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SIGNAL TRANSDUCTION

Differential effect on U937 cell differentiation by targeting transcriptional factors implicated in tissue- or stage-specific induced integrin expression

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The inhibition of transcription factor functions was used to define their role in phorbol ester-induced cellular differentiation of a monocytic cell line, U937. We demonstrate a differential effect on cell adhesion and differentiation: antisense or competitive binding with double-stranded oligonucleotides antagonized the functions of AP-1, NF-κB, and PU.1 transcriptional factors. In the presence of phorbol 12-myristate 13-acetate (PMA), U937 cells attached to the plastic surface and cells were characterized by marked expression of β2-integrin molecules on the cell surface. We show that the in vivo differentiation of U937 cells appears to occur normally in the absence of AP-1 activity. In contrast, the addition to the cell culture of phosphorothioate oligonucleotides that contained the NF-κB or PU.1 binding sites significantly inhibited U937 differentiation. The absence of NF-κB led to pleiotropic effects with a clear reduction in the expression of integrin and other lineage-specific myeloid antigens on the cell surface. In contrast, the absence of PU.1 had a more restricted effect on integrin expresion on the cell surface, probably as a result of blockage of CD18 gene expression. **Published by Elsevier Science Inc.**

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Introduction

U937, a histiocytic lymphoma cell line with monoblastic characteristics, can be induced to differentiate towards morphologically mature macrophage-like cells after treatment with phorbol esters [1–3]. Phorbol esters such as phorbol-12-myristate 13-acetate (PMA) have pleiotropic effects on cells [4]. Phorbol exerts its biologic effects by altering gene expression through the activation of PKC [5,6] and modulating the activity of transcriptional factors that bind cis elements, such as NF-κB [7–9], AP-1 [10,11], AP-2 [12,13], AP-3 [14], and Ets [15]. In this process, the expression of adhesion receptors changes notably: members of the β2-integrin family increase during differentiation in U937 and HL-60 cells [2,6]. The CD11/CD18 (β2-integrin) family consists of three heterodimeric cell surface receptors (CD11a/CD18, CD11b/CD18, and CD11c/CD18) that show restricted expression on leukocytes [16]. Members of this family are activated by PMA and a variety of other physiologic inflammatory stimuli, and play a critical role in leukocyte-endothelium interactions during immune and inflammatory reactions. These molecules are also responsible for the establishment of firm adhesion and transendothelial migration [17].

The level of expression of β2-integrins is under critical control during myeloid differentiation and inflammation. Some elements of this control are exerted at the transcriptional level, and a coordinated transcriptional up-regulation of the integrin subunits CD11/CD18 take places. Impairment of the expression of CD18 in an inherited immunodeficiency (Leu-CAM deficiency) alters the stoichiometry and abolishes cell surface expression [17].

The molecular events that ultimately influence the tissue- and differentiation-specific transcription of these genes during macrophage differentiation are not totally clear. The genes encoding both CD18 and CD11 subunits share similarities in the organization of their promoters and in the nature of the transcriptional factors, supporting a role for some of them in lineage commitment and coordinated expression during differentiation [15]. In this context, comparisons of the integrin promoters for CD11a, CD11b, CD11c, and CD18 reveals the presence of AP-1 and Ets-binding motifs [15,18–24]. These nuclear factors may be responsible for coordinated expression of these proteins during myeloid differentiation. Induction of these cells with PMA increases the transcriptional activity of AP-1 [25], NF-κB [8–11], and PU.1 [26]. In the present study, we used double-stranded (ds)

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phosphorothioate oligonucleotides that containied AP-1, Ets, or κB consensus sequences to inhibit sequence-specific DNA-binding proteins during differentiation in U937 cells as a model of monocytic maturation. We show that whereas U937 cells may be induced to differentiate in the absence of apparent AP-1 activity, the sequestration of Ets factors and NF-κB has a profound effect on cell attachment and the expression of β2-integrin molecules.

Materials and methods

Cell culture, reagents, and induction

U937 monoblastic cells were obtained from the ATCC (Rockville, MD). Cells were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 5% FCS, 2 mM glutamine, and 50 μg/mL gentamicin at 37°C in a humidified atmosphere with 5% CO_2. To induce differentiation, U937 cells were resuspended in fresh medium (4×10^5/mL), cultured for 12 hours, and then treated with PMA (20 ng/mL) (Sigma, St. Louis, MO) for up to 5 days. Bisindolylmaleimide GF109203X [27] (Boehringer Mannheim, Barcelona, Spain) was used at a concentration of 5 μM, curcumin [28] (Sigma) at a concentration of 2.5–5 μM, and staurosporine [29,30] (Sigma) at a concentration of 50 nM. The cells were cultured for 0.5–1.5 hours before induction with PMA. Every 24 hours the culture medium was changed and a new dose of inhibitors was added. At the concentrations used, the inhibitors did not affect cell viability.

Phosphorothioate oligodeoxynucleotides

All oligodeoxynucleotides used in antisense and decoy studies were phosphorothioate, and were synthesized in solid phase according to the modified phosphoramidate method on a Beckman model Oligo 1000 automated DNA synthetizer (Beckman Instruments Inc., Palo Alto, CA). For stepwise thiathion phosphite linkages, the oxidation step was in 0.2 M [3H]-1,2-benzodithiole-3-one 1,1-dioxide (Sigma) in acetonitrile [31]. Oligonucleotides were analyzed by polyacrylamide gel electrophoreis on denaturing gels and material judged to be at least >90% full-length was used. Oligonucleotides were depyrogenated by ultrafiltration. Sequences of the antisense and sense oligonucleotides used on this study are depicted in Table 1. Oligonucleotide concentrations were calculated assuming 1 OD_{260}unit = 33 μg/mL. Phosphorothioate oligonucleotides for competitive binding experiments (decoy experiments) were obtained in the same way. Double-stranded oligonucleotides were formed at a final concentration of 10 mM in TE pH8 by heating at 95°C for 3 minutes and cooling slowly during 90 minutes to room temperature.

Cell transfections and incubations with oligonucleotides

In transfections with liposomes, the cells were grown in T-75 flasks until 1×10^6 cel/mL. At this time, 5 mL of cells were washed in serum-free Opti-MEM medium (Gibco) prewarmed to 37°C. The cells were then resuspended in Opti-MEM containing 10 μg/mL DOTMA/DOPE solution (Lipofectin) (Gibco) and the appropriate sterilized oligonucleotide at a concentration of 10 μM. The cells were incubated for 4 hours at 37°C, and the medium was removed and replaced with normal growth medium containing 20 ng/mL PMA and a fresh dose of oligonucleotide. Every 24 hours the culture medium was changed and a new dose of oligonucleotide was added. Both basal and PMA-treated cells were pretreated with DOTMA/DOPE for 4 hours at 37°C in the absence of oligonucleotides.

Transfections by electroporation were performed with a BTX600 electroporator, as previously described [32]. U937 cells (1 to 2×10^7) were electroporated (100 V, 2950 μF, 186 Ohms) in 500 μL RPMI containing 10 μM of appropriate oligonucleotide. Both basal and PMA-treated cells were electroporated in the absence of oligonucleotide. Every 24 hours the culture medium was changed and a new dose of oligonucleotide was added.

Incubations of cells with antisense or sense oligonucleotides were performed by adding the corresponding oligonucleotide to the culture medium 1-4 hours before induction with PMA. In decoy experiments, the double-stranded phosphorothioate oligonucleotides were added to the cells 2–4 hours before induction with PMA. Every 24 hours the culture medium was changed and a new dose of oligonucleotide was added. At the concentrations used, the oligonucleotides did not affect cell viability.

