Phorbol-ester-mediated expression of the collagen type I pro- α 2 gene in mouse 3T3-L1 cells and its absence in a phorbol 12-myristate 13-acetate-non-responsive variant

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We have previously isolated a phorbol 12-myristate 13-acetate (PMA)-non-responsive variant line (VT-1) from mouse 3T3-L1 cells [Shimizu, Fujiki, Sugimura & Shimizu (1986) *Cancer Res.* **46**, 4027–4031]. Differential hybridization of cDNAs obtained from PMA-treated 3T3-L1 and VT-1 cells resulted in the isolation of a number of unique cDNA clones, including one with a high degree of sequence similarity to the type I pro- α 2 collagen gene (COL1A2) [Amagai, Inokuchi, Nishikawa, Shimizu & Shimizu (1989) *Somat. Cell Mol. Genet.* **15**, 153–158]. Here we examined the expression of type I collagen pro- α 2 [pro- α 2(I)] mRNA and production of type I collagen in these two cell lines. In quiescent cells, the pro- α 2(I) steady-state mRNA levels were four times greater in 3T3-L1 cells than in VT-1 cells. PMA addition caused the steady-state levels of pro- α 2(I) mRNA to be six times greater in 3T3-L1 cells than in VT-1 cells, with a maximum at 30–60 min. The pro- α 2(I) protein levels in the extracellular matrix or culture media of 3T3-L1 cells were substantially elevated by PMA treatment, but no significant increase was detected in VT-1 cells. The correlation of collagen expression with a PMA-mediated mitogenic response suggests a new role for collagen as an early component of mitogenic signal transduction.

INTRODUCTION

Treatment of various cell types with the tumour promoter phorbol 12-myristate 13-acetate (PMA) induces immediate morphological changes [1,2] and initiation of mitogenesis [3]. PMA apparently exerts its effect on cellular morphology by influencing the expression and organization of cytoskeletal and extracellular-matrix (ECM) components, such as actin and fibronectin [1,2,4,5]. The mitogenic signal transduction of PMA is at least in part dependent on the activation of protein kinase C (PKC), the Ca^{2+} - and phospholipid-dependent protein kinase [6–9]. Activation of PKC is associated with increased expression of a number of cellular proto-oncogenes, such as *c-myc*, *c-fos* and *c-jun*, and leads to subsequent cell division [10–16]. Despite numerous studies, the mitogenic signal-transduction mechanism of PMA is not yet completely understood.

Using the mitotic shake-off method we have previously isolated a PMA-non-responsive cell line, VT-1, from PMA-responsive mouse 3T3-L1 cells [17]. The only apparent difference between 3T3-L1 and VT-1 cells are those lesions involved with PMA responsiveness. The VT-1 cells grow normally in serum-supplemented media, but are unable to initiate DNA synthesis and cell division in response to PMA treatment. Differential hybridization of cDNAs isolated from 3T3-L1 and VT-1 cells treated with PMA for 20 min resulted in a number of unique cDNA clones, including type I and type III procollagen cDNA clones [18].

We report here that there was a substantial difference in basal levels and PMA-induced expression of type I α 2 collagen gene (COL1A2) in 3T3-L1 and VT-1 cells. VT-1 cells expressed type I α 2 procollagen [pro- α 2(I)] mRNA at a level 4 times lower than that in 3T3-L1 cells. Furthermore, PMA treatment of 3T3-L1 cells promoted a significant increase in the steady-state levels of

pro- α 2(I) mRNA and procollagen protein, in addition to its incorporation into the ECM. Virtually no significant stimulation of collagen-gene transcription and collagen synthesis was observed in the PMA-non-responsive VT-1 cells. Our results suggest a correlation between the PMA-induced mitogenic response and the expression of collagen, the genes for which form a family known to play a role in maintaining the morphological integrity of the cell. The possible role of collagen in mitogenic signal transduction is discussed.

