RIG-I from waterfowl and mammals differ in their abilities to induce antiviral responses against influenza A viruses

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The retinoic acid-induced gene I (RIG-I) plays a crucial role in sensing viral RNA and IFN-β production. RIG-I varies in length and sequence between different species. We assessed the functional differences between RIG-I proteins derived from mammals and birds. The transfection of duck caspase recruitment domains (CARDs) and duck RIG-I (dCARDs and dRIG-I) and goose CARDs and goose RIG-I (gCARDs and gRIG-I) into chicken DF-1 cells increased the production of IFN-β mRNA and IFN-stimulated genes and decreased influenza A virus (IAV) replication; whereas human CARDs and RIG-I (hCARDs and hRIG-I) and mouse CARDs and RIG-I (mCARDs and mRIG-I) had no effect. In human 293T and A549 cells, hCARDs had the strongest IFN-inducing activity, followed by mCARDs, dCARDs and gCARDs. The IFN-inducing activity of hRIG-I was stronger than that of mRIG-I, dRIG-I and gRIG-I, in that order. The results showed that, although the ability of dCARDs to activate IFN was stronger than that of gCARDs in DF-1, 293T and A549 cells, dRIG-I had a weaker ability to activate IFN than gRIG-I in DF-1 cells with or without IAV infection. These data suggest that RIG-I proteins from different species have different amino acid sequences and functions. This genetic and functional diversity renders RIG-I flexible, adaptable and capable of recognizing many viruses in different species.

INTRODUCTION

Type I IFNs and other pro-inflammatory cytokines are produced when host pattern recognition receptors, such as the Toll-like receptors and the retinoic acid-induced gene I (RIG-I)-like receptors, recognize pathogen-associated molecular patterns including nucleic acids or other structural components of microbes (Creagh & O’Neill, 2006; Kawai & Akira, 2009). RIG-I, as a cytoplasmic sensor of viral dsRNA and ssRNA, can be triggered by viral RNA (vRNA). RIG-I is composed of two N-terminal caspase recruitment domains (CARDs), a central DExD/H box helicase/ATPase and a C-terminal regulatory domain (Hausmann et al., 2008). Under normal conditions, RIG-I is in an inactive, closed conformation (Takahasi et al., 2008). Viral ssRNA or short dsRNA bearing a 5’-triphosphate can bind to the DExD/H box helicase domain at its C terminus, which causes RIG-I to undergo a conformational change that exposes the CARDs to the cytoplasm (Saito et al., 2007). The exposure of the RIG-I N-terminal CARDs leads to the Lys63-linked ubiquitination of Lys172. The ubiquitinated CARDs then allow RIG-I to bind to MAVS (also called IPS-1, CARDsif or VISA) (Kawai et al., 2005; Meylan et al., 2005; Xu et al., 2005). Other components of the pathway are also recruited to this multi-protein signal complex, which activates TANK-binding kinase 1 and IκB kinase (Meylan et al., 2005; Xu et al., 2005). The activation of these kinases results in the phosphorylation and activation of interferon regulatory factor (IRF)-3 and IRF-7, which form homo- and heterodimers, translocate into the nucleus and bind to IFN-stimulated response elements to stimulate the expression of type I IFN genes and a set of IFN-inducible genes (Honda et al., 2005; Nakhaei et al., 2006).

RIG-I has been identified in many species including mammals (humans, mice, pigs and rabbits), birds (ducks, geese, pigeons and zebra finches) and fish (trout and carp). The signalling pathways activated by human RIG-I (hRIG-I) and mouse RIG-I (mRIG-I) are well known (Loo & Gale, 2011). As a natural reservoir of most influenza A strains, waterfowl have a crucial role in the ecology and evolution of influenza A viruses (IAVs) (Webster et al., 1992). A switch with hRIG-I and mRIG-I, duck RIG-I (dRIG-I) and goose RIG-I (gRIG-I) also induce type I IFN after influenza infection and poly I: C transfection (Barber et al., 2010; Sun et al., 2013). Although dRIG-I and gRIG-I share similar CARDs and a helicase domain with hRIG-I and mRIG-I, the amino acids

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One supplementary figure and two supplementary tables are available with the online Supplementary Material.
Ser8 and Thr170, which are critical for phosphorylation, and Thr55 and Lys172, which are essential for interaction and polyubiquitination by the tripartite motif containing 25 (TRIM25) in humans, are not conserved in mice, ducks or geese. Recently, Miranzo-Navarro & Magor (2014) demonstrated that although TRIM25-mediated ubiquitination occurs at Lys167 and Lys193, the activation of dRIG-I is independent of the anchored ubiquitination, suggesting that the RIG-I signalling pathway functions differently in waterfowl and mammals.

