

Role of Regulatory Domain Mutants of PKC Isoforms in *c-fos* Induction

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Protein kinase C (PKC) is a multi-gene family of serine-threonine protein kinases, composed of at least 10 kinases.¹ Classical and novel PKC isoforms are activated by diacylglycerol (DAG) and induce survival pathways in various cells. A tandem repeat of cysteine-rich motif referred to as the C1a and C1b domain in the regulatory domain is thought to be responsible for this activation. Atypical PKC isoforms, which lack of the C1b domain, cannot be activated by DAG.¹ A DAG analog 12-O-tetradecanoylphorbol-13-acetate (TPA) is known to activate PKC, resulting in diverse cellular functions such as cell proliferation, differentiation and tumorigenesis. PKC can activate extracellular signal-regulated kinase (ERK) through mitogen-activated protein (MAP) kinases. Activated ERK translocates to the nucleus and regulates the activity of many transcription factors.² Ternary complex factor (TCF) is one of the most studied transcription factors in this pathway,³ and is involved in *c-fos* transcription.⁴ *c-fos* is a proto-oncogene belonging to the immediate early gene family of transcription factors, and is involved in cell proliferation, differentiation and progression of cancer.⁵ The serum response element (SRE) in the *c-fos* promoter is required for the induction of *c-fos* gene.⁶ TCF and another transcription factor, serum response factor (SRF) bind to SRE and activate the transcription.

The Rho family small GTPases including Rho proteins (RhoA and RhoB), Rac1, and Cdc42, are members of the Ras superfamily of GTPases. These GTPases regulate many biological processes, including cell proliferation, differentiation, cytoskeletal regulation, membrane trafficking, cell adhesion, cell polarization, and transcriptional activation.⁷ Rho family GTPases can activate serum response factor (SRF), resulting in cell proliferation. Recently we have shown that the regulatory domain mutants of specific PKC isoforms induced apoptosis in breast cancer cells.⁸ Here we studied the effect of regulatory domain mutants of PKC isoforms in transcriptional activation of *c-fos* gene.

We and several investigators demonstrated that TPA induces transcriptional activation of the *c-fos* gene in a PKC-mediated pathway.^{3,11} To explore the role of the regulatory domain mutants of PKC isoforms in this pathway, we used expression plasmids, pHANI-PKC α -REG, pHANI-PKC δ -REG, pHANI-PKC ϵ -REG and pHANI-PKC ζ -REG which encode regulatory domain mutants of PKC α , PKC δ , PKC ϵ and PKC ζ respectively. Western blot analyses after transient expression of these mutants in NIH3T3 mouse fibroblast cells, showed expected sizes of each constructs and similar

