PKC alpha regulates Sendai virus-mediated interferon induction through HDAC6 and β -catenin

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Recognition of viral RNA by cytoplasmic retinoic acid inducible gene I (RIG-I)-like receptors initiates signals leading to the induction of type I interferon (IFN) transcription via transcription factors such as interferon regulatory factor 3 (IRF3) and nuclear factor κ B (NF- κ B). Here, we describe a new signalling pathway that involves protein kinase C alpha (PKC α), histone deacetylase 6 (HDAC6) and beta-catenin (β -catenin), which is essential for IFN gene induction following virus infection. Knockdown of PKC α in various human cells, including primary cells, inhibited Sendai virus (SeV)-mediated IFN induction and enhanced virus replication. In the absence of this pathway IRF3 becomes activated, but does not bind to its promoter and is thus unable to support transcription. Mechanistically, SeV infection induced the activation of PKC α , which promoted its interaction with HDAC6 and enhanced its deacetylation activity in a phosphorylation-dependent manner. Further downstream, HDAC6 caused deacetylation of β -catenin and enhanced its nuclear translocation and promoter binding. In the nucleus, β -catenin acted as a co-activator for IRF3-mediated transcription. Our findings suggest an important role of a novel signalling pathway mediated by PKC α –HDAC6– β -catenin in controlling IRF3-mediated transcription.

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Introduction

Cellular innate immunity to virus infection is primarily provided by the sensing of viral double-stranded RNA (dsRNA) in the cytoplasm by retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). RIG-I has been shown to be particularly important for sensing RNA viruses such as Sendai virus (SeV), vesicular

stomatitis virus (VSV) and influenza virus, among others (Takeuchi and Akira, 2008). Upon binding to viral RNA, RIG-I forms a complex with its adaptor mitochondrial anti-viral-signalling protein (MAVS, also known as VISA, CARDIF and IPS-1) and other signalling adaptors on the mitochondrial surface. RIG-I binding to MAVS initiates a complex signal transduction process that leads to the transcriptional upregulation of a series of anti-viral genes including type I interferons (IFNs). Recent studies have shown that a number of cellular as well as viral gene products can modulate this signalling pathway in order to regulate the activation of the downstream transcription factors nuclear factor κ B (NF- κ B) and interferon regulatory factor 3 (IRF3). For example, ubiquitination of RIG-I by TRIM25 and Riplet (also known as RNF135) is essential for the upregulation of RLR signalling (Gack *et al*, 2007).

Calcium activated protein kinases (PKCs) are present ubiquitously in most cells and perform a large range of functions (Newton, 2003; Rosse *et al*, 2010). However, involvement of PKC in RLR signalling pathways has never been described. Among the three major groups of PKCs, the classical PKCs, PKC α , β 1 and β 2 are activated by calcium and diacylglycerol, and involved in various signal transduction pathways. PKC α controls endosomal trafficking in various cell types; calcium homeostasis and angiogenesis in vascular endothelial cells and degranulation and thrombosis in platelets (Konopatskaya and Poole, 2010). In terms of immune signalling, PKC α has been implicated in IgG class switching, and T-cell receptor expression and signalling (Trushin *et al*, 2003; Pfeiffer *et al*, 2006; von Essen *et al*, 2006). Based on pharmacological inhibition, classical PKCs have been reported to be important for Toll-like receptor (TLR) signalling pathways (Zhou *et al*, 2006; Johnson *et al*, 2007). However, the target(s) of PKC or the mechanism(s) of inhibition have not been described. Furthermore, uncertainty exists regarding the specific PKC isoform(s) involved in this process.

Like many other transcriptional signalling pathways, proteins involved in innate anti-viral response are modulated by reversible protein acetylation (Nusinzon and Horvath, 2005). Several histone acetylases such as CBP/p300, PCAF have been shown to associate and act as a co-activator for IRF-mediated transcription (Masumi and Ozato, 2001; Paulson *et al*, 2002; Suhara *et al*, 2002). Although histone deacetylases (HDACs) are associated with transcriptional repression, in the case of IFN and IRF signalling, they have been shown to be essential for transcription. Pharmacological inhibition of HDACs causes downregulation of IFN-stimulated genes (ISGs; Nusinzon and Horvath, 2003; Chang *et al*, 2004; Klampfer *et al*, 2004; Sakamoto *et al*, 2004), and IRF7- and IRF5-mediated transcription of cytokines (Caillaud *et al*, 2002; Feng *et al*, 2010). Ablation of histone deacetylase 6 (HDAC6) by siRNA has been shown to inhibit

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TLR3-mediated IFN- β induction (Nusinzon and Horvath, 2006). However, the upstream signalling pathway leading to HDAC6 activation and its downstream target in anti-viral signalling have remained elusive. HDAC6 is a member of the class II histone deacetylase family enzymes that is present in the cytoplasm, and involved in multiple cellular processes (Cohen and Yao, 2004; Yang and Gregoire, 2005; Boyault *et al*, 2007). Lysine deacetylation activity of HDAC6 has been demonstrated for a large variety of cytoplasmic protein substrates such as tubulin (Serrador *et al*, 2004), Hsp90 (Kovacs *et al*, 2005), Cortactin (Zhang *et al*, 2007) and β -catenin (Li *et al*, 2008). Functional consequences of each of these deacetylation events affects stress response, cell motility and endocytosis.

β -Catenin, a key effector molecule in Wnt signalling pathway (Nelson and Nusse, 2004), has been recently described as a co-activator of IRF3 (Yang *et al*, 2010). β -Catenin interacts with IRF3 to promote recruitment of p300 to the IFN- β promoter. However, the mechanism by which β -catenin is activated after virus infection remains unclear. In the study described here, we show that deacetylation of β -catenin by HDAC6 enhances its nuclear translocation and enhances IRF3-mediated transcription. Infection of cells with SeV leads to the activation of PKC α by self-phosphorylation, which then interacts and mediates phosphorylation of HDAC6 to enhance its activity. Pharmacological inhibition or loss of PKC α in human cells leads to incomplete activation of RIG-I signalling pathway and loss of type I IFN induction leading to increased viral replication. We provide, for the first time, a mechanistic link between PKC α , HDAC6 and β -catenin

activation and demonstrate the importance of this parallel signalling pathway in transcriptional activity of IRF3 and IFN- β induction.

