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Transcriptional Regulation of Fibronectin Gene by Phorbol Myristate Acetate in Hepatoma Cells: A Negative Role for NF-κB

Byung-Heon Lee,[1] **Min-Seung Kim,**[1] **Jung-Hwa Rhew,**[2] **Rang-Woon Park,**[2] **Benoit de Crombrugghe,**[3] **and In-San Kim**[2]*

[1]Department of Biochemistry, School of Medicine, Dongguk University, Kyongju, Kyongbuk 780-714, Korea
[2]Department of Biochemistry, School of Medicine, Kyungpook National University, Taegu 700-422, Korea
[3]Department of Molecular Genetics, M.D. Anderson Cancer Center, University of Texas, Houston, Texas 77030

Abstract The transcriptional regulation of the fibronectin (FN) gene in hepatoma cells by phorbol myristate acetate (PMA) was investigated. PMA increased the synthesis and mRNA levels of FN and its promoter activity in Hep3B hepatoma cells. The PMA-induced activation of FN expression was blocked by a protein kinase C (PKC) inhibitor and did not require a new protein synthesis. Deletion analysis revealed that the sequence between positions −69 and +136 of the FN gene was responsible for the PMA induction. Two PMA-inducible nuclear protein complexes were found to bind to a putative NF-κB site at −41 and were identified as a p65/p50 heterodimer and a p50/50 homodimer of NF-κB family. Mutations in the −41 NF-κB site, however, did not block the PMA induction of the FN promoter but rather enhanced it. Overexpression of p65 increased the FN promoter activity. While overexpression of p50 alone did not affect the promoter activity, it decreased the p65-induced activation of the FN promoter. Mutations in the −41 NF-κB site attenuated the p50-mediated suppression of the p65 transactivation of the FN promoter. Deletion of the sequence between +1 and +136 decreased the basal and PMA-induced activities of the FN promoter. This study shows that PMA induces the transcription of the FN gene in hepatoma cells via the PKC pathway. The DNA sequence between +1 and +136 is responsible, at least in part, for the PMA-induced activation of the FN gene, while the −41 NF-κB binding site plays as a negative regulatory element for it. In addition, this study is the first to show a role for NF-κB p65 in the transcriptional activation of the FN gene. J. Cell. Biochem. 76:437–451, 2000.

Key words: fibronectin; hepatoma; NF-κB; phorbol myristate acetate; protein kinase C

Fibronectin (FN) is a structurally and functionally diverse glycoprotein and exists in a soluble protomeric form in blood plasma, named plasma FN and in an insoluble multimeric form in the extracellular matrix (ECM), named cellular FN. FN is a substratum for cell adhesion and migration playing crucial roles in various biological processes, such as wound healing, hemostasis, cell differentiation and development, oncogenic transformation, and metastasis [Hynes, 1990].

In normal liver, there are slight deposits of FN in portal tracts and space of Disse [Clement et al., 1986]. The first change in the ECM after chronic injury is the deposition of FN in the space of Disse [Martinez-Hernandez and Amenta, 1993]. At this stage, hepatocytes are the cells

Abbreviations used: CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; FN, fibronectin; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PBS, phosphate-buffered saline, PKC; protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonylfluoride; TBP, TATA-binding protein; TGF-β, transforming growth factor-β.

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*Correspondence to: In-San Kim, Department of Biochemistry, School of Medicine, Kyungpook National University, 101 Dongin-Dong, Jung-Gu, Taegu 700–422, Korea. E-mail: iskim@kyungpook.ac.kr

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responsible for the synthesis of FN. The FN deposition is followed by the deposition of collagen type I and other ECM components suggesting that FN may act as the organizer of the ECM in the fibrotic liver [Martinez-Hernandez and Amenta, 1993]. In addition, deposition of FN by endothelial cells from injured liver accelerates the conversion of normal hepatic stellate cells (also known as Ito cells) to myofibroblast-like cells that have greatly increased synthetic activity for a wide variety of matrix proteins [Jarnagin et al., 1994]. The accumulation of ECM in the space of Disse by hepatic stellate cells, combined with that by the injured hepatocytes, is accompanied by loss of fenestrae from sinusoidal endothelial cells and causes portal hypertension. It has been reported that hepatic stellate cells on an individual basis produce more matrix than hepatocytes and the activation of these cells is an invariant feature of chronic liver injury that ultimately lead to fibrosis [Benyon and Arthur, 1998]. In the liver, however, the hepatocyte cytoplasm volume surpasses hepatic stellate cell cytoplasm volume by several orders of magnitude. Even a small contribution from hepatocytes thus could represent the major contribution to the deposition of matrix [Martinez-Hernandez and Amenta, 1995].

Accumulation of FN has also found in fibrotic septa and capsules of tumor nodules in hepatocellular carcinoma as well as in fibrotic septa in liver cirrhosis [Oyama et al., 1989; Tavian et al., 1994; Koukoulis et al., 1995; Matsui et al., 1997]. While normal hepatocytes synthesize plasma FN but rarely synthesize cellular FN [Kornblihtt et al., 1983], hepatocytes near fibrotic septa in liver cirrhosis and most hepatoma cells in hepatocellular carcinoma produce cellular FN suggesting the participation of cellular FN in the hepatic fibrogenesis and in the malignant phenotypes of hepatoma [Tavian et al., 1994; Matsui et al., 1997]. Yet, little is known about the signaling pathways and regulatory mechanisms involved in the upregulation of FN expression in hepatocytes and hepatoma cells during these processes.

Expression of the FN gene is regulated by various factors including transforming growth factor-β (TGF-β), dexamethasone and viral proteins such as adenovirus E1A [Dean et al., 1988; Nakajima et al., 1992]. Intracellular signal pathways mediated by cAMP and protein kinase C (PKC) have been shown to play roles in the regulation of the FN gene expression [Dean et al., 1988, 1989; Studer et al., 1993; Rovin et al., 1995; Lee et al., 1996, 1997, 1998]. Regulatory elements and transcription factors responsible for the regulation of the FN gene expression by the cyclic AMP pathway and several factors have been described [Kornblihtt et al., 1996]. Cyclic AMP-responsive elements (CRE) at position −160 and −170 of the rat and human FN promoter, respectively, have been shown to mediate the basal and cAMP-induced expression of the FN gene [Bowlus et al., 1991; Miao et al., 1993]. Among many CRE-binding proteins described, ATF-2 binds to the CRE of the human FN gene and cooperates with NF-1 and CP1 that bind to an adjacent CCAAT element for the transcription of the FN gene in hepatocytes [Srebrow et al., 1993; Alonso et al., 1996]. A G10 stretch at −104 and two Sp1 binding sites at −104 and −50 of the rat FN gene have been shown to be involved in the adenovirus E1A-induced repression of the rat FN gene through the binding of G10BP, a negative regulator of Sp1 transcription factor [Nakajima et al., 1992; Suzuki et al., 1995]. In addition, an AP-1 site at −453 of the rat FN gene is recently shown to mediate the angiotensin II-induced transcriptional activation of the FN gene [Tamura et al., 1998]. It is still unknown, however, about the regulatory mechanisms responsible for the regulation of the FN gene expression by the PKC pathway. Here we studied the role of phorbol 12-myristate 13-acetate (PMA), a well-known PKC activator, in the expression of FN in hepatoma cells and regulatory elements and transcription factors responsible for the PMA action.

