PKACs attenuate innate antiviral response by phosphorylating VISA and priming it for MARCH5-mediated degradation

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Abstract

Sensing of viral RNA by RIG-I-like receptors initiates innate antiviral response, which is mediated by the central adaptor VISA. How the RIG-I-VISA-mediated antiviral response is terminated at the late phase of infection is enigmatic. Here we identified the protein kinase A catalytic (PKAC) subunits α and β as negative regulators of RNA virus-triggered signaling in a redundant manner. Viral infection up-regulated cellular cAMP levels and activated PKACs, which then phosphorylated VISA at T54. This phosphorylation abrogated virus-induced aggregation of VISA and primed it for K48-linked polyubiquitination and degradation by the E3 ligase MARCH5, leading to attenuation of virus-triggered induction of downstream antiviral genes. PKACs-deficiency or inactivation by the inhibitor H89 potentiated innate immunity to RNA viruses in cells and mice. Our findings reveal a critical mechanism of attenuating innate immune response to avoid host damage at the late phase of viral infection by the house-keeping PKA kinase.

Author summary

VISA is a central adaptor protein required for innate immune response to RNA virus. Phosphorylation of VISA by protein kinase A leads to its polyubiquitination and degradation by the E3 ligase MARCH5 at the late phase of viral infection, which provides a critical control mechanism for the host to avoid excessive and harmful immune response.

Introduction

Innate immune response is the first line of host defense against invading microbial pathogens. The structurally conserved components of microbes called pathogen-associated molecular patterns (PAMPs) are recognized by host pattern-recognition receptors (PRRs), which initiates signaling pathways that lead to induction of type I interferons (IFNs), proinflammatory cytokines and other downstream effector genes [1]. During RNA virus infection, viral RNAs,
including the invading viral RNAs and RNA intermediates produced during viral replication, act as PAMPs that are mostly recognized by the cytoplasmic RIG-I-like receptor (RLR) family members including RIG-I and MDA5 [2]. Although RIG-I and MDA5 sense distinct types of viral RNAs, they utilize a common adaptor protein called VISA (also known as MAVS, IPS-1 or Cardiff) to transmit signals [3–6]. Upon binding to viral RNAs, RLRs are recruited to VISA located on the mitochondrial outer membrane, and this induces aggregation and activation of VISA [7]. VISA then acts as a central platform for recruitment of downstream signaling components, including TRAF2/3/5/6, cIAP1/2 and WDR5 [6, 8–10]. In these complexes, TRAF6 functions redundantly with TRAF2 and TRAF5 to activate IRF3 and IKK [11]. These processes lead to eventual transcription of downstream antiviral genes, including type I IFNs, proinflammatory cytokines and other effectors [12, 13].

Protein phosphorylation and dephosphorylation play important roles in innate immune responses to RNA viruses by regulating the activation and deactivation of multiple RLR-mediated signaling components, such as RIG-I, VISA, TRAF3, TBK1 and IRF3 [14–18]. In some cases, the enzymes that are responsible for their modifications are unknown. The RLR-mediated signaling pathways are also heavily regulated by other post-translational modifications, such as ubiquitination, sumoylation, methylation and [19–23]. How post-translational modifications cross-talk to regulate innate antiviral response remains enigmatic.

Protein kinase A (PKA) is one of the first identified protein kinases, which is critically important for many divergent cellular processes, such as metabolism, cell cycle, cell migration, differentiation and apoptosis. PKA exists as a tetrameric holoenzyme with two regulatory subunits and two catalytic subunits in its inactive form. Cyclic adenylyl monophosphate (cAMP) causes dissociation of the inactive holoenzyme into a dimer of regulatory subunits bound to four cAMP and two free monomeric catalytic subunits [24]. Four regulatory subunits (PKARI\(\alpha\), PKARI\(\beta\), PKARI\(\alpha\) and PKARI\(\beta\)) and three catalytic subunits (PKAC\(\alpha\), PKAC\(\beta\) and PKAC\(\gamma\)) have been identified in humans. PKAC\(\alpha\) and PKAC\(\beta\) are ubiquitously expressed in most examined tissues, but PKAC\(\gamma\) is specifically expressed in testis. Human PKAC\(\alpha\) and PKAC\(\beta\) are highly homologous, which share ~93% sequence identity at the amino acid level [25].

