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Functional analysis of Rousettus aegyptiacus "signal transducer and activator of transcription 1" (STAT1)

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ABSTRACT

Bats are now known as the source of several diseases in humans, but few studies regarding immune responses and factors associated with bats have so far been reported. In this study, we focused on STAT1, one of the critical components in interferon (IFN)-signaling and antiviral activity, which is often targeted by viral proteins to reduce antiviral activity and increase viral replication. We found that Rousettus aegyptiacus STAT1 (bat STAT1) is phosphorylatable and translocates to the nucleus when stimulated with human IFN-α (hIFN-α). Furthermore, phosphorylation of bat STAT1 and inhibition of nuclear translocation was observed in IFN-stimulated cells infected with the HEP-Flury strain of rabies virus, in the same manner as in other mammals. Additionally, quantitative real-time RT-PCR revealed that bat STAT1 mRNA was highly expressed in the liver, while low in muscle and spleen. © 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Recently, zoonotic pathogens have been recognized as major sources of emerging and reemerging infections in humans [1]. Bats are divided into two orders, Megachiroptera and Microchiroptera, and bats, especially fruit bats of Megachiroptera, are currently being focused on as a source of emerging infections such as rabies virus (RV), Nipah virus, Hendra virus, severe acute respiratory syndrome (SARS) virus, and Ebola virus [1–5]. Of these viruses, RV, which belongs to the family, genus Lyssavirus, is spreading the most worldwide and has a wide host range [6]. In mammals, RV infection causes 100% mortality, but some bats have shown no clinical signs with detectable antibody titers and were able to resist infection and become a carrier when infected with RV [7–10]. Although immunological studies of bats may provide important clues to help clarify the relationships between host immune factors and viruses, few studies have been performed on the immune system of bats [6,11–13].

Type I IFN, one of the most important antiviral immune factors, is associated with the ability to reduce virus replication by inducing cellular gene expression of numerous antiviral factors, termed interferon-stimulated genes (ISGs) [14–17]. Following virus infection in vivo, the IFN response is critical in restricting virus spread before the onset of adaptive immune system responses to control the infection [14–17]. Newly synthesized IFN-α/β is secreted and binds to the type-I IFN receptors on the surface of infected and neighboring cells, inducing the Janus kinase/signal transducer and activator of a transcription (Jak/STAT) signaling cascade [15,18,19]. STAT1 and STAT2 are activated by phosphorylation and form heterodimers that associate with a third factor, IFN regulatory factor-9 (IRF-9) [15,18,19]. The resulting complex, IFN-stimulated gene factor 3 (ISGF3), translocates to the nucleus and induces the transcription of ISGs through a promoter element IFN-stimulated response element (ISRE). In contrast, only natural killer (NK) cells and T cells secrete type-II IFN, IFN-γ, in response to virus infections [15]. IFN-γ signaling also involves a Jak/STAT signaling cascade. When activated by phosphorylation, STAT1 forms a homodimeric complex, γ-activated factor (GAF), which binds to promoters containing a γ-activated sequence (GAS) element after translocation to the nucleus, inducing the transcription of various genes involved in the regulation of innate and adaptive immune responses [14,15,20].

Viruses that require cellular machinery for their replication have evolved different strategies to counteract IFN signaling. Many viruses are known to target STAT1, thereby inhibiting the expression of ISGs, to invade host cells. For example, Henipavirus V protein has been demonstrated to subvert IFN responses by sequestering STAT1 in high-molecular-mass cytoplasmic complexes by binding to STAT1 [19,21–23], while Rhabdovirus P...
protein binds to STAT1 and retains it in the cytoplasm [24,25]. Furthermore, Respirovirus C protein inhibits both STAT1 and STAT2 tyrosine phosphorylation [21,26,27]. As STAT1 performs pivotal roles in IFN signaling and is commonly targeted by various viruses, including viruses found in bats, we studied the function of STAT1 in bats, which are now considered to be a major source of emerging infections. In this study, we used Rousettus aegyptiacus, a kind of fruit bat, and examined its ability to phosphorylate and localize STAT1 in normal situations and in RV infection, then measured the expression profile of its mRNA in tissues.

