Inducible control of transgene expression with ecdysone receptor: gene switches with high sensitivity, robust expression, and reduced size

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The ecdysone receptor (EcR)-based gene regulation system is a tool for controlling gene expression. To improve the sensitivity of this system, we evaluated many two-hybrid format synthetic gene constructs in which the GAL4 DNA binding domain was fused to the ligand binding domain of the Choristoneura fumiferana *EcR mutant V390I/Y410E (GEvy), and various activation domains—VP16, p53, p65, or E2F1—were fused to the EF domains of chimeric human RXR. These gene switches were assayed in NIH3T3 cells, HEK293 cells, and in mouse quadriceps in the presence of the nonsteroidal inducer RG-115819 or GS™-E. All of the two-hybrid format constructs had no or very low background in the "off" condition and high luciferase reporter gene expression levels in "on" conditions. Extremely high sensitivity was achieved, with EC_{50} values in the subnanomolar range and with maximal induction at 10 nM RG-115819. Co-expression of both receptor genes with encephalomyocarditis virus (EMCV) or eIF4G internal ribosome entry site (IRES) sequences gave robust induction levels. To reduce the size of the switch construct, we tested single receptor formats, in which any of 14 different activation domains were fused to GEvy. We identified several switches with acceptable levels of basal and maximal induction levels. The gene switches described here provide receptor configuration options suitable for gene function studies, therapeutic protein production in cell culture, transgenic mouse models, and gene/cell therapy.*

INTRODUCTION

Precise spatial and temporal modulation and control over levels of transgene expression by external application of a small molecule is an extremely powerful technology for gene function analysis, therapeutic protein production, and gene and cell therapy applications. Several transcriptional regulation systems (gene switches) have been developed to control mammalian gene expression in vitro and in vivo. These include: (*i*) Tet repressor-based, tetracycline-inducible; (*ii*) FKB12- and FRB-based, rapamycin-inducible; (*iii*) progesterone receptor-based, RU486-inducible; (*iv*) estrogen receptor-based, tamoxifen-inducible; (*v*) Pip repressor-based, streptogramin-inducible; (*vi*) MphR(A) protein-based, macrolide antibiotic-inducible; and (*vii*) ecdysone receptor (EcR)-based, ecdysone-inducible (see Reference 1 for a recent review).

The nuclear hormone receptor EcR and its heterodimeric partner ultraspiracle (USP) regulate growth, molting, and development in insects

(2). The mammalian ortholog of USP is retinoid X receptor (RXR). An ecdysteroid-dependent transcription activation system was developed by making a chimeric *Drosophila melanogaster* EcR (DmEcR) with its activation domain (AD) replaced with a VP16 AD and its DNA binding domain (DBD) mutated to recognize only the glucocorticoid receptor response element. This chimeric receptor (VgEcR), when cotransfected with recombinant RXR (rRXR), induced luciferase reporter expression in the presence of 1 μM muristerone A (3). The VgEcR/rRXR system was reconfigured to express bicistronically using a single promoter with internal ribosome entry site (IRES) sequence (4). Subsequent studies replaced DmEcR with *Bombyx mori* EcR (BmEcR), which eliminated the requirement for exogenous rRXR (5). In further studies, VgEcR and BmEcR were combined to form a hybrid EcR by joining the N-terminal AD and DBD of VgEcR with the C-terminal hinge and ligand binding domain (LBD) of BmEcR (6). This hybrid receptor did not require exogenous RXR and

was responsive to the nonsteroidal ecdysone agonist GS™-E ligand. Although these versions of EcR-based gene regulation systems possessed several desirable characteristics, they showed low fold inductions due to high basal (uninduced) and low induced levels of expression. In a recent study, the EcR-based gene regulation system was further improved by making a two-hybrid format switch, in which a GAL4 DBD was fused to the LBD of *Choristoneura fumiferana* EcR (CfEcR) and VP16 AD was fused to the EF domains of *Mus musculus* RXR (7).

The EcR-based gene regulation system has several advantages: (*i*) low basal expression (8); (*ii*) ecdysteroids are not present in mammalian cells and many safe nonsteroidal inducers are available (9); (*iii*) EcR mutants can differentially respond to different chemotypes (10); and (*iv*) EcR mutants can independently and simultaneously regulate two or more genes (11). To further improve the system for high sensitivity, we constructed two-hybrid format switches to examine the effects of various modifications on sensitivity, including synthetic genes codon-optimized for mouse and human expression, a mutant EcR LBD, IRES sequences, and different activation domains. In these constructs, GAL4 DBD was fused to EcR LBD, and ADs were fused to a chimeric human RXR LBD. To avoid the need for an exogenous supply of RXR and to reduce switch size, we also evaluated single receptor format switches by testing many chimeric proteins containing ADs from different transcription factors fused to GAL4 DBD and EcR LBD. By making these modifications, we obtained highly sensitive and robust switches suitable for in vitro, in vivo, and virus-based applications.