In this report, we identified PKAC\(\alpha\) and PKAC\(\beta\) as two redundant negative regulators of RNA virus-triggered induction of downstream antiviral genes. Viral infection activated PKACs, which in turn phosphorylated VISA at T54, leading to impairment of VISA aggregation and its K48-linked polyubiquitination and degradation by the E3 ligase MARCH5. We also showed that PKACs-deficiency or inactivation potentiated innate immunity to RNA viruses in cells and mice. Our findings reveal a critical mechanism of attenuating innate immune response at the late phase of viral infection and establish an un-described function for PKA in innate antiviral response.

**Results**

**PKAC\(\alpha\) and PKAC\(\beta\) negatively regulate VISA-mediated signaling**

VISA is a central adaptor protein in innate immune response to RNA virus. To identify potential kinases that regulate VISA-mediated signaling, we screened a cDNA library contains 352 kinase clones. We found that PKAC\(\alpha\) and PKAC\(\beta\) markedly inhibited VISA-mediated activation of the IFN-\(\beta\) promotor in HEK293 cells (Fig 1, panel A). PKAC\(\alpha\) and PKAC\(\beta\) also dose-dependently inhibited Sendai virus (SeV)-induced activation of the IFN-\(\beta\) promotor and ISRE, an enhancer motif for activated IRF3. Overexpression of PKAC\(\alpha\) and PKAC\(\beta\) activated NF-\(\kappa\)B (Fig 1, panel B), which is consistent with previous reports that PKAC\(\alpha\) and PKAC\(\beta\) can phosphorylate p65 on S276 [26, 27]. Overexpression of PKAC\(\alpha\) and PKAC\(\beta\) also inhibited SeV-
induced transcription of downstream genes such as IFNB1, ISG15 and IKBA (Fig 1, panel C). In contrast, the testis specific PKA catalytic subunit PKACγ, the PKA regulatory subunits PKARIα and PKARIβ, or the catalytic inactive mutants of PKACα (K73A) and PKACβ
PKACα and PKACβ can inhibit RNA virus-triggered and VISA-mediated induction of downstream antiviral genes. We next determined whether endogenous PKACα and PKACβ are involved in regulation of virus-induced signaling. We found that knockdown of either PKACα or PKACβ by RNAi had no marked effects on SeV-induced activation of the IFN-β promoter, ISRE and NF-κB in reporter assays. However, simultaneous knockdown of both PKACα and PKACβ markedly potentiated SeV-induced activation of the IFN-β promoter, ISRE and NF-κB (Fig 2, panel A). Consistently, simultaneous but not individual knockdown of PKACα and PKACβ potenti-ated SeV-induced transcription of IFNB1 and CXCL10 genes (Fig 2, panel B).

Because PKACα and PKACβ are highly conserved at both amino acid and mRNA sequence levels, we constructed two more RNAi plasmids (PKACs-RNAi #1 and #2), each of them simultaneously target both of human PKACα and PKACβ mRNAs. Simultaneous knockdown of the two catalytic subunits PKACα and PKACβ (referred below as PKACs) by these two RNAi plasmids potenti-ated SeV-induced activation of the IFN-β promoter, ISRE and NF-κB (Fig 2, panel C), as well as SeV or vesicular stomatitis virus (VSV)-induced transcription of IFNB1, ISG56 and TNFA genes (Fig 2, panel D&E). The degrees of potentiation were corre-lated to the knockdown efficiencies of the RNAi plasmids (Fig 2, panel C, D&E). Knockdown of PKACs also potenti-ated IFN-β promoter activation triggered by poly(I:C) transfected into HEK293 cells (Fig 2, panel F). In addition, knockdown of PKACs altered markedly increased SeV or VSV-induced phosphorylation of TBK1, IRF3 and IκBα (Fig 2, panel G&H). However, knockdown of PKACs had no marked effects on IFN-α-induced activation of STAT1/2 and IFN-γ-induced activation of the IRF1 promoter (Fig 2, panel I). These data suggest that PKACα and PKACβ negatively regulate RNA virus-induced expression of downstream genes in a redundant manner.