2. Materials and methods

2.1. Cells and viruses

Primary bat kidney (BatK) cells were prepared according to a previous report using the kidney of a matured female R. aegyptiacus [28]. Before collecting kidneys, whole blood was collected from the heart of the bats under diethyl ether anesthesia. All the experiments using animals were conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agriculture and Life Sciences, the University of Tokyo. BatK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% fetal calf serum (FCS). Mouse neuroblastoma cells (NA) were cultivated in Eagle’s minimum essential medium (EMEM) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% FCS. Human embryonic kidney 293 (293T) cells were maintained in DMEM with 2 mM L-glutamine and 10% FCS. The RV HEP-Flury strain (provided by Dr. K. Nakamichi, National Institute of Infectious Disease, Japan) was used.

2.2. Reagents and antibodies

Recombinant human interferon alpha 2a (hIFN-α 2a) was purchased from ProSpec (Charlotte, NC, USA), rabbit anti-STAT1 and anti-phospho-STAT1 (Tyr701) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-actin clone C4 was acquired from Millipore (Billerica, MA, USA). Anti-Flag M2 monoclonal antibody and IgG 1 Isotype Control from Sigma (St. Louis, MO, USA), and anti-STAT1 (diluted 1:500), anti-actin (diluted 1:1000), IgG 1 isotype control (diluted 1:1000), or HRP-conjugated anti-Flag (diluted 1:1000) for 2 h. The blots were washed and further incubated for 1 h with the following secondary antibodies, HRP-conjugated goat anti-rabbit IgG (diluted 1:5000), HRP-conjugated mouse anti-goat IgG (diluted 1:5000), or HRP-conjugated sheep anti-mouse IgG (diluted 1:5000). Signals were detected using the Enhanced Chemiluminescence (ECL) Detection Kit (GE Healthcare).

2.5. Immunoprecipitation

The lysate samples prepared for Western blotting were incubated at 4 °C with 3 mg of anti-Flag M2 monoclonal antibody for 2 h. Immune complexes were precipitated by incubation with Protein G Sepharose for 1 h at 4 °C, washed three times, and denatured in Laemmli sample buffer. Immunoprecipitated proteins were analyzed by Western blotting using HRP-conjugated goat anti-rabbit IgG and anti-phospho-STAT1 antibodies, as described above.

2.7. Quantitative real-time RT-PCR

RNA was isolated from 15 mg of spleen, 20 mg of kidney, 30 mg of liver, 30 mg of muscle, and 60 mg of heart, brain, and lung, which were obtained from three apparently healthy mature female R. aegyptiacus. The temperature program consisted of cDNA synthesis and a pre-denaturation step at 50 °C for 30 min and 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 2.5 min. The RT-PCR products were then inserted into the pFLAG-CMV-2 (Sigma) vector using EcoRV and SphI sites to generate pFlag-STAT1. The STAT1 expression plasmid was transfected into 293 T cells soon after RV infection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

2.4. Western blotting

About 6 × 10^6 293T cells were seeded onto 6-well plates, grown to 80% confluence, mock-infected or infected with RV at a multiplicity of infection (MOI) of 1, and incubated for 1 h. After washing the infected cells, 10% FCS DMEM was added and transfected with pFlag-STAT1 or empty vector. At 24 h post-transfection, cells were either not stimulated or stimulated with 10 kU/ml of IFN-α for 30 min, washed with phosphate-buffered saline (PBS) and lysed in 500 µl of buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% NP-40, and protease inhibitor cocktail (Roche, Basel, Switzerland). After sonication, samples were centrifuged (15,000 rpm, 10 min) and the supernatant was collected in new tubes. To equalize protein concentration of the samples, we performed protein quantification using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Tokyo, Japan) according to the manufacturer’s protocol. Equalized samples were lysed in Laemmli sample buffer and boiled for 3 min. Proteins were analyzed on a 7.5% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. After blocking with buffer containing 5% skim milk and 0.1% Tween 20 in PBS for 1 h, the membrane was incubated with primary antibody, anti-STAT1 (diluted 1:1000 with PBS containing 2% skim milk and 0.1% Tween 20), anti-phospho-STAT1 (diluted 1:500), anti-actin (diluted 1:1000), IgG 1 isotype control (diluted 1:1000), or HRP-conjugated anti-Flag (diluted 1:1000) for 2 h. The blots were washed and further incubated for 1 h with the following secondary antibodies, HRP-conjugated goat anti-rabbit IgG (diluted 1:5000), HRP-conjugated mouse anti-goat IgG (diluted 1:5000), or HRP-conjugated sheep anti-mouse IgG (diluted 1:5000). Signals were detected using the Enhanced Chemiluminescence (ECL) Detection Kit (GE Healthcare).

