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Effect of phorbol esters on activity of *Ha-ras* gene promoter in HeLa cells[1]

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KEY WORDS protein kinase C; *ras* genes; phorbol esters; HeLa cells

ABSTRACT

AIM: To study the role of phorbol esters (PMA) in the activity of promoter of *Ha-ras* in HeLa cells. **METHODS**: After treatment with PMA, the growth rate of HeLa cells was measured by MTT assay and the expression of *Ha-ras* gene was detected by reversed transcriptase polymerase chain reaction. The regulation fragment of *Ha-ras* was constructed into the plasmid enhanced yellow fluorescent protein (EYFP) which did not contain promoter element. The recombinant plasmid pRasEYFP was transient transfected to HeLa cells. Using the *EYFP* gene as reporter, the activity of promoter of *Ha-ras* after treatment of PMA was assayed. **RESULTS**: The growth rate of PMA-treated HeLa cells was markedly reduced compared with untreated cells. The expression of *Ha-ras* gene was obviously decreased and the activity of promoter of *Ha-ras* was decreased by 34.0 % and 26.7 % respectively in HeLa cells after treated with PMA (100 μg/L) for 48 h and 72 h. In the meanwhile the activity of protein kinase C (PKC) was decreased by 76.3 % and 73.2 % compared with the control cells, respectively. **CONCLUSION**: PMA play an important role in regulation of the activity of promoter of *Ha-ras* in HeLa cells. The molecular mechanism may be through the PKC pathway.

INTRODUCTION

Being a proto-oncogene product, ras proteins are cytoplasmic GTP binding proteins with intrinsic GTPase activity. They are critical components of signaling pathways leading from cell-surface receptors to control of cellular proliferation and differentiation. In mammal cells, there are three types of *ras* genes: c-*Ha-ras*, c-K_i-*ras*, and *N-ras* gene. A *ras* gene can be mutated to an oncogenic form by single point mutations, and such mutated *ras* genes are most frequently detected oncogenes in human tumors. Overexpression of even the normal form of *ras* can cause oncogenic transformation. So it is important to understand how *ras* genes are regulated. Phorbol esters (PMA) can stimulate membrane phospholipases (phospholipase C or A2) resulting in the formation of diacylglyceride, free arachidonic acid, and increased amounts of arachidonic acid metabolites. As a tumor promoting agent phorbol esters play an important role in tumorigenicity, but the effect of phorbol esters on the regulation of *ras* oncogene in signal transduction pathway remained unclear. Protein kinase C (PKC) is the major intracellular receptor for tumor-promoting phorbol esters[1]. It is believed that a wide array of cellular responses mediated by these tumor promoting agents on cell growth, differentiation, gene expression, and tumor promotion are mediated via PKC. As an important signal transduction enzyme, PKC conveys signals generated by ligand-receptor interaction at the cell surface to the nucleus and influences numerous cellular functions including cell proliferation and tumorigenesis. It correlates with products of some oncogenes, for example *ras* in signal transduction pathway. Our work also indicates that antisense expression of PKCα improved sensitivity to anticancer drugs in human lung LTEPa-2 cells and inhibition of PKCα dramatically reduced the *Ha-ras* mRNA level[2-4]. To provide further insight into the relationship between the specific biological role of PMA and expression of oncogene *ras* in signal transduction pathways, in this paper we choose HeLa cells as model, using EYFP (enhanced yellow fluorescent

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protein), a kind of green fluorescent protein as reporter to investigate the role of PMA on the activity of promoter of *Ha-ras* and the PKC activity.

MATERIALS AND METHODS

Reagents Total regulation fragment of *Ha-ras* was generously provided by Prof Wonkeun LEE (Conell University, USA). Nucleic acid restriction and lipofect transfection agents were purchased from GIBCO (Gaithersburg, USA). Phorbol esters were provided by Sigma (Saint Louis, USA). Plasmid EYFP was purchased from Clontech (Palo Alto, USA).

