Ectopic Expression of *CXCR5/BLR1* Accelerates Retinoic Acid- and Vitamin D_3-Induced Monocytic Differentiation of U937 Cells

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The product of the *blr1* gene is a CXC chemokine receptor (CXCR5) that regulates B lymphocyte migration and has been implicated in myelomonocytic differentiation. The U937 human leukemia cell line was used to study the role of *blr1* in retinoic acid-regulated monocytic leukemia cell growth and differentiation. *blr1* mRNA expression was induced within 12 hr by retinoic acid in U937 cells. To determine whether the early induction of *blr1* might regulate inducible monocytic cell differentiation, U937 cells were stably transfected with *blr1* (U937/*blr1* cells). Ectopic expression of *blr1* caused no significant cell cycle or differentiation changes, but caused the U937/*blr1* cells to differentiate faster when treated with either retinoic acid or 1α,25-dihydroxyvitamin D_3. Treated with retinoic acid, U937/*blr1* cells showed a greater increase in the percentage of CD11b expressing cells than vector control cells. Retinoic acid also induced a higher percentage of functionally differentiated *blr1* transfectants as assessed by nitroblue tetrazolium reduction. U937/*blr1* cells underwent moderate growth inhibition on treatment with retinoic acid. Similar results occurred with 1α,25-dihydroxyvitamin D_3. Because *blr1* was induced early during cell differentiation and because its overexpression accelerated monocytic differentiation, it may be important for signals controlling cell differentiation. Exp Biol Med 227:753–762, 2002

Regulation of hematopoietic cell differentiation and growth arrest has been studied using a variety of lineage specific leukemic cell lines. The U937 human monoblastic leukemia cell line provides an *in vitro* model for studying inducible monocytic differentiation. The differentiation of U937 cells is arrested at the monoblastic step, but this block in their development can be reversed with various physiological and pharmacological inducers of differentiation. All-*trans* retinoic acid (RA), a metabolic derivative of vitamin A, and 1α,25-dihydroxyvitamin D_3 (VD_3), the active metabolite of vitamin D_3, induce the differentiation of U937 monoblasts to monocyte/macrophage cells. RA and VD_3 can also induce monocytic differentiation of other leukemia cell lines (1–3). RA can also cause myeloid differentiation (2). RA is used to treat patients with acute promyelocytic leukemia (APL) and induces granulocytic differentiation of APL cells as well as several myeloid leukemia cell lines (4–7). Gene regulation by RA in U937 cells is thus of interest in understanding the mechanism of action of RA on leukemic cells.

The biological effects of RA and VD_3 are mediated largely through their respective nuclear receptors, retinoid receptors (RARs and RXRs) and the vitamin D receptor (VDR). These receptors belong to the steroid/thyroid hormone receptor superfamily of nuclear receptors. RAR, RXR, and VDR are ligand activated transcription factors that control gene transcription by binding as homo- or heterodimers to specific DNA response elements in the regulatory regions of target genes (8). RAR and VDR form heterodimers with RXR. RAR/RXR heterodimers are activated by the all-*trans* form of RA, and VDR/RXR is activated by VD_3. In addition, RXR is also capable of forming RXR/RXR homodimers, which are activated by the all-*trans* RA metabolite, 9-*cis* RA. The targets of these ligand-receptor complexes are of relevance to the mechanism of action of RA or VD_3.

The *blr1* gene is a RA regulated gene in some cellular contexts (9). *blr1* encodes the putative G protein-coupled receptor, Burkitt's lymphoma receptor 1, designated CXCR5, and was originally identified by subtractive hybridization of cDNA libraries of an Epstein Barr virus immortalized lymphoblastoid cell line from a Burkitt's lymphoma cell line (10). Recently, the CXC chemokines, BCA-1 and BLC, were identified as ligands for *blr1*, thus, *blr1* is a CXC chemokine receptor (11,12). Murine knockouts, which depleted mice of *blr1*, indicated that *blr1* is

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involved in directing migration of B lymphocytes (13). The role of *blr1* in myeloid and monocytic cells, however, is not well defined.

blr1 may regulate hematopoietic cell growth and differentiation. RA induces *blr1* mRNA expression in several myeloid-derived human leukemia cell lines, including HL-60, U937, and NB4, prior to the onset of growth arrest or functional differentiation (9), suggesting that *blr1* might play a role in myelomonocytic differentiation. Furthermore, ectopic expression of *blr1* in HL-60 myeloblastic cells caused an increase in activated mitogen activated protein kinase (MAPK). In wild-type HL-60 cells, RA caused an increase in MAPK activation, which is required for induced myeloid differentiation. *blr1* transfected HL-60 cells underwent functional differentiation and growth arrest on RA treatment faster than cells transfected with vector alone. This is presumably in part because increasing MAPK activation predisposed the cell toward differentiation. Thus, through activation of MAPK, *blr1* gene expression might play a role in inducible myeloid differentiation.

Because myeloid and monocytic cells derive from a common precursor, the potential regulatory involvement of *blr1* in myeloid differentiation motivates interest in its possible role in differentiation of a monocytic lineage cell. To determine whether *blr1* signaling regulates RA- and VD_3-induced U937 monocytic differentiation, U937 cells were stably transfected with an expression vector that constitutively expressed *blr1* (U937/*blr1* cells). The results indicated that ectopic expression of *blr1* in U937 cells accelerated RA- or VD_3-induced differentiation, measured by CD11b expression and by inducible oxidative metabolism. Ectopic expression of *blr1* in U937/*blr1* cells caused moderate growth inhibition in response to RA but not VD_3. Without RA or VD_3, *blr1* transfected cells had cell cycle kinetics similar to wild-type U937 cells. These findings suggest that *blr1* plays a regulatory role in inducible monocytic differentiation of these leukemic cells.

Materials and Methods

Cell Culture. Human U937 myeloid leukemia cells were grown in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% non–heat inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY) in a 5% CO_2 humidified atmosphere at 37°C. The cultures were initiated at a density of 0.2 or 0.1 × 10^6 cells/ml in 10 ml cultures every two or three days, respectively. Cell viability was determined by 0.2% trypan blue exclusion and routinely exceeded 95%. For experimental cultures, cells were suspended at a density of 0.2 × 10^6 cells/ml in 30 ml RPMI 1640 medium plus 10% FBS and treated with 1 μM all-*trans* RA (Sigma Chemical Company, St. Louis, MO) or 0.5 μM 1α,25-dihydroxyvitamin D_3 (Solvay Duphar B.V., Weesp, the Netherlands), where indicated.

