Transcriptional activation of the Cdk inhibitor p21 by vitamin D₃ leads to the induced differentiation of the myelomonocytic cell line U937

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The hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃, acting through its cognate nuclear receptor (vitamin D₃ receptor, VDR) will induce myeloid leukemic cell lines to terminally differentiate into monocytes/macrophages. Because VDR acts by transcriptionally regulating responsive genes in a ligand-dependent manner, we sought target genes of the receptor that initiate the differentiation process in response to ligand. We screened a cDNA library prepared from the myelomonocytic U937 cell line with probes generated from either 1,25-dihydroxyvitamin D₃-treated or untreated cells. We report here that a candidate clone that hybridized differentially is the Cdk inhibitor p21.WAF₁.CIP₁. Furthermore, we show that p21 is transcriptionally induced by 1,25-dihydroxyvitamin D₃ in a VDR-dependent, but not p53-dependent, manner, and we identify a functional vitamin D response element in the p21 promoter. Transient overexpression of p21 and/or the related Cdk inhibitor p27 in U937 cells in the absence of 1,25-dihydroxyvitamin D₃ results in the cell-surface expression of monocyte/macrophage-specific markers, suggesting that ligand-modulated transcriptional induction of the p21 gene facilitates the induced differentiation of this monoblastic cell line. We believe that this is the first report demonstrating that the ectopic overexpression of a Cdk inhibitor such as p21 or p27 directly leads to a terminal differentiation program.

[Key Words: Vitamin D₃; p21.WAF₁.CIP₁; U937 cells; vitamin D₃ receptor; cell cycle arrest; differentiation; vitamin D response element]

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The fat-soluble vitamin D₃ metabolite, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], is a major regulator of mineral homeostasis and bone formation/remodelling (Ross et al. 1994). This ligand can also elicit potent growth inhibitory and differentiation effects on a variety of cell types [Reichel et al. 1989]. For example, 1,25(OH)₂D₃ can induce normal and leukemic hematopoietic cells to differentiate into cells displaying characteristics consistent with a more mature monocyte/macrophage phenotype, including a decrease or cessation in their proliferation [Abe et al. 1981; Bar-Shavit et al. 1983; Mangelsdorf et al. 1984; Munkel et al. 1986; for review, see Moore 1987].

An examination of the role of 1,25(OH)₂D₃ during hematopoiesis was prompted by two observations: (1) Hematopoietic cells contain the vitamin D₃ receptor [Mangelsdorf et al. 1984]; and (2) osteoclasts arise from the fusion of circulating mononuclear precursor cells and therefore represent a terminal stage of mononuclear phagocyte differentiation [Kahn and Simmons 1975].

Abe and colleagues (1983) showed that 1,25(OH)₂D₃ at concentrations in the nanomolar range were sufficient to induce fusion of mouse alveolar macrophages. This same group first demonstrated that a myeloid leukemic cell line, mouse M1, could be induced to differentiate along the macrophage lineage by 1,25(OH)₂D₃ [Abe et al. 1981]. Subsequently, they and others showed that the human promyelocytic leukemia cell line HL60 and the human myelomonocytic cell line U937 can also be induced to terminally differentiate by 1,25(OH)₂D₃ [Ollson et al. 1983]. A variety of other compounds, such as phorbol esters, dimethylsulfoxide (DMSO), and retinoic acid, induce the differentiation of these cell lines (for review, see Collins 1987); however, HL-60 cells differentiate into granulocytes upon exposure to DMSO or retinoic acid [Collins et al. 1978; [Breitman et al. 1980], whereas they differentiate into cells exhibiting distinct monocyte/macrophage characteristics when treated with 1,25(OH)₂D₃ [McCarrthy et al. 1983].