MATERIALS AND METHODS

Cell Cultures

Hep3B (ATCC HB 8064) and HepG2 (ATCC HB 8065) human hepatocellular carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), 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.

Radioactive Labeling and Immunoprecipitation of FN

Radioactive labeling of cells with [^{35}S]methionine, the extraction of cell layer FN, and immu-

noprecipitation of FN were performed as previously described [Lee et al., 1996, 1997]. Samples containing the immunoprecipitated FN were analyzed by SDS-PAGE using a 6% gel and subsequent fluorography.

Northern Blot Hybridizations

Isolation of total cellular RNA and subsequent hybridization with a radiolabeled cDNA probe were performed as described previously [Lee et al., 1997]. As cDNA probes, FH1 human FN cDNA [Kornblihtt et al., 1983] and 18S ribosomal RNA cDNA were used.

Oligonucleotides

Oligonucleotides were synthesized from commercial companies, Life Technologies and Bioneer (Taejeon, Korea). The sequences of oligonucleotides used in this study are listed in Table I.

Plasmid Construction

The plasmids pF1900CAT, pF414CAT, pF166CAT, and pF123CAT containing the 5′ flanking region of the rat FN gene sequence fused to the chloramphenicol acetyltransferase (CAT) reporter gene were provided by Dr. Kinichiro Oda (Science University of Tokyo, Tokyo, Japan) and previously described [Nakajima et al., 1992]. To construct the plasmid pGL2F1900 containing the rat FN gene fused to the luciferase reporter gene, pF1900CAT was cleaved with *Pst*I and the 2.1-kb *Pst*I fragment containing the rat FN gene sequence between positions −1908 and +136 was inserted into the *Pst*I site of pBluescript II KS (−) (Stratagene, San Diego, CA) to generate pBS-F1900. Then, pBS-F1900 was cleaved with *BamH*I and *Hind*III, and the 2.1-kb *BamH*I-*Hind*III fragment was inserted into the *Bgl*II-*Hind*III site of pGL2-Basic (Promega, Madison, WI) to generate pGL2F1900.

For construction of 5′ sequential deletion constructs, pF414CAT, pF166CAT, and pF123CAT were cleaved with *Bgl*II and *Hind*III. Each *Bgl*II-*Hind*III fragment containing the sequence from −414, −166, and −123 to +136 of the rat FN gene was inserted into the *Bgl*II and *Hind*III site of pGL2-Basic to generate pGL2F414, pGL2F166, and pGL2F123, respectively. For construction of pGL2F69, pGL2F1900 was cleaved with *BssH*II at the position −69 of the FN gene, filled-in with Klenow enzyme, and then cleaved with *Hind*III. The 217-bp *BssH*II-*Hind*III fragment containing the sequence from −69 to +136 of the FN gene was inserted into the *Sma*I-*Hind*III site of pGL2-Basic.

For construction of a site-directed mutant construct pGL2F1900m κB containing point mutations in the −41 NF-κB site (5′-GGGACTGTCC-3′ to 5′-G**ac**ACTGTCC-3′), an oligonucleotide FNm κB encompassing the sequence from −59 (*Bss*SI site) to +1 (*Blp*I site) of the rat FN gene containing those mutations was synthesized and dephosphorylated by an alkaline phosphatase. Another piece of insert was cleaved from pGL2F1900 with *Xho*I and *Bss*SI. A vector DNA was made by digestion of pGL2F1900 with *Xho*I and *Blp*I. The 1.8-kb *Xho*I-*Bss*SI fragment from pGL2F1900 and the 59-bp FNm-κB oligonucleotide with *Bss*SI and *Blp*I sites at each end were ligated into the *Xho*I and *Blp*I sites of the vector DNA. To construct pGL2F69m κB, pGL2F1900m κB was cleaved with *BssH*II, filled-in with Klenow enzyme, and then cleaved with *Hind*III. The *BssH*II-*Hind*III fragment was inserted into the *Sma*I-*Hind*III site of pGL2-Basic.

For construction of pGL2hF510 containing the human FN gene sequence from -510 to +69 fused to the luciferase reporter gene, pFN-CAT (−510) containing the human FN gene sequence fused to the CAT reporter gene (pro-

TABLE I. Oligonucleotides Used in This Study

Oligonucleotide	Sequence[a]
−69/−27	5′-GCGCAGGGCCTCGTGGGGGGGCGGGAAGGGACTGTCCCATAT-3′
−50/−27	5′-GGGCGGGAAGGGACTGTCCCATAT-3′
−50/−27mκB	5′-GCGGGAAG**ac**ACTGTCCCATAT-3′
Consensus NF-κB	5′-AGTTGAGGGGACTTTCCCAGGC-3′
Consensus Egr-2	5′-GGATCCAGCGGGGGCGAGCGGGGGCG A-3′
Consensus Ets-2	5′-TCGGGCTCGAGATAAACAGGAAGTGGTC-3′
Consensus Sp1	5′-ATTCGATCGGGGCGGGGCGAGC-3′
Oct-1	5′-GGCCTGGGTAATTTGCATTTCTAAAA-3′

[a]Underlining indicates target sites; lowercase boldface letters indicate introduced mutations.

vided by Dr. Douglas C. Dean, Washington University, St. Louis, MI) was cleaved with *Pvu*II at position −510 and *Pst*I at position +69. The 579-bp *Pvu*II-*Pst*I fragment was inserted into the *EcoR*V and *Pst*I site of pBluescript KS (−) to generate pBS-hF510. Then, pBS-hFN510 was cleaved with *Kpn*I and *BamH*I and the resulting fragment was inserted into the *Kpn*I and *Bgl*II site of pGL2-Basic to generate pGL2hF510.

CMV-p65 and CMV-p50 used as expression vectors for p65 and p50 subunit of NF-κB, respectively, were provided by Dr. Patrick A Baeuerle (Tularik Inc., South San Francisco, CA) and described previously [Schmitz and Baeuerle, 1991].

Transient Transfection of DNA

DNA transfection was performed by liposome method using a LipofectAmine™ reagent (Life Technologies). Cells (2×10^5 cells per dish) were plated the day before transfection onto 35-mm culture dishes and grown to an approximate 70% confluence. Cells were transfected with a total 1 μg of DNA containing 0.6 μg of FN-reporter construct, 0.1 μg of pSV-β-galactosidase (Promega) as an internal control, and 0.3 μg of pGL2-Basic as a carrier. For each transfection, 1 μg of DNA diluted into 100 μl with serum-free medium was mixed with 6 μl of lipofectAmine™ reagent diluted into 100 μl with serum-free medium, and incubated at room temperature for 30 min to allow DNA-liposome complexes to form. The complexes were diluted with 0.8 ml of serum-free medium and added onto the cells. Cells were incubated with the complexes for 6 h at 37°C in a CO_2 incubator. After incubation, the transfection mixture was removed and cells were incubated overnight with a fresh medium containing 10% serum for recovery. Then, cells were incubated with the indicated reagents in a medium containing 0.5% serum for 24 h and harvested at 48 h from the start of transfection.