2.6. Immunofluorescence staining and confocal microscopy

The lyophilized samples prepared for Western blotting were incubated at 4 °C with 3 mg of anti-Flag M2 monoclonal antibody and 4 mg of anti-phospho-STAT1 antibody for 2 h. Immune complexes were precipitated by incubation with Protein G Sepharose for 1 h at 4 °C, washed three times, and denatured in Laemmli sample buffer. Immunoprecipitated proteins were analyzed by Western blotting using HRP-conjugated goat anti-Flag, rabbit anti-STAT1, or anti-phospho-STAT1 antibodies, as described above.

The lysis samples prepared for Western blotting were incubated at 4 °C with 3 mg of anti-Flag M2 monoclonal antibody and 4 mg of anti-phospho-STAT1 antibody for 2 h. Immune complexes were precipitated by incubation with Protein G Sepharose for 1 h at 4 °C, washed three times, and denatured in Laemmli sample buffer. Immunoprecipitated proteins were analyzed by Western blotting using HRP-conjugated goat anti-Flag, rabbit anti-STAT1, or anti-phospho-STAT1 antibodies, as described above.

About 2 × 10^4 BatK cells were seeded to 4-well chamber slide, grown to 80% confluence, mock-infected or infected with RV at an MOI of 0.1, and incubated for 1 h. After washing the infected cells, 10% FCS DMEM was added. At 24 h post-infection, BatK cells were mock-treated or treated with 10 kU/ml of IFN-α for 30 min, washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.5% TritonX-100 in PBS for 15 min. The intracellular distribution of STAT1 was analyzed using a rabbit anti-STAT1 antibody at a dilution of 1:500, followed by incubation with Alexa-594-conjugated anti-rabbit IgG antibody (Invitrogen) at a dilution of 1:1000. The viral protein was stained using FITC anti-rabies monoclonal globulin at a dilution of 1:400. Confocal laser microscopy was performed using an LSM510 (40× objective; Carl Zeiss, Oberkochen, Germany).

The lysis samples prepared for Western blotting were incubated at 4 °C with 3 mg of anti-Flag M2 monoclonal antibody and 4 mg of anti-phospho-STAT1 antibody for 2 h. Immune complexes were precipitated by incubation with Protein G Sepharose for 1 h at 4 °C, washed three times, and denatured in Laemmli sample buffer. Immunoprecipitated proteins were analyzed by Western blotting using HRP-conjugated goat anti-Flag, rabbit anti-STAT1, or anti-phospho-STAT1 antibodies, as described above.

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aequatus. RNA was isolated from each samples as the same manner as described in Section 2.3. The levels of STAT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression were detected using the Thermal Cycler Dice TP8000 (TaKaRa, Tokyo, Japan), One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and 10 μM of each specific primer sets shown in Table 1, according to the manufacturer’s protocol. About 100 pg of total RNA from each tissue was used for the real-time RT-PCR. The temperature program consisted of a reverse transcription step at 42 °C for 5 min and 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s, and one cycle of a dissociation step at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. In each run, tenfold serial dilutions of each RNA sample were tested in duplicate to establish a standard curve. GAPDH was used as a positive control and for normalization of expression levels. The data were determined by the standard 2nd derivative max (ΔΔCt) method.

3. Results

3.1. Bat STAT1 is phosphorylated and translocates into the nucleus after IFN-stimulation

We cloned bat STAT1 and registered its sequence in GenBank (AB524020). Searching the domain by InterProScan revealed that the STAT1 protein contains the putative conserved functional domains typical of STAT proteins: N-terminal protein interaction (2–122), coiled-coil (136–316), DNA-binding (317–568), SH (569–712), and transcription activation domains (713–749). Conserved tyrosine and serine phosphorylation sites were also found at the 701st and 727th amino acid residues, respectively. In addition, the overall similarity at the amino acid level between the R. aequatus STAT1 and those of the other animals are as follows: the highest identity, 97% with Equus caballus; 96% with Bos taurus, Canis familiaris, Homo sapiens, and Sus scrofa; 93% with Mus musculus and Rattus norvegicus; 83% with Gallus gallus; 80% with Xenopus laevis; and the lowest identity, 61% with Danio rerio. Each domain in R. aequatus STAT1 shared high homology with those of other mammals. We thus hypothesized that bat STAT1 has the same function as that of others.