Cell culture Cells were cultured and maintained in DMEM (Life Technologies, Inc) supplemented with 10 % calf serum at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

Plasmid construction[5] The cohesive ends of the regulation fragment of *Ha-ras* cut with *Hind* Ⅲ is subcloned into *Hind* Ⅲ sites of pEYFP-1 plasmid (Fig 1). The constructed plasmid was named pRasEYFP. The recombinant plasmid with the *Ha-ras* fragment in the sense orientation was confirmed by *Xho* Ⅰ.

MTT assay Cells (4×10^3 cells per well) were incubated in 96-well plates. MTT (5 g/L) 10 μL was added to each well. The reaction was stopped after 4 h of incubation by adding 100 μL of HCl 0.01 mol/L-10 % SDS, and the optical density at 570 nm was determined. Each point was determined by averaging the data of triplicate wells.

RNA extraction and reversed transcriptase polymerase chain reaction (PCR) analysis HeLa cells (provided by our lab) were treated with PMA for 48 h and 72 h respectively. Then the untreated cells and treated cells were harvested. Using Tripure Isolation Reagent (Boehringer Mamnheim) the RNA were extracted according to the procedure of the manufacturer. RNA concentrations were determined spectrophotometrically. cDNA was synthesized with AMV reverse transcriptase (Promega) using 1 μg RNA extracted from the cells according to the protocol recommended by the manufacturer under the following conditions: 25 ℃ 10 min, 42 ℃ 60 min, and 94 ℃ 5 min. Then the PCR reaction was carried out. For *Ha-ras* the 5′ primer sequence was 5′-TGA GGA GCG ATG ACG GAA TA-3′ and the 3′ primer sequence was 5′-GTA TCC AGG ATG ATG TCC AAC AG-3′. The primers for β_2-microglobulin were 5′-AAG ATG AGT ATG CCT GCC GT-3′ (5′ primer) and 5′-ATG CTG CTT ACA TGT CTC GAT-3′ (3′ primer). PCR was performed as follows: 94 ℃ for 3 min followed by 25 cycles at 95 ℃ for 50 s, 57 ℃ for 50 s, and 72 ℃ for 20 s. The PCR products were separated by electrophoresis on 1.5 % agarose.

Transient transfection Cells were seeded at 1×10^4 cells per dish onto 35-mm plates and allowed to recover for 24 h before transfection. Plasmids pEYFP-1 and pRasEYFP were purified and resolved in TE buffer then were transfected into HeLa cells using lipofectAMINE agent available from GIBCO according to the manufacturer's instruction.

Flow cytometry Transfected HeLa cells were harvested by centrifugation, washed twice with PBS, and resolved in PBS. Then the fluorescent intensity was analysed by flow cytometer (Coulter, USA).

PKC activity assay[6,7] HeLa cells were treated with PMA (100 μg/L) for 48 h and 72 h respectively. Then the untreated cells and treated cells were harvested and lysed. PKC was extracted and the PKC activity was assured by measuring the phosphorylation of Histone Ⅲ, a specific PKC substrate. PKC activity was expressed as $Bq \cdot min^{-1} \cdot g^{-1}$(protein).

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by t test, values of $P < 0.05$ were considered statistically significant.

RESULTS

Construction of recombinant plasmid pRasEYFP The promoter fragment of *Ha-ras* was cut with *Hind* Ⅲ and ligated with pEYFP-1 *Hind* Ⅲ fragment. The recombinant plasmid was transformed to competent DH5α bacteria (provided by our lab). The recombinant plasmid was named pRasEYFP (Fig 1).

Effect of PMA on the growth rate of HeLa cells HeLa cells were maintained in DMEM supplemented with 10 % calf serum. The growth curve assay indicated that the growth rate of HeLa cells treated with PMA (100 μg/L) was markedly reduced compared with untreated cells (Fig 2).