1,25(OH)₂D₃ transduces its signal directly through a regulable, DNA-binding transcription factor, the vitamin D₃ receptor (VDR), which is a member of a large
superfamily collectively known as nuclear hormone receptors (Evans 1988). Thus ligand-inducible effects on cell growth and differentiation are initiated through the direct activation or repression of target genes by VDR. The identities of such genes, however, have remained elusive. Several investigators have reported that a variety of genes are either up-regulated or down-regulated in these cells in response to the hormone. Such genes include the cellular oncogenes c-myc (Reitsma et al. 1983; Grosso et al. 1985), N-ras, p53, c-fms (Sariban et al. 1985), protein kinase C (Obeid et al. 1990), and c-jun and junB (Datta et al. 1991). None of these genes, however, have been shown to be direct targets for VDR regulation; more likely, they represent intermediate or endproducts of the differentiation process.

Attempts at isolating regulated early genes from HL60 and U937 cells during induced differentiation have been met with limited success. By applying cDNA subtraction cloning techniques to an HL60 cloned variant cell line called IF10, Bories et al. (1989) reported the identification of a serine protease called myeloblastin, whose down-regulation by phorbol esters resulted in growth arrest of promyelocytic leukemia cells. The same group was also able to clone by subtraction two related cDNAs encoding fructose-1,6-bisphosphatase (Solomon et al. 1988): One is activated by 1,25(OH)2D3 early in HL60 differentiation, the second is activated by the hormone in peripheral blood monocytes. It is unclear, however, whether induction of fructose-1,6-bisphosphatase is a direct effect of VDR at the level of transcription initiation or an indirect, downstream effect. Thus, the number of genes shown to be directly regulated by VDR, and the number of characterized vitamin D response elements, remains quite small, making it difficult to propose accurate models for how VDR recognizes and regulates target genes and how those genes induce a biological switch resulting in monocytic–macrophagic differentiation.

To isolate putative vitamin D3-inducible target genes during myeloid cell differentiation, we established a differential screen whereby we enriched for genes that would be among the earliest regulated following addition of ligand. This approach, in which RNA expressed during a short pulse of ligand is selectively isolated (i.e., nascent RNA), has been successfully used in the isolation of a series of interleukin-2 (IL-2)-inducible genes in T lymphocytes (Beadling et al. 1993). We report here that one candidate clone that was differentially expressed in response to 1,25(OH)2D3 is the Cdk inhibitor p21WAF1/CIP1 (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994). Induction of p21 mRNA occurs within 2 hr of 1,25(OH)2D3 addition and in the presence of cycloheximide. Other Cdk inhibitors, such as p27 and Ink4 family members, are also induced by the ligand. Using a p21 promoter–reporter construct, we demonstrate that the p21 gene is transcriptionally activated by 1,25(OH)2D3 in a VDR-dependent, but p53-independent, manner, and we describe a functional vitamin D response element within the p21 promoter that mediates this induction. Transient overexpression of p21 and/or the related cyclin-dependent kinase (Cdk) inhibitor p27 in U937 cells in the absence of 1,25(OH)2D3 results in the cell-surface expression of monocyte/macrophage–specific markers, suggesting that ligand-modulated transcriptional induction of the p21 gene directly results in the induced differentiation of this monoblastic cell line.

