Molecular identification and antiviral function of the guanylate-binding protein (GBP) genes in the Chinese tree shrew (Tupaia belangeri chinesis)

Tianle Gu\textsuperscript{a,b,}, Dandan Yu\textsuperscript{a,c,d,}, Yu Fan\textsuperscript{a,c,d,}, Yong Wu\textsuperscript{a,b,}, Yu-Lin Yao\textsuperscript{a,b,}, Ling Xu\textsuperscript{a,c,d,}*, Yong-Gang Yao\textsuperscript{a,b,c,d,*}

\textsuperscript{a} Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, 650223, China
\textsuperscript{b} Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, Yunnan, 650204, China
\textsuperscript{c} Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming, 650223, China
\textsuperscript{d} Kunming Primate Research Center of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, 650223, China

\textbf{A B S T R A C T}

Following viral detection and interferons (IFNs) production, several hundreds of IFN-stimulated genes (ISGs) are subsequently induced to act as direct antiviral effectors or regulators of the IFN signaling. The guanylate-binding protein (GBP) family belongs to IFN-inducible GTPases defending the host against a diverse group of invading pathogens such as parasites, bacteria and viruses. The Chinese tree shrew (\textit{Tupaia belangeri chinesis}) has been increasingly used as an experimental animal to primates in studying viral infectious diseases. Hitherto, the tree shrew GBP family has not been characterized. In this study, we identified five tree shrew GBP genes (\textit{tGBP1}, \textit{tGBP2}, \textit{tGBP4}, \textit{tGBP5} and \textit{tGBP7}) and characterized their antiviral activities. All these \textit{tGBPs} were ubiquitously expressed in heart, spleen, intestines, kidney, liver, lung and brain tissues of the tree shrew. IFN-\gamma treatment of tree shrew primary renal cells (TSPRCs) significantly induced the mRNA expression of \textit{tGBPs}. Infections with Newcastle disease virus (NDV), encephalomyocarditis virus (EMCV) and type 1 herpes simplex virus (HSV-1) enhanced \textit{tGBPs} mRNA expression in TSPRCs, but had no effect on the localization of \textit{tGBPs} proteins in the cytoplasm. \textit{tGBP1}, but not the other four \textit{tGBPs}, showed antiviral activity against vesicular stomatitis virus (VSV) and HSV-1 infections. Taken together, this study provided the first-hand information of the GBP family members in the Chinese tree shrew, which might assist the development of tree shrew animal model for infectious diseases.

\section{1. Introduction}

The interferon (IFN)-mediated innate immune response provides a robust first line of defense against invading pathogens (McNab et al., 2015). After the production of IFN that was induced by the pathogen detection via pattern recognition receptors (PRRs), it induces expression of hundreds of IFN-stimulated genes (ISGs), leading to a remarkable antiviral state, effectively against positive-, negative-, and double-stranded RNA viruses, DNA viruses, and intracellular bacteria and parasites (Akira et al., 2006; Goubau et al., 2013; Schneider et al., 2014). The guanylate-binding protein family (GBP) belongs to a superfamily of the IFN-induced GTPases that includes the immunity-related GTPases (IRGs), the Myxoma proteins (Mx) and the very large inducible GTPases (VLI Gs or GVINs) (Kim et al., 2012; MacMicking, 2004). The human GBP (hGBP) genes are located within one gene cluster on chromosome 1, whereas the mouse Gbp (mGBP) genes are located in two clusters on chromosomes 3 and 5 (Kim et al., 2011; Olzewski et al., 2006). GBP family has been shown to be important mediators of host defense \textit{in vivo} against bacterial pathogens and parasites (Meunier and Broz, 2016; Pilla-Moffett et al., 2016). There is evidence that GBPs are also involved in the host innate immunity to viral infections (Pilla-Moffett et al., 2016). Overexpression of hGBP1 could control vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), hepatitis C virus (HCV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) replication in cell lines (Carter et al., 2005; Itsui et al., 2009; Li et al., 2016; Lu et al., 2008; Zou et al., 2017). A splice variant of hGBP3 mediated anti-influenza activity through repression of the viral polymerase complex (Nordmann et al., 2012). mGBP4 could
negatively regulate Sendai virus (SeV)-triggered IFN-I production by directly targeting IRF7 and inhibiting its function (Hu et al., 2011). hGBP5 had an antiviral activity to human immunodeficiency virus type 1 and influenza virus infections (Feng et al., 2017; Hotter et al., 2017; Krapp et al., 2016). Recently, Biering et al. (2017) discovered that the replication complexes of positive-sense RNA viruses were marked by the LC3 conjugation system of autophagy and targeted by GBPs to inhibit viral replication. All these lines of evidence suggested that GBPs played active roles in antiviral function.

