Developmental and Comparative Immunology 34 (2010) 598–602

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

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

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ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form 5 January 2010
Accepted 5 January 2010
Available online 15 January 2010

Keywords:
Fruit bats
Rousettus aegyptiacus
STAT1
Rabies virus
Phosphorylation
Nuclear translocation

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.

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 *Rhabdoviridae* 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

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doi:10.1016/j.dci.2010.01.004

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 diethylether 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 murine myeloma were purchased from Sigma (St. Louis, MO, USA) and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, HRP-conjugated mouse anti-goat IgG, HRP-conjugated sheep anti-mouse IgG, and Protein G Sepharose were all obtained from GE Healthcare (Milwaukee, WI, USA). Fluorescein isothiocyanate (FITC) anti-rabies monoclonal globulin (Centocor, St. Louis, MO, USA) was provided by Dr. K. Nakamichi (National Institute of Infectious Diseases, Tokyo, Japan).

2.3. Construction of the mammalian expression vector and transfection

Total cellular RNA was isolated from BatK cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The STAT1 gene was amplified by RT-PCR using 200 pg of RNA isolated from BatK cells with 10 μM of primers, STAT1vectorF and STAT1vectorR (Table 1). 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 *Eco*RV and *Sal*I sites to generate pFlag-STAT1. The STAT1 expression plasmid was transfected into 293T 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).

2.5. 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 anti-Flag, rabbit anti-STAT1, or anti-phospho-STAT1 antibodies, as described above.

2.6. Immunofluorescence staining and confocal microscopy

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 postinfection, 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).

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.*