**Results**

**A differential screen for vitamin D-inducible target genes**

We set out to isolate and clone 1,25(OH)2D3-regulated genes during the induced differentiation of U937 cells. To do so, we prepared probes by isolating RNA from cells untreated or treated with 1 x 10^-7 M 1,25(OH)2D3 for 4 hr. A 4-hr pulse of the ligand is sufficient to commit U937 cells to differentiate along the monocyte/macrophage pathway [data not shown]. In both the presence (+) and absence (-) of 1,25(OH)2D3, cycloheximide was included to prevent further protein synthesis and 4-thiouridine was added to enrich for nascent mRNA transcripts using organomercury chromatography; both strategies were employed to increase the likelihood of an immediate early transcript induced by 1,25(OH)2D3 represented in the probes. In addition, a plasmid cDNA library was generated from the induced cells [also treated for 4 hr with ligand, cycloheximide, and 4-thiouridine]. Eventually, 100,000 colonies were replica plated and differentially screened with (-) and (+) probes. Several colonies were identified from the differential screen that failed to hybridize to the (-) probe but gave strong signals with the (+) probe [Fig. 1]. One clone, C3, was further characterized because upon sequencing, it was found to be identical to the Cdk inhibitor p21 [WAF1, CIP1, CAP20] (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994). Northern analysis of RNA from U937 cells untreated or treated with 1,25(OH)2D3 for 4 hr indicated that p21 transcripts were undetectable in the absence of ligand but were strongly induced after only 4 hr [Fig. 2]. RNA prepared after 4 hr of hormone treatment in the presence of cycloheximide also hybridized strongly to the p21 probe [Fig. 2], indicating that de novo protein synthesis is not required for 1,25(OH)2D3 induction of p21 mRNA, and suggesting that this is an immediate-early activation of p21 transcription by VDR.

**Time course of p21, p27, and CD14 induction by 1,25(OH)2D3**

1,25(OH)2D3-mediated induction of p21 was examined more closely by comparing RNA and protein expression over a time frame of 0–96 hr following addition of 1,25(OH)2D3 to U937 cells [Fig. 3]. The related Cdk inhibitor p27Kip1 [Poljak et al. 1994a,b; Toyoshima and Hunter 1994] was also included in this analysis [p57Kip2 [Lee et al. 1995; Matsuoka et al. 1995], another member of this family, has a highly restricted expression pattern and was not detectable in U937 cells]. Whereas p21 and p27 mRNA induction was very rapid [2 hr for p21 and 4
Figure 1. Representative set of differential colony screen autoradiographs. Filters were screened pairwise from 96-well plates with cDNA probes generated from U937 cells that were untreated (−) or treated (+) with 1,25(OH)2D3 for 4 hr. For both, cycloheximide (10 μg/ml) was added to cells for 4 hr. Clones A4, C1, C3, D6, E9, F5, H5, and H8 hybridized with the (+) probes but not at all or to a lesser degree with the (−) probes.

The time course of protein induction following 1,25(OH)2D3 treatment was much more gradual (Fig. 3B); an increase in the accumulation of p27 protein was not apparent until at least 48 hr after addition of hormone. The gap between the early mRNA induction of Cdk inhibitors and the late appearance of corresponding proteins suggests that a post-transcriptional or post-translational regulation is imposed on Cdk inhibitors. The effect of p21 and p27 induction in response to 1,25(OH)2D3 was also examined functionally by assessing cyclin/Cdk activity by using histone H1 as a substrate for the kinase. Cdk2-associated kinase activity actually increased from 4 to 24 hr following 1,25(OH)2D3 treatment and was totally extinguished at 72 hr (Fig. 3C). This correlated with a FACS analysis of 1,25(OH)2D3-treated U937 cells. As presented in Table 1, it was not until the 72-hr time point that the vast majority of U937 cells are arrested in G1. Presumably, this long time course is attributable to the fact that an asynchronous population of cells are initially exposed to the ligand and a majority of cells needed to complete a cycle before they could be arrested in G1. In addition, the cell population at G2/M phase accumulated at 24 hr (23%) and then decreased at 48 hr (13%). This delay in G2/M in the 1,25(OH)2D3-treated cells implies that cells need to cycle through G2/M to arrest at G1. Note also that RNA for the monocyte/macrophage-specific marker CD14 was first detected 8 hr following addition of ligand and increased gradually following the time course as more cells withdrew from cell cycle and committed to differentiation (Fig. 3A). The rapid and sustained increase in histone H1 kinase activity prior to growth arrest and differentiation is consistent with a proposal that p21 can also function as an assembly factor of cyclin-Cdk complexes at low stoichiometries (Zhang et al. 1994).

