Nuclear β-Arrestin1 Functions as a Scaffold for the Dephosphorylation of STAT1 and Moderates the Antiviral Activity of IFN-γ

Wei Mo,1,5 Liang Zhang,1,6 Guohua Yang,1 Jianwei Zhai,1 Zhonghua Hu,1 Yuelei Chen,1 Xu Chen,2 Lijian Hui,3 Ruimin Huang,4 and Gengxi Hu1,*

1State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
2Department of Ophthalmology, Zhongshan Hospital, Fudan University, Shanghai 200032, China
3Research Institute of Molecular Pathology, A-1030 Vienna, Austria
4Department of Neurology, Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA
5These authors contributed equally to this work
6Correspondence: gxhu@sibs.ac.cn

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SUMMARY

Signal transducers and activators of transcription 1 (STAT1) is activated by tyrosine phosphorylation upon interferon-γ (IFN-γ) stimulation. Phosphorylated STAT1 translocates into nucleus to initiate the transcription of IFN-γ target genes that are important in mediating antiviral, antiproliferative, and immune response. The inactivation of STAT1 is mainly accomplished via tyrosine dephosphorylation by the nuclear isoform of T cell protein tyrosine phosphatase (TC45) in nucleus. Here we show that β-arrestin1 directly interacts with STAT1 in nucleus after IFN-γ treatment, and accelerates STAT1 tyrosine dephosphorylation by recruiting TC45. Consequently, β-arrestin1 negatively regulates STAT1 transcription activity as well as the IFN-γ-induced gene transcription. Application of β-arrestin1 siRNA significantly enhances IFN-γ-induced antiviral response in vesicular stomatitis virus (VSV)-infected cells. Our results reveal that nuclear β-arrestin1, acting as a scaffold for the dephosphorylation of STAT1, is an essential negative regulator of IFN-γ signaling and participates in the IFN-γ-induced cellular antiviral response.

INTRODUCTION

IFN-γ was discovered on the basis of antiviral activity (Isaacs and Lindenmann, 1957). During virus infection, IFN-γ induces activation of the transcription factor STAT1 (Stark et al., 1998), which controls the expression of antiviral related genes. IFN-γR−/− and STAT1−/− mice are extremely susceptible to viral infections (Durbin et al., 1996; Muller et al., 1994).

STAT1, a member of STAT family, is essential for gene activation in response to IFN-γ stimulation (Darnell et al., 1994; Durbin et al., 1996). Cells and tissues from STAT1−/− mice are unresponsive to IFN-γ (Durbin et al., 1996). Upon IFN-γ treatment, STAT1 becomes tyrosine phosphorylated at residue 701 by Janus kinases (JAKs). Activated STAT1 dimerize and then translocate into nucleus to bind the promoters of IFN-γ response genes with IFN-γ activation site (GAS) and to activate their transcription (Shuai et al., 1993a, 1993b). Serine phosphorylation at residue 727 is required for maximal transcription activity of STAT1, though it is not essential for STAT1 nuclear translocation (Wen et al., 1995).

How the activity of the JAK-STAT pathway is restrained and balanced is crucially important to understand its physiological function. Abnormal activation of the pathway is implicated in various pathological conditions. For example, aberrant STAT activation contributes to the process of malignant transformation (Bowman et al., 2000); several hematopoietic disorders are caused, at least in part, by perturbations of this pathway (Ward et al., 2000); and impaired STAT1 activation may contribute to IFN-γ treatment failure in viral hepatitis patients (Gao, 2005). Several distinct mechanisms such as receptor-mediated endocytosis (Strous et al., 1996) and CIS/SOCS/SSI family proteins (Yoshimura, 1998) have been proposed to negatively regulate the pathway. Although great progress has been made toward understanding of STAT1 activity regulation in cytoplasm, much less is known about the molecular events of STAT1 inactivation and functional interaction with other proteins in nucleus. Tyrosine dephosphorylation is considered the final and key event in inactivation of STAT1 in nucleus (Haspel and Darnell, 1999; Haspel et al., 1996). Blockage of STAT1 dephosphorylation by sodium orthovanadate, a phosphatase inhibitor, leads to nuclear accumulation of all the tyrosine phosphorylated STAT1 and its subsequent persistence (Haspel and Darnell, 1999). TC45 is the major nuclear STAT1 protein tyrosine phosphatase (PTPase) (ten Hoeve et al., 2002). TC45 null mouse embryonic fibroblasts (MEFs) exhibit prolonged tyrosine phosphorylation (ten Hoeve et al., 2002). However, the detailed regulatory mechanisms remain to be elicited.