Results

PKC is involved in IRF3 signalling pathway

To characterize the involvement of PKC in virus-mediated IFN induction, we used several well-characterized chemical inhibitors of PKC in luciferase reporter assays. IRF3-dependent gene induction was monitored by measuring ISG56 promoter driven luciferase activity. RL24 cells (Zhu *et al*, 2010) were infected with SeV (10 m.o.i.) in the presence of various concentrations of PKC inhibitors (Figure 1). Pan-PKC inhibitor Gö6850 and classical PKC inhibitor Gö6976 inhibited ISG56-luciferase activity in a dose-dependent manner (Figure 1A and B), whereas a PKC β -specific inhibitor did not show any appreciable inhibition (Figure 1C). SeV-mediated induction of endogenous ISG56 protein was similarly inhibited in HEK293 cells in presence of these inhibitors (Figure 1D). Other than virus, the RIG-I/MDA5 signalling pathway can also be activated by polyinosinic acid:polycytidylic acid (poly(I):poly(C)) transfection (Binder *et al*, 2011; Loo and Gale, 2011), which was also inhibited similarly by PKC inhibitors (Supplementary Figure S1A and B).

One of the hallmarks for classical PKC activation is the signal-dependent auto-phosphorylation of its C-terminal hydrophobic domain (Keränen *et al*, 1995). To further investigate the involvement of PKC in IRF3 signalling pathway,

Figure 1 Involvement of PKC α in IRF3 signalling pathway. (A–C) Inhibition of gene induction in the presence of PKC inhibitors. RL24 cells were pretreated with pan-PKC inhibitor Gö6850 (A), conventional PKC inhibitor Gö6976 (B) and PKC β inhibitor (PKC β Inh.) (C) at the indicated concentrations for 1 h, then stimulated with SeV at 10 m.o.i. for another 8 h. Firefly and Renilla luciferase activities were measured with the Dual-Glo luciferase kit. ISG56 promoter activity was expressed as fold induction relative to non-stimulated cells after normalizing to Renilla luciferase. (D) PKC inhibitors decrease endogenous ISG56 protein induction by SeV. HEK293 cells (2×10^5 cells/well in 24-well plate) were treated with 0.5% DMSO or PKC inhibitors for 1 h, and then stimulated with SeV (10 m.o.i.) for another 8 h as indicated. Cell lysates were analysed by western blotting for ISG56 and β -actin expression levels. (E) PKC is phosphorylated upon SeV infection. HEK293 cells (2×10^5 cells) were stimulated with SeV (10 m.o.i.) for the indicated time periods. Cell lysates were analysed by western blotting for phospho-pan PKC, PKC α , ISG56 and β -actin antibodies. (F) PKC α translocates from cytoplasm to plasma membrane during SeV infection. HEK293 cells (5×10^5 cells) were stimulated with SeV (10 m.o.i.) for the indicated time periods. Cell cytoplasm and plasma membrane fractions were isolated and detected by immunoblotting with PKC α and β -actin antibodies.

we measured PKC activation following SeV infection using phospho-PKC (pan) antibody. As shown in Figure 1E, SeV infection caused PKC phosphorylation, which peaked at 4 h post-infection. Kinetically, PKC phosphorylation preceded ISG56 induction (6 and 8 h post-infection), indicating that this step might be important for gene induction. Similarly, PKC phosphorylation was observed in the early phases of several other RNA (Coxsackie virus B (CVB) and VSV) and DNA (Herpes simplex virus 1 (HSV1)) virus infections (Supplementary Figure S2), which indicates that this pathway might be activated by a number of diverse viruses. To further confirm SeV-mediated PKC α activation, we monitored its cellular localization following SeV treatment. Upon SeV infection, PKC α showed significant translocation from the cytosolic to the plasma membrane fraction (Figure 1F), further supporting its activation by viral infection.

Classical PKC is activated by Ca²⁺ and diacylglycerol. Therefore, we hypothesized that Ca²⁺ signalling may be important for PKC activation and IRF3 signalling following virus infection. To test this, we pretreated RL24 cells with the cell permeable intracellular Ca²⁺ chelator BAPTA-AM, followed by either SeV infection or poly(I):poly(C) transfection. BAPTA-AM showed a dose-dependent inhibition of ISG56-luciferase activity (Supplementary Figure S3A and C). We also tested the effect of increasing intracellular Ca²⁺ using thapsigargin (Jin *et al*, 2006), a specific inhibitor of SERCA channels that results in depletion of ER-derived Ca²⁺ stores and net increases in intracellular Ca²⁺ levels. In contrast to our findings with BAPTA-AM, we found that thapsigargin treatment enhanced ISG56-luciferase activity (Supplementary Figure S3B and D). Taken together, these results indicate that classical PKCs, most likely PKC α , are activated by Ca²⁺ signalling in response to virus infection and are essential for virus-mediated IFN induction.

Loss of PKC α affects SeV-mediated gene induction

In the next series of experiments, we confirmed isozyme-specific involvement of PKCs in the IRF3 signalling pathway using siRNA-mediated knockdown of PKC α or PKC β . HEK293 cells were transfected with siRNAs against PKC α or PKC β and type I IFN induction determined by ISG56-luciferase reporter assay in SeV-infected cells. As shown in Figure 2A, specific knockdown of PKC α , but not PKC β , inhibited SeV-mediated induction of ISG56-luciferase activity. The inhibition of endogenous ISG56 and ISG60 (another IRF3-dependent gene; Sarkar and Sen, 2004; Fensterl and Sen, 2010) protein induction was confirmed by performing immunoblotting in siRNA-transfected HEK293 cells infected with SeV (Figure 2B). Knockdown of PKC α and PKC β was confirmed by immunoblotting (Figure 2B) and by quantitative RT-PCR (Supplementary Figure S4A), and infection with SeV was confirmed by immunoblotting with anti-SeV C-protein (Figure 2B).

