



PIASy Regulation of STAT5 Activity: Different Reporter Vectors, Different Responses

ABSTRACT

Luciferase reporter vectors are widely used to study regulation of gene expression because they are sensitive, easy to quantify and detectable over a broad dynamic range. However, presence of cryptic *dis*-acting regulatory sequences in some vectors can induce anomalous responses when activated by undesired transcription factors. Here we used dual-reporter assays with firefly and *Renilla* luciferases to investigate whether PIASy regulates the transcriptional activity of STAT5 in insulinoma INS-1 cells. Unexpectedly, we found that the expression of *Renilla* luciferase encoded by the phRL-null Vector was highly regulated by PIASy, an E3-SUMO ligase that inhibits STATs. Analysis of the pRL-null Vector backbone revealed the presence of three previously undetected regulatory elements for STAT5. The pGL4.70[*hRluc*] Vector, from which these elements and those for many other transcription factors have been removed, was found to be a more reliable means for assessing PIASy function. These data highlight the limitation of the phRL Vector backbone and the enhanced performance of the pGL4 Vector backbone. The results emphasize the need to perform appropriate controls to determine whether differences in the expression of reporter genes result from variability in transfection efficiency or from diversity in the response of the promoter and promoterless vectors.

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INTRODUCTION

Regulation of gene expression is commonly studied by measuring the activity of a reporter protein under the control of promoters and enhancer elements of eukaryotic genes. Most of the reporter vectors have been generated from pBR322 or pUC-derived backbones. They bear a pMB1 origin of replication, an antibiotic resistance gene, a eukaryotic polyA-addition signal and an intron for splicing, which is often necessary for optimal transcription (1). Luciferases are the preferred reporter proteins because of their high sensitivity, wide dynamic range of response and high signal-to-background ratio. Firefly (FL) and *Renilla* (RL) luciferases differ in their substrate and their enzymology (2). The simultaneous expression of both luciferases allows differences in transcription induction to be distinguished from variability in transfection efficiency. Other reporter vectors rely on the expression of β -galactosidase, alkaline phosphatase, or enhanced green fluorescent protein (EGFP).

The PIAS family of protein inhibitors of activated Signal Transducers and Activators of Transcription (STAT) was first discovered in a yeast two-hybrid screen for interacting partners of STATs (3). Humans and small rodents express five PIAS proteins, named PIAS1, PIAS3, the α and β splice variants of PIASx, and PIASy (4). Overexpression studies, in particular, have shown that PIAS1 binds activated STAT1 dimers and inhibits their DNA-binding activity (3,5). Furthermore, PIAS proteins have been shown to act as SUMO-ligases that mediate sumoylation of transcription factors such as p53 and c-Jun (6,7). In this study, we investigated whether PIASy inhibits STAT5 activity using a Dual-Luciferase[®] reporter assay in insulinoma INS-1 cells (8).

MATERIALS AND METHODS

Cell culture, cDNA cloning and transfection. INS-1 cells were cultured as previously described (8) in RPMI-1640 with GlutaMAX[™] supplemented with 10% heat-inactivated FBS, 50 μ M β -mercaptoethanol, 100U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Mouse PIASy was inserted into pEGFP-N1 vector as previously described (8). 4 \times 10⁶ INS-1 cells were electroporated with an Amaxa nucleoporator (Amaxa Biosystems, Cologne, Germany) as previously described (9). Electroporated cells were cultured in 35mm wells and grown for 3 days before being processed for dual-reporter assays.

Luciferase assays. INS-1 cells were transfected with pGL3-Basic Vector (FL; Cat.# E1751) under the control of a β -casein kinase promoter (CAS) containing 8 gamma-interferon-activated sequence-like elements (GLE) for STAT5 binding (8GLE-CAS-FL). Relative light units (RLU) were normalized by co-expression of *Renilla* luciferase (RL) from promoterless phRL-null (Cat.# E6231) or pGL4.70 [hRluc] (Cat.# E6881) Vectors, designated RL/phRL and RL/pGL4, respectively. In addition to 8GLE-CAS-FL and RL/phRL or RL/pGL4, cells were co-transfected with the EGFP-N1 Vector (Clontech, Palo Alto, CA) with or without PIASy-EGFP, also in the N1 vector. After transfection, cells were cultured for 2 days in complete medium and then in low serum medium for 18 hours. On day 3, cells were stimulated with 20nM of human growth hormone (hGH) for 20 minutes and cultured again in serum-free media for an additional 6 hours. Cells were harvested in 500 μ l Passive Lysis Buffer (Cat.# E1941), and 10 μ l was used for measurement of luciferase activ-

Simultaneous expression of firefly and *Renilla* luciferase allows differences in transcription induction to be distinguished from variability in transfection efficiency.