Northern Analysis. Total RNA was isolated using TriReagent (Molecular Research Center, Inc., Gincinnati, OH) and resolved on 1.0% agarose/6.0% formaldehyde gels. The RNA was transferred to nylon membrane (Amersham, Arlington Heights, IL) and baked at 80°C for 2 hr. Blots were probed for *blr1* expression using a radioactively labeled 713 base pair polymerase chain reaction (PCR) amplified fragment. Following reverse transcription of 10 μg of RNA isolated from RA-treated U937 cells, *blr1* was PCR amplified using *blr1*-specific primers (5'-AGACAGTGACCAGTCTGGTG-3', 5'-GGAAAATCATCTCTGCCCTG-3'), resolved on a 1% agarose gel, and gel purified. The purified *blr1* PCR fragment was radioactively labeled with ^{32}P-dATP using the Random Primed Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Probes were used at a concentration of 1.5 × 10^6 cpm/ml prehybridization/hybridization solution. Prehybridization/hybridizations were performed using ZipHyb hybridization buffer (Ambion, Inc., Austin, TX) for 16 hr. Blots were autoradiographed using BioMax MS film (Eastman Kodak, Rochester, NY) with intensifying screens at –80°C for <1 to 4 days. Northern analysis of *mdr15* expression was performed as previously described (9).

Transfection. The *blr1* open reading frame was subcloned into the pIRES expression plasmid (Clontech, Palo Alto, CA) as described (9). The pIRES/*blr1* plasmid was transfected into the JM101 *E. coli* strain, and a large scale plasmid preparation was performed using the Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA).

A total of 20 × 10^6 U937 cells were washed twice with fresh RPMI 1640 medium and then resuspended in 250 μl RPMI 1640 plus 10% FBS. To the cell suspension, 200 μl of sucrose buffer (272 mM sucrose, 7 mM Na_3PO_4, pH 7.4) and 15 μg pIRES/*blr1* plasmid DNA in 50 μl TE was added. The cells were electroporated using 300 V and a capacitance of 500 μF and then resuspended in 10 ml of RPMI 1640 supplemented with 20% non–heat inactivated FBS. After 48 hr cells were collected by centrifugation, resuspended in RPMI 1640 medium plus 10% FBS containing 1 mg/ml active G418 (Sigma Chemical Company, St. Louis, MO), and then distributed in 24-well plates at 1 × 10^5 cells/well. Medium was exchanged every two to three days. After approximately 19 days of culture, a viable, G418-resistant cell population that stably expressed *blr1* emerged. Vector control cells were established using the same procedure except that 15 μg of pIRES plasmid DNA was electroporated into U937 cells. The U937 vector control cells grew and differentiated in response to RA and VD_3 with kinetics indistinguishable from wild-type U937 cells. The stable transfectants were maintained in 1 mg/ml active G418.

Western Analysis. At the indicated times, 1 × 10^6 cells were harvested, fixed in 90% methanol, and stored at –20°C until used for Western analysis. Fixed cells were collected by centrifugation and lysed by boiling in SDS buffer 5 min prior to gel electrophoresis. Lysates were resolved on 10% SDS-polyacrylamide gels for 15 hr at 75V. Following electrophoresis, proteins were transferred to ni-

trocellulose membrane. Blots were incubated with antiactive ERK1 and ERK2 antibodies that detect the Thr^{183} and Tyr^{185} phosphorylation of the Thr-Glu-Tyr motif in ERK1 and ERK2 (V6671 rabbit polyclonal antibody, Promega, Inc., Madison, WI) or anti-ERK1 and ERK2 antibodies that detect the phosphorylated and unphosphorylated forms of ERK1 and ERK2 (C-14 [SC154 rabbit polyclonal antibody] Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 0.5 mg/ml in phosphate-buffered saline plus 0.05% Tween 20 (PBST) for 1 hr at room temperature. Blots were then incubated with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL) for 30 min at room temperature. Detection was performed using the chemiluminescent ECL kit (Amersham, Arlington Heights, IL).

Growth and Differentiation Assays. Cell growth was measured by cell density and by distribution in the cell cycle. Differentiation was measured by CD11b expression and by inducible oxidative metabolism. For experimental cultures, cells were initiated at a density of 0.2×10^6 cells/ml and treated with 1μM all-*trans* RA or 0.5 μM 1,25-dihydroxyvitamin D_3, where indicated. Cell density was determined by counting cells using a hemacytometer when cells were initiated in culture and every 24 hr thereafter. Viability was determined by trypan blue exclusion. Cell cycle distribution was determined by harvesting and resuspending 0.5×10^6 cells in 500 μl of hypotonic propidium iodide solution (50 mg/L propidium iodide, 1 g/L sodium citrate, and 0.1% Triton X-100). Propidium iodide stained nuclei were stored at 4°C in the dark until analyzed by flow cytometry. Flow cytometric analysis was performed using a multiparameter dual laser fluorescence-activated cell sorter with 200 mW of 488 nm excitation (EPICS; Coulter Electronics, Hialeah, FL). Differentiation was determined by expression of the CD11b myelomonocytic cell surface marker. CD11b expression was measured by flow cytometry using a FITC-conjugated CD11b murine monoclonal antibody (Beckman-Coulter, Fullerton, CA). A total of 0.2×10^6 cells were harvested and incubated with 97.5 μl normal goat serum and 2.5 μl FITC-conjugated CD11b antibody for 30 min on ice in the dark. Cells were washed with normal goat serum three times, resuspended in 400 μl phosphate-buffered saline, and then analyzed by flow cytometry. Untreated cells were used to set a 95% negative threshold. The percentage above the threshold was reported as positive. All histograms shown represent the same number of total, negative plus positive, events. Functional differentiation was assayed by 12-O-tetradeconoylphorbol 13-acetate (TPA)-inducible oxidative metabolism. A total of 0.2×10^6 cells were harvested daily and resuspended in 200 μl of 2 mg/ml nitroblue tetrazolium (NBT) in PBS containing 200 ng/ml TPA. The cell suspension was incubated for 20 min at 37°C with occasional vortexing. Cells were counted using a hemacytometer, and the percentage of cells that expressed intracellular blue formazan was determined. Untreated stock cultures were used for analysis of all zero hour time points.