To further explore the effects of PKC α knockdown on other IRF3 target genes, we measured endogenous mRNA induction of a number of selected primary response genes following SeV infection. Although some of these genes can be induced by type I IFN, they are also directly induced via IRF3 following SeV infection (Sarkar and Sen, 2004; Elco *et al*, 2005). Among them, inhibition of IFN- β induction was strongest after PKC α ablation (Figure 2C). Induction of other genes such as OASL, ISG56, ISG60, IP-10 and Viperin (Cig5)

was also significantly inhibited in samples from PKC α knock-down cells (Figure 2D–H). These results confirmed a specific role for PKC α in the induction of type I IFN and other genes after SeV infection.

PKC α knockdown affects IRF3-mediated transcription without affecting its activation

In order to understand the steps involved in PKC α -mediated modulation of IRF3 signalling, we generated stable cells (HEK293 and HT1080) in which PKC α was downregulated by shRNA. Several shRNA constructs targeting different regions of the PKC α -coding sequence were transfected into HEK293 cells followed by puromycin selection. Puromycin-resistant pools of cells derived from individual shRNA construct were tested for PKC α and PKC β expressions (Figure 3A). Cells obtained from shRNA clone#14 and clone#15 showed specific knockdown of PKC α protein and used for further experiments. As expected, SeV-mediated gene induction was inhibited in both cell lines compared with cells generated with a control shRNA construct (HEK293-derived cells are shown in Figure 3B and HT1080-derived cell lines are shown in Supplementary Figure S6A).

In the innate immune signalling pathway, IRF3 activation is carried out by Ser/Thr phosphorylation of its C-terminal activation domain via TBK1 or IKK ϵ , followed by its dimerization and nuclear translocation (Takeuchi and Akira, 2008). From here on, we define 'IRF3 activation' as its Ser396 phosphorylation and nuclear translocation only. We characterized the kinetics of IRF3 activation using these parameters upon SeV infection, which showed maximum activation at 6 h post-infection (Supplementary Figure S5A and C). However, we did not observe inhibition of IRF3 phosphorylation at Ser-396 or IRF3 nuclear translocation in any of the PKC α knock-down cells (Figure 3C and D, HEK293-derived cells). PKC α knockdown HT1080 cells also did not show any defect in IRF3 activation (Supplementary Figure S6B and C). Similar results were obtained when PKC α was transiently downregulated by siRNA transfection (Supplementary Figure S5B and D). Similar observations have been made regarding TLR3-dependent innate immune signalling (Sarkar *et al*, 2004). In the case of TLR3 signalling, incompletely activated IRF3 translocated to the nucleus, but failed to bind its corresponding promoter element and support transcription. Thus, we tested the recruitment of IRF3 to the ISG56 promoter in PKC α knockdown cells by chromatin immunoprecipitation (ChIP) (Figure 3E). Quantitative PCR was used to detect DNA recovered from antibody-bound chromatin segments. As shown in Figure 3E, SeV infection-induced IRF3 promoter binding was largely abrogated in PKC α knockdown cells compared with vector control cells, while control RNA Pol II binding was unaffected. These results indicate that in the absence of PKC α , although RIG-I-mediated activation of IRF3 was not affected, the ability of IRF3 to induce gene transcription is severely attenuated due to its failure to stably bind the promoter.

Enhanced replication of VSV in the absence of PKC α

Replication of VSV is highly sensitive to type I IFNs. We attempted to establish the physiological relevance of PKC α in IFN induction using VSV as a model system. We found a significantly higher level of VSV replication in PKC α knock-down HT1080 cells compared with control cells (Figure 4). Cells derived from two different PKC α shRNA constructs

Figure 2 Silencing PKC α , not PKC β , decreases SeV activated IRF3 signalling and gene induction. (A) PKC α knockdown decreases SeV-induced ISG56 promoter activity. HEK293 cells were co-transfected with PKC siRNA or control siRNA, together with ISG56 firefly luciferase reporter and β -actin Renilla luciferase. After 48 h, transfected cells were stimulated with SeV (10 m.o.i.) for 16 h, and luciferase activities were measured. (B) PKC α knockdown decreases SeV-induced ISG56 protein expression. HEK293 cells (2×10^5 cells) were transfected with PKC siRNA or control siRNA. Forty-eight hours post-transfection, cells were stimulated with SeV for 8 h. Whole cell lysates were analysed by western blotting using antibodies against ISG56, ISG60, PKC α , PKC β , SeV C and β -actin. (C–H) PKC α knockdown decreases SeV activated IRF3 regulated gene expression. HEK293 cells were transfected and stimulated as in (B), followed by total RNA extraction and analysis by quantitative RT-PCR for IFN- β (C), OASL (D) ISG56 (E) ISG60 (F), IP-10 (G) and Viperin (H) mRNA.

(shRNA#14 and shRNA#15) were used for these experiments (Supplementary Figure S6A). Infection of PKC α knockdown HT1080 cells with a GFP-tagged VSV (Das *et al*, 2006) resulted in increased GFP fluorescence compared with vector control cells (Figure 4A) and enhanced production of GFP by immunoblotting (Figure 4B). Therefore, PKC α is essential for

the induction of anti-viral IFN response, and ablation of PKC α results in increased virus replication.