Viral infection induces PKACs activation

To investigate how PKACs function following viral infection, we determined whether viral infection triggers the accumulation of cellular cAMP. We found that SeV infection caused a transient decrease of cellular cAMP level at the early phase of infection (3 h) but marked increase at the late phase of infection (Fig 3, panel A). The cAMP levels in RIG-I-deficient cells at the late phase of infection were not increased (Fig 3, panel B), suggesting that the increase of cAMP level at the late phase of viral infection was dependent on RIG-I-mediated signaling. In addition, SeV infection induced the phosphorylation of PKACs on T197 (Fig 3, panel C), which is a hallmark of PKACs activation [29, 30]. These results suggest that viral infection leads to increase of cellular cAMP levels and activation of PKACs at the late phase of infection in a RIG-I-dependent manner.

Additionally, we determined the effects of exogenous cAMP on virus-induced expression of downstream antiviral genes. We found that introduction of exogenous cAMP into the cells abolished SeV-induced transcription of IFNB1 gene, and knockdown of PKACs reversed the effects of exogenous cAMP (Fig 3, panel D). These results suggest that viral infection induced increase of cAMP levels and activation of PKACs, which in turn inhibit virus-triggered induction of downstream antiviral genes in a negative feedback manner.

PKACs catalyze phosphorylation of VISA at T54

We next determined the molecular mechanisms responsible for the inhibitory effects of PKACs on virus-triggered induction of downstream genes. In transient transfection and co-immunoprecipitation experiments, PKACα interacted with VISA, while PKACβ interacted...
Fig 2. Simultaneous knockdown of PKACα and PKACβ potentiates RNA virus-triggered signaling. (A) Left panels: Knockdown efficiencies of PKACα and PKACβ by RNAi. For the upper three panels, HEK293 cells were transfected with Flag-PKACα/β, HA-β-actin, and the indicated RNAi plasmids for 36 h, and then analyzed by immunoblots with anti-Flag or anti-HA.
with VISA, TRAF3 and TRAF6. Neither PKACα nor PKACβ interacted with RIG-I (Fig 4, panel A). Cellular fractionation experiments indicated that PKACs were localized in the cytosol and at the mitochondria (Fig 4, panel B). Endogenous co-immunoprecipitation experiments indicated that PKACs were constitutively associated with VISA before and after SeV infection (Fig 4, panel C). In reporter assays, knockdown of PKACs enhanced upstream components RIG-I-, MDA- and VISA- but not downstream components TBK1-, IRF3- or IRF7-mediated ISRE activation (Fig 4, panel D). H89 is a specific and potent PKA inhibitor [31], which completely reversed the inhibitory effects of PKACs on VISA-mediated activation of the IFN-β promoter (Fig 4, panel E). In the same experiments, H89 had no effects on the inhibition of VISA-mediated activation of the IFN-β promoter by DYRK2, a kinase that negatively regulates virus-triggered signaling by targeting TBK1 for phosphorylation [16]. Collectively, these results suggest that PKACs inhibit virus-triggered induction of downstream antiviral genes by targeting VISA. Consistently, overexpression of PKACα or PKACβ but not their kinase inactive mutants caused a shift of VISA to higher molecular weight species (Fig 5, panel A). These higher molecular weight species of VISA were recognized by an antibody to phosphorylated serine and/or threonine (p-S/T) and removed by treatment with lambda phosphatase (λ-PPase) (Fig 5, panel B). These results suggest that PKACs phosphorylate VISA.

We next determined the residues of VISA that are phosphorylated by PKACs. Prediction by GPS3.0 program indicates that VISA contains four consensus PKA phosphorylation residues, including T54, S100, T234 and S238. Among the phosphorylation sites, T54 is highly conserved in mammals and the only residue located in the N-terminal CARD-like domain of VISA. Mutagenesis indicated that PKACα phosphorylated wild-type VISA and the VISA mutants VISA(S100A), VISA(T234A) and VISA(S238A) but not VISA(T54A) (Fig 5, panel C). Reporter assays indicated that mutation of S100, T234 and S238 of VISA to either alanine (A) or aspartic acid (D) had no marked effects on its ability to activate the IFN-β promoter. However, mutation of T54 of VISA to D, which mimics its phosphorylation, dramatically impaired its ability to activate downstream signaling. Unexpectedly, mutation of T54 of VISA to A, which mimics its un-phosphorylated form, also impaired its activity, though to a lesser degree (Fig 5, panel D). We further investigated the functions of the T54 mutants of VISA by reconstituting them into VISA-deficient HEK293 cells. We found that VISA(T54D) completely lost...
the ability to mediate SeV-triggered induction of downstream *IFNB1* and *ISG56* genes, while VISA(T54A) partially maintained the ability in comparison to wild-type VISA (Fig 5, panel E). Consistently, SeV-induced phosphorylation of TBK1 and IRF3 was partially and completely impaired in VISA(T54A)- and VISA(T54D)-reconstituted cells respectively in comparison to wild-type VISA-reconstituted cells (Fig 5, panel F). These results suggest that T54 is probably the target residue of PKACs.