Luciferase and β-Galactosidase Assays

Cells were washed twice with phosphate-buffered saline (PBS) and incubated with 250 μl of a lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% [v/v] Triton X-100, 1 mM dithiothreitol [DTT]) at room temperature for 10 min. The lysates were transferred to microcentrifuge tubes and cell debris was removed by centrifugation at 10,000*g* for 2 min. 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 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2 mM ATP, 1 mM DTT). The reaction mixture was incubated with 100 μl of the luciferase assay buffer containing 100 μM luciferin (Analytical Luminescence Laboratory, Bedford, MA) and then luminescence was measured in a luminometer (Berthold, Wildbad, Germany). β-Galactosidase assays were carried out using 10 μl of the cell lysates and a Galacto-Light™ chemiluminescent reporter assay system (Tropix, Ann Arbor, MI) according to the manufacturer's directions. The luciferase activity was normalized by the β-galactosidase activity for the correction of the transfection efficiency.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using a procedure adapted from a previously described method [Schreiber et al., 1989]. Cells were grown to a 70–80% confluence on 100-mm culture dishes and incubated with the indicated reagent. After incubation, cells were washed two times with ice-cold PBS, scraped in 1.5 ml of PBS/plate, transferred to a microcentrifuge tube, and pelleted by centrifugation at 10,000*g* for 15 sec at 4°C. Cell pellets were resuspended in 400 μl of cold Buffer A (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 0.5 mM phenylmethylsulfonylfluoride [PMSF], 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin)/plate and placed on ice for 15 min. The tubes were added with 25 μl of 10% Nonidet P-40, vortexed vigorously for 10 sec, centrifuged at 10,000*g* for 30 sec at 4°C, and the supernatant was removed. The nuclear pellet was resuspended in 25 μl of Buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin)/plate and incubated with shaking for 15 min at 4°C. Samples were centrifuged for 5 min as before and the supernatant (containing nuclear extract) was collected. The nuclear extract was frozen in small aliquots (5–10 μl) on dry-ice and stored at −70°C. Protein concentration was determined using a Bio-Rad™ protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Oligonucleotides were end-labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) by incubation with a Klenow enzyme at 37°C for 30 min. A labeled oligonucleotide probe was separated from unincorporated nucleotides using Sephadex G50 columns (Amersham Pharmacia Biotech). The nuclear extract (2–4 μg protein) was incubated with 5 fmol (~30,000 cpm) of a labeled probe for 20 min at room temperature in 15 μl of binding buffer containing 25 mM HEPES (pH 7.9), 10% glycerol, 0.05% Nonidet P-40, 1 mM EDTA, 0.5 M PMSF, 0.5 mM DTT, 2 μg poly(dI-dC), and 75 mM NaCl at a final concentration. Electrophoresis was carried out at 4°C for 2.5 h at 150 volts in 0.5 × TBE buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA) using a 4% nondenaturating polyacrylamide gel. Gels were dried and exposed to an X-ray film overnight at −70°C. For the competition assays, the nuclear extract was pre-incubated with an unlabeled oligonucleotide at room temperature for 15 min prior to the addition of a labeled probe. For the supershift assays, the nuclear extract was pre-incubated with 1 μl of antiserum directed against NF-κB family transcription factors (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C for 15 min prior to the addition of a labeled probe.

RESULTS

PMA Stimulates FN Gene Expression in Hepatoma Cells

We have previously showed that PMA stimulates FN synthesis in human lung fibroblasts through the activation of PKC pathway [Lee et al., 1996]. To investigate whether the expression of FN is regulated by PMA in hepatoma cells, we treated Hep3B human hepatocellular carcinoma cells with various concentrations of PMA for 24 h. PMA increased, maximally at 1 ng/ml, both the steady-state levels of FN mRNA and FN synthesis in Hep3B cells (Fig. 1A and B, respectively).

To investigate the involvement of transcriptional regulation in the PMA stimulation of FN expression, a FN promoter-luciferase reporter construct containing the rat FN gene sequence from −1908 to +136 (pGL2F1900) was generated and transfected into Hep3B cells. PMA dose-dependently increased the activity of pGL2F1900 maximally by 5.8-fold at a concentration of 10 ng/ml compared to the untreated control (Fig. 1C). PMA also increased the activity of the FN promoter by three-fold in HepG2 human hepatocellular carcinoma cells (Fig. 1D). In addition, when a FN promoter-luciferase construct containing the human FN gene sequence from −510 to +69 (pGL2hF510) was transfected into Hep3B cells, PMA increased the human FN promoter activity by six-fold in the cells (Fig. 1E). These results show that PMA stimulates FN expression by activating the transcription of the FN gene in hepatoma cells. The rat FN promoter was used for the following experiments, since it has a relatively longer sequence than the human FN promoter and its regulatory elements have been studied in detail.

Stimulation of FN Gene Expression by PMA Occurs via the PKC Pathway and Does Not Require a New Protein Synthesis

To investigate whether the effect of PMA on FN gene expression is mediated through the PKC pathway in Hep3B cells, a PKC inhibitor bisindolylmaleimide (Sigma Chemical Co., St. Louis, MO) was used to block the activation of the PKC. Bisindolylmaleimide at 1 μM efficiently blocked the PMA-induced increase of FN mRNA levels (Fig. 2A) and of the promoter activity of pGL2F1900 (Fig. 2B). In addition, 4α-phorbol 12,13 didecanoate (Sigma Chemical Co.), a phorbol ester that does not activate PKC, had no effect on the promoter activity of pGL2F1900 excluding the nonspecific effect of phorbol esters (data not shown). These results indicate that the stimulation of FN gene expression by PMA occurs via the activation of PKC pathway in hepatoma cells.

Cycloheximide, a protein synthesis inhibitor, did not affect the PMA-induced increase of FN mRNA levels (Fig. 2C). This result suggests that the stimulation of FN gene expression by PMA does not require a new protein synthesis and may be mediated by the activation of a pre-existing protein(s).

Identification of the PMA-Responsive Region in the FN Promoter

The nucleotide sequence containing the 5′-flanking sequence of the rat FN gene between −1908 and +136 has been reported [Nakajima et al., 1992]. It contains several cis-regulatory elements or binding sites for transcription factors such as putative AP-1 site at −453, AP-2

