Over-expression of protein kinase B α enhances recombinant protein expression in transient systems

Elham Ettehadieh, Sharon Wong-Madden, Teri Aldrich, Kelly Lane & Arvia E. Morris*

Immunex Corporation, Department of Cell Sciences, 51 University St. Seattle, WA 98101, USA

(* Author for correspondence; E-mail: morrisa@immunex.com; Fax: +1 206-903-0923)

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Abstract

As more genes are being identified through genomic techniques, the need to rapidly express recombinant proteins for functional studies has become increasingly acute. Transient expression of recombinant protein using COS-1, CV-1 and 293 cells is widely used to address this need. To improve the robustness of host cells for transient expression, the effect of over-expression of Protein Kinase B α has been explored. In this report we demonstrate that over-expression of Protein Kinase B α can improve transient recombinant protein expression 40% to >200% depending on the protein being expressed and the cell line used.

Introduction

COS-1, CV-1 and 293 cells are widely used in transient transfection systems for rapid expression of recombinant proteins in mammalian cells. Using expression plasmids capable of replication in the host cells can optimize the level of recombinant protein expression. An inherent limitation of these systems is that the viability of the host cells is often difficult to maintain, and low viability may result in loss of recombinant protein titer over time. Recent studies have shown that Protein Kinase B α (PKB α) plays a central role in mediating cellular responses to a variety of stimuli including growth factors, heat shock, hypoxia, chemokines and hyperosmolarity (Coffer et al., 1998; Vanhaesebroeck and Alessi, 2000). It has been shown that PKB α is a regulator of cellular metabolic functions as well as apoptotic response (Coffer et al., 1998). Since PKB α is an important regulator of cellular functions, we are investigating the possibility of improving transient transfection systems by over-expression of PKB α . We have examined three host cell lines, COS-1, CV-1EBNA (derivative of CV-1) and 293M (derivative of 293) by transient co-transfection of a reporter gene with a wild-type PKB α . Data suggest that all the host cells benefit by over-expression of PKB α as demon-

strated by an improved recombinant protein titer. To determine whether the increased protein production is limited to transient co-transfection, stable clonal lines of CV-1EBNA and COS-1 cells that over-express PKB α were developed. We found that transient protein titers are improved when the hosts CV-1EBNA and COS-1 are stably over-expressing PKB α . The mechanism responsible for this improved performance is being investigated.

Materials and methods

Tissue culture

The 293M cells are a derivative of 293 human embryonic kidney cells. The CV-1EBNA cell line is a derivative of the CV-1 fibroblast like African Green Monkey cell line (ATCC, Manassas, VA). CV-1EBNA cells constitutively express EBNA driven from the CMV immediate early enhancer/promoter. COS-1 is a cell line established from CV-1 cells which constitutively expresses SimianVirus 40 large T antigen (ATCC). CV-1EBNA and COS-1 cells are grown in Dulbecco's Modified Eagles Medium/F12 (DMEM/F12)(JRH, Lenexa, KS) with the addition

of 1% penicillin streptomycin (100x stock; GibcoBRL, Grand Island, NY), 1% Glutamax (GibcoBRL) and 5% fetal bovine serum (FBS) (Hyclone, Logan, VT). The 293M cells are grown in DMEM (Hyclone) with the addition of 1% penicillin streptomycin, 1% Glutamax and 10% FBS. For transient transfections, cell lines were transfected using standard DEAE/dextran (Sigma, St. Louis, MO) protocols which included a 10% dimethylsulfoxide (DMSO) (Sigma) shock and chloroquine (Sambrook et al., 1989). The DMSO shock was not given to the 293M cells. Subsequent to the transfection, the cells were cultured in growth media containing low serum (0.5%). For co-transfection experiments, equal amounts of each plasmid were used. To create cell lines with stably integrated pcDNA3 (Invitrogen, Carlsbad, CA) or pcDNA3PKB α , COS-1 or CV-1EBNA cells were transfected with either plasmid using lipofectamine (GibcoBRL). Cells were selected in media containing G418, and G418 resistant cells were either pooled or plated in 96-well microtiter plates for individual cell cloning. Recombinant protein expression was monitored by ELISA or Western blotting. All expression studies were done at least twice in triplicate. Representative results are shown.

