

# 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>-induced Myeloid Cell Differentiation Is Regulated by a Vitamin D Receptor–Phosphatidylinositol 3-Kinase Signaling Complex

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## Summary

1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (D<sub>3</sub>) promotes the maturation of myeloid cells and surface expressions of CD14 and CD11b, markers of cell differentiation in response to D<sub>3</sub>. To examine how these responses are regulated, THP-1 cells were grown in serum-free medium and incubated with D<sub>3</sub>. This was associated with rapid and transient increases in phosphatidylinositol 3-kinase (PI 3-kinase) activity. Furthermore, induction of CD14 expression in response to D<sub>3</sub> was abrogated by (a) the PI 3-kinase inhibitors LY294002 and wortmannin; (b) antisense oligonucleotides to mRNA for the p110 catalytic subunit of PI 3-kinase; and (c) a dominant negative mutant of PI 3-kinase. In THP-1 cells, induction of CD11b expression by D<sub>3</sub> was also abrogated by LY294002 and wortmannin. Similarly, LY294002 and wortmannin inhibited D<sub>3</sub>-induced expression of both CD14 and CD11b in peripheral blood monocytes. In contrast to CD14 and CD11b, hormone-induced expression of the Cdk inhibitor p21 in THP-1 cells was unaffected by either wortmannin or LY294002. These findings suggest that PI 3-kinase selectively regulates D<sub>3</sub>-induced monocyte differentiation, independent of any effects on p21.

Pretreatment of THP-1 cells with antisense oligonucleotides to the vitamin D receptor (VDR) mRNA abrogated both activation of PI 3-kinase in response to D<sub>3</sub> and hormone-induced CD14 expression. Moreover, both Western blots and in vitro kinase assays carried out on immunoprecipitates of the VDR showed that D<sub>3</sub> treatment brought about formation of a complex containing both PI 3-kinase and the VDR. These findings reveal a novel, nongenomic mechanism of hormone action regulating monocyte differentiation, in which vitamin D<sub>3</sub> activates a VDR- and PI 3-kinase-dependent signaling pathway.

**Key words:** myeloid cell • differentiation • vitamin D<sub>3</sub> • phosphatidylinositol 3-kinase

The steroid hormone 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (D<sub>3</sub>)<sup>1</sup> plays critical roles in regulating numerous cellular and physiological responses. In addition to its well-established importance in plasma calcium homeostasis (1, 2) and bone resorption (3, 4), D<sub>3</sub> also plays a functional role in the hemopoietic system. D<sub>3</sub> modulates the expression of

several genes in promonocytic cell lines, thereby regulating their differentiation (5). Indeed, when myeloid leukemia cells such as HL60, U937, THP-1, and M1 are incubated with D<sub>3</sub>, they differentiate into cells expressing functional properties and differentiation markers of monocytes and/or macrophages, including CD14 and CR3 (6–9).

The pleiotropic effects of D<sub>3</sub> are principally mediated through the intracellular vitamin D receptor (VDR). The VDR is a member of the superfamily of nuclear steroid, thyroid, and retinoic acid receptors. As such, it functions as a ligand-dependent transcription factor, regulating the activation of vitamin D-responsive target genes (10). At the molecular level, the VDR activates its target genes via in-

<sup>1</sup>Abbreviations used in this paper: D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; ECL, enhanced chemiluminescence; IGF, insulin-like growth factor; MAP, mitogen-activated protein; MFI, mean fluorescence intensity; PI 3-kinase, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol 3-phosphate; RT, reverse transcription; S-oligo, phosphorothioate-modified oligonucleotide; VDR, vitamin D receptor; VDRE, vitamin D-responsive element.

teractions with specific DNA sequences designated as vitamin D-responsive elements (VDRE) (11–13), a mode of signaling referred to as “genomic action.”

CD14 is a 55-kD glycoprotein originally described as a differentiation marker of monocytes (14). Extensive research has demonstrated that CD14 is involved in mediating responses to several bacterial cell wall products, most notably bacterial LPS (15–18). CD14 has other pleiotropic effects including, but not limited to, regulating IL-2-induced monocyte tumoricidal activity (19), monocyte resistance to HIV (20), and the adhesive capacity of the molecule LFA-1 (21, 22). As CD14 is inserted into the cell membrane through a glycosylphosphatidylinositol anchor (23), the mode of signal transduction through this molecule has been intensively investigated. Recent reports indicate a *Drosophila* Toll-like membrane protein acts as a cell surface coreceptor to mediate signaling after ligand binding to CD14 (24, 25).

CD14 is undetectable on the surface of monocytic precursors, and increases dramatically during their differentiation into monocytes (26–28). Therefore, surface expression of CD14 is an excellent model in which to study the mechanisms of myeloid cell maturation regulated by  $D_3$ . Induction of CD14 expression in response to  $D_3$  occurs at the level of gene transcription and requires new protein synthesis (28, 29). However, a curious paradox of this and other systems is that VDRE sequences have not been identified in the promoter regions of many  $D_3$ -inducible genes, including CD14 (10, 28). Hence, the regulatory events leading to CD14 expression in response to  $D_3$  remain largely unknown.

The  $\beta_2$  integrin CR3 is another classical marker of monocyte differentiation that is involved in cell adhesion and also functions as a complement receptor (6–9). CD11b, the  $\alpha$  subunit of CR3, is a 160-kD glycoprotein that associates noncovalently with a  $\beta_2$  subunit partner, CD18. CD11b–CD18 is expressed by mature myeloid cells, and is widely used as an early monocyte differentiation marker (30). Vitamin  $D_3$  has been shown to increase cell surface expression of CD11b by HL-60 (30, 31), U937 (31), and THP-1 cells (8).

In various attempts to define the molecular mechanisms regulating cellular responses to  $D_3$ , efforts have been made to identify alternative signaling pathways to the classical mode of genomic action. Indeed, during the past decade, experimental evidence for “nongenomic” signaling has challenged the concept of exclusive VDR-mediated genomic action. For example,  $D_3$  has been shown to stimulate the rapid formation of second messengers including ceramides, cAMP, inositols, and calcium, and to activate a variety of protein kinases including protein kinase C, Raf, mitogen-activated protein (MAP) kinase, and Src family kinases (10, 32–34). However, the physiological importance of nongenomic signaling by itself or relative to VDR-mediated genomic action is still unclear. Moreover, it is not known whether  $D_3$  uses the classical VDR for nongenomic signaling, or whether an alternative receptor system is involved.

Phosphatidylinositol 3-kinase (PI 3-kinase), a lipid kinase

composed of a Src homology 2 domain-containing regulatory subunit (p85) and a 110-kD catalytic subunit (p110), catalyzes the formation of inositol phospholipids phosphorylated at the D3 position of PI. Although PI 3-kinase is known to be important in a wide variety of cellular processes, including intracellular trafficking, organization of the cytoskeleton, cell growth and transformation, and prevention of apoptosis (35, 36), its potential role in  $D_3$ -induced monocyte differentiation remains largely unknown. In this regard, in a recent series of experiments from this laboratory that examined monocyte differentiation, it was observed that expression of a dominant negative mutant of PI 3-kinase in U937 cells abrogated  $D_3$ -induced CD14 expression (Herrera-Velit, P., Z. Hmama, and N. Reiner, unpublished data). This finding provided direct evidence to suggest that monocyte differentiation in response to  $D_3$  may be PI 3-kinase dependent. This study investigated the role of PI 3-kinase and the VDR in regulating monocyte differentiation in response to  $D_3$ . The results obtained show that  $D_3$ -induced expression of the monocyte differentiation markers CD14 and CD11b requires PI 3-kinase, and that hormone treatment induces formation of a signaling complex in which the VDR associates with PI 3-kinase. Activation of PI 3-kinase represents a novel pathway for  $D_3$  signaling regulating monocyte differentiation, and suggests a mechanism of action for the VDR distinct from classical genomic signaling.

## Materials and Methods

**Reagents and Chemicals.** RPMI 1640, HBSS, and penicillin/streptomycin were from StemCell Technologies, Inc. Wortmannin, 1- $\alpha$ -phosphatidylinositol, PMSF, leupeptin, pepstatin A, and aprotinin were purchased from Sigma Chemical Co. LY294002 and microcystin were from Calbiochem Corp. Protein A-agarose and electrophoresis reagents were purchased from Bio-Rad Laboratories. [ $\gamma$ - $^{32}$ P]ATP was from Nycomed Amersham Plc.

**mAbs.** The following mAbs were used: 3C10 (mouse IgG<sub>2b</sub>, anti-CD14 mAb; a gift from Dr. W.C. Van Voorhis, University of Washington, Seattle, WA); W6/32 (mouse IgG<sub>2a</sub>, anti-HLA class I mAb; American Type Culture Collection); UB93-3 (mouse mAb to PI 3-kinase; Upstate Biotechnology Inc.) and 9A7 (rat IgG<sub>2b</sub>, anti-VDR; Chemicon International). Other Abs used included anti-CD14 and anti-CD11b, both murine IgG<sub>1</sub> (SC-7328 and SC-1186, respectively; Santa Cruz Biotechnology, Inc.). Murine IgG<sub>1</sub> isotype control MG100 was from Caltag Laboratories.

**Cell Lines.** The promonocytic cell line THP-1 was from the American Type Culture Collection. The promonocytic cell line U937 transfected with cDNA, encoding the entire coding region of either wild-type bovine PI 3-kinase subunit p85 $\alpha$  (Wp85 $\alpha$ ) or mutant bovine p85 $\alpha$ , ( $\Delta$ p85 $\alpha$ ) has been described (37). The mutant has a deletion of 35 amino acids from residues 479–513 of bovine p85 $\alpha$  and the insertion of two other amino acids (Ser-Arg) in the deleted position. Mutant p85 $\alpha$  competes with native p85 for binding to essential signaling proteins, thereby acting as a dominant negative mutant (38). THP-1 and U937 cell lines were cultured in RPMI 1640 supplemented with 10% FCS (HyClone), 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Cell density was maintained at a concentration of  $<5 \times 10^5$ /ml.