β-arrestins are ubiquitous multifunctional scaffold proteins. In addition to their well-established functions in the desensitization and endocytosis of diverse cell surface receptors (Perry and Lefkowitz, 2002), β-arrestins also serve as scaffolds to regulate
the activities or subcellular distribution of multiple signaling molecules, such as the kinase Akt, Src family kinases, and MDM2 in cytoplasm (DeWire et al., 2007). Recently, a nuclear function of β-arrestin1 has been reported in regulating gene expression by recruiting histone acetyltransferase p300 to specific gene promoters (Kang et al., 2005). However, in addition to modulating histone acetylation, whether nuclear β-arrestin1 can act as a scaffold for the phosphorylation of transcription factors and regulate signaling transcription is unknown.

Here we show that, in response to IFN-γ, nuclear β-arrestin1 directly interacts with transcription factor STAT1 and negatively regulates STAT1 transcription activity as well as the expression of IFN-γ response genes by attenuating STAT1 tyrosine phosphorylation. We further demonstrate that β-arrestin1 specifically accelerates STAT1 tyrosine dephosphorylation via recruiting TC45 to form the STAT1-β-arrestin1-TC45 complex in nucleus. Moreover, knockdown of β-arrestin1 by siRNA enhances the IFN-γ-induced antiviral response in VSV-infected cells. Our results collectively demonstrate a scaffold function of β-arrestin1 in nucleus as an essential negative regulator of STAT1 and IFN-γ signaling, which moderates the antiviral activity of IFN-γ.

RESULTS

β-Arrestin1 Directly Interacts with STAT1

To identify regulators of JAK-STAT pathway, a yeast two-hybrid screening was performed to find out which proteins interact with STAT1, by using deletion mutant of STAT1 without transactivation domain (TAD) as the bait. Several positive clones were identified to encode β-arrestin1. The kinase Akt, Src family kinases, and MDM2 in cytoplasm (DeWire et al., 2007). Recently, a nuclear function of β-arrestin1 has been reported in regulating gene expression by recruiting histone acetyltransferase p300 to specific gene promoters (Kang et al., 2005). However, in addition to modulating histone acetylation, whether nuclear β-arrestin1 can act as a scaffold for the phosphorylation of transcription factors and regulate signaling transcription is unknown.

Here we show that, in response to IFN-γ, nuclear β-arrestin1 directly interacts with transcription factor STAT1 and negatively regulates STAT1 transcription activity as well as the expression of IFN-γ response genes by attenuating STAT1 tyrosine phosphorylation. We further demonstrate that β-arrestin1 specifically accelerates STAT1 tyrosine dephosphorylation via recruiting TC45 to form the STAT1-β-arrestin1-TC45 complex in nucleus. Moreover, knockdown of β-arrestin1 by siRNA enhances the IFN-γ-induced antiviral response in VSV-infected cells. Our results collectively demonstrate a scaffold function of β-arrestin1 in nucleus as an essential negative regulator of STAT1 and IFN-γ signaling, which moderates the antiviral activity of IFN-γ.

C Terminus of β-Arrestin1 Binds to the Linker Domain of STAT1

To map the binding region of β-arrestin1 to STAT1 protein, colP assay was performed with exogenous Myc-ST1 and HA-tagged deletion mutants of β-arrestin1 (Figure 2A). The mutants without the C terminus of β-arrestin1, Arrb1 (1–180), Arrb1 (1–240), and Arrb1 (1–320), lost their affinities to STAT1 (Figure 2B). In contrast, N-terminal deletion mutants of β-arrestin1 protein, Arrb1 (181–418), Arrb1 (241–418), and Arrb1 (318–418), efficiently interacted with STAT1 (Figure 2B). On the other hand, binding affinities of STAT1 deletion mutants to β-arrestin1 protein were also detected (Figure 2C). STAT1 (310–750), STAT1 (480–750), and STAT1 (372–580) could bind to β-arrestin1 protein. The overlapping region (residues 480–580) of these deletion mutants is located inside the linker region.
domain (residues 488–576) of STAT1. Moreover, STAT1 deletion mutant without the linker domain (STAT1<sup>D</sup>LD) lost its affinity to β-arrestin1 (Figure 2D). The above results suggest that the C terminus of β-arrestin1 and the linker domain of STAT1 contribute to the interaction between β-arrestin1 and STAT1.