MATERIALS AND METHODS

Plasmid Constructs

The constructs made can be grouped into three types as depicted in Figure 1: (*i*) a two-hybrid format; (*ii*) a two-hybrid IRES format; and (*iii*) a single receptor format. DNA sequences

encoding chimeric receptors were cloned in pRL-CMV or pRL-SV40 (Promega, Madison, WI, USA) for cytomegalovirus (CMV) or simian virus 40 (SV40) promoter-driven expression, respectively. For two-hybrid format constructs, the nucleotide (nt) sequence encoding amino acid (aa) 1–147 of GAL4 (G; nt 1116–1556 of pBIND from Promega) was cloned 5′ to either the nucleotide sequence encoding aa 206–541 (DEF domains) of CfEcR (E; GenBank® accession no. U29531) or a nucleotide sequence encoding aa 206–541 of EcR with V390I and Y410E mutations (Evy) to obtain GE or GEvy. Similarly, a nucleotide sequence encoding 87 aa of VP16 AD (V; nt 412–672 of pVP16 from BD Biosciences Clontech, Mountain View, CA, USA) or other ADs (see below) were placed 5′ to the sequence of a chimeric RXR LBD [R; helices 1–8 of locust RXR LBD replaced with helices 1–8 of human RXRβ, Hs-LmR(EF) of Reference 10] to obtain the heterodimeric partner VR. Synthetic GE, GEvy, and VR sequences (sGE, sGEvy, and sVR) optimized for mouse and human expression were designed in collaboration with GeneArt GmbH (Regensburg, Germany). For the single receptor format constructs, synthetic V, G, and Evy sequences (sVGEvy) were cloned behind a CMV promoter (Figure 1). All constructs were assembled by standard cloning methods and confirmed by DNA sequencing.

The following IRES sequences were evaluated: 570-bp encephalomyocarditis virus (EMCV) IRES (EI; nt 2334–2903 of pERV3 from Stratagene, La Jolla, CA, USA), 357-bp eukaryotic initiation factor eIF4G IRES (FI; nt 1–357, GenBank accession no. NM_004953) (12), and 101-bp eIF4G IRES (nt 257–357 of GenBank accession no. NM_004953).

Inducible luciferase reporter plasmid pFRLuc containing five copies of the GAL4 response element and synthetic TATA sequences, used for cell culture experiments, was purchased from Stratagene. Inducible secreted alkaline phosphatase (SEAP) reporter plasmid pTTR-SEAP containing six copies of the GAL4 response element and TTR TATA, used for mouse studies, was described previously (11).

The constitutively expressed luciferase (pCMV-Luc, positive control) and *Renilla* luciferase (pRL-CMV, internal control) constructs were obtained from Promega.

Activation Domains

The following 14 ADs were examined: 73 aa VP16-based activation domain from pACT plasmid (Act; nt 1188–1325 from Promega); 47 aa VP16 AD (V-47; nt 2719–2859 of pFB-ERV from Stratagene); four copies of 7 aa (DDFDLDL) core sequence of VP16 (V-4 × 7) (13); two copies of 12 aa (PADALDDFDLDM) core sequence of VP16 (V-2 × 12) (14); four copies of 12 aa core sequence of VP16 (V-4 × 12) (14); four copies of aa 143–160 of Oct-2 (Oct-2Q; 4 × Q18; GenBank accession no. NM_002698) (15); two copies of aa 438–479 of Oct-2 (Oct-2P) (15); aa 380–437 of E2F1 (E2F1; GenBank accession no. NM_005225) (16); aa 297–413 of E2F4 (E2F4; GenBank accession no. BC033180) (17); aa 1–48 of HIV-1 Tat (Tat; GenBank accession no. NC_001802) (16); aa 399–499 of CCAAT-box transcription factor (CTF; GenBank accession no. X12492) (16); two copies of aa 263–391 of Sp1 (2 × Sp1; GenBank accession no. NM_138473) (15); aa 1–92 of p53 (p53; GenBank accession no. BC003596); and aa 366–549 of p65 subunit of NF-κB (p65, nt 706–1266 of pCMV-AD from Stratagene).