**Isolation and Culture of Human Monocytes.** Peripheral blood mononuclear cells were isolated as described previously (39). Monocytes were allowed to adhere for 1 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Nonadherent cells were removed by three washes with HBSS. Adherent cells were immediately treated and used for cell surface phenotype analysis.

**Cell Surface Phenotype Analysis.** To measure the expression of surface molecules, cells were incubated with specific mouse mAb or irrelevant isotype-matched IgG (10 µg/ml) for 30 min, then washed twice and labeled with FITC-conjugated F(ab')<sub>2</sub> sheep anti-mouse IgG (Sigma Chemical Co.) for 30 min. Cells were then washed twice and fixed in 2% paraformaldehyde in staining buffer. All staining and washing procedures were performed at 4°C in HBSS containing 0.1% NaN<sub>3</sub> and 1% FCS. Cell fluorescence was analyzed using a Coulter Elite flow cytometer. Relative fluorescence intensities of 5,000–10,000 cells were recorded as single-parameter histograms (log scale, 1,024 channels, 4 log decades), and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI indices which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.

**In Vitro PI 3-Kinase Assay.** Cell lysates for analysis of PI 3-kinase were prepared in 20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM NaF, 100 nM microcystin, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin. Aliquots of lysates adjusted for protein concentration (300–500 µg protein) were incubated for 2–4 h at 4°C with UB93-3 mAb (anti-PI 3-kinase), and immune complexes were adsorbed onto protein A-agarose for 30–60 min. The complexes were washed twice with lysis buffer and three times with 10 mM Tris-HCl, pH 7.4. PI 3-kinase activity was measured as described (22, 40). In brief, immunoprecipitates were incubated for 10 min at 4°C with 10 µg of sonicated (3 times for 20 s in an ultrasonic cell disrupter; Branson Sonic Power Co.) 1- $\alpha$ -phosphatidylinositol in 10 µl of 30 mM Hepes, to which was added 40 µl of kinase assay buffer (30 mM Hepes, 30 mM MgCl<sub>2</sub>, 200 µM adenosine, 50 µM ATP, and 10 µCi of [ $\gamma$ -<sup>32</sup>P]ATP). Reactions were carried out for 15 min at room temperature, and stopped by the addition of 100 µl of 1 N HCl and 200 µl of chloroform/methanol (1:1, vol/vol). Lipids were separated on oxalate-treated silica TLC plates using a solvent system of chloroform/methanol/water/28% ammonia (45:35:7.5:2.5, vol/vol/vol/vol). Plates were exposed to X-ray film at –70°C. Incorporation of radioactivity into lipids was measured by excising the corresponding portions of the TLC plate, followed by liquid scintillation counting. Alternatively, cell lysates were incubated overnight at 4°C with 9A7 mAb (anti-VDR), and immune complexes were adsorbed onto protein A-agarose, washed in lysis buffer containing 200 mM NaCl, and assayed for PI 3-kinase activity as described above.

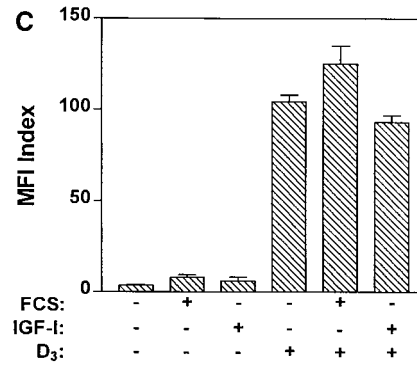
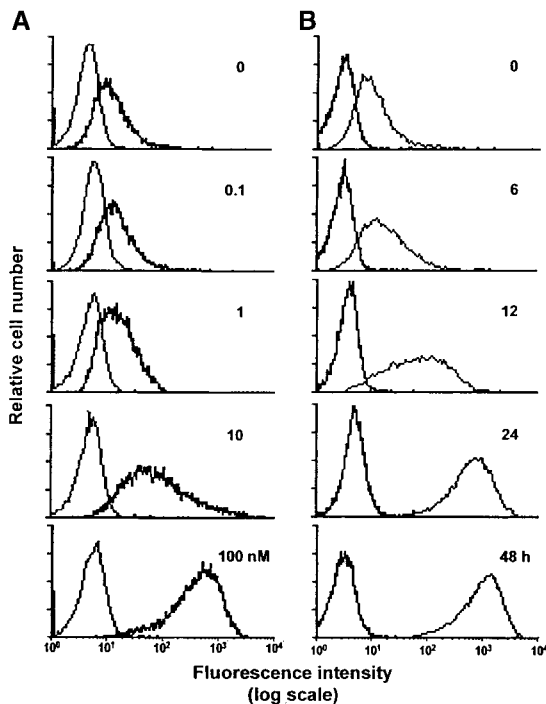
**RNA Isolation and Reverse Transcription PCR.** RNA isolation, cDNA synthesis, and PCR conditions were as described previously (41). Sequences (5' to 3') of oligonucleotide primers used in PCR amplifications were as follows: CD14 sense, CCC AAG CTT GGG CAG AGG TTC GGA AGA CTT ATC G; CD14 antisense, GGG GTA CCC CTT GAC CGT GTC AGC ATA CTG CC (29); Cdk inhibitor p21 sense, TTC TCC TTT TCC TCT CTC C; p21 antisense, TCT ACT CCC CCA TCA TAT ACC;  $\beta$ -actin sense, CAC CCC GTG CTG CTG ACC GAG GCC;  $\beta$ -actin antisense, CCA CAC GGA GTA CTT GCG CTC AGG (41). Controls included in the reverse transcription (RT)-PCR reactions were no RNA and RNA without RT, and different cycle numbers of PCR reactions were performed to ensure linear cDNA amplification.

**Sense and Antisense Oligonucleotides.** Phosphorothioate-modified oligonucleotides (S-oligos) to VDR and p110 subunit of PI 3-kinase were synthesized by Life Technologies, Inc. The oligonucleotides were phosphorothioate-modified to limit intracellular degradation, and purified by high-performance liquid chromatography to remove incomplete synthesis products. 21-mer sequences, including the presumed translation initiation site of human cDNA sequences corresponding to the VDR (42) and the  $\alpha$  isoform of the p110 subunit of PI 3-kinase (43), were made in both sense and antisense orientations with the following sequences: VDR sense, 5'-ATG GAG GCA ATG GCG GCC AGC-3'; VDR antisense, 5'-GCT GGC CGC CAT TGC CTC CAT-3'; p110 sense, 5'-ATG CCT CCA AGA CCA TCA TCA-3'; and p110 antisense, 5'-TGA TGA TGG TCT TGG AGG CAT-3'. The sequences were selected by screening for uniqueness using Blast 227, and were also tested for lack of secondary structure and pairing by using Primer Software (v. 2.0). 3–7  $\times$  10<sup>6</sup> THP-1 cells were suspended in 500 µl RPMI containing 2.5% lipofectAMINE (Life Technologies, Inc.) and 5 µM S-oligo, and incubated on a rotary shaker for 4 h at 37°C. In addition, fluorescent-modified antisense and sense and flow cytometry were used to monitor S-oligo incorporation into cells. After this incubation, the medium was brought up to 5–10 ml, and cells were cultured for an additional 18 h at 37°C and 5% CO<sub>2</sub>.

**Immunoprecipitation and Western Blotting.** To examine a potential association between the VDR and PI 3-kinase, D<sub>3</sub>-treated cells were lysed in the buffer used to prepare cell lysates for PI 3-kinase assay, then immunoprecipitated with specific mAb to VDR. Immunoprecipitates were washed with lysis buffer containing 200 mM NaCl to minimize nonspecific protein-protein interactions. Samples were then analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI 3-kinase subunit as described previously (40). To measure the effects of cell treatments with antisense S-oligos on protein levels of VDR, whole-cell lysates prepared by boiling in Laemmli buffer were subjected to SDS-PAGE and immunoblotting with the mAb 9A7. Membranes were developed by enhanced chemiluminescence (ECL) as described (44).

## Results

**D<sub>3</sub>-induced CD14 Expression in THP-1 Cells.** Initial experiments involving immunofluorescence and flow cytometric analysis defined the experimental conditions for D<sub>3</sub>-induced CD14 expression by THP-1 cells. THP-1 cells maintained in complete medium expressed nearly undetectable levels of CD14 on the surface, and treatment with hormone caused a dose- and time-dependent increase in CD14 expression (Fig. 1). A response was obtained in the presence of as little as 0.1 nM D<sub>3</sub> (Fig. 1 A), and at 100 nM, virtually all cells expressed CD14 at an ~50-fold increase in MFI. The kinetics of induction of CD14 in response to 100 nM D<sub>3</sub> is shown in Fig. 1 B. CD14 expression increased progressively with time and was maximal (55-fold increase in MFI index, average of 2 separate experiments) after 48 h. Since a 24-h exposure to 100 nM D<sub>3</sub> was sufficient to induce a high level of surface CD14 (53-fold increase in MFI index, average of 2 separate experiments) in nearly 100% of cells, subsequent experiments were carried out under these conditions.



