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Regulation of fibronectin gene expression by cyclic AMP and phorbol myristate acetate in HT-1080 human fibrosarcoma

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Abbreviations: FN, fibronectin; CRE, cAMP-responsive element; PKC, protein kinase C; MEM, minimal essential medium; BSA, bovine serum albumin; PMA, phorbol-12-myristate-13-acetate; Bt_2cAMP, dibutyryl cAMP

Abstract

We studied the regulation of fibronectin (FN) gene expression by cAMP and phorbol-12-myristate-13-acetate (PMA) in HT-1080 human fibrosarcoma cells. Dibutyryl cAMP increased FN synthesis and mRNA levels, while PMA inhibited the cAMP-induced FN synthesis. In transient transfection assays, cAMP increased FN promoter activity, while PMA paradoxically enhanced the cAMP-induced promoter activity. Stable transfection experiments, however, showed that neither cAMP or PMA alone nor together affected FN promoter activity. These results suggest that PMA antagonizes the cAMP-induced FN gene expression and that both the action of cAMP and the inhibition of its action by PMA may occur at the posttranscriptional level in HT-1080 cells.

Keywords: cAMP, fibronectin, HT-1080, phorbol myristate acetate, protein kinase C

Introduction

The expression of fibronectin (FN), an adhesive glycoprotein present in the extracellular matrix, is of great importance in development, wound healing, fibrogenesis, and neoplastic transformation (For reviews, see Hynes, 1990). A number of factors have been reported to regulate FN expression. These include transforming growth factor-β, glucocorticoids, viral proteins such as HTLV-I Tax and adenovirus E1A, and transcription factors such as E2F1 (Dean *et al.*, 1988; Nakajima *et al.*, 1992; Taylor *et al.*, 1992; Jordan-Sciutto *et al.*, 1997). The cAMP pathway has been shown to be an important signaling pathway involved in FN expression (Dean *et al.*, 1988, 1989). It has been reported to activate the transcription of FN gene through the cAMP-responsive element (CRE) in FN promoter (Dean *et al.*, 1989; Bowlus *et al.*, 1991). The protein kinase C (PKC) pathway, another important signaling pathway, has also been shown to regulate FN expression (Cagliero *et al.*, 1991; Studer *et al.*, 1993; Lee *et al.*, 1996).

Regulation of FN expression by cAMP and PKC pathways show different patterns depending on cell type. Cyclic AMP stimulates FN synthesis in fibrosarcoma cells but inhibits it in granulosa cells (Dean *et al.*, 1988; Dean *et al.*, 1989; Bernath *et al.*, 1990). Phorbol-12-myristate-13-acetate (PMA), a PKC activator, stimulates FN expression in lung fibroblasts and renal mesangial cells but inhibits it in endothelial cells (Cagliero *et al.*, 1991; Studer *et al.*, 1993; Lee *et al.*, 1996). These reports suggest cell-type specific regulation of FN expression. In addition, cAMP and PKC pathways show synergistic or antagonistic interactions in the regulation of the expression of cellular genes including the FN gene. Cyclic AMP and PMA interact synergistically to regulate the expression of the chorionic gonadotropin gene in choriocarcinoma cells (Anderson *et al.*, 1988). Cyclic AMP inhibits the PKC-induced stimulation of FN expression in human lung fibroblasts, while it alone has no effect (Lee *et al.*, 1997). In this regard, we studied the regulation of FN gene expression by cAMP and PMA and their interaction in HT-1080 human fibrosarcoma cells in which FN expression has been reported to be induced by cAMP (Dean *et al.*, 1988; Dean *et al.*, 1989).

Materials and Methods

Cell cultures

A human fibrosarcoma cell line HT-1080 (ATCC CCL 121) was obtained from the American Type Culture Collection. Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.1% nonessential amino acids. Cells were incubated at 37˚C in a 5% CO_2 air environment.