In this study, we examined the ability of the CARDs of RIG-I and the full-length RIG-Is encoded by humans, mice, ducks and geese to induce type I IFN in chicken DF-1 cells, human A549 and human 293T cells during IAV infection from mice, swine and avian species. Results showed that the ability of waterfowl RIG-Is to induce the expression of antiviral genes was stronger than mammalian RIG-Is in chicken DF-1 cells during IAV infection. However, mammalian RIG-Is induced greater expression of antiviral genes than waterfowl RIG-Is in human 293T and A549 cells during IAV infection. Waterfowl RIG-I interfered with the replication of IAV more efficiently than mammalian RIG-Is in DF-1 cells; whereas, mammalian RIG-Is were more efficient than waterfowl RIG-I in A549 and Madin–Darby canine kidney (MDCK) cells.

RESULTS

Protein expression of RIG-I and CARDs in chicken DF-1 cells and human 293T cells

RIG-Is and CARDs tagged with EGFP were generated and transfected into 293T or DF-1 cells. The detection of EGFP-tagged RIG-I and CARDs using fluorescence microscopy indicated that significant levels of EGFP-tagged RIG-I and CARDs accumulated in 293T and DF-1 cells (Fig. 1a, b). The fluorescence intensity of waterfowl-derived RIG-I and CARDs was significantly higher than that of mammalian-derived RIG-I and CARDs (Fig. 1a, b). The expression of EGFP-tagged CARDs and RIG-I proteins derived from humans, mice, ducks and geese was also detected in DF-1 cells (Fig. 1c) and 293T cells (Fig. 1d) using Western blotting with anti-EGFP antibody. The expression of human CARDs (hCARDs) and mouse CARDs (mCARDs) proteins was significantly lower than the expression of both duck CARDs (dCARDs) and goose CARDs (gCARDs) and hRIG-I, mRIG-I, dRIG-I and gRIG-I in DF-1 and 293T cells. These data suggest that waterfowl-derived RIG-Is and CARDs are expressed more efficiently than mammal-derived RIG and CARDs in both DF-1 and 293T cells.

Waterfowl RIG-Is induce the expression of antiviral genes more strongly than mammalian RIG-Is in chicken DF-1 cells during IAV infection

To evaluate the differences in IFN-inducing activity between mammal-derived and waterfowl-derived RIG-Is and CARDs variants in chicken DF-1 cells, cells were transfected with EGFP-tagged RIG-I and CARDs expression plasmids (Fig. 1a, c) and then infected with influenza viruses or mock treated. The mRNA expression of IFN-β and the antiviral IFN-stimulated genes Mx1 and PKR were measured using quantitative real-time PCR (qRT-PCR) at 8 h post-infection (p.i.) with SW731 (A/White/Scotland/2007 (H1N1)), ZB07 (A/Chicken/Scotland/ZB/2007 (H9N2)) or WSN (A/WSN/33 (H1N1)) viruses. Transfection with dCARDs, gCARDs, dRIG-I and gRIG-I alone induced significantly more IFN-β, Mx1 and PKR synthesis compared with empty vector transfection with or without IAV infection (Fig. 1a–f). However, hCARDs, mCARDs, hRIG-I and mRIG-I transfection did not increase the mRNA production of IFN-β, Mx1 or PKR compared with empty vector transfection with or without IAV infection (Fig. 1a–f). Transfection with mRIG-I significantly inhibited IFN-β, Mx1 and PKR mRNA production only in the WSN infection group (Fig. 2b, d, f). Although dCARDs induced more IFN-β, Mx1 and PKR mRNA production than observed with gCARDs, dRIG-I induced significant less IFN-β, Mx1 and PKR mRNA production than gRIG-I (Fig. 2a–f). Infection with the ZB07, WSN and SW731 viruses also reduced the gRIG-I-mediated increases of Mx1 and PKR mRNA expression compared with the mock-treated group. The inhibitory effects of WSN were the strongest (Fig. 2d, f). These observations suggest that transfected waterfowl RIG-I and CARDs have a greater ability to induce antiviral gene expression than mammalian RIG-I and CARDs in DF-1 cells during IAV infection. dCARDs were more efficient in inducing IFN-β mRNA production than gCARDs.