Plasmid constructs

The reporter plasmids encoding Fc receptor 1, Fc receptor 2, Reporter 2 or a fluorescent reporter consisted of the cDNA for these reporters cloned into the expression plasmid pDC409 (Giri et al., 1994). pDC409 includes the Epstein Barr Virus origin of replication and the SV40 Virus origin of replication. Expression of recombinant protein is driven by an expression cassette that includes the HIV trans-activator Tat driving the expression of the Tar promoter sequence. The reporter plasmid for Cytokine 1, pDC412 Cytokine 1, consisted of the cDNA for this protein cloned into pDC412. pDC412 expression plasmid is similar to pDC409, except that the multiple cloning site has been modified. Expression of Reporter 1 was driven by the SV40 promoter/enhancer. The cDNA for hamster PKB α was cloned using RT-PCR from Chinese hamster ovary cell RNA. An RT-PCR fragment-encoding hamster PKB α was cloned into the expression vector pcDNA3.

Western blots

For Western blot analysis, cells were washed with PBS and lysed in cell lysis buffer (New England Bio-

labs, Beverly, MA). Lysates from equal numbers of cells were separated by electrophoresis using 4–12% Bis-Tris gels (Novex/Invitrogen) under conditions recommended by the supplier. After running the gels, proteins were transferred to nitrocellulose, and PKB α antibody (Transduction Laboratories, Lexington, KY) was used to detect PKB α .

Results and discussion

Transient co-transfection, COS-1, CV-1EBNA and 293M

Initial studies were performed to determine whether co-expression of PKB α with reporter proteins would improve their expression in transient assays. The 293M cells and COS-1 cells were transfected with equal amounts of the pcDNA3PKB α and pDC409 Fc receptor 1 or pcDNA3 control vector and pDC409 Fc receptor 1. The Fc receptor 1 titers were higher in the transfections that included pcDNA3PKB α with a 260% improvement for the 293M cells and an 80% improvement for the COS-1 cells (Table 1). Overall the titers for the 293M cells were higher indicating that Fc receptor 1 is expressed better in 293M cells. Transient expression of two reporters, Reporter 1 and Fc receptor 2, was examined in CV-1EBNA cells. In this case Reporter 1 expression was improved 200% and Fc receptor 2 expression was improved by 50% (Table 1). Thus CV-1EBNA transient expression appears to be improved with over-expression of PKB α , but the extent of the improvement varies depending on which reporter is used.

Selection of clones over-expressing PKB α

Since the transient expression of reporter constructs was improved with co-transfection of the pcDNA3PKB α , we decided to determine whether CV-1EBNA or COS-1 cells stably expressing PKB α also had improved expression compared with CV-1EBNA or COS-1 cells transfected with an empty pcDNA3 plasmid or parental cells. Pools and individual clones of CV-1EBNA with stably integrated pcDNA3 or pcDNA3 PKB α were screened by Western blot for expression of PKB α . One clone, KD4, expressed more PKB α than the parental CV-1EBNA cells or a clone PF1 that was transfected with an empty vector, pcDNA3 plasmid (Figure 1A). Western blot analysis of COS-1 cells that were transfected with pcDNA3 PKB α identified two clones, CKE5 and

Table 1. Transient Co-transfection with and without PKB α expression plasmid

Cell line	Reporter	Titer+pcDNA3	Titer+pcDNA3PKB α	% increase in reporter expression
293M	Fc receptor 1	37.7 $\pm$ 1.9 μ g ml $^{-1}$	136.3 $\pm$ 15.5 μ g ml $^{-1}$	260%
COS-1	Fc receptor 1	7.6 $\pm$ 1.3 μ g ml $^{-1}$	14 $\pm$ 2.7 μ g ml $^{-1}$	80%
CV-1EBNA	Reporter 1	155.7 $\pm$ 2.6 U ml $^{-1}$	474.1 $\pm$ 23.0 U ml $^{-1}$	200%
CV-1EBNA	Fc receptor 2	16.0 $\pm$ 4.4 μ g ml $^{-1}$	23.9 $\pm$ 1.5 μ g ml $^{-1}$	50%

Figure 1. Western blot analysis of endogenous and recombinant PKB α expression in various cell lines and clones. Arrows indicate the major PKB α . Black dots indicate putative degraded PKB α .