Transient Transfections

Mouse NIH3T3 and human HEK293 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (obtained from Invitrogen, Carlsbad, CA, USA). On the day of transfection, NIH3T3 cells were plated in a 96-well plate at a density of 6000 cells/well. The cells were transfected with 40 ng receptor construct(s) and 160 ng pFRLuc reporter plasmid using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). Constitutively expressed *Renilla* luciferase (RLuc) reporter plasmid (pRL-CMV; 2 ng/well) was

cotransfected and used as an internal control to normalize the data. DMEM medium with 10% FBS containing the inducer RG-115819 (RheoGene, Norristown, PA, USA) at 0, 0.01, 0.1, 1, 10, 100, or 1000 nM concentration was added 4 h posttransfection. HEK293 cells were also transfected under the same conditions, except cells were plated (15,000 cells/well) 2 days before transfection. Forty-eight hours after transfection, the media was discarded, and the cells were lysed with 50 µL passive lysis buffer (Promega). Extracts (50 µL) were assayed using Dual Luciferase Assay kit (Promega) on a plate reader equipped with injectors (Dynex Technologies, Chantilly, VA, USA).
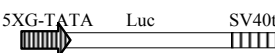
Two-Hybrid Format: sGEvy+sVR
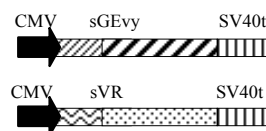
Two-Hybrid IRES Format: sVR-I-sGEvy

Single Receptor Format: sVGEvy

Inducible Reporter: pFRLuc

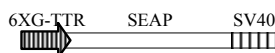**Inducible Reporter: pTTR-SEAP**

Figure 1. Diagram of *Choristoneura fumiferana* ecdysone receptor (CfEcR)-based two-hybrid format and single receptor format constructs and reporter genes. Receptor proteins encoded by synthetic genes GAL4 DNA domain binding domain fused to ecdysone receptor CfEcR V390I/Y410E mutant ligand binding domain (sGEvy), VP16 activation domain fused to chimeric retinoid X receptor (RXR) ligand binding domain (sVR), and VP16 activation domain fused to GEvy (sVGEvy) were produced under the control of the human cytomegalovirus (CMV) promoter. The luciferase (Luc) and secretable alkaline phosphatase (SEAP) were driven by a synthetic minimal promoter flanking five copies of GAL4 response element (5 × G-TATA) and TTR minimal promoter flanking six copies of GAL4 response element (6 × G-TTR), respectively.

Data are expressed as luciferase activity (relative light units) in 50 μL extract. Similar results were observed when ratios of Luc/RLuc were compared (data not shown). Each construct was assayed in two to six independent experiments and replicated three times in each experiment. Since the number of experiments varied for different constructs, the standard error was used to present the variation. EC_{50} value for each construct was estimated using GraphPad Prism® (GraphPad Software, San Diego, CA, USA).

In Vivo Assays

Adult C57BL/6 mice were anesthetized, and plasmid DNA [125 μg receptor(s) and 25 μg of 6× GAL4-TTR-SEAP reporter] were injected into the quadriceps. Electrode conductivity gel was applied, an electrode (1 × 1 cm) was placed on the hind leg and electroporated with 200 V/cm eight times for 20 ms/pulse at 1-s time intervals. The transverse electrical field direction was reversed after the animals received half of the pulses. Animals were treated with 5 mg of GS-E in dimethyl sulfoxide (DMSO) by intraperitoneal injection 3 days after electroporation. The SEAP activity in mouse sera was evaluated at 0, 3, 6, 8, 10, 13, 15 and 17 days after ligand administration by using the Great Escape Chemiluminescence kit (BD Biosciences Clontech). Five animals were used per treatment group.
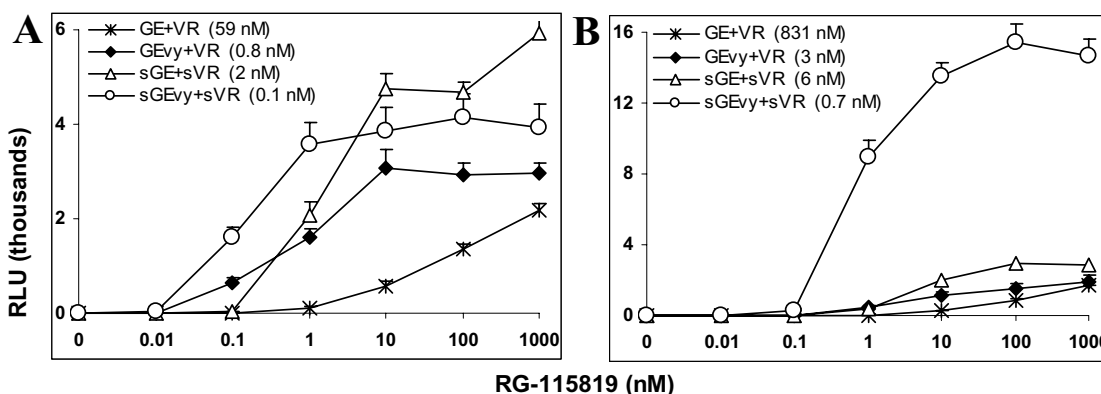

