubated with CK2 α in the buffer containing 100 μ M unlabeled ATP, 5 μ Ci of [γ - 32 P]ATP, and different concentrations of DMAT, as described in Materials and Methods. (Top) It was analyzed by autoradiography after the reaction. (Bottom) The loading protein was stained with Coomassie blue.

FIG. 4. Dephosphorylation of threonine 770 and serine 854 and 855 results in the activation of RIG-I. (A) 293T cells were transfected with the indicated luciferase construct and plasmids containing RIG-I or its variants. The luciferase assay was performed 18 h after transfection. Values are shown as the means $\pm$ SD from one representative experiment. The results were obtained from three independent experiments. (B) 293T cells were transfected with the plasmids containing RIG-I or its variants for 24 h. Total RNA was extracted and analyzed by real-time PCR for the expression of IFN- β and actin. (C) Bacterially expressed His-tagged RIG-I fragments were incubated with GST-CK2 in buffer containing 100 μ M unlabeled ATP and 5 μ Ci of [γ - 32 P]ATP. SDS-PAGE was performed with the above-described mixture after the reaction. (Top) Phosphorylation was analyzed by autoradiography. (Bottom) The loading protein was stained with Coomassie blue. (D) The bacterially expressed His-tagged RIG-I RD and its mutants were incubated with GST-CK2 in buffer containing 100 μ M unlabeled ATP and 5 μ Ci of [γ - 32 P]ATP. SDS-PAGE was performed with the above-described mixture after the reaction. (Top) Phosphorylation was analyzed by autoradiography. (Bottom) The loading protein was stained with Coomassie blue. (E) 293T cells were transfected with the plasmids containing the Flag-tagged RIG-I RD and its mutants for 24 h. The ectopic protein was immunoprecipitated by anti-Flag antibody and immunoblotted with antiphosphoserine or antiphosphothreonine and anti-Flag antibody. (F, top) 293T cells were pretreated with increasing concentrations of the serine/threonine-specific phosphatase inhibitor OA for 2 h, followed by infection with SeV for 8 h. Total RNA was extracted and analyzed by real-time PCR for the expression of IFN- β . (Bottom) The same experiment was then performed with HUVEC. (G) Phosphopeptide sequences identified by mass spectrometry. *, phosphorylation sites.

conformation changes that allow interaction of the CARDS of VISA/IPS-1/Cardif/MAVS (2, 15). To test whether the RD interacts with the CARD and whether phosphorylation is involved in the interaction, the RD or its mutants were cotrans-

fected with the CARD. We show that both the wild-type RD and its aa698 mutant interact with the CARD, while mutation at aa 770 and 854 to 855 led to the failure to interact with the CARD (Fig. 5A). These observations suggest that phosphory-

FIG. 5. Amino acids 770 and 854 to 855 are essential for the intermolecular interaction and multimerization of RIG-I. (A) 293T cells were transfected with the plasmids containing the myc-tagged CARD and Flag-tagged RD or their mutants for 24 h. Total protein was immunoprecipitated with anti-Flag antibody. The immunoprecipitates were then analyzed by immunoblotting with anti-RIG-I and anti-Flag antibodies. Expression of the ectopic genes was analyzed by immunoblotting with anti-Flag and anti-RIG-I antibodies. (B) 293T cells were transfected with the plasmids containing Flag-tagged RIG-I and its mutants for 24 h. Total protein was extracted and subjected to native PAGE. (Top) Immunoblotting was performed with antibody against RIG-I after transfer onto the membrane. (Bottom) Expression of the ectopic genes was measured by immunoblotting with anti-Flag antibody. (C) 293T cells were pretreated with CK2 inhibitor DMAT or the phosphatase inhibitor OA, followed by virus infection with SeV or AdV. Cells were collected after infection for 8 h. Total protein was extracted and subjected to native PAGE, followed by an immunoblot assay with antibody against RIG-I.