To determine whether PKACs indeed target T54 of VISA for phosphorylation, we generated a rabbit polyclonal antibody to a peptide containing phosphorylated T54 (p-VISA-T54). Immunoblot analysis indicated that PKACs but not their kinase inactive mutants caused phosphorylation of VISA at T54 (p-VISA-T54). The phosphorylation of VISA at T54 was dramatically enhanced following SeV infection for 10 hours, while knockdown of PKACs impaired the phosphorylation of VISA at T54 and increased induction of RIG-I (Fig 5, panel H). In addition, knockdown of PKACs potentiated wild-type VISA and its S100A, T234A and S238A mutants but not T54A mutant mediated activation of the IFN-β promoter (Fig 5, panel I). Taken together, these results suggest that PKACs inhibit SeV-triggered induction of downstream genes by direct phosphorylation of VISA at T54.
Fig 4. PKACs target VISA in the virus-triggered pathways. (A) VISA interacts with PKACα and PKACβ. HEK293 cells were transfected with the indicated plasmids for 20 h before co-immunoprecipitation and immunoblot analysis with the indicated antibodies. (B) Distribution of PKACs. HEK293 cells were infected with SeV (MOI = 1) for the indicated times and then fractionated the subcellular fractions were equilibrated to equal volumes and analyzed by immunoblots with the indicated antibodies. (C) Endogenous VISA is associated with PKACs in the mitochondria. HEK293 cells were infected with SeV (MOI = 1) for the indicated times. The mitochondria were isolated by cell fractionation and the mitochondrial lysates were subjected to immunoprecipitation and immunoblot analysis with the indicated antibodies. (D) Effects of PKACs knockdown on ISRE activation by various components. HEK293 cells were transfected with ISRE luciferase and PKACs RNAi plasmids for 36 h, then transfected with the indicated expression plasmids for 20 h before luciferase assays were performed. (E) Effects of H89 on PKACs-mediated inhibition of VISA activity. HEK293 cells were transfected with the IFN-β promoter luciferase and the indicated expression plasmids for 20 h, then transfected with VISA plasmid for 20 h and treated with H89 (10 μg/mL) or left untreated for 8 h before luciferase assays were performed.
Fig 5. PKACs phosphorylate VISA at T54. (A) Effects of PKACs and their kinase inactive mutants on VISA modification. HEK293 cells were transfected with HA-VISA and increased amounts of the indicated expression.
PKACs impair VISA aggregation and promote its degradation by MARCH5

It has been demonstrated that VISA forms prion-like aggregates on the mitochondrial membrane to activate innate immune response after viral infection [7]. We next investigated whether phosphorylation of VISA at T54 by PKACs impairs the formation of VISA aggregates. Co-immunoprecipitation experiments indicated that the self-association of VISA(T54D) was markedly decreased compared to wild-type VISA, VISA(T54A), VISA(S100A), or VISA(S100D) (Fig 6, panel A). Reconstitution experiments indicated that SeV-induced aggregation of VISA(T54D) was decreased in comparison to wild-type VISA and VISA(T54A) (Fig 6, panel B). In addition, knockdown of PKACs increased the formation of VISA aggregates following SeV infection. HEK293 cells were transfected with HA-VISA, Flag-tagged PKACα and Flag-PKACβ and their mutants for 20 h. Cell lysates were analyzed by immunoblots with the indicated antibodies. (C) Effects of PKACα on VISA and its mutants. HEK293 cells were transfected with the indicated expression plasmids for 20 h and then analyzed by immunoblots with the indicated antibodies. (D) Effects of VISA and its mutants on IFN-β promoter activation. HEK293 cells were transfected with the IFN-β promoter luciferase and Flag-VISA or its mutants plasmids for 20 h before luciferase assays were performed. The blot shows the expression levels of the transfected VISA and its mutants. (E) Effects of VISA and its mutants on SeV-induced transcription of IFNB1 gene. VISA-deficient HEK293 cells reconstituted with VISA or its mutants were infected with SeV (MOI = 1) for the indicated times before qPCR analysis. (F) Effects of VISA and its mutants on SeV-induced aggregation of VISA(T54D) was decreased in comparison to wild-type VISA and VISA(T54A) (Fig 6, panel B). In addition, knockdown of PKACs increased the formation of VISA aggregates following SeV infection (Fig 6, panel C). Confocal microscopy indicated that VISA(T54A) formed aggregates more dramatically than wild-type VISA at the early phase of infection (2–8 h) and some aggregates remained even at the late phase of infection (24 h). Interestingly, VISA(T54D) colocalized with mitochondria but did not form aggregates before and after viral infection. In addition, VISA(T54D) was also not degraded after viral infection (Fig 6, panel D&E). These results suggest that PKACs-mediated phosphorylation of VISA at T54 impairs its aggregation and activation.