Figure 2. Dose-responsive activation of gene expression by *Choristoneura fumiferana* ecdysone receptor (CfEcR)-based two-hybrid format constructs. (A) NIH3T3 and (B) HEK293 cells were transiently transfected with plasmids encoding receptor fusion proteins GAL4 DNA binding domain (G) fused to CfEcR ligand binding domain (E) and VP16 activation domain (V) fused to chimeric human retinoid X receptor (RXR) (R). Luciferase reporter plasmid pFRLuc was co-introduced along with the receptor constructs. Luciferase activity in cells was measured following 48 h incubation of cells with the indicated concentration of inducer RG-115819. Six to sixteen replicate assays were performed, and mean values (in relative light units, RLU) ± SEM were plotted. EC_{50} values for each switch are shown in brackets next to the receptor constructs. Evy, CfEcR V390I/Y410E mutant; s, synthetic gene.
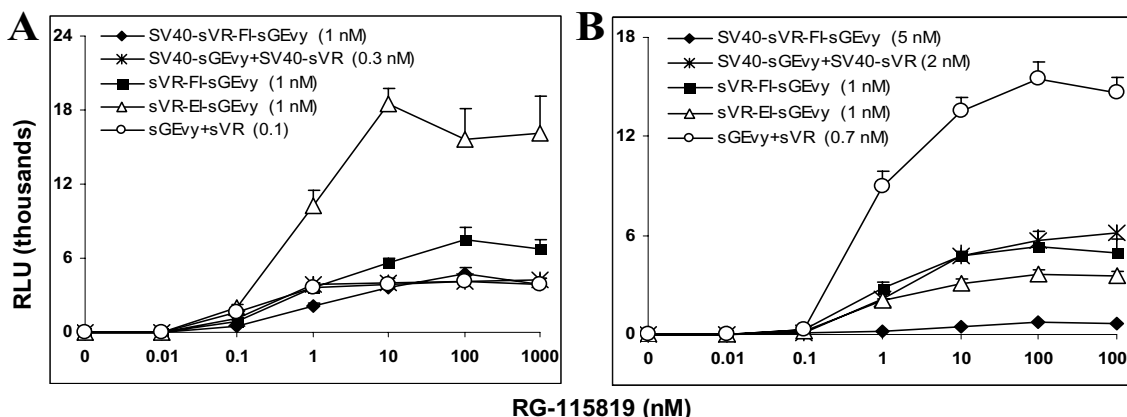

Figure 3. Dose-responsive activation of gene expression by *Choristoneura fumiferana* ecdysone receptor (CfEcR)-based bicistronic constructs. (A) NIH3T3 and (B) HEK293 cells were transiently transfected with bicistronic plasmids or receptors on separate plasmids as shown, along with the luciferase reporter plasmid pFRLuc. Luciferase activity in cells was measured following 48 h incubation of cells with the indicated concentration of inducer RG-115819. Six to sixteen replicate assays were performed, and mean values (in relative light units, RLU) ± SEM were plotted. The EC_{50} value for each switch is shown in brackets next to the receptor constructs. SV40-sVR-FI-sGEvy, simian virus 40 (SV40) promoter-driven eIF4G internal ribosome entry site (IRES) bicistronic construct; SV40-sGEvy+SV40-sVR, SV40 promoter-driven sGEvy and sVR on separate plasmids; sVR-FI-sGEvy, cytomegalovirus (CMV) promoter-driven eIF4G IRES bicistronic construct; sVR-EI-sGEvy, CMV promoter-driven encephalomyocarditis virus (EMCV) IRES bicistronic construct; and sGEvy+sVR, CMV promoter-driven sGEvy and sVR on separate plasmids.