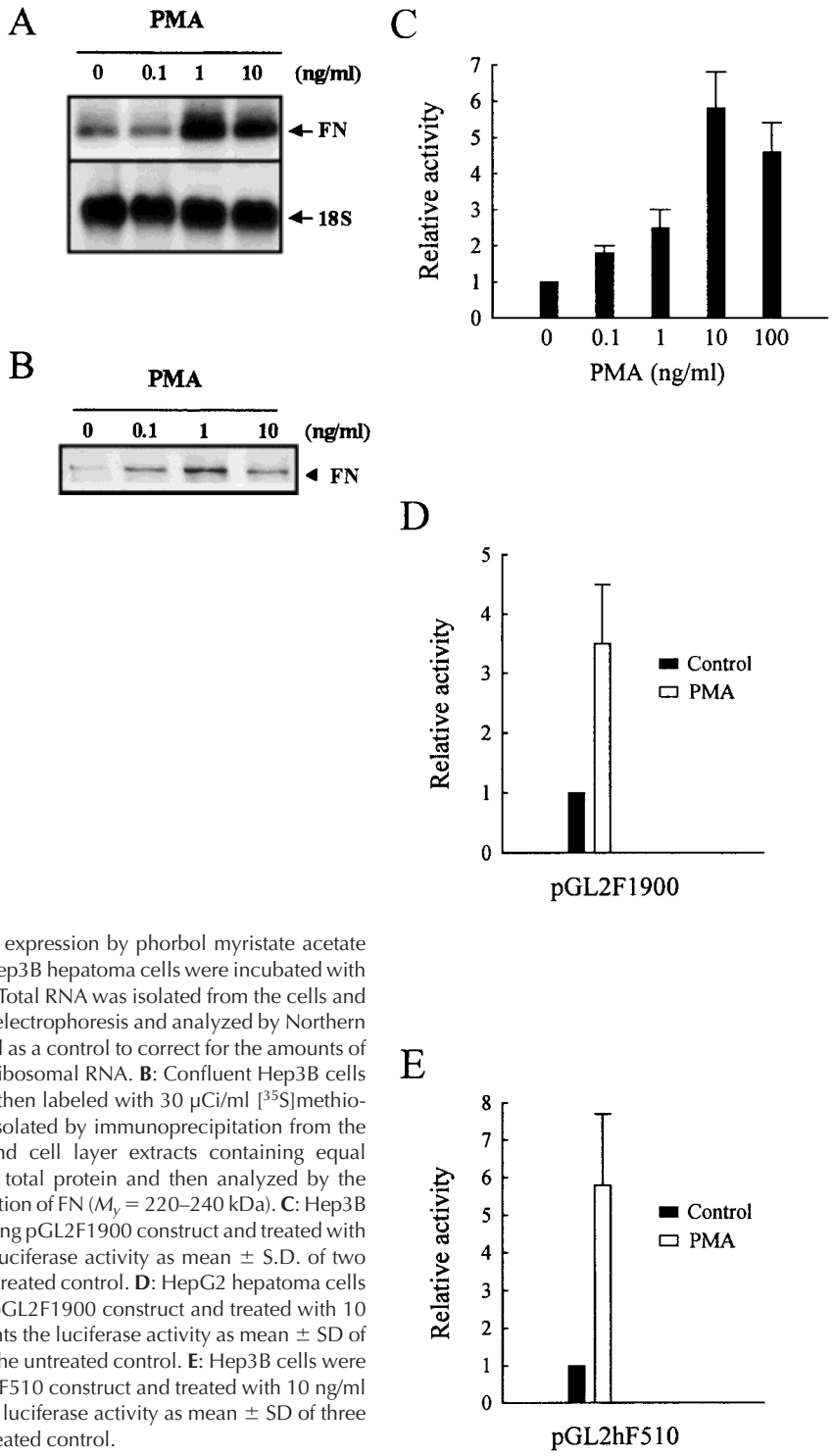

Fig. 1. Stimulation of fibronectin gene expression by phorbol myristate acetate (PMA) in hepatoma cells. **A**: Confluent Hep3B hepatoma cells were incubated with various concentrations of PMA for 24 h. Total RNA was isolated from the cells and 10 μg of each sample were subjected to electrophoresis and analyzed by Northern blot hybridization. A 18S probe was used as a control to correct for the amounts of RNA loaded. FN, fibronectin; 18S, 18S ribosomal RNA. **B**: Confluent Hep3B cells were incubated with PMA for 24 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 and then analyzed by the SDS-PAGE. Arrowhead indicates the location of FN (M_y = 220–240 kDa). **C**: Hep3B cells were transfected with DNA containing pGL2F1900 construct and treated with PMA for 24 h. Each bar represents the luciferase activity as mean ± S.D. of two different experiments relatively to the untreated control. **D**: HepG2 hepatoma cells were transfected with DNA containing pGL2F1900 construct and treated with 10 ng/ml of PMA for 24 h. Each bar represents the luciferase activity as mean ± SD of three different experiments relatively to the untreated control. **E**: Hep3B cells were transfected with DNA containing pGL2hF510 construct and treated with 10 ng/ml of PMA for 24 h. Each bar represents the luciferase activity as mean ± SD of three different experiments relative to the untreated control.

site at −364, CRE at −160, and two GC boxes or Sp1 sites at −104 and −50 [Nakajima et al., 1992]. To locate the cis-regulatory element(s) that confers PMA responsiveness, a series of 5′ deletion through the 5′-flanking region of the FN gene was generated (Fig. 3A). Deletion of the 5′ flanking region to −414 did not have a significant effect on the basal promoter activity. Deletion to -166 increased the basal activity by 74% relative to that of pGL2F1900. Deletion to −123 decreased the basal activity by 67% relative to that of pGL2F1900 (Fig. 3A), suggesting

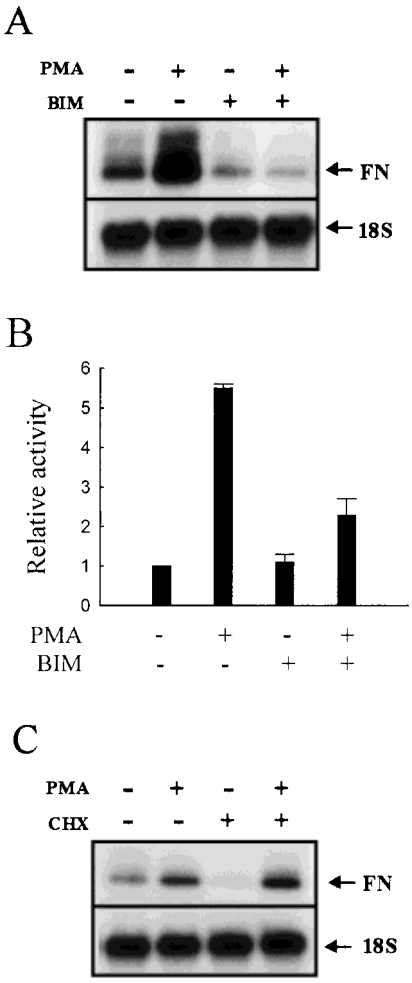

Fig. 2. Stimulation of FN gene expression by phorbol myristate acetate (PMA) occurs via the protein kinase C pathway and does not require a new protein synthesis. **A**: Hep3B cells were pre-incubated with 1 μM of bisindolylmaleimide for 2 h, followed by treatment with 10 ng/ml of PMA for 24 h. Northern blot hybridizations were performed as described in Figure 1. BIM, bisindolylmaleimide; FN, fibronectin; 18S, 18S ribosomal RNA. **B**: Cells were transfected with DNA containing pGL2F1900 construct and treated with the indicated reagents as described above. Each bar represents the luciferase activity as mean ± SD of two different experiments relatively to the untreated control. **C**: Cells were pre-incubated with 10 μg/ml of cycloheximide for 1 h, followed by treatment with 10 ng/ml of PMA for 24 h. Northern blot hybridizations were performed as described in Figure 1. CHX, cycloheximide.

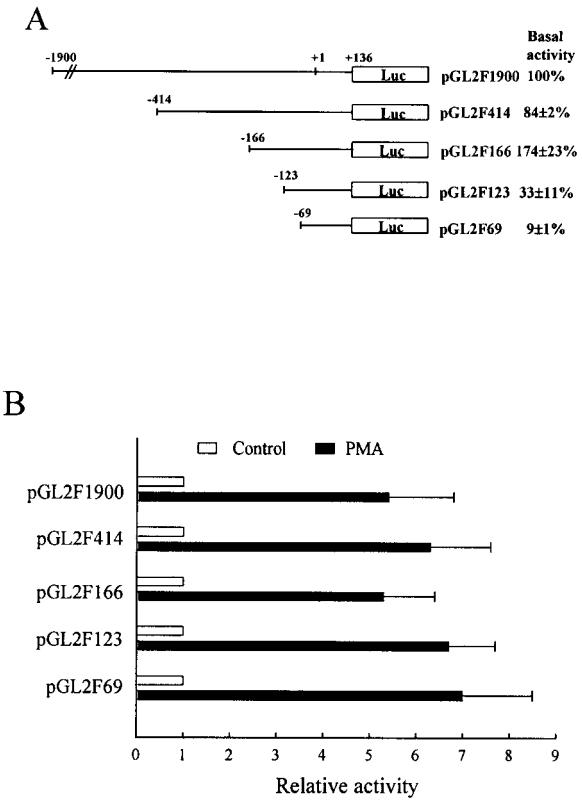

Fig. 3. 5′-sequential deletion analysis for the phorbol myristate acetate (PMA)-induced promoter activity. **A**: A diagram of 5′-sequential deletion constructs fused to the luciferase. The transcriptional start site is shown as +1. The basal activity of each construct is represented as percentage, with the activity of the longest construct listed as 100%. **B**: Hep3B cells were transfected with DNA containing each deletion construct and treated with 10 ng/ml of PMA for 24 h. Each bar represents the luciferase activity relative to the untreated control of each construct as mean ± SD of three different experiments.