MATERIALS AND METHODS

Chemicals

Supplies for cell culture were purchased from GIBCO (Grand Island, NY, U.S.A.). PMA and cycloheximide were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Collagenase (activity 85 units/mg; non-specific proteolytic activity 6 units/mg) was from Calbiochem (San Diego, CA, U.S.A.). PMA (100 $\mu\text{g}/\text{ml}$) was dissolved in dimethyl sulphoxide (DMSO) and stored at -20°C until use.

Cell culture

PMA-non-responsive VT-1 variant cells were isolated from mouse fibroblast 3T3-L1 cells (American Type Culture Collection, Rockville, MD, U.S.A.) by the mitotic shake-off method using dihydrotelocidin B and vinblastine sulphate [17]. Unless otherwise specified, cells were grown to about 80% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-bovine serum (FBS), growth-arrested for 5–6 days in DMEM containing 5% FBS; cells were tested regularly for PMA-responsiveness using [^3H]thymidine-incor-

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; ECM, extracellular matrix; PKC, protein kinase C; pro- α 2(I), type I α 2 procollagen; COL1A2, type I α 2 collagen gene; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal-bovine serum; PBS, Dulbecco's phosphate buffer without CaCl_2 and MgCl_2 ; $1\times\text{SSC}$, 0.15 M-NaCl/0.015 sodium citrate, pH 7.0; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; FGF, fibroblast growth factor; TRE, PMA-responsive element; TGF- β , transforming growth factor β .

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poration assays as previously described [17]. The cultures were treated with DMSO (0.1%), PMA (100 ng/ml) or FBS (10%). Cycloheximide (10 μ g/ml) was added just before PMA addition. After the treatment, cultures were placed on ice, washed twice with Dulbecco's phosphate-buffered saline without CaCl_2 and MgCl_2 (PBS), detached from plates with Teflon-coated razor blades, washed with PBS and stored at -70°C until use.

RNA isolation and Northern analysis

RNA was isolated using guanidine thiocyanate and CsCl centrifugation [19]. Total RNA was separated by 1.5% agarose-gel electrophoresis in the presence of 2.2 M-formaldehyde and transferred to Nytran filters (Schleicher and Schuell, Keene, NH, U.S.A.) using $10\times\text{SSC}$ ($1\times\text{SSC}$ is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0). Filters were prehybridized at 42°C and hybridized in the presence of 50% (v/v) formamide at 42°C to a ^{32}P -labelled cDNA probe prepared by the random-priming method (Pharmacia, Piscataway, NJ, U.S.A.). The 2.2 kb *Eco*R1 insert of TIE-5 cDNA in pUC9 (18) was used to detect pro- $\alpha 2(\text{I})$ mRNA. The filters were washed twice for 15 min at room temperature in $6\times\text{SSC}/0.1\%$ SDS, twice for 15 min at 37°C in $1\times\text{SSC}/1\%$ SDS, and once for 2 h at 65°C in $0.1\times\text{SSC}/0.1\%$ SDS, and exposed to Konica type A X-ray film with two intensifying screens at -70°C for 1 day. The autoradiograms were quantified by using a densitometer (E-C Apparatus Corp., St. Petersburg, FL, U.S.A.).