Mammalian RIG-Is induce the expression of antiviral genes more strongly than waterfowl RIG-Is in human cells during IAV infection

To assess the differences in IFN-inducing activity between mammalian and waterfowl RIG-Is in 293T cells, cells were transfected with EGFP-tagged RIG-I and CARDs expression plasmids (Fig. 1b, d) and were then infected with influenza viruses or mock treated. The mRNA expressions of IFN-β, Mx1 and PKR were detected using qRT-PCR at 8 h p.i. hCARDs and mCARDs induced increased IFN-β mRNA production compared with dCARDs and gCARDs in the presence or absence of IAV infection (Fig. 3a). hCARDs had the strongest ability to induce IFN-β expression, followed by mCARDs, dCARDs and gCARDs (Fig. 3a). hRIG-I had the most pronounced ability to induce IFN-β, followed by mRIG-I, dRIG-I and gRIG-I (Fig. 3b). Although gRIG-I transfection increased IFN-β synthesis (Fig. 3b), this increase of IFN-β expression was no greater than that observed with transfection with empty vector after IAV infection (Fig. 3b). gRIG-I blocked IFN-β mRNA production during WSN or SW731 infection (Fig. 3b). Because of the low expression of Mx1 and PKR in 293T cells (data not shown), we also performed the transfections and
Avian and mammalian RIG-Is differ in antiviral response

Fig. 1. Expression levels of RIG-Is and CARDs in DF-1 cells (a) and 293T cells (b). After 24 h, fluorescence microscopy was used to confirm RIG-Is and CARDs expression. (i) Vector, (ii) hCARDs, (iii) mCARDs, (iv) dCARDs, (v) gCARDs, (vi) hRIG-I, (vii) mRIG-I, (viii) dRIG-I and (ix) gRIG-I. Bars, 100 μm. After 24 h, for DF-1 cells (c) or 293T cells (d) transfected with EGFP-tagged RIG-I and CARD expression plasmids or empty vector, cell lysates were separated by SDS-PAGE and probed with anti-EGFP and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. The relative fluorescence intensities were analysed with Image-Pro-Plus (Media Cybernetics). Results are representative of two independent experiments. Data represent means ± SEM from three wells per group. *P ≤ 0.05 vs dCARDs, †P ≤ 0.05 vs dRIG-I, ‡P ≤ 0.05 vs gRIG-I.