In our experiments, we routinely found that PKACα and PKACβ but not their kinase inactive mutants caused down-regulation of VISA (Fig 5, panel A), while knockdown of PKACs up-regulated the levels of endogenous VISA (Fig 5, panel H). Kinetic experiments indicated that the levels of VISA were gradually down-regulated from 2–24 h after viral infection, and knockdown of PKACs slowed SeV-triggered down-regulation of VISA (Fig 7, panel A). We therefore tested the hypothesis that phosphorylation of VISA at T54 by PKACs impairs its aggregation and primes it for polyubiquitination and proteasomal degradation. We found that overexpression of PKACs enhanced K48- but not K63-linked polyubiquitination of VISA (Fig 7, panel B). Conversely, knockdown of PKACs inhibited SeV-induced K48- but not K63-linked polyubiquitination of VISA (Fig 7, panel C). Reconstitution experiments indicated that SeV-induced K48-linked polyubiquitination and degradation of VISA(T54A) was impaired in comparison to wild-type VISA (Fig 7, panel D). These results suggest that phosphorylation of VISA at T54 by PKACs primes it for K48-linked polyubiquitination and degradation.
Fig 6. PKACs impair VISA aggregation. (A) Oligomerization of VISA and its mutants. HEK293 cells were transfected with the indicated expression plasmids for 20 h before co-immunoprecipitation and immunoblot analysis with the indicated antibodies. (B) Effects of T54 mutation on SeV-induced aggregation of VISA in the mitochondria. Crude PKA negatively regulate innate antiviral response.
Previous studies have identified AIP4, MARCH5 and RNF5 as E3 ubiquitin ligases that catalyze K48-linked polyubiquitination of VISA [32–34]. We found that individually knockdown of the examined three E3 ligases did not affect the phosphorylation of VISA by PKACα. However, knockdown of MARCH5 but not RNF5 or AIP4 dramatically inhibited the degradation of VISA mediated by PKACα (Fig 7, panel E). In addition, knockdown of MARCH5 but not RNF5 and AIP4 inhibited the synergistic activation of the IFN-β promoter induced by SeV and PKACs knockdown (Fig 7, panel F). Previously, it has been demonstrated that MARCH5 targets K7 and K500 of VISA for K48-linked polyubiquitination and degradation [33]. We found that PKACα caused degradation of wild-type VISA but not VISA(T54A) or VISA(K7/500R), in which either the PKACs-mediated phosphorylation or MARCH5-mediated K48-linked polyubiquitination residues are mutated (Fig 7, panel G). Collectively, these results suggest that PKACs-mediated phosphorylation of VISA at T54 primes it for K48-linked polyubiquitination and degradation by MARCH5.

PKACs inhibit antiviral response in vivo

Finally, we investigated whether PKACs regulate innate antiviral response in immune cells and in vivo. We found that induction of Ifnb1, Ifna4 and Il6 mRNAs by either SeV or encephalomyocarditis virus (EMCV) in bone marrow-derived dendritic cells (BMDCs) was markedly increased by knockdown of PKACs (Fig 8, panel A). In addition, mice treated with the PKA inhibitor H89 produced higher levels of serum cytokines including IFN-α4, IFN-β and IL-6 upon EMCV infection (Fig 8, panel B) and were more resistant to EMCV-induced death (Fig 8, panel C). These results suggest that PKA negatively regulates innate immune responses to RNA viruses in mice.