RESULTS AND DISCUSSION

Evaluation of Gene Switches Based on a Synthetic EcR V390I/Y410E Mutant

Recent work (7) aimed at overcoming the drawbacks of earlier versions of EcR-based gene switches (3–6) resulted in an improvement in the switch for low basal and high inducible expression by using a two-hybrid format where GAL4 DBD was fused to the CfEcR LBD (GE) and VP16 AD was fused to the *Mus musculus* RXR EF domain. However, the system required a 10 μM concentration of inducer to achieve maximum induction. To improve the sensitivity of the system, in this study we used CfEcR mutant V390I/Y410E (Evy) that has increased sensitivity to diacylhydrazine inducers like RG-115819 and GS-E (unpublished results). The inducer RG-115819 has potency in mammalian cell cultures similar to potency of GS-E that was used in earlier studies (6,7). We prepared CMV promoter-driven GE, GEvy, and VP16 AD fused to chimeric RXR construct (VR; helices 1–8 of locust RXR LBD replaced with helices 1–8 of human RXRβ) (10) and transfected mouse NIH3T3 and human HEK293 cells (hereafter referred to as 3T3 and 293 cells, respectively) with inducible reporter plasmid pFRLuc. The dose responses of GE+VR and GEvy+VR with inducer RG-115819

are shown in Figure 2. Both switches had undetectable or very low basal expression and similar maximal levels of luciferase reporter gene induction. However, GEvy had an EC_{50} of 0.8 and 3 nM, while GE had an EC_{50} of 59 and 831 nM in 3T3 and 293 cells, respectively (Figure 2). Thus, GEvy was 74–277 times more sensitive than GE.

Since the GE, GEvy, and VR sequences are of nonmammalian origin, we asked whether the switch could be further improved by optimizing gene sequences for mouse and human expression. GE, GEvy, and VR sequences were synthesized using codons for high expression in mouse and human cells. In addition, regions of very high (>80%) or very low (<30%) GC content and *cis*-acting motifs for internal TATA boxes, ribosomal entry sites, RNA secondary structures, cryptic splice sites, and poly(A) sites were avoided in optimizing the sequence. We evaluated two switches based on these synthetic genes. For the synthetic sGE+sVR switch, the maximum induction of luciferase reporter gene was 2.7- and 1.7-fold higher than the one observed for native (nonsynthetic) GE+VR in 3T3 and 293 cells, respectively (Figure 2). The synthetic sGE+sVR switch had EC_{50} of 2 and 6 nM in 3T3 and 293 cells, respectively (Figure 2). These values are 37 and 139 times less than the EC_{50} values observed for GE+VR in 3T3 and 293 cells, respectively, showing that

the sGE+sVR switch is more sensitive than GE+VR switch. The synthetic sGEvy+sVR switch also had increased sensitivity compared to the GEvy+VR switch (compare EC_{50} values in Figure 2). The maximum induction observed for the sGEvy+sVR switch was similar to the GE+VR switch in 3T3 cells, but was 7-fold higher in 293 cells. It is unclear why sGEvy+sVR showed 7-fold higher maximal induction levels in 293 cells, even though both switches had similar maximum induction levels in 3T3 cells. These results show that the system based on the sGEvy is extremely sensitive with EC_{50} values in the subnanomolar range, with high maximal induction. Because DNA sequence optimization improved switch performance, all subsequent constructs described below were made using the synthetic sGEvy and sVR sequences.

IRES Constructs

For applications such as tissue-specific expression, it is desirable to express both sGEvy and sVR receptors using a single promoter. IRES sequences derived from viruses and eukaryotic mRNAs have been used to co-express two genes from a single bicistronic messenger RNA (mRNA) (18). Initially, we created and tested two constructs transcribed from the SV40 promoter using eukaryotic initiation factor eIF4G IRES (FI; 339-bp) (12). Constructs SV40-sGEvy-FI-sVR and
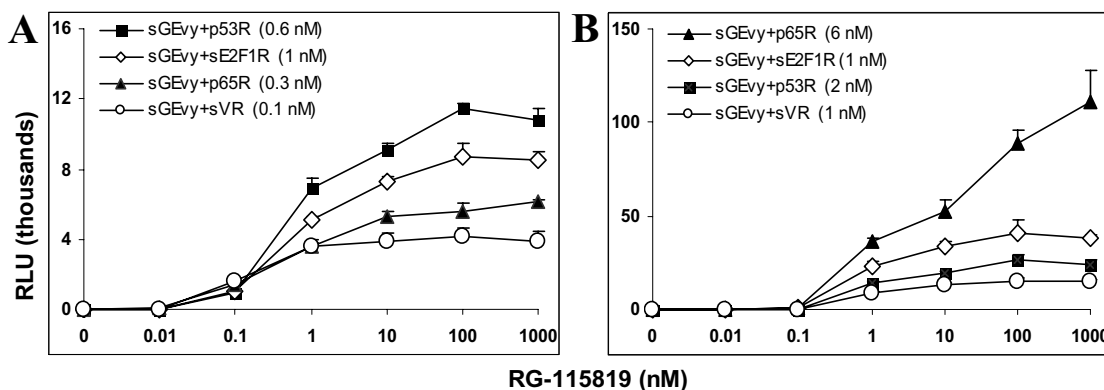