Results

RA-Induced *blr1* Expression During Differentiation of U937 Cells. Previous studies established that RA induces *blr1* expression by 48 hr of RA or TPA treatment in U937 cells (9). To further characterize the kinetics of *blr1* expression in U937 cells, Northern blot analysis was performed on cells that were left untreated for 12 hr or treated with 1 μM RA for 12, 24, 48, 72, and 96 hr. The expression of *blr1* mRNA increased when U937 cells were induced to undergo monocytic differentiation with RA (Fig. 1A). Although untreated cells show no detectable *blr1* expression, RA treatment causes induction of *blr1* mRNA within 12 hr. The level of mRNA decreases by 24 hr, levels off by 48 hr,

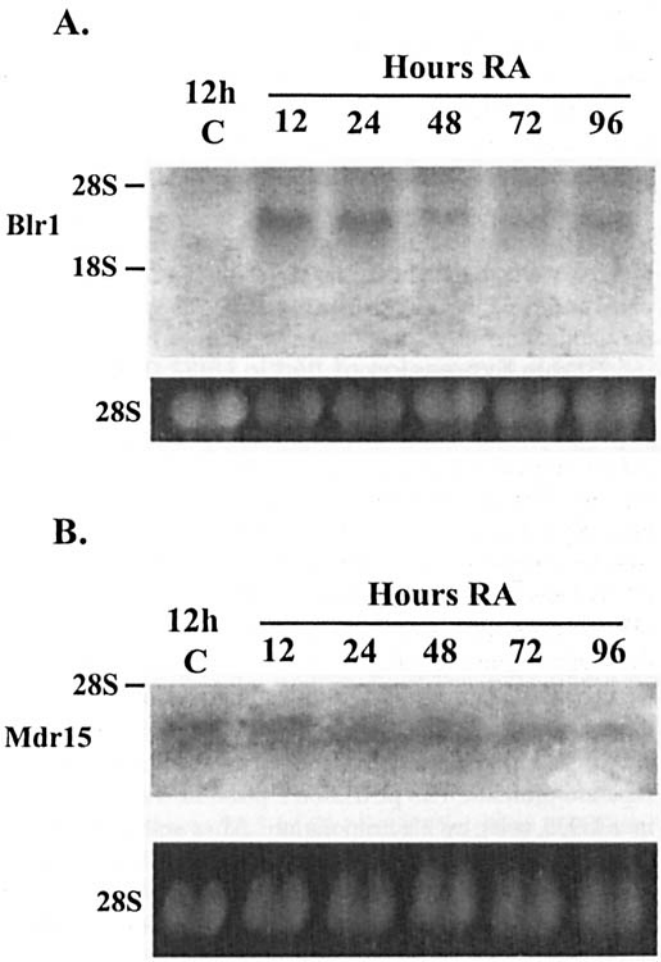

Figure 1. (A) Time-dependent induction of *blr1* mRNA by RA and VD_3 in U937 cells. U937 cells were left untreated for 12 hr or treated with 1 μM RA for 12, 24, 48, 72, and 96 hr. Total RNA was isolated and subjected (25 μg RNA/lane) to Northern blot analysis. The membrane was hybridized with a ^{32}P-labeled *blr1* cDNA probe and followed by exposure to film for one day. Comparable loading was determined by ethidium bromide staining of the 28S rRNA band, as shown in the lower panel. (B) Northern analysis of *mdr15* expression. U937 cells were left untreated for 12 hr or treated with 1 μM RA for 12, 24, 48, 72, and 96 hr. Total RNA was isolated and subjected (25 μg RNA/lane) to Northern blot analysis using a ^{32}P-labeled *mdr15* cDNA probe followed by exposure to film for 14 days.

and is detectable through 96 hr of RA treatment. The maximal level of expression is observed after 12 hr of RA treatment, a time prior to onset of differentiation in U937 cells.

As previously reported, an alternate splice form, monocyte derived receptor 15 (MDR15), can be expressed from the *blr1* gene (14). *blr1* and *mdr15* cDNA sequences are identical except in their 5' region. The two transcripts originate from different start sites in exon 1, which results in *mdr15* being shorter than *blr1* by 45 codons. While the 5' primer used to generate the *blr1* PCR fragment that was used as the probe for the Northern analysis in Figure 1A was specific to *blr1*, the 3' primer was common to both sequences. As a result, the 3' end of the probe could potentially recognize both *blr1* and *mdr15* on the Northern blot. To determine the relative contributions of *mdr15* and *blr1* mRNA in the Northern analysis, a Northern blot was probed with a radiolabeled probe specific to *mdr15*. The *mdr15* probe was hybridized to the Northern blot under identical hybridization conditions and exposed to film for the same amount of time, but no signal was detected. Increasing the exposure time to film from 1 day to 14 days revealed weak *mdr15* expression (Fig. 1B) in the U937 cells that was comparable in both untreated and RA-treated cells. Because the *mdr15* probe revealed *mdr15* expression only after prolonged exposure to film and because there was no induction by RA, we concluded that the differential gene expression observed using the *blrl* probe (Fig. 1A) was specific for the *blr1* splice form.

Stable Expression of *blr1* in U937 Cells. To determine whether the early induction of *blr1* mRNA was important for inducible monocytic differentiation, the *blr1* gene was stably transfected into U937 cells. The *blr1* open reading frame, containing the 372 amino acid sequence that encodes a CXC chemokine receptor, was cloned into the pIRES expression vector (pIRES/*blr1*), which contains the neomycin resistance gene (9). Transcription from the pIRES/*blr1* expression vector produces a continuous RNA transcript containing *blr1*, the internal ribosomal entry site (IRES), and neomycin (NEO) selectable marker sequences, thus the mRNA transcript appears larger than the endogenous *blr1* transcript. *Blr1* and NEO are translated into separate proteins. The pIRES/*blr1* plasmid was transfected into U937 cells by electroporation. After selection with 1 mg/ml G418, a viable cell population emerged after 19 days of culture. *Blr1* is not expressed in wild-type U937 cells, but transfection with the pIRES/*blr1* plasmid results in constitutive expression of *blr1*.