the presence of an important regulatory element for the basal activity within the sequence from −166 to −123. In support of these results, a previous study reported that the sequence between −164 and −90 is important for the basal expression of the rat FN gene and this activity is mediated by CRE at −160 [Miao et al., 1993]. Further deletion to −69 decreased the basal promoter activity by 90% relative to that of pGL2F1900 (Fig. 3A), suggesting an additional regulatory element for the basal activity within the sequence from −123 to −69.

PMA increased the promoter activities of various 5′-deletion constructs as well as that of pGL2F1900 by five- to seven-fold compared to the untreated control of each construct (Fig. 3B). A construct with deletion to −69 (pGL2F69) still showed the PMA-induction of the promoter activity. These results suggest that the sequence downstream of the position −69 of the FN gene is an essential region required for the PMA induction of the FN promoter.

The NF-κB Site at −41 Is a Binding Site for the PMA-Induced Proteins

Sequence analysis of the PMA-responsive region between −69 and +136 revealed potential

binding sites for transcription factors such as Egr-2 (5′-GGGGGGGCGG-3′) between −54 and −45, Sp-1 (5′-GGGCGG-3′) between −50 and −45, Ets-2 (5′-GGGAAG-3′) between −46 and −41, and NF-κB (5′-GGGACTGTCC-3′) between −41 and −32 (Fig. 4A). To investigate whether PMA induces the binding of nuclear proteins to these sequence motifs, EMSA was performed. The sequences of synthetic oligonucleotides used for EMSA are shown in Table I. When a −69/−27 oligonucleotide containing the sequence between −69 and −27 of the FN gene was used as a probe, two DNA-protein complexes consisting of an upper major complex and a lower minor complex were induced by PMA (Fig. 4B, lanes 1 and 2). Binding of nuclear proteins to a Oct-1 probe used as an internal control was not different between PMA-treated and untreated cells showing equal amounts of nuclear proteins from each condition were used for EMSA (Fig. 4B, lanes 3 and 4).

To determine which of the sequence motifs functions as a binding site for the PMA-induced DNA-protein complexes, competition assays using consensus NF-κB, Egr-2, Ets-2, and Sp1 oligonucleotides as competitors were performed. The induced DNA-protein complexes were efficiently competed by the consensus NF-κB oligonucleotide but not by the consensus Egr-2, Ets-2, and Sp1 oligonucleotides even at a 100-fold molar excess concentration (Fig. 4C, lanes 3,4 vs. 5–10). These results suggest that the NF-κB binding site at -41 may be a binding site for the PMA-induced DNA-protein complexes. To study further, a −50/−27 oligonucleotide containing the sequence between −50 and −27 flanking the −41 NF-κB site was used as a probe. Binding of the PMA-induced DNA-protein complexes was competed by the −50/−27 itself and by the consensus NF-κB, but not by a −50/−27mκB oligonucleotide containing point mutations in the −41 NF-κB site (Fig. 4D, lanes 3–6 vs. 7,8). No binding complexes were observed when the −50/−27mκB was used as a probe (Fig. 4D, lanes 9,10). Similar pattern of DNA-protein complexes was observed when the consensus NF-κB oligonucleotide was used as a probe (Fig. 4E, lanes 1,2). The induced DNA-protein complexes were efficiently competed by the consensus NF-κB and less efficiently by the −50/−27, but not by the −50/−27mκB (Fig. 4E, lanes 3–6 vs. 7,8). These results show that the NF-κB site at −41 is a binding site for the PMA-induced DNA-protein complexes.

Identification of the PMA-Induced Proteins that Bind to the −41 NF-κB Site of the FN Promoter

To identify the nature of the PMA-induced DNA-protein complexes, supershift assays using antibodies against p65, p50, p52, c-Rel, and Rel-B subunits of NF-κB family were performed. An antibody against the amino terminal domain (corresponding to amino acids 3–19) of p65 supershifted the upper DNA-protein complex (Fig. 5, lane 3). An antibody against the amino terminal domain (corresponding to amino acids 4–22) of p50 supershifted both upper and lower DNA-protein complexes (Fig. 5, lane 5). While clearly there was a loss of the original DNA-protein complexes (Fig. 5, open arrowheads), the supershifted complexes appeared as diffuse bands rather than clear bands (Fig. 5, filled arrowheads). Antibodies against the carboxy terminal domain of p65 (corresponding to amino acids 531–550) and of p50 (corresponding to amino acids 338–357) as well as antibodies against other members of NF-κB family such as p52, c-Rel, and Rel-B had no effect (Fig. 5, lanes 4,6 and 7–9). In addition, an antibody against FN used as a nonspecific control had no effect (Fig. 5, lane 10). These results indicate that the upper DNA-protein complex is composed of both p65 and p50 subunits, most likely a p65/p50 heterodimer, and that the lower complex is composed of only p50 subunit, most likely a p50/p50 homodimer.

Effect of PMA on the Activities of Wild-Type and Mutant FN Promoter

To examine the functional importance of the −41 NF-κB site for the PMA response, mutant FN promoter constructs containing point mutations in the −41 NF-κB site were generated (Fig. 6A). Unexpectedly, mutations in the −41 NF-κB site of pGL2F1900 did not affect either the basal or PMA-induced promoter activities compared to the wild-type promoter (Fig. 6B). Furthermore, mutations in the −41 NF-κB site of pGL2F123 and pGL2F69 enhanced the PMA-induced activities compared to each Wild-type promoter (Fig. 6B). These results led us to hypothesize that the −41 NF-κB site may be a negative regulatory element rather than a positive regulatory element for the transcriptional regulation of the FN gene in response to PMA