Immunofluorescence analysis

After induction with PMA for 48–72 hours, cells were harvested, washed twice in ice-cold phosphate-buffered saline (PBS), and incubated at 4°C with the following antibodies marked with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): anti-CD11b-PE, anti-CD11c-PE, anti-CD15-FITC, anti-CD33-PE, anti-CD14-PE, anti-CD11a-FITC, and anti-CD18-FITC. All were purchased from Becton Dickinson (San Jose, CA). As a negative control, we used an irrelevant PE- or FITC-conjugated monoclonal antibody. Cells were analyzed by flow cytometry using a Facsort cytometer (Beckton Dickinson). A minimun of 10×10^3 cells were analyzed in each experiment.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
AP-1	5′GTGACTCATGACTCATGACTCATGACTC 3′
	3′ACTGAGTACTGAGTACTGAGTACTGAGT 5′
NF-κB	5′GCATTAGGGGGCTTCCACGGCCTGA 3′
	3′GTAATCCCCCGAAGGTGCCGGACTT 5′
SP-1	5′GGTCAGGACCCGCCCCTTCTGGTCCG 3′
	3′CAGTCCTGGGCGGGGAAGACCAGGCT 5′
CRE	5′GGTGATTGCCTGACGTCAGAGAGCAT 3′
	3′CACTAACGGACTGCAGTCTCTCGTAT 5′
antisense c-fos[1]	5′GCCCGAGAACATCATCGT 3′
Sense c-fos[1]	5′ACGATGATGTTCTCGGGC 3′
antisense c-jun[1]	5′CGTTTCCATCTTTGCAGT 3′
sense c-jun[1]	5′ACTGCAAAGATGGAAACG 3′
AP-1ppt[1]	5′GCCCCCTCTGACTCATGCTGACA 3′
	3′GGGGGAGACTGAGTACGACTGTT 5′
NF-κB-ppt[1]	5′GTACATTAGGGGGGCTTCCACGGCCTG 3′
	3′ATGTAATCCCCCCGAAGGTGCCGGACT 5′
PU.1-ppt[1]	5′GCTGAAAGAAGGGCAGAAAAGGAGAAGTAG 3′
	3′GACTTTCTTCCCGTCTTTTCCTCTTCATCT 5′
AP-1ppt-mut[1]	5′GCCCCCTCTAGCAGATGCTGACA 3′
	3′GGGGGAGATCGTCTACGACTGTT 5′
NF-κB-ppt-mut[1]	5′GTACATTAGCTATGAGAACACGGCCTG 3′
	3′ATGTAATCGATACTCTTGTGCCGGACT 5′
PU.1-ppt-mut[1]	5′GCTGAAAGAAGGGCAGCCCGGGAGAAGTAG 3′
	3′GACTTTCTTCCCGTCGGGCCCTCTTCATCT 5′

[1]Phosphorothioate oligonucleotides.

Both basal and PMA-treated cells were pretreated with DOTMA/DOPE for 4 hours at 37°C or electroporated in the absence of oligonucleotides.

Immunoprecipitation analysis

Cells (5×10^6) were incubated for 1 hour in 1 mL [^{35}S] methionine-free RPMI (Gibco) supplemented with 10% dialysed fetal calf serum, and [^{35}S]-methionine (250 μCi) (Amersham) with antisense or sense oligonucleotides and then stimulated with PMA (20 ng/mL) during 1 hour. Cells were washed in PBS and lysed in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1% bovine serum albumin. After lysis, supernatants were cleared by ultracentrifugation at 10,000 *g* for 1 hour. Lysates were then adjusted for equal incorporation of [^{35}S]-methionine in trichloroacetic acid-precipitable counts and processed by immunoprecipitation. Lysates were precleared with 3 μl normal mouse serum and then with 100 μl protein-A-sepharose 10% (w/v) (Pharmacia, Uppsala, Sweden). Precleared lysates were incubated with 10 μg of a monoconal antibody anti-human c-FOS, Ab-1, (Oncogene Science Inc., NY), overnight at 4°C. Immune complexes were isolated with protein-A Sepharose for 1 hour on ice, then washed in lysis buffer. Pellets were resuspended in 40 μL SDS sample buffer and loaded on a 12% SDS-PAGE gel under reducing conditions.

Electrophoretic mobility shift assay

Cells lines were grown to log phase before harvest for nuclear protein extraction. Nuclear proteins were extracted 6–24 hours after induction with PMA and were prepared as previously described [32] with modifications [33]. Briefly, 10×10^6 cells were collected, washed twice in PBS, and resuspended in 400 μL cold buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine, 0.15 mM spermine) with 25 μL NP-40 10% and allowed to swell for 15 minutes on ice. The nuclei were collected by centrifugation at $400 \times g$ for 5 minutes, washed once in buffer A, and resuspended in 50 μL of buffer C (20 mM HEPES, pH 7.9, 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). All buffers contained aprotinin (2 μg/mL), leupeptin (2 μg/mL), and pepstatin (1 μg/mL) as additional protease inhibitors. The tubes were vigorously rocked on a shaking platform at 4°C for 30 minutes and centrifuged at $12,000 \times g$ for 5 minutes at 4°C, and the supernatant