Two lines of evidence indicate that functional *blr1* was stably transfected in G418-resistant cells. First, G418-resistant cells were analyzed for expression of the *blr1*/NEO mRNA transcript by Northern blot analysis. To determine whether G418 resistant cells expressed *blr1*/NEO mRNA, total RNA was harvested from the untreated G418-resistant U937 cells and analyzed for *blr1* expression. Total RNA from U937 cells treated with RA for 48 hr was used as a positive control. The *blr1*/IRES/NEO mRNA transcript was expressed in the G418-resistant cells (Fig. 2A). Stable transfectants, named U937/*blr1* cells, were maintained in 1 mg/ml G418.

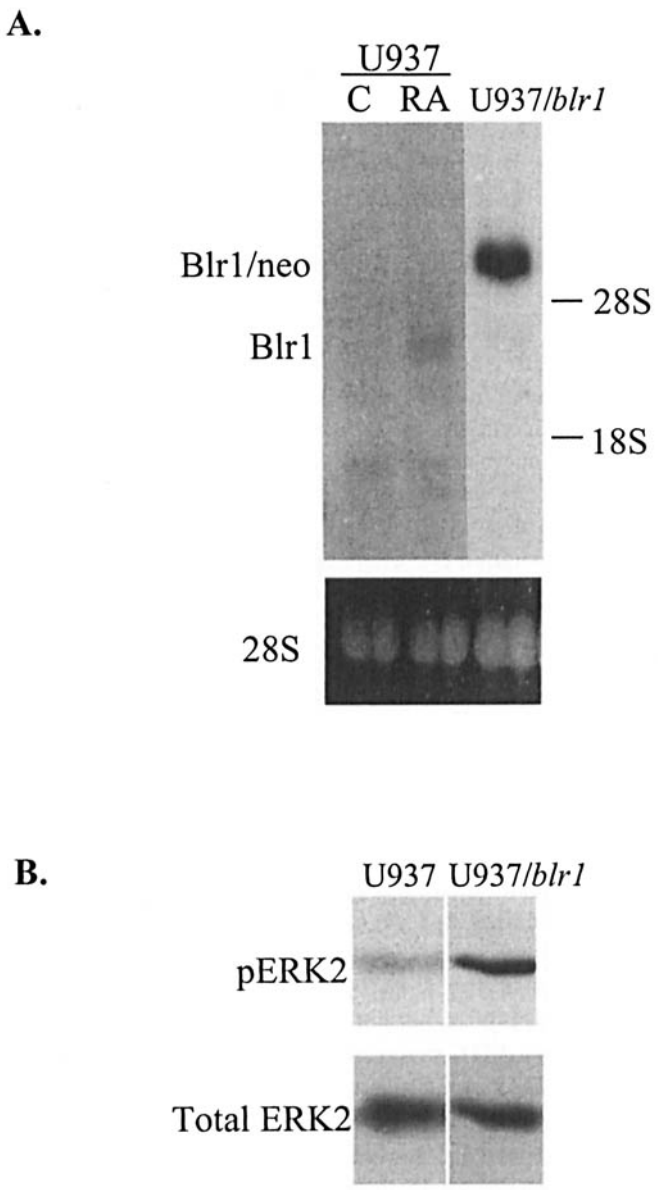

Figure 2. Ectopic expression of *blr1* in U937 cells. U937 cells were transfected with *blr1* cDNA using the pIRES expression plasmid. (A) Northern blot analysis confirming expression of the *blr1*/IRES/NEO transcript in transfected U937 cells. Total RNA was isolated for Northern analysis (25 μg/lane) from G418-resistant U937 cells. The Northern blot was probed for *blr1* mRNA expression using a ^{32}P-labeled *blr1* cDNA probe. The right side of the figure (U937/*blr1*) is a 3.5 hour exposure of the blot to film, whereas the left side (U937, C, RA) is a 3 day exposure of the film. Comparable loading of the lanes was determined by ethidium bromide staining of the 28S rRNA band on the gel. (B) Western analysis for phosphorylated ERK2 (pERK2) and for total ERK2 protein. The gel was loaded with 1×10^6 cells per lane from untreated stock cultures of U937 and U937/*blr1* cells. Blots were probed with an antibody specific for phosphorylated ERK2 protein (upper panel), and then stripped and reprobed with an antibody for total ERK2 protein (lower panel). The upper panel shows increased expression of phosphorylated ERK2 while the lower panel shows the lanes were loaded with comparable amounts of protein. The results in Fig. 2B are representative of four experiments.

The second means for testing whether G418-resistant U937 cells were functional *blr1* transfectants was Western blot analysis for enhanced ERK2 phosphorylation. One of the few known molecular consequences of *blr1* expression is increased phosphorylation of the extracellular signal-regulated kinase, ERK2 (9). This was used as a test of *blr1* transfection. Stable overexpression of *blr1* in U937 cells enhanced activation of ERK2, determined by its phosphorylation at Thr^{183} and Tyr^{185}. Western blot analysis using an antibody specific for the Thr^{183}-Glu^{184}-Tyr^{185} motif characterizing activated ERK1 or ERK2 is shown in Figure 2B. (U937 cells express predominantly ERK2 relative to ERK1 protein. Western analysis of whole cell lysates for total ERK protein reveals only the 42 kD ERK2 protein and no detectable 44 kD ERK1 protein. Consistent with this observation, Western analysis for activated ERK protein detects only activated 42 kD ERK2 protein). The U937/*blr1* cells express more of the phosphorylated form of ERK2 compared with U937 cells (Fig. 2B, upper panel). The blot was stripped and reprobed for total ERK2 protein to ensure that the amount of protein in each lane was comparable (Fig. 2B, lower panel). The significance of phosphorylated ERK2 in U937 cells has not been established; however, ERKs have been shown to mediate a variety of cellular responses, including mitogenesis, differentiation, and survival in hematopoietic cells (15–19).

Accelerated Differentiation in U937/*blr1* Cells Treated with RA and VD_3. To determine whether overexpression of *blr1* affected U937 cell differentiation, U937/*blr1* cells were treated with the differentiation inducers, RA and VD_3. Ectopic expression of *blr1* accelerated functional differentiation in RA- and VD_3-treated U937/*blr1* cells. Differentiation was measured by expression of the CD11b surface antigen and by reduction of nitroblue tetrazolium (NBT), which is indicative of inducible oxidative metabolism, a functional differentiation marker for mature myelomonocytic cells.