Transcriptional activation of p21 by the vitamin D3 receptor

The fact that mRNAs for several Cdk inhibitors were strongly induced after only 4 hr of 1,25(OH)2D3 treatment in the presence of cycloheximide suggests that induction could be a direct effect on the transcription of these genes by the VDR. To directly address this question for p21, a human p21 reporter construct, WWP-p21-Luc (El-Deiry et al. 1993), containing a 2.4-kb upstream genomic fragment that includes the p21 pro-

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Northern

hrs after addition of 1, 25(OH)2D3

0 2 4 8 12 24 48 72 96

p21

p27

actin

Western

hrs after addition of 1, 25(OH)2D3

0 4 8 12 24 48 72 96

p21

p27

Cdk2-associated H1 kinase activity

hrs after addition of 1, 25(OH)2D3

0 4 8 12 24 48 72 96

H1

The p21 promoter contains a functional vitamin D response element

Nuclear receptors, such as VDR, act as direct signal

Table 1. Cell cycle distribution of 1,25(OH)2D3-treated U937 cells

|         | 0    | 12   | 24   | 48   | 72   |
|---------|------|------|------|------|------|
| +1,25(OH)2D3 |      |      |      |      |      |
| G1      | 31.2 | 39.0 | 35.3 | 56.2 | 79.8 |
| S       | 64.6 | 53.5 | 41.5 | 30.9 | 9.7  |
| G1/M    | 4.2  | 7.5  | 23.2 | 12.9 | 10.5 |
| -1,25(OH)2D3 |     |      |      |      |      |
| G1      | 31.2 | 35.2 | 35.4 | 35.7 | 34.6 |
| S       | 64.6 | 63.0 | 55.3 | 57.7 | 58.7 |
| G1/M    | 4.2  | 1.8  | 9.2  | 6.6  | 6.7  |

U937-4 cells were treated with 1,25(OH)2D3 for the indicated times and analyzed for DNA content by propidium iodide staining. Data were processed using the Multicycle program (Phoenix Flow System).
The effect of 1,25(OH)₂D₃ on the accumulation of Cdk inhibiting Ink4 family mRNAs. A Northern blot is presented of RNA from exponentially growing U937 cells in the absence and at various times (in hours) following the addition of 1,25(OH)₂D₃ (1 x 10⁻² M), hybridized to probes corresponding to p15, p16, and p18 genes. Equal amounts of RNA loaded in each lane was confirmed by hybridization of the same filter to an actin probe. (4 + CHX) A 4-hr induction in the presence of cycloheximide.

Recently, it was shown that the increased expression of p21 is closely correlated to the induction of differentiation in several cell types and that this can occur independently of p53, a tumor suppressor that transcriptionally activates p21 (Jiang et al. 1994; Steinman et al. 1994; Halevy et al. 1995; Macleod et al. 1995; Parker et al. 1995; Skapek et al. 1995). Given that 1,25(OH)₂D₃ induces U937 cells to differentiate and that p21 is transcriptionally activated by 1,25(OH)₂D₃, we wondered whether the overexpression of p21 or p27 independent of a hormone or agent was able to induce these cells to differentiate to monocyte/macrophages. A cytomegalovirus (CMV)-driven p21 or p27 expression plasmid, together with a CMV-driven lacZ expression plasmid, was used to transiently cotransfect U937 cells, and 80 hr later, cells were harvested and analyzed by FACS for both CD14 and CD11b expression; both CD14 and CD11b are monocyte/macrophage-specific cell-surface markers in U937 cells.
Vitamin D₃ induction of the p21WAF1/CIP1 gene

