Volume 260, number 2, 281-284 FEB 08069 January 1990

Expression of protein kinase C I in NIH 3T3 cells increases its growth response to specific activators

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Received 28 October 1989; revised version received 8 December 1989

In order to investigate the effects of protein kinase C (PKC) expression on cellular growth and morphology, we established mouse fibroblast cell populations which expressed the rat *pkc-γ* gene under the control of a retroviral promoter. NIH 3T3 stable transfectants displayed a three-fold increase in total PKC levels. These cells appeared morphologically unaltered but exhibited a stronger mitogenic response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and cardiolipin (CL) as well as enhanced growth in semisolid medium in the presence of TPA. Thus, at these enzyme levels, PKC conferred growth advantages to NIH 3T3 cells only in response to specific activators.

Signal transduction; Tumor promotion; Phorbol ester; Cardiolipin

1. INTRODUCTION

Protein kinase C (PKC), as described by Nishizuka and colleagues (reviewed in [1,2]), consists of a family of Ca^{2+} and phospholipid-dependent serine-threonine kinases which exhibit strong amino acid sequence homology and structural similarity [3–5]. Diacylglycerol (DAG) is an endogenous activator of different subspecies including types I, II and III [6]. An early event in signal transduction is an increase in the hydrolysis of phosphatidyl inositol 2,4 bisphosphate [7,8], yielding phosphoinositol 1,4,5 trisphosphate (IP_3) and DAG. The fact that many growth factors induce an increase in DAG has led to the speculation that PKC plays a pivotal role in the mechanism of signal transduction [9]. On the other hand, there are indications that PKC might not be necessary for the transmission of some growth signals [10–12]. Moreover, overproduction of PKC in two different cell types induced varying degrees of morphological changes [13,14] which ranged from subtle differences in growth [14] to focus formation and anchorage independence [13].

In order to further define the effect of PKC expression on cell growth and morphology, we transfected the gene coding for rat PKC-I into NIH 3T3 cells. The response of these cells to specific activators of PKC was analyzed with respect to mitogenic activity and cloning efficiency.

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2. MATERIALS AND METHODS

2.1. *Plasmids*

The 2.2. kb *NcoI-XbaI* fragment containing the full coding sequence of rat *pkc-γ* was isolated from PMT 2-PKCI [3] and subcloned into the *Bam*HI site of vectors CIX-I and LTR-2 to yield the constructs pAC20 and pAC24, respectively. CIX-I was generated from pLJ [15] by removing the 1.4 kb *DraI* fragment which inactivates the polyoma middle T gene. Plasmids LTR-2 and pSV2neo have been described [16,17].

2.2. *Analysis of PKC expression*

The relative level of endogenous and transfected PKC was determined by Western blot analysis [18] using specific antibodies. Polyclonal antisera directed against either PKC-I (γ) or PKC-II (β) were a gift of Dr. B. Straulovic (Syntex). Tumor cell line HA1984 which overexpresses PKC-II was a gift of Dr. S. Aaronson. PKC-III (α) was identified with antibody MC5 (Amersham) which did not recognize the PKC-I isoform (data not shown).

2.3. *PDBu binding*

Cells were plated in 1 cm wells in triplicate and allowed to proliferate until confluence. They were washed three times with DMEM and after incubation for 30 min, DMEM containing 50 nM [^{3}H]PDBu (26.6 Ci/mmol, Amersham) was added at 37°C for 1 h. Cells were rinsed twice with PBS and twice with cold 5% TCA. TCA insoluble material was resuspended in 0.25 N NaOH and the amount of bound [^{3}H]PDBu was correlated to the number of cells in replica wells.

2.4. *Cell culture*

Growth of NIH 3T3 cells and calcium-phosphate transfections were conducted as described elsewhere [19]. Cells were selected with killer HAT medium or medium supplemented with 0.5 mg/ml G418 (Gibco). For agar growth assays, cells were resuspended in medium containing 0.3% agar (Difco) [20]. TPA (Sigma) was added at the indicated concentrations. Mitogenic assays were performed as described in [21]. Medium was supplemented with TPA or cardiolipin (Sigma) plus 3 μCi/mmol of [^{3}H]thymidine (50–90 mCi/mmol, New England Nuclear).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

3. RESULTS

3.1. *Transfection of expression vectors containing the rat* pkc-γ *gene into NIH 3T3 cells does not induce the transformed phenotype*

Expression of PKC-I with the help of the adenovirus late promoter has been reported for COS cells [3]. In order to achieve higher expression levels in murine fibroblasts the complete coding sequence of *pkc-γ* was placed under the control of the LTR sequences of the Moloney murine leukemia virus, yielding plasmids pAC20 and pAC24, respectively (fig.1). Plasmids PMT2-PKCI, pAC20 and pAC24 were transfected into NIH 3T3 cells using 10–0.01 μg DNA per plate. PMT2-PKCI plasmid DNA was cotransfected with pSV2*neo* DNA at a 10:1 molar ratio.