Effect of PMA on mRNA expression of *Ha-ras* in HeLa cells After HeLa cells were treated with PMA (100 μg/L) for 48 h and 72 h, the level of mRNA of *Ha-ras* was obviously decreased after treated with PMA for 48 h and 72 h in HeLa cells (Fig 3).

Effect of PMA on the activity of the promoter of *Ha-ras* in HeLa cells The fluorescent

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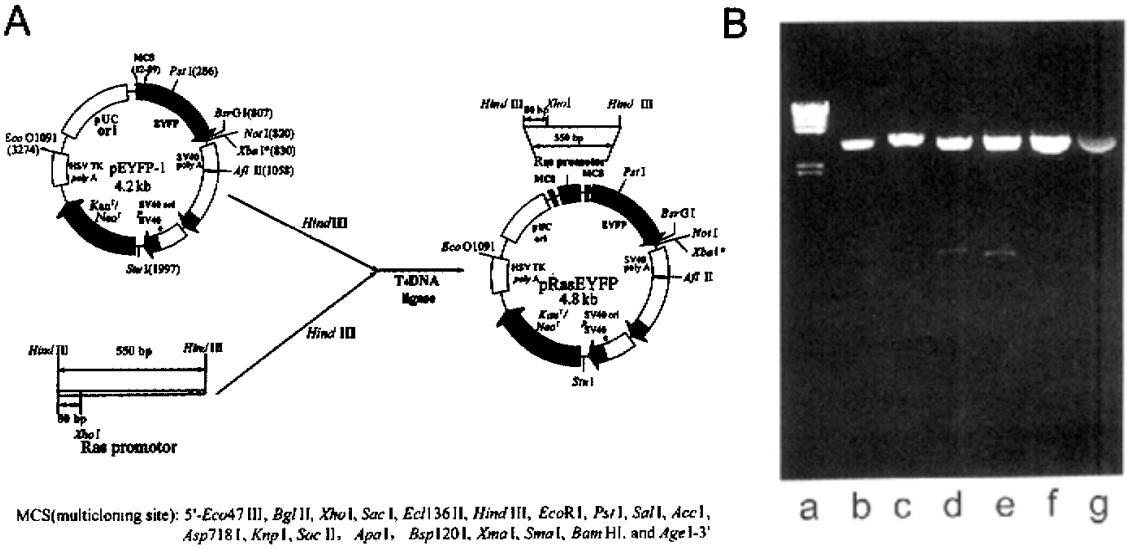

MCS(multicloning site): 5'-*Eco*47 III, *Bgl* II, *Xho* I, *Sac* I, *Ecl*136 II, *Hind* III, *Eco*R I, *Pst* I, *Sal* I, *Acc* I, *Asp*718 I, *Kpn* I, *Sac* II, *Apa* I, *Bsp*120 I, *Xma* I, *Sma* I, *Bam* HI, and *Age* I-3'

Fig 1. Construction of eukaryotic expression vector pRasEYFP. A) Schedule of construction of plasmid pRasEYFP. B) Identification of plasmid pRasEYFP by restriction endonucleases. a) λDNA *Hind* Ⅲ Marker; b) pRasEYFP/*Hind* Ⅲ; c) pRasEYFP/*Xho* Ⅰ; d) pSarEYFP (inserted reversely)/*Hind* Ⅲ; e) pSarEYFP (inserted reversely)/*Xho* Ⅰ; f) pEYFP/*Hind* Ⅲ; g) pEYFP/*Xho* Ⅰ.

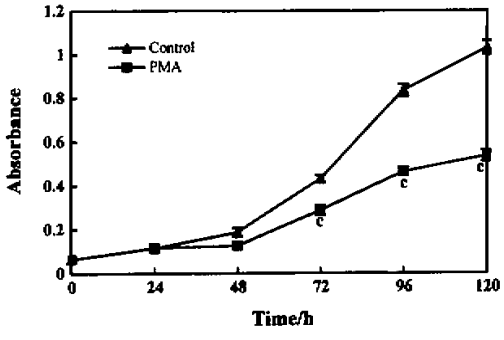

Fig 2. Effect of PMA on the growth rate of HeLa cells. $n=3$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control.