Figure 4. Dose-responsive activation of gene expression by *Choristoneura fumiferana* ecdysone receptor (CfEcR)-based two-hybrid format constructs with p53, p65, and E2F1 activation domains. (A) NIH3T3 and (B) HEK293 cells were transiently transfected with cytomegalovirus (CMV) promoter-driven plasmids containing a synthetic GAL4 DNA binding domain fused to a synthetic CfEcR V390I/Y410E mutant ligand binding domain (sGEvy) and a p53, p65, E2F1, or VP16 activation domain fused to synthetic chimeric human retinoid X receptor (RXR) (p53R, p65R, E2F1R, or sVR, respectively). Luciferase reporter plasmid pFRLuc was co-introduced along with the receptor constructs. Luciferase activity in cells was measured following 48 h incubation of cells with the indicated concentration of inducer RG-115819. Six replicate assays were performed, and mean values (in relative light units, RLU) ± SEM were plotted. EC_{50} values for each switch is shown in brackets next to the receptor constructs.

SV40-sVR-FI-sGEvy were designed to determine the effect of position of the genes on the bicistronic mRNA. The construct in which sVR was the first cistron showed higher induction levels compared to the construct in which GEvy was the first cistron (data not shown). Generally, cap-dependent translation of the first cistron is more efficient than the reinitiation of translation at the IRES (18). Therefore, sVR was placed as the first cistron in all subsequent constructs. Results obtained in 3T3 cells clearly indicated that the construct in which sVR was the first cistron (SV40-sVR-FI-sGEvy) induced luciferase activity to similar levels as did plasmids SV40-sGEvy+SV40-sVR (Figure 3A). In 293 cells, however, the construct was less robust than SV40-sGEvy+SV40-sVR (Figure 3B). A construct with a shorter 101-bp version of eIF4G IRES (12) also behaved similarly to the above 339-bp version in both cell lines (data not shown).

In addition to eIF4G IRES, we also used the EMCV IRES (EI; 570-bp) (19) and made CMV promoter-driven sVR-EI-sGEvy. For comparison, we also made CMV promoter-driven eIF4G IRES construct sVR-FI-sGEvy. We chose EMCV IRES because it is widely used (18), and eIF4G because it has been shown to perform better than EMCV IRES in several cell lines (20). Interestingly, CMV promoter-driven constructs in which sVR and sGEvy are co-expressed using either

eIF4G or EMCV IRES showed lower EC_{50} values (i.e., higher sensitivity) and higher level induction compared to SV40 promoter-driven eIF4G IRES construct SV40-sVR-FI-sGEvy (Figure 3). Particularly noteworthy is that sVR-EI-sGEvy transcribed from the CMV promoter had 4-fold higher maximal induction than sGEvy+sVR transcribed from CMV promoter on two separate plasmids in 3T3 cells. We observed higher induction in 3T3 cells with EMCV IRES and in 293 cells, with eIF4G IRES. The differences observed for constructs in 3T3 and 293 cells are not uncommon and appear to be due to inconsistencies between cell types in the translation from the IRES sequences.

Evaluation of p53, p65, and E2F1 Activation Domains

We investigated three human ADs— p53, p65, and E2F1—to evaluate the RG-115819-dependent activation of the two-hybrid format switch and to further enhance switch performance. The VP16 sequence in VR was replaced with p53, p65, or E2F1 to create p65R, p53R, and E2F1R. They were introduced into 3T3 and 293 cells with partner sGEvy. The ADs did affect the dose-response curves (Figure 4). p53, p65, and E2F1 were highly effective in stimulating higher induction than VP16 AD in both 3T3 and 293 cells. These results indicate the versatility of CfEcR-based gene switch

to function with ADs derived from different transcription factors.