The Chinese tree shrew is a squirrel-like animal inhabiting in the tropical shrubs or forests of South and Southeast Asia (Fan et al., 2013; Xu et al., 2013b; Yao, 2017; Zheng et al., 2014). This animal has a small body size, a low-cost of maintenance, a short reproductive cycle, and a close affinity with primates (Fan et al., 2013; Xu et al., 2013a; Yao, 2017). It has a susceptibility to a wide range of human pathogens, making it as a promising and viable experimental animal for human diseases, including metabolic diseases, brain aging, neurological and psychiatric diseases, and cancer (Fan et al., 2016; Li et al., 2018; Wei et al., 2017; Xiao et al., 2017; Yao, 2017). In the past decades, great efforts have been made to establish infectious disease animal models using the Chinese tree shrew (Tsukiyama-Kohara and Kohara, 2014; Xiao et al., 2017; Yao, 2017). For example, many reports have suggested that tree shrew is susceptible to hepatitis B virus (HBV) and HCV (Guo et al., 2018; Walter et al., 1996; Wang et al., 2012; Xie et al., 1998; Yan et al., 1996). Besides, it has been shown that tree shrew is readily infected by many other viruses, such as HSV-1 (Li et al., 2016a, 2016b), Coxackie virus A16 (Li et al., 2014) and influenza H1N1 virus (Yang et al., 2013). However, lack of basic knowledge regarding the immune system of tree shrew has disabled our efforts to create a stable and successful animal model for infectious disease using this species (Yao, 2017).

In this study, we identified and characterized five tGBP genes and examined their tissue expression pattern in adult Chinese tree shrew. We also investigated tGBP expression pattern in tree shrew primary renal cells (TSPRCs) in response to different virus infections and tree shrew interferon-γ (tIFNγ) treatment. The cellular localization and antiviral activity of these tGBPs were also examined. Our results indicated that GBPs have a closer relationship to hGBPs than to mGBPs, and tGBP1 has a potent antiviral activity to VSV infection.

2. Materials and methods

2.1. Experimental animals and cell culture

The Chinese tree shrews (n = 5) were purchased from the experimental animal core facility of Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). After lethally anesthetized by pentobarbital, seven different tissues including heart, liver, spleen, lung, kidney, small intestine and brain of each tree shrew were collected. This study was approved by the Institutional Animal Care and Use Committee of KIZ, CAS.

The preparation and culture of TSPRCs was described in our previous studies (Xu et al., 2016; Yu et al., 2014). In brief, the kidney tissues were cut into small pieces and digested in a volume of 25 mL solution containing 1 mg/mL DNase (Sigma, AMPD1-1 KT) and 5 mg/mL collagenase type IV (Invitrogen, 17104019) for 45 min at 37 °C. After three washes with cold phosphate-buffered saline (PBS; biological debris). The BCA protein assay kit (Beyotime, P0013) was used to determine the protein concentration. A total of 20 μg protein for each

2.2. RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using RNeasy Total RNA Kit (TIANGEN, DP419) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized by using random primer and M-MLV reverse transcriptase (Promega, M1701). RT-qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, 1725124) supplemented with gene-specific primers (Table S1) on a CFX Connect Real-Time System (Bio-Rad, USA), as had been described in our previous studies (Xu et al., 2015, 2016; Yao et al., 2019; Yu et al., 2014).

We serially diluted the PCR product of the respective amplicon and used the 10⁻³ - 10⁻¹ dilutions to generate the standard curve for the primer pair to quantify the target gene. The Ct values were measured relative to the corresponding standard curve. The tree shrew housekeeping gene β-actin was used as the reference gene for normalization.

2.3. Molecular clone and construction of overexpression vector

We designed the primers for cloning and amplification of tGBPs based on the predicted tGBP sequences of the Chinese tree shrew genome (http://www.treeshrewdb.org/) (Fan et al., 2014). In order to get a relatively intact mRNA sequence, rapid amplification of cDNA ends (RACE) was used to amplify the 5'-UTR and 3'-UTR using the SMARTer RACE cDNA Amplification Kit (Clontech, 634923) in accordance with the manufacturer's instruction. The full-length mRNA of GBPs were amplified using the cDNA prepared from total RNA of TSPRCs that treated with tIFNγ for 8 h. Purified PCR products were cloned into the pMD 19-T simple vector (TaKaRa, 3271). We sequenced five positive clones to get the consensus sequence for each insert.

We constructed overexpression vectors for each tGBP and tIFNγ using the gene-specific primer pairs (Table S1). The PCR product was purified with the TIANquick Midi Purification Kit (TIANGEN, DP204) and was cloned into Flag-tagged pCMV-3Tag-8 plasmid. All the constructs were confirmed by sequencing. Overexpression vectors for tMALS (Flag-tagged tMVAS) and tMITA (MYC-tagged tMITA) had been described in our previous studies (Xu et al., 2015, 2016) and were used as a positive control in this study.

2.4. Phylogenetic analysis

The protein sequences of human GBPs and mouse GBPs were retrieved from the GenBank (https://www.ncbi.nlm.nih.gov/): hGBP1 (NP_002044.2), hGBP2 (NP_004111.2), hGBP3 (AAI40838.1), hGBP4 (NP_443173.2), hGBP5 (NP_443174.1), hGBP6 (AA117314.1), hGBP7 (NP_997281.2), mGBP1 (NP_034389.2), mGBP2 (AAD39746.1), mGBP3 (AAH19195.1), mGBP4 (NP_032646.2), mGBP5a (AAN22821.2), mGBP5b (AA37668.1), mGBP6 (XP_01247688.1), mGBP7 (NP_663520.2), mGBP8 (NP_083785.3), mGBP9 (NP_766365.1), mGBP10 (NP_001034735.2), mGBP11 (NP_001034736.3). The protein sequences were aligned by using Muscle 3.8 (Edgar, 2004). The unrooted neighbor-joining (NJ) tree were constructed by MEGA 6.0 (Tamura et al., 2013) with the Poisson model. A total of 1000 bootstrap replications were performed to determine the confidence of tree branch positions.