STAT1 is known to be phosphorylated and translocate into the nucleus after IFN-stimulation[15,18,19]. First, to examine whether bat STAT1 was phosphorylatable following IFN stimulation, Western blotting of samples from the transfected 293T cells. As the anti-STAT1 and anti-phospho-STAT1 antibody detected both the transfected Flag-tagged protein and the endogenous one in 293T cells, we performed Western blotting with both lysate and immunoprecipitated samples. The phosphorylation of bat STAT1 was observed in only IFN-stimulated samples (Fig. 1A, lanes 2 and 6; Fig. 1B, lane 2). The upward migration of bands detected by anti-STAT1 and anti-phospho-STAT1 antibody in the pFlag-STAT1 transfected samples are derived from Flag-tagged bat STAT1 (Fig. 1A).

We next confirmed whether bat STAT1 could be translocated into the nucleus by IFN stimulation. Bat STAT1 localized in the cytoplasm in the IFN-un-treated BatK cells (Fig. 2a–c). However, the nuclear localization of bat STAT1 was observed in the IFN-treated

| Primer name | Sequence (5’-3’) | Application |
|-------------|------------------|-------------|
| STATvectorF | GAGATATCCGATGCTCACCAGTTGCTAGCTT | Vector construction |
| STATvectorR | GCGCTGACATATACCTGCTACTGCT | Vector construction |
| RT-STAT1f | CCATGCTGATCAGAAGCCCTTCATC | Real-time RT-PCR |
| RT-STAT1r | CAGTTCGACATACACCAAGACA | Real-time RT-PCR |
| RT-GAPDHf | TGCGAGTAGCATCGGCACGACT | Real-time RT-PCR |
| RT-GAPDHR | GTCCAGGAGGCTTGCTGAC | Real-time RT-PCR |

Fig. 1. Western blotting of samples from the transfected 293T cells. (A) 293T cells were mock-infected (lanes 1, 2, 5, and 6) or infected (lanes 3, 4, 7, and 8) with IFN at an MOI of 1 and transfected with pFlag-STAT1 (lanes 1–4) or empty vector (lanes 5–8). At 24 h post-transfection, cells were mock-treated (lanes 1, 3, 5, and 7) or treated (lanes 2, 4, 6, and 8) with 10 kU/ml of IFN-α for 30 min. The expression of STAT1, phosphorylated STAT1, and Flag were detected by Western blot analysis with specific antibodies. Actin was used as a loading control. (B) The same lysates were immunoprecipitated with Flag antibody. The expression of STAT1, phosphorylated STAT1, and Flag were detected by the immunoblot analysis with specific antibodies. IgG was used as a loading control.

BatK cells (Fig. 2d–f). These results suggest that bat STAT conserves the function to be phosphorylated and translocate into the nucleus by hIFN-α stimulation.

3.2. RV infection does not affect phosphorylation but inhibits nuclear translocation of bat STAT1

To compare the function of STAT1 between bats and other animals, we examined the function of bat STAT1 using RV, whose natural reservoir is bats [1,6]. RV infection does not affect phosphorylation of STAT1 but inhibits nuclear localization after IFN stimulation in humans [24,25]. As shown in Fig. 1, regardless of prior infection with RV, phosphorylation of bat STAT1 was detected in cells stimulated with IFN-α (Fig. 1A, lanes 2, 4, 6, and 8; Fig. 1B, lanes 2 and 4).

Notably, phosphorylation of STAT1 was observed in unstimulated RV-infected cells (Fig. 1A, lanes 3 and 7; Fig. 1B, lane 3), suggesting that IFN secretion was induced by RV infection. The level of STAT1 phosphorylation was highest in RV-infected cells stimulated with IFN-α (Fig. 1A, lane 4; Fig. 1B, lane 4). Additionally, in the only RV-treated cells, STAT1 localized in the cytoplasm in both infected and uninfected cells as it would normally (Fig. 2g–i). On the other hand, although STAT1 localized in the nucleus in uninfected cells (Fig. 2j–l, arrows), STAT1 remained in the cytoplasm in RV-infected cells even after IFN treatment in the RV-treated cells (Fig. 2j–l, arrows). These results suggest that bat STAT1 localizes in the same manner as that of humans when infected with RV.