A

B

C

Figure 6. A VDR binding site resides within the p21 promoter. [A] Above is the sequence of the human p21 promoter from -788 to -756, containing a putative VDRE. Also shown is a consensus VDRE, a directly repeating hexameric element spaced by 3 nucleotides (DR3). R is purine and N is any base. Below is an electrophoretic mobility-shift assay of the putative p21 VDRE using overexpressed VDR and GST-RXR proteins (Cheskis and Freedman 1994). End-labeled oligonucleotides containing either the sequence from the p21 promoter (p21-788 to -756) [lanes 1-10], or a known VDRE from the mouse osteopontin promoter (DR3 VDRE) [lanes 11-20] were used as probes, together with increasing amounts of VDR and a fixed amount of GST–RXR [25 ng]. VDR indicates a presumed homodimer of the receptor, and VDR–RXR represents heterodimers of the two receptors. [B] Gel shift assay comparing GST–RXR and VDR binding to the p21 element and a mutated site. Endlabeled oligonucleotides containing the indicated sequences from the p21 promoter (p21 VDRE) or a mutant version containing a 1-bp change in each half-site (G to T; p21 VDRE mut) were used as probes. [C] Deletion of p21 VDRE abolishes 1,25(OH)₂D₃ induction of p21 transcription. A 15-bp deletion of the sequence corresponding to the putative VDRE shown in A was introduced into the p21 promoter (-778 to -765), the resulting luciferase reporter, called p21(Δ-778 to -765)-Luc, was used to transiently cotransfect 10-1 cells with CMV–VDR in the absence and presence of 1,25(OH)₂D₃, as described in Fig. 4.

proteins and, thus, appropriate markers for differentiation [Breard et al. 1980]. A portion of cells from each individual transfection was also stained for β-galactosidase and used to normalize for transfection efficiency. Under conditions where cells were maintained subconfluent following transfection, CD11b and CD14 expression were induced well above background in cells transfected with p21 or p27 but not with the CMV vector alone (Fig. 7). When p21 and p27 were coexpressed following transient transfection, the percentage of transfected cells that stained positively for CD11b and CD14 was ~45% and 35%, respectively. A similar proportion of cells also exhibited morphological characteristics of monocytes after transfection with the p21 and p27 expression plasmids, in that the cells appeared larger, contained vacuolated cytoplasms with villous membranes and irregular nuclei, and were somewhat adherent [M. Liu, unpubl.]. Amino-terminal deletions of p21 and p27
Discussion

The role of Cdk inhibitors in differentiation

Hormonal ligands such as 1,25(OH)₂D₃ and retinoids are well-known growth inhibitors and inducers of differentiation. We have shown here that one transcriptionally activated target gene of the vitamin D₃ receptor encodes a protein, p21, involved in blocking cell cycle progression. In this work we have observed that in myeloid leukemic cells, which are poised to differentiate, p21 and/or p27 overexpression in the absence of 1,25(OH)₂D₃ is sufficient to drive these cells to express CD14 and CD11b surface antigens typically detected in mature monocyte/macrophages following addition of the ligand (Fig. 7). This observation suggests that in this cellular context, hormonal agents like 1,25(OH)₂D₃ induce genes that mediate G₁ arrest to initiate an induction leading to terminal differentiation. Simply arresting these cells in G₁ with p21 and p27 is apparently sufficient to activate the differentiation pathway. Consistent with this, several groups have reported that p21WAF1/CIP1 expression is up-regulated during the differentiation of a number of cell types in vitro [Jiang et al. 1994; Steinman et al. 1994; Haley et al. 1995; Macleod et al. 1995; Missiro et al. 1995; Parker et al. 1995; Zhang et al. 1995], and is involved in various developmental processes in the mouse, including those of the outer layers of epidermis and olfactory neurons [Parker et al. 1995]. In addition, MyoD activates the p21 gene to arrest the cell cycle during muscle differentiation [Haley et al. 1995]. We believe, however, that this is the first report demonstrating that the ectopic overexpression of a Cdk inhibitor such as p21 or p27 can lead directly to a differentiation program.

