12-*O*-tetradecanoyl phorbol 13-acetate induces the expression of B7-DC, -H1, -H2, and -H3 in K562 cells

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Abstract. Induction of the B7 family molecules by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) has been reported, however, the mechanism by which TPA up-regulates these molecules remains poorly understood. In this study, the expression of B7-DC, -H1, -H2, and -H3 in response to TPA was markedly induced in K562 cells. TPA also induced activation of ERK, p38 mitogen-activated protein kinase (MAPK), JNK, phosphatidylinositol-3-kinase (PI-3K), or nuclear factor (NF)-κB. Pre-treatments with protein kinase C (PKC) inhibitors significantly inhibited TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA as well as TPA-induced phosphorylation of ERK, p38 MAPK, JNK, and PI-3K. TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA was abrogated by pre-treatments with inhibitors of ERK and p38 MAPK. However, inhibition of PI-3K and JNK only caused decrease of TPA-induced B7-DC mRNA and B7-H3 mRNA, respectively. TPA-induced degradation of IκB-α was markedly abrogated by treatments with PKC inhibitors, but not by treatments with inhibitors of ERK, p38 MAPK, JNK, or PI-3K. NF-κB inhibitors significantly attenuated the expression of B7-DC, -H1, -H2, and -H3 mRNA in response to TPA. These results suggest that TPA induces the expression of B7-DC, -H1, -H2, and -H3 mRNA in K562 cells via activation of PKC, ERK, p38 MAPK, and NF-κB. Distinctly, the expression of B7-DC mRNA and -H3 mRNA in response to TPA is also PI-3K- and JNK-dependent, respectively.

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Introduction

The B7 family co-signaling molecules are members of the immunoglobulin superfamily of type I transmembrane proteins and have important roles in the inhibitory or the stimulatory regulation of T cell responses. The most well-known T cell co-signaling molecules of the B7 family are B7-1 (CD80) and B7-2 (CD86), which are expressed mainly by dendritic cells, Langerhans cells, activated macrophages, and B cells and have been interacted with the co-stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (cytotoxic T lymphocyte antigen-4) on T cells (1,2). Recently, several new molecules of the B7 family (B7-DC, -H1, -H2, -H3, and -H4) have been reported. B7-H1 is expressed on the surface of macrophages, B cells, dendritic cells, and some cancer cells and has been found to deliver not only inhibitory signals through a receptor, programmed death-1 (PD-1) but also stimulatory signals through a yet unidentified receptor other than PD-1 (3-5). Like B7-H1, B7-DC inhibits lymphocyte activation through PD-1 and also mediates a stimulatory regulation of T cell responses through a yet unknown mechanism (6,7). B7-H2 is a ligand of inducible co-stimulator (ICOS) expressed on antigen-prime T cells and is expressed by macrophages, B cells, and some cancer cells (8-10). B7-H2-ICOS signal has been reported to have an important role in regulating Th2-type immunity (11,12). B7-H3 is known to co-stimulate proliferation of both $CD4^+$ and $CD8^+$ T cells through an unknown receptor and has also been reported to down-modulate Th-1-mediated immune responses (13,14). Lastly, B7-H4, which is ubiquitously expressed in lymphoid and non-lymphoid tissues, functions as a negative regulator of T cell responses through receptors on activated B and T cells (15,16). B and T lymphocyte attenuator (BTLA) has been identified and suggested to be a receptor for B7-H4 (17).

Hematopoiesis, which is a complex and multi-step process, takes place in specific organs during development. During differentiation of hematopoietic precursors into mature blood cells, the precise and appropriate regulation of essential gene expression occurs. It can be postulated that the regulation of expression of the B7 family molecules may be also essential and important in differentiation of hematopoietic progenitors. In addition, Chen *et al* (18) demonstrated that the B7 family

molecules were up-regulated in chondrogenic-, but not in osteogenic- or adipogenic-differentiated mesenchymal stem cells. However, the mechanisms involved in the regulation of expression of the B7 family molecules during differentiation of multi-potential progenitors are poorly understood. It has been reported that 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) induces primary human $CD34^+$ bone marrow progenitor cells to differentiate into functional dendritic cells and up-regulates the expression of the B7-2 co-stimulatory molecule (19). Expression of the B7 family molecules has been shown to be induced by interferon-γ, TNF-α, or TPA (3,14,19-21). Human chronic myelogenous K562 cells have been widely used as a model for the study of leukemia differentiation. K562 cells are differentiated into cells with megakaryocytic characteristics when exposed to TPA, a well-known protein kinase C (PKC) activator (22,23).

In the present study, we investigated the effect of TPA on the expression of B7-DC, -H1, -H2, -H3, or -H4 in K562 cells and the associated signaling mechanism. We found that the exposure of TPA into K562 cells induced the expression of B7-DC, -H1, -H2, and -H3, but not that of B7-H4. Although some of the molecular events linked to TPA-associated differentiation are known in K562 cells, the regulatory signaling mechanism responsible for the TPA-induced expression of B7 family co-signaling molecules is not fully known. Our data suggest that the increased expression of B7-DC, -H1, H2, and -H3 in response to TPA is at least, in part mediated through activation of PKC and the nuclear factor-κB (NF-κB) transcriptional factor. Interestingly, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (PI-3K) are involved in TPA-induced expression of B7-DC mRNA, but not that of B7-H1, -H2, or -H3 mRNA.

Materials and methods

Cell culture. K562, U937, HL60, and THP-1 cell lines were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Typically, $3x10^5$ cells/ml were seeded in T-25 flasks as 4-ml cultures, and maintained in the tissue culture incubator for 12-16 h before the addition of TPA or other reagents.