**IFN-γ Treatment Enhances the β-Arrestin1-STAT1 Interaction in Nucleus**

Interaction of STAT1 with other binding partners has been shown to be regulated by IFN-γ stimulation, as in the case of its interaction with PIAS1 (Liu et al., 1998) and TRADD (Wesemann et al., 2004). Therefore, we examined whether the β-arrestin1-STAT1 interaction was regulated by IFN-γ treatment. As shown in Figure 3A, endogenous β-arrestin1-STAT1 interaction in HeLa, HEK293, and RAW264.7 cells was significantly enhanced upon IFN-γ stimulation. Furthermore, the fractionated cytoplasmic and nuclear extracts of HEK293 cells treated by IFN-γ were immunoprecipitated with anti-β-arrestin antibody. In the cytoplasm fraction, there was almost no detectable STAT1 in β-arrestin-immunoprecipitates in the presence or absence of IFN-γ. However, in the nuclear fraction, increased STAT1 protein (by a factor of five) was detected in β-arrestin-immunoprecipitates induced by IFN-γ (Figure 3B). These results show that the β-arrestin1-STAT1 interaction is enhanced in nucleus instead of in cytoplasm after IFN-γ treatment. Given that β-arrestin1 is localized in both the nucleus and the cytoplasm at steady state, while β-arrestin2 is predominantly distributed in the cytoplasm (Wang et al., 2003b), and that no obvious nuclear translocation of β-arrestin was detected after IFN-γ treatment (Figure 3B), we reasoned that it was nuclear β-arrestin1 that interacted with STAT1. It was confirmed by immunoprecipitation using nuclear extracts, which showed that there was increased β-arrestin1 protein in STAT1-immunocomplex after IFN-γ treatment, while both β-arrestin2 and β-arrestin1 cytoplasmic mutant (Q394L), located exclusively in cytoplasm (Wang et al., 2003b), had no such interaction with STAT1 (Figure 3C). Further, we reasoned the enhancement of interaction was presumably due to IFN-γ-induced tyrosine/serine phosphorylation or nuclear translocation of STAT1. To test this hypothesis, three STAT1 mutants, S727A, Y701F, and L407A, were generated. S727A and Y701F could not be serine or tyrosine phosphorylated, respectively. L407A could not translocate into nucleus (McBride et al., 2002; Shuai et al., 1993b; Wen et al., 1995). coIP assay in nuclear extracts of U3A cells that lack endogenous STAT1 showed that, after IFN-γ treatment, both STAT1 and S727A had higher affinities to β-arrestin1, while Y701F and L407A did not increase the precipitation of β-arrestin1 (Figure 3D), indicating that both tyrosine phosphorylation and nuclear localization of STAT1 are required for IFN-γ-induced enhancement of β-arrestin1-STAT1 interaction. What’s more, tyrosine phosphorylation of STAT1...
could directly enhance its interaction with β-arrestin1 (Figure 3E). These observations strongly support that β-arrestin1 can efficiently interact with tyrosine phosphorylated STAT1 in nucleus in IFN-γ-stimulated manner.

**β-Arrestin1 Accelerates STAT1 Dephosphorylation**

Since the tyrosine phosphorylation of STAT1 is functionally important for efficient signaling in IFN-γ signaling pathway (Stark et al., 1998), we explored whether the β-arrestin-STAT1

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**Figure 3. IFN-γ Treatment Enhances the Interaction of β-Arrestin1 with STAT1 in Nucleus**

(A) Endogenous interaction of β-arrestin1 with STAT1 in HeLa, HEK293, or RAW 264.7 cells after IFN-γ treatment for 30 min.

(B) Immunoprecipitation and immunoblot of cytoplasmic or nuclear extracts from HEK293 cells after IFN-γ treatment for 30 min.

(C) Immunoprecipitation and immunoblot of nuclear extracts from HEK293 cells expressing Myc-STAT1 and HA-β-arrestin1 or HA-β-arrestin2 or HA-β-arrestin1 Q394L in the absence or presence of IFN-γ for 30 min.

(D) Immunoprecipitation and immunoblot of nuclear extracts from U3A cells expressing Flag-β-arrestin1 and Myc-STAT1 or Myc-STAT1 mutants in the absence or presence of IFN-γ for 30 min.

(E) Endogenous STAT1 and tyrosine-phosphorylated STAT1 were immunoprecipitated from β-arrestin null MEFs in the absence or presence of IFN-γ with their antibodies, respectively. The immunoprecipitates were eluted and equal amount proteins were used in GST pull-down assay.