Discussion

Proper and efficient innate immune response at the early phase of infection is critical for clearance of viruses, while timely termination of innate antiviral response at the late phase of infection is important for avoiding harmful immune damage and death of the host. How the innate antiviral response is delicately regulated has been heavily investigated in the past decade. In this report, we found that the house-keeping kinase PKA played an essential role in attenuating innate immune response to RNA virus by inactivating the central adaptor protein VISA in the virus-triggered signaling pathways.

Overexpression of PKACα and PKACβ, but not PKACγ, the PKA regulatory subunits PKARα and PKARβ, or the catalytic inactive mutants of PKACα (K73A) and PKACβ (K73A) markedly inhibited SeV-triggered and VISA-mediated induction of downstream antiviral genes. Interestingly, knockdown of either PKACα or PKACβ had no marked effects on SeV-triggered signaling, but simultaneous knockdown of both PKACα and PKACβ dramatically potentiated
PKAs promote VISA degradation by MARCH5.

(A) Knockdown of PKACs inhibits SeV-induced VISA degradation. HEK293 cells were transfected with the indicated RNAi plasmids and selected with puromycin, then infected with SeV (MOI = 1) for the indicated times. Cell lysates were analyzed by immunoblots with the indicated antibodies.

(B) PKACs promotes K48- but not K63-linked polyubiquitination of VISA. HEK293 cells were transfected with the indicated plasmids for 20 h, followed by co-immunoprecipitation and western blotting.

(D) Reconstitution in VISA-deficient HEK293 cells

(F) IFN-β analysis

Fig 7. PKACs promote VISA degradation by MARCH5. (A) Knockdown of PKACs inhibits SeV-induced VISA degradation. HEK293 cells were transfected with the indicated RNAi plasmids and selected with puromycin, then infected with SeV (MOI = 1) for the indicated times. Cell lysates were analyzed by immunoblots with the indicated antibodies. (B) PKACs promotes K48- but not K63-linked polyubiquitination of VISA. HEK293 cells were transfected with the indicated plasmids for 20 h, followed by co-immunoprecipitation and western blotting.
immunoblotting analysis. (C) Knockdown of PKACα inhibits SeV-induced K48-linked polyubiquitination of VISA. PKACα-RNAi stably-transduced HEK293 cells were infected with SeV (MOI = 1) for the indicated times before co-immunoprecipitation and immunoblotting analysis. (D) Mutation of T54 of VISA to alanine impairs its K48-linked polyubiquitination induced by SeV. VISA-deficient HEK293 cells reconstituted with VISA or its mutants were infected with SeV (MOI = 1) for the indicated times before co-immunoprecipitation and immunoblot analysis. (E) Effects of knockdown of AIP4, MARCH5 or RNF5 on PKACα-induced phosphorylation and degradation of VISA. HEK293 cells were transfected with the indicated RNAi plasmids and selected with puromycin, then transfected with HA-VISA and increased amounts of Flag-PKACα for 20 h before immunoblot analysis with the indicated antibodies. For the second blot from the top, the same samples were treated with λ-PPase before immunoblot analysis. (F) Effects of knockdown of PKACs on activation of the IFN-β promoter triggered by SeV and knockdown of three E3 ligases. HEK293 cells were transfected with the IFN-β promoter luciferase and the indicated RNAi plasmids for 48 h before luciferase assays were performed. (G) Effects of PKACα on the levels of VISA and its mutants. HEK293 cells were transfected with Flag-VISA or its mutants and increased amounts of Flag- PKACα plasmid for 20 h before immunoblot analysis with the indicated antibodies.

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SeV-triggered induction of downstream genes. These results suggest that PKACα and PKACβ play redundant roles in inhibiting innate antiviral response, which is consistent with their redundant roles in regulation of many other cellular processes.