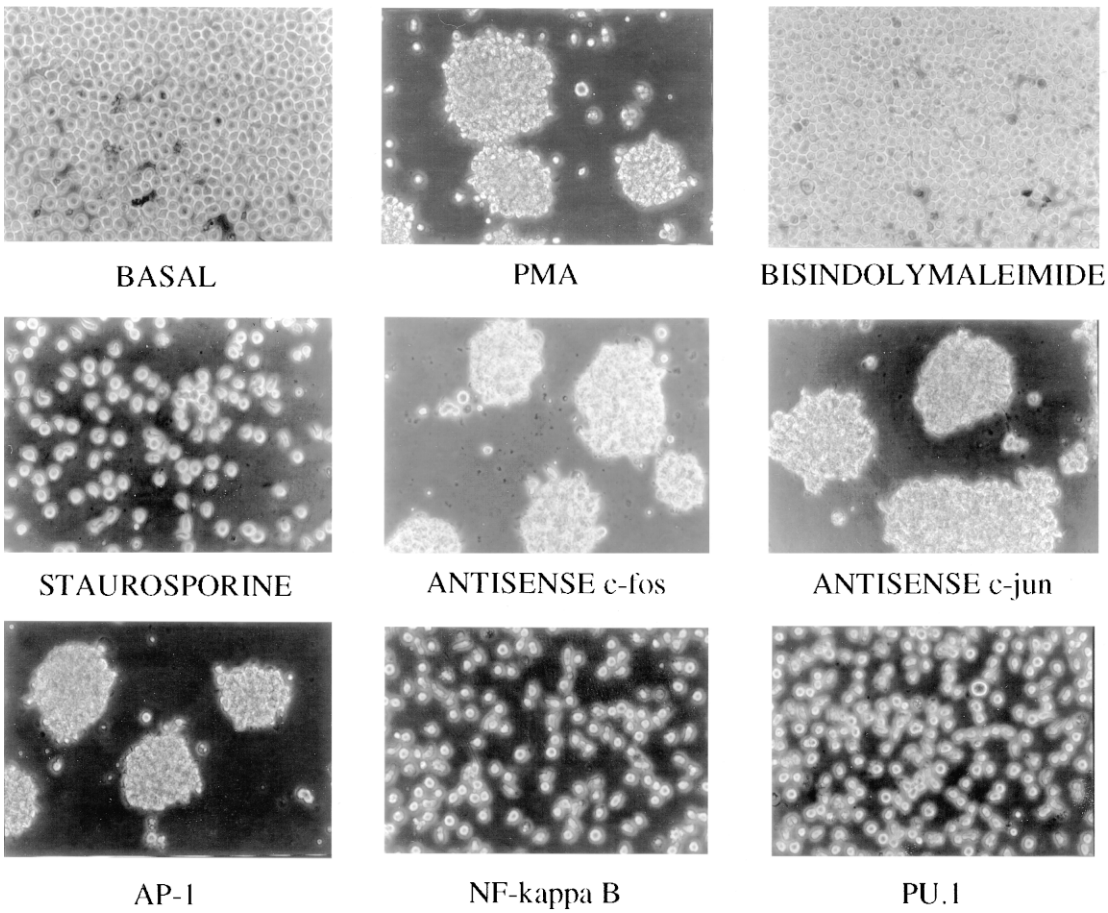

Figure 1. Effect of different treatments on PMA-induced differentiation of U937 cells. Cells were pretreated with each agent, bisindolylmaleimide GF109203X (5 μM), staurosporine (50 nM), phosphorothioate antisense c-fos oligonucleotide (10 μM), phosphorothioate antisense c-jun oligonucleotide (10 μM), double-stranded phosphorothioate AP-1 oligonucleotide (7.5 μM), double-stranded phosphorothioate NF-κB oligonucleotide, or double-stranded phosphorothiate PU.1 oligonucleotide, and then stimulated with PMA (20 ng/mL). The effects on morphologic changes and homotypic adhesion are compared. Phase-contrast photomicrophographs were made after 72 hours of PMA induction. Results from one of three experiments.

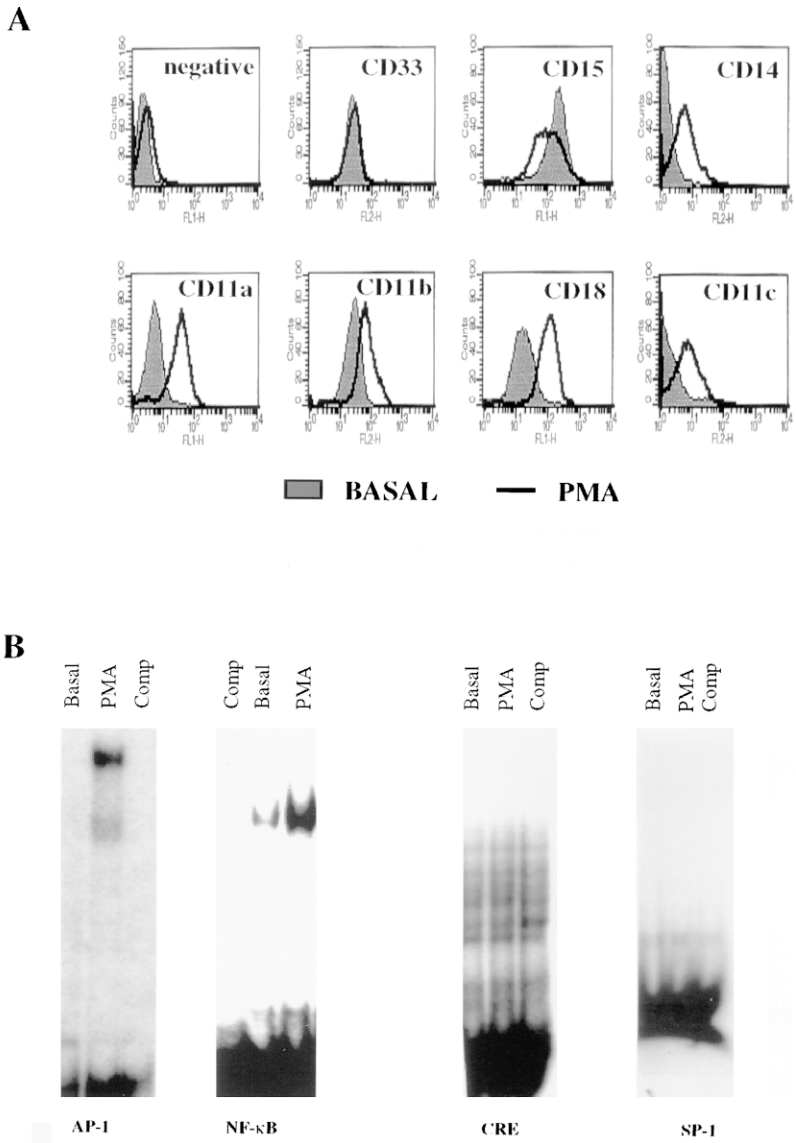

Figure 2. (A) Expression of cell surface antigens in U937 cells after differentiation with PMA. Immunofluorecence flow cytometry was performed on untreated cells (Basal) or cells induced to differentiate with PMA (20 ng/mL) during 72 hours. Cells were washed and stained with either FITC- or PE-labelled mAbs against the following surface markers: CD11a, CD11b, CD11c, CD18, CD14, CD15, and CD33. Stained cells were analyzed on a FACSort cytometer. Black areas show nonstimulated cells and white areas show staining after PMA induction. (B) Induction by PMA of transcriptional factor binding activity in U937 cells. Radiolabeled oligonucleotides AP-1, NF-κB, SP-1, and CRE (Table 1) were incubated with nuclear extract (3 μg) prepared from U937 cells (Basal lanes), or U937 cells induced to differentiate along the monocytic pathway with PMA during 24 hours (PMA lanes). The last binding reactions were performed in the presence of a 100-fold excess of the appropriate unlabeled oligonucleotide (Comp lanes).

was aliquoted and rapidly frozen on liquid N_2 and stored at −80°C. The protein concentration of the extracts was determined with the Bradford protein assay (Bio-Rad, Madrid, Spain).

Binding activity was assessed using DNA oligomers, some of them corresponding to the promoter region of β2-integrins (Table 1). Complementary oligodeoxynucleotides containing the required binding sequence were synthesized in our laboratory as described above. Probes were annealed with complementary strand by heating to 95°C for 3 minutes, then slowly cooling to room temperature.