2.5. Western blot and immunofluorescence assay

Transfection was performed using X-tremeGENE HP (Roche, 06366546001) following the manufacturer's instruction. Cells were harvested and lysed on ice in RIPA lysis buffer (Beyotime, P0013), followed by centrifugation at 12,000 g at 4 °C for 10 min to remove cell debris. The BCA protein assay kit (Beyotime, P0012) was used to determine the protein concentration. A total of 20 μg protein for each
The culture supernatants containing tIFN-γ were collected and infected for use.

For immunoﬂuorescence assay, cells seeded in chamber slides (Thermo, 154526) were washed three times with PBS and were ﬁxed with 4% paraformaldehyde. The ﬁxed cells were permeated with 0.2% Triton X-100 for 15 min. After three washes (each 5 min) with PBS, cells were incubated with the primary antibody against Flag (EnoGene, E12-001-1) to detect the Flag-tagged proteins, and against β-actin (EnoGene, E12-041-1) to detect endogenous β-actin overnight at 4 °C, respectively. After three washes with PBS, the membranes were incubated for 1 h with anti-mouse secondary antibody (1:10000, KPL, USA) at room temperature. The epitope was visualized by using an ECL western blot detection kit (Millipore, WBKPS0500).

For immunofluorescence assay, cells seeded in chamber slides (Thermo, 154526) were washed three times with PBS and were ﬁxed with 4% paraformaldehyde. The ﬁxed cells were permeated with 0.2% Triton X-100 for 15 min. After three washes (each 5 min) with PBS, cells were incubated with the primary antibody against Flag to detect the Flag-tagged proteins overnight at 4 °C. After another round of three washes with PBS, cells were incubated with the ﬂuorescence labeled anti-mouse secondary antibody (Invitrogen, A-21207) for 1 h. Nuclei were stained by DAPI (Roche, 10236276001). Intact cells were imaged by using an Olympus Fluoview™ 1000 confocal microscope (Olympus).

2.6. Virus infections

The vesicular stomatitis virus (tagged by GFP; VSV-GFP), type 1 herpes simplex virus (HSV-1), Newcastle disease virus (NDV) and Encephalomyocarditis virus (EMCV) had been described in our previous study (Xu et al., 2016). Briefly, the VSV-GFP was obtained from Xinwen Chen, Wuhan Institute of Virology, Wuhan, China, the HSV-1 was introduced from Jumin Zhou’s laboratory at KIZ, the NDV was obtained from the China Institute of Veterinary Drug Control, Beijing, and the EMCV (Zhang et al., 2007) was a kind gift from Hanchun Yang, China Agricultural University, Beijing. For viral infections, cells were seeded in 24-well plates (5 × 10^4 cells/well) or in the chamber slides (2 × 10^4 cells/well), and were washed three times with PBS. Cells were then incubated with NDV at a multiplicity of infection of 1 (MOI = 1), HSV-1 (MOI = 1), VSV-GFP (MOI = 0.01) or EMCV (MOI = 1), respectively, for 1 h in DMEM without FBS, and were rinsed and cultured in 24-well plates (5 × 10^4 cells/well) or in the chamber slides ranging from 1733 bp to 1884 bp, and the predicted proteins had a molecular weight ranged from 67 kD to 72 kD (Table S2). In the tGBPs cluster (Fig. 1B).

3. Results

3.1. Identiﬁcation of five GBP genes in the Chinese tree shrew

There were 7 members (hGBP1 - hGBP7) and 11 members (mGBP1 - mGBP11) of GBPs in human and mouse, respectively (Olszewski et al., 2006). Based on the genome sequence of the Chinese tree shrew and the tree shrew database (http://www.treeshrewdb.org) (Fan et al., 2013, 2014), we were able to identify ﬁve GBPs (tGBP1, tGBP2, tGBP4, tGBP5, tGBP7; the naming of each tGBP was based on the clustering pattern of GBPs with hGBPs and mGBPs in the phylogenetic tree and their chromosomal localization) in tree shrew (Table S2). The coding region of tGBPs ranged from 1733 bp to 1884 bp, and the predicted proteins had a molecular weight ranged from 67 kD to 72 kD (Table S2). In the unrooted NJ tree, tGBPs had a closer relationship to hGBPs than to mGBPs (Fig. 1A). All five tGBPs were mapped to one gene cluster on one chromosome based on the updated assembly genome of the Chinese tree shrew (authors’ unpublished data), which resembled the human GBPs cluster (Fig. 1B).

There were conserved motifs in the N-terminal of tGBPs, including the GxxxxGK(S/T), DxxG and TLRD/TVRD motifs that belong to the GTPases domain. The C-terminal of tGBPs had a relatively low sequence identity among different members, whereas the N-terminal of different tGBPs seemed to be more conserved. Only tGBP1, tGBP2 and tGBP5 contained a CaaX motif (Fig. 2), which was said to be responsible for its isoprenylation (Degrandi et al., 2007; Olszewski et al., 2006).