3.3. Expression profile of R. aequatus STAT1 in various tissues

STAT1 normally exists in an inactive, latent form in the cytoplasm and is activated by binding of IFNs to each receptor
Thus, some level of basal expression of STAT1 is necessary for a prompt response to a signal. We next analyzed the expression of bat STAT1 mRNA in various tissues using quantitative real-time RT-PCR. As shown in Fig. 3, bat STAT1 mRNA was detected in all tissues tested. However, differences were observed in the expression levels among tissues. The expression level was relatively low in muscle and spleen, whereas it was markedly high in the liver.

4. Discussion

STAT1 is highly conserved among animals, and as expected, the homology of bat STAT1 is very high with that of other animals, especially horses and dogs. In addition, we showed bat STAT1 is phosphorylated and translocates into the nucleus after hIFN-α stimulation (Figs. 1 and 2). These results suggest that the function of bat STAT1 is the same as other known STAT1.

Although Hagmaier et al. stated that STAT1 of bats had no cross-reactivity with that of humans [29], our results indicated that STAT1 and phospho-STAT1 of R. aegyptiacus has cross-reactivity with antibodies against human STAT1 (Figs. 1 and 2). This difference in antibody reactivity to bat STAT1 between the two studies might have been caused by the differences in the antibodies used or the difference in bat species. Although Hagmaier et al. used Tb-1-Lu, cells derived from Tadarida brasiliensis of Microchiroptera, we used cloned STAT1 from the primary bat kidney cells obtained from R. aegyptiacus of Megachiroptera.

Fig. 2. Confocal imaging of STAT1 in bat cells infected with RV. The IFN-induced nuclear translocation of STAT1 in bat cells is prevented in RV-infected cells. BatK cells were mock-infected (a–f) or infected (g–l) with RV at an MOI of 0.1. At 24 h postinfection, cells were stimulated (d–f and j–l) or unstimulated (a–c and g–i) with 10 kU/ml of IFN-α for 30 min, fixed, and stained with STAT1 antibody as described in Section 2. The arrows point to RV-infected cells and the arrowheads point to uninfected cells. RV was visualized using FITC-labeled RV antibody as described in Section 2s. The scale bars correspond to 20 μm.
Fig. 3. Quantitative real-time RT-PCR analysis of STAT1 gene expression in various tissues. Expression of STAT1 mRNA in various tissues of Rousettus aegypticus was analyzed using real-time RT-PCR. Data are averaged from three different bats with standard deviations. The data were normalized to GAPDH gene expression using the 2nd derivative max (ΔΔCt) method.

RV P protein is known to inhibit STAT1 nuclear translocation by binding to the coiled-coil and DNA-binding domains of STAT1 in human cells, but it does not inhibit phosphorylation of human STAT1 [30]. We hypothesized that the clinical sign differences between bats and humans [7,9,10] might be caused by RV–STAT1 interaction differences among species. However, bat STAT1 in RV-infected cells was phosphorylated but could not translocate to the nucleus, in the same manner as that of humans [24,25]. This result seems reasonable as the putative coiled-coil and DNA-binding domains of RV protein, of bat STAT1 showed high sequence similarity with those of other mammals and suggests that other factors related to the development of clinical signs exist when infected by RV.

Also, we compared the expression level of bat STAT1 among various tissues. Generally, the expression level of STAT1 tends to be high in tissues that are related to the immune system, such as the thymus and spleen [31,32]. However, our data showed that the expression level was high in liver, and it was relatively low in spleen, which is thought to show the highest expression. In humans, NK cells, which produce IFN-γ, are known to be more abundant in liver, and the liver has plasmacytoid dendritic cells (pDCs), a major source of IFN-α [33]. The fact that the basal expression level of bat STAT1 in liver is extremely high suggests the liver of bats consists of immune-related cells or includes other immune-related factors at a high rate, and play an important role in antiviral responses in bats. However, since we could not clarify the reason, further studies on the localization of immune factors are needed.

Our findings support previous research showing that immune responses of bats have some similarities with those of mammals [11–13], and led to the hypothesis that STAT1 might not be a critical factor for the differing clinical signs following infection with zoonotic viruses [7,9,10]. However, the target positions of STAT1 are different among viruses, and the structure and function of bat STAT1 might be different among bats. Thus, further studies on bat immune systems are needed to clarify the relationships and responses between hosts and viruses.

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