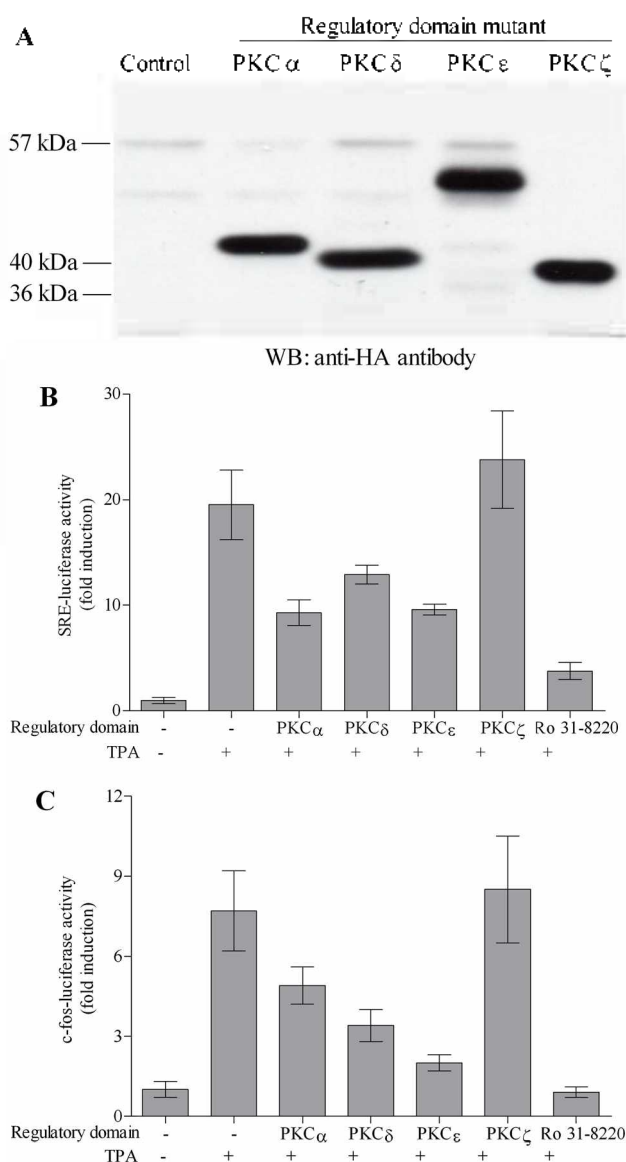


Figure 1. Role of regulatory domain mutants of PKC isoforms in TPA-mediated *c-fos* induction. COS-7 cells were transfected with pHANI-PKC α -REG, pHANI-PKC δ -REG, pHANI-PKC ϵ -REG, pHANI-PKC ζ -REG or pcDNA3 and cellular proteins were extracted by cell lysis. Western blotting was performed using anti-HA-antibody (A). NIH3T3 cells were cotransfected with pSRE-luc (B) or pfos-WT-luc (C) reporter plasmid (1 μ g) and pHANI-PKC α -REG, pHANI-PKC δ -REG, pHANI-PKC ϵ -REG, pHANI-PKC ζ -REG or empty control vector. The cells were then serum starved for 24 hours and treated with either 0.1% DMSO or 100 ng/mL TPA for 3 hours and assayed for luciferase activities.

expression levels (Fig. 1A).

Using transient transfection assays with SRE luciferase-reporter plasmid (pSRE-luc) in NIH3T3 mouse fibroblast cells, we found that TPA-induced SRE activation was partially blocked by the regulatory domain mutants of PKC α , PKC δ and PKC ϵ but not by the regulatory domain mutant of PKC ζ (Fig. 1B). These results suggest that regulatory domain mutants of PKC isoforms act as a dominant-negative form in our transient transfection assays. Failure to block TPA-mediated signaling pathway by the regulatory domain mutant of PKC ζ is consistent with previous reports that PKC ζ lacks TPA binding domain and thus is not responsive to DAG or TPA.¹² TPA-induced SRE activation was only partially inhibited (30-50%) by individual regulatory domain mutants and was further inhibited dramatically by the combination of the regulatory domain mutants of PKC α , PKC δ and PKC ϵ (data not shown), and non-selective PKC inhibitor Ro 31-8220 (Fig. 1B). Use of c-fos luciferase

reporter plasmid (pfos-WT-luc) further supports these findings (Fig. 1C).

Since PKC and Rho family small GTPases were reported to be involved in SRE activation,¹³ we examined the effect of regulatory domain mutants of PKC isoforms in this pathway. NIH3T3 cells were cotransfected with luciferase reporter plasmids (pfos-WT-luc or pSRE-luc), constitutively active mutants of Rho family GTPases (pcDNA3B-cdc42Hs-Q61L or pcDNA3-rac1-Q61L or pCMV-rhoA-Q63L), and regulatory domain mutants of PKC isoforms (pHAN1-PKC α -REG, pHAN1-PKC δ -REG, pHAN1-PKC ϵ -REG and pHAN1-PKC ζ -REG) or empty control vector (pcDNA3). SRE activation or c-fos induction mediated by the constitutively active mutants of Rho family small GTPases was mostly inhibited by the regulatory domain mutants of PKC α , PKC δ , PKC ϵ and PKC ζ (Fig. 2). These results suggest that PKC isoforms are the downstream effectors of Rho family GTPases and the regulatory domain mutants of PKC iso-