2.7. IFN-γ (tIFN-γ) treatment

The HEK293 cells were seeded in 6-well plates (1 × 10^5 cells/well) and were transfected with pCMV-3tag-8 (empty vector) and pCMV-tIFN-γ-3tag-8 using X-tremeGENE HP (Roche, 06366546001) following the manufacturer’s instruction. After 6 h of transfection, cells were washed three times with PBS, and switched to growth medium for 48 h. The culture supernatants containing tIFN-γ were collected and ﬁltered with 0.45 μm ﬁlter for use.

For tIFN-γ stimulation assay, the TSPRCs were seeded in 24-well plates (5 × 10^4 cells/well with 500 μL growth medium) for growth for 12 h, then the culture supernatant containing tIFN-γ was added to growth medium (100 μL/well). The TSPRCs were harvested at the indicated times post tIFN-γ treatment.

2.8. Knockout of tGBP1

We used the CRISPR-Cas9 system to knock out the tGBP1 gene in TSR6 cells (Ma et al., 2018; Ran et al., 2013). The sgRNAs (tGBP1-sgRNA-F 5′-ACACCATTGTCAGAGGCTGGT 3′/tGBP1-sgRNA-R 5′-AACACATTAGGCTGGTAAATC 3′) targeting the coding region of the tGBP1 gene were designed by the CRISPR Design Tool (http://bioinfogp.cnb.csic.es/tools/breakingcas/index.php) (Olveros et al., 2016). The sgRNA pair was annealed and cloned into the pX330-T7 vector (a kind gift from Dr. Ping Zheng, KIZ) expressing mCherry. The TSR6 cells were transfected with the pX330-T7 vector carrying the sgRNAs. Transfected cells expressing mCherry were sorted by ﬂow cytometry and cultured for 48 h. Single cells were manually picked with a pipette tip for expansion for around three weeks.

The AxyPrep Multisource Genomic DNA Miniprep Kit (Axxygen, 26817KCI) was used to extract genomic DNA of the single TSR6 cells with potential knockout of tGBP1. The gene region spanning the sgRNA targeting sites was ampliﬁed by using primer pair (tGBP1-sgF 5′-CGATAACAGCCTCATG 3′/tGBP1-sgR 5′-ACTGGTCTACCCACACCC3′). The PCR products were sequenced using the primer tGBP1-sgF and analyzed by the DNAStar Lasergene 7.1 (DNAS Inc., Madison, WI, USA) to screen for mutation(s). We were able to pick up a cell clone with a frameshift mutation (c.74_75insA) to disrupt the translation of tGBP1 protein. We used the human GBP1 monoclonal antibody (SANTA CRUZ, sc-53857) to probe the tGBP1, but this antibody was unfortunately not workable in our assay.

2.9. Statistical analysis

Statistical analysis was determined by using the unpaired Student’s t-test with Prism software (GraphPad). Significant values were indicated as *, p < 0.05 and **, p < 0.01. Values were presented as mean ± standard error of mean (SEM).

3. Results

3.1. Identiﬁcation of five GBP genes in the Chinese tree shrew

The mRNA expression levels of tGBPs were analyzed in seven tissues from healthy adult tree shrews. tGBPs mRNA were ubiquitously detected in all examined tissues at a very low level according to the RT-qPCR. However, we could not obtain full-length tGBP mRNA sequences using these cDNAs as the template for PCR, suggesting that the full-length tGBP mRNA were expressed at extremely low level. There was a seemingly tissue-speciﬁc pattern for the mRNA levels of tGBPs in healthy animals. In particular, tGBP1 had a relatively higher mRNA level in lung compared to other tissues (Fig. 3A). tGBP2 exhibited a
higher mRNA level in heart and spleen tissues compared to other tissues (Fig. 3B). tGBP4 was highly expressed in heart, spleen, liver and lung tissues (Fig. 3C). tGBP5 had a higher mRNA level in spleen tissues relative to the other tissues (Fig. 3D), whereas tGBP7 was highly expressed in heart and lung tissues (Fig. 3E). The different tissue expression pattern of tGBPs might be relevant to the specific roles of tGBPs in the immune system and further studies should be performed to characterize the underpinnings of the unique expression pattern.

3.3. Upregulation of tGBPs in TSPRCs by tree shrew IFN-γ (tIFN-γ)

The GBP genes belong to the dynamin-like GTPases superfamily and are prominently induced by IFN-γ in human and mouse (Meunier and Broz, 2016; Pilla-Moffett et al., 2016). We investigated whether the tGBPs could be induced by tIFN-γ. As the IFNs exerted their function in an autocrine or paracrine manner (Borden et al., 2007), we overexpressed the tIFN-γ in HEK293T cells and collected the culture supernatant to treat TSPRCs. tIFN-γ treatment significantly upregulated the mRNA expression levels of tGBPs as early as at 4 h. Notably, the most predominantly induced tGBP5 was upregulated by nearly 70 folds as early as 9 h post infection (hpi) (Fig. 5A). EMCV infection significantly induced tGBPs expression at 12 hpi, and tGBP1 and tGBP5 had an earlier upregulation at 9 hpi (Fig. 5B). HSV-1 infection continuously enhanced the mRNA expression of tGBPs at 6 hpi (Fig. 5C). Among these three viruses, NDV infection had the most pronounced effect (Fig. 5). These results suggested that tGBPs were upregulated by viral infections and might play active roles in antiviral immunity.