Drugs and materials. Antibodies against phospho-ERK (p-ERK), ERK, phospho-p38 MAPK (p-p38 MAPK), p38 MAPK, phospho-c-Jun N-terminal kinases (p-JNK), JNK, phosphor-AKT (p-AKT), AKT, phospho-p70S6 kinase (p-p70S6K), and p70S6K were purchased from Cell Signaling (Beverly, MA). Antibodies against inhibitory κB-α (IκB-α), p65 NF-κB, ß-actin, and ß-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-human B7-DC, -H1, -H2, -H3, and goat IgG isotype were purchased from R&D systems (Minneapolis, MN). Rabbit anti-goat FITC-conjugated IgG (Fc) was purchased from AbD-serotec (Oxford, JE). PD98059, SB203580, GF109203X, and Go6983 were purchased from Biomol (Plymoth, PA). MG132, BAY11-7085 (BAY), benzyloxycarbonyl-Leu-Leu-phenylalninal (ZLLF) and SP600125 were obtained from Calbiochem (La Jolla, CA). Cycloheximide (CHX), TPA, vitamin E, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO).

Reverse transcription-polymerase chain reaction (RT-PCR). The expression values of B7-DC, -H1, H2, and -H3 were quantified by semi-quantitative RT-PCR analysis, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Total cellular RNA was isolated from cells and reverse-transcribed using a random hexadeoxynucleotide primer (Perkin Elmer, Branchburg, NJ), and reverse transcriptase (Perkin Elmer) in 20 μl volume. PCR amplification was performed by using 0.25 μl of AmpliTaq DNA polymerase (Perkin Elmer), 1 μl of each 10 mM deoxyribonucleotide triphosphosphate, 6.25 μl of GeneAmp 10X PCR buffer II (Perkin Elmer), 4 μl of 25 mM $MgCl_2$, 1 μl of each specific sense and anti-sense primer at 25 μM, and water with the hot-start method to enhance the sensitivity and specificity of amplification. The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows: B7-DC forward 5'-CAA TGA TGC AGG AGG GGA TGA A-3', reverse 5'-CCA AGT GAG GGA CGA AGG ACA GTA-3', 422 bp; B7-H1 forward 5'-TCT TAC CAC TCA GGA CTT G-3', reverse 5'-AAA CAA TTA GAC CTG GCT G-3', 399 bp; B7-H2 forward 5'-CGT GTA CTG GAT CAA TAA GAC GG-3', reverse 5'-TGA GCT CCG GTC AAA CGT GGC C-3', 417 bp; B7-H3 forward 5'-CTG AGG TGT TCT GGC AGG AT-3', reverse 5'-CAC CAG CTC TTT GGT GTC TG-3', 362 bp; and GAPDH forward 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse 5'-CGG CCA TCA CGC CAC AGT TT-3', 300 bp.

Measurement of reactive oxygen species (ROS) generation. DCFH-DA was used to detect intracellular generation of ROS by TPA. DCFH-DA is a stable, nonfluorescent, and nonpolar compound that can diffuse through cell membranes. Once inside the cell, the acetyl groups are primarily cleaved by cytosolic enzymes to form the polar nonfluorescent dicholorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in PBS ($1x10^6$ cells/ml). Cell suspension (500 μl) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM, and incubated at 37˚C for 20 min. After the addition of TPA, cells were incubated at 37˚C for various times. Then ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells with a FACSCalibur flow cytometer (BD Biosciences, San Jose CA). Mean fluorescence intensity was obtained by histogram statistics using the CellQuest program.

Western blot analysis. For phospho-protein detection, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl). To isolate cytosolic and nuclear proteins, cells were homogenized in ice-cold hypotonic buffer (10 mM HEPES,

