Product Application Focus

Stable Luciferase Reporter Cell Lines for Signal Transduction Pathway Readout Using GAL4 Fusion Transactivators

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ABSTRACT

While GAL4 fusion activators have been widely used for dissecting signal transduction pathways in transient assays, there has been surprisingly little reported on utilizing cell lines with stably integrated fusion activators. To avoid problems with the efficiency and reproducibility inherent to transient transfection, we describe here the generation and characterization of HeLa reporter cell lines, which contain a stably integrated luciferase gene responsive to stably integrated and constitutively expressed GAL4-CREB or GAL4-Elk1 fusion activators. These cell lines exhibited extremely low basal luciferase expression but robust response to various extracellular stimuli or the expression of signaling molecules that resulted in elevated MAP kinase or PKA activities. This integrated two-component reporter system allows one to focus specifically on particular signaling pathway endpoints and the altered transactivation activity of either Elk1 or CREB. With the procedures described here, many novel cell-based assays can be developed by generating new reporter cell lines with medically important but difficult-to-transfect cell types, and by using different reporter genes or different fusion transactivator genes.

INTRODUCTION

Signal transduction is essential for cellular growth and differentiation and for the coordination of cellular activities of a diverse range of cell types in multicellular organisms. Perturbations of signaling events have been linked to many human diseases, and, hence, many of the signaling molecules are potential targets for drug development (1,8,16). Reporter genes have been widely used in signal transduction studies because many signal transduction pathways regulate gene expression by regulating the activity of transcription factors. For example, cAMP-dependent protein kinase (PKA) pathway and several of the mitogen-activated protein kinase (MAPK) pathways all rely on the nuclear translocation of an activated protein kinase (e.g., MAPK or PKA) to transmit signals from outside the cell to the transcription machinery in the cell nucleus (1,8,16). One reporter gene system makes use of this property of signal transduction and utilizes a GAL4 or LexA fusion transactivator as a sensor for these protein kinases and signaling pathways (11,15,19,21). The key component in these reporter systems is the plasmid that expresses the fusion transactivator protein. While the DNA-binding domain enables a fusion activator to bind to the promoter of a reporter gene, it is the activation moiety that confers the ability to activate reporter gene expression in a signaling pathway-specific manner. Fusion transactivators consisting of the DNA-binding

domain of yeast GAL4 protein or *E. coli* LexA and the activation domains of other transcription activators including CREB, Elk1, c-Jun, and CHOP/GADD153 have been used successfully to study the functions of genes involved in PKA and Ras/MAPK (11,15,18,19,21).

As compared with reporter constructs using a native promoter or synthetic enhancer elements, reporter systems using a fusion transactivator are much more specific and robust. These systems measure the activation of an individual transcription factor; the background expression of the reporter gene is extremely low because mammalian cells do not have endogenous transcription factors that can efficiently bind at the GAL4 sites in the reporter plasmid. While these features are attractive to applications involving the analysis of large numbers of samples (e.g., high-throughput drug screening), the necessity for transient transfection of the reporter and activator plasmids, and the inherent variability in transfection efficiency, precluded the use of this type of assays for high-throughput screening. We report here the generation and characterization of stable cell lines expressing GAL4-Elk1 or GAL4-CREB constitutively and expressing luciferase upon activation of these fusion proteins. As these cell lines have "built-in" sensors, the effect of extracellular stimulus on the respective signaling pathways can be studied simply by treating the cells with these stimuli and assaying for luciferase activity. Many

medically important cell types are difficult to be transfected and studied with transient transfection. Stable cell lines with integrated two-component reporter systems will allow the analysis of the activation or inhibition of upstream signaling molecules in these cells because one just has to transfect at a sufficiently large scale and then use positive drug selection to generate the desired lines.

MATERIALS AND METHODS

Plasmids

Fusion transactivator plasmids pFA2-Elk1, pFA2-CREB, or pFA2-cJun and control plasmids including pFC-MEK1 and pFC-PKA are from PathDetect® trans-Reporting Systems (Stratagene, La Jolla, CA, USA) (21). Plasmid DNAs used for transfection were purified from *E. coli* XL1-Blue cells with the Qiagen® Maxi Prep kit (Qiagen, Valencia, CA, USA) or by CsCl₂ banding. To facilitate the stable integration of pFR-Luc, a hygromycin expression cassette was excised from pTK-Hygro (Clontech Laboratories, Palo Alto, CA, USA) and inserted into the *Nde*I site of pFR-Luc. The resulting plasmid was named pFR-Luc^{Hyg}.