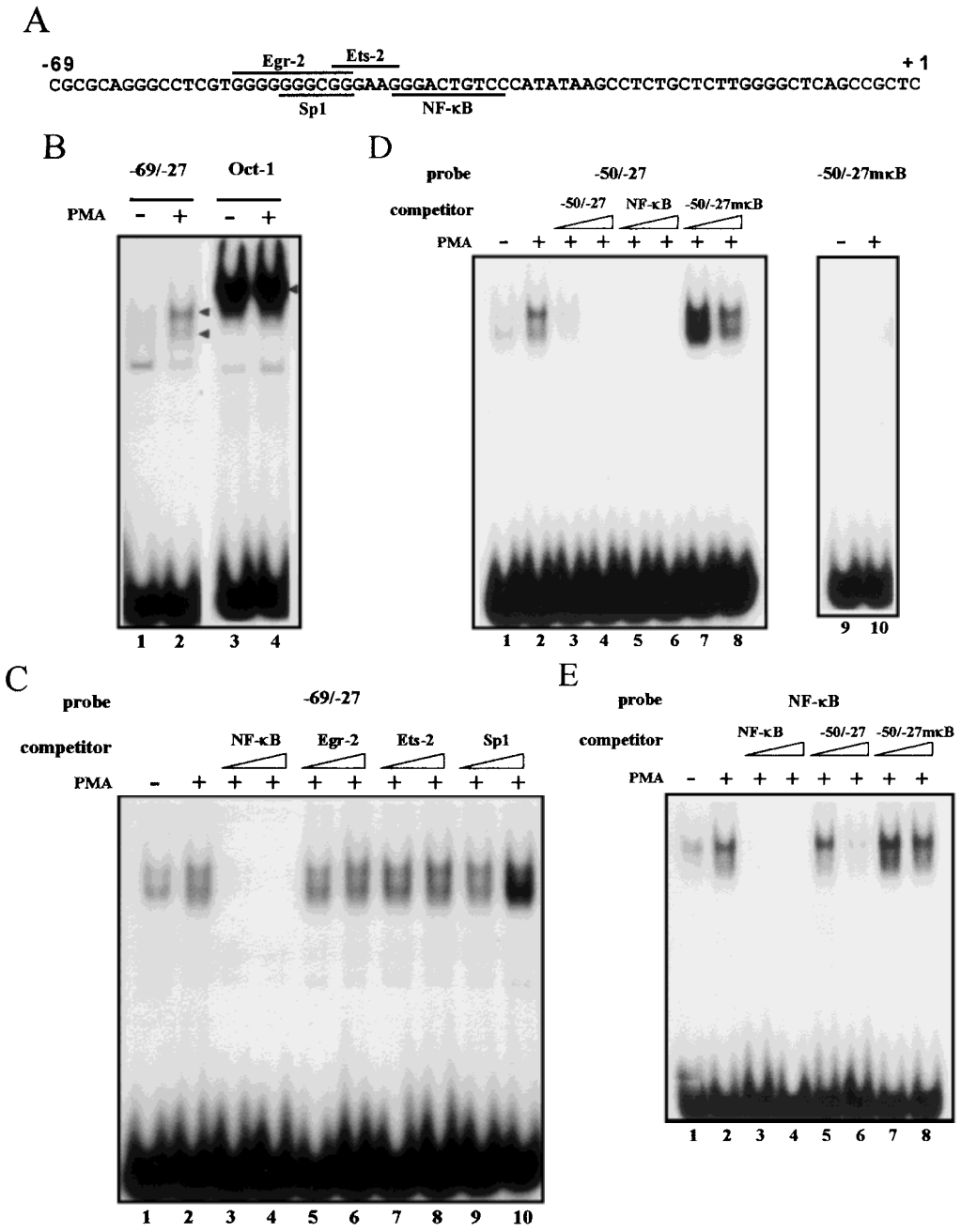

Fig. 4. An NF-κB site at −41 is a binding site for the phorbol myristate acetate (PMA)-induced proteins. **A**: Nucleotide sequences of the fibronectin gene from −69 to +1. Potential binding sites for Egr-2, Ets-2, Sp1, and NF-κB are underlined. **B**: Hep3B cells were incubated with 10 ng/ml of PMA for 24 h and then nuclear extracts were prepared for the electromobility shift assay. A labeled −69/−27 oligonucleotide was incubated with the nuclear extracts containing the same amounts of proteins. The Oct-1 oligonucleotide was used to correct for the amounts of proteins loaded. The positions of specific DNA-protein complexes are indicated with arrow heads. **C**: The binding of nuclear proteins to the labeled −60/−27 probe was competed with unlabeled NF-κB, Egr-2, Ets-2, and Sp1 consensus oligonucleotides given in 50-fold and 100-fold molar excess. **D**: In **lanes 1–8**, the binding of nuclear proteins to a labeled −50/−27 probe was competed with the indicated unlabeled oligonucleotides given in 50-fold and 100-fold molar excess. In **lanes 9,10,** a labeled −50/−27mκB oligonucleotide was incubated with the nuclear extracts. **E**: The binding of nuclear proteins to a labeled NF-κB consensus oligonucleotide was competed with the indicated unlabeled oligonucleotides given in 50-fold and 100-fold molar excess.

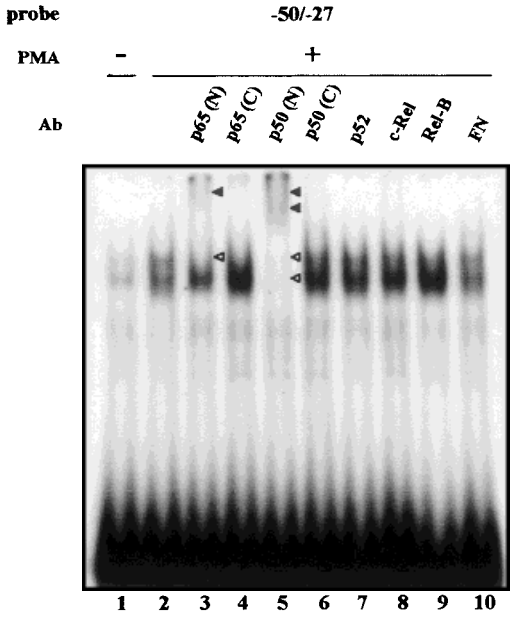

Fig. 5. Identification of the phorbol myristate acetate (PMA)-induced proteins that bind to the −41 NF-κB site. Nuclear extracts were prepared from Hep3B cells incubated with 10 ng/ml of PMA for 24 h. The nuclear extracts were pre-incubated with the indicated antibodies against NF-κB family before the incubation with a labeled −50/−27 oligonucleotide. An anti-human fibronectin antibody was used as a nonspecific negative control. The original and shifted positions of DNA-protein complexes are indicated with open and filled arrowheads, respectively. N, amino terminal; C, carboxy terminal.

in hepatoma cells. This hypothesis was supported by the following experiments.

To examine whether p65 and p50 subunits of NF-κB play roles for the transcriptional regulation of FN gene in hepatoma cells, p65 and p50 expression vectors were co-transfected with FN promoter constructs. Overexpression of p65 increased the activity of pGL2F1900 by 36-fold (Fig. 6C). While overexpression of p50 alone did not affect the promoter activity, a combined expression of p50 and p65 suppressed the p65-induced promoter activity by more than 80% (Fig. 6C). Mutations in the −41 NF-κB site, however, attenuated the p50-mediated suppression of the p65-transactivation of the FN promoter (Fig. 6C). These results provide a further support for the function of the −41 NF-κB site as a negative regulatory element for the transcription of FN gene by PMA in hepatoma cells. In addition, mutations in the −41 NF-κB site did not affect the p65-induced promoter activity (Fig. 6C), suggesting the presence of a potential p65-responsive element(s) at the position upstream of the −41 NF-κB site.

Involvement of the Sequence Between +1 and +136 in the PMA-Induced FN Promoter Activity

To study the presence of potential positive regulatory elements within the sequence downstream to the transcriptional initiation site (+1) of the FN gene, 3′-deletion constructs, pGL2F1900Δ+1/+136 and pGL2F69Δ+1/+136, which lack the sequence between positions +1 and +136 were generated (Fig. 7A). Deletion of the sequence between +1 and +136 decreased the PMA-induced activity of pGL2F1900 by 52% and nearly abolished that of pGL2F69 compared to the wild-type promoter of each construct (Fig. 7B). Deletion of the sequence also decreased the basal promoter activity of pGL2F1900 and pGL2F69 by 50% and 80%, respectively (Fig. 7B). These results suggest that the sequence between +1 and +136 plays a role, at least in part, in the basal and PMA-induced expression of the FN gene.