The data are representative of four similar experiments.
β-Arrestin1 Negatively Regulates STAT1 Activity

**β-Arrestin1 is Required for TC45-Mediated Tyrosine Dephosphorylation of STAT1**

Given that TC45 is clearly the primary PTPase responsible for the dephosphorylation of STAT1 (ten Hoeve et al., 2002), we investigated whether TC45 was involved in β-arrestin1-promoted STAT1 dephosphorylation. WT and TC-PTP null MEFs were transiently transfected with β-arrestin1 followed with IFN-γ treatment. Overexpression of β-arrestin1 (by ~5-fold over endogenous β-arrestin1) led to an accelerated STAT1 tyrosine dephosphorylation in WT MEFs (decline ~75% after 60 min of IFN-γ treatment), but not in TC-PTP null MEFs (Figure 5A), suggesting β-arrestin1 may regulate STAT1 dephosphorylation through TC45. Further, we explore the synergy between β-arrestin1 and TC45 in promoting the tyrosine dephosphorylation of STAT1 in β-arrestin1 null MEFs followed IFN-γ stimulation. As shown in Figure 5B, overexpression of TC45 (by ~3-fold) led to a limited acceleration of STAT1 tyrosine dephosphorylation (decline ~20% after 60 min of IFN-γ treatment), while the effect of TC45 was significantly augmented by coexpression of β-arrestin1 (decline ~50% after 60 min of IFN-γ treatment) (Figure 5B), indicating that β-arrestin1 is required for TC45-induced tyrosine dephosphorylation of STAT1. The relation between STAT1, β-arrestin1, and TC45 was further examined. We first verified whether recombinant GST-β-arrestin1 could interact with endogenous TC45 and STAT1. Affinity purification following incubation of GST or GST-β-arrestin1 with nuclear extracts of β-arrestin null MEFs untreated or treated with IFN-γ revealed a copurification of TC45 and STAT1 with GST-β-arrestin1, but not GST, suggesting an interaction of endogenous TC45 and STAT1 with β-arrestin1 (Figure 5C). Furthermore, the interaction could be enhanced (by ~5-fold) by IFN-γ treatment (Figure 5C). Immunoprecipitation experiments were performed to confirm the results obtained from GST pull-downs. As shown in Figure 5D, immunoprecipitation of STAT1 or TC45 from nuclear extracts of WT MEFs led to a colP of the other two proteins, suggesting the existence of STAT1/β-arrestin1/TC45 signaling complex in nucleus. Similarly, IFN-γ stimulation enhanced the association of TC45 with STAT1 by a factor of five. As we know that β-arrestin1 can serve as a scaffold protein to coordinate protein-protein interactions (Lefkowitz and Shenoy, 2005; Luttrell et al., 1999), an immunoprecipitation experiment using nuclear extracts from WT and β-arrestin null MEFs was performed to determine whether β-arrestin1 was essential for the formation of the STAT1/TC45 signaling complex. Consistent with results in Figure 5D, in WT MEFs, IFN-γ stimulation increased the amount of STAT1 in TC45 immunocomplex. In contrast, immunoprecipitation in nuclear extracts of β-arrestin null MEFs revealed a dramatic reduction in the amount of STAT1 interacting with TC45. In addition, reintroduction of β-arrestin1 to β-arrestin null MEFs could restore the STAT1/TC45 signaling complex (by ~80%) during IFN-γ stimulation (Figure 5E), indicating that β-arrestin1 is an essential scaffold interaction could affect IFN-γ-induced STAT1 phosphorylation. IFN-γ-treated wild-type (WT) and β-arrestin null MEFs were analyzed at different time points to measure the time-dependent tyrosine phosphorylation at residue Tyr701. In WT MEFs, the level of tyrosine-phosphorylated STAT1 reached the peak at 30 min of IFN-γ treatment, declined ~80% after 90 min, and became barely detectable after 120 min. In contrast, in the β-arrestin-null MEFs, STAT1 tyrosine phosphorylation reached its maximum at 60 min, remained at ~70% of the maximal level after 90 min of IFN-γ treatment, and was sustained for much longer time periods (Figure 4A). No significant difference in IFN-γ-induced STAT1 serine phosphorylation (Ser727) was observed between the WT and β-arrestin-null MEFs. Moreover, ectopic expression of β-arrestin1 (by ~2-fold over endogenous β-arrestin1 in WT MEFs), instead of β-arrestin2, in β-arrestin null MEFs led to a large decrease in STAT1 tyrosine phosphorylation (by ~70% after a 90 min exposure to IFN-γ) and caused a similar kinetic pattern of STAT1 phosphorylation as that in WT MEFs (Figure 4B), indicating that β-arrestin1 may negatively regulate IFN-γ-mediated STAT1 tyrosine phosphorylation.