Cell Lines and Cell Culture

HeLa cells were obtained from ATCC (Manassas, VA, USA) (ATCC CCL2). Other cell lines described in this work, including HLR, HLR-Elk1, and HLR-CREB, are commercially available from Stratagene. All cell lines were kept and cultured in DMEM supplemented with 1% L-Glutamine, 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), antibiotic antimycotic liquid (Life Technologies, Rockville, MD, USA), and hygromycin and/or neomycin as appropriate according to standard protocols.

Transient Transfection

Transfections were performed in 24-well dishes using LIPOFECTAMINE™ (Life Technologies) following the manufacturer's guidelines unless otherwise described. Briefly, cells were seeded at

approximately 0.5×10^5 cells/well and incubated overnight in a 37°C incubator (5% of CO₂). DNA lipid mixtures were prepared according to the manufacturer's recommendations, incubated at room temperature for 20–40 min and overlaid onto the cells (0.25 mL/well). Equal volume of medium containing 1% FBS was added to each well 5 h after transfection. Medium was replaced 18–24 h after transfection with fresh DMEM containing 0.5% FBS. Cell lysates were collected 48 h after transfection and assayed for luciferase activity.

Stable Transfection

HeLa cells were plated in 100-mm tissue culture dishes at 5×10^5 cells/dish the day before transfection. pFR-Luc^{Hyg}, pFA2-Elk1, or pFA2-CREB (1–10 µg plasmid DNA/transfection) was introduced into the appropriate cells using calcium phosphate precipitation method with the Mammalian Transfection Kit from Stratagene per the manufacturer's instruction. HLR cells were selected with hygromycin (0.2 mg/mL) only while stable cells containing both pFR-Luc and a fusion transactivator plasmid were selected using both G418 (0.5 mg/mL G418) and hygromycin (0.2 mg/mL). About two weeks after transfection, single colonies were transferred to 24-well plates and sequentially expanded to 12-well plates, 6-well plates, and finally T-25 flasks. The clones were then tested by transient transfection as discussed in the Results and Discussion section and, if positive, frozen down for storage.

Stimulation of HLR-Elk1 and HLR-CREB Cells with Extracellular Stimuli

HLR-Elk1 or HLR-CREB cells (0.5×10^5) were plated on 24-well plates in DMEM containing 10% FBS. The sera used to culture cells may contain many kinds of growth factors that are able to activate certain signal transduction pathways in the cells, especially those involved in cell proliferation (e.g., MAPK pathway). To reduce the background reporter gene expression, it is often necessary to starve cells of serum before treating the cells with extracellular stimuli. We usually starve the cells

by culturing the cells for 18–24 h in low-serum medium (DMEM containing 0.5% FBS) before treating the cells with extracellular stimuli such as epidermal growth factor (EGF). Cells were then harvested and lysed for luciferase activity assays 5–8 h after the treatment.

Luciferase Assay

Cell lysates were prepared and tested for luciferase activity using Stratagene's Luciferase Assay Kit according to the instruction manual.

RESULTS AND DISCUSSION

Establishment of Stable Cell Lines

pFR-Luc encodes the firefly luciferase gene under the control of a synthetic promoter containing five direct repeats of the 17-mer GAL4 binding site (Figure 1). Luciferase expression from pFR-Luc depends on the binding and activation of a transactivator containing GAL4 DNA-binding domain such as GAL4-VP16 or GAL4-Elk1. To select for cell lines containing pFR-Luc and a GAL4 fusion transactivator plasmid, pFR-Luc was first introduced into HeLa cells. After transfection of HeLa cells with pFR-Luc^{Hyg}, a total of 11 hygromycin-resistant clones was expanded and tested by transfecting the cells with a plasmid expressing GAL4-VP16 or a control plasmid expressing GAL4 dbd only. One clone (no. 1C3) showed the highest luciferase expression when transfected with GAL4-VP16 plasmid but very low luciferase expression with control plasmid. Luciferase expression in this clone was also highly responsive to PKA or MEK1 when co-transfected with pFA-CREB or -Elk1, respectively (data not shown). It was designated as HLR (HeLa luciferase reporter cell line) and was used to make the double-stable cell lines described below.