CD11b is an early cell surface differentiation marker for mature myeloid and monocytic cells. Undifferentiated U937 cells do not express CD11b, whereas mature monocytic U937 cells express high levels of CD11b. The level of CD11b expression was compared during differentiation of U937 and U937/*blr1* cells. Cells were treated with or without 1 μM RA or 0.5 μM VD_3, and analyzed for CD11b expression by flow cytometry at the indicated times. Figure 3 indicates that after 24 hr of RA treatment, the percentage of U937/*blr1* cells expressing CD11b is higher than the percentage of vector control cells expressing CD11b. Figure 3A shows the flow cytometric histograms and the emergence of the induced CD11b positive cells. Figure 3B shows the percentage of positive cells in the vector control and *blr1* transfected cells. The percentage of CD11b-positive U937/*blr1* cells was approximately twice that of vector control cells. The higher percentage of CD11b-positive cells is maintained through 72 hr of RA treatment. Likewise, there was a higher percentage of CD11b-positive cells in U937/*blr1* cultures treated with VD_3 within 72 hr of VD_3 treatment. Thus, on exposure to differentiation inducers, there was a higher percentage of differentiated U937/*blr1* cells compared with vector control cells. Expression of *blr1* thus accelerated RA- and VD_3–induced differentiation.

Oxidative metabolism, measured by cellular ability to reduce NBT to the blue/black precipitate, formazan, is a marker for functional differentiation characteristic of mature monocytic cells. Ectopic expression of *blr1* in U937 cells accelerated their functional maturation. NBT-reducing activity was examined in U937 vector control and U937/*blr1* cells, following treatment with or without 1 μM RA or 0.5 μM VD_3. Differentiation is expressed as the percentage of formazan positive cells. RA and VD_3 induced a greater percentage of NBT-reducing cells in *blr1* transfectants compared with vector control cells (Fig. 4). By 48 hr RA or VD_3 alone caused a moderate increase in the percentage of NBT-reducing U937 vector control cells. By comparison, U937/*blr1* cells treated with RA or VD_3 showed a higher percentage of functionally differentiated cells by 48 hr. The higher percentage of functionally differentiated U937/*blr1* cells was more prominent by 72 hr. This functional differentiation data corroborates the CD11b cell surface data. These results show that ectopic expression of *blr1* accelerated U937 cell differentiation induced by RA or VD_3 compared to vector control cells.

Proliferative Features of U937/*blr1* Cells. The growth properties of U937/*blr1* cells were compared to those of vector control cells. Ectopic expression of *blr1* had no apparent effect on cell cycle or differentiation. U937 vector control and U937/*blr1* cells were initiated at a density of 0.2×10^6 cells/ml and analyzed for cell number at 24 hr intervals. U937 vector control and U937/*blr1* cells doubled at the approximately the same rate during exponential steady-state growth (Fig. 5). To determine the duration of G_1, S, and G_2/M cell cycle phases for U937 and U937/*blr1* cells, cells were harvested 48 hr after initiation of cell culture when they were undergoing log-linear growth. The distribution of cells in the cell cycle was analyzed by flow cytometry and used to determine the G_1, S, and G_2/M phase durations (20). The exponential doubling time for U937 vector control cells was 22.9 hr, and the doubling time for U937/*blr1* cells was 22.3 hr (Table 1). The phase durations for G1, S, and G2/M were also similar. Expression of *blr1* also had no apparent effect on cell differentiation. There was no increase in the percentage of cells capable of oxidative metabolism either when onset of differentiation typically occurs if induced or later (see Fig. 4). Likewise, there were no changes in CD11b cell surface marker expression (see Fig. 3). These results indicate that ectopic expression of *blr1* alone neither perturbs the cell cycle nor induces differentiation of U937 cells.

Inhibition of U937 Cell Growth by *blr1* in the Presence of RA but not VD_3. Ectopic expression of *blr1* in U937 cells significantly inhibits cell growth in the presence of RA but not VD_3. U937 vector control and