Since the overall level of tyrosine-phosphorylated STAT1 is determined by the balance of phosphorylation and dephosphorylation events (Haspel et al., 1996), prolonged tyrosine phosphorylation of STAT1 in β-arrestin null MEFs may result from either an increase in JAK kinase activity or a decrease in PTPase activity toward STAT1. To monitor the rate of STAT1 dephosphorylation, a pulse-chase strategy (Haspel and Darnell, 1999; Haspel et al., 1996) was employed in which staurosporine, a protein kinase inhibitor, was added to cells pretreated with IFN-γ for 30 min, abruptly blocking the continuous phosphorylation of STAT1 by JAKs. The residual level of tyrosine-phosphorylated STAT1 was then determined at several later time points. In WT MEFs, tyrosine phosphorylation of STAT1 declined more than 85% after only 30 min of staurosporine treatment (Figure 4D). In contrast, STAT1 phosphorylation remained at approximately 75% of the maximal level after 30 min of staurosporine treatment in the β-arrestin null MEFs (Figure 4D). These results indicate that the prolonged tyrosine phosphorylation of STAT1 in β-arrestin null MEFs is due to a dephosphorylation event. Further, we used sodium orthovanadate, a nonspecific PTPase inhibitor (Haspel and Darnell, 1999), to confirm the contribution of dephosphorylation event. Cells were treated with IFN-γ for 30 min followed by the addition of orthovanadate and continued incubation for the indicated time periods. Orthovanadate almost eliminated the difference in the tyrosine phosphorylation of STAT1 between both MEFs (Figure 4E), indicating that β-arrestin1 accelerates STAT1 inactivation through PTPase-mediated dephosphorylation. Given the nuclear localization of β-arrestin1-STAT1 complex, the tyrosine dephosphorylation of nuclear STAT1 was examined. Ectopic expression of β-arrestin1, but not β-arrestin2, restored the dephosphorylation of nuclear STAT1 in β-arrestin null MEFs (Figure 4F), which was consistent with the previous results derived from the whole-cell lysate. To test whether these findings were an artifact of the particular cells, we repeated the pulse-chase experiment in HeLa cells. Our data showed that specific siRNAs reduced expression of β-arrestin1 by ~80% and β-arrestin2 by ~90% (Figure 4H). Depletion of β-arrestin1, but not β-arrestin2 in HeLa cells treated with IFN-γ, led to persistent STAT1 phosphorylation, which remained at ~70% of the maximal level after 90 min of staurosporine treatment (Figure 4G). These data coincided well with results derived from MEFs. In summary, β-arrestin1 accelerates the tyrosine dephosphorylation of IFN-γ-activated STAT1.

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Haspel et al., 1996)

long time periods (Figure 4A). No significant difference in IFN-γ-induced STAT1 serine phosphorylation (Ser727) was observed between the WT and β-arrestin-null MEFs. Moreover, ectopic expression of β-arrestin1 (by ~2-fold over endogenous β-arrestin1 in WT MEFs), instead of β-arrestin2, in β-arrestin null MEFs led to a large decrease in STAT1 tyrosine phosphorylation (by ~70% after a 90 min exposure to IFN-γ) and caused a similar kinetic pattern of STAT1 phosphorylation as that in WT MEFs (Figure 4B), indicating that β-arrestin1 may negatively regulate IFN-γ-mediated STAT1 tyrosine phosphorylation.

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β-Arrestin1 Negatively Regulates STAT1 Activity Induced by IFN-γ

Since β-arrestin1 impairs STAT1 tyrosine phosphorylation in response to IFN-γ treatment and the activity of STAT1 is dependent on tyrosine phosphorylation (Shuai et al., 1993a), we assessed the effect of β-arrestin1 on STAT1 transcription activity by the reporter with four GAS elements, which is activated by STAT1. In HeLa cells, ectopic expression of β-arrestin1 (by ~3- to 8-fold) significantly inhibited IFN-γ-induced STAT1 transcription activity in a dose-dependent way (by about 25%–60%) (Figure 6A). Our data from immunoprecipitation assay indicate that the C terminus of β-arrestin1 is required for interaction with TC45 (see Figure S1 available online). As expected, expression of Arrb1C (β-arrestin1 amino acids 181–418, which binds to both STAT1 and TC45), had a similar inhibition as WT β-arrestin1 on STAT1 reporter activity. In contrast, expression of Arrb1N (β-arrestin1 amino acids 1–180, which binds to neither STAT1 nor TC45) had no effect (Figure 6B), indicating binding to STAT1 and TC45 is necessary for β-arrestin1 to inhibit STAT1 transcription activity. On the other hand, application of β-arrestin1 siRNA augmented STAT1 reporter activity in the presence of IFN-γ (Figure 6C).