Enzyme-linked immunosorbent assay (ELISA) of FN

Confluent cells in 24-well culture plates were incubated with serum-free MEM containing 1% bovine serum albumin

(BSA) and the indicated reagents. The cell layer FN was extracted using a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% SDS, 1 mM EDTA and 1 mM PMSF. The total amount of FN in the medium and cell layer was determined by ELISA and then normalized by the cellular DNA content measured by fluorophotometric assay as described previously (Kim *et al.*, 1992). Microtiter plates were coated with 100 ng of purified human plasma FN. The first antibody was a 1:800 dilution of goat anti-human FN antibody (Sigma). The second antibody was a 1:3,000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma). The enzyme substrate was o-phenylenediamine, and the absorbance was read at 492 nm. The standard curve using purified human plasma FN was linear between 30 ng and 300 ng.

Radioactive labeling and immunoprecipitation of FN

Confluent cells in 35-mm culture dishes were incubated with serum-free MEM containing 1% BSA and reagents and then labeled for 2 h with methionine-free MEM containing 30 μCi/ml [^{35}S]methionine (Amersham) and the corresponding reagents. The medium was collected and the cell layer FN was extracted with a lysis buffer as described above. Immunoprecipitation of FN from the combined samples of medium and cell layer extracts containing equal amounts of radioactive total protein was performed as described previously (Lee *et al.*, 1996). An excess (3 μg) of goat anti-human FN antibody was added to the combined samples of medium and cell layer extracts containing the same amounts of radioactivity and then incubated at 4˚C for 1-2 h. The immune complexes were incubated with 30 μl of protein A-sepharose beads (Pharmacia) for 1 hour. Sepharose beads and adsorbed proteins were solubilized in 30 μl of an electrophoresis sample buffer by heating at 100˚C for 4 min. Samples containing the isolated FN were analyzed either by SDS-PAGE using a 6% gel and subsequent fluorography or by counting the radioactivity (cpm) of the solution with a β-counter.

Northern blot hybridizations

Confluent cells in 100-mm culture dishes were incubated with serum-free MEM containing 1% BSA and reagents. Total cellular RNA was isolated with acid guanidinium thiocyanate-phenol-chroloform, fractionated by electrophoresis on a 0.8% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and hybridized with a cDNA probe as described previously (Sambrook *et al.*, 1989). The cDNA probe was labeled with [α-^{32}P] dCTP (Amersham) using a megaprime DNA labeling system (Amersham). FH1 human FN cDNA (Kornblihtt *et al.*, 1983) and pHcGAP human glyceraldehyde-3-phosphate dehydrogenase cDNA (Tso *et al.*, 1985) were used as probes.

Plasmid constructions

To construct the plasmid pGL2F1900 containing DNA sequences from -1908 to +136 of the rat FN gene fused to the luciferase reporter gene, the plasmid pF1900CAT containing the same DNA sequences fused to the CAT gene (provided by Dr. Kinichiro Oda, Science University of Tokyo) was cleaved with *Pst*I. The 2.1 kb *Pst*I fragment of the FN gene was inserted into the *Pst*I site of pBluescript II KS(-) (Stratagene) to generate pBS-F1900. Finally, the insert was cleaved with *Bam*HI-*Hind*III from pBS-F1900 and inserted into the *Bgl*II-*Hind*III site of pGL2-Basic vector (Promega) to generate pGL2F1900.