Single Receptor Switch

To assess whether the CfEcR-based switch could activate gene expression in mammalian cells in the absence of exogenous RXR and also to reduce the switch size, we fused the VP16 AD (V) to GEvy and tested it in 3T3 and 293 cells. Basal expression was unacceptably high in both cell types, resulting in only 4- to 9-fold induction (Figure 5). However, the maximal induction levels from this single receptor format switch were higher than the two-hybrid format sGEvy+sVR, suggesting that the CfEcR-based single receptor switch could be used in mammalian cells, provided that basal expression could be reduced to acceptable levels. It is well established that the transactivation of a given AD is a product of its intrinsic activation potential and the context in which it is placed. To address the possibility that use of other ADs in place of VP16 could reduce basal expression, we made single receptor format switches with 14 different ADs that were previously characterized as transactivators (see Materials and Methods) and generated dose-response curves in 3T3 and 293 cells with the RG-115819 inducer at 0, 0.01, 0.1, 1, 10, 100, and 1000 nM concentrations. We found that switches with V-2 × 12, Act, V-47, E2F1, p53,
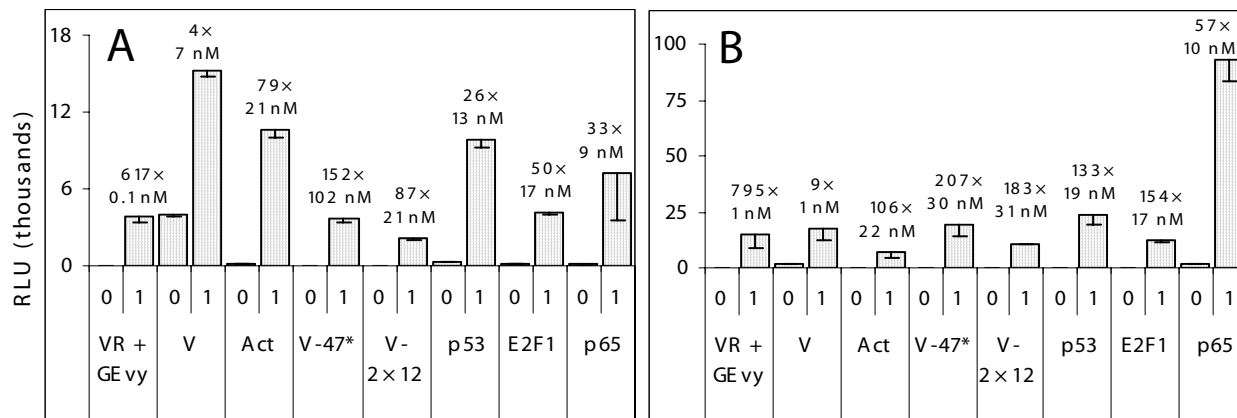