3.5. Cellular location of tGBPs

The cellular location was important for GBP proteins to exert their host defense function against pathogen infection (Biering et al., 2017; Choi et al., 2016; Park et al., 2016; Wandel et al., 2017; Yamamoto et al., 2012). We investigated cellular location of tGBPs in TSPRCs with or without viral infections. The tGBPs were widely distributed in the cytoplasm of TSPRCs, and this localization pattern was not altered by NDV or HSV-1 infection (Fig. 6). Overexpression of tGBP7 showed seemingly granular structures irrespective of viral infections, which might be the aggregation of overexpressed tGBP7 protein (Fig. 6) (Britzen-Laurent et al., 2010).

3.6. Overexpression of tGBP1 repressed VSV replication in TSPRCs

The GBPs of human and mouse have been shown to be involved in

alterations of tGBPs in response to different virus infections in TSPRCs. Either the RNA virus (NDV or EMCV) or DNA virus (HSV-1) infection stimulated tGBPs mRNA expression (Fig. 5). NDV infection significantly and persistently upregulated all five tGBPs as early as 9 h post infection (hpi) (Fig. 5A). EMCV infection significantly induced tGBPs expression at 12 hpi, and tGBP1 and tGBP5 had an earlier upregulation at 9 hpi (Fig. 5B). HSV-1 infection continuously enhanced the mRNA expression of tGBPs at 6 hpi (Fig. 5C). Among these three viruses, NDV infection had the most pronounced effect (Fig. 5). These results suggested that tGBPs were upregulated by viral infections and might play active roles in antiviral immunity.
the response against viral infection (Pilla-Moffett et al., 2016). We examined the antiviral function of tGBPs upon VSV and HSV-1 infections. TSPRCs were transiently transfected with respective tGBP expression vector. All 5 tGBPs could be successfully overexpressed in TSPRCs (Fig. 7A). As had been reported before (Xu et al., 2015, 2016), overexpression of tMAVS or tMITA (as a positive control) inhibited VSV and HSV-1 infection, respectively (Fig. 7B–D). Ectopic expression of tGBP1 significantly inhibited the replication of VSV and had a mild effect on HSV-1 replication in TSPRCs (Fig. 7B–D). However, the other tGBPs had no apparent inhibitory effect on VSV and HSV-1 replications (Fig. 7B–D).

Fig. 2. Multiple protein sequence alignment of five tGBPs. Black boxes indicated identical residues, and gray boxes indicated similar residues. The three conserved motifs within the GTP-binding site and the carboxy-terminal CaaX motif were marked in red box and blue box, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. The mRNA levels of tGBPs in seven tissues of the Chinese tree shrews. The mRNA levels of (A) tGBP1, (B) tGBP2, (C) tGBP4, (D) tGBP5 and (E) tGBP7 in each tissue from five healthy adult tree shrews were measured by quantitative real-time PCR, with normalization to β-actin.

the response against viral infection (Pilla-Moffett et al., 2016). We examined the antiviral function of tGBPs upon VSV and HSV-1 infections. TSPRCs were transiently transfected with respective tGBP expression vector. All 5 tGBPs could be successfully overexpressed in TSPRCs (Fig. 7A). As had been reported before (Xu et al., 2015, 2016), overexpression of tMAVS or tMITA (as a positive control) inhibited VSV and HSV-1 infection, respectively (Fig. 7B–D). Ectopic expression of tGBP1 significantly inhibited the replication of VSV and had a mild effect on HSV-1 replication in TSPRCs (Fig. 7B–D). However, the other tGBPs had no apparent inhibitory effect on VSV and HSV-1 replications (Fig. 7B–D).
3.7 Knockout of tGBP1 promoted VSV replication in a tree shrew renal cell line

We further confirmed the antiviral activity of tGBP1 by knocking out this gene in the TSR6 cell line, which was immortalized from TSPRCs in our recent study (Gu et al., 2019). Using the CRISPR/Cas9 technique, we successfully disrupted the tGBP1 coding frame by introducing mutation c.74_75insA (Fig. 8A). As expected, the VSV replication was significantly increased in the TSR6 cells with tGBP1 knockout relative to wild-type cells (Fig. 8B and C). Collectively, these results based on the overexpression and knockout of tGBP1 supported its antiviral activity.

4. Discussion

In recent years, the Chinese tree shrew has received much attention in the field for its usage as a small and viable experimental animal (Li et al., 2018; Yao, 2017). The closer affinity of the tree shrew to primates as compared to that of rodents to primates had opened a new avenue for replacing primates by tree shrew in biomedical researches in certain aspects (Xiao et al., 2017; Yao, 2017), especially considering the recent breakthrough for gene editing (Ma et al., 2018) and transgenic

Fig. 4. Upregulation of tGBP mRNA expression in tree shrew primary renal cells (TSPRCs) with and without tIFN-γ treatment. The TSPRCs were stimulated by culture supernatants of HEK293T cells transfected with pCMV-3tag-8 (empty vector) or pCMV-tIFN-γ-3tag-8 for the indicated times. The relative mRNA levels of tGBPs were determined by quantitative real-time PCR, with normalization to β-actin. The fold values (relative to the controls at each time point) were presented as mean ± SEM. The experiments were repeated three times, with consistent results.