Transient and stable transfection of DNA

DNA transfection was performed by using the $CaPO_4$ precipitation method modified by Chen and Okayama (Chen and Okayama, 1987). Cells (4×10^5 cells per dish) were plated the day before transfection onto 100-mm culture dishes and grown to an approximate 70% confluence. Cells were transfected with 4 μg of pGL2F 1900, 1 μg of pSVβ-gal as an internal control, and 3 μg of pGL2-Basic vector as a carrier. After incubation with the DNA precipitate for 5 h and subsequently with 15% glycerol for 2 min, cells were recovered overnight with a fresh medium containing 10% serum. Cells were then incubated with each reagent in a medium containing 0.5% serum for 24 h and harvested at 48 h from the start of transfection. For stable transfection, cells grown in 60-mm culture dishes were transfected with 7 μg of pGL2F1900, 0.7 μg of pSVβ-gal, and 0.7 μg of pSVNeo as a selection marker. Transfected clones were selected by subculture in a medium containing 400 μg/ml of geneticin (GIBCO/BRL). All selected clones were pooled and used for the following experiments.

Luciferase and β-galactosidase assays

Cells were lysed with 250 μl of a lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% (v/v) Triton X-100, and 1 mM dithiothreitol (DTT)). For luciferase assays, 100 μl of the cell lysates were mixed with 500 μl of a luciferase assay buffer (15 mM potassium phosphate, pH 7.8, 25 mM glyglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM DTT). The reaction mixture was incubated with 100 μl of a luciferase assay buffer containing 100 μM luciferin (Analytical luminescence laboratory) and luminescence was measured by using a luminometer (Berthold). For β-galactosidase assays, 10 μl of the cell lysates were used and activity was measured with a Galacto-Light™ chemiluminesent reporter assay system (Tropix) according to the manufacturer's directions.

Results

Treatment of HT-1080 cells for 24 h with dibutyryl cAMP (Bt_2cAMP), a cAMP analogue, increased FN synthesis in a dose-dependent manner with a maximum 3.5-fold increase, whereas treatment with PMA, a PKC activator, did not affect FN synthesis at any concentration tested (Figure 1A). To examine the time-course of FN synthesis, cells were treated for various periods of time with either 1 mM Bt_2cAMP or 100 ng/ml PMA. Bt_2cAMP increased FN synthesis time-dependently from 3 hours to 48 h, while PMA did not (Figure 1B). The effect of these reagents on FN synthesis does not seem to be a nonspecific effect on total protein synthesis, since neither Bt_2cAMP nor PMA affect total protein synthesis (data not shown).

To study the interaction between cAMP and PMA, cells were treated for 48 h with Bt_2cAMP and PMA in combination. PMA inhibited the Bt_2cAMP-induced stimulation of FN synthesis, but when used alone had no effect (Figure 2A). To test whether the regulation by cAMP and PMA occurs at the mRNA levels, the steady-state levels of FN mRNA were determined. In agreement with the results of FN synthesis, Bt_2cAMP increased FN mRNA levels, while PMA inhibited the Bt_2cAMP-induced increase of FN mRNA levels (Figure 2B).

To investigate the regulation of FN expression by cAMP and PMA and their interaction at the transcriptional level, the plasmid containing 1900 bp of FN promoter fused to the luciferase reporter gene was transiently transfected into HT-1080 cells. Bt_2cAMP increased the FN promoter activity by 4.5-fold, while