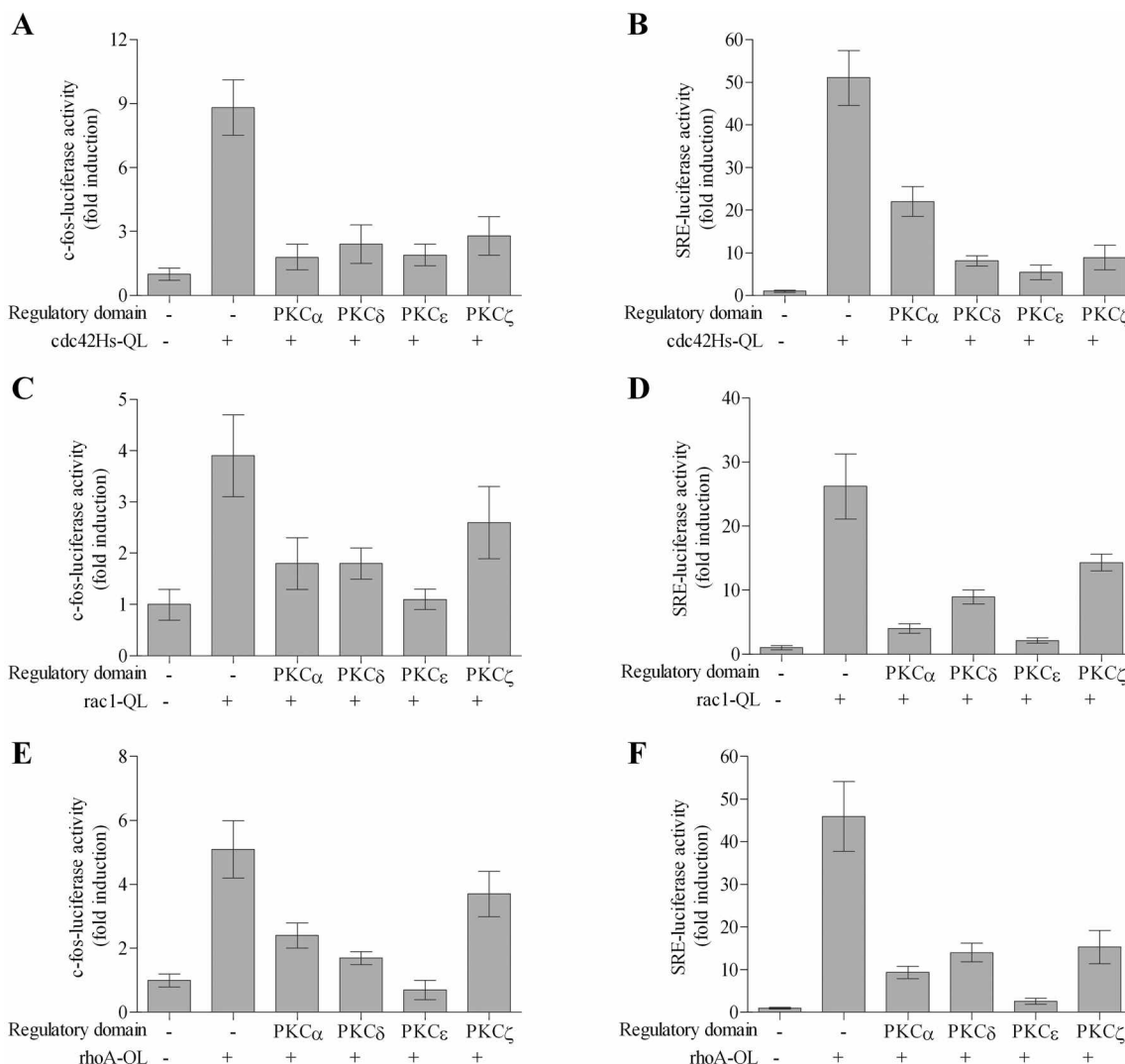


Figure 2. Role of regulatory domain mutants of PKC isoforms in GTPases-mediated c-fos induction. NIH3T3 cells were cotransfected with pfos-WT-luc or pSRE-luc reporter plasmid (1 μ g), pHAN1-PKC α -REG, pHAN1-PKC δ -REG, pHAN1-PKC ϵ -REG, pHAN1-PKC ζ -REG or empty control vector and pcDNA3B-cdc42Hs-Q61L, pcDNA3-rac1-Q61L, pCMV-rhoA-Q63L or empty control vector. The cells were then serum starved for 24 hours and treated with 0.1% DMSO and assayed for luciferase activities.

forms inhibited c-fos induction by blocking the Rho-SRF pathway.

In conclusion, the results of this study suggest that regulatory domain mutants of PKC isoforms inhibited TPA or constitutively active mutants of Rho family GTPases mediated transcriptional activation of c-fos gene by blocking the activation of endogenous PKC isoforms. This observation further provides the evidence that PKC isoforms might be a good target for developing anti-cancer agent in tumor growth.

Experimental Section

Plasmids. Luciferase reporter plasmids, pSRE-luc and pfos-WT-luc and constitutively active mutant of rhoA, pCMV-rhoA-Q63L were described previously.³ Expression vectors for the regulatory domains of PKC isoforms, pHAN1-PKC α -REG pHAN1-PKC β -REG and pHAN1-PKC ζ -REG were also described previously.⁸ pHAN1-PKC δ -REG expression plasmid was constructed by subcloning the EcoRI fragment of regulatory domain of PKC δ ⁹ into pHAN1 vector.⁸ pcDNA3-rac1-Q61L, pcDNA3B-cdc42Hs-Q61L were also described previously.¹⁰

Cell transfection. NIH3T3 cells were grown in DMEM containing 10% calf serum. Triplicate of 1×10^5 cells in 35 mm plates were transfected by lipofectin (Gibco BRL) with 2 μ g of reporter plasmid, 2 or 5 μ g of expression vectors, and 1 μ g of pCMV- β -gal. pcDNA3 plasmid DNA was added to the transfections as needed to achieve the same amount of plasmids per transfection. COS-7 cells were grown in DMEM containing 10% FBS. 2×10^5 cells in 60 mm plates were transfected by lipofectin (Gibco BRL) with 5 μ g of expression vectors or control vector pcDNA3.