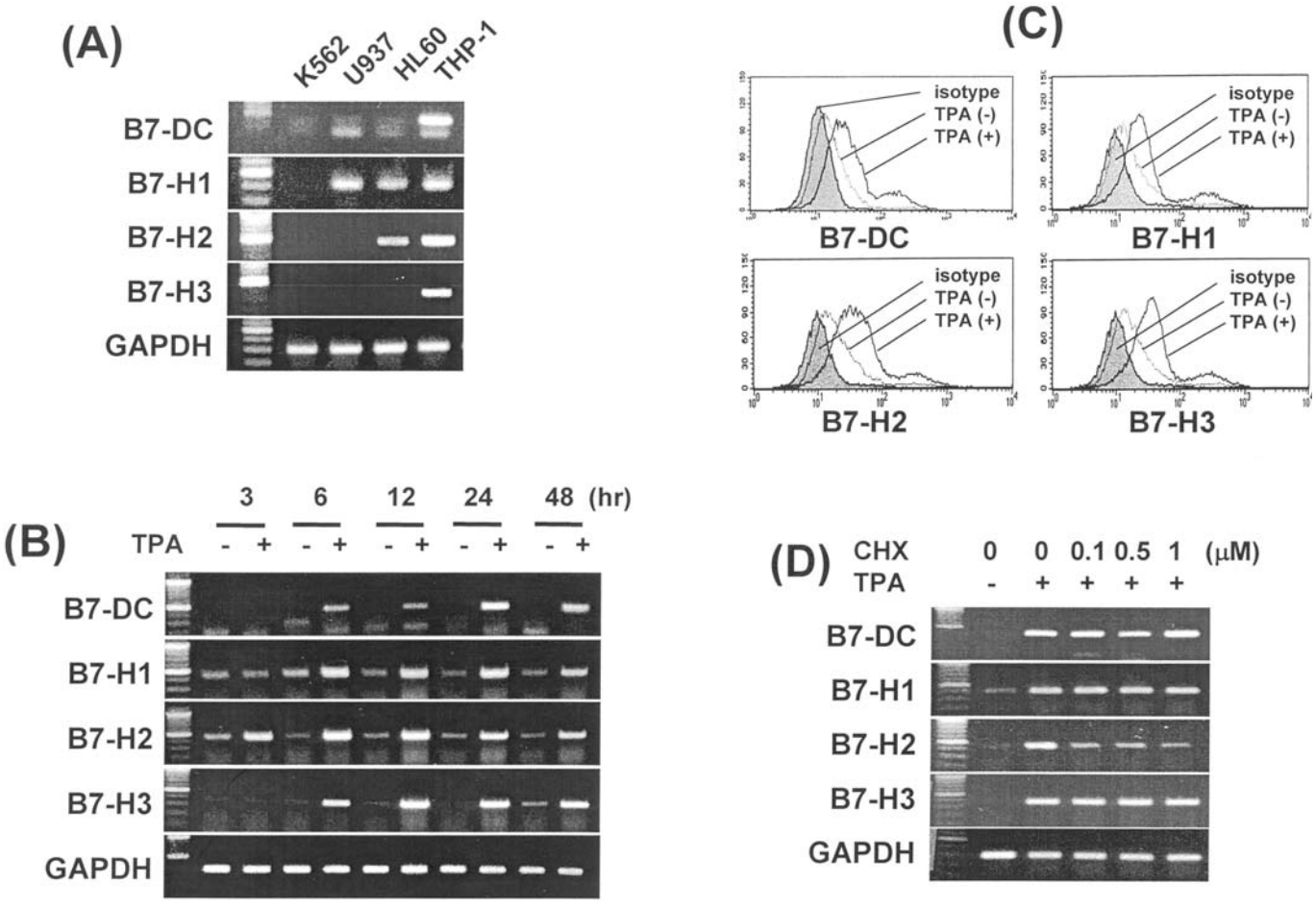

Figure 1. Expression of B7-DC, -H1, -H2, and -H3 in K562 cells treated with TPA. (A) Total RNAs were extracted from K562, U937, HL60, and THP-1, and analyzed by RT-PCR with specific primers for B7-DC, -H1, -H2, and -H3. GAPDH was analyzed to verify similar cDNA loading. (B) K562 cells were treated with TPA for the indicated times. Total RNAs were extracted from cells and analyzed by RT-PCR with specific primers for B7-DC, -H1, -H2, -H3, and GAPDH. (C) K562 cells were treated with TPA for 24 h and then cells were harvested and assayed for the cell surface expression of B7-DC, -H1, -H2, and -H3 as described in Materials and methods. (D) The effect of CHX on TPA-induced B7-DC, -H1, -H2, and -H3 mRNA expression in K562 cells. Cells were pre-treated with the indicated concentrations of CHX for 1 h and then stimulated with TPA for 24 h.

10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, 200 nM aprotinin) for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a cytosolic fraction. The pellets were homogenized in ice-cold nuclear extract buffer [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, 200 nM aprotinin] for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a nuclear fraction. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as ERK (1:2000), ERK (1:2000), p-JNK (1:1000), JNK (1:2000), p-p38 MAPK (1:1000), p38 MAPK (1:2000), p-AKT (1:1000), AKT (1:2000), p-p70S6K (1:1000), p70S6K (1:2000), p65 NF-κB (1:2000), IκB-α (1:2000), ß-actin (1:10000), and ß-tubulin (1:5000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Analysis of expression of the surface B7-DC, -H1, H2, and -H3. Cells ($1x10^6$) were washed in PBS containing bovine serum albumin (BSA) and sodium azide, and then incubated with the appropriate antibodies diluted in 1% BSA and 0.02% sodium azide in PBS for 30 min at 4˚C. After washing, FITC-conjugated rabbit anti-goat antibody was added and suspension was incubated at 4˚C for 30 min. Cells were resuspended in 400 μl of PBS and analyzed by BD FACSVantage flow cytometer (Becton Dickinson Biosciences, San Jose, CA). The data from flow cytometry were analyzed by WinMDI 2.8 software. Negative control was prepared by incubating with an isotype-matched control antibody.

Results

TPA induces B7-DC, -H1, -H2, and -H3 mRNA expression in K562 cells. The endogenous expression level of B7-DC, -H1, -H2, -H3, or -H4 was analyzed in human K562, U937, HL60,