DISCUSSION

This study shows that the activation of PKC signaling pathway by PMA induces the transcription of the FN gene in hepatoma cells and that the DNA sequence between +1 and +136 is responsible, at least in part, for the PMA-induced activation of the FN gene, while the −41 NF-κB binding site plays as a negative regulatory element for the PMA response. In addition, this study shows that NF-κB p65 plays a role in the transcriptional activation of the FN gene in hepatoma cells.

PKC phosphorylates and activates various protein substrates and also regulates the expression of many target genes [Nishizuka, 1986]. Several isozymes of PKC are constitutively expressed in Hep3B hepatoma cells and by PMA treatment PKC-α is translocated from cytosol to membrane or nuclear fraction, while PKC-δ and ϵ are translocated from membrane to nuclear fraction [Hsu et al., 1998]. In many cases, the regulation of gene expression by PKC is mediated through the AP-1 element recognized and activated by the AP-1 (Jun/Fos) transcription factor [Angel et al., 1987]. The DNA sequence of the 5′-upstream region of the rat FN gene has been described previously [Nakajima et al., 1992]. There is an AP-1 element at −453 suggesting its possible role in the expression of the FN gene by PKC. In addition, as described previously [Lee et al., 1996], the FN gene has other possible regulatory elements

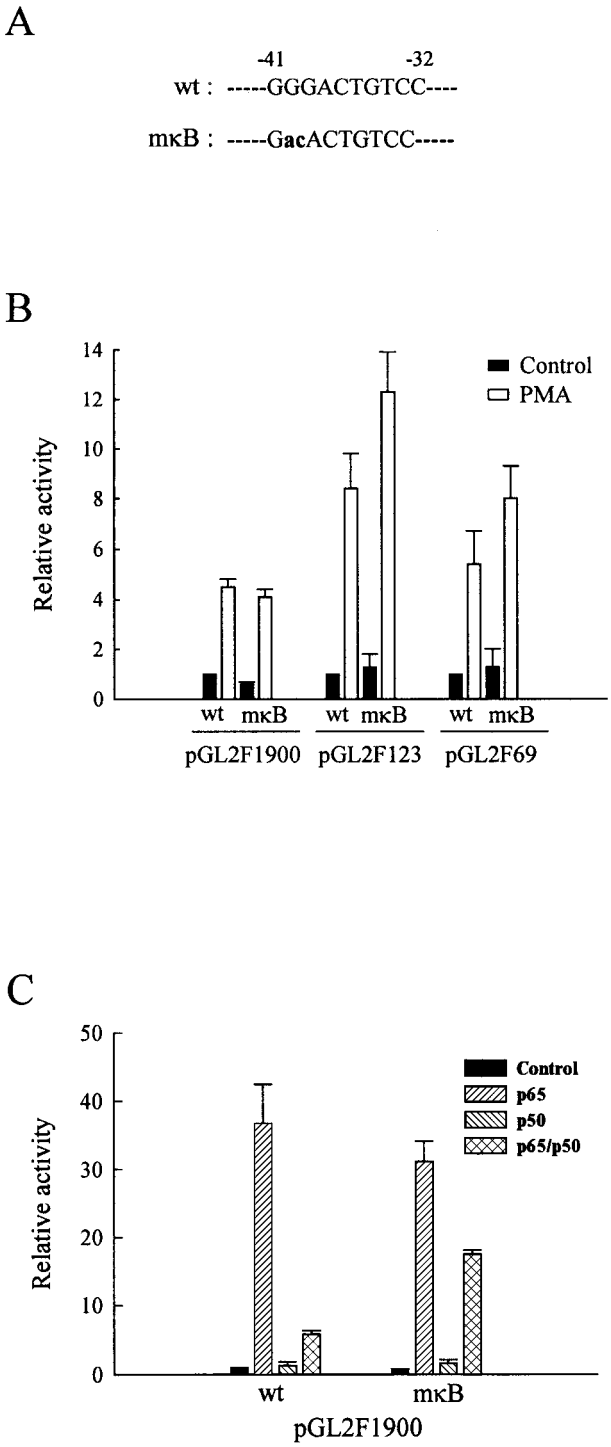

Fig. 6. Effect of mutations in the −41 NF-κB site on the phorbol myristate acetate (PMA)-induced (B) or p65 and/or p50-induced (C) fibronectin promoter activity. **A**: Diagram of point mutations at the −41 NF-κB site. Lowercase boldface letters indicate the introduced mutations. wt, wild-type; mκB, mutant NF-κB. **B**: Hep3B cells were transfected with DNA containing either wild-type or mutant construct and treated with 10 ng/ml of PMA for 24 h. Each bar represents the luciferase activity relative to the untreated control of the wild-type promoter of each construct as mean ± SD of three different experiments. **C**: Cells were transfected with DNA containing the indicated FN reporter construct and 0.1 μg of p65 and/or 0.1 μg of p50 expression construct. Each bar represents the luciferase activity relative to the untreated control of the wild-type construct as mean ± SD of three different experiments.

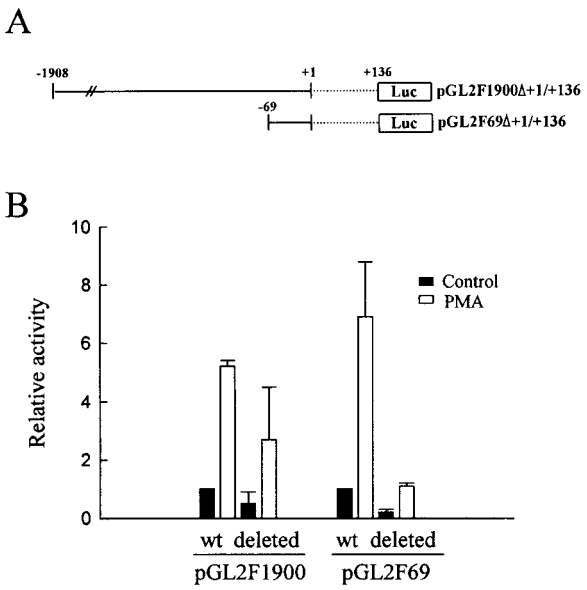

Fig. 7. Effect of a deletion of the sequence from +1 to +136 on the phorbol myristate acetate (PMA)-induced fibronectin promoter activity. **A**: Diagram of fibronectin-luciferase reporter constructs containing deletions of the sequence from +1 to +136. A dotted line and triangle symbol indicate a deletion of the sequence. **B**: Hep3B cells were transfected with DNA containing either wild-type or deletion construct and treated with 10 ng/ml of PMA for 24 h. Each bar represents the luciferase activity relative to the untreated control of the wild-type construct of each construct as mean ± SD of three different experiments.

such as CRE at −160 and AP-2 at −364 that may be involved in the PKC-induced FN expression. Our results, however, showed that deletion of the FN gene sequence including the AP-1 and CRE elements did not affect the PMA-induced promoter activity, while deletion of the CRE significantly decreased the basal promoter activity excluding the involvement of these elements in the PMA-induced activity. Next, analysis of DNA-binding proteins and mutation studies showed that PMA induced binding of protein complexes to the NF-κB site at −41 and this element unexpectedly acted as a functionally negative regulator for the PMA response (described below). Further deletion analysis revealed that the sequence between +1 and +136 was responsible for the activation of the FN gene by PMA. It remains, however, to identify a specific positive regulatory element(s) and a transacting factor(s) for the PMA response within this region (+1/+136) of the FN gene.