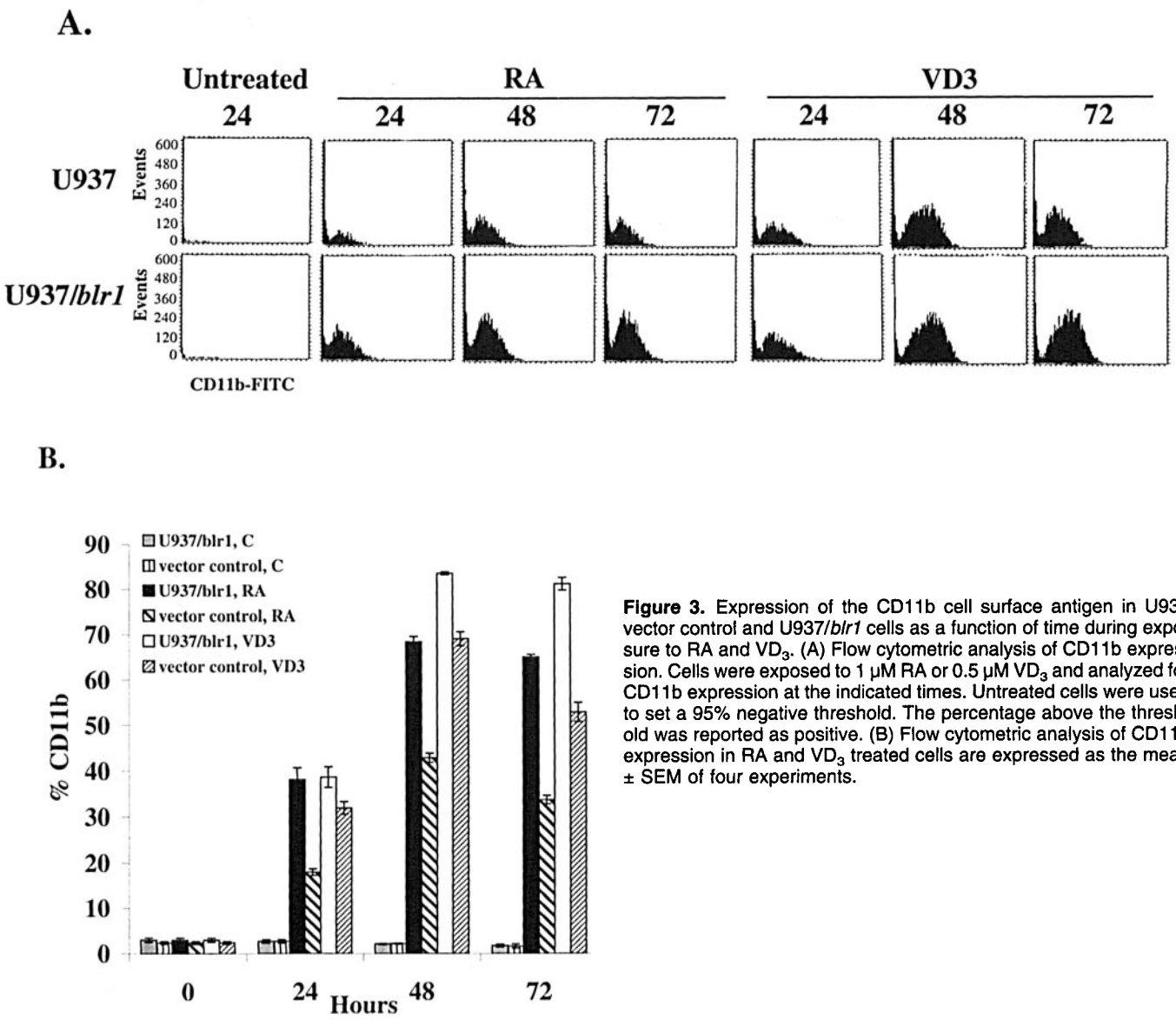

Figure 3. Expression of the CD11b cell surface antigen in U937 vector control and U937/*blr1* cells as a function of time during exposure to RA and VD_3. (A) Flow cytometric analysis of CD11b expression. Cells were exposed to 1 μM RA or 0.5 μM VD_3 and analyzed for CD11b expression at the indicated times. Untreated cells were used to set a 95% negative threshold. The percentage above the threshold was reported as positive. (B) Flow cytometric analysis of CD11b expression in RA and VD_3 treated cells are expressed as the mean ± SEM of four experiments.

U937/*blr1* cells were cultured with or without 1 μM RA or 0.5 μM VD_3, for 96 hr. Cell growth was determined by hemacytometer cell counts at the indicated times. The concentrations of RA and VD_3 used caused little growth inhibition of the vector control cells. Although untreated U937/*blr1* cells grew at the same rate as the vector control U937 cells, RA-treated U937/*blr1* cells (Fig. 6A) underwent modest growth inhibition. There was an insignificant effect on cell growth in VD_3-treated U937/*blr1* cells (Fig. 6B). Table II shows the distribution in the G1, S, and G2/M phases for these cells. Distribution in the cell cycle was determined by measuring the DNA content of propidium iodide-stained nuclei by flow cytometry. Viability assessed by trypan blue exclusion was greater than 95% in all cultures, and treated U937/*blr1* cells showed no apparent loss of viability. After 72 hr of RA treatment U937 vector control and U937/*blr1* cells had the same percentage in each phase of the cell cycle. At 96 hr there was a slight accumulation of RA-treated *blr1* transfectants in the G_1/G_0 phase of the cell cycle, consistent with their modest growth inhibition, compared with RA-treated U937 vector control cells or untreated cells. After 72 and 96 hr of VD_3 treatment U937/*blr1* and vector control cells showed similar modest G_1/G_0 accumulation, indicating no *blr1*-attributable difference between them, but there was no significant reduction in cell growth. This indicates that although there is no significant difference in growth rate due to VD_3, there are cell cycle effects. The only case in which growth inhibition occurred was in RA-treated cells expressing *blr1*. RA induced modest G1 enrichment and caused growth inhibition in cells expressing *blr1*. In the case of VD_3 the cell cycle effects are independent of *blr1* expression. VD_3 induced G1-enrichment but it was uncoupled from any apparent growth inhibition. Ectopic expression of *blr1* thus enabled RA but not VD_3 to retard cell growth.

Discussion

The identification of genes that regulate RA- and VD_3-induced differentiation of hematopoietic cells is of rel-

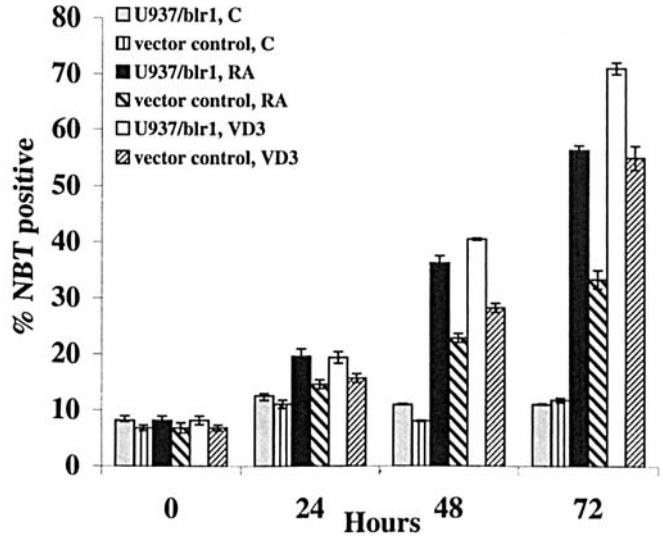

Figure 4. Functional differentiation of RA- and VD_3 -treated U937 vector control and U937/*blr1* cells. U937 vector control and U937/*blr1* cells were treated with 1 μM RA or 0.5 μM VD_3. Differentiation was determined by incubating 0.2 × 10^6 cells with 2 mg/ml NBT containing 200 ng/ml TPA at 37°C for 20 min. At least 200 cells in each sample were counted to determine the percentage of cells expressing the blue/black precipitate, formazan. Values are the mean ± SEM of four experiments.