These PKC constructs did not induce transformed foci beyond the rate of spontaneous focus formation measured in cells transfected with vector DNAs alone. It has been suggested that alterations in growth of NIH 3T3 cells are dectectable only in the absence of non-transfected cells [14]. We, therefore, screened transfected cells grown under selective pressure for morphological changes. No reproducible differences in phenotypes could be detected when compared to cells transfected with vector DNAs only.

3.2. *Expression of the rat* pkc-γ *gene in transfected NIH 3T3 cells leads to a three-fold increase in the total number of phorbol ester receptors*

We estimated the expression levels of PKC-I in these cells by Western blot analysis. As shown in fig.2, NIH 3T3 cells transfected with vector DNAs alone did not express the PKC-I or PKC-II isoforms. However, the cell populations which contained either one of the three different *pkc-γ* plasmids produced easily detectable levels of PKC-I. Thus, whereas NIH 3T3 cells contain only PKC-III, rat PKC-I can be efficiently expressed in these cells without changing cellular morphology.

Since PKC-I and PKC-III bind DAG or phorbol esters with similar affinities [22], binding of PDBu to cells which express PKC-I and endogenous PKC-III can be used to estimate the increase in the total level PKC enzyme. The additional presence of PKC-I led to a two- to three-fold increase in total number of high affinity receptors for PDBu (table 1).

3.3. *Production of PKC-I in NIH 3T3 transfectants results in an increased mitogenic response to specific enzyme activators*

Phorbol esters act as mitogens in several cell systems via activation of PKC [2,6]. We compared the mitogenic response which could be elicited by different concentrations of TPA in *pkc-γ* transfectants with that of cells transfected with vector DNA only. As shown in fig.3A, in the presence of 50–200 nM TPA NIH 3T3 cells containing pAC24 gave a three- to four-fold larger

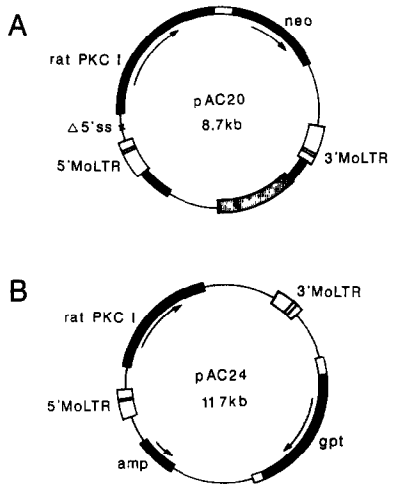

Fig.1. Structure of plasmids pAC20 and pAC24. PKC-I is expressed under the control of the Monoley murine leukemia virus LTR sequences (MoLTR). The *neo* and *gpt* genes were used for selection of transfected NIH 3T3 cells.

mitogenic response than control cells. In addition, we analyzed the increase in [^{3}H]thymidine incorporation elicited by cardiolipin which has been suggested to specifically activate PKC-I [22]. Cardiolipin concentrations in the range of 10–100 μg/ml also generated an enhanced [^{3}H]thymidine incorporation in the *pkc-γ* transfectants (fig.3B).

3.4. *Higher PKC levels in NIH 3T3 cells lead to increased cloning efficiencies in semisolid medium in the presence of TPA*

NIH 3T3 cells transfected with pAC24 grew in semisolid medium with similar cloning efficiency as cells harboring vector sequences only. As shown in table 2, however, in the presence of increasing concen-

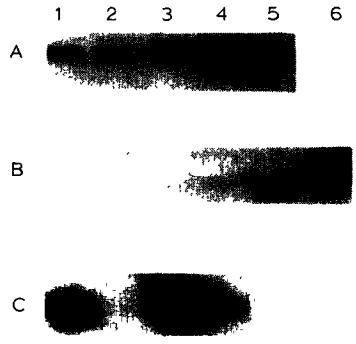

Fig.2. Analysis of the expression of PKC-I, PKC-II, and PKC-III. Western blot analysis was performed with antibodies against PKC-III (A), PKC-II (B) and PKC-I (C). 1, cell line 25-41, expressing PKC-I from plasmid pAC20; 2, cell line 25-91 containing vector CIX-I; 3, cell line 25-61 expressing PKC-I from plasmid pAC24; 4, cell line 25-77 expressing PKC-I from plasmid PMT2-PKCI; 5, cell line 25-89 containing vector LTR-2; 6, tumor cell line HA1984 overexpressing PKC-II as determined by Northern blot hybridization (data not shown).