Luciferase reporter assay. Six hours after transfection, cells were fed with new media (DMEM with 10% calf serum), left overnight, then serum-starved for 24 hours in DMEM with 0.5% calf serum. For TPA experiments, cells were treated with either or TPA for 3 hours to make a final concentration of 100 ng/mL. DMSO was used as a control. Luciferase Assays were performed with serum-starved or TPA-treated cells using the Luciferase Assay System (Promega). Luciferase activities were normalized by β -gal activities. β -gal assays were performed using the β -Galactosidase Enzyme Assay System (Promega).

Transient expression of PKCs in COS-7 cells. Six hours

after transfection, cells were fed with DMEM with 10% fetal bovine serum and left overnight. Cells were then transferred to 10 cm plates and grown for 24 hours before protein extraction. Cellular proteins were extracted by cell lysis in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol) containing protease inhibitors (10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 0.1 mM PMSF) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na₃VO₄, 10 mM β -glycerophosphate). Fifty microgram aliquots of total cell extracts were subjected to SDS-PAGE. Proteins were then transferred to Immobilon-P (Millipore) at 60V for 3 hours at 4 °C. Membranes were subsequently blocked with 5% dry milk in TBS-T (20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20), and then probed with an anti-HA antibody HA.11 (Covance) with 1:1000 dilution. Immunoblots were visualized with the Enhanced Chemiluminescence Western Blotting System (Amersham).

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