Since deletion of the sequence between +1 and +136 abolished approximately half of the PMA-induced activity in the native promoter pGL2F1900, it is suggested that there may be other unidentified elements or mechanism for the PMA response. One of the possible mechanisms is that PMA may activate general transcription machinery leading to the increased transcription of the FN gene. It has been shown that a component of general transcription machinery, possibly a TFIID-associated protein, is induced in T cells by PMA and responsible for the induction of HIV-1 transcription by PMA [Sakaguchi et al., 1991]. In addition, PMA has been shown to induce the phosphorylation of the TATA-binding protein (TBP) via activation of the mitogen-activated protein kinase pathway and stimulate specific interactions of TBP with other proteins in U937 leukemic cells [Biggs et al., 1998]. It remains to be studied whether PMA actually induces a component of general transcription machinery such as TBP for the regulation of the FN gene in hepatoma cells. Taken these considerations and the action of PKC on a number of target proteins together, the transcriptional regulation of the FN gene by PKC seems to be mediated by a complex mechanism involving multiple cis-regulatory elements and transcription factors.

NF-κB transcription factors have been shown to activate the expression of many genes in response to various stimuli including cytokines such as tumor necrosis factor-α, PKC activators such as PMA, and viruses [Baeuerle and Baichwal, 1997; Barnes and Karin, 1997]. The consensus element for NF-κB binding sites is 5′-GGGRNNYYCC-3′ and the target genes for NF-κB are mainly involved in immune and inflammatory responses including genes for inflammatory cytokines such as interleukin-2 and interleukin-8 and for inflammatory adhesion molecules such as E-selectin [Baeuerle and Baichwal, 1997; Barnes and Karin, 1997]. In the regulation of the target genes, a p65/p50 heterodimer has been shown as a classical transactivator of NF-κB family and within this complex the function of p50 is to improve the DNA binding of p65, which is otherwise a weak binder, and consequently help p65 act as a more effective transactivator within p65/p50 [Grimm and Baeuerle, 1993; Schmitz and Baeuerle, 1991]. Unlike most other known cases of the NF-κB-mediated regulation of the target genes, the −41 NF-κB binding site (5′-GGGACTGTCC-3′) of the FN gene acts as a negative regulatory element for the PMA response and this activity seems to be mediated by p50 subunit of NF-κB

family. The results from our study demonstrated that mutations in the −41 FN NF-κB site did not eliminate the PMA-induced activation of the FN promoter but rather enhanced it, whereas these mutations abolished the binding of the PMA-induced DNA-protein complexes composed of a p65/p50 heterodimer and a p50/p50 homodimer. In addition, co-expression of p50 and p65 suppressed the p65 alone-induced transactivation of the FN promoter and this p50-mediated suppression was largely blocked by the mutations in the -41 NF-κB site. Furthermore, the −41 NF-κB site had a preferential affinity for the p50 subunit in DNA-protein binding assays (data not shown). In the regulation of the FN gene, therefore, p50 does not appear to function as a helper subunit but may actively inhibit the transactivation potential of p65 and that of PMA by preferentially binding to the −41 NF-κB site as a p50/p50 homodimer or a p65/p50 heterodimer. The function of NF-κB site as a negative regulatory element and the negative actions for p50 against p65 action have been reported in the regulation of other genes. A NF-κB site of the rat androgen receptor gene functions as a negative regulator mediated by a p50 homodimer in liver cells [Supakar et al., 1995]. Overexpression of p50 blocks the p65 alone-mediated transactivation of the vascular cell adhesion molecule-1 gene in endothelial cells [Ahmad et al., 1995] and that of interleukin-8 gene in T lymphocytes [Kunsch and Rosen, 1993]. In macrophages, p50 inhibits the lipopolysaccharide-induced transcription of tumor necrosis factor-α gene by binding to NF-κB sites of the gene as p50 homodimers [Baer et al., 1998]. It appears to be, therefore, that the transcriptional regulation of a gene mediated by specific subsets of NF-κB family proteins differs depending on the specific gene and NF-κB binding motifs present.

NF-κB p65-mediated activation of the FN gene in hepatoma cells is a novel finding from this study providing a new target gene for NF-κB transcription factors. Until now, NF-κB has been shown to be involved in the transcriptional regulation of inflammatory adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1 in endothelial cells [Schindler et al., 1994; Ahmad et al., 1995; Aoudjit et al., 1997]. Since our results showed that mutations in the −41 FN NF-κB site did not affect the p65-induced activation of FN transcription, it could be suggested that a cis-responsive element(s) for the p65 transactivation is located at a position upstream of the −41 FN NF-κB site. Indeed, sequence analysis of the upstream region showed several potential NF-κB sites in the rat FN promoter. The p65-mediated transactivation may be mediated by either p65/p65 homodimers or complexes of p65 with an endogenous NF-κB family. It may also occur through complexing of p65 with other transcription factors expressed in hepatoma cells distinct from NF-κB family. A study to identify the p65-responsive element in the rat FN promoter and the nature of proteins binding to the site is undergoing in our laboratory. Taken together, it is conceivable that NF-κB transcription factors play as a dual regulator, positive and negative regulator, acting on the potential p65-responsive element and the −41 NF-κB site of the FN gene, respectively.

Accumulation of FN in the fibrotic septa and capsules of tumor nodules has been a characteristic finding of hepatoma [Oyama et al., 1989; Tavian et al., 1994; Koukoulis et al., 1995; Matsui et al., 1997]. In this study, PKC and NF-κB p65 were shown to upregulate the expression of the FN gene in hepatoma cells suggesting their roles as intracellular signal molecules in the deposition of FN in hepatoma. NF-κB p65 also activates the expression of the FN gene in normal hepatocytes (data not shown). Recently, NF-κB has been shown to be activated by TGF-β signaling [Li et al., 1998; Sakurai et al., 1998, 1999]. TGF-β has been well known to stimulate the synthesis of matrix including FN and implicated as a major fibrogenic factor responsible for the deposition of matrix in liver fibrosis [Benyon and Arthur, 1998]. The down-stream signaling molecules for the TGF-β action are still unclear. These findings, combined with our finding of the induction of FN expression by NF-κB, suggest that the TGF-β-induced FN expression may be mediated by NF-κB leading to the deposition of FN in the liver. Furthermore, the regulation of FN expression by NF-κB may be involved in liver regeneration. It has been reported that NF-κB is important for the liver regeneration. A mice lacking the p65 component of NF-κB dies of massive degeneration of the liver by apoptosis [Beg et al., 1995]. NF-κB is rapidly activated in liver regeneration after partial hepatectomy [Fausto et al., 1995]. FNs including the EIIIA$^+$ FN isoform have been shown to be increased in regenerating

liver [Caputi et al., 1995], suggesting a role for FN in cell proliferation. Indeed, FN matrix has been shown to play roles in the cell survival and cell cycle progression [Globus et al., 1998; Sechler and Schwarzbauer, 1998; Manabe et al., 1999]. Taken together, the results of this study suggest that the regulation of FN expression by NF-κB and PKC may play roles in the various physiological and pathological processes in the liver.

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