Figure 5. Induction of luciferase reporter gene by single receptor format switches. (A) NIH3T3 and (B) HEK293 cells were transiently transfected with plasmid encoding receptor fusion protein consisting an activation domain (one of the 14 shown), synthetic GAL4 DNA binding domain (G), and synthetic CfEcR V390I/Y410E mutant ligand binding domain (Evy). Luciferase reporter plasmid pFRLuc was co-introduced along with the receptor construct. Luciferase activity in cells was measured following 48 h incubation of cells with 0 or 1 μM inducer RG-115819. Six to twelve replicate assays were performed, and mean values (in relative light units, RLU) ± SEM were plotted. Fold-inductions and EC_{50} values are shown above the bar for the 1 μM. sGEvy+sVR, cytomegalovirus (CMV) promoter-driven sGEvy and sVR were on separate plasmids. See Materials and Methods section for the description of activation domains.
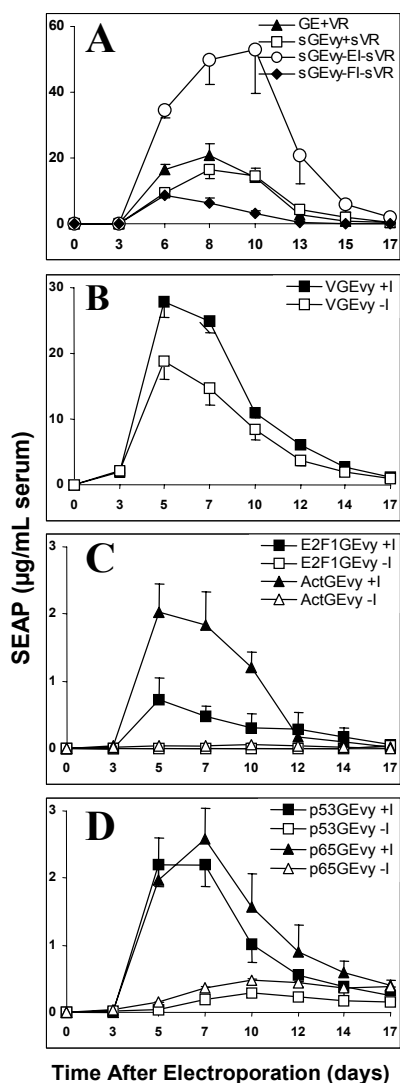
Figure 6. In vivo evaluation of two-hybrid format and single receptor format constructs. Cytomegalovirus (CMV) promoter-driven two-hybrid format constructs GEvy+VR or sGEvy+sVR, two-hybrid internal ribosome entry site (IRES) format construct sVR-EI-sGEvy [containing encephalomyocarditis virus (EMCV) IRES] or sVR-FI-sGEvy (containing eIF4G IRES), and single receptor switch format constructs VGEvy, ActGEvy, E2F1GEvy, p53GEvy, or p65GEvy were electroporated into the quadriceps of C57BL/6 mice along with the reporter plasmid pTTR-SEAP. Animals were treated with 5 mg inducer GS-E in dimethyl sulfoxide by intraperitoneal injection 3 days after electroporation. Secretable alkaline phosphatase (SEAP) activity in mouse serum was evaluated at the indicated days after electroporation. The open and filled squares in (B–D) are SEAP activities in the absence and presence of inducer (I), respectively. Constructs shown in panel A had no SEAP activity in the absence of inducer. Values are the average from five animals ± SEM.

and p65 ADs had acceptable fold-induction (ratio of activity at 1 to 0 μM) and EC_{50} values compared to two-hybrid format sGEvy+sVR (Figure 5; only basal expression and activity at 1 μM are shown), and switches with V-4 × 7, V-4 × 12, Oct-2Q, Oct-2P, Tat, CTF, E2F4, 2 × Sp1 ADs had basal and induced levels too low to be of any practical use (data not shown). The single receptor format switches, due to their reduced size and graded activation potential, should have utility in virus-based applications.

In Vivo Switch Optimization

Selected two-hybrid format and single receptor format switches were tested in C57BL/6 mice by electroporating the receptor-containing plasmids into muscle tissue as described in the Materials and Methods section. CMV promoter-driven native GEvy+VR and synthetic sGEvy+sVR constructs induced SEAP activity to similar levels in response to GS-E (Figure 6A). Reporter activation with EMCV IRES construct sVR-EI-sGEvy was 4- and 6-fold greater than was observed for the construct sGEvy+sVR and the eIF4G IRES construct sVR-FI-sGEvy, respectively (Figure 6A). These results clearly indicated that co-expression of sGEvy and sVR using EMCV IRES is highly effective in muscle tissue.

The single receptor switch sVGEvy showed both high basal and high induction in vivo (Figure 6B) similar to what was observed in 3T3 cells. Among the four other single receptor switches tested, ActGEvy and E2F1GEvy had the lowest basal activity followed by p65GEvy and p53GEvy (Figure 6, C and D). However, the induction levels of these single receptor switches were approximately 15% of the levels observed for sGEvy+sVR. In 3T3 cells, E2F1, p53, and p65 ADs, either in two-hybrid format (Figure 3A) or in single receptor format (Figure 4A) showed higher levels of induction than sGEvy+sVR. It appears, under the experimental conditions used, that VP16AD has a higher activation potential in muscle cells than the other ADs tested.

In conclusion, our results showed that two-hybrid format switches with

the synthetic CfEcR V390I/Y410E mutant were responsive to subnano-molar concentrations of RG-115819, had undetectable or very low basal expression, and reached maximum induction at 10 nM RG-115819. The tightness of the system is particularly noteworthy because a leaky system reduces efficiency and is unsuitable for cytotoxic gene expression. The single receptor format testing of different ADs resulted in switches with graded transactivation potential. Our results indicate that the CfEcR-based system is versatile to a variety of receptor format modifications and that the constructs can be optimized for each cell culture model. Improvements presented here may advance the ability to regulate transgene and native gene expression. Example applications include up- or down-regulation of native gene expression using engineered zinc fingers and inducible small interfering RNA (siRNA).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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