We also examined the effect of β-arrestin1 on the induction of IFN-γ downstream target genes including Gbp1, Gbp2, Ipl0, and Gsg15 (Schroder et al., 2004). Quantitative real-time RT-PCR data showed that IFN-γ-induced expression of the four genes was significantly increased in β-arrestin1-null MEFs compared with WT MEFs (Figure 6D). In addition, restoration of β-arrestin1, but not β-arrestin2, in β-arrestin1-null MEFs inhibited IFN-γ-induced Gbp1, Gbp2, Ipl0, and Gsg15 expression by about 50% (Figure 6E). Expression of Arrb1C similarly inhibited transcription of these genes, whereas expression of Arrb1N had no effect (Figure 6E). To assess the extent of involvement of β-arrestin in IFN-γ-stimulated genes more comprehensively, an Affymetrix-based analysis of WT and β-arrestin null MEFs was conducted after stimulation with IFN-γ for 0 or 6 hr. As shown in Table S1, induction of a major subset of IFN-γ-stimulated genes was much stronger in β-arrestin null MEFs than in WT MEFs, especially of some antigen processing-related genes such as Ciita, Tap1, Tap2, Dma, and Dmb and some antiviral-related genes such as Oas1 and Oas2. Our data collectively support the idea that β-arrestin1 functions as a negative regulator of IFN-γ signaling by accelerating the STAT1 inactivation.

DISCUSSION

β-Arrestin1, a Negative Regulator of STAT1

As an important transcription factor, STAT1 is tightly regulated. Upon IFN-γ treatment, STAT1 undergoes an activation-inactivation cycle (Haspel et al., 1996). Great progress has been made toward understanding of STAT1 activation in cytoplasm. However, the precise mechanism of the inactivation has not been fully investigated. STAT1 inactivation includes both maintaining STAT1 latency and terminating activated STAT1 by dephosphorylation. Nuclear dephosphorylation by tyrosine phosphatases represents the most important mechanism by which cells negatively control STAT1 activity (Haspel and Darnell, 1999; Haspel
The results from this study reveal an unanticipated function of \( \beta \)-arrestin1 as a regulator of STAT1 dephosphorylation via direct interaction with STAT1. Upon IFN-\( \gamma \) stimulation, the physical association of \( \beta \)-arrestin1 to STAT1 accelerates its dephosphorylation and downregulates STAT1 transcription activity, thereby exerting a negative feedback control of STAT1 function.

TC45 is regarded as the most important tyrosine phosphatase in the process of STAT1 dephosphorylation (ten Hoeve et al., 2002). However, in our experiments, the tyrosine dephosphorylation of STAT1 was accelerated in a limited degree by overexpression of TC45 in the absence of \( \beta \)-arrestin1, and the efficient performance of TC45 function required the presence of \( \beta \)-arrestin1 (Figure 5B). The finding may be contributed to a much more efficient association of TC45 with IFN-\( \gamma \)-activated STAT1 (Figure 5E). Recent study has shown that a radical reorientation of tyrosine phosphorylated parallel STAT1 dimers is required for easy access to TC45 (Mertens et al., 2006). Whether \( \beta \)-arrestin1 is involved in this process and which mechanisms underlie \( \beta \)-arrestin1 recruitment of TC45 are worthy of further investigation.

It is not expected that dephosphorylation was observed after prolonged IFN stimulation of \( \beta \)-arrestin null MEFs and TC-PTP null MEFs, suggesting the existence of other minor PTPases such as SHP-2 in STAT1 dephosphorylation (Venema et al., 1998; Wu et al., 2002). However, overexpression of SHP-2 could accelerate STAT1 dephosphorylation in \( \beta \)-arrestin null MEFs (Figure 5C), indicating that the efficient performance of these PTPases seems to be independent of \( \beta \)-arrestin1.

\textbf{Difference between \( \beta \)-Arrestin1 and \( \beta \)-Arrestin2 in Modulation of STAT1 Dephosphorylation}

\( \beta \)-arrestins are multifunctional signaling molecules. Although \( \beta \)-arrestin1 and \( \beta \)-arrestin2 share a high degree of homology and play similar functions in GPCR signal transduction, some differences between them have been reported, such as in their binding affinities to different classes of GPCR (Luttrell and Lefkowitz, 2002), their association with different binding partners (McDonald et al., 2000), and their subcellular localization (Wang et al., 2003b). Our study also shows \( \beta \)-arrestin1, but not \( \beta \)-arrestin2, specifically negatively regulates tyrosine phosphorylation of STAT1 (Figure 4B). Although exogenous \( \beta \)-arrestin2 shows a similar ability to interact with STAT1 in HEK293 cells (Figure 1B), more credible data from endogenous coIP assay suggest a higher affinity of \( \beta \)-arrestin1 to STAT1 than \( \beta \)-arrestin2 in HEK293 cells (Figure 1C). IFN-\( \gamma \) stimulation enhances the \( \beta \)-arrestin1-STAT1 interaction, which is mainly found in nucleus (Figure 3B); moreover, the interaction of STAT1 with \( \beta \)-arrestin1, but not \( \beta \)-arrestin2 or the \( \beta \)-arrestin1 cytoplasmic mutant (Q394L), could be detected and enhanced in nuclear extracts by IFN-\( \gamma \) stimulation (Figure 3C). Since IFN-\( \gamma \) stimulation did not cause \( \beta \)-arrestin1 or \( \beta \)-arrestin2 translocation into nucleus (Figure 3B) and \( \beta \)-arrestin1 is present in both the cytoplasm and the nucleus while \( \beta \)-arrestin2 is predominantly distributed in cytoplasm, their functional difference is presumably due to their different subcellular distribution.