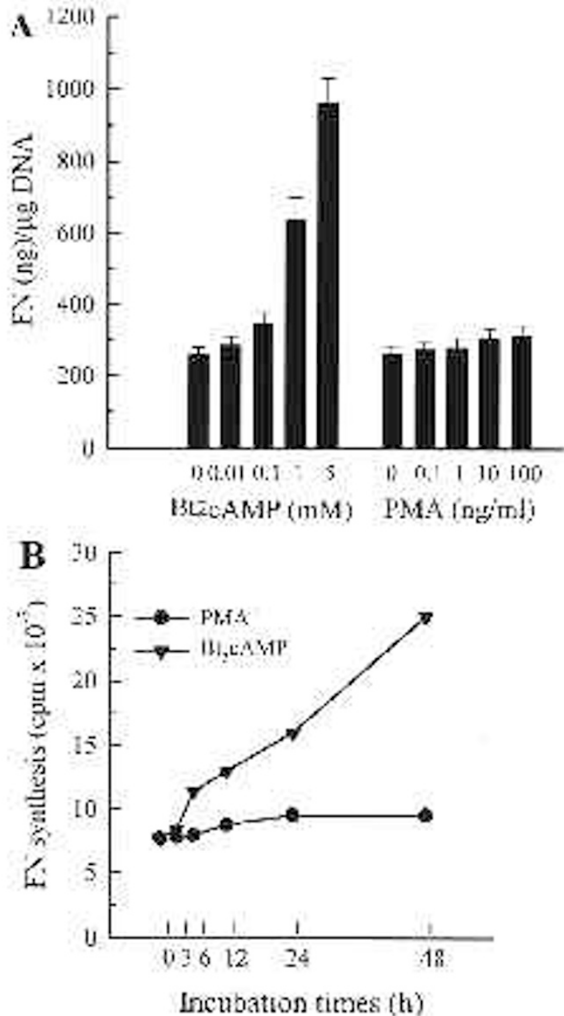

Figure 1. Dose- and time-response of FN synthesis by Bt_2cAMP and PMA in HT-1080 cells. (A) Confluent cells were incubated with various concentrations of either Bt_2cAMP or PMA for 24 h. FN synthesis was determined by measuring the total amount of FN in the medium and cell layer extracts by ELISA and then normalizing it with the cellular DNA content. Data are given as a mean ± S.D. of four different samples. (B) Confluent cells were incubated with either Bt_2cAMP (1 mM) or PMA (100 ng/ml) for the indicated periods of time and then labeled with 30 μCi/ml [^{35}S]methionine (1,000 Ci/mmol) for 2 h. FN was isolated by immunoprecipitation from the combined fractions of the medium and cell layer extracts containing equal amounts (5×10^6 cpm) of radioactive total protein. Samples containing the isolated FN were analyzed by counting the radioactivity (cpm) of the solution with a β-counter.

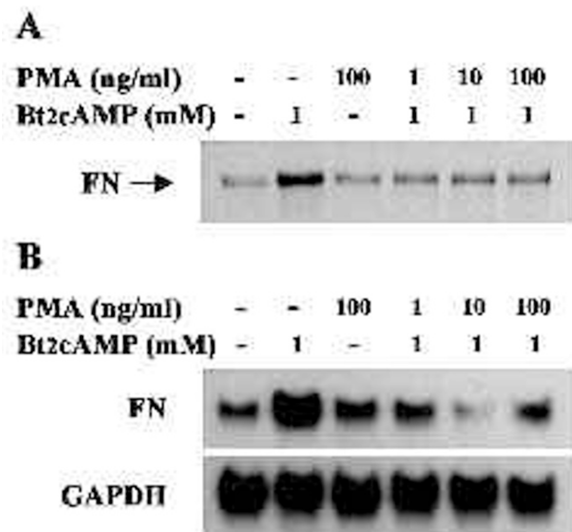

Figure 2. Inhibition of Bt_2cAMP -induced stimulation of FN synthesis and mRNA levels by PMA in HT-1080 cells. **(A)** Confluent cells were incubated with Bt_2cAMP or PMA for 48 h and then labeled with 30 μCi/ml [^{35}S]methionine (1,000 Ci/mmol) for 2 h. FN was isolated by immunoprecipitation from the combined fractions of the medium and cell layer extracts containing equal amounts (5×10^6 cpm) of radioactive total protein. The isolated FN was analyzed by SDS-PAGE and fluorography. The arrow indicates the location of FN (*Mr.* = 220-240 kDa). **(B)** Total RNA was isolated and 30 μg of each sample were subjected to electrophoresis and analyzed by Northern blot hybridization. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