pFA2-Elk1 or -CREB were then stably transfected into HLR cells and selected with G418. The resulting clones were screened by transient transfection with positive control vectors expressing MEK1 or PKA, respectively. Clones that showed the highest luciferase expression were selected and tested

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further by other criteria such as background luciferase expression, growth rate, and viability. After testing total of nine clones for pFA2-Elk1 and 24 clones for pFA2-CREB, the best-performing ones were selected and designated as HLR-Elk1 and HLR-CREB, respectively. The stable integration of the fusion transactivator plasmids in these two cell lines was also confirmed by PCR with primers specific to GAL4-Elk1 or GAL4-CREB (not shown).

Use of HLR-Elk1 and HLR-CREB Cell Lines for Gene Function Analysis

To confirm the involvement of MEK1 protein in the signaling path-

ways converging at Elk1, HLR-Elk1 cells were transiently transfected with plasmids expressing MEK1 proteins. Luciferase expression in the cells was enhanced over 50-fold by a constitutively active form of MEK1. This effect is a result of its kinase activity because a kinase-deficient form of MEK1 not only failed to activate luciferase expression significantly but also inhibited the effect of the active MEK1 by 75%. Similar experiments can be performed with other genes of unknown function to determine if they are involved in signaling pathways converging at Elk1 or CREB.

As with almost all cell-based assays involving transient transfection, the results of this type of experiment are affected dramatically by the amount of

the plasmid DNA used for transfection. Figure 2 shows a typical dose response of luciferase expression to the amount of pFC-PAK plasmid used to transfect HLR-CREB cells. Too much or too little plasmid both resulted in decreased luciferase expression. HLR-Elk1 cells showed a similar dose-response curve: luciferase expression peaked at 10 ng/well but decreased by 80% at 100 ng/well of pFC-MEK1 (not shown). To achieve the optimal result, the amount of the expression plasmid for the gene of interest should be optimized.

When studying gene functions with transient transfection assays, the effect of the gene of interest tends to be underestimated, especially if there is substantial basal reporter expression or if

A. Fusion *Trans*-activator Plasmids:

B. Reporter

C. Positive Control

Figure 1. Components of PathDetect trans-Reporting. (A) Pathway-specific fusion *trans*-activator vectors are derived from pFA-CMV (Stratagene). The coding DNA sequences for activation domain of human Elk1 (residues 307–428) (11,12) or rat CREB (residues 1–283) (5) were fused in frame with the DNA-binding domain of the yeast *trans*-activator GAL4 (residues 1–147) (9,13). (B) pFR-Luc vector. The reporter vector was constructed by cloning the entire coding sequence of *Photinus pyralis* (firefly) luciferase (3) downstream of a basic promoter element (TATA box) derived from Adenovirus E1b promoter and joined to five tandem repeats of the 17-bp GAL4-binding element. The backbone of pFR-Luc is derived from pUC-19. (C) Positive control plasmids for PathDetect Elk1 and CREB systems are mammalian expression vectors encoding a constitutively active form of MEK1 (10,20) or the catalytic subunit of murine protein kinase A (17), respectively, under the control of cytomegalovirus (CMV) promoter.

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Table 1. Stimulation of Luciferase Expression by Extracellular Stimuli in HLR-Elk1 and HLR-CREB Cells

Cell Lines	Extracellular Stimuli	Luciferase Activity (RLU)
HLR-Elk1	DMEM	1827
	EGF (100 ng/mL)	12 002
	PMA (50 ng/mL)	120 205
	EGF + PMA	169 004
HLR-CREB	DMEM	995
	DMSO	1047
	cAMP (10 mM)	6942
	AC Toxin (40 μ g/mL)	18 769
	Forskolin (50 μ M)	1455
	PMA (50 ng/mL)	45 396
	PMA + Forskolin (10 μ M)	80 893
	PMA + Forskolin (50 μ M)	124 639
	PMA + Forskolin (100 μ M)	189 239