Infections in a human lung adenocarcinoma epithelial cell line (A549 cells). Fig. 4(a, b) shows that hRIG-I, mRIG-I, dRIG-I, gRIG-I, hCARDs, mCARDs, dCARDs and gCARDs had similar IFN-β-inducing ability in A549 and 293T cells. Fig. 4(c, e) shows that hCARDs induced the highest mRNA levels of Mx1 and PKR, followed by mCARDs, dCARDs and gCARDs. hRIG-I had the strongest ability to induce Mx and PKR, followed by mRIG-I, dRIG-I and gRIG-I (Fig. 4d, f). Although gCARDs facilitated IFN-β, Mx1 and PKR mRNA production (Fig. 4a, c, e), gRIG-I inhibited IFN-β, Mx1 and PKR mRNA synthesis compared with empty vector transfection after IAV infection (Fig. 4b, d, f).
**Fig. 2.** Waterfowl RIG-Is and CARDs induce innate immune genes more strongly than mammalian RIG-Is and CARDs in DF-1 cells during IAV infection. CARDs-transfected (a) and RIG-I-transfected (b) DF-1 cells responded to IAV infection (m.o.i. = 1) by increasing the expression of chicken IFN-β relative to empty vector-transfected and mock-treated cells. DF-1 cells were transfected with EGFP-tagged plasmids or empty vector and infected with SW731, WSN or ZB07 viruses, or mock treated, 24 h later. qRT-PCR was performed on extracted RNA at 8 h p.i. CARDs-transfected (c) and RIG-I-transfected (d) DF-1 cells responded to IAV infection by an increase of chicken Mx1 expression relative to empty vector-transfected and mock-treated cells. DF-1 cells were transfected with EGFP-tagged plasmids or empty vector and infected with SW731, WSN or ZB07 viruses, or mock treated, 24 h later. qRT-PCR was performed on extracted RNA at 8 h p.i. CARDs-transfected (e) and RIG-I-transfected (f) DF-1 cells respond to IAV infection by an increase of chicken PKR expression relative to empty vector-transfected and mock-treated cells. Results are representative of two independent experiments. Data represent means ± SEM from three wells per group. *P ≤ 0.05 vs vector; **P ≤ 0.005 vs vector.
These data suggest that transfected mammalian RIG-Is and CARDs had stronger abilities to induce the expression of antiviral genes in 293T and A549 cells during IAV infection than the waterfowl RIG-Is and CARDs. In addition, gRIG-I could negatively regulate the IFN-β signalling pathway in 293T and A549 cells during IAV infection.

**Waterfowl RIG-Is reduce the replication of IAV more efficiently than mammalian RIG-Is in DF-1 cells**

We evaluated the effects of the mammalian and waterfowl CARDs and RIG-Is on viral replication in DF-1 cells. Cells were transfected with EGFP-tagged RIG-I and CARDs plasmids or empty vector alone and then were challenged with ZB07, WSN or SW731 each at an m.o.i. of 0.01. At 24 and 48 h p.i., the viral titres in the cell cultures were determined as TCID₅₀ ml⁻¹. No differences were observed between the ZB07, WSN or SW731 viral titres in DF-1 cells transfected at 24 or 48 h p.i. with hCARDs, mCARDs, hRIG-I, mRIG-I or empty vector (Fig. 5a–f). However, significantly decreased ZB07, WSN and SW731 viral titres were observed in DF-1 cells transfected with dCARDs, gCARDs, dRIG-I and gRIG-I at both 24 and 48 h p.i. compared with empty vector-transfected cells (Fig. 5a–f). As with their IFN-inducing activities, in DF-1 cells dCARDs, gCARDs decreased the ZB07, WSN and SW731 viral titres to a lower level than observed with dRIG-I and gRIG-I (Fig. 5a–f). Despite the increase in IFN-β mRNA seen with dCARDs transfection, there were no differences between the ZB07 and WSN viral titres in dCARDs- and gCARDs-transfected DF-1 cells. dCARDs decreased the SW731 titre to a lower level than that observed with gCARDs (Fig. 5a). These results suggest that waterfowl RIG-Is reduce IAV replication more efficiently than mammalian RIG-Is in DF-1 cells and gCARDs have a weaker ability to inhibit IAV replication than dCARDs.

**Mammalian RIG-Is reduce IAV replication more efficiently than waterfowl RIG-Is in A549 cells**

The effects of mammalian and waterfowl RIG-Is on viral multiplicity were evaluated in A549 cells. Cells were transfected with EGFP-tagged CARDs and RIG-I expression plasmids or empty vector alone and were then challenged with ZB07, WSN or SW731 each at an m.o.i. of 0.01. The viral titres were determined as TCID₅₀ at 24 and 48 h p.i. hCARDs and mCARDs exhibited the most significant inhibitory effects on the multiplication of ZB07, WSN and SW731 at both 24 and 48 h (Fig. 6a, c, e). In contrast, dCARDs only slightly decreased WSN and SW731 viral titres at 24 h and ZB07 titres at 24 and 48 h (Fig. 6a, c, e). No differences were observed between the ZB07, WSN or SW731 viral titres in A549 cells transfected with gCARDs at 24 or 48 h compared with those transfected with empty vector (Fig. 6a, c, e). Consistent with the results from the hCARDs and mCARDs transfections, hRIG-I and mRIG-I suppressed the replication of the three influenza viruses at 24 and 48 h (Fig. 6b, d, f). No inhibitory effects on viral titres were observed in dRIG-I- and gRIG-I-transfected cells. Similar results were also observed in transfection and viral multiplication experiments performed in MDCK cells (Fig. S1, available in the online Supplementary Material). These data suggest that mammalian RIG-Is and CARDs reduce the replication of IAV in mammalian cells more efficiently than observed with waterfowl RIG-Is and CARDs.