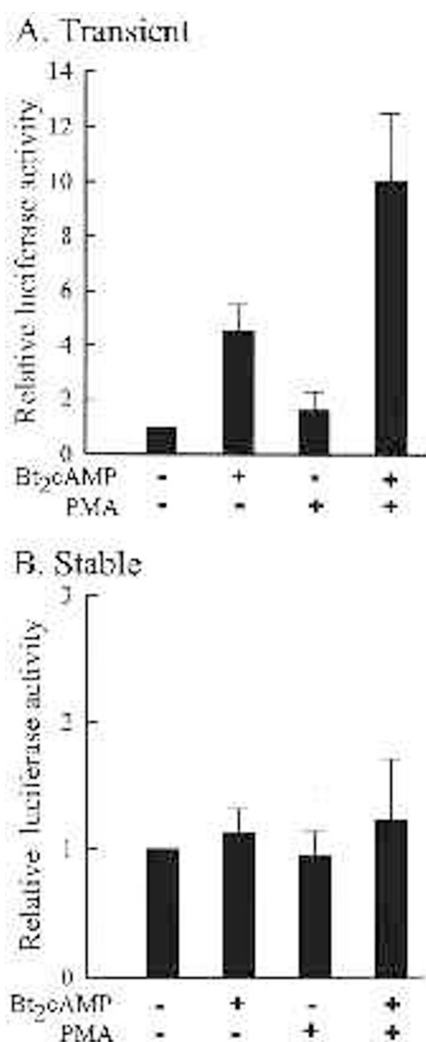

Figure 3. Effect of Bt_2cAMP and PMA on the activity of transiently and stably transfected FN promoter in HT-1080 cells. **(A)** Cells were transfected with 4 μg of pGL2F1900, 1 μg of pSVβ-gal, and 3 μg of pGL2-Basic vector as described in 'Methods and Materials'. After 48 h of treatment with Bt_2cAMP (1 mM) and PMA (100 ng/ml), cells were harvested and the luciferase activity was measured and normalized by β-galactosidase activity to correct transfection efficiency. Each bar represents promoter activity expressed relatively to the control value. Data are given as a mean ± S.D. of three different experiments. **(B)** Cells were transfected with 7 μg of pGL2F1900, 0.7 μg of pSVβ-gal, and 0.7 μg of pSVNeo and then stably transfected cells were selected by culturing in the presence of 400 μg/ml of geneticin. Stably transfected cells were treated with Bt_2cAMP (1 mM) and PMA (100 ng/ml) for 48 h and the activities of luciferase and β-galactosidase were determined. Each bar represents promoter activity expressed relatively to the control value. Data are given as a mean ± S.D. of three different experiments.

PMA alone did not show any significant effect (Figure 3A). PMA, on the contrary to its inhibitory effect on the cAMP-induced FN synthesis and mRNA levels, enhanced the cAMP-induced FN promoter activity up to 10-fold (Figure 3A).

Since the effect of PMA on the cAMP-induced transiently transfected promoter activity was different from that of PMA on the cAMP-induced FN synthesis and mRNA levels, stable transfection experiments were performed. However, neither Bt_2cAMP nor PMA affected stably transfected FN promoter activity (Figure 3B). The combined treatment of PMA and Bt_2cAMP did not affect stably transfected promoter activity, either (Figure 3B).

Discussion

This study shows that PMA antagonizes cAMP-induced FN gene expression and that both the induction of FN gene expression by cAMP and the inhibition of cAMP action by PMA may occur at the posttranscriptional level in HT-1080 human fibrosarcoma cells. Cyclic AMP stimulated FN synthesis and mRNA levels and the activity of a transiently transfected FN promoter, but had no effect on the activity of a stably transfected promoter. The discrepancy between the protein/mRNA levels and the promoter activity suggests that cAMP probably stimulates FN expression through posttranscriptional regulation in HT-1080 cells. PMA inhibited the cAMP-induced FN synthesis and mRNA levels but enhanced the cAMP-induced transiently transfected FN promoter, while having no effect on the stably transfected promoter. The differential effect of PMA on cAMP-induced FN protein/mRNA levels and promoter activity suggests that the PMA-induced signaling pathway antagonistically interacts with the cAMP pathway in regulating FN expression probably at the posttranscriptional level in HT-1080 cells.