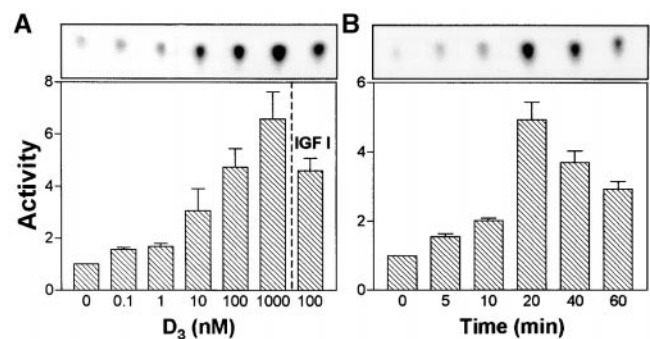
**Figure 1.** Induction of CD14 expression in response to D<sub>3</sub>. (A) THP-1 cells were incubated in RPMI 1640 containing 10% FCS for 24 h with the indicated concentrations of D<sub>3</sub>. (B) Cells were incubated in the same medium with 100 nM D<sub>3</sub> for the indicated times. (C) Serum-starved cells were incubated for 24 h either in serum-free medium, or in medium supplemented with either 10% FCS, IGF-I (100 ng/ml), D<sub>3</sub> (100 nM), 10% FCS + D<sub>3</sub>, or IGF-I + D<sub>3</sub>. At the end of incubation, cells were washed with staining buffer and labeled for 30 min at 4°C with either 3C10 (anti-CD14) mAb or irrelevant IgG2b. Cells were washed and stained with FITC-conjugated F(ab)<sub>2</sub> sheep anti-mouse IgG. Samples were washed and fixed in 2% paraformaldehyde before flow cytometric analysis. Results in (A) and (B) are expressed as histograms of fluorescence intensity. Histograms displaced to the right represent cells stained with 3C10, and histograms on the left represent cells stained with irrelevant IgG2b. Data presented correspond to one of two independent experiments yielding similar results. In C, results are means ± SEM (*n* = 3 independent experiments) of MFI indices which correspond to the following ratio: MFI of cells incubated with specific Ab/MFI of cells stained with irrelevant Ab.

Other experiments examined whether the CD14 response of cells to D<sub>3</sub> (100 nM, 24 h) was serum dependent. Results obtained in three independent experiments (Fig. 1 C) using RPMI 1640 alone showed that THP-1 cells were fully responsive for CD14 induction (MFI index = 104.4 ± 5.1, mean ± SEM). Addition of FCS had only a limited additive effect (MFI index = 125.0 ± 14.1). Conversely, addition of insulin growth factor (IGF)-I a serum component known to influence cell differentiation (30), to D<sub>3</sub> minimally inhibited CD14 expression. These observations suggest that in THP-1 cells, D<sub>3</sub> is capable of inducing CD14 in the absence of any serum factors, and that D<sub>3</sub> delivery to cell surface is independent of serum vitamin D binding protein (45, 46).

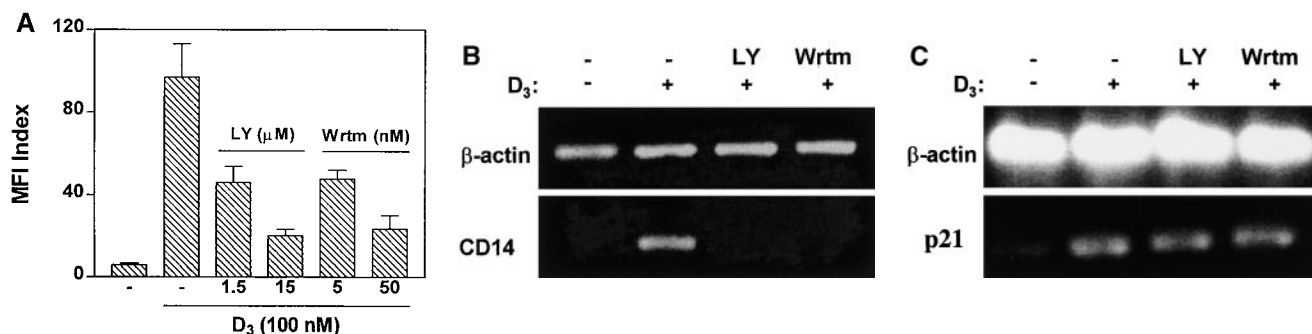
**Treatment of THP-1 Cells with D<sub>3</sub> Activates PI 3-Kinase.** In light of the important roles played by PI 3-kinase in regulating the differentiation of a variety of cell types (47–49), the possibility that D<sub>3</sub>-induced monocytic differentiation involves PI 3-kinase was investigated. As shown in Fig. 2 A, incubation of serum-starved THP-1 cells with 10 nM D<sub>3</sub> brought about a significant increase in PI 3-kinase activity (3.07 ± 1.17 fold increase, mean ± SEM, *n* = 3), with a maximum response observed at 1 μM (6.59 ± 1.47 fold increase). Although PI 3-kinase activity was also significantly increased in IGF-I (100 ng/ml)-treated cells (Fig. 2 A), this was not associated with increased CD14 expression (Fig. 1 C). Time course experiments using 100 nM D<sub>3</sub> (Fig. 2 B) showed that PI 3-kinase activation was detectable by as early as 5 min and reached a maximum level (4.94 ± 0.72 fold increase) by 20 min.

**D<sub>3</sub>-induced Expression of CD14 and CD11b are PI 3-Kinase Dependent.** To address whether PI 3-kinase activation is required for D<sub>3</sub>-induced monocytic differentiation, serum-

starved THP-1 cells were incubated with either wortmannin or LY294002 for 20 min before addition of hormone. Inhibitors were used at concentrations known to be relatively selective for inhibition of PI 3-kinase (48, 50). Incubation with inhibitors alone had no effect on basal expression of CD14 (data not shown). Preincubation with 5 nM wortmannin led to 65.7 ± 5.6% inhibition of D<sub>3</sub>-induced CD14 expression (mean ± SEM, *n* = 3; Fig. 3 A). Increasing the concentration of wortmannin to 50 nM inhibited CD14 expression by 86.0 ± 8.5%. LY294002, an inhibitor of PI 3-kinase that acts via a distinct mechanism from that



**Figure 2.** D<sub>3</sub> induces PI 3-kinase activation in THP-1 cells. Serum-starved (6 h) cells were stimulated with D<sub>3</sub>, followed by detergent lysis and immunoprecipitation with anti-PI 3-kinase Ab. PI 3-kinase activity was assayed as described in Materials and Methods. Spots corresponding to phosphatidylinositol 3-phosphate (PIP) were cut and subjected to scintillation counting. In A, cells were stimulated with a range of concentrations of D<sub>3</sub> (0–1,000 nM) or with IGF-I (100 ng/ml) for 20 min. In B, cells were stimulated with 100 nM D<sub>3</sub> for 0–60 min. The upper rectangles in both panels show PIP spots obtained in one representative experiment. The graphical data shown are the means ± SEM of values obtained in three separate experiments. Activities are expressed as fold increase relative to control (untreated) cells.



**Figure 3.** Wortmannin and LY294002 attenuate D<sub>3</sub>-induced CD14 expression, but not expression of Cdk inhibitor p21. Serum-starved THP-1 ( $4 \times 10^6$ ) cells were incubated for 20 min at 37°C and 5% CO<sub>2</sub> in medium alone, with wortmannin (Wrtm), or with LY294002 (LY). D<sub>3</sub> (100 nM) was then added for 24 h at 37°C. (A) Aliquots of cells ( $\sim 0.5 \times 10^6$ ) from each treatment were washed with staining buffer and labeled with anti-CD14 mAb or irrelevant mAb, followed by FITC-conjugated secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. The results shown are the means  $\pm$  SEM of values obtained in three separate experiments. (B and C) Total RNA was extracted from the cells remaining, and RT-PCR was carried out for CD14 (B) and p21 (C) as described (reference 41).  $\beta$ -actin was analyzed to control for loading. Negative controls consisting of no RNA and RNA without RT were included, and these produced no signals (data not shown). The data shown are from one of two independent experiments that yielded similar results.

of wortmannin, when used at 1.5  $\mu$ M reduced D<sub>3</sub>-induced CD14 expression by  $66.8 \pm 11.9\%$ . CD14 expression decreased further in the presence of 15  $\mu$ M LY294002 ( $88.1 \pm 5.1\%$ ). In contrast to abrogation of D<sub>3</sub>-induced CD14, neither wortmannin nor LY294002 had significant effects on the expression of HLA class I molecules (data not shown), indicating that the effects of neither wortmannin nor LY294002 were due to nonspecific toxicity. The PI 3-kinase inhibitors LY294002 and wortmannin also attenuated D<sub>3</sub>-induced cell surface expression of CD11b by THP-1 cells. While incubation with inhibitors alone had no effect on basal levels of CD11b expression, preincubation with 5 nM wortmannin inhibited the response to D<sub>3</sub>

**Table I.** Effects of PI 3-Kinase Inhibitors on Vitamin D<sub>3</sub>-induced Surface Expression of CD11b by THP-1 Cells

Treatment	CD11b (MFI index)
Control	1.19 $\pm$ 0.17
Vit D <sub>3</sub>	2.77 $\pm$ 0.78
15 $\mu$ M LY alone	1.17 $\pm$ 0.17
50 nM wrtm alone	1.13 $\pm$ 0.15
1.5 $\mu$ M LY + vit D <sub>3</sub>	2.01 $\pm$ 0.41
5.0 nM wrtm + vit D <sub>3</sub>	2.13 $\pm$ 0.37
15 $\mu$ M LY + vit D <sub>3</sub>	1.38 $\pm$ 0.22
50 nM wrtm + vit D <sub>3</sub>	1.19 $\pm$ 0.14

Serum-starved THP-1 cells were incubated for 20 min at 37°C and 5% CO<sub>2</sub> in either medium alone, wortmannin (wrtm), or LY294002 (LY). D<sub>3</sub> (vit D<sub>3</sub> [100 nM]) was then added for 24 h at 37°C. Cells from each treatment were washed with staining buffer, labeled with anti-CD11b mAb or irrelevant mAb followed by FITC-conjugated secondary Abs, and flow cytometric analyses were performed. The results shown are the means  $\pm$  SEM of values obtained in three separate experiments. Results are expressed as MFI indices which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.

by  $41 \pm 7\%$  (Table I). A higher concentration of 50 nM increased inhibition to  $100 \pm 12\%$ . LY294002 inhibited D<sub>3</sub>-induced CD11b surface expression by  $48 \pm 10\%$  at a concentration of 1.5  $\mu$ M, and by  $88 \pm 14\%$  at a concentration of 15  $\mu$ M (Table I). Surface expression of CD14 and CD11b was also examined in human peripheral blood monocytes. As shown in Table II, LY294002 and wortmannin markedly inhibited D<sub>3</sub>-induced expression of both CD14 and CD11b in these cells.