Cells (5×10^4) were first starved in DMEM containing 0.5% FBS for 18–24 h. They were then treated with various reagents or combinations of reagents as indicated. Numbers in brackets show the final concentrations of the reagents. Luciferase activity was expressed as relative light unit (RLU), an arbitrary readout unit from a luminometer. Values represent the averages of three separate transfection samples.

the transfection efficiency is low (e.g., if the gene of interest stimulated a 20-fold increase of luciferase over basal expression but only 5% of cells got transfected). The net effect on the reporter assay could only be about a 2-fold change because the signal from the transfected cells will be diluted by the basal expression from all the non-transfected cells.

Activation of GAL4 Fusion Transactivators by Extracellular Stimuli

Overexpression of a protein (e.g., MEK1) usually gives rise to a more dramatic effect than treatment of the cells with extracellular stimuli. To test if the stable reporter cell lines respond well to extracellular stimuli, HLR-Elk1 and HLR-CREB cells were treated directly with reagents known to activate MAPK or PKA (Table 1).

EGF and phorbol 12-myristate 13-acetate (PMA) are well-documented MAPK activators. They both stimulated luciferase expression in HLR-Elk1 cells: a 5- to 7-fold increase for EGF and a 50-fold increase for PMA. This is a much stronger response as compared with the less than 2-fold induction of luciferase expression by EGF and 3- to 10-fold by PMA observed with a human astrocytoma cell line established by co-transfection of pFR-Luc and pFA2-Elk1 plasmids (14).

The effects of various cAMP-elevating agents on luciferase expression from HLR-CREB cells were evaluated as well. AC toxin gave about a 7-fold increase of luciferase expression. Although PMA has no effect on cellular cAMP level, it stimulated luciferase expression in HLR-CREB cells dramatically (about 45-fold). This effect of PMA has been observed with cell lines with a luciferase gene under the control of *cis*-acting cAMP-response element (CRE) element and is believed to be a result of crosstalk between cAMP and other signaling pathways (4,14). Forskolin itself did not have much effect on luciferase expression. However, it enhanced the effect of PMA dramatically in a dose-dependent manner (Table 1). A practical implication of these results is that PMA can be used to enhance the effect of extracellular stimuli such as forskolin or EGF if they

Figure 2. Activation of GAL4-CREB by PKA in HLR-CREB cells. Various amounts of pFC-PKA (solid diamonds) or pCMV-Script (open diamonds) as shown on the x-axis were transfected into HLR-CREB cells (5×10^4). The DNA amount in each well was balanced by adding different amounts of an unrelated plasmid (pBluescript[®]). Cells were harvested and assayed for luciferase activity 48 h after transfection as described in Materials and Methods.

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themselves cannot elicit strong enough readout signal.

Cell-Based Assay Development

Cell-based assays with engineered mammalian cells are increasingly used for high-throughput applications because they are relatively easy to be adapted to automated assays, are more physiologically relevant than biochemical assays, and can be used to monitor complicated pathways underlining various disease conditions.

HLR can serve as the reporter cell line for any assay systems that utilize the GAL4 DNA-binding domain such as mammalian two-hybrid assays (2). Similar cell lines have also been used for bioassays of endocrine activities or endocrine disrupter activities present in various commercial chemicals using GAL4 fusions of the hormone-binding domains of steroid hormone receptors (6,7). HLR-Elk1 and HLR-CREB cells contain "built-in" sensors for MAPK or PKA. It should be fairly straightforward to adapt these cell lines to high-throughput, cell-based assays for drug (inhibitors) screening efforts and panel assays for possible oncogenic activities (activators) of various commercial compounds.

With the procedures described here, many novel assays can be developed by building new reporter cell lines with medically important but difficult-to-transfect cell types and with different reporter genes or different fusion transactivator genes. For example, Sharif and Sharif (14) have recently introduced pFR-Luc and pFA2-Elk1 into U-373MG (a human astrocytoma cell line) and selected a stable cell line that can be used for high-throughput evaluation of protein kinase C inhibitors based on Elk1 activation. We have recently introduced pFR-Luc and pFA-cJun into HeLa cells and found it responded very well to JNK (c-Jun N-terminal kinase) activation.

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