Synergistic or antagonistic interactions between cAMP and PMA on the FN expression depending on cell-types have been reported (Anderson *et al.*, 1988; Lee *et al.*, 1997). Previously it has been shown that cAMP alone has no effect on FN expression but inhibits the PMA-induced stimulation of FN expression in lung fibroblasts (Lee *et al.*, 1997). The present study, on the contrary, shows that PMA alone has no effect on FN expression but inhibits the cAMP-induced stimulation of FN expression in HT-1080 fibrosarcoma cells. The mechanism of the cell-type specific regulation of FN expression is still unknown.

Cyclic AMP has been reported to stimulate FN expression by activating transcription possibly through the CRE site at -170 and two putative CRE-like sites at -260 and -415 in FN promoter, based on the results of transient transfection experiments in HT-1080 cells (Dean *et al.*, 1989; Bowlus *et al.*, 1991). In agreement with the previous reports (Dean *et al.*, 1989; Bowlus *et al.*, 1991), this study shows that cAMP stimulates the activity of transiently transfected FN promoter. This study, however, shows that cAMP has no effect on the activity of stably transfected FN promoter. Several possible explanations could be considered for the discrepancy in the promoter activity

in transient and stable transfections. One of them is that the transient transfection in which the target gene is in an episomal state and the stable transfection in which the target gene is integrated into cellular chromatin may have different general transcription factor requirements or rate-limiting steps for transcription (Natesan *et al.*, 1997). Whatever the mechanism, the promoter activity in stable transfection would reflect more physiological situation than that in transient transfection. In fact, the results of this study show that PMA enhances the cAMP-induced FN promoter activity in transient transfection assays that is quite opposite to its inhibitory effect on cAMP-induced FN protein synthesis and mRNA levels. In this regard, it is unlikely that the results of transient transfection reflect the endogenous FN expression, at least in HT-1080 cells.

It has been shown that the deletion of CRE at -170 does not eliminate the cAMP-responsiveness of FN promoter (Bowlus *et al.*, 1991). Furthermore, the FN CRE (-170)-binding factor that cooperates with the adjacent CCAAT box-binding protein to stimulate FN transcription in the liver has been shown to be an heterodimer of a 43-kDa protein and ATF-2 (Muro *et al.*, 1992; Srebrow *et al.*, 1993). ATF-2 itself lacks a constitutive activating domain but interacts with transcriptional activators like adenovirus E1A and HTLV-1 Tax, recruiting these factors to CRE-containing promoters (Liu and Green, 1990). ATF-2, however, has been shown to be a cAMP-independent transcription factor, unlike the cAMP-dependent trans-cription factor CREB (Flint and Jones, 1991). Taken together, contrary to previous reports, cAMP may stimulate FN expression, not by activating the transcription of the FN gene, but by acting through a posttranscriptional mechanism in HT-1080 cells.

Posttranscriptional regulation of the FN expression in HT-1080 cells has been observed in other conditions. The basal levels of FN synthesis and mRNA are lower in HT-1080 cells than in nontumorigenic revertants of the cells in which the mutated N-ras oncogene is underexpressed, whereas FN promoter is more active in HT-1080 cells than in revertant cells (Chandler and Bourgeois, 1991; Chandler *et al.*, 1994). Transforming growth factor-β and dexamethasone increase FN synthesis in HT-1080 cells partly through increasing the stability of FN mRNA (Dean *et al.*, 1988). Possible mechanism(s) of the posttranscrip-tional regulation of FN expression observed in this study may include an alteration in the cytoplasmic stability of FN mRNA and/or in the alternative splicing of FN mRNA by cAMP or PMA. Further studies including nuclear run-on assays would be required to obtain more direct evidence for the posttranscriptional regulation and understanding of its possible mechanisms.

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