Our experiments suggest that PKACs inhibit innate antiviral response by targeting the central adaptor protein VISA, which is mostly localized at the mitochondria. Consistent with previous reports [35], we found that a fraction of PKACs was located in the mitochondria, and associated with VISA constitutively before and after viral infection. Overexpression of PKACs phosphorylated VISA at T54 and caused its degradation, whereas knockdown of PKACs inhibited SeV-induced phosphorylation of VISA at T54 and up-regulated its protein level. These results suggest that PKACs negatively regulate SeV-triggered induction of downstream genes by phosphorylating VISA at T54. Consistently, reconstitution experiments indicated that mutation of T54 of VISA to D, which mimics its phosphorylated status, abolished VISA activity, and this mutant failed to mediate SeV-triggered signaling and induction of downstream antiviral genes. Unexpectedly, mutation of T54 of VISA to A, which mimics its un-phosphorylated status, partially inhibited its activity. The exact reasons responsible for this observation is currently unknown. The simplest explanation is that mutation of T54 of VISA to A causes its conformational changes that partially affect its activity. In fact, there are numerous cases that mutation of T to A or D does not act in an opposite way. For examples, both S366A and S366D mutants of STING/MITA, or both S527A and S527D mutants of TBK1 have greatly reduced ability to mediate IFN-β induction [16, 36].

Our experiments suggest that phosphorylation of VISA by PKACs causes its inactivation by at least two processes. Firstly, the self-association, as well as SeV-induced aggregation of VISA (T54D), was decreased in comparison to wild-type VISA or VISA(T54A), whereas knockdown of PKACs increased VISA aggregation. Since virus-induced aggregation of VISA is an essential event for its activation, PKACs may inhibit virus-triggered signaling by phosphorylating VISA and impairing its aggregation. Secondly, phosphorylation of VISA by PKACs increased its K48- but not K63-linked polyubiquitination, whereas knockdown of PKACs decreased SeV-induced K48-linked polyubiquitination of VISA. In addition, SeV-induced K48-linked polyubiquitination and degradation of VISA(T54A) were abolished in comparison to wild-type VISA. These results suggest that phosphorylation of VISA at T54 by PKACs primes it for K48-linked polyubiquitination and degradation. We further showed that the E3 ubiquitin ligase MARCH5 but not RNF5 or AIP4 was responsible for mediating PKACs-primed K48-linked polyubiquitination of VISA. This is consistent with previous reports that MARCH5 is a mitochondrial-associated E3 ligase that negatively regulates virus-triggered induction of downstream genes at the late phase of infection [33]. It is possible that multiple E3 ligases are involved in regulation of VISA-mediated signaling in distinct cellular compartments and/or different phases of viral infection.
Previously, the involvement of PKA in the regulation of innate immune response has not been reported. We found that SeV infection caused decrease of cAMP levels at the early phase of infection, but induced increase of cAMP levels at the late phase of infection, which was correlated to the increased PKA activity at the late phase of infection. Since virus-induced increase of cAMP levels at the late phase of infection was abrogated in Rig-I−/− cells, we conclude that virus-triggered induction of cAMP and PKA activity is dependent on RIG-I.

Fig 8. PKA negatively regulates innate antiviral response in mice. (A) Knockdown of PKACs inhibits SeV- and EMCV-induced transcription of downstream antiviral genes in BMDCs. BMDCs were transfected with PKACs siRNA for 36 h, then infected with SeV (MOI = 1) or EMCV (MOI = 1) for the indicated times before qPCR analysis. (B) H89 potentiates EMCV-induced serum cytokine levels in mice. Mice (n = 6) were infected i.p. with EMCV at 1×10⁷ pfu per mouse for 12 h before serum cytokines were measured by ELISA. (C) H89 inhibits EMCV-induced death in mice. Mice (n = 8) were infected i.p. with EMCV at 1×10⁵ pfu per mouse, and the survival rates of mice were observed and recorded for 2 weeks.

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mediated pathways. It has been well established that binding of a ligand to a G-protein coupled receptor (GPCR) activates adenylyl cyclase (AC), which catalyzes the synthesis of cAMP from ATP. It is possible that the RIG-I pathways directly or indirectly through GPCR activate an AC, which leads to induction of cAMP. In light of the observation that PKACs are constitutively associated with VISA in the mitochondria, our results suggest that virus-triggered induction of cAMP modulates VISA activity in a temporal manner. In un-infected cells, basal cAMP maintains VISA activity at a steady level. At the early phase of infection, cAMP levels are down-regulated, which decreases PKACs activity and promotes VISA activity for efficient induction of downstream antiviral genes. At the late phase of infection, cAMP levels and PKACs activity are increased, which inactivates VISA and attenuates innate antiviral response. Therefore, PKA attenuates innate antiviral response in a feed-back negative regulatory manner.