Analysis of labelled proteins in medium and extracellular matrix

Quiescent cultures (10 cm dish) were treated with PMA (100 ng/ml), DMSO (0.1%), FBS (10%) and/or actinomycin D (2.5 μ g/ml) for 6 h at 37°C . After treatment, labellings were done in methionine-free DMEM containing [^{35}S]methionine (20 $\mu\text{Ci}/\text{ml}$; 144 Ci/mmol; NEN, Boston, MA, U.S.A.) or in DMEM containing [^3H]proline (2 $\mu\text{Ci}/\text{ml}$; 22 Ci/mmol; ICN, Irvine, CA, U.S.A.) for 3 h at 37°C . The medium was saved and freeze-dried in the presence of 1 mM-phenylmethanesulphonyl fluoride (PMSF). To analyse proteins of the ECM, labelled cells were washed with 10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl and extracted with 1 M-urea solution containing 10 mM-Tris/HCl (pH 7.4), 1 mM-dithiothreitol (DTT)/10 mM-EDTA/1 mM-PMSF [20]. After swirling for 5 min on ice, cells were scraped off and vortex-mixed for 5 min. Supernatants were collected after centrifugation at 13000 g for 20 min. ECM fractions (70 μl each) were treated with 5 units of collagenase (85 units/mg of protein) in 50 mM-Tris/HCl (pH 7.4)/12.5 mM- CaCl_2 /1.25 mM-PMSF for 5 h at room temperature. Aliquots of medium and ECM fractions were analysed by 5% or 10% (w/v)-PAGE in the presence of 2 M-urea and 0.2% SDS [21] and fluorographed using 1 M-sodium salicylate [22]. The dried gels were exposed to Konica type A X-ray film. Molecular-mass markers (Sigma Chemical Co.) used were myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine plasma albumin (66 kDa), egg albumin (45 kDa) and lactate dehydrogenase (36.5 kDa); prestained molecular-mass markers were α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa) and lactate dehydrogenase (36.5 kDa).

RESULTS

Pro- $\alpha 2(\text{I})$ steady-state mRNA expression

In confluent quiescent cells, steady-state pro- $\alpha 2(\text{I})$ mRNA levels were significantly different between the parental 3T3-L1 cells and the variant VT-1 cells (Figs. 1a, 1b and 1e). The basal

level in 3T3-L1 cells was 4-fold greater than in VT-1 cells, where, by comparison, the amounts of pro- $\alpha 2(\text{I})$ mRNA were negligible. When growth-arrested 3T3-L1 cells were treated with PMA, pro- $\alpha 2(\text{I})$ mRNA levels increased to levels six times greater than those found in VT-1 cells under the same conditions (Figs. 1a, 1b and 1e). Two transcripts, of 5.2 and 5.7 kb, were present. As previously reported, more than one collagen transcript is often seen, owing to different 3'-polyadenylation sequences [23–25]. Maximal pro- $\alpha 2(\text{I})$ mRNA levels were observed at 30 min after treatment. Although a slight increase of pro- $\alpha 2(\text{I})$ mRNA was seen in VT-1 cells (Figs. 1b and 1e), the maximum expression of

Fig. 1. Increase of pro- $\alpha 2(\text{I})$ mRNA by PMA in 3T3-L1 and VT-1 cells

Quiescent 3T3-L1 (a and c) and VT-1 (b and d) cells were exposed to PMA (100 ng/ml) for 0, 10, 20, 30 and 60 min (lanes 1–5 respectively) and to 0.1% DMSO for 30 min (lane 6). Total RNA (10 μ g) was analysed for pro- $\alpha 2(\text{I})$ expression by Northern blotting. The sizes of the pro- $\alpha 2(\text{I})$ transcripts detected (5.2 and 5.7 kb) were determined from the location of the ribosomal 18 S and 28 S rRNA. To ensure that equal amount of total RNA were loaded in each lane, the gel was stained with ethidium bromide (1 μ g/ml) (c and d), photographed, and the quantities of 18 S and 28 S rRNA were determined. Lanes 1–6 are as described above. The autoradiogram depicted above (e) was analysed by densitometry. The RNA for the Northern analysis was derived from non-treated and PMA-treated 3T3-L1 cells (□) and VT-1 cells (●). Values in the presence of DMSO (control) in arbitrary units were 37.5 for 3T3-L1 and 6 for VT-1.

Fig. 2. Increase of pro- α 2(I) mRNA in the presence of PMA or serum
Quiescent 3T3-L1 cells (a) and VT-1 (b) cells were exposed to DMSO (0.1 %) (lane 1), PMA (100 ng/ml) (lane 2) or FBS (10 %) (lane 3) for 30 min. Total RNA was analysed for pro- α 2(I) expression by Northern blotting.