One-hundred-nanogram amounts of double-stranded oligonucleotides were labelled by filling-in with Klenow fragments and [α-^{32}P] dCTP or dATP (3000 Ci/mmol). A quantity amounting to 0.5 ng (3×10^4 cpm) of labeled oligomers was then incubated at 4°C for 20 minutes with 2–6 μg of nuclear extract in 20 μL of binding buffer containing 5 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 0.2 mM EDTA, 15 mM KCl, 15% glycerol, 1 mM DTT, and 2 μg poly(dI-dC). In certain experiments, supershift analysis was performed using c-fos- and jun-specific antisera, kindly provided by Dr. Rodrigo Bravo (Bristol-Myers-Squibb Research Institute, Princeton, NJ). For these experiments, the cellular extract was preincubated for 30 minutes at 4°C with 0.5 μL of antisera before the labeled probe was added. In competition experiments, a 100-fold

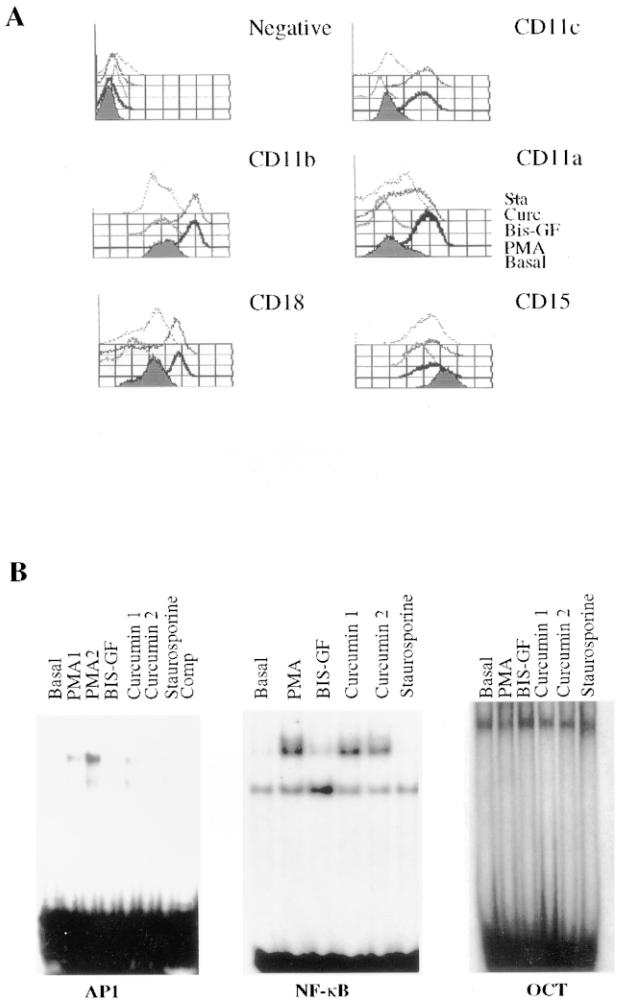

Figure 3. (A) Changes in the expression of cell surface differentiation antigens in PMA-treated U937 cells treated with PKC inhibitors or curcumin. U937 cells were pretreated with bisindolylmaleimide GF109203X (Bis-GF) (5 μM), staurosporine (50 nM), or curcumin (5 μM) 1 hour before PMA induction. The inhibitors were added every 24 hours thereafter. Seventy-two hours after PMA induction, the cells were washed and stained with FITC- or PE-mAbs. Stained cells were analyzed on a FACScan cytometer. The order of the curves is the same as for CD11a. (B) Effects of PKC inhibitors and curcumin on AP-1 and NF-κB binding activity in PMA-treated U937 cells. Radiolabeled oligonucleotides AP-1 and NF-κB (Table 1) were incubated with nuclear extract (3–5 μg) prepared from U937 cells (Basal lanes), PMA-treated U937 cells in the absence of unlabeled specific competitor DNA (lanes PMA 1 and 2; 1 and 20 ng of PMA, respectively) or in the presence of a 100-fold excess of unlabeled specific competitor (comp lanes), bisindolylmaleimide GF109203X-pretreated U937 cells treated with 20 ng/mL PMA (Bis lanes), curcumin-pretreated U937 cells with 20ng/mL PMA (lanes curcumin 1 and curcumin 2; 0.5 μM and 5 μM curcumin, respectively), or staurosporine-pretreated U937 cells treated with 20 ng/mL PMA (Staurosporine lanes). The inhibitors were added 1 hour before induction with PMA, and nuclear extracts were obtained 6–24 hours after induction with PMA. Nuclear extracts were also incubated with radiolabeled oligonucleotide oct-1 (Table 1), to verify that the amount of extract used in each reaction was the same. Results from one of three experiments.

excess of unlabeled oligomer was incubated at 4°C with the cellular extract for 15 minutes before the radioactive probe was added. An oligonucleotide containing the oct-1 binding site was used to control the quality of nuclear extracts (Table 1). Reactions were electrophoresed through 4%–6% low-ionic-strength acrylamide gels (29/1), 0.5 × TBE (1 × TBE is 89 mM Tris-base, 89 mM boric acid, and 2mM EDTA), fixed in 10% acetic acid, dried, and exposed on XAR film (Kodak) at −80°C.

Results

Expression of CD11/CD18 molecules during U937 cell differentiation

Analysis of adhesion, attachment to plates and integrin expression was used to evaluate differentiation of U937 cells. In the absence of PMA, nondifferentiated U937 cells did not attach to the plastic surface, and remained in an undifferentiated state without homotypic adhesion (Fig. 1). PMA induced rapid adhesion to the plastic surface accompanied by a significant increase in cell surface expression of CD11/CD18 molecules and the myelomonocytic antigen CD14 (Fig. 2A). In contrast, CD33 expression was not modified and CD15 expression was clearly down-modulated. These antigens were studied to rule out any effect on cell viability during oligonucleotide treatment. The increase in CD11/CD18 expression in response to PMA correlated with changes in binding activity of nuclear transcriptinal factors. AP-1 and NF-κB binding activity was strongly increased in PMA-treated cells, whereas binding for CRE and SP-1 factors was not (Fig. 2B).

Inhibition of PKC and changes in adhesion properties and integrin expression

Two PKC inhibitors, staurosporine [29,30] and bisindolylmaleimide GF109203X [27], were investigated for their ability to inhibit U937 differentiation. Cells were incubated for 60 minutes in the presence or absence of 50 nM of staurosporine and 5 μM of bisindolylmaleimide, respectively, and then with 20 ng/mL PMA. Cellular fractions were separated for immunofluorescence studies with monoclonal antibodies, and CD11/CD18 expression was analyzed after 72 hours.

Figure 1 shows the effect of PKC inhibitors on the morphology and adhesion of U937 cells. The differentiated phenotype of U937 cells after PMA treatment was completely blocked in the presence of staurosporine or bisindolylmaleimide, and the effect on cell morphology and homotypic adhesion correlated with the absence of changes in CD11/CD18 expression (Fig. 3A).

The effect of PKC inhibitors on NF-κB and AP-1 binding activities was also studied. Figure 3B shows that selective blockage of PKC-dependent pathways abrogated NF-κB and AP-1 PMA-induced activities: PMA induced NF-κB and AP-1 activity in electrophoretic mobility shift assays