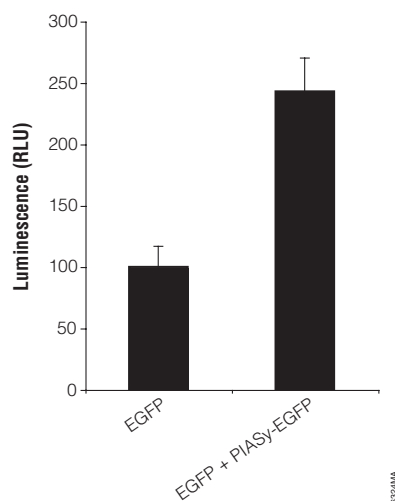


Figure 1. Differential transactivation of the firefly and Renilla luciferases in phRL-null Vector backbone. INS-1 cells were simultaneously transfected with 8GLE-CAS-FL and a plasmid encoding EGFP in the presence or absence of PIASy-EGFP. To control transfection efficiency, cells were also co-transfected with RL/phRL. RLU of FL activity was normalized to RL activity. Each experiment was performed in triplicate and repeated at least 3 times.

ity in a Berthold luminometer (Berthold, Bundoora, Australia). RLU were calculated by dividing the FL signal by the corresponding RL signal from cells transfected with either RL/phRL or RL/pGL4. Experiments were performed in triplicate, and the data represent the mean of at least 3 independent experiments.

Western blot. INS-1 cells were treated as described above for luciferase assays. Following cell lysis, 10 μ l of protein extracts were separated by 10% SDS-PAGE. After protein transfer, nitrocellulose filters were probed with a goat anti-GFP antibody followed by horseradish peroxidase-conjugated rabbit anti-goat IgG. Chemiluminescence was developed using the Supersignal West Pico kit (Pierce, Rockford, IL) and detected with a LAS 3000 Bioimaging System (Fuji, Tokyo, Japan).

RESULTS AND DISCUSSION

To test whether PIASy affects the transcriptional activity of STAT5, INS-1 cells were transfected with FL driven by a bovine β -casein promoter with 8 GLEs (8GLE-CAS-

FL) together with the RL in the promoterless phRL-null Vector (RL/phRL) and EGFP, and with or without PIASy-EGFP. Expression of EGFP was used to monitor transfection efficiency in addition to RL. Following stimulation with hGH to activate STAT5, cells co-transfected with PIASy-EGFP displayed a ~2.5-fold increase in RLU relative to cells transfected with EGFP (Table 1 and Figure 1). Analysis of the light values showed that, while PIASy reduced FL activity by 68%, it inhibited RL activity by 87.5%. Thus, after normalization for RL activity, the FL activity appeared to be increased in cells co-expressing EGFP + PIASy-EGFP compared to cells expressing EGFP. These results were surprising since PIAS1 has been reported to inhibit STAT1 protein. However, the apparent increase in FL activity was not due to PIASy-enhanced transcription of 8GLE-CAS-FL by STAT but rather to the differential repression of FL and RL transcription.

The most likely explanation for these findings is that phRL contains cryptic regulatory or enhancer elements that allow STATs, and thereby PIAS proteins, to modulate expression of the luciferase gene. Alternatively, PIASy may inactivate a distinct STAT inhibitor or modulate the activity of other transcription factors that interact with regulatory elements found in the phRL Vector. Several studies have already stated that cryptic promoter elements in the vector sequences flanking the luciferase reporter gene could lead to spurious luciferase expression (10–13). Therefore, luciferase reporter vectors are engineered to minimize such effects. However, in some cases cryptic regulatory elements or enhancers are part of the vector backbone and are required for its replication in bacteria or for optimal expression in eukaryotic cells.

Examination of the phRL Vector revealed the presence of three STAT5 regulatory elements, one in the RL cDNA, one in the vicinity of the synthetic poly(A) signal/transcription pause site, and one in the ampicillin resistance cDNA.

Promega has developed a new RL construct in the pGL4 Vectors that minimizes responses due to unspecific regulatory elements. In the pGL4 Vectors, many transcription factor binding sites have been removed, and the RL cDNA contains fewer cryptic regulatory elements. Indeed, use of RL/pGL4 improved the signal-to-background ratio

Table 1. Differential Transactivation of phRL and pGL4 Reporter Vectors by PIASy.