**DISCUSSION**

We examined the differences in the expression of RIG-I and CARDs proteins derived from mammals and birds and examined their IFN-inducing activity and ability to suppress the replication of influenza viruses in chicken.
cells and human cells. qRT-PCR was used to analyse the ability of these RIG-Is and CARDs variants to induce IFN expression in DF-1, 293T and A549 cells. In DF-1 cells, after IAV infection, waterfowl RIG-Is and CARDs had more pronounced abilities to induce the expression of antiviral genes than the mammalian proteins. Conversely, in 293T and A549 cells during IAV infection, mammalian RIG-I and CARDs had more pronounced abilities to induce the expression of antiviral genes than waterfowl RIG-I and CARDs. Waterfowl RIG-Is interfered with the

Fig. 4. Mammalian RIG-Is and CARDs induced type I IFN more strongly than waterfowl in A549 cells during IAV infection. qRT-PCR at 8 h p.i. with SW731, WSN or ZB07 viruses showed that CARDs-transfected (a) and RIG-I-transfected (b) A549 cells responded to IAV infection (m.o.i. = 1) with increased expression of IFN-β compared with empty vector-transfected and mock-treated cells. qRT-PCR shows that IAV infection in CARDs-transfected (c) and RIG-I-transfected (d) A549 cells increases expression of Mx1 compared with empty vector-transfected and mock-treated cells. PKR expression in CARDs-transfected (e) and RIG-I-transfected (f) A549 cells with IAV infection is also increased compared with to empty vector-transfected and mock-treated cells. Results are representative of two independent experiments. Data represent mean ± SEM from three wells per group. *P < 0.05 vs mCARDs, **P < 0.05 vs dCARDs, ***P < 0.05 vs gCARDs, ****P < 0.05 vs vector.
replication of IAV more efficiently than mammalian RIG-Is did in DF-1 cells; whereas, mammalian RIG-Is reduced the replication of IAV more efficiently than did waterfowl RIG-I in A549 and MDCK cells. Therefore, RIG-Is from waterfowl and mammals had different abilities to induce an antiviral response against IAV.

Previous studies revealed that duck and goose CARDs activate IFN-β in chicken cells, suggesting that chicken cells contain all the proteins required for the activation and downstream signalling of RIG-I (Miranzo-Navarro & Magor, 2014; Sun et al., 2013). Recent in vitro studies demonstrated that human TRIM25 did not interact with,
ubiquitinate or activate human CARDs that were co-transfected into chicken DF-1 cells (Miranzo-Navarro & Magor, 2014). Similarly, dCARDs, gCARDs, dRIG-I and gRIG-I activated IFN-β production in chicken DF-1 cells; whereas, hCARDs, mCARDs, hRIG-I and mRIG-I did not. This suggests that only waterfowl-derived RIG-Is are functional in chicken DF-1 cells and some factors necessary for mammal-derived RIG-I activation are missing or unrecognizable in chicken cells. For example, RIPLET, which ubiquitinates Lys849 and Lys851 in the C-terminal domain of RIG-I (Gao et al., 2009; Oshiumi et al., 2009, 2010), is absent from chicken and duck genomes (Magor et al., 2013; Rajsbaum et al., 2012).

SW731, WSN and ZB07 have different IFN-inducing activities (Figs 2a, b and 3a, b). Sequence alignment showed that three viruses have different lengths of the non-structural domain at 24 h p.i., the viral titres in the cell cultures were determined as TCID₅₀. This was repeated using the WSN virus (c, d) and the ZB07 virus (e, f). Results were analysed using the independent sample t-test (n=3). Results are representative of two independent experiments. Data represent means ± SEM from three wells per group. *P≤0.05 vs vector; **P≤0.005 vs vector; ***P≤0.05 vs dCARDs; $P≤0.05 vs dRIG-I.