lation at aa 770 and aa 854 to 855 is required for the intermolecular interaction between the RD and CARD. It has been proposed that dissociation of intermolecular interaction is necessary for the multimer formation (2, 15). To determine whether the mutations at these sites affected multimerization, we transfected the mutants and the wild type of RIG-I into cells. Multimerization was examined by immunoblot assay with the antibody against RIG-I with native PAGE and showed that

the active forms of the aa770 and aa854 to 855 mutants formed more multimers and less monomers (Fig. 5B). In contrast, the inactive form of the aa162 mutant was mostly retained in monomer form. The ratio of multimer to monomer for the aa698 mutant was similar to that of wild-type RIG-I, clearly demonstrating that aa 770 and aa 854 to 855 of RIG-I are essential for its intermolecular interaction and multimerization. It seems that overexpression of wild-type RIG-I and the

aa162 mutant also led to the formation of multimers, although to a lower extent compared with the overexpression of the aa770 and aa854 to 855 mutants. However, the mechanism behind the phenomenon is currently unknown. To verify the specificity of the multimer and the effect of the phosphorylation state on the multimer formation, we pretreated 293T cells with pharmacological inhibitors for CK2 and phosphatase, followed by virus infection. The multimerization was examined by immunoblot assay with the antibody against RIG-I with native PAGE. We show that endogenous RIG-I stays as a monomer before virus infection. Virus infection induces the multimer formation. Inhibition of CK2 by a pharmacological compound enhances the formation of the multimers, and inhibition of phosphatase results in the accumulation of monomers after virus treatment (Fig. 5C). The result is consistent with the data shown in Fig. 5B, indicating that dephosphorylation at aa 770 and aa 854 to 855 leads to the activation of RIG-I and subsequently results in multimer formation.

HCV infection results in the dephosphorylation of RIG-I. In order to verify if RIG-I is phosphorylated, we generated a panel of antibodies against aa 770 and 854 to 855. It showed that synthetic phosphorylated, but not nonphosphorylated, peptide blocks the recognition of antibody to phosphorylated recombinant RIG-I, indicating that the anti-phospho-antibody is specific for threonine 770 and serine 854 to 855. (Fig. 6A). These antibodies were subsequently used to determine whether RNA virus infection induces the dephosphorylation of RIG-I. 293T cells and HUVEC were infected with Sendai virus, and total cellular proteins were extracted at different time points after infection. As shown in Fig. 6B, virus infection led to the dephosphorylation of RIG-I at aa 770 and 854 to 855, starting at 2 h after infection, while RIG-I protein levels were increased during the infection. We next tested whether a second virus, HCV, will also induce RIG-I dephosphorylation, as HCV has been shown to activate RIG-I-dependent signaling (15). Huh7 cells showed increased expression of the RIG-I protein and reduced phosphorylation at aa 770 and 854 to 855 following infection with HCV (Fig. 6C). We then addressed whether CK2 is involved in the RIG-I-mediated anti-HCV response. CK2 was knocked down in Huh7 cells by siRNA, and cells were infected with modified HCV. Total RNA was extracted and subjected to real-time PCR (RT-PCR) for HCV NS5A gene analysis, which has been considered an indicator for the replication of HCV in cells (10, 21). As shown in Fig. 6D, the silence of CK2 resulted in decreased expression of the HCV NS5A gene, indicating that inhibition of CK2 enhances the RIG-I-mediated antiviral response.

DISCUSSION

It has become evident that the helicase domain of RIG-I is not solely important for its function, and the C-terminal domain (CTD) and CARD, which recognizes foreign RNA and mediates the signaling, respectively, are essential for the function of RIG-I. The CTD almost overlaps with the repressor domain (RD) (2, 15). This RD region holds the following two distinct functions: RNA ligand recognition and signal suppression. Mutagenesis on the basic concave surface inactivated RNA recognition, but none of the mutations rendered RIG-I constitutively active, suggesting that the RNA recognition sur-

face and the RNA-suppressive surface do not overlap (2). The mechanism of the repression of RIG-I signaling by the CTD itself has not yet been determined. Our results demonstrate that threonine at aa 770 and serine at aa 854 to 855 in the RD are essential amino acids in the regulation of RIG-I activity. In resting cells, the RD of RIG-I interacts with CK2, leading to the phosphorylation at aa 770 and 854 to 855. The phosphorylation of the RD leads to the interaction with the CARD, thus inactivating RIG-I, and the intermolecular interaction between the RD and RIG-I could be disassociated during virus infection. In addition to the dephosphorylation modification, RIG-I is also modified in other ways, such as ubiquitination (6) and ISGylation (8). This proposed mechanism for the inactivation and activation of RIG-I is illustrated in Fig. 7.