**Table II.** Effects of PI 3-kinase Inhibitors on Vitamin D<sub>3</sub>-induced Surface Expression of CD14 and CD11b by Peripheral Blood Monocytes

Treatment	CD14 (MFI index)	CD11b (MFI index)
Control	1.09	1.27
Vit D <sub>3</sub>	1.98	1.80
15 $\mu$ M LY alone	1.03	1.08
50 nM wrtm alone	1.21	1.25
1.5 $\mu$ M LY + vit D <sub>3</sub>	1.37	1.63
5.0 nM wrtm + vit D <sub>3</sub>	1.54	1.54
15 $\mu$ M LY + vit D <sub>3</sub>	1.07	1.24
50 nM wrtm + vit D <sub>3</sub>	1.20	1.20

Peripheral blood monocytes were allowed to adhere to plastic dishes for 1 h at 37°C and 5% CO<sub>2</sub> in serum-free medium. Cells were then washed with warm HBSS, and the remaining adherent monocytes were incubated for 20 min at 37°C and 5% CO<sub>2</sub> in either medium alone, wortmannin (wrtm), or LY294002 (LY). D<sub>3</sub> (vit D<sub>3</sub> [100 nM]) was then added for 24 h at 37°C. Cells from each treatment were washed with staining buffer and labeled with either anti-CD14 mAb, anti-CD11b mAb, or irrelevant mAb, followed by FITC-conjugated secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in Materials and Methods. Results are expressed as MFI indices which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG. The results shown are based on 10,000 recorded events for each treatment condition performed on cells from 1 individual donor.

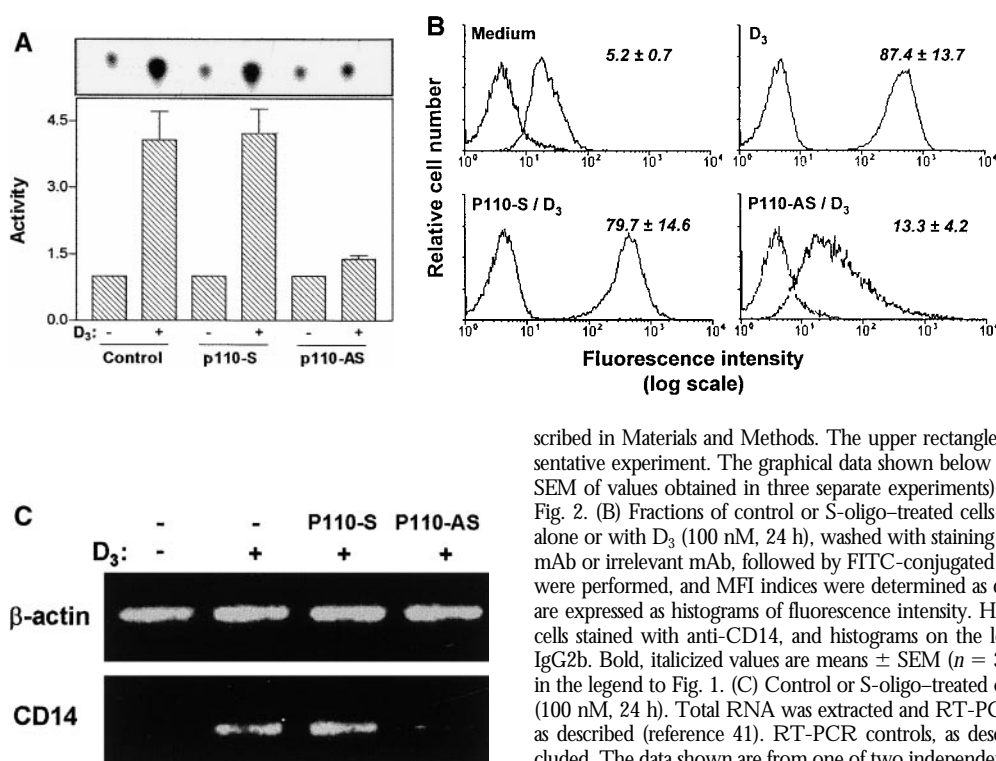
Inhibition of PI 3-kinase has been shown to affect the transport of some cell surface molecules (51), and thus was a possible mechanism to explain the effects of PI 3-kinase inhibitors on  $D_3$ -induced CD14 expression. However, when total RNA was isolated and RT-PCR was performed using primers for *CD14* and  $\beta$ -actin, the results showed that mRNA levels for CD14 were markedly reduced in cells incubated with either wortmannin or LY294002 (Fig. 3 B). These findings indicate that inhibition of PI 3-kinase results in attenuation of  $D_3$ -induced CD14 gene expression at a pretranslational level.

Treatment of immature myeloid cells with  $D_3$  induces the expression of the Cdk inhibitor p21, and the latter has been shown to regulate gene expression and to promote myeloid cell differentiation (52). Experiments were carried out to examine whether the induction of p21 in response to hormone also involved PI 3-kinase. As shown in Fig. 3 C, THP-1 cells expressed low levels of p21 mRNA in the basal state. As expected, p21 expression was significantly induced in response to incubation of cells with  $D_3$ . However, in contrast to the findings with CD14 and CD11b, preincubation of cells with either wortmannin or LY294002 had no effect on hormone-induced expression of p21. These findings suggest that PI 3-kinase selectively regulates monocyte differentiation, independent of any effects on p21.

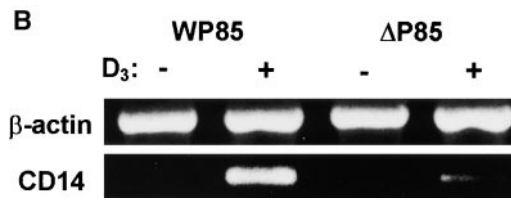
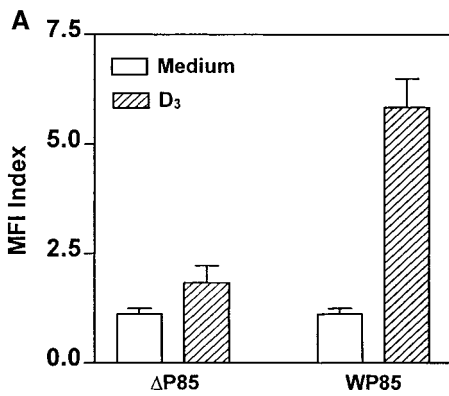
The role of PI 3-kinase in regulating monocyte differentiation was investigated further by inhibiting the synthesis of the p110 catalytic subunit of PI 3-kinase. THP-1 cells were incubated in the presence of antisense S-oligo complementary to the p110 translation initiation region (including the ATG initiation codon), and then assayed for

both PI 3-kinase activation and CD14 expression in response to  $D_3$ . Flow cytometric analysis of cells exposed to fluorescein-modified antisense S-oligo, under the same conditions used for unmodified S-oligos, revealed that THP-1 cells readily incorporated foreign DNA (data not shown). As shown in Fig. 4 A, antisense S-oligo to p110 mRNA significantly attenuated  $D_3$ -induced PI 3-kinase activity (% inhibition =  $87.1 \pm 5.6$ , mean  $\pm$  SEM,  $n = 3$ ), whereas at the same concentration, the control sense S-oligo had no apparent effect on the PI 3-kinase response. In parallel with this effect on PI 3-kinase activation, pretreatment with antisense S-oligo (and not with the control, sense oligo) almost completely inhibited hormone-induced surface expression of CD14 (% inhibition =  $91.7 \pm 3.9$ , mean  $\pm$  SEM of three experiments; Fig. 4 B). In addition, RT-PCR results shown in Fig. 4 C demonstrated that  $D_3$ -induced mRNA levels for *CD14* were markedly diminished in cells treated with antisense S-oligo to p110 mRNA.

The PI 3-kinase requirement for  $D_3$ -induced CD14 expression was also examined in U937 cells transfected with a dominant negative mutant of p85 ( $\Delta p85$ ). Stable transfection of these cells with  $\Delta p85$  resulted in a significant reduction of stimulated PI 3-kinase activity (22, 37). Exposure of  $\Delta p85$  U937 to  $D_3$  (100 nM, 48 h) led to only a marginal increase in surface expression of CD14 above baseline. In contrast, cells transfected with wild-type p85 showed significant induction of CD14 expression (Fig. 5 A). In addition, as observed with THP-1 cells, RT-PCR experiments showed that attenuation of PI 3-kinase in U937 cells resulted in markedly reduced response to  $D_3$  for induction of CD14 mRNA (Fig. 5 B). Taken together, these findings



**Figure 4.** Antisense S-oligo to the p110 subunit of PI 3-kinase inhibits  $D_3$ -induced CD14 expression.  $7 \times 10^6$  THP-1 cells were suspended in 500  $\mu$ l of 2.5% lipofectAMINE/RPMI 1640 alone (Control), or containing 5  $\mu$ M of either sense (S) or antisense (AS) S-oligo to p110. Cells were then incubated on a rotary shaker for 4 h at 37°C. The incubation was brought up to 10 ml of medium, and cells were cultured for an additional 18 h at 37°C and 5%  $CO_2$ . (A)  $3 \times 10^6$  cells were stimulated with 100 nM  $D_3$  for 20 min and assayed for PI 3-kinase assay as described in Materials and Methods. The upper rectangle shows PIP spots obtained in one representative experiment. The graphical data shown below represent PI 3-kinase activities (means  $\pm$  SEM of values obtained in three separate experiments), calculated as described in the legend to Fig. 2. (B) Fractions of control or S-oligo-treated cells ( $\sim 1 \times 10^6$ ) were incubated in medium alone or with  $D_3$  (100 nM, 24 h), washed with staining buffer, and then labeled with anti-CD14 mAb or irrelevant mAb, followed by FITC-conjugated secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. Results are expressed as histograms of fluorescence intensity. Histograms displaced to the right represent cells stained with anti-CD14, and histograms on the left represent cells stained with irrelevant IgG2b. Bold, italicized values are means  $\pm$  SEM ( $n = 3$ ) of MFI indices, calculated as described in the legend to Fig. 1. (C) Control or S-oligo-treated cells ( $\sim 3 \times 10^6$ ) were incubated with  $D_3$  (100 nM, 24 h). Total RNA was extracted and RT-PCR was carried out for *CD14* and  $\beta$ -actin as described (reference 41). RT-PCR controls, as described in the legend to Fig. 3, were included. The data shown are from one of two independent experiments that yielded similar results.