Besides p21 and probably p27, other putative target genes of VDR may also encode other cell cycle inhibitors, such as the so-called INK4 family members p15, p16, and p18. In fact, mRNAs corresponding to this class of Cdk inhibitors also appear to be induced by 1,25(OH)₂D₃ [Fig. 4]. Moreover, the strongest expression of the cell-surface differentiation markers detected in the transient overexpression experiment presented in Figure 7 occurred when both p21 and p27 were cotransfected, suggesting that multiple cell cycle inhibitors might be required for complete differentiation. This is reminiscent of a report that coexpression of p16 and p21 can augment muscle-specific gene expression typically activated by MyoD [Skapek et al. 1995]. At the same time, this class of molecules may be functionally redundant, because it was recently demonstrated that p21−/− mice, although impeded in their ability to arrest in G₁, develop normally both at gross anatomical and histological levels [Deng et al. 1995]. It should be emphasized that other differentially expressed candidate genes identified by our screen might also play key roles in normally carrying out the differentiation program. For example, some of these genes might regulate the steady-state expression of the Cdk inhibitors [Pagaon et al. 1995]; others might down-regulate cyclin or Cdk expression as is often observed in terminal differentiating cells [Li Yokawa et al. 1994]. Alternatively, other genes induced by 1,25(OH)₂D₃ and VDR might cooperate with p21 and p27 to drive myeloid cells to terminally differentiate. These same genes could also be targets of other inducers. Moreover, whereas the differential screen we set up here was designed to isolate 1,25(OH)₂D₃-inducible genes, many target genes could
be subjected to transcriptional repression by VDR. An example of a gene down-regulated by VDR is IL-2, where the receptor antagonizes the positive transcription factors NFATp and Jun/Fos, resulting in repression of IL-2 transcription and a suppression of activated T cells by 1,25(OH)₂D₃ (Alroy et al. 1995).

We view Cdk inhibitors as central targets during induced differentiation, as it appears that several other inducers of myeloid cell differentiation up-regulate p21 and p27 expression (Sherr and Roberts 1995). These agents include TPA, sodium butyrate, DMSO, and retinoids, as well as serum. Therefore, the p21 promoter probably contains multiple response elements for several differentiation signals. These elements could be scattered throughout the promoter or might overlap to create potential combinatorial or synergistic responses. As an example of the latter, it was shown recently that a functional serum response element overlaps with a p53 binding site (Macleod et al. 1995). We also have observed p21 induction in response to retinoids. Activation by retinoic acid persists when the p21 VDRE is deleted (M. Liu and L.P. Freedman, unpubl.), suggesting that other hormone response elements besides the VDRE that we have identified here reside in the p21 promoter and reinforcing the notion that p21 gene transcription is regulated by multiple cis-acting elements responsive to a variety of inducing signals.

Another level of p21 regulation is reflected by MyoD during skeletal muscle cell differentiation. MyoD is proposed to be phosphorylated by Cdk4, resulting in a block of its transcriptional enhancing activity. Unphosphorylated MyoD appears to induce p21 expression, which blocks Cdk activity, thereby maintaining MyoD in a transcriptionally active form and resulting in a positive feedback loop for p21 expression (Halevy et al. 1995). This might lead one to speculate that any activator that induces the transcription of a Cdk inhibitor such as p21 may also be subject to negative regulation by a Cdk. Consistent with this, phosphorylation appears to modulate to varying degrees the activities of several steroid and nuclear receptors (Tsai and O'Malley 1994). Thus, it would be interesting to test whether VDR’s transcriptional activity is affected by Cdks.

The kinetics of p21/p27 induction and the onset of growth arrest and differentiation

Induction of p21 and p27 mRNA expression following 1,25(OH)₂D₃ treatment (Fig. 3A), implying that asynchronous cells must complete a cycle and become arrested in G₁ before they can commit to differentiate; such cells will increase over time. In fact, only at 72 hr following 1,25(OH)₂D₃ addition did we find the majority of cells arrested at G₁, as assayed by FACS (Table 1). The kinetics of p21 and p27 mRNA induction, as well as the expression of the CD14 differentiation marker, suggests that the p21/p27 induction occurs hours before growth arrest is completed and differentiation begins.