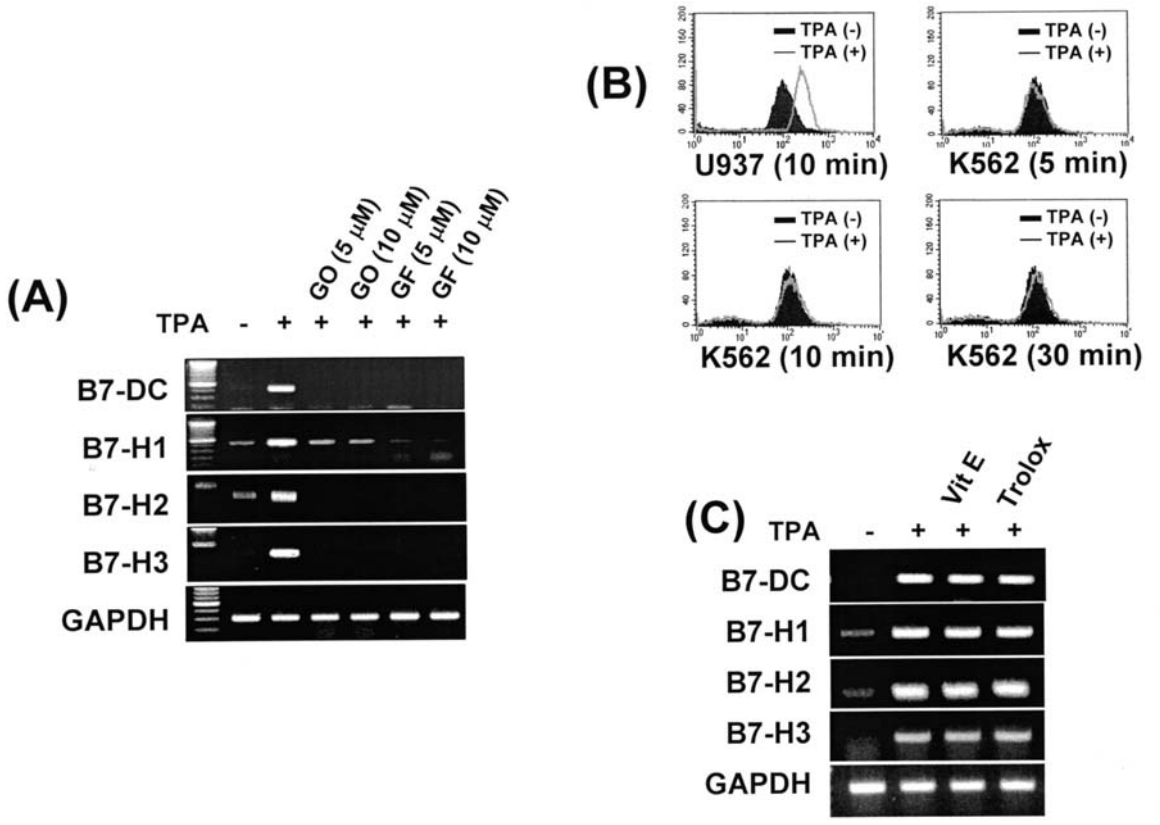

Figure 2. TPA induced PKC activation, but did not induce ROS generation in K562 cells. (A) Activation of PKC was critical for B7-DC, -H1, -H2, and -H3 mRNA expression in K562 cells. Cells were pre-treated with the indicated concentrations of Go6983 (GO) and GF109203X (GF), and then treated with TPA for 24 h. Total RNAs were extracted from K562 cells and analyzed by RT-PCR with specific primers for B7-DC, -H1, -H2, -H3, and GAPDH. (B) TPA did not induce ROS generation in K562 cells. U937 and K562 cells were pre-loaded with 20 μM DCFH-DA for 20 min at 37˚C, and then TPA was added to the cells and incubated for the indicated times at 37˚C. Cells were harvested and assayed for DCF fluorescence as described in Materials and methods. (C) K562 cells were pre-treated with 100 μM vitamin E (Vit E) or 100 μM Trolox for 1 h and then treated with TPA for 24 h. Total RNAs were extracted from K562 cells and analyzed by RT-PCR with specific primers for B7-DC, -H1, -H2, -H3, and GAPDH.

and THP-1 cells by RT-PCR using respective primers. As shown in Fig. 1A, THP-1 leukemia cells expressed B7-DC, -H1, -H2, and -H3 mRNA. While U937 leukemia cells expressed only B7-DC and -H1 mRNA, HL60 leukemia cells expressed only B7-H1 and -H2 mRNA. Notably, K562 leukemia cells did not express B7-DC, -H1, -H2, or -H3 mRNA. We did not detect B7-H4 mRNA in the cell lines tested. K562 cells are pluripotent and TPA has been known to induce differentiation of these cells into megakaryotic-like cells. To analyze whether TPA could induce expression of these B7 family molecules in K562 cells, TPA was exposed to cells for 2 days. As shown in Fig 1B, TPA treatment induced a significant increase of expression of B7-DC, -H1, -H2, and -H3 mRNA in a time-dependent manner, which was sustained for up to 2 days. However, we failed to detect B7-H4 mRNA expression in response to TPA. Kinetic data demonstrated that a significant induction of B7-H2 mRNA was detected at 3 h and B7-DC, B7-H1, or B7-H3 mRNA was detected at 6 h following TPA treatment. To verify whether protein of B7-DC, -H1, -H2, or -H3 is expressed on the surface of K562 cells, flow cytometry analysis was carried out. As shown in Fig. 1C, B7-DC, -H1, -H2, and -H3 protein were strongly induced at 24 h after TPA treatment. In analysis of the cell surface expression, negative control was prepared by incubating with an isotype-matched control antibody. To determine whether the increased expression of B7-DC, -H1, -H2, or -H3 mRNA was dependent on new protein synthesis, K562 cells were pre-treated with CHX, a protein synthesis inhibitor, for 1 h before TPA treatment. Interestingly, pre-treatment with CHX attenuated TPA-induced expression of B7-H2 mRNA, but not that of B7-DC, -H1, or -H3 mRNA, suggesting that only B7-H2 mRNA expression by TPA in K562 cells required *de novo* protein synthesis (Fig. 1D).