Table 2. Expression of recombinant protein from CV-1EBNA clones over-expressing PKB α

Cell line	Reporter 1
PF1 vector clone	622.7 $\pm$ 26.8 U ml $^{-1}$
KD4 PKB α clone	1755.0 $\pm$ 377.9 U/ml

CKA9, which over-expressed PKB α compared with the parental COS-1 cells (Figure 1B). The Western blot analysis of clones from both cell lines demonstrated that there is heterogeneity in the expression of PKB α in the cell pools (data not shown).

Expression of reporter constructs in CV-1EBNA and COS-1 cells over-expressing PKB α

To determine whether expression of reporters in the KD4 derivative of CV-1EBNA is improved compared with clones transfected with the empty expression

plasmid, this cell line was used to express Reporter 1. For Reporter 1 there was an approximate 150% improvement in expression of the reporter compared with controls (Table 2). Improvement of 30% to >200% have been found with additional reporters (data not shown). It was also found that COS-1 clones, which over-expressed PKB α , gave improved titers for expression of recombinant protein in transient assays compared with COS-1. Transient expression of Cytokine 1 was investigated in COS-1 cells, a pool of COS-1 cells stably transfected with pcDNA3, a pool of COS-1 cells stably transfected with pcDNA3PKB α and the clone, CKE5, stably over-expressing PKB α . The expression of Cytokine 1 was improved by about 40% in CKE5 compared with the COS-1 cells or either of the pools (Table 3). For Reporter 2, a 127% improvement was observed (Table 3). Similar results have been observed using at least five additional recombinant proteins including one recombinant anit-

Table 3. Expression of recombinant protein from COS-1 clone and pools of cells over-expressing PKB α

Cell line	Cytokine 1 ^a	Reporter 2 ^b
COS-1	1.9 $\pm$ 0.5 μ g ml ⁻¹	.76 $\pm$.06 μ g ml ⁻¹
COS-1 vector pool	1.0 $\pm$ 0.2 μ g ml ⁻¹	na ^c
COS-1 PKB α pool	1.2 $\pm$ 0.3 μ g ml ⁻¹	na
CKE5	2.7 $\pm$ 0.1 μ g ml ⁻¹	1.73 $\pm$.15 μ g ml ⁻¹

^a six-well dish

^b roller bottle

^c na=not assayed

body. The improved expression with the CKE5 cells has been observed for at least 12 weeks.

In an attempt to understand the mechanism responsible for improved expression in KD4 and CKE5 cells, several parameters were compared to the parental cell lines. The doubling times for each cell line were found to be similar: 21.1 $\pm$ 1.1 hours for COS-1 and 25.0 $\pm$ 2.3 hours for CKE5; and 27.9 $\pm$ 2 hours for CV-1EBNA and 29.5 $\pm$ 1.8 hours for KD4. Transfection efficiency was measured by FACS analysis of a fluorescent reporter expression at 24 hours after transfection. The transfection efficiency for the CV-1 and KD4 cells was similar, 11.1% and 7.8%, respectively. For the COS-1 and CKE5 cells, the transfection efficiencies were also similar to each other at 23.5% and 26.4%, respectively.

In this study we have found that over-expression of PKB α in 293M, CV-1EBNA and COS-1 cells improves transient expression of recombinant reporter proteins. This effect can be observed by co-transfecting with PKB α or stably expressing PKB α in the host cell line. The improvement in expression varies between 30% and >200%

depending on the cell line and reporter construct used. Improved expression was found to be independent of the enhancer or promoter used in the expression cassette, indicating the generality of the effect. Improved expression was also independent of scale because it was observed in six-well dishes as well as roller bottles. Since PKB α regulates a variety of cellular functions, it will be interesting to determine whether the results are due to improved cell viability, transcription, translation or changes in cellular metabolism or combinations of any of these. Initial investigations have shown that over-expression of PKB α does not dramatically change cell growth rate or transfection efficiency. It has been shown that over-expression of Bcl2 can improve transient expression (Fujita et al., 1996), but these results have not been repeated. It could be that the level of over-expression of endogenous cellular proteins is important for the phenotype observed. It would be interesting to study these systems further with inducible expression systems.

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