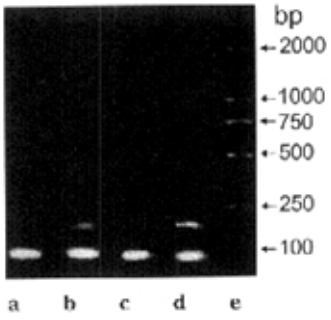

Fig 3. Effect of PMA 100 μg/L on the expression of *Ha-ras* in HeLa cells. a) 72 h with PMA; b) 72 h without PMA; c) 48 h with PMA; d) 48 h without PMA; e) DNA Marker.

intensity of EYFP was decreased under fluorescent microscope in PMA-treated HeLa cells compared with the untreated cells. Compared with control cells the fluorescent intensity was decreased by 34.0 % and 26.7 % respectively after treated with PMA for 48 h and 72 h (Fig 4).

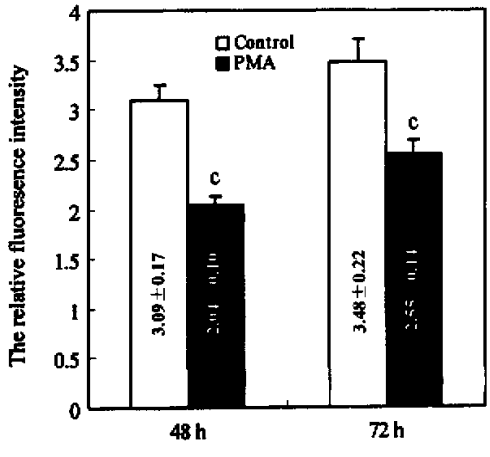

Fig 4. Effect of PMA on the activity of the promoter of *Ha-ras* in HeLa cells. $n=3$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control group.

Effect of PMA on the activity of PKC in

HeLa cells Cells in logarithmic phase were harvested and the activity of PKC was investigated. The activity of PKC was decreased by 76.3 % and 73.2 % compared with the control cells after treated with PMA (100 μg/L) for 48 h and 72 h respectively (Fig 5).

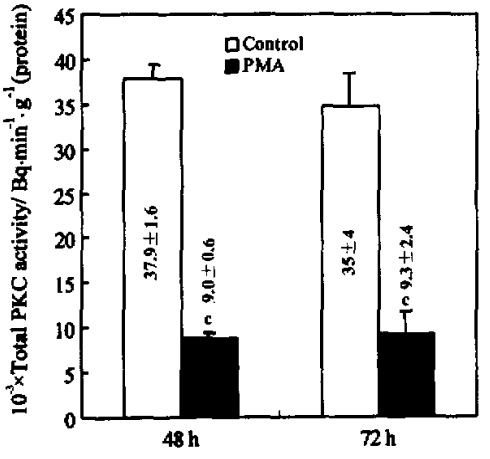

Fig 5. Effect of PMA on the activity of PKC in HeLa cells. $n=3$. $\bar{x} \pm s$. $^cP<0.01$ *vs* **control group.**

DISCUSSION

Being a tumor-promoting agent, PMA is widely used as a tool to investigate the tumorigenicity. But the molecular mechanism of PMA, especially the relationship between PMA and the expression of oncogene *ras* in PKC related transduction pathways are not clear. Our results indicated that the HeLa cells persistently treated by PMA exhibited a decreased growth rate and the expression of *Ha-ras* gene was decreased subsequently compared with control cells. Using *EYFP* as reporter, we observed that treatment of PMA (100 μg/L) resulted in down-regulation of *EYFP*. The regulation is under the control of the upstream regulation element of *Ha-ras*. The result provided an evidence that inhibition of *Ha-ras* promoter reduced the *Ha-ras* mRNA level. It indicated first that PMA signal was involved in the transcriptional regulation of the *Ha-ras* gene. It was demonstrated that PKC probably served as a receptor for the tumor-promoting phorbol esters[1]. PMA plays a dual effect on PKC. It appears to show a positive action that initially activates PKC, then a negative action that initiates degradation of the enzyme during sustained activation[8]. In this study PKC activity was assayed and we observed that the PKC activity was inhibited in HeLa cells persistently treated with PMA (100 μg/L) for 48 h and 72 h.