It has been well known that phosphorylation and dephosphorylation of protein play a key role in cellular signaling. Upon RNA virus infection, the downstream molecule of IRF3 could be activated by phosphorylation, which activates the transcription of IFN- β through either the TLR3 pathway or RIG-I pathway (1, 17, 26). It is interesting that virus infection also induces the dephosphorylation of RIG-I that leads to the activation of its antiviral signaling. This phenomenon indicates a novel mechanism involving phosphatases in antiviral responses that regulate RIG-I-mediated signaling during virus infection. Searching for the specific phosphatase that recognizes and dephosphorylates RIG-I will be helpful in finalizing the regulation of the antiviral pathway.

Structural analysis reveals that the RNA binding sequence of RIG-I resides mainly in the CTD that nearly overlaps with a previously identified repressor domain (aa 723 to 925) (15). It has been recently reported that a short, blunt-end, double-stranded 5'-triphosphate RNA structure contained in the pan-handle of negative-strand viral genomes confers full RIG-I ligand activity to a RNA molecule (19). However, the RNA recognition does not alter the RIG-I activity (25). Intriguingly, the CK2 phosphorylation sites, aa 770 and aa 854 to 855, are also present in the same region, suggesting that RNA binding to the CTD of RIG-I may induce a conformation change that allows phosphatase to be accessible to these amino acids and hydrolysis to the phospho residues, leading to the activation of RIG-I. It may be interesting for further study to check whether mutation at these sites could alter the affinity to virus RNA using either single-stranded or double-stranded RNA.

The involvement of CK2 in antiviral signaling has not been extensively explored, although it has been implicated in some viral infections (3, 4, 9, 12, 14). Our study demonstrates that CK2 plays a role in RIG-I-mediated signaling. We show that CK2 phosphorylates aa 770 and 854 to 855 in the RD of RIG-I, leading to inactivation. Inhibition of CK2 by a chemical compound enhances RNA virus-induced expression of IFN- β , while inhibition or silencing of CK2 enhances the RIG-I-mediated antiviral response. It has been noticed that synthetic siRNAs can be potent inducers of interferons and inflammatory cytokines. Cytosolic RNA binding proteins like PKR, RIG-I, and Mda5 are the mediators for this response (18). However, the immunostimulatory activity of formulated siRNAs and the associated toxicities are dependent on the nucleotide sequence (7). Although we have not found a nonspecific immunoresponse in the experiments with siRNA in our study, it should not be excluded that other undetected immu-

FIG. 6. HCV infection results in the dephosphorylation of RIG-I. (A) Specificity of the anti-phospho-antibody. Bacterially expressed RIG-I was incubated with (lane 1) or without (lane 2) CK2α in a buffer containing 100 μM ATP. The mixture was subjected to SDS-PAGE after 1 h of incubation at 37°C, followed by immunoblotting with antibodies against phosphorylated S854 to 855 and T770 peptides of RIG-I, in the presence of either related phosphorylated peptide or nonphosphorylated peptide. (Bottom) The anti-phospho-antibody was generated by the synthetic peptides. (B) 293T cells (top) and HUVEC (bottom) were infected with SeV for the indicated periods of time. Total protein was extracted and subjected to SDS-PAGE, followed by immunoblotting with antibodies against phosphorylated S854 to 855 and T770 peptides of RIG-I, RIG-I, and actin. (C) Huh7 cells were infected with HCV for the indicated periods of time. Total protein was extracted and subjected to SDS-PAGE, followed by immunoblotting with antibodies against phosphorylated S854 to 855 and T770 peptides of RIG-I, RIG-I, and actin. (D, top) Huh7 cells (1×10^5) were transfected with CK2α-RNAi double-stranded oligonucleotides (2 μg/ml). A total of 36 h after transfection, cells were infected with HCV. Total RNA was extracted at 24 h postinfection and analyzed by real-time PCR for the expression of HCV NS5A and CK2α. (Bottom) Result from Western blotting with specific anti-CK2α and anti-actin antibodies for normalization.