Fig. 5. Upregulation of mRNA levels of tGBPs in tree shrew primary renal cells (TSPRCs) in response to virus infections. The TSPRCs were infected by (A) NDV (MOI = 1), (B) EMCV (MOI = 1) or (C) HSV-1 (MOI = 1) for the indicated time points. The mRNA level of each tGBP was determined by quantitative real-time PCR, with normalization to β-actin. Data shown were fold changes of each tGBP relative to the respective control. The experiments were repeated three times with similar results.
GBPs were successfully identified in the Chinese tree shrew. These tGBPs shared the common features with hGBPs and mGbps. The most conserved GTP binding motif TLRD/TVRD and GxxxxGK(S/T) (Degrandi et al., 2007; Olszewski et al., 2006; Pilla-Moffett et al., 2016) were presented in all tGBPs. In accordance with hGBPs and mGbps, tGBP1, tGBP2 and tGBP5 had a CaaX motif in the C-terminal parts (Olszewski et al., 2006). Further analysis demonstrated that the tGBPs in tree shrew had a closer relationship with hGBP1 and mGbp2, since the respective tGBP was examined using confocal microscope. Data shown were representative of three independent experiments.

GBPs play important roles in host defense against bacterial, parasitic and viral pathogens (Meunier and Broz, 2016; Pilla-Moffett et al., 2016). However, the GBP family were only well characterized in human and mouse (Degrandi et al., 2007; Olszewski et al., 2006). Homologs of GBPs were not detected in any invertebrate species, indicating that GBPs were most likely arose in the ancestor of chordates and functioned in the conservation and divergence of GBPs in tree shrew.

GBPs were indeed induced by many cytokines and proinflammatory cytokines, and were mainly induced by IFN-γ (Degrandi et al., 2007; Tripal et al., 2007). Similarly to hGBPs and mGbps, tGBPs were strongly induced by IFN-γ. In addition, mRNA levels of tGBPs could be stimulated by virus infections, suggesting that tGBPs are involved in the antiviral immune response. It has been reported that GBPs were recruited to the pathogen-containing vacuoles (PCVs) to destroy the integrity of PCVs, thus to expose the pathogens to host or to eliminate it (Choi et al., 2014; Kravets et al., 2016). Previous studies showed that hGBP1 and hGBP5 were consistently located in cytoplasm in the resting state or upon different virus infections (Feng et al., 2017; Itsui et al., 2009; Zou et al., 2017). Porcine GBP1 also showed a similar cellular location in response to classical swine fever virus infection (Li et al., 2016c). Consistent with these observations, we found that tGBPs were located in the cytoplasm and viral infections had no apparent effect on the cellular localization of tGBPs.

Fig. 6. Cellular localization of tGBPs in tree shrew renal cells (TSPRCs) with or without viral infections. Cells were transiently transfected with the indicated Flag-tagged tGBP expression vector for 24 h, then were infected with NDV (MOI = 1) or HSV-1 (MOI = 1) for 12 h. After fixation and immunostaining, cellular localization of the respective tGBP was examined using confocal microscope. Data shown were representative of three independent experiments.

manipulation of the Chinese tree shrews (Li et al., 2017; Xu et al., 2017). We and others have described the unique and common features of the tree shrew immune genes in the recent years (Jiang et al., 2018; Luo et al., 2018; Mu et al., 2014; Xu et al., 2016; Yao et al., 2019; Yu et al., 2018; Mu et al., 2014; Xu et al., 2016; Xu et al., 2013a). In this study, we characterized the GBPs in the Chinese tree shrew, and we added another case showing the conservation and divergence of GBPs in tree shrew.

GBPs play important roles in host defense against bacterial, parasitic and viral pathogens (Meunier and Broz, 2016; Pilla-Moffett et al., 2016). However, the GBP family were only well characterized in human and mouse (Degrandi et al., 2007; Olszewski et al., 2006). Homologs of GBPs were not detected in any invertebrate species, indicating that GBPs were most likely arose in the ancestor of chordates and functioned in the conservation and divergence of GBPs in tree shrew.

GBPs were successfully identified in the Chinese tree shrew. These tGBPs shared the common features with hGBPs and mGbps. The most conserved GTP binding motif TLRD/TVRD and GxxxxGK(S/T) (Degrandi et al., 2007; Olszewski et al., 2006; Pilla-Moffett et al., 2016) were presented in all tGBPs. In accordance with hGBPs and mGbps, tGBP1, tGBP2 and tGBP5 had a CaaX motif in the C-terminal parts (Olszewski et al., 2006). Further analysis demonstrated that the tGBPs in tree shrew had a closer relationship with hGBP1 than with mGbps. First, the chromosomal location and gene organization of tGBPs were more similar to hGBPs compared to mGbps (Kim et al., 2011; Olszewski et al., 2006; Pilla-Moffett et al., 2016). Second, the phylogenetic clustering pattern of tGBPs with hGBPs also supported this conclusion. These observations were in line with the opinion that the Chinese tree shrew had a close relationship with primates (Fan et al., 2013; Xu et al., 2013a).