Fig. 3. Effect of cycloheximide on pro- α 2(I) mRNA increase and degradation
Total RNA from quiescent 3T3-L1 cells exposed to PMA (100 ng/ml) (a and c), or PMA and cycloheximide (10 μ g/ml) (b and d) for 0.5, 1, 2, 6 and 12 h (lanes 1–5 respectively) were isolated and analysed for pro- α 2(I) mRNA by Northern blotting. Before blotting, the gel was stained with ethidium bromide as described above (c and d).

Fig. 4. Pro- α 2(I) mRNA increase by PMA during proliferation and after growth arrest
Total RNA from 3T3-L1 cells were isolated when cells were subconfluent (a) and after 5 days growth arrest in FBS (5%) (b). Total RNA was extracted after 30 min of treatment with no drug (lane 1), PMA (100 ng/ml) (lane 2) or FBS (10 %) (lane 3).

Fig. 5. ECM components of 3T3-L1 and VT-1 cells
ECM components from 3T3-L1 (a) and VT-1 (b) cells were analysed by a SDS/urea/5 %-PAGE. Cells were treated for 30 min with DMSO (0.1 %) (lanes 1 and 2), PMA (100 ng/ml) (lanes 3 and 4) or FBS (10 %) (lanes 5 and 6). ECM components treated with collagenase are shown in lanes 2, 4 and 6. Arrows indicate collagenase-sensitive proteins. Abbreviation: *M*, molecular mass.

pro- α 2(I) mRNA in 3T3-L1 cells was significantly higher than that in VT-1 cells. Induction of pro- α 2(I) mRNA was dependent on PMA concentrations, and it was maximum at 100 ng/ml (results not shown). The expression of pro- α 2(I) mRNA in response to serum was equivalent in both cell lines (Fig. 2). Thus the basal levels of pro- α 2(I) mRNA was greater in quiescent 3T3-L1 cells than in VT-1 cells, and steady-state levels of pro- α 2(I) mRNA increased in response to PMA in quiescent 3T3-L1 cells and to a much lesser extent in VT-1 cells.

To determine whether *de novo* protein synthesis is needed for the increase of pro- α 2(I) mRNA, 3T3-L1 cells were treated with cycloheximide and PMA. PMA again stimulated pro- α 2(I) mRNA expression, although the maximum level was reached at 60 min in this particular experiment. The pro- α 2(I) mRNA induction by PMA and serum was consistently seen, but the kinetics differed slightly among separate sets of experiments. Cycloheximide had little effect on the level of pro- α 2(I) mRNA in response to PMA (Figs. 3a and 3b). In a separate experiment, PMA induction of *c-fos*, *c-jun* and *c-myc* mRNA was clearly observed (results not shown) in 3T3-L1 cells and VT-1 cells. These mRNAs were stabilized in the presence of cycloheximide (results not shown) in 3T3-L1 cells. Thus increased pro- α 2(I) mRNA expression requires no protein synthesis *de novo*, and the mRNA is not stabilized by cycloheximide.

When proliferating 3T3-L1 cells were treated with PMA or serum, no increase of pro- α 2(I) mRNA was detected (Fig. 4a), whereas a typical increase was seen in the fully confluent quiescent 3T3-L1 cells (Fig. 4b). The level of pro- α 2(I) mRNA even slightly decreased in proliferating cells after fresh serum addition. Thus PMA induction of pro- α 2(I) mRNA expression is specific to the confluent quiescent stage of 3T3-L1 cells.

Collagen protein expression

In order to determine whether increased pro- α 2(I) mRNA expression is reflected at the protein level in the presence of

Fig. 6. Collagen components in media and the ECM of 3T3-L1 and VT-1 cells

Cells were treated with 0.1% DMSO (lane 1), PMA (100 ng/ml) (lane 2), actinomycin D (2.5 μ g/ml) (lane 3) and PMA and actinomycin D (lane 4) and labelled with [3 H]proline for 3 h as described in the Materials and methods section. Media from 3T3-L1 cells (a) and VT-1 (b) and ECM from 3T3-L1 (c) and VT-1 (d) were analysed by SDS/urea/10%-PAGE. Arrows indicate collagen bands.