\textbf{The Nuclear Function of \( \beta \)-Arrestin1}

\( \beta \)-arrestins are multifunctional signaling molecules. Recent study from Lefkowitz and coworkers suggested that \( \beta \)-arrestin-interacting proteins, which were determined by mass spectrometry-based proteomics approaches, distribute not only in the cytoplasm but also in the nucleus as well as other subcellular compartments (Xiao et al., 2007). Moreover, a function of \( \beta \)-arrestin1 in the nucleus, where it binds and recruits histone acetylase p300 to specific genome promoters and increases gene transcription, has been reported (Kang et al., 2005). In the present study, we show that \( \beta \)-arrestin1 recruits a tyrosine phosphatase TC45 to STAT1 in nucleus responding to IFN-\( \gamma \) stimulation and affects STAT1 phosphorylation, which negatively regulated IFN-\( \gamma \) signaling pathway and target genes transcription. This result provides a mechanism of \( \beta \)-arrestin1-mediated regulation of signaling pathway: (1) in addition to modulating histone acetylation, \( \beta \)-arrestin1 can also function as a scaffold for dephosphorylation of activated STAT1 in nucleus; and (2) \( \beta \)-arrestin1-mediated regulation of signaling pathway in nucleus is not restricted to GPCR stimulation. In short, our work reveals another nuclear function of \( \beta \)-arrestin1 and further illustrates the biological significance of the nuclear distribution of \( \beta \)-arrestin1.

\textbf{IFN-\( \gamma \) and Other Extracellular Ligand Regulation of \( \beta \)-Arrestin1-STAT1 Interaction}

In mammals, the JAK-STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors, such as IFN-\( \gamma \), interferon-\( \alpha \) (IFN-\( \alpha \)), colony-stimulating factor (CSF), and epidermal growth factor (EGF) (Improt et al., 1994; Ruff-Jamison et al., 1993; Silvennoinen et al., 1993). Besides IFN-\( \gamma \), \( \beta \)-arrestin1 also negatively regulated the gene induction and antiviral response to IFN-\( \alpha \) (Figure S3). It seems that \( \beta \)-arrestin1 may be involved in the antiviral function of both IFN-\( \alpha \) and IFN-\( \gamma \). Moreover, these extracellular ligand stimulations also enhanced the \( \beta \)-arrestin1-STAT1 interaction (data not shown), indicating the physiological significance of the
interaction and the possibility of β-arrestin1 as a universal regulator of these pathways. However, stimulation of β2-adrenergic receptor (β2AR) did not affect the interaction of β-arrestin1 with STAT1 in our immunoprecipitation assay (data not shown). Whether other GPCR stimulation can affect the interaction or there exists a crosstalk between GPCR and IFN-γ pathway are worthy of further investigation.

**β-Arrestin1, a Potential Drug Target for Antivirus Therapy**

It has been reported that virus can interfere with the interferon-induced JAK-STAT signaling and impair the STAT-mediated response (Heim et al., 1999; Lin et al., 2006). Interestingly, we found that in the first 8 days after transfection of hepatocytes in mouse with a replication-competent, overlength, linear hepatitis B virus (HBV) genome by hydrodynamic injection, the level of β-arrestin1 protein in mouse liver where HBV specifically attacked increased and then returned to baseline levels by day 10, which was coincident with the kinetics of viraemia (Yang et al., 2002). Conversely, no obvious change of β-arrestin1 protein level in spleen where HBV did not attack was observed during infection (data not shown). These data imply that β-arrestin1 may be directly related to virus infection and replication. The antiviral function of β-arrestin1 is worthy of further investigation applying the genetic modified mouse model. On the other hand, knockdown of endogenous β-arrestin1 could

**Figure 6. β-Arrestin1 Inhibits STAT1 Transcriptional Activity and the Induction of IFN-γ Target Genes in Response to IFN-γ**

(A–C) Reporter assay analysis of STAT1 activation in HeLa cells expressing increasing concentrations of Flag-β-arrestin1 (A) or deletion mutants of β-arrestin1 (B) or β-arrestin siRNA (C) in the absence or presence of IFN-γ. (D and E) Quantitative real-time RT-PCR analysis of relative Gbp1, Gbp2, Ip10, and Isg15 mRNA after IFN-γ stimulation in WT and β-arrestin null MEFs (D) or β-arrestin null MEFs expressing deletion mutants of β-arrestin1 (E).