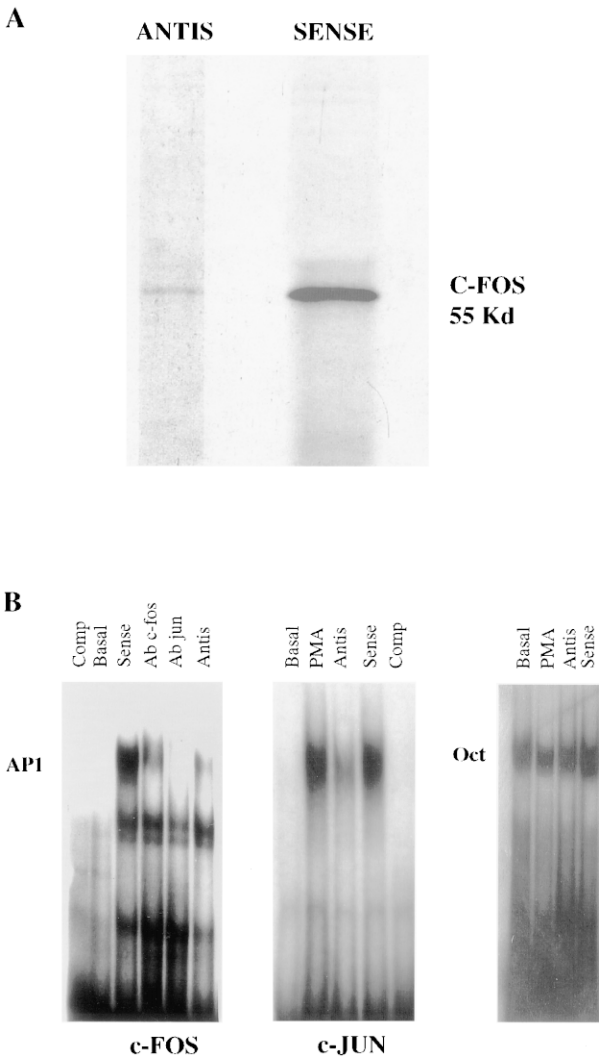

Figure 4. (A) Effect of antisense c-fos oligonucleotides on fos protein induction. Immunoprecipitation of fos protein was performed on lysates from cells that were treated with sense or antisense oligonucleotides and then stimulated with PMA, as described in the Materials and methods section. A faint band at 55 Kd can be detected in the antisense line (ANTIS) in comparison with the sense line (SENSE). This experiment was repeated three times. (B) Alterations in AP-1 binding activity in U937 cells incubated with antisense-c-fos or antisense-c-jun phosphorothioate oligonucleotides. Radiolabeled oligonucleotide AP-1 was incubated with nuclear extract (3–5 μg) prepared from U937 cells (Basal lanes), PMA-treated U937 cells in the absence of unlabeled specific competitor DNA (PMA lane) or in the presence of a 100-fold excess of unlabeled specific competitor (Comp lanes), U937 cells incubated with sense c-fos or sense c-jun phosphorothioate oligonucleotide and then with PMA (Sense lanes), and U937 cells incubated with antisense c-fos or c-jun phosphorothioate oligonucleotide and then PMA (Antisense lanes). Supershifts were performed with the radiolabeled probe, which was added to nuclear extract (5 μg) from PMA-treated U937 cells preincubated for 30 minutes at 4°C with 0.5 μL rabbit polyclonal antisera against c-fos (lane Ab c-fos), or jun-family (lane Ab jun). Each nuclear extract was also incubated with radiolabeled oligonucleotide oct-1 (Table 1), to verify that the amount nuclear extract used in each reaction was the same. Results from one of three experiments.

(EMSA), and the binding of both factors was strongly reduced in PKC inhibitor-treated cells. We next studied the contribution of AP-1 and NF-κB binding activities to changes in adhesion properties and β2-integrin expression. Ets binding activity was investigated because other studies of the regulatory region of CD11c and CD18 suggested that this element is involved in PMA-induced macrophage differentiation [13,26,34].

AP-1 binding activity is not required for PMA-induced U937 differentiation

Previous studies have shown that PMA treatment of U937 cells is associated with c-fos and c-jun induction [35,36]. AP-1 sites have been identified within CD11c and CD18 promoters. This nuclear factor may contribute to the up-regulation of surface-coordinated expression of these subunits. We used different oligonucleotide strategies to block AP-1 activity and monitored β2-integrin expression and U937 cell adhesion after either antisense or double-strand sequence-specific phosphorothioate oligonucleotides treatment. Two antisense-specific oligonucleotides for c-fos and c-jun were tested and the two corresponding sense oligonucleotides were used as controls (Table 1). Exposure to the jun and fos antisense oligonucleotides during 4 hours before the addition of PMA did not significantly reduce CD11/CD18 expression (not shown). In fact, differentiation of U937 cells appeared to occur normally, with morphologic features indicative of macrophage differentiation at 72 hours post-PMA treatment (Fig. 1). This suggested that c-jun and and c-fos induction was not essential for U937 cell differentiation. We then examined the expression of c-jun and c-fos mRNA (not shown), c-fos protein, and AP-1 activity (Fig. 4). We found that c-fos protein expression was inhibited in immunoprecipitation assays and SDS-PAGE analyses when antisense oligonucleotides were used, in contrast to the findings with sense oligonucleotides (Fig. 4A). Furthemore, the binding of AP-1 to its cognate DNA enhacer element was evaluated using gel-shift analysis with a radiolabeled AP-1 consensus sequence (Table 1).

DNA binding activity was examined in PMA-treated and nonstimulated cells. Treatment with PMA for 24 hours increased the intensity of the highest band (Fig. 4B), but did not alter the intensity of the lowest band. The specificity of these bands was analyzed using a 100-fold excess of unlabeled wild-type oligonucleotide (Fig. 4B). The formation of complexes was abrogated in the presence of wild-type but not the mutant oligonucleotides (not shown). AP-1 binding activity was also modified by the polyclonal antisera specific for c-fos and jun proteins (Fig. 4B). Preincubation with anti-jun antisera totally abrogated the uppermost band. In contrast, incubation with anti-c-fos antisera partially reduced the complex in the band shift assay. The lower band was not affected by any treatment.

In antisense experiments, U937 cells were previously exposed to 10 μM antisense or sense oligonucleotides for 1–4