PMA, the ECM was analysed for newly synthesized type I collagen in both 3T3-L1 and VT-1 cells. The ECM components were extracted from [35 S]methionine-labelled cells and were analysed by SDS/urea/PAGE (Fig. 5). Among several proteins detected in 3T3-L1 cells, elevated levels of proteins of molecular mass 155, 185, 190 and 205 kDa were found after PMA and serum treatment (Fig. 5a). Collagenase treatment resulted in the selective digestion of these proteins. Other proteins were not altered by collagenase treatment. These properties are characteristic for type I collagen [20]. Western analysis using rabbit anti-(mouse type I collagen) antibody also confirmed these bands as type I collagen (results not shown).

Synthesis of collagen was stimulated by addition of PMA or serum in 3T3-L1 cells, but only by serum in VT-1 cells (Fig. 5b). These results reflect the presence or absence of pro- α 2(I) mRNA shown in Fig. 2.

To confirm that the increase in the collagens is contingent upon mRNA synthesis, we monitored total collagen synthesis in the presence of actinomycin D. By using [3 H]proline the medium and the ECM from [3 H]proline-labelled cells was examined (Fig. 6). In 3T3-L1 cells, PMA induced collagen secretion into the medium and enhanced accumulation into the ECM (Figs. 6a and 6c). Two proteins, of molecular masses 140 and 150 kDa, were found in the media. The differences in molecular mass of collagen found in the media and in the ECM appear to represent processed and unprocessed collagens. The synthesis of these proteins was inhibited by actinomycin D, confirming the ex-

pression of pro- α 2(I) mRNA. In VT-1 cells, no increase in collagen synthesis was observed in the presence of PMA in either the medium or the ECM (Figs. 6b and 6d). The basal level of collagen in VT-1 cells was slightly lower than that in the parental 3T3-L1 cells. These results are consistent with the basal pro- α 2(I) mRNA levels shown in Fig. 1.

DISCUSSION

The present study demonstrated that the overall expression of type I procollagen differed between PMA-responsive and PMA-non-responsive cells. Furthermore, addition of PMA to growth-arrested 3T3-L1 cells caused a substantial increase in pro- α 2(I) mRNA expression (Fig. 1). Both of these results imply that type-I-procollagen expression is associated with the cell's mitogenic responsiveness to PMA.

When a full complement of growth factors are present (i.e. 10% FBS), both 3T3-L1 and VT-1 cells express pro- α 2(I) mRNA and collagen protein to equivalent levels (Figs. 2 and 5). This result suggests that, although growth-arrested VT-1 cells express very low levels of pro- α 2(I), the growth-factor-associated machinery necessary for pro- α 2(I) expression at the same level of parental 3T3-L1 cells is intact. This finding also suggests that an alternative signalling pathway may be used by PMA for inducing the expression of pro- α 2(I). This pathway may be related to the lesions in VT-1 cells.

Growth factors, particularly fibroblast growth factor (FGF), can be sequestered into the ECM [26–28]. Serum and polypeptide mitogens such as insulin, epidermal growth factor and FGF have been shown to act synergistically with PMA to stimulate DNA synthesis in quiescent 3T3 cells [29]. 3T3-L1 cells may harbour a growth-potentiating composite of the ECM and be PMA-responsive, owing to their ability to sequester appropriate amounts of growth factors to substantiate the mitogenic action of PMA. The consequence of a decreased production of collagen may be an incomplete or unbalanced matrix formation. Since the growth-arrested VT-1 cells have an overall decreased level of collagen production and exhibit a loss of the typical 2:1 ratio of pro- α 1(I) to pro- α 2(I), their matrix may be altered and thus not be fully capable of sequestering growth factors. Therefore, a sufficient amount of mitogen may not be present to co-operate with PMA to induce a complete mitogenic response.