Role of PKC or ROS in TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA. Because TPA is a well-known PKC activator, we hypothesized that TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA might be associated with activation of PKC. As shown in Fig. 2A, pre-treatments with Go6983 and GF109203X, inhibitors of PKC, significantly blocked not only differentiation of K562 cells (data not shown) but also expression of B7-DC, -H1, -H2, and -H3 mRNA in response to TPA. These results suggest that the increased expression of B7-DC, -H1, -H2, and -H3 mRNA by TPA correlated with activation of PKC. It has been shown that TPA treatment of leukemia cells induces generation of ROS in U937 cells (24). We thus determined whether treatment of K562 cells with TPA leads to ROS generation. K562 cells were incubated with DCFH-DA, and ROS-mediated oxidation of the fluochrome was assessed by flow cytometry. As shown in Fig. 2B, TPA treatment significantly induced ROS generation at 10 min in U937 cells, however, compared with control cells,

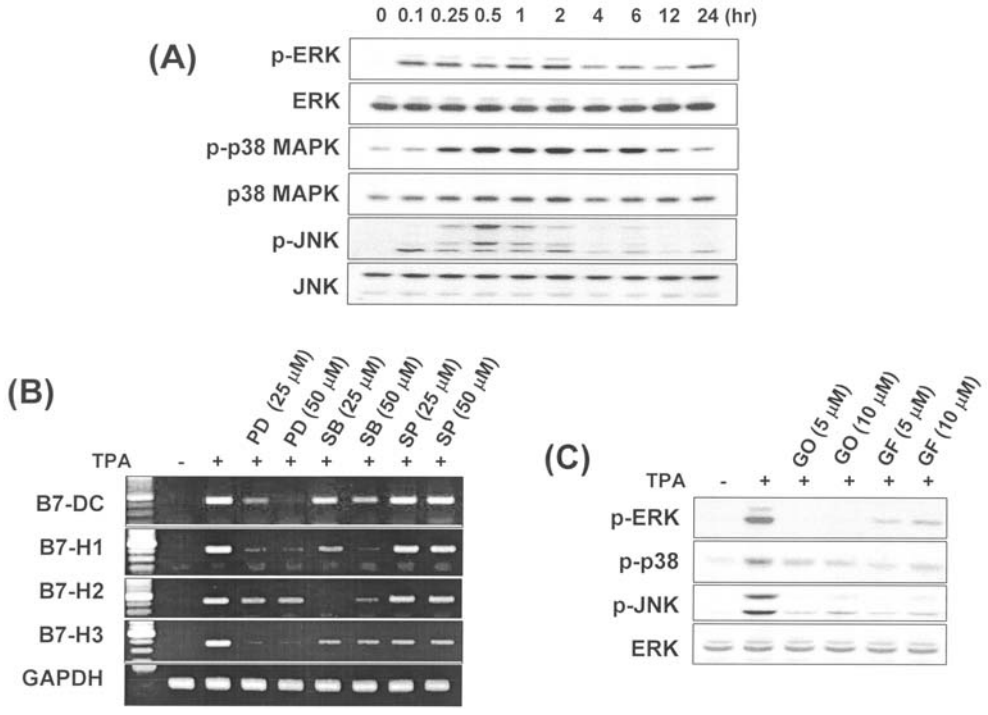

Figure 3. Role of MAPKs on TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA. (A) K562 cells were treated with TPA for the indicated times. At each time, whole cell lysates were prepared and used for p-ERKs, ERKs, p-p38 MAPK, p38 MAPK, p-JNK, or JNK Western blotting with respective antibodies. (B) Cells were pre-treated with the indicated concentrations of PD98059 (PD), SB203580 (SB), and SP600125 (SP) for 1 h and then treated with TPA for an additional 24 h. Total RNAs were prepared and used for B7-DC, -H1, -H2, -H3, or GAPDH by RT-PCR. (C) Cells were pre-treated with the indicated concentrations of Go6983 (GO) and GF109203X (GF) for 1 h and then treated with TPA for an additional 30 min. Whole cell lysates were prepared and used for p-ERK, p-p38 MAPK, p-JNK and ERK Western blotting with respective antibodies.

treatment with TPA did not induce ROS generation in K562 cells. Consistent with this result, pre-treatments with well-known antioxidants, such as vitamin E and Trolox had no effect on TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA in K562 cells.

Role of MAPKs on TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA. MAPKs, consisting of ERK, p38 MAPK, and JNK, are activated during TPA-induced megakaryocytic differentiation (25,26). To determine whether MAPKs are involved in TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA, we first measured the effects of TPA on activation of ERK, p38 MAPK, and JNK using respective antibodies that specifically recognize the active phosphorylated forms. As shown in Fig. 3A, treatment with TPA resulted in phosphorylation (activation) of these kinases. Activation of these kinases became apparent at 0.1-0.25 h. Stripping and subsequently reprobing the same blot with antibodies against total ERK, p38 MAPK, and JNK demonstrated no change of total protein levels of each kinase, indicating that TPA led to activation of pre-existing ERK, p38 MAPK, and JNK. We next tested the role of these signaling proteins in TPA-induced expression of the B7 family mRNA tested. As shown in Fig. 3B, pre-treatment with PD98059 (an inhibitor of ERK) and SB203580 (an inhibitor of p38 MAPK) for 1 h inhibited TPA-induced B7-DC, -H1, -H2, or -H3 mRNA expression. In addition, pre-treatment with SP600125 (an inhibitor of JNK) only attenuated TPA-induced expression of B7-H3 mRNA. These results suggest that though ERK, p38 MAPK, and JNK are activated by TPA, ERK and p38 MAPK mediate TPA-induced B7-DC, -H1, -H2, and -H3 mRNA expression and JNK mediated only TPA-induced B7-H3 mRNA expression. To investigate whether PKC regulates activation of ERK, p38 MAPK, and JNK, we tested the effects of PKC inhibitors on activation of these kinases. As shown in Fig. 3C, the data of inhibition of TPA-induced activation of these kinases by pre-treatments with Go6983 and GF109203X strongly suggest that TPA-induced activation of ERK, p38 MAPK, and JNK is PKC-dependent.