Our experiments suggest that PKA is not only important for negative regulation of innate antiviral response in cells, it is also essential for attenuating innate antiviral response in mice. We found that inhibition of PKA by the specific inhibitor H89 markedly potentiated SeV- and EMCV-induced expression of type I IFNs and IL-6 in the sera, and potentiated EMCV-induced death of infected mice. Because Prkaca and Prkacb double knockout is lethal in mice [37], we are currently unable to directly determine the effects of PKACα and PKACβ deficiency on innate antiviral response in animals. Nevertheless, our studies provide solid evidences for the feed-back negative regulation of VISA-mediated innate antiviral response by the house-keeping kinase PKA, and certainly help to understand how innate immune response is terminated at the late phase of viral infection to avoid host damage.

Materials and methods

Ethics statement

All animal experiments were performed in accordance with the Wuhan University animal care and use committee guidelines.

Reagents and antibodies

Lipofectamine 2000 (Invitrogen); RNase inhibitor (Thermo); SYBR (Bio-Rad); mouse monoclonal antibodies against Flag, HA, and β-actin (Sigma), TBK1, phospho-TBK1 (Ser172) and phospho-Ser/Thr (Abcam), phospho-PKA substrate and phospho-IRF3 (Ser396) (Cell Signaling Technology), rabbit polyclonal antibodies against IRF3 (Santa Cruz Biotechnology) and rabbit polyclonal antibodies against VISA (Bethyl) were purchased from the indicated manufacturers; mouse anti- PKACα/β antisera were raised against recombinant human full-length PKACβ; SeV, EMCV and HSV-1 were previously described [38, 39]; HEK293 and THP1 cells (ATCC) were purchased from the indicated manufactures; HEK293T cells were originally provided by Dr. Gary Johnson (National Jewish Health).

Constructs

NF-κB, ISRE, STAT1/2, IFN-β promotor, and IRF1 promoter luciferase reporter plasmids, mammalian expression plasmids for Flag- or HA-tagged RIG-1, MDA5, VISA, MITA, TBK1 and IRF3 were previously described [40–42]. Flag- or HA-tagged PKACα, PKACβ, PKACγ and their mutants were constructed by standard molecular biology techniques.

RNAi

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.retro RNAi plasmid (Oligoengine). The targeting sequences are as following. Human
PKACα: 5′-GGGTGATGCTGGTGAA ACA-3′; Human PKACβ: 5′-GAAGAGTCATGTTGG TAAA-3′. The targeting sequences for both human PKACα and PKACβ (PKACs): #1: 5′-GAA GGTCCAGTGAGCCCCA-3′; #2: 5′-TAGCCAAAGCCCAAGAAGA-3′. siRNA oligonucleotides sequence for both mouse PKACα and PKACβ (PKACs): 5′-TAGCCAAAGCCCAAGAAGA TAAA-3′.

Transfection and reporter assays
Cells (1×10^5) were seeded in 24-well plates and transfected the following day by standard calcium phosphate precipitation. In the same experiment, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.01 μg of pRL-TK or pRL-SV40 (Renilla luciferase) reporter plasmid was added to each transfection [43, 44]. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega).

Co-immunoprecipitation and immunoblot analysis
For co-immunoprecipitation, cells (1×10^7) were lysed in 1 mL NP-40 lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). For direct analysis of protein expression, cells were lysed with SDS-PAGE loading buffer followed by ultra-sonication. Co-immunoprecipitation and immunoblot analysis were performed as previously described [45, 46].

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)
SDD-AGE was performed as previously described [47].

Ubiquitination assays
Ubiquitination assays were performed as previously described [48, 49].

Statistics
GraphPad Prism software was used for all statistical analyses. Quantitative data displayed as histograms are expressed as means ± SD (represented as error bars). Data were analyzed using a Student’s unpaired t test or multiple t test. The number of asterisks represents the degree of significance with respect to p values. Statistical significance was set at a p < 0.05. Mice in each sample group were selected randomly in mouse experiments. The sample size (n) of each experimental group is described in the figure legend.

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