PKC α interacts with and activates HDAC6

Having recognized the importance of PKC α in the induction of anti-viral innate immune responses, we sought to identify

Figure 3 PKC α knockdown does not affect IRF3 phosphorylation, nuclear translocation, but inhibits IRF3 DNA binding, and downstream ISG expression upon SeV infection. (A) HEK293 cells (2×10^5 cells) were transfected with the indicated PKC α -shRNA-pLKO.1 plasmids and control pLKO.1 vector using Fugene 6. The cell lysates from puromycin-resistant cells were analysed by western blotting for PKC α , PKC β and β -actin. (B) SeV-induced ISG protein expressions were decreased in PKC α stable knockdown cells. Puromycin-resistant pools obtained from PKC α shRNA constructs #14 and #15 and control vector transfected HEK293 cells were stimulated with SeV for 8 h. Whole cell lysates were analysed by immunoblotting for ISG56, ISG60, PKC α and β -actin expressions. (C) IRF3 phosphorylation by SeV is not affected in PKC α knockdown cells. PKC α stable knockdown HEK293 cells and vector control HEK293 cells were stimulated with SeV for 5 h, and analysed by western blotting using antibodies against phospho-IRF3 (Ser396) and IRF3. (D) SeV induced IRF3 nuclear translocation in PKC α knockdown cells. PKC α stable knockdown cells (shRNA#14) and control HEK293 cells (3×10^5 cells) were stimulated with SeV at the indicated concentrations for 6 h. Nuclear (NE) and cytoplasmic extracts (CP) controls were isolated and analysed by immunoblotting for IRF3. Antibody against nuclear protein DRBP76 was used for loading controls of nuclear fractions, while the same fractions were also western blotted with α -tubulin antibody as fractionation controls. (E) IRF3 binding to ISG56 promoter is impaired in PKC α stable knockdown cells. PKC α stable knockdown cells (shRNA#14) and control cells (1×10^7 cells) were stimulated with SeV for 6 h. The IRF3 binding to ISG56 promoter (ISRE) DNA and ISG56 exon 2 DNA was analysed by ChIP assay (left panel); whereas the RNA polymerase II binding to ISG56 promoter DNA and ISG56 exon 2 DNA as controls was also analysed in parallel (right panel). IgG binding to DNA was not observed in all above ChIP experiments. The results were expressed as fold change with respect to IRF3/RNA polymerase II binding to ISG56 promoter DNA in non-stimulated control 293 cells (*) after normalization with corresponding input DNA.

its target in the RIG-I signalling pathway. In a series of co-immunoprecipitation assays, we tested the interaction of PKC α with various known components of RIG-I signalling. PKC α was not found to interact with RIG-I, MDA5, MAVS, TRAF3, IKK ϵ , TBK1 or IRF3 (Supplementary Figure S7). Moreover, the observation that the loss of PKC α did not affect IRF3 activation, pointed us to search for unknown targets of PKC α that might be affecting transcription without affecting IRF3 activation via the RIG-I pathway.

Besides phosphorylation and ubiquitination, deacetylation has been shown to play an important role in IRF-dependent transcription (Caillaud *et al*, 2002; Sakamoto *et al*, 2004; Nusinzon and Horvath, 2006; Feng *et al*, 2010). Previously, Nusinzon and Horvath (2006) observed that specific knock-

down of HDAC6 impaired dsRNA-mediated IFN induction. However, no specific target protein has been identified to date, and the signalling pathway leading to HDAC activation has not been described. In the next series of experiments, we explored the possibility that HDAC6 might be involved in PKC α -mediated modulation of IRF3 transcription. In co-immunoprecipitation assays, HDAC6 was found to interact with PKC α in a signal-dependent manner (Figure 5A). Inhibition of HDAC6 activity using the HDAC6-specific chemical inhibitor CAY10603 not only inhibited ISG56 induction by SeV (Figure 5B), but also showed very similar phenotypes as was observed by PKC α inhibition. In the next series of experiments, we explored the mechanism of HDAC6 activation by PKC α . Phosphorylation-dependent HDAC6 activation

Figure 4 Enhanced VSV replication in PKC α knockdown HT1080 cells. **(A)** PKC α stably knockdown (shRNA #14 and #15) and vector control HT1080 cells (2×10^5 cells/well in 24-well plate) were infected with VSV-GFP at the indicated m.o.i. Forty-two hours post-infection, the GFP signals were photographed under fluorescent microscope. **(B)** Cell lysates from similarly infected HT1080 cells were analysed by western blotting for GFP, PKC α and β -actin.

has been previously described in ciliary disassembly mediated by aurora A kinase (Pugacheva *et al*, 2007). We tested whether PKC α directly phosphorylated HDAC6 using an *in vitro* kinase assay (Figure 5C). Due to the unavailability of an anti-HDAC6 antibodies capable of immunoprecipitating endogenous HDAC6, we tested exogenously expressed FLAG-tagged HDAC6 phosphorylation by endogenous PKC α . PKC α knockdown and vector control HEK293 cells were transfected with FLAG-HDAC6, followed by immunoprecipitation of HDAC6 with anti-FLAG antibody. Incubation of the immunoprecipitated samples with radiolabelled ATP in kinase buffer resulted in the increase in HDAC6 phosphorylation with SeV treatment (Figure 5C, lanes 3 and 4). This phosphorylation was reduced in PKC α knockdown cells (Figure 5C, lanes 1 and 2), demonstrating PKC α -dependent phosphorylation. However, there was remaining HDAC6 phosphorylation in untreated PKC α knockdown and control cells, which might be due to the incomplete knockdown

and/or the basal levels of PKC activity found in most cell lines.

One of the best-characterized substrates for HDAC6 is α -tubulin. We measured changes in HDAC6 activity by monitoring the levels of acetylated α -tubulin upon SeV infection. As shown in Figure 5D, SeV infection induced tubulin deacetylation (Figure 5D, lanes 5 and 6), which was abrogated in PKC α knockdown cells (Figure 5D, lanes 2 and 3). Finally, we tested the effects of HDAC6 inhibition or overexpression on IRF3 DNA binding by ChIP assay. As shown in Figure 5E, pretreatment of cells with HDAC6 inhibitor significantly ($P = 0.0124$) inhibited SeV-mediated IRF3 binding to ISG56 promoter, while overexpression of HDAC6 enhanced this binding compared with the vector control. These results suggested that upon SeV infection, PKC α recruits and activates HDAC6 by phosphorylation, which is needed for IRF3-dependent transcription.