The p21 and p27 time course also suggests that both of these Cdk inhibitors are required for complete cell cycle arrest, as judged by correlating p21 and p27 protein levels following 1,25(OH)₂D₃ addition with Cdk2-associated histone H1 kinase activity (Fig. 3B,C). That the kinase activity was not extinguished completely until 72 hr postaddition may reflect an additive or synergistic effect of both Cdk inhibitors, and the fact that it is only after 72 hr that the vast majority of cells are arrested in G₁ (Table 1).

Surprisingly, we observed an initial increase in Cdk2-associated histone H1 kinase activity following addition of 1,25(OH)₂D₃ (Fig. 3C), which corresponded to the peak of p21 mRNA levels (Fig. 3A). A similar observation has been reported in both wild-type and p53⁻/⁻ mouse embryonic fibroblasts where p21-associated histone H1 kinase activity peaked coordinately with p21 mRNA levels following serum stimulation (Macleod et al. 1995). These observations may reflect the functions of p21 and related proteins that are not yet completely understood. It has been proposed that at low levels, p21 may act to promote the association of cyclin and Cdk subunits, and only after a high enough stoichiometry is achieved can it act to inhibit the complex (Zhang et al. 1994).

Conclusions

The model presented in Figure 8 highlights a key role for p21 in facilitating the differentiation of myeloid cells in response to inducers such as 1,25(OH)₂D₃ in a p53-independent manner. This occurs through an activation of p21 transcription by VDR, most likely in a heterodimeric complex with RXR, at a VDRE within the p21 promoter. However, the related Cdk inhibitor p27 appears to also facilitate differentiation and is also induced by 1,25(OH)₂D₃, and coexpression of p21 and p27 has an enhanced effect on U937 differentiation. In addition, other Cdk inhibitors appear to be induced by 1,25(OH)₂D₃ in these cells. We presume that non-Cdk inhibitor targets that drive the differentiation process may also be induced by the ligand–receptor complex, and we are currently characterizing our collection of additional differentially expressed clones for such genes.

Materials and methods

Library construction and differential screening

U937 cells (clone 4), provided by K. Nilsson (Ollson et al. 1983), routinely maintained in charcoal-stripped fetal bovine serum
Figure 8. A model for vitamin D induction of p21 and other putative target genes leading myeloid cell differentiation. Gene X denotes any other potential VDR target gene regulated in response to 1,25(OH)₂D₃ and involved in facilitating the differentiation process (see text for details).

[FBs (Bio Gemini), were set at a density of 0.5×10⁶ cells/ml and cultured in the presence or absence of 1×10⁻⁷ M 1,25(OH)₂D₃ (a gift from M. Uskokovic, Hoffman-LaRoche, Nutley, NJ), 10 μg/ml of cycloheximide, and 2.5 mCi/ml of [³²P]thymidine for a total of 4 hr. Total cellular RNA was isolated by the guanidium thiocyanate/phenol method. Thiol-labeled nascent transcripts were then purified as described (Beadling et al. 1993). Finally, poly[A]⁺ RNA was obtained by one-round oligo(dT) selection (Invitrogen). Five micrograms of the 1,25(OH)₂D₃-treated nascent mRNA was used to prepare a pBluescript SK+ plasmid cDNA library (Stratagene), and portions of the library were used to transform escherichia coli XL2-blue MRF' competent cells. For differential colony screening, nascent ³²P-labeled cDNA from treated or untreated cells was synthesized using Superscript reverse transcriptase (BRL). Hybridization was carried out for 72 hr at 42°C in 50% formamide, 6× SSPE, 5× Denhardt's solution, 1% SDS, 100 μg/ml of poly[A], and 100 μg/ml of poly[C], with a final probe concentration of 2.0×10⁶ cpm/ml. The final wash was done at 50°C with 0.1× SSPE and 0.1% SDS for 30 min. After the primary screen (1.0×10³ unamplified clones), ~4000 colonies that exhibited differential hybridization to the treated versus untreated probes were then picked and grown in 96-well plates. Duplicate dot blots were prepared with a replicator beaded lid (TSP, Nunc) on HATF membranes (Millipore) and hybridized again with the ± cDNA probes as in the primary screen. Candidates after the secondary screen were then isolated for Northern analysis. To measure Cdk2-associated histone H1 kinase activity, equal amounts of 1,25(OH)₂D₃-treated cell extracts from each time point were immunoprecipitated with a Cdk2 antibody (Pharmingen), and the immunoprecipitates were then assayed for HH1 kinase activity as described previously (Koff et al. 1993).