**Figure 5.** CD14 response to D<sub>3</sub> in U937 cells transfected with wild-type bovine p85α (WP85) or dominant negative mutant Δp85α (Δp85). (A) Cells were stimulated with either 100 nM D<sub>3</sub> or medium alone for 48 h. Cells were washed with staining buffer, and then labeled with anti-CD14

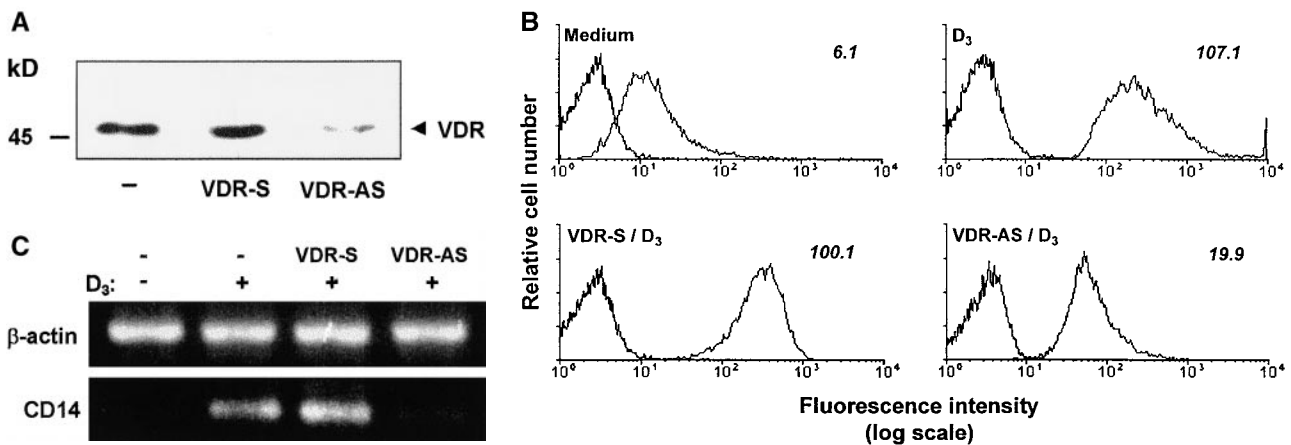
mAb or irrelevant mAb, followed by secondary FITC-conjugated Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. The data shown are the means ± SEM of values obtained in three separate experiments. (B) Cells were stimulated with D<sub>3</sub> or medium alone for 24 h, then total RNA was extracted and RT-PCR was carried out for *CD14* and *β-actin* as described (reference 41). No signals were obtained with controls consisting of no RNA and RNA without RT (data not shown). The data shown are from one of two independent experiments that yielded similar results.

strongly suggest that PI 3-kinase plays a central role in D<sub>3</sub>-induced monocyte differentiation, and indicate that PI 3-kinase activation is required to induce CD14 expression.

**Induction of CD14 in Response to D<sub>3</sub> Requires the VDR.** Many responses to D<sub>3</sub>, such as induction of expression of osteopontin, osteocalcin, calbindin, and 24-hydroxylase, are brought about by a mechanism involving binding of the VDR to a specific VDRE in the corresponding promoters (53). Since no VDRE has been identified within the CD14 gene promoter (28), this raised the question as to whether the VDR plays any role in regulating D<sub>3</sub>-induced CD14 expression. To address this question, THP-1 cells were incubated overnight with antisense S-oligo specific to VDR mRNA. This resulted in significant attenuation of the level of VDR protein in THP-1 cells as detected by Western blotting (Fig. 6 A). In contrast, pretreatment of cells with the control, sense S-oligo had no apparent effect on the

level of VDR protein. In parallel with the reduction of VDR protein, D<sub>3</sub>-induced surface expression of CD14 was markedly attenuated (Fig. 6 B; in two separate experiments an average decrease of 86.8% was observed). In addition, cells were treated with S-oligo antisense specific for VDR mRNA before D<sub>3</sub>, followed by RNA extraction and RT-PCR. The results showed that the induction of CD14 mRNA was markedly attenuated in antisense-treated cells (Fig. 6 C). Taken together, these findings strongly suggest that the VDR is essential for D<sub>3</sub>-induced CD14 gene expression.

**Association of PI 3-Kinase with the VDR.** Given the evidence that both PI 3-kinase and the VDR were essential for induction of CD14 in response to D<sub>3</sub>, the question examined next was whether the VDR is required for activation of PI 3-kinase. THP-1 cells were treated with antisense S-oligos as described above to reduce VDR protein expression before incubation with D<sub>3</sub> (100 nM, 20 min).



**Figure 6.** VDR antisense S-oligo inhibits D<sub>3</sub>-induced CD14 expression.  $5 \times 10^6$  THP-1 cells were suspended in 500  $\mu$ l of 2.5% lipofectAMINE/RPMI 1640 alone (control), or containing 5  $\mu$ M of either sense (S) or antisense (AS) S-oligo to the VDR. Cells were then incubated on a rotary shaker for 4 h at 37°C. The volume was then brought up to 7 ml, and cells were cultured for an additional 18 h at 37°C and 5% CO<sub>2</sub>. (A)  $0.5 \times 10^6$  cells were boiled in Laemmli buffer and subjected to SDS-PAGE and immunoblotting with mAb to VDR. Membranes were developed by ECL as described (reference 44). The data shown are from one of two independent experiments that yielded similar results. (B) Aliquots of control or S-oligo-treated cells ( $\sim 1 \times 10^6$ ) were incubated with D<sub>3</sub> (100 nM, 24 h), washed with staining buffer, and then labeled with anti-CD14 mAb or irrelevant mAb followed by FITC-conjugated, secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. Results are expressed as histograms of fluorescence intensity. Histograms displaced to the right represent cells stained with anti-CD14, and histograms on the left represent cells stained with irrelevant IgG2b. Bold, italicized values in each frame are averages ( $n = 2$ ) of MFI indices, determined as in the legend to Fig. 1. (C) Control or S-oligo-treated cells ( $\sim 3 \times 10^6$ ) were incubated with D<sub>3</sub> (100 nM, 24 h). Total RNA was extracted and RT-PCR was carried out for *CD14* and *β-actin* as described (reference 41). Controls consisting of no RNA and RNA without RT were included, and no signals were obtained (data not shown). The data shown are from one of two independent experiments that yielded similar results.

Cell lysates were then prepared and assayed for PI 3-kinase activity. The results in Fig. 7 A show that pretreatment with antisense to VDR almost completely abrogated (92.9% inhibition, average of two separate experiments) PI 3-kinase activation in response to  $D_3$ . In contrast, treatment with control sense S-oligos to VDR had no inhibitory effect.

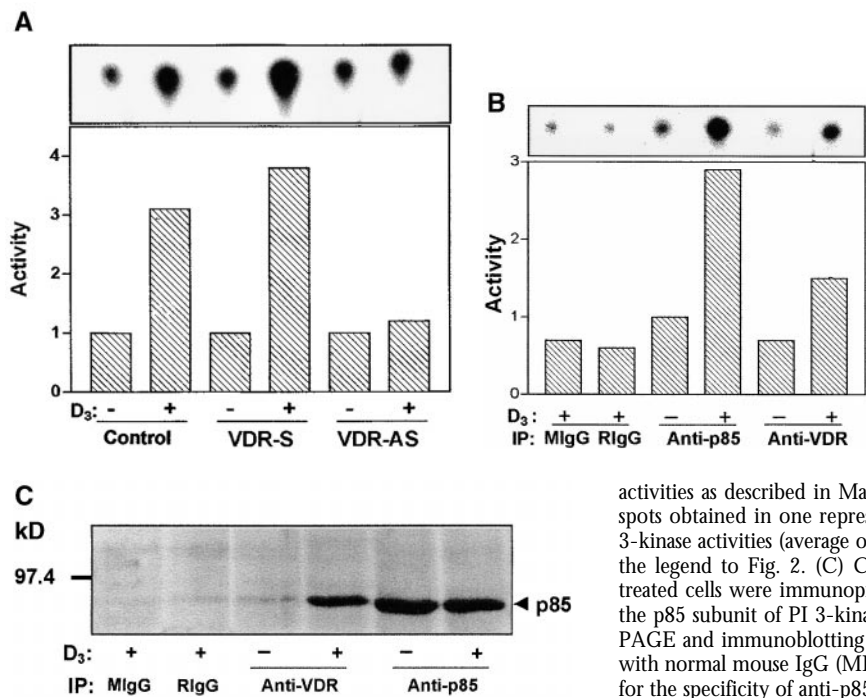
The evidence that the  $D_3$  receptor was required for both PI 3-kinase activation and CD14 expression in response to  $D_3$  suggested the possibility that this might involve a signaling complex containing both the VDR and PI 3-kinase. To test this hypothesis, THP-1 cells were incubated with  $D_3$ , and immunoprecipitates prepared with mAb to the VDR were assayed for PI 3-kinase activity. The results shown in Fig. 7 B indicate that immunoprecipitates of the VDR prepared from cells activated with  $D_3$  contained PI 3-kinase activity. To examine further whether the VDR associates with PI 3-kinase upon  $D_3$  stimulation, aliquots from anti-VDR and anti-p85 (PI 3-kinase) immunoprecipitates of  $D_3$ -treated cells were subjected to SDS-PAGE and Western blotting. Blots were probed with mAb to p85 and developed by ECL. The results shown in Fig. 7 C indicate that Abs to p85 reacted in anti-VDR immunoprecipitates with a band that presumably corresponds to the p85 subunit of PI 3-kinase. This association was only observed in cells treated with hormone, and not in untreated cells. Taken together, the findings suggest that  $D_3$  treatment induces the formation of a signaling complex containing the VDR and PI 3-kinase. This results in activation of the lipid kinase, and is required for monocyte differentiation and induction of CD14 expression.