β -Catenin is the downstream effector for PKC α -HDAC6-mediated IRF3 modulation

The multifunctional protein β -catenin has been recently identified as a co-activator of IRF3 (Yang *et al*, 2010) and was found to participate in signals induced by the nucleic acid sensor LRRFIP1. HDAC6 is known to be necessary to deacetylate and enhance β -catenin nuclear translocation in EGF receptor signalling pathway (Li *et al*, 2008). Based on these previous observations, we tested whether β -catenin is involved in PKC α -HDAC6-mediated modulation of IRF3. To that end, we measured nuclear translocation of β -catenin after SeV infection. Similar to the results obtained using dsRNA (Yang *et al*) we found significant β -catenin nuclear translocation upon SeV infection (Figure 6A). In an effort to establish the link between PKC α -HDAC6 and β -catenin, we performed co-immunoprecipitation studies. We found that endogenous PKC α and β -catenin co-immunoprecipitated, indicating that these components were present in a complex (Figure 6B), albeit not in a signal-dependent manner. Li *et al* described the unique deacetylation of β -catenin on Lys-49 induced by HDAC6. To determine if this was the case for IRF3 signalling, we immunoprecipitated β -catenin from SeV-infected cells and immunoblotted with anti-acetylated β -catenin (K49) antibody. As shown in Figure 6C, there was an appreciable reduction in Lys-49 acetylated β -catenin upon SeV infection in vector control HEK293 cells (Figure 6C, lanes 4–6, intensity ratio of acetyl- β -catenin/ β -catenin = 0.45, 0.39 and 0.19). However, this deacetylation was absent in PKC α knockdown cells (Figure 6C, lanes 1–3, acetyl- β -catenin/ β -catenin = 0.35, 0.35 and 0.49). This confirmed our hypothesis that PKC α controls HDAC6 activation, which causes β -catenin deacetylation. Furthermore, when we tested SeV-mediated β -catenin nuclear translocation in PKC α knockdown cells, it was abrogated (Figure 6D). This indicated that β -catenin deacetylation and nuclear translocation occurs in a PKC α -dependent manner. We also found that in the presence of the HDAC6 inhibitor CAY10603, SeV-mediated β -catenin nuclear translocation was inhibited. Finally, in a ChIP assay we tested the recruitment of β -catenin to the ISG56 promoter. As shown in Figure 6E, β -catenin was recruited to the ISG56 promoter upon SeV infection, which was absent in PKC α knockdown cells. These results demonstrate that β -catenin may be the target of PKC α -HDAC6 to mediate the modulation of IRF3 signalling.

Figure 5 PKC α interacts and activates HDAC6 upon SeV infection. (A) Interaction of PKC α with HDAC6. HEK293 cells (1×10^6 cells/well in 6-well plate) were transfected with FLAG-tagged HDAC6 and HA-tagged PKC α with or without SeV stimulation as indicated. Whole cell lysates were immunoprecipitated either with anti-FLAG or with anti-HA antibodies. The immunoprecipitates and cell lysate control samples were analysed by western blotting using anti-HA or anti-FLAG antibodies. (B) HDAC6 inhibitor inhibits SeV-induced ISG56 production. HEK293 cells (2×10^5 cells) were pretreated with 0.5% DMSO or CAY10603 at the indicated concentrations for 1 h, and then stimulated with SeV for 8 h. Cell lysates were analysed for ISG56 and β -actin expressions. (C) HDAC6 is phosphorylated by PKC α . PKC α stable knockdown cells (shRNA#14) and control cells (1×10^6 cells each) transfected with FLAG-tagged HDAC6 for 48 h were stimulated or not stimulated with SeV for 3 h. The HDAC6 was immunoprecipitated using anti-FLAG antibody, washed with lysis buffer and kinase buffer, and incubated with γ - 32 P ATP at 30°C for 30 min. The reactions were subjected to SDS-PAGE and phosphorylated HDAC6 was visualized by autoradiography of the dried SDS-PAGE gel. Part of the same immunoprecipitated samples was blotted with anti-FLAG antibody as controls. (D) PKC α -dependent increase in HDAC6 enzyme activity upon SeV infection. PKC α stable knockdown cells (shRNA#14) and control cells (2×10^5 cells) were stimulated with SeV at the indicated m.o.i. for 5 h. Cell lysates were analysed by western blotting using anti-acetyl- α -tubulin and anti- α -tubulin antibodies. (E) HDAC6 influences IRF3 binding to the ISG56 promoter. As indicated, HEK293 cells (1×10^7 cells) were either pretreated with DMSO or CAY10603 (0.5 μ M), or transfected for 48 h with vector or HDAC6, followed by SeV infection (6 h). IRF3 binding to ISG56 promoter or ISG56 exon2 (control) was analysed by ChIP assay.

Involvement of PKC α -HDAC6- β -catenin pathway in SeV-mediated gene induction in primary epithelial cells

To investigate the relevance of the PKC α -HDAC6- β -catenin signalling pathway in modulating RIG-I-IRF3 signalling in different cell types, we tested the sensitivity of IRF3 signalling pathway to PKC α and HDAC6 inhibition in a number of human cells. Human primary keratinocytes showed inhibition of ISG56 induction by SeV when treated with both PKC and HDAC6 inhibitors (Figure 7A). Similar results were obtained with HT1080, a cell line derived from human fibrosarcoma, immortalized normal human fibroblasts BJ-Tert (Hein *et al*, 2009) and immortalized normal human keratinocytes (Spardy *et al*, 2008; Supplementary Figure S8). We also observed enhanced β -catenin nuclear translocation after SeV infection in all these three cell types (Supplementary Figure S9), indicating the involvement of β -catenin in all different cell types. Finally, using human primary keratinocytes we established the specific involvement of PKC α in anti-viral signalling. siRNA-mediated transient knockdown of PKC α in primary human keratinocytes strongly inhibited ISG56 induction following SeV treatment (Figure 7B).

Together, these results establish a novel signalling pathway which along with the RIG-I-MAVS-TRAF3-IKK ϵ /TBK1 plays an important role in SeV-mediated gene induction

(Figure 7C). After SeV infection, PKC α is activated via a yet unknown mechanism possibly involving calcium. Activated PKC α causes HDAC6 activation by phosphorylation. HDAC6 mediates deacetylation of β -catenin, enhances its nuclear translocation where it binds to IRF3 and help recruit CBP/p300 to start transcription.