noresponses may cause the effects related to our current study. However, the finding that CK2 is involved in the RIG-I-mediated antiviral response has been identified in multiple ways, in addition to the experiment with siRNA. The results are consistent and support each other. Moreover, we find that CK2 is involved in HCV infection. HCV infection reduces the phosphorylation of RIG-I and activates its antiviral signaling. Our study shows that CK2 suppresses the RIG-I-mediated anti-HCV response through phosphorylation of RIG-I at aa 770

and 854 to 855. During the submission of this paper, Gack et al. reported that mutation at aa 170 in the CARD region rendered RIG-I inactive (5). Both aa 170 and 162 are potential PKC phosphorylation sites, according to the bioinformatics analysis. Amino acid 170 mediates the interaction between RIG-I and its adaptor VISA/IPS-1/Cardif/MAVS and, thus, transduces the signal. We also find that aa 162 is a key site in mediating the interaction with its adaptor (data not shown). These data suggest that the phosphorylation state in the CARD region

FIG. 7. Model of the regulation of RIG-I activity during RNA virus infection. In resting cells, the RD region of RIG-I is phosphorylated by CK2 α and interacts with the CARD to form a closed, inactivated conformation. The RD is dephosphorylated when cells are infected by RNA virus. The recognition and binding of RNA by RIG-I may induce the conformational change that allows the access of the phosphatase. Infection simultaneously activates the phosphatase that dephosphorylates RIG-I at aa 770 and 854 to 855. Dephosphorylation in the RD of RIG-I results in the disassociation of intermolecular interaction between the CARD and RD. Such a disassociation leads to the formation of an opened, activated conformation, followed by multimerization and polyubiquitination.

may affect the intramolecular interaction and not alter the intermolecular interaction, since the aa162 mutant still holds the ability to form the multimer when overexpressed. However, the phosphorylation state in the RD region specifically controls the intermolecular interaction. Together with our finding, it suggests that phosphorylation and dephosphorylation could be other events that control the activity of RIG-I and its signaling during virus infection. It also indicates that phosphorylation and dephosphorylation at a different region of RIG-I is controlled by a different kinase and phosphatase, which play different roles in RIG-I signaling.

Based on what we have found, we conclude that regulation of RIG-I activity during the infection of RNA virus includes the following several events. The recognition and binding of RNA make up the first step, which leads to the conformational change of RIG-I. Infection simultaneously activates the phosphatase that subsequently interacts with conformational changed RIG-I, and finally, dephosphorylation of RIG-I at aa 770 and 854 to 855 leads to the formation of multimers and thus activation. It is also possible that conformational change upon binding of a RIG-I ligand may allow a phosphatase to gain access to the phosphorylated sites of RIG-I (aa 770 and 854 to 855). This phosphatase may be constitutively active and may not require additional activation by the virus. However, the detailed mechanisms await further study.

In summary, our data disclose a novel mechanism that regulates the activity of RIG-I during RNA virus infection. Phosphorylation and dephosphorylation at aa 770 and aa 854 to 855 are essential events to regulate the activity of RIG-I. Phos-

phorylation by CK2 and dephosphorylation by phosphatase is RIG-I specific during RNA virus infection. Bioinformatics analysis shows that the amino acid identity between human and mouse RIG-I is nearly 80%. Protein function analysis shows that human and mouse RIG-I are structurally similar. However, the phosphorylated sites that we found and other groups found are not conserved in the human and mouse. T770 is the only one conserved in the RD domain of human and mouse RIG-I, indicating that amino acid 770 may play a key role in the regulation of RIG-I activity. This is the first molecular evidence to demonstrate the involvement of CK2 and phosphatase in RIG-I-mediated antiviral signaling. Meanwhile, our data also uncover the long-standing question of how autorepression takes place in RIG-I, which help us to further understand the structure and function of RIG-I at the molecular level.

ACKNOWLEDGMENT

This work was supported by a grant (2010CB911801) from the National Basic Research Program, China.

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