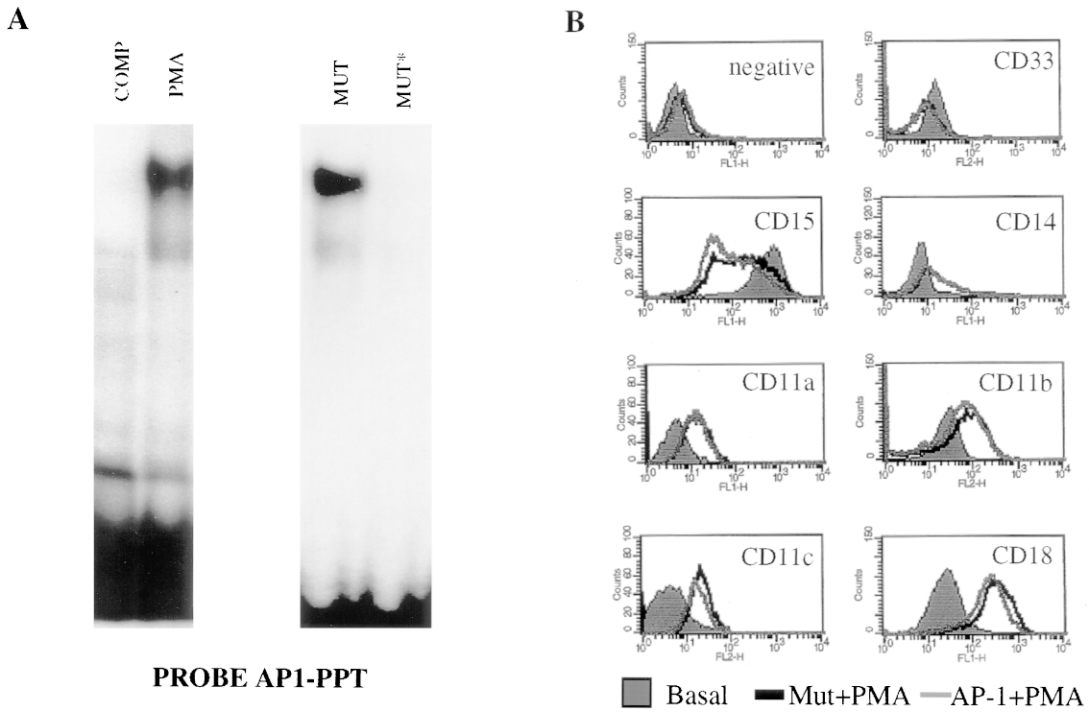

Figure 5. (A) Characterization of AP-1 binding activity to the AP-1 double-stranded phosphorothioate oligonucleotide in PMA-treated U937 cells. Nuclear extract from PMA- treated U937 cells was incubated with the radiolabeled phosphorothioate oligonucleotide AP1-ppt (Table 1) in the absence of unlabeled specific competitor DNA (PMA lane) or in the presence of a 100-fold excess of unlabeled specific competitor (COMP lane), in the presence of a 100-fold excess of unlabeled nonspecific competitor AP-1-ppt-mut (MUT lane). Nuclear extract from PMA-treated U937 cells was also incubated with the radiolabeled phosphorothioate oligonucleotide AP-1-ppt mut (MUT* lane). (B) Expression of cell surface differentiation antigens in U937 cells incubated with the AP-1 double-stranded phosphorothioate oligonucleotide and PMA. U937 cells were transfected with 7.5 μM of either AP-1-ppt double-stranded phosphorothioate oligonucleotide or Ap-1-ppt-mut double-stranded oligonucleotide. After 4 hours, cells were induced with PMA (20 ng/mL). Every 24 hours the culture medium was replaced and a new dose of oligonucleotide was added; 72 hours after PMA induction, the cells were washed, stained and analyzed on a FACSort cytometer.

hours at 37°C. Cells were transfected using either electroporation or liposome treatment and were then treated with PMA for 24 hours. As shown in Figure 4B, AP-1 activity increased after PMA induction in cells transfected with sense oligonucleotides. Antisense c-fos and c-jun oligonucleotides partially affected AP-1 activity. In both cases, U937 cell differentiation and the pattern of expresson of CD11/CD18 were identical to those in PMA-treated cells (Figs. 1 and 2A). These results suggest that during anti-c-fos or anti-c-jun treatment, residual AP-1 activity was always detected as a result of the complexity of the protein complexes that determine AP-1 binding activity. Furthemore, low binding activity was seen in some experiments in nonstimulated cells, which could not be removed in antisense experiments. For this reason we used double-stranded phosphorothioate oligonucleotides containing the site for AP-1 binding to eliminate residual AP-1 activity (Table 1, probe AP-1-ppt). In EMSA assays, the wild-type AP-1 sequence bound the AP-1 complex, whereas a mutated sequence did not (Fig. 5A). With 7.5 μM of either wild-type AP-1 or mutated AP-1, U937 cells differentiated as seen in controls, and the characteristic changes in morphologic and β2-integrin expression occurred (Figs. 1 and 5B)

We also tested curcumin as a specific inhibitor of AP-1 activity. This treatment significantly reduced AP-1 activity in EMSA assays (Fig. 3B, Curc2 lane). The results obtained were similar to those found with the decoy or antisense strategies, and in contrast to the effect of PKC inhibitors, U937 cell differentiation and increment of β2-integrin expression were not blocked (Fig. 3A).

U937 differentiation is specifically inhibited after sequestering of transcriptional PU.1 and NF-κB factors

We tested the induction of β2-integrin molecules on the cell surface of U937 cells after treatment with of NF-κB or PU.1 double strand phosphorothioate oligodeoxynucleotides (Table 1). The specificity of the oligonucleotide for NF-κB was assessed by mobility-shift assays using wild-type or mutated sequences (Fig. 6A). For PU.1, we used the sequence described previously [34] and the specificity of the oligonucleotide for PU.1 was assased by mobility-shift assays (Fig. 7A). We transfected or exposed U937 cells to 7.5 μM oligo-

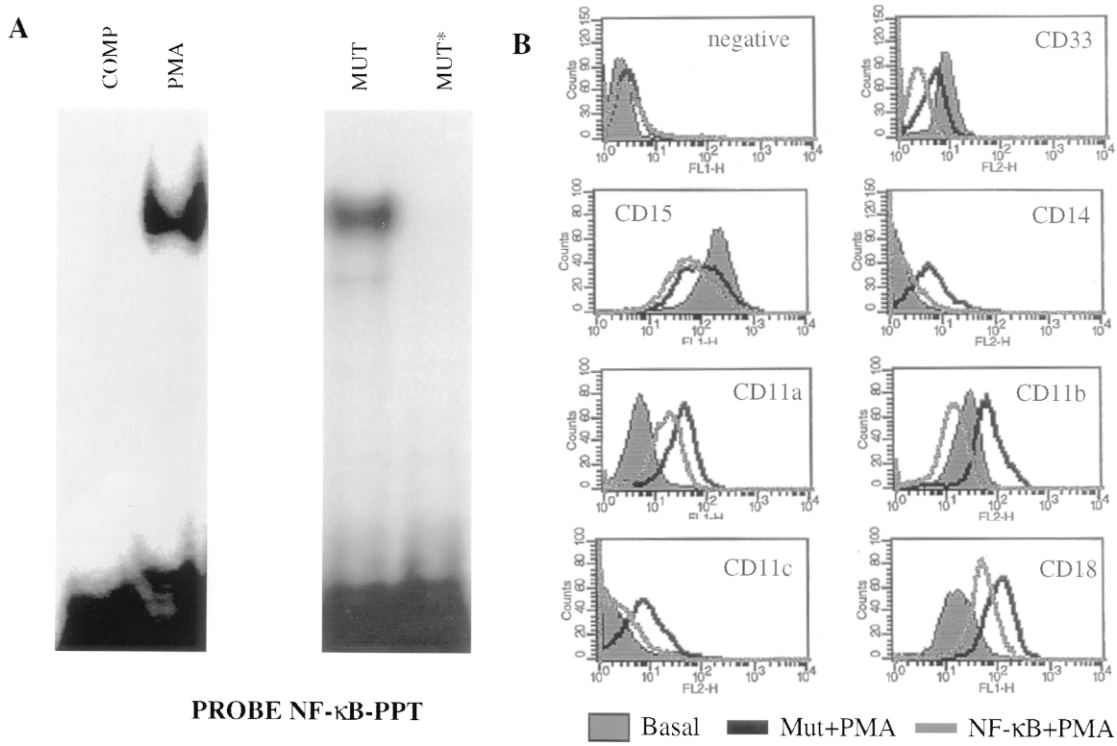

Figure 6. (A) NF-κB binding activity in PMA-U937 cells. Electrophoretic mobility shift assays (EMSA) showing the protein-DNA complexes that form the NF-κB double-stranded phosphorothioate oligonucleotide. Nuclear extract from PMA-treated U937 cells was incubated with the radiolabeled phosphorothioate oligonucleotide NF-κB-ppt (Table 1) in the absence of unlabeled specific competitor DNA (PMA lane), in the presence of a 100-fold excess of unlabeled specific competitor (COMP lanes),or in the presence of a 100-fold excess of unlabeled nonspecific competitor NF-κB-ppt-mut (MUT lane). Nuclear extract from PMA-treated U937 cells was also incubated with the radiolabeled phosphorothioate oligonucleotide NF-κB-ppt mut (MUT* lane). (B) Variation in the expression of cell surface differentiation antigens in U937 cells incubated with the NF-κB double-stranded phosphorothioate oligonucleotide and PMA. U937 cells were transfected with 7.5 μM of either NF-κB-ppt double-stranded phosphorothioate oligonucleotide or NF-κB-ppt-mut double-stranded oligonucleotide. After 4 hours, cells were induced with PMA (20 ng/mL). Every 24 hours the culture medium was changed and a new dose of oligonucleotide was added; 72 hours after PMA induction, the cells were washed, stained and analyzed on a FACSort cytometer.

nucleotides for 1–2 hours at 37°C and then cultured the cells for 72 hours with PMA. Exposure to the oligonucleotide was not toxic and did not affect cell viability. The wild-type NF-κB (NF-κB-ppt) clearly reduced CD11b expression to below the levels in nonstimulated cells, and led to more moderate reductions in CD11a,c, CD18, and CD14 expression. In contrast, the down-modulation of CD15 expression was unaffected (Fig. 6B). The mutated sequence had no effect on β2-integrin expression (Fig. 6B). Cell attachment and homotypic adhesion were also apparently blocked by NF-κB oligodeoxynucleotide treatment (Fig. 1).