**Fig. 6.** Mammalian RIG-Is interfere with replication of IAV more efficiently than waterfowl RIG-I in A549 cells. CARDs-transfected (a) and RIG-I-transfected (b) A549 cells were infected with SW731 at an m.o.i. of 0.01, 24 h later. At 24 and 48 h p.i., the viral titres in the cell cultures were determined as TCID₅₀. This was repeated using the WSN virus (c, d) and the ZB07 virus (e, f). Results were analysed using the independent sample t-test (n=3). Results are representative of two independent experiments. Data represent means ± SEM from three wells per group. *P≤0.05 vs vector; **P≤0.005 vs vector; ***P≤0.05 vs dCARDs; $P≤0.05 vs dRIG-I.
protein1 (NS1): SW731, 219 aa; WSN, 230 aa; ZB07, 217 aa (data not shown). The most pronounced IFN synthesis observed in ZB07-infected DF-1 and 293T cells (Figs 2a, b and 3a, b) may due to the weak suppression of IFN by NS1. However, mRIG-I, but not mCARD, blocked the IFN-β, Mx1 and PKR mRNA synthesis only in WSN-infected DF-1 cells (Fig. 2a–f). Another study reported that only avian influenza virus-derived NS1 can bind to chicken TRIM25, thereby suppressing the induction of IFN (Rajsbaum et al., 2012). Mouse-adapted IAV NS1 specifically binds to mouse RIPLET in mouse cells (Rajsbaum et al., 2012). NS1 or other WSN viral proteins might specifically interact with mRIG-I to suppress the induction of IFN in DF-1 cells.

Although gRIG-I induced slightly greater IFN-β production in DF-1 cells than dRIG-I with or without IAV infection, the expression of gRIG-I induced significantly more Mx1 and PKR expression (Fig. 2b, d, f). This suggests that gRIG-I might induce Mx1 and PKR in an IFN-independent manner. dCARDs share 92.98% amino acid identity with gCARDs; however, dCARDs induced significantly greater IFN-β production and IFN-stimulated gene expression than gCARDs in both DF-1 and 293T cells with or without IAV infection. An alignment of the dRIG-I and gRIG-I sequences revealed that the phosphorylation sites for inactivation are not conserved between ducks (Ser8) and geese (Gly8). Ser8 phosphorylation negatively regulates hRIG-I, possibly by preventing TRIM25 binding (Gack et al., 2010; Nistal-Villan et al., 2010). However, it is unclear whether the Ser8Gly mutation in gRIG-I is responsible for its pronounced IFN-inducing activity.

Recent in vitro studies demonstrated that dRIG-I interacts with the unanchored Lys63-linked polyubiquitin chains generated by hTRIM25 in 293T cells (Mirano-Navarro & Magor, 2014). Sun et al. (2013) demonstrated that Newcastle disease virus (NDV) infection in gRIG-I-infected 293T/17 cells resulted in upregulated activity of the IFN-β promoter and IRF-3 and IFIT1 mRNA levels, but decreased viral titres. Consistent with these reports, in this study dCARDs, gCARDs, dRIG-I and gRIG-I promoted IFN expression in mock-treated 293T and A549 cells. However, the ability of dCARDs and gCARDs to induce IFN production was significantly less pronounced than that of hCARDs and mCARDs. The ability of dRIG-I and gRIG-I to induce IFN production was also significantly reduced compared with hRIG-I and mRIG-I. Surprisingly, gRIG-I blocked IFN-β, Mx1 and PKR mRNA production during influenza infection, which is almost the opposite effect reported for gRIG-I in 293T cells after NDV infection (Sun et al., 2013). One possible cause of this inhibitory effect is that gRIG-I could compete with endogenous hRIG-I to bind influenza vRNA. However, gRIG-I does not activate the IFN pathway as effectively as hRIG-I, resulting in competitive inhibition.