Ignatz & Massague [20,30] proposed that the cellular action of TGF- β is mediated by changes in the ECM, as evidenced by increased fibronectin and collagen synthesis in a variety of fibroblastic and epithelial cell lines. The increased collagen synthesis was observed several hours after TGF- β addition, in contrast with the early induction of collagen synthesis that was observed after PMA addition in our study. The results of Blatti *et al.* [4] demonstrated a synergistic effect between growth factors on the expression of fibronectin and β -actin, and they proposed that coexpression of fibronectin and cytoskeletal actin may be a significant early event during the transition of cells from a resting to a proliferating state. Similarly, the early induction of pro- α 2(I) mRNA by PMA in growth-arrested fibroblast cells implicates collagen as a potential component involved in stimulating cell proliferation.

In fibroblasts and other anchorage-dependent cells, certain genes involved in cell proliferation are regulated by cell density. Expression of *c-myc* mRNA is low in growth-arrested 3T3 cells, but a sharp increase is seen after mitogenic stimulation [31]. The ability of PMA to induce collagen synthesis is also contingent upon the cellular growth state (Fig. 4). Previous studies report a PMA-mediated down-regulation of pro- α 2(I) mRNA and its synthesis [32,33]. In these other systems, cells are either actively proliferating [32] or exposed to PMA for extended periods of

time [33]. In proliferating or subconfluent 3T3-L1 cells, pro- $\alpha 2(I)$ mRNA remains at a basal level even after PMA treatment (Fig. 4). When 3T3-L1 cells are in a density-dependent growth arrest, PMA is able to induce pro- $\alpha 2(I)$ mRNA expression (Figs. 2a and 2b). This result implies that pro- $\alpha 2(I)$ mRNA expression may be associated with density-dependent PMA-induced initiation of cell division.

Various upstream regions, such as the serum-responsive element and the PMA-responsive element (TRE), and their respective binding proteins, such as the serum responsive factor and AP-1, have been shown to be involved in gene regulation when cells are in the presence of serum or PMA [34,35]. Since pro- $\alpha 2(I)$ -gene expression was PMA-sensitive in our system, a TRE element may be located in its 5' flanking region or downstream of the 5' start site. A sequence search using the GCG Sequence Analysis Package Ver. 6 [36] revealed several TRE-like sequences within the available sequence data for COL1A2 [37]. Two were localized in the first intron and enhancer. This finding was confirmed by Sherwood *et al.* [38]. Localization of TRE elements within regulatory domains of COL1A2 implies that PMA-induced collagen-gene transcription may be regulated by a combination of up- and down-stream PMA-responsive *cis*-acting elements.

Interestingly, when TGF- β stimulates collagen gene transcription, a *trans*-acting factor interacts with a segment of the promoter between -350 and -300 overlapping a nuclear factor 1-binding site [39]. More recently, de Crombrughe and his colleagues reported that mouse COL1A2-gene expression is regulated by a complex of two DNA-binding proteins, A (39 kDa) and B (41 kDa), which bind to a CCAAT motif at -80 of the promoter region [40]. As PKC was shown to accumulate in the nuclei of mouse fibroblasts after PMA stimulation [41], the rather intriguing possibility that either one of these protein factors may be regulated by phosphorylation via activated PKC is raised. Because of the reduced level of PKC activation by PMA, VT-1 cells may be unable to modulate the protein factors and fail to induce COL1A2 gene expression.

Thus low levels of pro- $\alpha 2(I)$ mRNA and procollagen protein, in conjunction with the inability of PMA to induce wild-type levels of expression of the COL1A2 gene, may be in part responsible for the PMA-non-responsiveness of VT-1 cells. The ability of PMA to induce a mitogenic response is associated with its ability to enhance collagen synthesis in 3T3-L1 cells. In summary, the PMA-mediated response in VT-1 and 3T3-L1 cells appears to be contingent upon a collagen-gene-activation pathway, the status of the ECM, the cell-growth state and the activation of PKC.

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