## Discussion

The steroid hormone vitamin  $D_3$  is known to induce immature, myeloid precursor cells to differentiate into ma-

ture monocytic cells. This process is accompanied by high-level expression of mRNA and protein for CD14, and other markers such as CD11b (27–29). How these  $D_3$ -initiated events are regulated is not completely understood. Cellular responses to  $D_3$  have primarily been attributed to activation of the VDR, which acts as a transcription factor modulating the expression of a variety of genes (53). In contrast to genomic signaling, several reports have implicated alternative, nongenomic mechanisms of action for  $D_3$  involving initiation of signaling at the cell membrane (54–56). The objective of this study was to examine signaling events in response to  $D_3$  in order to define further how this hormone regulates myeloid cell differentiation.

The principal conclusions drawn from the experiments reported are that  $D_3$ -induced expression of CD14 and CD11b requires both PI 3-kinase and the VDR. Moreover, this process appears to involve the formation of a PI 3-kinase–VDR signaling complex. The conclusion that  $D_3$ -induced CD14 expression is PI 3-kinase dependent is based on several lines of evidence, including: (a) *in vitro* kinase assays with immunoprecipitated PI 3-kinase, showing that incubation of cells with  $D_3$  leads to activation of the enzyme (Fig. 2); (b)  $D_3$ -induced CD14 expression is abrogated in THP-1 cells incubated with the PI 3-kinase inhibitors, wortmannin and LY294002 (Fig. 3); (c) an antisense strategy to downregulate PI 3-kinase also attenuated  $D_3$ -induced CD14 expression (Fig. 4); and (d) expression of a dominant negative mutant of PI 3-kinase ( $\Delta p85$ ) in U937 cells also completely abrogated  $D_3$ -induced expression of both CD14 mRNA and protein (Fig. 5). Similarly, CD11b induction in response to  $D_3$  was also attenuated by wortmannin and LY294002 in both THP-1 cells and in human peripheral blood monocytes. Taken together, these findings establish that  $D_3$ -induced CD14 and CD11b expres-



**Figure 7.** The VDR is required for  $D_3$ -induced PI 3-kinase activation. (A)  $3 \times 10^6$  THP-1 cells were treated overnight with 5  $\mu$ M of either sense (S) or antisense (AS) S-oligo to VDR as described in the legend to Fig. 6. Cells were then stimulated for 20 min with 100 nM  $D_3$ , and lysates were prepared and assayed for PI 3-kinase activities as described in Materials and Methods. The upper rectangle shows PIP spots obtained in one representative experiment. The data shown below are PI 3-kinase activities (averages of values obtained in two separate experiments), calculated as described in the legend to Fig. 2. (B) Cells were stimulated with  $D_3$  as in A and cell lysates were prepared and incubated overnight at 4°C either with 9A7 mAb (anti-VDR) or with mAb specific for the p85 subunit of PI 3-kinase. Immune complexes were adsorbed onto protein A and assayed for PI 3-kinase activities as described in Materials and Methods. The upper rectangle shows PIP spots obtained in one representative experiment. The data shown below are PI 3-kinase activities (average of two separate experiments), calculated as described in the legend to Fig. 2. (C) Cell lysates prepared from either control cells or  $D_3$ -treated cells were immunoprecipitated with specific mAbs to either the VDR or the p85 subunit of PI 3-kinase. Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting with Abs to p85 (reference 40). Immunoprecipitates with normal mouse IgG (MlgG) and normal rat IgG (RlgG) were used to control for the specificity of anti-p85 and anti-VDR Abs, respectively.



sion are regulated by PI 3-kinase, and that they are consistent with a nongenomic mechanism of hormone action.

Expression of the Cdk inhibitor p21 is induced by  $D_3$ , and this is associated with the differentiation of immature myeloid cells, including the expression of CD14 (52). Therefore, it was possible that inhibition of PI 3-kinase may have abrogated hormone-induced expression of p21 proximally, leading to secondary, indirect effects on the expression of CD14 and CD11b. However, the findings that neither wortmannin nor LY294002 inhibited the induction of p21 while at the same time they inhibited the expression of CD14 and CD11b (Fig. 3, Tables I and II) indicated that this was not the case. These findings suggest that PI 3-kinase selectively regulates these monocyte differentiation markers independent of any effects on p21. They also suggest that  $D_3$ -induced signaling initiated through the VDR appears to involve at least two separate pathways, one leading to p21 induction that is independent of PI 3-kinase, and a second pathway that is PI 3-kinase dependent and which regulates at least the induction of CD14 and CD11b. Moreover, although p21 has been shown to be involved in regulating monocyte differentiation (52), results from the present study suggest that it is not sufficient by itself for induction of monocyte differentiation in response to  $D_3$ , and that other, PI 3-kinase- and VDR-regulated elements are required.

Induction of CD14 expression in response to  $D_3$  is regulated at the level of gene transcription (28, 29) and the *CD14* promoter, particularly from bp -128 to -70, has been shown to be critical for CD14 expression in response to  $D_3$  (28). However, this region does not contain a canonical VDRE (28). Rather, the *CD14* promoter contains two GC boxes that bind the nonreceptor, transcription factor Sp1, and this interaction is believed to be essential for CD14 expression (28, 57). In light of this molecular data, activation of *CD14* transcription would not be expected to be a direct response to  $D_3$  signaling through the VDR. Nevertheless, experiments that used VDR antisense S-oligo to downregulate expression of the endogenous VDR before  $D_3$  stimulation showed that the VDR is required for  $D_3$ -induced expression of CD14 mRNA (Fig. 6). The obligate requirement for the VDR in this response was also supported by the finding that activation of PI 3-kinase in response to  $D_3$  was markedly attenuated in cells treated with VDR antisense (Fig. 7 A). Moreover, a signaling complex containing both the VDR and PI 3-kinase was identified in  $D_3$ -treated cells (Fig. 7, B and C), suggesting that a functional cytosolic VDR is a prerequisite for PI 3-kinase activation in response to  $D_3$ . These findings appear to identify a novel, nongenomic mechanism of action for the VDR. It is of interest to note that the hormone-induced formation of a VDR-PI 3-kinase signaling complex is reminiscent of the finding that activation of the Raf-MAP kinase pathway by  $D_3$  in keratinocytes involves an association of the VDR with the adaptor protein p66<sup>shc</sup> (58). Together, these findings suggest that other models of nongenomic signaling involving the VDR may yet be identified.

The observations that PI 3-kinase regulates CD14 expression in response to  $D_3$  and that the VDR is directly in-

involved in this process do not exclude the possibility of a component of genomic action by the VDR, despite the absence of a canonical VDRE within the *CD14* promoter. One possibility to consider is that upon incubation of cells with  $D_3$ , the VDR may translocate to the nucleus towards  $D_3$ -responsive elements, and enhance the activity of the known CD14 transcription factor Sp1. Two observations lend support to this hypothesis: (a) the finding of transcriptional synergism between the VDR and Sp1 using a construct of the VDRE, the binding sites for Sp1, and a luciferase reporter gene (59); and (b) the presence of a VDRE-like sequence at the distal Sp1 site within the *CD14* gene (60).

The findings that  $D_3$ -induced monocyte differentiation for CD14 and CD11b expression requires PI 3-kinase are consistent with previous reports in which this lipid kinase has been implicated in regulating the differentiation of various cell types, including immature myeloid cells (49, 61-63). For example, indirect data, based solely on the use of inhibitors, suggested a potential role for PI 3-kinase in regulating the differentiation of the myeloid leukemia cells HL-60 (63). In addition, when FDC-P1 myeloid cells that express the M-CSF receptor *c-fms* were treated with M-CSF, there occurred the rapid formation of a complex between *c-fms* and several signal transduction proteins, including PI 3-kinase (49). Other studies have suggested that the differentiation of FDC-P1 cells is regulated by the activities of both phospholipase C- $\gamma$  and PI 3-kinase in response to M-CSF stimulation (61). In addition, stimulation of type III receptor tyrosine kinases in the same cell line was observed to lead to PI 3-kinase-dependent monocytic differentiation (62). Thus, the steroid hormone  $D_3$  appears to share with M-CSF the property of PI 3-kinase activation during the induction of monocyte differentiation.

It is of interest to compare  $D_3$  with phorbol esters that also act to promote monocyte differentiation. For example, PMA induces the expression of the monocyte differentiation markers CR3 (CD11b/CD18) and p150.95 (64), and modulates several functional properties of myeloid precursor cells, such as intracellular adhesion molecule 1-dependent adhesion (22), phagocytosis (44, 65), and bactericidal activity (66). However, PMA does not activate PI 3-kinase (Hmama, Z., and N. Reiner, unpublished data), and PMA-induced cell differentiation is resistant to PI 3-kinase inhibition (22). Consistent with the findings presented above, although low concentrations of  $D_3$  induce an ~50-fold increase in CD14 surface expression, optimal doses of PMA resulted in only marginal increases (Hmama, Z., and N. Reiner, unpublished data). Regarding signaling mechanisms regulating PMA-induced cell differentiation, several reports have implicated the protein kinase C-Raf-MAP kinase pathway (33, 67, 68). MAP kinase activity has been also shown to be activated by  $D_3$  in keratinocytes, enterocytes, and in the promyelocytic cell lines HL60 and NB4 (34, 58, 69). To examine whether the MAP kinase pathway may be involved in THP-1 cell differentiation,  $D_3$ -treated cells were examined for tyrosine phosphorylation of p42 and p44 MAP kinase isoforms, and assayed for MAP

kinase activity using myelin basic protein as substrate. The results obtained (data not shown) indicated that MAP kinase is not activated in response to D<sub>3</sub> in THP-1 cells.