Although mRIG-I protein has 76.57% amino acid identity with hRIG-I, hRIG-I induced significantly more IFN-β production in 293T and A549 cells compared with mRIG-I with or without IAV infection. mCARDs also has 75.09% amino acid identity with hCARDs, but hCARDs induced significantly more IFN production and IFN-stimulated gene expression in 293T and A549 cells than mCARDs regardless of IAV infection. We determined that hCARDs and dCARDs have conserved phosphorylation sites (Ser8 and Ser168 in duck; Ser8 and Thr170 in humans), but mCARDs have Asn8 and Val170. In addition, in mCARDs the amino acid Thr55, which plays a critical role in the interaction, is displaced by serine (Gack et al., 2008). Lys172, the site that is ubiquitinated by TRIM25 in humans, is not conserved in mRIG-I. This might be responsible for the observations that mCARDs and mRIG-I had reduced abilities to induce IFN than hCARDs and hRIG-I (Jiang et al., 2012; Zeng et al., 2010).

In this study, the effects of RIG-Is and CARDs variants on virus replication were evaluated in DF-1, A549 and MDCK cells. ZB07, WSN and SW731 titres in hCARDs-, mCARDs-, hRIG-I- and mRIG-I-transfected DF-1 cells confirmed the inability of hCARDs, mCARDs, hRIG-I and mRIG-I to function in chicken DF-1 cells. The inability of dRIG-I and gRIG-I to function in chicken DF-1 cells was also demonstrated by the ZB07, WSN and SW731 titres in dRIG-I- and gRIG-I-transfected A549 and MDCK cells. This suggests that the RIG-I signalling pathway has changed during evolution, possibly because of selection pressure from viruses that disrupted this regulation. Both birds and mammals evolved from reptiles 200 million years ago. RIG-Is derived from humans and mice are not functional in chicken DF-1 cells. RIG-Is derived from ducks and geese are functional in human 293T cells, but do not function as efficiently as hRIG-I. This might be due to the alteration of the RIG-I amino acid sequence during evolution and the emergence of novel signal transducers, such as RIPLET. However, waterfowl retain part of the original signal transduction system. Therefore, waterfowl RIG-I are still partially functional in human 293T cells. These data suggest that RIG-I from individual species can only activate the IFN signalling pathway effectively in their own cellular systems or in very similar systems (dRIG-I and gRIG-I in DF-1; hRIG-I and mRIG-I in A549, 293T and MDCK cells). These results suggest that attention should be paid to elucidating whether transgenes taken from different species can function in evolutionary distant hosts.

METHODS

Plasmids. The hRIG-I ORF was cloned from cDNA isolated from human A549 cells using the primers 5’-ATGACCCAGCGAGGAC-3’ and 5’-CTATTGGCATTTCTCTCTGATCA-3’ based on the human RIG-I sequence (GenBank accession no. AF038963). The mRIG-I ORF was cloned from mouse lung cDNA using the primers 5’-ATGACCCAGCGAGCGCGGC-3’ and 5’-CTATTGGCATTTCTCTGATCA-3’ based on the mouse RIG-I sequence (GenBank accession no. AY553221). The dRIG-I ORF was obtained from duck (Anas platyrhynchos) spleenic cDNA using the primers 5’-ATGACCCAGCGAGGAC-3’ and 5’-CTATTGGCATTTCTCTGATCA-3’ based on the duck RIG-I sequence (GenBank accession no. EU363349.1). Finally, the gRIG-I ORF was amplified from goose spleenic
cDNA using the primers 5'-ATGACGGCGGAGAAAAGC-3' and 5'-CTAACATGGGTTACAGGTGAC-3' based on goose RIG-I sequence (GenBank accession no. F804977). The homologous arms of the mammalian expression vector pEFGP-N1 were added to the 5' and 3' termini of human, mouse, duck, and goose full-length RIG-I and CARDs from human, mouse, duck and goose RIG-I using PCR with the primers shown in Table S1. Human, mouse, duck and goose full-length RIG-I and CARDs were cloned into pEFGP-N1 (NovoRec) by homologous recombination in accordance with the manufacturer’s instructions.

**Viruses.** The SW731, WSN or ZB07 viruses, kind gifts from Dr Jinhua Liu (China Agricultural University), were amplified in chicken embryos. The titres were determined as TCID<sub>50</sub> by monitoring the cytopathic effect of end-point dilutions on MDCK cells in the presence of 1 μg ml<sup>-1</sup> tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical). All virus experiments were performed under biosafety level 2 conditions with investigators wearing appropriate protective equipment and complying with the general biosafety standards of the Microbiological and Biomedical Laboratories of the Ministry of Health of the People’s Republic of China (WS 233-2002).