Both ras protein and PKC function downstream of plasma membrane-associated tyrosine kinases in cellular signal transduction. Nevertheless the reports about their relative position and functional relationship in the cell are controversial. There are two possibilities about the relationship of ras protein and PKC. Either PKC or ras protein functions upstream or downstream of each other in a common pathway[9] or these two pathways coexist and function in a distinct, yet interdependent manner[10]. Cells that overexpress protein kinase C β Ⅰ are more susceptible to transformation by an activated *Ha-ras* oncogene. These works provide genetic evidence that PKC mediates some of the effects of c-*Ha-ras* oncogene on cell transformation. The optimum synergistic effects between c-*Ha-ras* and PKC require critical levels of their respective activities[11]. Our work indicated that specific elevation of the PKCα level directly affected the increase of growth and the transforming phenotype of cells. The increased expression of oncogene *Ha-ras* may be one of the molecular mechanisms of the effect of PKCα[12]. Activator protein-1 (Ap-1) consists of jun and fos families. It could recognize and bind phorbol esters response element (TRE) sequece in the promoter region of several genes, and finally activate gene transcription. PKC activation induces dephosphorylation of three specific sites in C-terminal of C-jun by enhanced phosphatase or inhibites C-jun protein kinase. This dephosphorylation is associated with increase of AP-1 binding activity[13]. We observed also that the effect of overexpression of PKCα could activate transcription factor AP-1[14]. Transient overexpression of PKCα, β Ⅱ, and γ mediated phorbol esters-induced transcriptional activation of the TRE in rat fibroblast 3Y1 cells.

In summary, that persistent PMA treatment suppressed transcriptional activation of the *Ha-ras* promoter by using *EYFP* as a reporter. This may have a correlationship with PKC activity. Loss of PKC activity may suppress the transcriptional activation of the *Ha-ras* promoter. The result first indicated that PKC maybe involved in a signaling pathway toward the transcriptional regulation of *Ha-ras* in HeLa cells.

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佛波醇酯对 HeLa 细胞 *Ha-ras* 基因启动子活性的影响[1]

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关键词 蛋白激酶 C；*ras* 基因；佛波醇酯类；HeLa 细胞

目的：研究佛波醇酯（PMA）对 HeLa 细胞 *Ha-ras* 基因启动子活性的影响. **方法**：利用 MTT 法检测 PMA 对 HeLa 细胞生长速率的影响，并通过逆转录聚合酶链反应法测定 HeLa 细胞 *Ha-ras* 基因表达水平，进一步将 *Ha-ras* 基因上游调控序列插入到不含启动子元件的 *EYFP* 基因的上游，构建了重组质粒 pRasEYFP，并将其瞬时转染到 HeLa 细胞中. 以 *EYFP* 作为报告基因分析了 PMA 长期处理对 *Ha-ras* 基因启动子活性的影响. 在此基础上对 PMA 处理的 HeLa 细胞中蛋白激酶 C（PKC）的活性进行了检测. **结果**：与未处理细胞相比，PMA 长期处理引起 HeLa 细胞生长速率明显降低. 经 PMA 处理（100 μg/L）48 小时和 72 小时，与未处理细胞相比 HeLa 细胞中 *Ha-ras* 基因的表达降低，*Ha-ras* 基因启动子活性分别下降了 34.0 % 和 26.7 %，同时 PKC 的活性分别降低了 76.3 % 和 73.2 %. **结论**：PMA 在 *Ha-ras* 基因启动子活性的调节中扮演着重要的角色. 其作用的机理可能与 PKC 信号通路有关.

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