The results obtained with a specific sequence corresponding to the PU.1 site of the CD11b promoter (PU.1-ppt, Table 1) differed from those with NF-κB oligonucleotide. Whereas adhesion to plastic and homotypic adhesion was apparently blocked by both treatments (Fig. 1), in PU.1-treated cells the expression of CD11b and CD11c was markedly inhibited. Moderate inhibition was seen for CD18, CD14, while CD11a expression was weakly inhibited. The levels of expression of CD33 and CD15 were similar to those in cells treated with the mutated sequence (Fig. 7B).

Our results also reveal a synergism between the transcription factors because the combination of either NF-κB/AP-1 and PU.1/AP-1 oligonucleotides reduced more significantly than either alone (Fig. 8).

Discussion

The exact role of the sequences in the promoter of CD11a, b, c, and CD18 involved in coordinated integrin induction and stage-specific maturation of the myeloid cell line U937 has not been fully resolved. The promoter regions for β2 [15] and αM [22–24,37,38], αL [21,39], and αX [18] contain common putative regulatory sequences. The aim of our study was to investigate the role of several transcriptional factors in PMA-induced differentiation in U937 cells. Protein kinase C (PKC) is activated after PMA treatment and is believed to modulate the activity of transcription factors that bind to these cis elements. The PKC inhibitors staurosporine and bisindolylmaleimide significantly reduce AP-1 and NF-κB induction, and completely block U937 cell differentiation induced by PMA. (Figs. 1 and 3A and 3B). Pre-

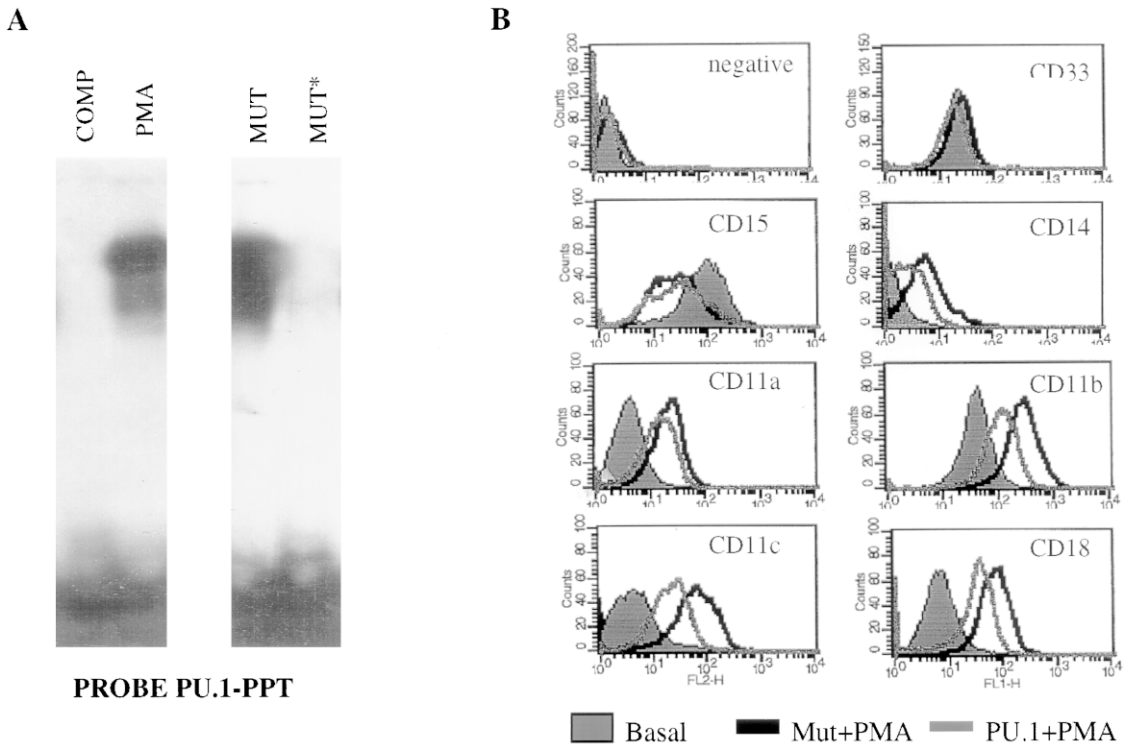

Figure 7. (A) PU.1 binding activity in PMA-U937 cells. Electrophoretic mobility shift assays (EMSA) showing the protein-DNA complexes that form the PU.1 double-stranded phosphorothioate oligonucleotide. Nuclear extract from PMA-treated U937 cells was incubated with the radiolabeled phosphorothioate oligonucleotide PU.1-ppt (Table 1) in the absence of unlabeled specific competitor DNA (PMA lane), in the presence of a 100-fold excess of unlabeled specific competitor (COMP lanes), or in the presence of a 100-fold excess of unlabeled nonspecific competitor PU.1-ppt-mut (MUT lane). Nuclear extract from PMA-treated U937 cells was also incubated with the radiolabeled phosphorothioate oligonucleotide PU.1-ppt mut (MUT* lane). (B) Expression of differentiation antigens in U937 cells incubated with the PU.1 double-stranded phosphorothioate oligonucleotide and PMA. U937 cells were transfected with 7.5 μM of either PU.1-ppt double-stranded phosphorothioate oligonucleotide or PU.1-ppt-mut double-stranded oligonucleotide. After 4 hours, cells were induced with PMA (20 ng/mL). Every 24 hours the culture medium was replaced and a new dose of oligonucleotide was added; 72 hours after PMA induction, the cells were washed, stained and analyzed.

vious studies have suggested that c-fos induction by phorbol esters is not necessary for HL60 or U937 differentiation [40,41]. In contrast, other studies have implicated AP-1 activity as a positive regulator of myeloid differentiation [42] and as a factor in the up-regulation of integrin expression [25]. Our results show that AP-1 activity is not sufficient to account for PMA induced β2-integrin transcriptional activation. The induction of c-fos and c-jun, and the increase in AP-1 binding activity are not necessary or obligatory for U937 cell differentiation (Fig. 1). Our results with antisense strategies showed that integrin activation and homotypic cell adhesion were not blocked. The reduction of c-fos protein (Fig. 4A), and in c-fos- and c-jun-containing complexes in mobility shift assays during U937 cell differentiation was significant (Fig. 4B). However, binding was partially inhibited, probably because other proteins of the fos and jun family contribute to the generation of AP-1 binding activity during diferentiation [25,43,44]. In fact, the patterns of inhibition were similar when the DNA binding reactions were supplemented with antisera to fos protein (Fig. 4B). Residual binding activity was always seen, probably because the reduction in c-fos-containing complexes during U937 cell differentiation was compensated by jun/jun dimers that also recognize the AP-1 element in the CD11c promoter [25]. This residual binding activity may contribute to the absence of inhibition of cell differentiation in antisense experiments.