Discussion

In this study, we provide evidence for a novel signalling pathway involving PKC α , HDAC6 and β -catenin in IRF3-mediated gene induction (summarized in Figure 7C). We not only show that virus infection induces the isozyme-specific activation of PKC α , but also show for the first time that this activation plays an important role in IRF3 activation. Activation of PKC α is a necessary prerequisite for the phosphorylation-mediated induction of HDAC6 activity, which subsequently mediates the deacetylation and nuclear translocation of β -catenin. Although HDAC6-mediated deacetylation of β -catenin has been shown for EGFR signalling, it has not been implicated in anti-viral signalling. Our studies reveal a new layer of regulation in the induction of anti-viral innate immune responses, which is induced via PKC α -HDAC6 and β -catenin. Our findings tie together some seemingly unrelated

Figure 6 Involvement of β -catenin in IRF3 signalling pathway. (A) Nuclear translocation of β -catenin upon SeV infection. HEK293 cells (3×10^5 cells) were stimulated with SeV (10 m.o.i.) for the indicated time periods. Nuclear and cytoplasmic extracts were analysed by western blotting for β -catenin and DRBP76. (B) β -Catenin immunoprecipitates with PKC α . Cell lysates from HEK293 cells (1×10^6 cells) with or without SeV stimulation were immunoprecipitated with anti-PKC α or control IgG; the immunoprecipitate and lysate control samples were analysed by western blotting with anti- β -catenin and anti-PKC α antibodies. (C) β -Catenin is deacetylated upon SeV infection. PKC α knockdown (shRNA#14) and vector control HEK293 cells (1×10^6 cells) were infected with SeV as indicated for 5 h. β -Catenin was immunoprecipitated from all samples with anti- β -catenin antibody followed by immunoblotting with anti-acetyl- β -catenin (K49) and anti- β -catenin antibodies. (D) β -Catenin nuclear translocation is decreased in PKC α knockdown cells. PKC α stable knockdown cells (shRNA#14) and control cells (3×10^5 cells) were stimulated with SeV at the indicated m.o.i. for 6 h, and nuclear and cytoplasmic extracts were analysed by western blotting as in (A). (E) β -Catenin binding to ISG56 promoter is impaired in PKC α stable knockdown cells. PKC α stable knockdown cells (shRNA#14) and control cells (1×10^7 cells) were stimulated with SeV for 6 h. The β -catenin binding to ISG56 promoter DNA (ISRE) and ISG56 exon 2 DNA as a control was analysed by ChIP assay. IgG binding to neither ISG56 promoter DNA (ISRE) nor ISG56 exon 2 DNA was observed. The results were expressed as fold changes with respect to β -catenin binding to ISG56 promoter DNA in non-stimulated control 293 cells (*) after normalization by corresponding input DNA.

prior observations, such as the essential role for HDAC6 in the induction of IFN- β gene expression (Nusinzon and Horvath, 2005) and the recently uncovered role for β -catenin mediating IRF3 signalling (Yang *et al*, 2010).

Both PKC α and HDAC6 have been reported to play roles in TLR3-mediated dsRNA signalling (Johnson *et al*, 2007). Based on our findings, it is logical to functionally connect these two observations and predict that the same pathway may be involved in TLR3-IRF3 signalling. It is also possible that a similar mechanism is involved in TLR4-mediated IRF3 activation, which has also been reported to require PKC activity (Zhou *et al*, 2006). Although HDAC activity and multiple PKC isoforms have been implicated in IFN signalling (Ivaska *et al*, 2003; Sakamoto *et al*, 2004; Redig and Platanius, 2007), it remains to be determined whether a similar PKC-HDAC-mediated pathway is necessary. It is possible that other

IRFs may need additional co-activators such as β -catenin for p300/CBP recruitment.

An outstanding issue arising from our findings is the mechanism by which PKC α is activated by SeV infection. As shown in Supplementary Figure S7, PKC α did not show detectable interaction with RIG-I, and siRNA-mediated knockdown of RIG-I did not affect SeV-mediated PKC α phosphorylation (although it inhibited ISG56 induction, Supplementary Figure S10). Several recent findings linking virus entry and activation of calcium-mediated signalling pathways may provide important clues. For example, entry of CVB into polarized endothelial cells has been shown to induce intracellular calcium release (Bozym *et al*, 2010) and similar observations have been made for several other viruses (Collins *et al*, 2004; Scherbik and Brinton, 2010). In fact SeV infection does alter the kinetics of Ca^{2+} release from

Figure 7 Involvement of PKC α -HDAC6- β -catenin signalling pathway in SeV-mediated IRF3 target gene induction in primary epithelial cells. (A) Inhibition of SeV-mediated ISG induction in primary human keratinocytes by PKC and HDAC6 inhibitors. Keratinocytes (3×10^5 cells) were pretreated with the indicated PKC and HDAC6 inhibitors or vehicle control for 1 h, and then stimulated with SeV for another 8 h at 10 m.o.i. Cell lysates were analysed by western blotting for ISG56, ISG60 and β -actin. (B) Inhibition of SeV-induced ISG56 in primary human keratinocytes by PKC α siRNA. Keratinocytes (1.5×10^5 cells) were transfected with PKC α and control siRNA (50 nM each) using Lipofectamine RNAiMAX. Forty-eight hours post-transfection, cells were stimulated with SeV at the indicated m.o.i. for 12 h. Cell lysates were analysed by western blotting for ISG56, PKC α and β -actin. (C) The schematic model for PKC α regulation of virus-mediated IRF3 activation and gene expression through HDAC6- β -catenin cascade.

ER-derived stores, when measured using thapsigargin treatment after 2–3 h post-infection (Supplementary Figure S11). For VSV, it has been demonstrated that virus particle size determines the nature of endocytosis and actin assembly that follows (Cureton *et al*, 2010). Therefore, it is possible that the PKC pathway is activated via calcium signalling during virus entry and/or intracellular trafficking. A corollary of this hypothesis would be that viruses of different sizes may give rise to varying extents of PKC phosphorylation, which may determine the outcome of IRF3 signalling. Furthermore, the route of endocytosis utilized by a given virus might also play an important role in determining the mechanism by which PKC α is activated and whether this activation leads to IRF3 induction.