The mechanism by which PI 3-kinase becomes activated in a complex with the VDR is not presently known. At least one well-established mechanism for activation of PI 3-kinase is known to involve interactions of the Src homology 2 domain of the p85 regulatory subunit with tyrosine phosphorylated proteins, including both receptor- and nonreceptor-protein tyrosine kinases (35). In the VDR, the only potential sites of phosphorylation correspond to Ser<sup>51</sup> (70) and Ser<sup>208</sup> (71), and the relevance of these residues in VDR-mediated D<sub>3</sub> signal transduction is unknown. Thus, it is not presently clear how a direct interaction of the VDR with the p85 subunit may lead to activation of PI 3-kinase. On the other hand, a multimolecular complex involving the VDR and a tyrosine-phosphorylated adaptor protein could potentially be involved in D<sub>3</sub>-induced activation of PI 3-kinase.

At present, it is not clear how VDR-activated PI 3-kinase would be targeted to the membrane compartment to mediate cellular responses, given the cytosolic and nuclear localization of the VDR. One possibility to consider is that there is a small, membrane-associated population of VDR molecules that induces translocation of PI 3-kinase to the

membrane compartment. A second possibility is that once activated, the kinase may simply diffuse to the vicinity of the inner leaflet of the plasma membrane. Third, it is also possible that VDR-activated PI 3-kinase may act on non-membrane-associated substrates to mediate its effects.

During the course of these studies, it was observed that IGF-I failed to induce CD14 surface expression (Fig. 1 C), despite its ability to activate PI 3-kinase (Fig. 2). Therefore, PI 3-kinase activation in response to D<sub>3</sub> appears to be necessary, but not sufficient to bring about CD14 expression. These findings suggest the possibility that induction of CD14 in response to D<sub>3</sub> may involve VDR-mediated signals that bifurcate to involve nongenomic effects of PI 3-kinase, perhaps acting in concert with a genomic mechanism of action. In this respect, cross-talk between nuclear receptor-mediated signaling and nongenomic actions have been described in D<sub>3</sub>-treated osteoblasts (72).

In summary, the findings presented demonstrate a novel pathway in which D<sub>3</sub> signaling for myeloid cell differentiation involves PI 3-kinase activation and signal complex formation with the VDR, which is itself shown to be required for cell differentiation. This pathway is essential for D<sub>3</sub>-induced expression of CD14 and CD11b, and attributes important functional roles to PI 3-kinase and the VDR in monocyte differentiation.

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## References

- Boyle, I.T., L. Miravet, R.W. Gray, M.F. Holick, and H.F. DeLuca. 1972. The response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxyvitamin D in nephrectomized rats. *Endocrinology*. 90:605-608.
- Nemere, I. 1995. Nongenomic effects of 1,25-dihydroxyvitamin D<sub>3</sub>: potential relation of a plasmalemmal receptor to the acute enhancement of intestinal calcium transport in chick. *J. Nutr.* 125:1695S-1698S.
- Holick, M.F., M. Garabedian, and H.F. DeLuca. 1972. 1,25-dihydroxycholecalciferol: metabolite of vitamin D<sub>3</sub> active on bone in anephric rats. *Science*. 176:1146-1147.
- Raisz, L.G., C.L. Trummel, M.F. Holick, and H.F. DeLuca. 1972. 1,25-dihydroxyvitamin: a potent stimulator of bone resorption in tissue culture. *Science*. 175:768-769.
- Takahashi, T., K. Nakamura, and S. Iho. 1997. Differentiation of myeloid cells and 1,25-dihydroxyvitamin D<sub>3</sub>. *Leuk. Lymphoma*. 27:25-33.
- Brackman, D., F. Lund-Johansen, and D. Aarskog. 1995. Expression of leukocyte differentiation antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D<sub>3</sub>: comparison with the maturation of normal monocytic and granulocytic bone marrow cells. *J. Leukoc. Biol.* 58:547-555.
- Oberg, F., J. Botling, and K. Nilsson. 1993. Functional antagonism between vitamin D3 and retinoic acid in the regulation of CD14 and CD23 expression during monocytic differentiation of U-937 cells. *J. Immunol.* 150:3487-3495.
- Schwende, H., E. Fitzke, P. Ambs, and P. Dieter. 1996. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D<sub>3</sub>. *J. Leukoc. Biol.* 59:555-561.
- Abe, E., C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, and T. Suda. 1981. Differentiation of mouse myeloid leukemia cells induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. *Proc. Natl. Acad. Sci. USA*. 78:4990-4994.
- Bouillon, R., W.H. Okamura, and A.W. Norman. 1995. Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16:200-257.
- Ozono, K., J. Liao, S.A. Kerner, R.A. Scott, and J.W. Pike. 1990. The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene

- enhancer. *J. Biol. Chem.* 265:21881–21888.
12. Darwish, H.M., and H.F. DeLuca. 1992. Identification of a 1,25-dihydroxyvitamin D<sub>3</sub>-response element in the 5'-flanking region of the rat calbindin D-9k gene. *Proc. Natl. Acad. Sci. USA.* 89:603–607.
  13. Zierold, C., H.M. Darwish, and H.F. DeLuca. 1994. Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D<sub>3</sub>) 24-hydroxylase gene. *Proc. Natl. Acad. Sci. USA.* 91:900–902.
  14. Todd, R.F., L.M. Nadler, and S.F. Schlossman. 1981. Antigens on human monocytes identified by monoclonal antibodies. *J. Immunol.* 126:1435–1439.
  15. Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and J.C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* 249:1431–1433.
  16. Medvedev, A.E., T. Flo, R.R. Ingalls, D.T. Golenbock, G. Teti, S.N. Vogel, and T. Espevik. 1998. Involvement of CD14 and complement receptor CR3 and CR4 in nuclear factor-kappa B activation and TNF production induced by lipopolysaccharide and group B streptococcal cell walls. *J. Immunol.* 160:4535–4542.
  17. Wooten, R.M., T.B. Morrison, J.H. Weis, S.D. Wright, R. Thieringer, and J.J. Weis. 1998. The role of CD14 in signaling mediated by outer membrane lipoproteins of *Borrelia burgdorferi*. *J. Immunol.* 160:5485–5492.
  18. Sellati, T.J., D.A. Bouis, R.L. Kitchens, R.P. Darveau, J. Pugin, R.J. Ulevitch, S.C. Gangloff, S.M. Goyert, M.W. Norgard, and J.D. Radolf. 1998. *Treponem treponema* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. *J. Immunol.* 160:5455–5464.
  19. Bosco, M.C., I. Espinosa-Delgado, T.K. Rowe, M.G. Malabarba, D.L. Longo, and L. Varesio. 1997. Functional role for the myeloid differentiation antigen CD14 in the activation of human monocytes by IL-2. *J. Immunol.* 159:2922–2931.
  20. Zybarth, G., N. Reiling, H. Schmidtmayerova, B. Sherry, and M. Bukrinsky. 1999. Activation-induced resistance of human macrophages to HIV-1 infection in vitro. *J. Immunol.* 162:400–406.
  21. Beekhuizen, H., I. Blokland, A.J. Corsel-van Tilburg, F. Koning, and R. van Furth. 1991. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. *J. Immunol.* 147:3761–3767.
  22. Hmama, Z., K.L. Knutson, P. Herrera-Velit, D. Nandan, and N.E. Reiner. 1999. Monocyte adherence induced by lipopolysaccharide involves CD14, LFA-1, and cytohesin-1. Regulation by Rho and phosphatidylinositol 3-kinase. *J. Biol. Chem.* 274:1050–1057.
  23. Haziot, A.S., S. Chen, E. Ferrero, M. Low, R. Silber, and S.M. Goyert. 1986. The monocyte differentiation antigen CD14 is anchored to the cell surface by a phosphatidylinositol linkage. *J. Immunol.* 141:547–552.
  24. Kirschning, C.J., H. Wesche, A.T. Merrill, and M. Rothe. 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* 188:2091–2097.
  25. Chow, J.C., D.W. Young, D.T. Golenbock, W.J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274:10689–10692.
  26. Ziegler-Heitbrock, H.W.L., and R.J. Ulevitch. 1994. CD14: cell surface receptor and differentiation marker. *Immunol. Today.* 14:121–125.
  27. Antal-Szalmas, P., J.A. Strip, A.J. Weersink, J. Verhoef, and K.P. Van Kessel. 1997. Quantitation of surface CD14 on human monocytes and neutrophils. *J. Leukoc. Biol.* 61:721–728.
  28. Zhang, D.E., C.J. Hetherington, D.A. Gonzalez, H.M. Chen, and D.G. Tenen. 1994. Regulation of CD14 expression during monocytic differentiation induced with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. *J. Immunol.* 153:3276–3284.
  29. Martin, T.R., S.M. Mongovin, P.S. Tobias, J.C. Mathison, A.M. Moriarty, D.J. Letrucq, and R.J. Ulevitch. 1994. The CD14 differentiation antigen mediates the development of endotoxin responsiveness during differentiation of mononuclear phagocytes. *J. Leukoc. Biol.* 56:1–9.
  30. Liu, Q., W. Ning, R. Dantzer, G.G. Freund, and K.W. Kelly. 1998. Activation of protein kinase C- $\zeta$  and phosphatidylinositol 3'-kinase and promotion of macrophage differentiation by insulin-like growth factor-I. *J. Immunol.* 160:1393–1401.
  31. James, S.Y., M.A. Williams, S.M. Kelsey, A.C. Newland, and K.W. Colston. 1997. The role of vitamin D derivatives and retinoids in the differentiation of human leukaemia cells. *Biochem. Pharmacol.* 54:625–634.
  32. Gniadecki, R. 1998. Nongenomic signaling by vitamin D. A new face of Src. *Biochem. Pharmacol.* 56:1273–1277.
  33. Kharbanda, S., A. Saleem, Y. Emoto, R.U. Stone, and D. Kufe. 1994. Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. *J. Biol. Chem.* 269:872–878.
  34. Marcinkowska, E., A. Wiedlocha, and C. Radzikowski. 1997. 1,25-Dihydroxyvitamin D<sub>3</sub> induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. *Biochem. Biophys. Res. Commun.* 241:419–426.
  35. Fry, M.J. 1994. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta.* 1226:237–268.
  36. Toker, A., and L.C. Cantley. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature.* 387:673–676.
  37. Herrera-Velit, P., K.L. Knutson, and N.E. Reiner. 1997. Phosphatidylinositol 3-kinase-dependent activation of protein kinase C- $\zeta$  in bacterial lipopolysaccharide-treated human monocyte. *J. Biol. Chem.* 272:16445–16452.
  38. Hara, K., K. Yonezawa, H. Sakae, A. Ando, K. Kotani, T. Kitamura, Y. Kitamura, H. Ueda, L. Stephens, and T.R. Jackson. 1994. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc. Natl. Acad. Sci. USA.* 91:7415–7419.
  39. Liu, M.K., P. Herrera-Velit, R.W. Brownsey, and N.E. Reiner. 1994. CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. *J. Immunol.* 153:2642–2652.
  40. Herrera-Velit, P., and N.E. Reiner. 1996. Bacterial lipopolysaccharide induces the association and coordinate activation of p53/56<sup>lyn</sup> and phosphatidylinositol 3-kinase in human monocytes. *J. Immunol.* 156:1157–1165.
  41. Nandan, D., and N.E. Reiner. 1997. TGF- $\beta$  attenuates the class II transactivator and reveals an accessory pathway of IFN- $\gamma$  action. *J. Immunol.* 158:1095–1101.
  42. Baker, A.R., D.P. McDonnell, M. Hughes, T.M. Crisp, D.J. Mangelsdorf, M.R. Haussler, J.W. Pike, J. Shine, and B.W. O'Malley. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA.* 85:1211–1215.