With decoy strategies it is possible to sequester AP-1 and to inhibit the expression of genes regulated by this transcription factor [45,46]. However, our data show that stimulation of β2-integrin expression by PMA was unaffected in cells exposed to phosphorothioate oligonucleotides containing the AP-1 site (Fig. 5B). Similar results were obtained with curcumin, a specific AP-1 inhibitor (Fig. 3A). The promoter of CD11 and CD18 genes share similarities in organization with the stromelysin gene and, in this case, the AP-1 site is not necessary for the PMA-response [47,48]. Our findings are consistent with other studies that showed that c-fos expression was not obligatory for U937 maturation, and that although the AP-1 binding site is involved in the activity of integrin promoters, macrophage differentiation in

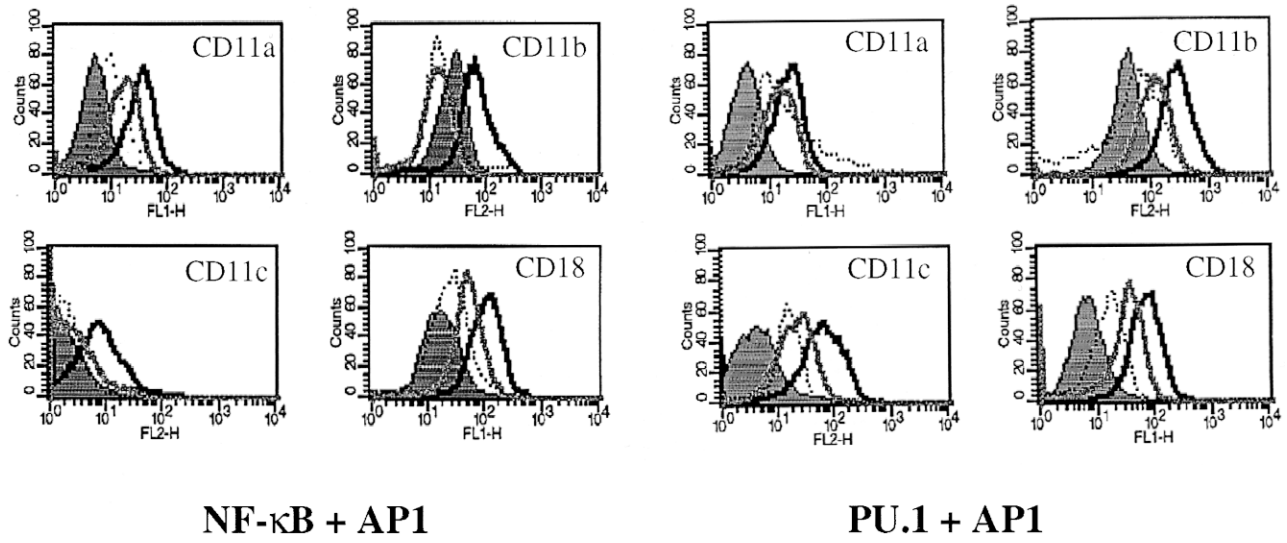

Figure 8. Sinergistic effects of decoy oligonucleotides on PMA-induced U937 cells. The combined use of either AP-1/NF-κB or AP-1 /PU.1 oligonucleotides evidenced a stronger reduction in integrin cell surface expression in comparison with the effect obtained with each oligonucleotide separately. ■ Basal, —— Mut+PMA, —— NF-κB+PMA, · · · · NF-κB+AP1+PMA (left); ■ Basal, —— Mut+PMA, —— PU.1+PMA, · · · · PU.1+AP1+PMA (right)

vivo normally occurs in the absence of the AP-1 activity [41,49–51].

Phorbol esters also induce both the translocation of NF-κB to the nucleus and the post-translational activation of PU.1 [26,52]. PU.1 elements are a common feature of the promoters of the CD11 and CD18 genes. The critical role for PU.1 in mediating gene expression is shown by the ability of double-stranded oligonucleotides containing the PU.1 binding site to inhibit specific gene expression [34]. However, contradictory results have been observed regarding the role of PU.1 binding during PMA-induced differentiation of the U937 cell line [26,53,54]. Our results suggest that PU.1 contributes significantly to differentiation in U937. The wild-type PU.1-binding phosphorothioate oligonucleotide, when added before PMA treatment, blocked adhesion properties (Fig. 1) and clearly reduced integrin expression in U937 cells, in contrast with the mutated oligonucleotide (Fig. 7B). PU.1 is a positive regulator of the CD18 and CD11b gene promoters, and disruption of the PU.1 binding sites significantly reduces promoter activity [55,56].

However, PU.1 appears to be a negative regulator of the CD11c promoter [57]. Our results also show a significant reduction in PMA-induced CD11c cell surface expression (Fig. 7B). In this context, the impaired expression of the CD18 gene could affect cell surface expression of the CD11 subunits, in a manner similar to that in inherited Leu-CAM deficiency [17].

Integrin expression was also inhibited by competitive binding of NF-κB factors. However, putative binding sequences have been identified only in the CD11a promoter, but have not been found for CD11b. Interestingly, the expression of CD11b fell to below basal levels (Fig. 6B). In agreement with our findings, antisense oligonucleotides to the p65 subunit of NF-κB block CD11b expression but do not inhibit gene expression [58]. We also found a strong inhibition in the expression of CD11a, CD11c, CD18, CD33, and CD14. It has been suggested that p65 NF-κB oligonucleotides may be blocking transcription of some factor(s) important for the release of intracellular granules containing preformed CD11b or, alternatively, p65 may be affecting the release of CD11b from granules by nontranscriptional mechanisms [58].

We suggest that NF-κB activation is a necessary event that affects the entire differentiation program in U937. Although both PU.1 and NF-κB oligonucleotides clearly affected cell surface adhesion, they appear to be responsible for only part of the induction of CD11/CD18 expression during U937 cell differentiation (Figs. 6B and 7B). These results may be explained by insufficient competitive binding of the nuclear factors under our experimental conditions. Alternatively, it is also possible that the developmental regulation of integrin expression is not fully under the control of a nuclear factor alone, and treatment with a single oligonucleotide is not sufficient to induce complete inhibition. Thus, the absence of nuclear factor may be compensated by the presence of other positive transacting factors [56]. For this reason, combinations of either NF-κB/AP-1 or Pu.1/AP-1 oligonucleotides in cotransfection experiments were also done. The combinations of NF-κB and AP-1 produced synergistic inhibition (Fig. 8). Both transcriptional factors target unique enhancer elements; however, its has been demonstrated that the cross-coupling of these tran-

scription factors pontentiates biologic functions in vivo through either κB or AP-1 enhancer elements [59]. In addition, Ets transcription factors also cooperate with AP-1 for transcriptional activation [60–62].

The development of stable oligonucleotide competitors makes it possible to study individual nuclear transcriptional factors by blocking the expression of genes involved in pathologic conditions. In this context, the presence of multiple regulatory elements in the gene promoter makes it necessary to obtain a detailed understanding of the real implication of nuclear factors in gene expression or repression. Therefore, reagents that target integrin expression can be used to study cellular differentiation and function, and may have potentially important therapeutic applications.

Acknowledgments

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