Along with IRF3, NF- κ B is also activated by RIG-I signalling. We observed inhibition of SeV-stimulated NF- κ B-mediated gene induction in PKC α knockdown cells (Supplementary Figure S4B–D). However, it remains to be determined whether this is also mediated by HDAC6- β -catenin-dependent downstream signalling. Moreover, in response to certain stimuli, the NF- κ B p65 subunit is known to be phosphorylated by PKC in order to modulate its transcriptional ability (Chen and Greene, 2004). Therefore, it is possible that the requirement of PKC α in the case of RIG-I-NF- κ B signalling may be to appropriately phosphorylate p65 and not activate HDAC6.

In the context of anti-viral innate immunity, components of Wnt- β -catenin signalling pathways, such as Glycogen synthase kinase 3 β (GSK3 β) and β -catenin have been shown to play important roles (Wang *et al*, 2008; Lei *et al*, 2010; Yang *et al*, 2010). However, a consensus picture has not emerged, likely as a consequence of the use of different experimental systems. Our findings that the PKC α -HDAC6- β -catenin pathway is required for IRF3 signalling in epithelial cells and fibroblasts may explain some of the contradictory findings reported in the literature. Similarly, PKC α has been reported to phosphorylate β -catenin in the context of Wnt5a signalling to exert a negative effect on Wnt/ β -catenin pathway (Gwak *et al*, 2006). However, our findings that the inhibition of HDAC6 activity impaired SeV-induced β -catenin nuclear translocation argues against a direct role for PKC α in β -catenin phosphorylation. Furthermore, in our study, PKC α activation does not act as a negative regulator, but rather as a positive regulator of β -catenin co-activator activity.

As we and others have shown, the phosphorylation and nuclear translocation of IRF3 are essential but not sufficient for activation of transcription (Sarkar *et al*, 2004; Clement *et al*, 2008; Noyce *et al*, 2009). Further events such as phosphorylation and/or co-activator activation are necessary to induce IRF3 transcriptional ability. These additional requirements for IRF3 transcriptional activity can be rationalized from the fact that unlike other IRFs, IRF3 is constitutively expressed in most cell types. Therefore, these extra co-activator activation requirements may provide an essential layer of control to prevent unintentional gene induction.

Induction of type I IFNs by epithelial cells plays an important role in attenuating a viral infection. As such, many viruses have evolved mechanisms to suppress type I IFN induction as a means to limit host defenses. It is interesting to speculate that the PKC-HDAC6- β -catenin pathway described herein is also targeted by viruses to abrogate

IRF3-mediated induction of type I IFNs. The fact that influenza virus infection has been shown to induce the proteolytic cleavage of HDAC6 (Husain and Harrod, 2009) may represent a novel evasion mechanism that may be shared by other viruses to suppress this powerful arm of the anti-viral response pathway.

In summary, we show that PKC α is an essential component in the induction of IRF3 by RLRs. PKC α induces the deacetylation of β -catenin by activation of HDAC6, which in turn increases the nuclear translocation of β -catenin. Once in the nucleus, β -catenin cooperates with IRF3 to bind respective promoter elements to enhance anti-viral gene transcription. These findings support a role for a novel signalling pathway in modulating anti-viral innate immune responses and IFN induction.

Materials and methods

Cells and reagents

HT1080, HEK293, HEK293-derived reporter cell line RL24 (Zhu *et al*, 2010) and BJ-Tert cells (Hein *et al*, 2009) were cultured in DMEM (Lonza, Rockland, ME) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and Penicillin/Streptomycin. Immortalized human keratinocytes (Spardy *et al*, 2008) and primary human keratinocytes (purchased from Invitrogen, Carlsbad, CA) were maintained in EpiLife medium (Invitrogen) containing human keratinocyte growth supplement (Invitrogen). SeV (Cantell Strain) was purchased from Charles River Laboratories (Wilmington, MA). PKC inhibitors Gö6850 and Gö6976 were from Enzo Life Science (Plymouth Meeting, PA) and PKC β inhibitor was from EMD Biosciences (Gibbstown, NJ). HDAC6 inhibitor CAY10603 was from Cayman Chemical (Ann Arbor, MI) (Kozikowski *et al*, 2008). All the inhibitors were added to the medium as DMSO stock solutions at their final working concentrations while keeping final DMSO concentrations below 0.5%, at which there were no significant effect on gene induction (Supplementary Figure S1C and D). poly(I):poly(C) (GE Healthcare, Piscataway, NJ) was transfected at 1 μ g/ml with Fugene6 (Roche, Indianapolis, IN). All primers were custom synthesized by Integrated DNA Technologies (Coralville, IA). Transfection reagent Fugene 6 and anti-GFP antibody were from Roche. Anti-phospho-IRF3 (Ser396), anti-phospho-pan-PKC and anti-acetyl- β -catenin (K-49) antibodies were from Cell Signaling Technology Inc. (Beverly, MA). Anti-PKC α and anti-PKC β antibodies were from BD Biosciences (San Jose, CA). Anti- β -actin, anti- β -catenin, anti-IRF7, anti-HA antibodies and protein A/G agarose beads were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-FLAG, anti-acetyl- α -tubulin antibodies and anti-FLAG beads were from Sigma-Aldrich (St Louis, MO). Anti-ISG56, anti-ISG60, anti-IRF3, anti-DRBP76, anti- α -tubulin and anti-SeV C antibodies have been described before (Sarkar and Sen, 2004; Peters *et al*, 2008). Enhanced GFP-Tagged VSV was a gift from Dr Asit K Pattnaik (Das *et al*, 2006). GFP-Tagged HSV1 (strain KOS) and Coxsackie virus B have been described before (Desai and Person, 1998; Bozym *et al*, 2011).

Transient and stable knockdown of PKC

HEK293 cells (3×10^5 cells/well) in 24-well plate were transfected using Lipofectamine RNAiMAX (Invitrogen) with the PKC α , PKC β and control negative stealth RNAi siRNAs (50 nM each) (Invitrogen). Cells were used 48 h post-transfection for downstream applications. To prepare PKC α stable knockdown HEK293 cells, the cells were transfected by Fugene 6 with PKC α -shRNA-pLKO.1 constructs (Sigma-Aldrich), and then selected with 1 μ g/ml puromycin. PKC α stable knockdown HT1080 cells were prepared by packaging the PKC α shRNA pLKO.1 into lentivirus followed by infection and puromycin selection of HT1080 cells.