- Sci. USA.* 85:3294–3298.
43. Volinia, S., I. Hiles, E. Ormondroyd, D. Nizetic, R. Antonacci, M. Rocchi, and M.D. Waterfield. 1994. Molecular cloning, cDNA sequence, and chromosomal localization of the human phosphatidylinositol 3-kinase p110 $\alpha$  (PIK3CA) gene. *Genomics*. 24:472–477.
  44. Nandan, D., and N.E. Reiner. 1995. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat1. *Infect. Immun.* 63:4495–4500.
  45. Van Baelen, H., K. Allewaert, and R. Bouillon. 1988. New aspects of the plasma carrier protein for 25-dihydroxycholecalciferol in vertebrates. *Ann. NY Acad. Sci.* 538:60–68.
  46. Cooke, N.E., and J.G. Haddad. 1989. Vitamin D binding protein (Gc-globulin). *Endocr. Rev.* 10:294–307.
  47. Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodera, and Y. Fukui. 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 269:18961–18967.
  48. Kaliman, P., F. Vinals, X. Testar, M. Palacin, and A. Zorzano. 1996. Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. *J. Biol. Chem.* 271:19146–19151.
  49. Rohrschneider, L.R., R.P. Bourette, M.N. Lioubin, P.A. Algate, G.M. Myles, and K. Carlberg. 1997. Growth and differentiation signals regulated by the M-CSF receptor. *Mol. Reprod. Dev.* 46:96–103.
  50. Wymann, M.P., G. Bulgarelli-Leva, M.J. Zvelebil, L. Pirola, B. Vanhaesebroeck, M.D. Waterfield, and G. Panayotou. 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* 16:1722–1733.
  51. Kundra, R., and S. Kornfeld. 1998. Wortmannin retards the movement of the mannose 6-phosphate/insulin-like growth factor II receptor and its ligand out of endosomes. *J. Biol. Chem.* 273:3848–3853.
  52. Liu, M., M.H. Lee, M. Cohen, M. Bommakanti, and L.P. Freedman. 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D<sub>3</sub> leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* 10:142–153.
  53. Christakos, S., M. Raval-Pandya, P.P. Wernyj, and W. Yang. 1996. Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D<sub>3</sub>. *Biochem. J.* 316:361–371.
  54. Berry, D.M., R. Anyochi, M. Bhatia, and K.A. Meckling-Gill. 1996. 1,25-dihydroxyvitamin D<sub>3</sub> stimulates expression and translocation of protein kinase C $\alpha$  and C $\delta$  via a nongenomic mechanism and rapidly induces phosphorylation of a 33-kDa protein in acute promyelocytic NB4 cells. *J. Biol. Chem.* 271:16090–16096.
  55. Bhatia, M., J.B. Kirkland, and K.A. Meckling-Gill. 1995. Monocytic differentiation of acute promyelocytic leukemia cells in response to 1,25-dihydroxyvitamin D<sub>3</sub> is independent of nuclear receptor binding. *J. Biol. Chem.* 270:15962–15965.
  56. Darwish, H.M., and H.F. DeLuca. 1996. Recent advances in the molecular biology of vitamin D action. *Prog. Nucleic Acid Res. Mol. Biol.* 53:321–344.
  57. Zhang, D.E., C.J. Hetherington, S. Tan, S.E. Dziennis, D.A. Gonzalez, H.M. Chen, and D.G. Tenen. 1994. Sp1 is a critical factor for the monocytic specific expression of human CD14. *J. Biol. Chem.* 269:11425–11434.
  58. Gniadecki, R. 1996. Activation of Raf-mitogen-activated protein kinase signaling pathway by 1,25-dihydroxyvitamin D<sub>3</sub> in normal human keratinocytes. *J. Invest. Dermatol.* 106:1212–1217.
  59. Liu, M., and L.P. Freedman. 1994. Transcriptional synergism between the vitamin D<sub>3</sub> receptor and other nonreceptor transcription factors. *Mol. Endocrinol.* 8:1593–1604.
  60. Perez-Fernandez, R., V. Arce, and L.P. Freedman. 1993. Delineation of a DNA recognition element for the vitamin D<sub>3</sub> receptor by binding site selection. *Biochem. Biophys. Res. Commun.* 192:728–737.
  61. Bourette, R.P., G.M. Myles, J.L. Choi, and L.R. Rohrschneider. 1997. Sequential activation of phosphatidylinositol 3-kinase and phospholipase C- $\gamma$ 2 by the M-CSF receptor is necessary for differentiation signaling. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:5880–5893.
  62. Kubota, Y., T. Angelotti, G. Niederfellner, R. Herbst, and A. Ullrich. 1998. Activation of phosphatidylinositol 3-kinase is necessary for differentiation of FDC-P1 cells following stimulation of type III receptor tyrosine kinases. *Cell Growth Differ.* 9:247–256.
  63. Marcinkowska, E., A. Wiedlocha, and C. Radzikowski. 1998. Evidence that phosphatidylinositol 3-kinase and p70<sup>S6K</sup> protein are involved in differentiation of HL-60 cells induced by calcitriol. *Anticancer Res.* 18:3507–3514.
  64. Noti, J.D., and B.C. Reinemann. 1995. The leukocyte integrin gene CD11c is transcriptionally regulated during monocyte differentiation. *Mol. Immunol.* 32:361–369.
  65. Hmama, Z., R. Gabathuler, W.A. Jefferies, G. de Jon, and N.E. Reiner. 1998. Attenuation of HLA-DR expression by mononuclear phagocytes infected with *Mycobacterium tuberculosis* is related to intracellular sequestration of immature class II heterodimers. *J. Immunol.* 161:4882–4893.
  66. Caron, E., J.P. Liautard, and S. Kohler. 1994. Differentiated U937 cells exhibit increased bactericidal activity upon LPS activation and discriminate between virulent and avirulent *Listeria* and *Brucella* species. *J. Leukoc. Biol.* 56:174–181.
  67. Kang, C.D., B.K. Lee, K.W. Kim, C.M. Kim, S.H. Kim, and B.S. Chung. 1996. Signaling mechanism of PMA-induced differentiation of K562 cells. *Biochem. Biophys. Res. Commun.* 221:95–100.
  68. Herrera, R., S. Hubbell, S. Decker, and L. Petruzzelli. 1998. A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells. *Exp. Cell Res.* 238:407–414.
  69. de Boland, A.R., and A.W. Norman. 1998. 1 $\alpha$ ,25(OH)<sub>2</sub>-vitamin D<sub>3</sub> signaling in chick enterocytes: enhancement of tyrosine phosphorylation and rapid stimulation of mitogen-activated protein (MAP) kinase. *J. Cell. Biochem.* 69:470–482.
  70. Hsieh, J.C., P.W. Jurutka, M.A. Galligan, C.M. Terpening, C.A. Haussler, D.S. Samuels, Y. Shimizu, N. Shimizu, and M.R. Haussler. 1991. Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. *Proc. Natl. Acad. Sci. USA.* 88:9315–9319.
  71. Jurutka, P.W., J.C. Hsieh, S. Nakajima, C.A. Haussler, G.K. Whitfield, and M.R. Haussler. 1996. Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. *Proc. Natl. Acad. Sci. USA.* 93:3519–3524.
  72. Farach-Carson, M.C., and A.L. Ridal. 1998. Dual 1,25-dihydroxyvitamin D<sub>3</sub> signal response pathways in osteoblasts: cross-talk between genomic and membrane-initiated pathways. *Am. J. Kidney Dis.* 31:729–742.