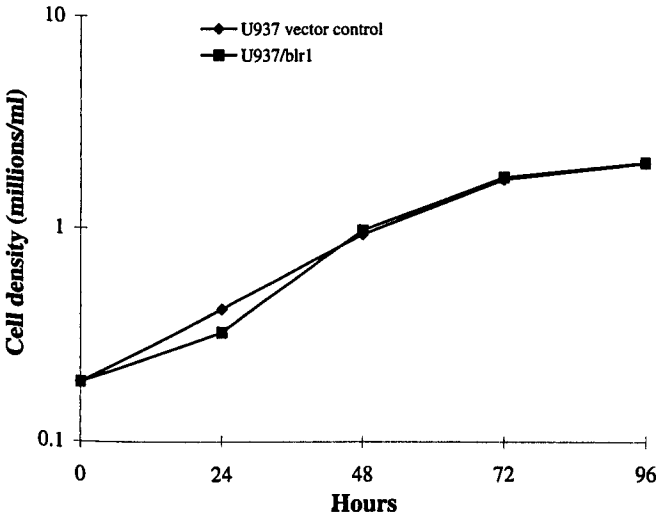

Figure 5. Comparison of growth rates of vector control U937 and U937/*blr1* cells. *blr1* transfectants (U937/*blr1*) and vector control cells were initiated in culture and counted at the indicated times using a hemacytometer. Cell density was plotted on a logarithmic axis as a function of time in culture. During logarithmic growth the slope ($\ln 2/T_D$, where T_D = doubling time) is comparable for both U937 and U937/*blr1* cells, indicating similar growth rates.

evance to a molecular understanding of hematopoiesis and differentiation therapy of leukemia. The early targets of such differentiation inducers are not well characterized. The present study demonstrates that the *blr1* gene is induced by RA during U937 cell differentiation prior to onset of any apparent differentiation and growth arrest. Furthermore, the results show that ectopically expressed *blr1* cooperates with the differentiation inducers, RA and VD_3, to accelerate differentiation of the human U937 monocytic leukemia cell

Table I. Comparison of the cell cycle kinetics of U937 vector control and U937/*blr1* cells

	T_D (hours)[A]	Phase durations (hours)[B]		
		G_1	S	G_2/M
U937 vector control cells	22.9	9.7	11.1	2.1
U937/*blr1* cells	22.3	8.2	11.6	2.5

[A] T_D = Doubling time

[B] Phase durations were calculated as previously described (20). The distribution of cells in the cell cycle was determined by flow cytometry using cells in steady-state exponential growth. The percentage of U937 vector control cells in G_1, S, and G_2/M was 51.0%, 42.5%, and 6.5%, respectively; the percentage of U937/*blr1* cells in G_1, S, and G_2/M was 49.1%, 42.7%, and 8.2%.

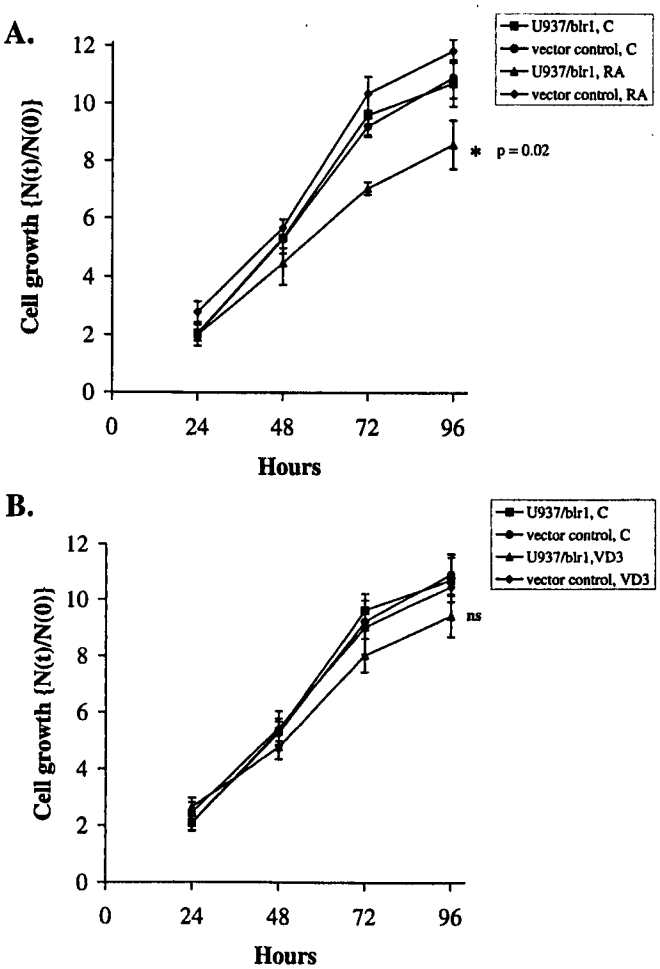

Figure 6. Growth rates of U937 vector control and U937/*blr1* cells treated with RA and VD_3. U937 vector control and U937/*blr1* cells were grown in the presence or absence of 1 μM RA (A) or 0.5 μM VD_3 (B). Relative cell density is shown as a function of time in culture. N(t)/N(0) is the number of cells at the indicated time, t, divided by the number of cells at time 0 hour. Values are the mean ± SEM of three experiments. Statistically significant differences from control values are indicated (*); ns, not statistically significant.

line. The ectopic *blr1* also retarded the growth of RA-treated cells. This suggests that the biological significance of the induction of *blr1* may be to initiate G protein-

Table II. Cell cycle distribution of RA- and VD_3-treated U937/*blr1* and U937 vector control cells

Hours after addition of inducer	% of cells in indicated cell cycle phase					
	72			96		
	G_1/G_0[A]	S	G_2/M	G_1/G_0	S	G_2/M
Cell line						
U937/*blr1* C	61.4 ± 0.9	33.3 ± 1.1	5.3 ± 0.4	67.9 ± 2.4	24.6 ± 6	7.5 ± 1.1
U937 vector control, C	61.5 ± 0.9	33 ± 0.7	5.5 ± 0.4	67.1 ± 0.9	26.9 ± 1.1	6.0 ± 0.4
U937/*blr1* RA	61.2 ± 0.7	32.6 ± 0.6	6.1 ± 0.1	72.2 ± 0.4	24.5 ± 0.3	3.3 ± 0.1
U937 vector control, RA	60.4 ± 1.3	33 ± 0.7	5.4 ± 0.6	65.5 ± 0.4	30.4 ± 0.1	4.1 ± 0.3
U937/*blr1* VD_3	73.4 ± 2.4	21 ± 2	3.3 ± 0.1	85.7 ± 1.2	10.5 ± 1.6	3.8 ± 0.4
U937 vector control, VD_3	74.3 ± 2.2	20.8 ± 2	4.1 ± 0.3	85.2 ± 0.6	11.6 ± 0.6	3.3 ± 0.1

[A] G_1/G_0 percentages of U937/*blr1* and U937 vector control cells treated with 1 μM RA or 0.5 μM VD_3 for the indicated times were measured by flow cytometric analysis of the percentage of propidium iodide-stained nuclei with G_1/G_0 DNA content. Values are the mean ± SEM of four experiments.

mediated signals that propel hematopoietic differentiation.