Role of PI-3K on TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA. To investigate whether PI-3K is also involved in TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA, we determined the effect of TPA on activation of PI-3K in K562 cells. As shown in Fig. 4A, treatment with TPA resulted in phosphorylation (activation) of AKT and p70S6K, which are known downstream effectors of PI-3K. Activation of AKT and p70S6K became apparent 15 min following treatment with TPA and was sustained for up to 6 and 24 h, respectively. To determine whether PKC also mediates activation of AKT and p70S6K in TPA-treated K562 cells, we evaluated the effects of PKC inhibitors (Go6983 and GF109203X) on activation of these kinases. As shown in Fig. 4B, pre-treatments of cells with PKC inhibitors for 1 h attenuated TPA-induced activation of AKT and p70S6K. In addition, pre-treatment with PKC inhibitors effectively suppressed TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA, while pre-treatment with LY294002, an inhibitor of PI-3K, for 1 h did not inhibit B7-H1, -H2, or -H3 mRNA expression, but attenuated B7-DC mRNA expression

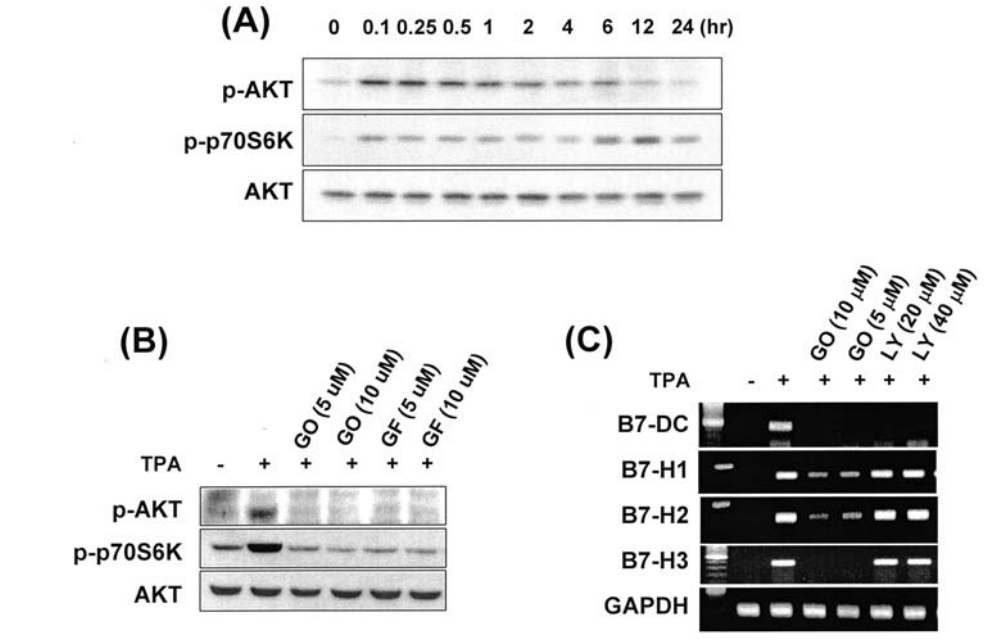

Figure 4. Role of PI-3K on TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA. (A) K562 cells were treated with TPA for the indicated times. At each time, whole cell lysates were prepared and used for p-AKT, AKT, and p-p70S6K Western blotting with respective antibodies. (B) Cells were pre-treated with the indicated concentrations of Go6983 (GO) and GF109203X (GF) for 1 h and then treated with TPA for an additional 30 min. Whole cell lysates were prepared and used for p-AKT, AKT, and p-p70S6K Western blotting with respective antibodies. (C) Cells were pre-treated with the indicated concentrations of Go6983 (GO) and LY294002 (LY) for 1 h and then treated with TPA for an additional 24 h. Total RNAs were prepared and used for B7-DC, -H1, -H2, -H3, or GAPDH by RT-PCR.