Hitherto, the tissue expression patterns of GBPs have not been fully evaluated in other species except for human and mouse (Degrandi et al., 2007; Kim et al., 2011; Olszewski et al., 2006). The expression profiles of GBPs in human and mouse had been compared by using the data form UniGene database (Olszewski et al., 2006), which showed that hGBP1 and mGbp2 in the spleen and hGBP4 and mGbp3 in the bone marrow had a similar expression pattern, respectively (Olszewski et al., 2006). We examined the tissue expression profiles of tGBPs in seven tissues from healthy adult tree shrews and provided the first-hand data for the tissue expression pattern of GBPs. The tGBPs were ubiquitously expressed in all seven tissues examined in this study, but there was a tissue-specific pattern for different members. The overall tissue expression pattern of tGBPs resembled that of human (Olszewski et al., 2006).

GBPs are ISGs that were induced by many cytokines and proinflammatory cytokines, and were mainly induced by IFN-γ (Degrandi et al., 2007; Tripal et al., 2007). Similarly to hGBPs and mGbps, tGBPs were strongly induced by IFN-γ. In addition, mRNA levels of tGBPs could be stimulated by virus infections, suggesting that tGBPs are involved in the antiviral immune response. It has been reported that GBPs were recruited to the pathogen-containing vacuoles (PCVs) to destroy the integrity of PCVs, thus to expose the pathogens to host or to eliminate it (Choi et al., 2014; Kravets et al., 2016). Previous studies showed that hGBP1 and hGBP5 were consistently located in cytoplasm in the resting state or upon different virus infections (Feng et al., 2017; Itsui et al., 2009; Zou et al., 2017). Porcine GBP1 also showed a similar cellular location in response to classical swine fever virus infection (Li et al., 2016c). Consistent with these observations, we found that tGBPs were located in the cytoplasm and viral infections had no apparent effect on the cellular localization of tGBPs.

Human GBP1 showed antiviral activity against various virus infections, such as VSV, EMCV, HCV, KSHV and HBV (Anderson et al., 1999; Itsui et al., 2009; Itsui et al., 2009; Lu et al., 2008; Zou et al., 2017). While the tGBP1 had an inhibitory effect on VSV and HSV-1 replications, the other tGBPs had no obvious effect on the replications of VSV and HSV-1. We speculated that tGBP1 and hGBP1 may share the same antiviral mechanism, as tGBP1 and hGBP1 had a closer affinity in the phylogenetic tree, and both presented similar antiviral spectrum (Anderson et al., 1999; Carter et al., 2005; Itsui et al., 2009). The antiviral activity of hGBP1 against the positive-strand RNA virus and DNA virus infections had been well depicted (Itsui et al., 2009; Zou et al., 2017). As VSV is a negative-strand RNA virus, further functional assays should be performed to
understand the antiviral function of tGBP1 against VSV infection. This pursuit will benefit for the understanding of the broad antiviral spectrum of GBP1.

In short, we identified five tGBPs and confirmed the conservation of GBP structure and function in the Chinese tree shrew. The updated information about the GBPs in the Chinese tree shrew will provide more information for the GBP family and for us to learn the innate immune restriction factors in this species.

Fig. 7. Overexpression of tGBP1 inhibited replication of VSV and HSV-1 in tree shrew renal cells (TSPRCs). (A) Western blot analysis of the overexpressed tGBPs in TSPRCs. (B) Flow cytometry analysis and (C) quantification of VSV replication in TSPRCs overexpressing the indicated vector. Cells (1 × 10^5) were transfected with the indicated vector (1 μg) for 12 h before being infected with VSV-GFP (MOI = 0.01) for 12 h. Percentage of 10,000 cells expressing GFP (GFP+ cells) was counted by flow cytometry. Overexpression of tMAVS was used as a positive control. (D) Quantification of HSV-1 replication in TSPRCs overexpressing the indicated vectors. TSPRCs (1 × 10^5) were transfected with the indicated vectors for 24 h, followed by infection with HSV-1 (MOI = 1) for 1 h. Cells were washed three times with PBS and switched to growth medium for 24 h. The copy number of HSV-1 DNA in the culture supernatant was analyzed by quantitative real-time PCR using the specific primers for HSV-1 genome as reported in previous study (Zhang et al., 2014). Overexpression of tMITA was used as a positive control. The data were representative of three independent experiments.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (U1402224 to Y-GY, 31700155 to LX), Yunnan Province (2016FB018 to LX) and CAS “Light of West China” Program (to LX).
Developmental and Comparative Immunology 96 (2019) 27–36

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.02.014.

References


interferon-induced GTPase, exerts an antiviral activity against classical swine fever virus depending on its GTPase activity. J. Virol. 90, 4412–4426.