Transient reporter transfections, protein-DNA binding, and oligonucleotide-directed mutagenesis
p53⁻/⁻ mouse embryonic fibroblast 10-I cells (kindly provided by Dr. A. Levine, Princeton University, NJ) were transfected by the calcium phosphate/DNA coprecipitation method using 2.0 μg of either WWP-p21-Luc or DM-p21-Luc reporters (provided by B. Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD) and 0.5 μg of CMV-VDR or pCMV [empty vector] per transfection (60-mm dish). After the precipitate was washed away, cells were fed with fresh medium containing 10% charcoal-stripped FBS and incubated with 1×10⁻⁸ M 1,25(OH)₂D₃ or its solvent (ethanol) for 48 hr. Total cellular extracts were then split and assayed for luciferase and β-galactosidase activities. Gel shift assays with VDR and RXR were carried out as described previously (Cheskis and Freedman 1994). VDR was overexpressed and partially purified from baculovirus-infected Sf9 cells, glutathione S-transferase (GST)-RXR was overexpressed and purified to homogeneity as described (Cheskis and Freedman 1994). For oligo-directed mutagenesis, a 43-base oligonucleotide was synthesized as the reverse complement of the p21 sense strand excluding 15 bases corresponding to the putative p21 VPDE (−778 to −765) [5'-GGGAAACAGAAGAATTGGACATACACCCTAACATCACCTGAAC-3'] and used to anneal and extend the wild-type single-stranded template generated from p21-Luc, a plasmid containing a 2.4-kb HindIII frag-
ment derived from WWP–Luc [El-Deiry et al. 1993] and inserted into the luciferase reporter pGL2–basic (Stratagene). p21–Luc and the deletion construct, called p21Δ(-778 to -765)Luc, were then used to transiently transfect 10-1 cells to test for 1,25(OH)2D3 responsiveness.

Overexpression of p21 and p27 and FACS analysis

Early log-phase-growing U937 cells were harvested and washed twice with ice-cold PBS (−Mg and Ca), resuspended in PBS at a density of 6 x 10^6 cells/ml, and electroporated at 960 mF and 250 V in 0.4-cm cuvettes. After 5 min electroporation at room temperature, the cells were diluted into 6 ml of RPMI 1640 plus 10% charcoal-stripped FBS. After an additional 14 hr, the medium was removed and the cells were refed with 8 ml of fresh medium and 1 ml of fresh medium was added every 24 hr prior to harvest. Approximately 1.0 x 10^6 cells were used for FACS analysis (Becton Dickinson) with FITC-conjugated CD11b or CD14 antibodies (Caltag). Cell viability was estimated by trypan blue dye exclusion, and an equal numbers of viable cells were used for β-galactosidase activity staining as described (MacGregor et al. 1987) to normalize for transfection efficiency. All CMV expression constructs were generously provided by J. Massagué (Memorial Sloan-Kettering Cancer, New York).

Cell cycle analysis

U937 cells (2 x 10^6) were washed in ice-cold 1 x PBS (−Mg/Ca, 1% BSA) and permeabilized with 70% ethanol at 4°C for ≥1 hour. After centrifugation, cell pellets were resuspended in 1 x 10^6/ml of 1 x PBS, treated with RNase A (0.08 mg/ml), and stained with propidium iodide (0.2 mg/ml) at 37°C for 30 min. Collected data from FACSscan (Becton Dickenson) were analyzed using the Multicycle program from Phoenix Flow System.

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