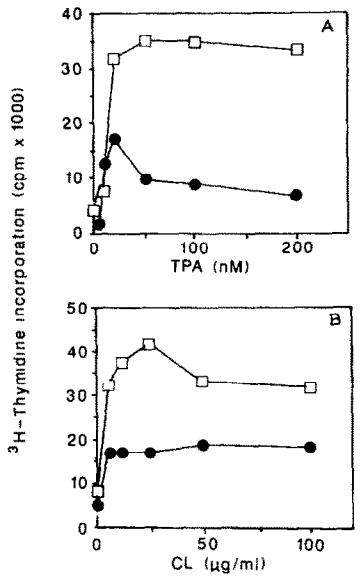

Fig.3. Mitogenic response to increasing concentrations of TPA (A) and cardiolipin (B) of cell line 25-61 expressing PKC-I from plasmid pAC24 (□) and of cell line 25-89 transfected with vector LTR-2 (●). These results correspond to one representative experiment out of three. Each value is the average of three measurements. Standard deviations were about 10% of the mean for each point.

Table 1

Phorbol ester binding in NIH 3T3 cells expressing PKC-I

Cell line	[^{3}H]PDBu binding (pmol/10^6 cells)	Increase
25-91	80 ± 19	
25-41	170 ± 32	2.1 ×
25-89	90 ± 70	
25-61	246 ± 26	2.7 ×

Cell lines transfected with the *pkc-γ* constructs pAC20 (25-41) and pAC24 (25-61) were assayed for phorbol 12,13 dibutyrate (PDBu) binding activity and compared to cells transfected with the respective vectors CIX-I (25-91) and LTR-2 (25-89). All assays are means ± S.D. of triplicate determinations as described in section 2. The increase in binding activity refers to the appropriate comparison with the control transfectants

Table 2

Cloning efficiency of NIH 3T3 cells overproducing PKC

TPA (nM)	25-61	25-89
0	2.7 ± 1.3	3.8 ± 1.2
10	5.5 ± 1.7	3.5 ± 1.2
20	6.8 ± 1.9	5.9 ± 1.5
50	17.4 ± 2.6	8.8 ± 1.9
100	19.3 ± 2.7	13.4 ± 2.4
200	21.5 ± 2.7	13.0 ± 2.2

Cell line 25-61 containing plasmid pAC24 and control transfectant containing vector LTR-2 were compared for their colony forming efficiencies in semisolid medium with and without TPA treatment. Values are mean ± SD

trations of TPA, pAC24 transfectants generated up to twice as many small colonies in soft agar as the control cell population. Thus, the level of PKC expression in the *pkc-γ* transfectants was sufficient to cause a pronounced effect on growth in soft agar but only in the presence of TPA.

4. DISCUSSION

Our data show that introduction of PKC-I in NIH 3T3 cells, which normally do not express this PKC subtype, can lead to pronounced changes in mitogenicity and anchorage-independent growth upon stimulation with the tumor promoter TPA, whereas growth in serum containing medium is unaltered. We could not detect any changes in morphology or cloning efficiency in our *pkc-γ* transfectants without the addition of phorbol esters. Persons et al. [14] have shown that constitutive expression of the same gene in NIH 3T3 cells resulted in subtle changes of morphology and in growth to higher saturation densities. The levels of high affinity receptors in those cell lines seemed to be in the same range or lower than in our overproducer cells. The different results in both studies might be due to clonal variability in the phenotype of NIH 3T3 cells. We, therefore, decided to restrict our analysis to mass cell populations transfected with the *pkc-γ* constructs and selected for the expression of the marker gene. Our results demonstrate that a certain level of increased PKC expression is not linked to an increase in anchorage-independent growth but that a strong tumor promoter such as TPA can evoke such a difference.

The effect of TPA may be substantially different from the action of other activators of PKC such as DAG, since TPA is hardly degraded and can exert a more prolonged action on PKC. Thus, chronic stimulation with TPA results in the degradation of PKC [5]. Therefore, the combined short- and long-term action of TPA on PKC might be responsible for the observed phenotypic changes in cells with originally higher enzyme levels. Nothing is known about the existence of specific, in vivo activators for the various subspecies of the enzyme. Such physiological activators might exert a dual action in vivo similar to the one of TPA in vitro and therefore might play an important role in tumour growth and dissemination. Cell lines overproducing PKC at moderate levels such as those described here might be ideal tools to be used in the search for these activators.

Acknowledgements: We wish to thank Dr. Stuart Aaronson for support and helpful discussions, and Dr. John Knopf for providing us with plasmid PMT2-PKCI.

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