<table>
<thead>
<tr>
<th>Gene</th>
<th>Usage</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>tGBP1</td>
<td>5' RACE</td>
<td>TTTATGGTTTTCACCAGTACCTTG</td>
</tr>
<tr>
<td></td>
<td>5' RACE nest</td>
<td>GCTGTATTACACTAAGGGTCTGTC</td>
</tr>
<tr>
<td></td>
<td>3' RACE</td>
<td>CTGTGATTGATAAATTCTACAGAC</td>
</tr>
<tr>
<td></td>
<td>3' RACE nest</td>
<td>AAAGAGCAATACTGTACACTT</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR F</td>
<td>TTTGATCGACCCACCTCACCG</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR R</td>
<td>AGTGGGTAAATGGGCTTGG</td>
</tr>
<tr>
<td></td>
<td>Vector construction F</td>
<td>ATGCctcagATGGCTCTAGACACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCctcagATGGCTCTAGACACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tGBP2</td>
<td>5' RACE</td>
<td>AGGGTGTGGTTTGGCTTGGGA</td>
</tr>
<tr>
<td></td>
<td>5' RACE nest</td>
<td>CGCAATAGCCACCCACGGC</td>
</tr>
<tr>
<td></td>
<td>3' RACE</td>
<td>GGAGATAGGGAAACGGATGGA</td>
</tr>
<tr>
<td></td>
<td>3' RACE nest</td>
<td>ATGGGAGAGACTATGCAGTGTTGT</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR F</td>
<td>TCAGCAGACTTCAAGCCAG</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR R</td>
<td>AATAGCACCACCCACCGC</td>
</tr>
<tr>
<td></td>
<td>Vector construction F</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tGBP4</td>
<td>5' RACE</td>
<td>CTTTTTCTATGTCGAGCTCT</td>
</tr>
<tr>
<td></td>
<td>5' RACE nest</td>
<td>TTCTTGTCAGCGGCTTGG</td>
</tr>
<tr>
<td></td>
<td>3' RACE</td>
<td>TATTTTAAATCCATGAGAAGCAGCA</td>
</tr>
<tr>
<td></td>
<td>3' RACE nest</td>
<td>CTGGTGATGCTCGCAT</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR F</td>
<td>TACCTAGGCCTAGCAGC</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR R</td>
<td>ACACACCTAGATGAGCTT</td>
</tr>
<tr>
<td></td>
<td>Vector construction F</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tGBP5</td>
<td>5' RACE</td>
<td>AATCTGGGTGTCCCTGGTTGGT</td>
</tr>
<tr>
<td></td>
<td>5' RACE nest</td>
<td>GCATACACCATCCAGATTCCT</td>
</tr>
<tr>
<td></td>
<td>3' RACE</td>
<td>CCAGGCTCTTCAGCGAAGGAA</td>
</tr>
<tr>
<td></td>
<td>3' RACE nest</td>
<td>CACATCGAGAACACTAGAATAGA</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR F</td>
<td>CCCTACTAGATGCAAGCGG</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR R</td>
<td>ACTCTTCTTCTGGGCTTG</td>
</tr>
<tr>
<td></td>
<td>Vector construction F</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tGBP7</td>
<td>5' RACE</td>
<td>TGACCAACATGTCAGTTGTCCTT</td>
</tr>
<tr>
<td></td>
<td>5' RACE nest</td>
<td>CTCTTGGAAGAATGACTGTTCTT</td>
</tr>
<tr>
<td></td>
<td>3' RACE</td>
<td>ACAGCACTACAGCCACCA</td>
</tr>
<tr>
<td></td>
<td>3' RACE nest</td>
<td>ATTTTGAAAGTTGGAACAGTAG</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR F</td>
<td>AGTTTGCTGTGCTAGATCC</td>
</tr>
<tr>
<td></td>
<td>RT-qPCR R</td>
<td>TCTTGGAACAGAGCAAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction F</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tIFNγ</td>
<td>Vector construction F</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
<tr>
<td></td>
<td>Vector construction R</td>
<td>ATGCgagATGGCCCTACAGACAT</td>
</tr>
</tbody>
</table>

The nucleotides marked in italic refer to the recognition site of restricted enzymes. RT-qPCR, quantitative real-time PCR.
Table S2. The characteristics of tree shrew GBPs

<table>
<thead>
<tr>
<th>Gene name</th>
<th>5' UTR (bp)</th>
<th>3' UTR (bp)</th>
<th>CDS (bp)</th>
<th>Full-length (bp)</th>
<th>Mw (kD)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>tGBP1</td>
<td>816</td>
<td>196</td>
<td>1733</td>
<td>2155</td>
<td>67.6</td>
<td>MK214685</td>
</tr>
<tr>
<td>tGBP2</td>
<td>127</td>
<td>1162</td>
<td>1764</td>
<td>3052</td>
<td>66.9</td>
<td>MK214686</td>
</tr>
<tr>
<td>tGBP4</td>
<td>323</td>
<td>728</td>
<td>1872</td>
<td>2922</td>
<td>71.3</td>
<td>MK214687</td>
</tr>
<tr>
<td>tGBP5</td>
<td>218</td>
<td>134</td>
<td>1770</td>
<td>2120</td>
<td>67.3</td>
<td>MK214688</td>
</tr>
<tr>
<td>tGBP7</td>
<td>126</td>
<td>719</td>
<td>1884</td>
<td>2728</td>
<td>71.4</td>
<td>MK214689</td>
</tr>
</tbody>
</table>

Mw, molecular weight.