Data are mean ± SE of three independent experiments. *p < 0.05 and **p < 0.01 versus control.
inhibit virus replication (Figure 7D). In summary, we suppose that β-arrestin1 is involved in virus-induced interference with the JAK-STAT signaling and IFN response and may be a drug target for antiviral therapy.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Antibodies, Reagents, and siRNAs**

Human β-arrestin1 and β-arrestin2 were cloned into modified pcDNA3 vector in-frame with HA or Flag tag at the N terminus. Mouse β-arrestin1 and β-arrestin2 were cloned into the same vector. GST-β-arrestin1 was created by PCR using Flag-β-arrestin1 as a template and cloned into pGEX-4T1 (Amer- sham Biosciences) in-frame with GST at the N terminus. Expression construct for human STAT1 was cloned into modified pcDNA3.1A (Clontech) in-frame with Myc tag at C terminus or modified pcDNA3 vector in-frame with HA tag at the N terminus. Human TC45 was cloned into modified pcDNA3 vector in-frame with HA or Flag tag at the N terminus. Antibodies, reagents, and siRNAs are shown in the Supplemental Data.

**Cell Culture and Transfection**

HEK293, RAW 264.7, and HeLa cells were purchased from the Type Culture Collection of Chinese Academy of Sciences, Shanghai. WT and β-arrestin null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). WT and null MEF cell lines were kindly provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA).

**Immunoprecipitation and GST Pull-Down Assay**

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% Nonidet P-40, 10% glycerol, 1 mM PMSF) for 1.5 hr at 4°C. Cell extracts were incubated at 4°C with antibody overnight. Immunocomplexes were immobilized on protein A (Amersham Biosciences) for 2 hr at 4°C and washed three times with lysis buffer. The immunoprecipitated complexes were separated by SDS-PAGE and blotted with different antibodies. GST-β-arrestin1 fusion protein was induced with 0.1 mM IPTG for 5 hr at 28°C and purified with glutathione agarose beads (BD Biosciences PharMin- gen). Recombinant His-STAT1 protein or nuclear extracts was incubated with GST-β-arrestin1 or GST for 2 hr at 4°C and then applied to the column. The GST fusion proteins were eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione). Samples were subjected to western blot analysis.

**Reporter Assay**

HeLa cells were cotransfected with a GAS-luciferase plasmid that contains four GAS elements upstream of a luciferase reporter gene, pRL-TK (Clontech), and other plasmids. Then, 24 hr after transfection (48 hr for siRNA), cells were induced by IFN-γ (100 U/ml) for 10 hr and lysed with passive lysis buffer (Promega), and then the reporter activity was analyzed with the Dual-Luciferase Report Assay System (Promega) and measured in a luminometer (Turner Biosystems Luminometer Model TD-20/20).

**Quantitative Real-Time RT-PCR**

Cells were incubated with IFN-γ (100 U/ml) for the indicated time. Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed with Revertase Ace (TOYOBO). To measure the mRNA level of endogenous genes induced by IFN-γ, a real-time PCR assay was performed on a DNA Engine Opticon 2 (MJ Research, Watertown, MA, USA) using SYBR Green (Invitrogen). Expression values were normalized with control β-actin. Primer pairs are listed in the Supplemental Data.

**Nuclear Extract Preparation**

Cells were incubated with IFN-γ (100 U/ml) for 30 min. Nuclear extracts were prepared as described previously (Gao et al., 2004).
Yeast Two-Hybrid Screening
A cDNA fragment encoding residues 1–683 of human STAT1 was inserted into the Gal4 DNA-binding domain vector pGBKT7. A human thymus cDNA library (CLONTECH Laboratories, Inc.) was screened according to protocols recommended by the manufacturer.

Viral Infection
Cells were plated in 24- or 96-well plates and pretreated with or without IFN-γ for 16 hr. Cells were then infected with VSV at a multiplicity of infection (MOI) of 1. At specified time points after infection, cells were stained and visualized with 0.05% crystal violet, and the absorbance was measured at 450 nm.

Statistical Analysis
Data are presented as mean ± SE. Student’s t-test was used to analyze two independent groups. For all tests, p < 0.05 was considered statistically significant.

SUPPLEMENTAL DATA
The Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/31/5/695/DC1/.

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REFERENCES


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