ISG56 reporter assay

HEK293 (3×10^5 cells/well) in 24-well plate were co-transfected by Lipofectamine 2000 (Invitrogen) with PKC siRNA or control siRNA (50 nM), ISG56 firefly luciferase reporter (0.4 μ g) and β -actin Renilla luciferase reporter (0.012 μ g). Twenty-four hours later, the cells

from each well were collected by trypsin digestion and seeded into six wells in white-wall 96-well plate. Forty-eight hours post-transfection, the cells were stimulated with SeV for 16 h, and luciferase activities were measured using the Dual-Glo luciferase assay system from Promega (Madison, WI). The results were expressed as fold induction of ISG56-luciferase relative to that of non-stimulated control siRNA-transfected cells after normalizing to Renilla luciferase.

Quantitative RT-PCR analysis of gene expression

Total RNA was isolated from transfected and/or stimulated cells by Trizol (Invitrogen), and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). For each sample, 1/20th of the cDNA synthesized from 1 μ g RNA was used and subjected to real-time PCR using SsoFast EvaGreen Supermix in a CFX96 Real Time System (Bio-Rad) according to the manufacturer's instructions. The primers used were IP-10 (5'-GTGGCATTCAAGGACTA CCTC-3'; 5'-GCCTTCGATTCTGGATTACAGACA-3'), OASL (5'-AGGGT ACAGATGGGACATCG-3'; 5'-AAGGGTTACAGATGAGGTTG-3') and Viperin (5'-CAGTGCTTGCATTGCTTTGT-3'; 5'-TCAGAAATGTAGG GCCAACCC-3'). The primers for human ISG56, ISG60 and IFN- β have been described before. Each PCR amplification was normalized to ribosomal protein L32 (RPL32; Zhu *et al*, 2010).

ChIP assay

ChIP was performed using the ChIP-IT Express kit from Active Motif according to the manufacturer's protocols. Briefly, 1×10^7 HEK293 cells in a 10-cm dish were crosslinked with 1% formaldehyde for 10 min. Cells were lysed and chromatin was sheared into 200–600 bp in length by sonication. For each sample, 25 μ g chromatin was mixed with protein G magnetic beads and anti-IRF3 (3 μ g) or anti- β -catenin (3 μ g) antibody and control anti-RNA polymerase II antibody and IgG to precipitate the target protein. The protein/DNA complex was uncrosslinked, DNA purified and together with input DNA subjected to quantitative PCR analysis. Primers used were ISRE (5'-GAATCCGCTAGCTTTAGTTTCAC-3'; 5'-CCCCAAGACAGT GTTATATAAGGG -3') and negative control primer ISG56 exon 2 (5'-CTCCTTGGGTTCGTCTACAA-3'; 5'-AATGAAATGTGAAAGTGGCTG ATA-3'). Each sample was normalized to corresponding input DNA and expressed as fold change with respect to untreated controls from ISRE samples (*).

Virus infection

HT1080 cells transfected with PKC α siRNA or stable PKC α knock-down HT1080 cells in 24-well plates were infected with VSV expressing enhanced GFP (VSV-GFP) at different m.o.i. Forty-two hours post-infection, the GFP signal was examined under fluorescence microscope and cells were harvested for western blotting analysis.

Subcellular fractionations

Cell plasma membrane and cytoplasmic fractions were isolated as described (Paone *et al*, 2008) with minor modifications. Following stimulation, cells were washed and cell pellets were resuspended in hypotonic buffer (20 mM HEPES (pH 8.0), 10 mM KCl, 1 mM MgCl₂, 20% glycerol) with protease inhibitors. The cell suspensions were sonicated, incubated on ice for 15 min and centrifuged (20 000 g for 1 h at 4°C). The supernatants were collected as cytoplasmic fraction. Pellets were resuspended in same volume of above hypotonic buffer plus 0.1% Triton X-100, incubated on ice for 30 min and centrifuged (20 000 g for 10 min at 4°C). The resultant supernatants were plasma membrane fractions. To isolate cytoplasmic and nuclear fractions, cells were suspended in the hypotonic buffer plus 0.1% Triton X-100, incubated on ice for 10 min, vortexed for 30 s, and centrifuged (12 000 g for 10 min at 4°C). The supernatants were used as cytoplasmic fractions. The pellet nuclei were thoroughly washed and lysed in lysis buffer (20 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1.5 mM MgCl₂, 12.5 mM β -glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF and $1 \times$ protease inhibitors).

Co-immunoprecipitation and western blotting

In all, 1×10^6 cells in 6-well plate transfected and/or treated with SeV were lysed in lysis buffer (see above). The cell lysates were incubated at 4°C with antibody plus protein A/G agarose beads or anti-FLAG beads overnight, washed three times with lysis buffer, and boiled in $1 \times$ SDS-PAGE loading buffer, separated by

SDS-PAGE and analysed by immunoblotting. All other cellular fractions and whole cell lysates were also boiled in 1 $\times$ SDS-PAGE loading buffer, separated by SDS-PAGE and analysed by immunoblotting.

HDAC phosphorylation assay

One million cells transfected with FLAG-HDAC6 and stimulated with SeV were lysed in lysis buffer. The cell lysates were incubated with anti-FLAG beads overnight and washed with lysis buffer for three times and kinase buffer once (25 mM Tris-HCl pH 7.5, 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄ and 10 mM MgCl₂). The washed immunoprecipitates were incubated with 5 μ Ci γ -³²P ATP in 30 μ l kinase buffer with 200 μ M ATP for 30 min at 30°C. The reactions were boiled in 1 $\times$ SDS-PAGE loading buffer, separated by SDS-PAGE and analysed by autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: JZ designed and performed most of the experiments, CBC designed and performed Ca²⁺ signalling experiments, SNS designed and performed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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