The early induction of *blr1* is common to inducible myeloid and monocytic differentiation. *blr1* expression was shown to be induced by RA during myeloid differentiation of the HL-60 human myeloblastic leukemia cell line and the acute promyelocytic leukemia-derived NB4 cell line (9). In HL-60 cells, *blr1* expression was induced by 12 hr of RA treatment, and its expression was insensitive to the protein synthesis inhibitor, cycloheximide. Likewise, *blr1* mRNA is induced after 12 hr of RA treatment in U937 cells, which is also when its cellular expression level is maximal. In U937, HL-60, and NB4 cells, *blr1* is induced well before cells undergo measurable differentiation changes, such as G_1/G_0 growth arrest or functional differentiation. *blr1* induction thus appears to be a common early event in RA- and VD_3-induced myeloid and monocytic differentiation.

The *blr1* gene encodes a seven-transmembrane-spanning, heterotrimeric G protein-coupled receptor, the constitutive overexpression of which facilitates U937 differentiation. When *blr1* was stably transfected into U937 monoblastic leukemia cells, the transfectants did not differentiate or grow differently compared with vector control transfected U937 cells. The effect of ectopically expressed *blr1* was more readily apparent when cells were treated with RA or VD_3. RA and VD_3 are fairly weak inducers of U937 differentiation when used singularly, but ectopic *blr1* expression enhances their effects. After exposure to RA or VD_3 for 24 hr expression of CD11b was augmented in U937/*blr1* cells compared with vector control U937 cells. CD11b expression was at least 2-fold higher in RA-treated U937/*blr1* cells. Inducible oxidative metabolism was also used to assess maturation of RA- and VD_3-treated U937 vector control cells and *blr1* transfectants. By 48 hr of RA or VD_3 treatment a higher percentage of NBT-reducing cells was detected in U937/*blr1* cells compared with vector control U937 cells. Ectopically expressed *blr1* also resulted in growth inhibition in RA- but not VD_3-treated transfectants, which was apparent by approximately 72 hr of treatment. The growth inhibition largely reflected a modest enrichment of G1 cells. Because ectopic expression of *blr1* had these biological consequences, these results further indicate that the amount of receptor is rate limiting, and a surplus of ligand appears to be in the serum supplemented medium.

The role of the increased ERK2 activation in *blr1* transfectants remains unclear due to the poorly understood role of ERK2 in U937 differentiation. Pretreatment of U937 cells with PD98059 blocked TPA-induced differentiation (21), suggesting that ERK2 activation is necessary for TPA-induced monocytic differentiation of U937 cells. However, activation of ERK2 was not sufficient to induce U937 differentiation because transfection of U937 cells with constitutively active MAPK kinase (MEK1) mutants did not elicit differentiation (22). In HL-60 cells RA caused an increase in ERK2 MAPK activity, which was necessary for RA-induced myeloid differentiation (15). Ectopic expression of *blr1* in HL-60 cells increased ERK2 activation and also enhanced induced differentiation (9). This suggests that activation of ERK2 in U937/*blr1* cells had a causal role in accelerating their differentiation. However, 24 hr of RA or VD_3 treatment of U937 or U937/*blr1* cells induced no enhancement of ERK2 activation detectable by Western analysis using an antibody specific for activated ERK2 (unpublished observations). It is not apparent why ectopic *blr1* expression resulted in ERK2 activation, but RA-induced *blr1* expression resulted in no change in ERK2 activation. Ectopic *blr1* thus caused enhanced ERK2 activation in U937 cells, which may have enhanced the effects of RA or VD_3, but other signaling effects due to *blr1* may well be significant.

The effect of ectopically expressed *blr1* on differentiation was more pronounced than the effect on growth arrest, suggesting that *blr1* is more important for eliciting differentiation than growth arrest. These effects fit the model for inducible differentiation of hematopoietic cells in which differentiation and growth arrest can be uncoupled, with specific signals propelling each. In HL-60 cells the cellular response to RA consists of a series of signal thresholds during which cell differentiation is elicited first, followed by RB hypophosphorylation, and ultimately, cell cycle-specific growth arrest (23). At the concentrations used in this study, RA and VD_3 induced moderate cellular responses in U937

cells; however, the constitutive overexpression of *blr1* potentiated the effects of RA and VD_3, causing cells to differentiate faster without inducing significant RB hypophosphorylation (unpublished observations) or prominent G_1/G_0 growth arrest. The finding that ectopic *blr1* expression increases the amount of activated ERK2 and also enhances RA-induced differentiation, but not G_1/G_0 growth arrest, is consistent with earlier findings that RA-induced differentiation of HL-60 cells is more strongly dependent on ERK2 activation than G_1/G_0 growth arrest is (24). This suggests a mechanism of U937 differentiation in which the chemokine receptor encoded by the *blr1* gene mediates a signal that facilitates cell differentiation but not significant G_1/G_0 growth arrest.

The results presented in this study support previous findings that suggest RA works partly through various heterotrimeric G protein-coupled receptors. P19 mouse embryonal carcinoma cells, which undergo endodermal differentiation in response to RA, acquire endodermal characteristics when transfected with constitutively active forms of $G_{\alpha 13}$ (25). During RA-induced differentiation of HL-60 cells, the heterotrimeric subunit, $G_{\alpha s}$, decreases, and ectopic expression of constitutively active $G_{\alpha s}$ blocks myeloid differentiation (26). Activation of the prostaglandin EP2 receptor with the agonist butaprost potentiated RA-induced differentiation of HL-60 cells, presumably through adenylyl cyclase activity (27). The downregulation of $G_{\alpha i2}$ by RA in F9 teratocarcinoma stem cells was shown to regulate their differentiation into primitive endoderm (28). The signaling pathways initiated by G proteins that are related to RA-induced differentiation in U937 cells are poorly understood. The results presented in this study indicate that *blr1* has a regulatory function in RA- and VD_3-induced U937 differentiation.

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