**Cell culture, transfections and infections.** DF-1, 293T, A549 and MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 0.6 μg ml<sup>-1</sup> penicillin, 60 μg ml<sup>-1</sup> streptomycin and 10% FBS (Gibco), in an atmosphere of 5% CO<sub>2</sub> at 37°C. For infection of transfected cells, 2.5 × 10<sup>3</sup> DF-1, 293T, A549 or MDCK cells were seeded into 24-well plates and maintained in DMEM plus 10% FBS overnight. DF-1, 293T and MDCK cells were transfected with 800 ng of EGFP-tagged plasmids with Lipofectamine 2000 (Invitrogen). A549 cells were transfected with the EGFP-tagged plasmids using FuGENE® HD (Promega). Then, 24 h p.i., cells were infected with SW731, WSN or ZB07 viruses. TPCK-treated trypsin (0.5 μg ml<sup>-1</sup>) was used in A549 and 2 μg ml<sup>-1</sup> in MDCK cells; a lower concentration (0.1 μg ml<sup>-1</sup>) was used for infection of DF-1 and 293T cells in view of their trypsin sensitivity (Lee et al., 2008).

**Western blotting.** Human 293T and chicken DF-1 cells were lysed in RIPA buffer containing 20 mM Tris base-HCl (pH 7.5), 150 mM NaCl, 1.0 mM EDTA (pH 8.0), 1.0 M EGTA (pH 8.0), 0.1% SDS, 0.5% DOC (Sigma), 1% NP-40 (Sigma), protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Proteins were separated using SDS-PAGE under reducing conditions and analysed using Western blotting with anti-EGFP (Beijing B&M Biotech Co.) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Beijing CoWin Biotech) antibodies followed by HRP-labelled secondary antibodies (Bio-RAD) (Chen et al., 2010).

**Quantitative real-time PCR.** Total RNA was extracted using TRIzol (Invitrogen) and treated with DNase I (Promega) to remove the contaminating DNA. Then, 1 μg RNA was reverse-transcribed into cDNA using a GoScript reverse transcription system (Promega) in a 20 μl reaction mixture. The cDNA was analysed using qRT-PCR with SYBR green Master I (Roche). The primers specific for chicken GAPDH, IFN-β, Mx1 and PKR have been described previously (Barber et al., 2010). The primers specific for human GAPDH, IFN-β, Mx1 and PKR were designed using Primer Express 3.0 and are shown in Table S2. qRT-PCR was performed under the following cycling conditions: 95°C for 10 min for activation, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. This was followed by one cycle of 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. The final step was needed to obtain a melting curve for the PCR products to determine the specificity of amplification. All control and infected samples were analysed in triplicate on the same plate. The relative mRNA abundances were calculated using the 2<sup>-ΔΔC<sub>T</sub></sup> method with GAPDH as a reference and plotted as fold changes compared with the mock-control samples (Livak & Schmittgen, 2001).

**Measurement of virus growth in avian and mammalian cells.** DF-1, A549 and MDCK cells transfected with EGFP-tagged plasmids were infected with SW731, WSN and ZB07 at an m.o.i. of 0.01 and cell cultures were collected at different times (24 and 48 h) after infection. The cell culture samples were centrifuged at 5000 g for 1 min and the supernatants were stored at −80°C until use. The viral contents in the supernatants were titrated using TCID<sub>50</sub> in MDCK cells (Reed & Muench, 1938).

**Statistical analysis.** All data analyses were performed using SPSS 16.0 software. Independent sample t-tests were used to determine significant differences between different plasmid-transfected groups. P-values ≤0.05 were considered significant.

**ACKNOWLEDGEMENTS**

This work was supported by the National Basic Research Program (973 Program 2013CB940000). We thank Dr Zhiyi Wan for critical reading of the manuscript. We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

**REFERENCES**


triggering RIG-I- and Mda5-mediated type I interferon induction. 