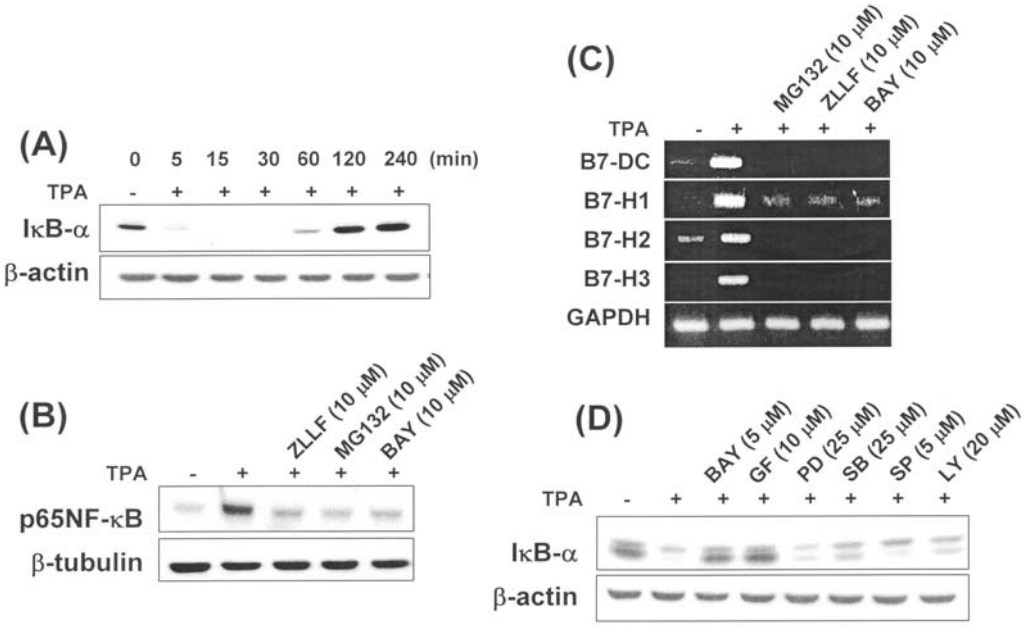

Figure 5. TPA induced activation of NF-κB, which is an important transcriptional factor mediating B7-DC, -H1, -H2, and -H3 mRNA expression in K562 cells. (A) Cells were treated with TPA for the indicated times. At each time, whole cell lysates were prepared and used for IκB-α and ß-actin Western blotting. (B) Cells were pre-treated with the indicated concentrations of ZLLF, MG132, or BAY, and then treated with TPA for an additional 15 min. Nuclear fraction was prepared and used for p65 NF-κB and ß-tubulin Western blotting with respective antibodies. (C) Cells were pre-treated with the indicated concentrations of ZLLF, MG132, or BAY, and then treated with TPA for an additional 24 h. Total RNAs were prepared and used for B7-DC, -H1, -H2, -H3, or GAPDH by RT-PCR. (D) Cells were pre-treated with the indicated concentrations of BAY, GF109203X (GF), PD98059 (PD), SB203580 (SB), SP600125 (SP), and LY294002 (LY) and then treated with TPA for an additional 15 min. Cell lysates were prepared and used for IκB-α and ß-actin Western blotting.

in response to TPA (Fig. 4C). These results suggest that though AKT and p70S6K are activated by TPA, activation of these kinases mediates only TPA-induced B7-DC mRNA expression, but not B7-H1, -H2, or -H3 mRNA expression.

Role of NF-κB on TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA. NF-κB signaling pathway has been implicated in expression of the B7 family molecules (20,27-29). To test whether NF-κB participates in TPA-induced expression of

B7-DC, -H1, -H2, or -H3 mRNA, we initially determined the effect of TPA on NF-κB activation in K562 cells. In the present study, the activation of NF-κB was assessed by measuring the degree of degradation of IκB-α, an inhibitory protein of NF-κB. Treatment with TPA induced NF-κB activation, which occurred within 5-60 min following TPA treatment (Fig. 5A). Using ZLLF, MG132, or BAY, inhibitors of NF-κB, we determined the role of NF-κB in TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA in K562 cells. To assure the effectiveness of ZLLF, MG132, or BAY as NF-κB inhibitors, cells were pre-treated for 30 min with these compounds and then treated with TPA for an additional 15 min, followed by measurement of the degree of nuclear translocation of p65 NF-κB. Pre-treatment with ZLLF, MG132, or BAY effectively suppressed TPA-induced nuclear translocation of p65 NF-κB (Fig. 5B). Furthermore, TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA was also largely inhibited by pre-treatment with ZLLF, MG132, or BAY (Fig. 5C). These results suggest that NF-κB is strongly involved in TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA. As mentioned above, TPA treatment resulted in activation of PKC, ERK, p38 MAPK, JNK, and PI-3K pathways. To determine whether TPA-induced activation of NF-κB is mediated through PKC, ERK, p38 MAPK, JNK, or PI-3K, K562 cells were pre-treated for 30 min with inhibitors of each kinase, and then treated with TPA for an additional 15 min. BAY and GF109203X suppressed IκB-α degradation in response to TPA, whereas PD98059, SB203580, SP600125, or LY294002 did not (Fig. 5D), suggesting that PKC pathway, but not ERK, p38, JNK, or PI-3K pathway, seems to regulate NF-κB activation in response to TPA in K562 cells.

Discussion

Human chronic myelogenous K562 cells are regarded as pluripotent hematopoietic progenitor cells and have been recognized to be a useful cell culture model in the study of differentiation. K562 cells differentiate into megakaryocytic or erythroid lineage, depending on the stimulus. K562 cells respond to TPA that causes activation of PKC with growth inhibition and induction of megakaryocytic-like phenotype (22,23). Expression of the B7 family co-signaling molecules is constitutive or inducible. The B7 family molecules can be up-regulated on human T cells, B cells, dendritic cells, macrophages, and other cells upon activation of various cytokines (3).

TPA activates PKC, a family of serine/threonine-specific kinase, which is involved in a pleiotypic set of processes including cell differentiation and growth (30). It has been reported that TPA induces the expression of co-stimulatory B7 family molecules, such as B7-1, B7-2, and B7-H3 in dendritic cells, chondrogenic-differentiated mesenchymal stem cells, and U937 cells, respectively (14,18,19). In this study, we have found that TPA-induced expression of B7-DC, -H1, -H2, and -H3 mRNA is dependent on PKC because pre-treatments with PKC inhibitors such as Go6983 and GF109203X effectively inhibit the expression of these molecules. It has been also known that TPA treatment with myeloid leukemia cells is associated with the generation of ROS and PKC is required for TPA-induced ROS generation (24). ROS play an important role of second messengers in various signal transductions such as growth, apoptosis, differentiation, and gene expression (31). In this study, however, TPA treatment did not induce ROS generation in K562 cells, suggesting that TPA-induced activation of PKC is not associated with ROS generation but is associated with B7-DC, -H1, -H2, or -H3 mRNA expression.