Vectors	Firefly Luciferase Activity	Renilla Luciferase Activity	Normalized RLU	% of Renilla Luciferase Inhibition by PIASy
RL/phRL + EGFP	14,000 \pm 1,000 \times 10 ³	3,800 \pm 430 \times 10 ³	3.7	
RL/phRL + PIASy-EGFP	4,400 \pm 0.08 \times 10 ³	470 \pm 85 \times 10 ³	9.4	88
RL/pGL4 + EGFP	6,900 \pm 1,000 \times 10 ³	5.20 \pm 1.7 \times 10 ³	1,300	
RL/pGL4 + PIASy-EGFP	4,000 \pm 340 \times 10 ³	4.7 \pm 0.7 \times 10 ³	860	11

The construct 8GLE-CAS-FL was co-transfected into INS-1 cells with EGFP with or without PIASy-EGFP. RLU were calculated by dividing the signal from transfection with 8GLE-CAS-FL or PIASy/8GLE-CAS-FL by the corresponding signal from co-transfection with RL/phRL or RL/pGL4. The experiment was performed in triplicate, and the data are from 3 independent experiments.

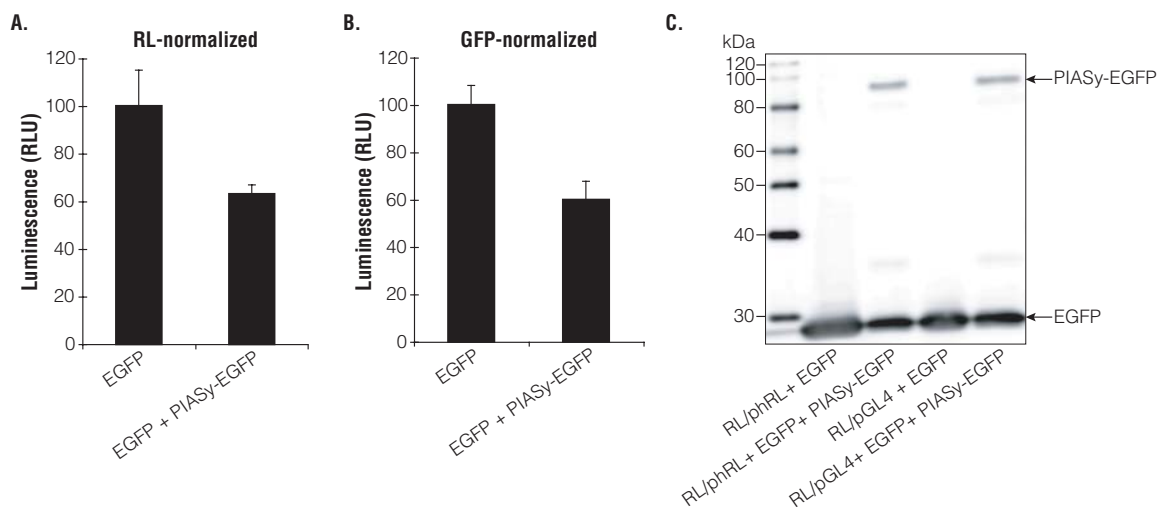


Figure 2. PIASy inhibits the transcription activity of STAT5. INS-1 cells were transfected as described in Figure 1 except that RL/pGL4 was used instead of RL/phRL to express RL. RLU of FL activity were normalized either to RL activity (**Panel A**) or to GFP expression (**Panel B**). Proteins from cell lysates prepared for luciferase assay were also separated by SDS-PAGE and analyzed by Western blot with anti-GFP antibody (**Panel C**).

due to a ~1,300 fold increase in the RLU compared to RL/phRL (Table 1). Moreover, it decreased the inhibition of RL activity by PIASy from 87.5% to 11%, as measured by dividing the absolute values of RL activity from cells expressing EGFP+PIASy-EGFP by the RL activity from cells expressing EGFP (Table 1). Consequently, the PIASy inhibitory role of STAT5 activity was now apparent (Figure 2, Panel B). To verify the reliability of these data independently, the FL activity was also normalized based on EGFP expression as measured by immunoblotting (Figure 2, Panel C). Consistent with the data obtained using RL/pGL4, this analysis indicated that PIASy represses FL activity by 40%.

We conclude that pGL4 is a significantly more reliable vector than phRL for investigating gene regulation. Whenever testing the activity of a transcription factor, the performance of preliminary internal controls that distinguish not only differences in transcriptional regulation from variability in the efficiency of transfection but also variability in the responses of the promoter and promoterless reporter vectors is also indicated. Following this strategy, we recently showed that PIASy inhibits STAT5b transcription activity and that the decoy tyrosine phosphatase ICA512/IA-2 can counteract this inhibition (14).

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ORDERING INFORMATION

Product	Size	Cat.#
pGL4.70[hRluc]	20µg	E6881

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We conclude:
pGL4 is a significantly more reliable vector than phRL for investigating gene regulation.