MAPKs have been shown to be required for the TPA-induced megakaryocytic differentiation of K562 cells (26,32). Previous studies have shown that TPA-induced activation of MAPKs is mediated by PKC activation (26,32,33). In this study, TPA-induced activation of ERK, p38 MAPK, or JNK in K562 cells is largely dependent on the PKC pathway because pre-treatment with Go6983 and GF109203X effectively inhibited TPA-induced activation of these kinases. Though expression of the B7 family molecules is induced by various stimuli which can activate MAPKs, the role of MAPKs has not been fully characterized. It has been reported that while IFN-γ-induced B7-H1 expression is not inhibited by pre-treatment with PD98059, SB203580, or SP600125 in A549 cells (21), it is the ERK-dependent manner in dermal fibroblast cells (34), suggesting that the induced expression of B7-H1 by IFN-γ appears to be cell-type specific. In this study, we have observed that TPA causes a marked increase in the level of phosphorylated ERK, p38 MAPK, and JNK in K562 cells. Interestingly, though phosphorylation of ERK, p38 MAPK, or JNK is induced by TPA, TPA-induced B7-DC, -H1, or -H2 mRNA expression was inhibited by pre-treatments with PD98059 and SB203580, but not SP600125. However, pre-treatment with SP600125 significantly attenuated TPA-induced B7-H3 mRNA expression only. These results suggest that ERK and p38 MAPK mediate B7-DC, -H1, -H2, or -H3 mRNA expression and JNK only mediates B7-H3 mRNA expression in response to TPA in K562 cells.

Although activation of PI-3K by TPA pathway has been demonstrated (35), the role of PI-3K has not been fully understood in TPA-induced megakaryocytic differentiation or TPA-induced expression of the B7 family molecules in K562 cells. Jacquel *et al* (32) showed that TPA induced PKC-, ERK-, JNK-, and p38-MAPK-dependent and PI-3K-independent differentiation in K562 cells. In this study, we have specifically found that PI-3K pathway appears to be at least, in part involved in TPA-induced B7-DC mRNA expression, but not B7-H1, -H2, or -H3 mRNA expression. This notion is based on the present findings that TPA induces activation of AKT and p70S6K and inactivation of PI-3K by LY294002 decreases TPA-induced B7-DC mRNA expression, but not B7-H1, -H2, or -H3 mRNA expression. It has been shown that TPA-induced activation of PI-3K is mediated by PKC (36). Consistent with this, we found that TPA-induced activation of AKT or p70S6K is largely dependent on the PKC pathway because pre-treatment with Go6983 and GF109203X effectively inhibited TPA-induced activation of AKT or p70S6K in K562 cells.

It has been reported that expression of the B7 family molecules including B7-1, B7-2, B7-H1, B7-DC, B7-H2, or B7-H3 by a variety of stimuli may be dependent on activation of transcriptional factors, such as STAT (signal transducer and activator of transcription), IFN-γ regulating factor-1, or NF-κB (14,20,21,27-29,34,37). However, though B7-H4 expression is induced on lymphoid cells, such as T cells, B

cells, and dendritic cells following activation (15,38), there is no evidence whether the inducible expression of this molecule is dependent on NF-κB or other transcriptional factors. Up-regulation of B7-H1 expression by IFN-γ, TNF-α, or LPS is known to be mediated through an IFN-γ regulating factor-1-, STAT 1/3-, or NF-κB-dependent manner (21,28,34,37). However, Liang *et al* (20) demonstrated that the expression of B7-DC, but not B7-H1 is controlled by NF-κB and STAT6. TNF-α, which is a well-known inflammatory cytokine, induces B7-H2 expression by alveolar epithelial type II cells in an NF-κB-dependent manner (29). Chapoval *et al* (14) demonstrated that co-treatment with phorbol ester plus ionomycin increased the expression of B7-H3 on dendritic cells, monocytes, and U937 cells, suggesting that the induced expression of B7-H3 could be NF-κB-dependent. Previous studies have shown that TPA can induce an increase in NF-κB activity in K562 cells (39-41). In the present study, we have demonstrated that TPA treatment markedly induces degradation of IκB-α, suggesting NF-κB activation by TPA in K562 cells. Furthermore, the present data showing that pre-treatment with ZLLF, MG132, or BAY effectively suppresses TPA-induced nuclear translocation of p65 NF-κB as well as TPA-induced up-regulation of B7-H1, -DC, -H2, and -H3 mRNA expression strongly suggest that NF-κB is an important transcriptional factor mediating TPA-induced expression of B7-DC, -H1, -H2, or -H3 probably by transcriptional up-regulation in K562 cells. Since TPA-induced expression of B7-DC, -H1, -H2, or -H3 mRNA is required for activation of PKC and NF-κB and the induced expression of B7-DC mRNA is dependent on activation of PKC, ERK, p38 MAPK, PI-3K, or NF-κB in response to TPA, therefore, we speculated that TPA-induced NF-κB activation might correlate with activation of PKC, ERK, p38 MAPK, and/or PI-3K. We have found that pre-treatments with BAY and GF109203X effectively inhibit degradation of IκB-α in TPA-treated K562 cells, whereas, inactivation of ERK, p38 MAPK, JNK, and PI-3K by PD98059, SB203580, SP600125, and LY294002, respectively, has no effect on TPA-induced IκB-α degradation. These results may suggest a possible cross-talk between PKC and NF-κB in mediating TPA-induced expression of B7-H1, -DC, -H2, and -H3 mRNA in K562 cells.

In conclusion, findings of the present study demonstrate that TPA up-regulates B7-DC, -H1, -H2, and -H3 mRNA expression in K562 cells via activation of PKC, ERK, p38 MAPK, and NF-κB. In addition, the expression of B7-DC mRNA and -H3 mRNA in response to TPA is also PI-3K- and JNK-dependent, respectively. Activation of PKC by TPA may involve in TPA-induced expression of the B7 family molecules tested, in part by activating NF-κB. Thus, the inducibility of B7-DC, -H1, -H2, or -H3 mRNA expression by TPA appears to be achieved by multiple signaling molecules. Further efforts to investigate other signaling pathways associated with TPA-induced expression of these co-signaling molecules are warranted.

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