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; Munker 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 (McCarthy 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

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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 p21^{WAF1, 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 1×10^{-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 p27^{Kip1} (Polyak et al. 1994a,b; Toyoshima and Hunter 1994) was also included in this analysis [p57^{Kip2} (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

hr for p27; Fig. 3A), the time course of protein induction following $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ treatment and was totally extinguished at 72 hr (Fig. 3C). This correlated with a FACS analysis of $1,25(\text{OH})_2\text{D}_3$ -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 G_1 . 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 G_1 . In addition, the cell population at G_2/M phase accumulated at 24 hr (23%) and then decreased at 48 hr (13%). This delay in G_2/M in the $1,25(\text{OH})_2\text{D}_3$ -treated cells implies that cells need to cycle through G_2/M to arrest at G_1 . 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].

Induction of *Ink4* Cdk inhibitors by $1,25(\text{OH})_2\text{D}_3$

In addition to Cip/Kip inhibitors, a second distinct family of Cdk inhibitors exists that includes $p16^{\text{Ink4A}}$ [Serrano et al. 1993], $p15^{\text{Ink4B}}$ [Hannon and Beach 1994], $p18^{\text{Ink4C}}$ [Guan et al. 1994; Hirai et al. 1995], and $p19^{\text{Ink4D}}$ [Chan et al. 1995; Hirai et al. 1995]. These so-called *Ink4* proteins inhibit cyclin D binding to Cdk4 and Cdk6 to block cell proliferation [Guan et al. 1994;

Hirai et al. 1995]. We examined the effect of $1,25(\text{OH})_2\text{D}_3$ on the expression of p15, p16, and p18 mRNA in U937 cells by Northern analysis. As is shown in Figure 4, mRNAs corresponding to these three *Ink4* family members were induced in response to the ligand in a time course similar to that observed with p21 and p27. p15 and p18 induction is also cycloheximide resistant. We do not yet know if up-regulation of the *Ink4* genes are direct responses to VDR, but these results suggest a coordinate induction of many Cdk inhibitors by $1,25(\text{OH})_2\text{D}_3$, perhaps contributing cooperatively to cell cycle arrest that leads to monocyte/macrophage differentiation. Along these lines, Reynisdóttir et al. (1995) recently reported that Kip/Cip and *Ink4* inhibitors act together to induce the cell cycle arrest of mink lung epithelial cells and human keratinocytes in response to transforming growth factor- β (TGF- β).

Transcriptional activation of p21 by the vitamin D_3 receptor

The fact that mRNAs for several Cdk inhibitors were strongly induced after only 4 hr of $1,25(\text{OH})_2\text{D}_3$ 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-

Figure 2. Northern blot analysis of clone C3 (p21) expressed in U937 cells. Twenty micrograms (per lane) of total RNA was isolated from exponentially growing cells not treated (0 hr) and from cells treated for 4 hr with $1,25(\text{OH})_2\text{D}_3$ in either the presence (+ CHX) or absence of cycloheximide.

A Northern **B** Western **C** Cdk2-associated H1 kinase activity

Figure 3. (A) Time course of p21, p27, and CD14 mRNA induction by 1,25(OH)₂D₃ in U937 cells. Northern blot analysis of total RNA isolated from exponentially growing U937 cells (time 0) and at various times (in hours) following addition of 1,25(OH)₂D₃ (1×10^{-7} M). Human p21 (clone C3 in this study), p27 (Polyak et al. 1994b), and CD14 (Goyert et al. 1988) probes were used. Equal amounts of RNA loaded in each lane was confirmed by hybridization of the same filter with an actin probe. (B) Induction of p21 and p27 proteins by 1,25(OH)₂D₃ in differentiating U937 cells. Whole cell lysates isolated from cells that were untreated or treated with 1,25(OH)₂D₃ for the indicated times were resolved by SDS-PAGE, and p21 or p27 protein levels were detected by Western blot analysis using antibodies directed against p21 and p27. (C) Inhibition of Cdk2-associated histone H1 kinase activity in differentiating U937 cells following 1,25(OH)₂D₃ addition. Cells and extracts were treated and prepared as in B. Note that the 12-hr time point was underloaded and does not represent a true decrease in H1 kinase activity.

motor and a truncated version of this reporter called DM-p21-Luc lacking the functional p53 binding site within the promoter were used to transiently transfect the mouse embryonic fibroblast cell line 10-1 (Harvey and Levine 1991) in the presence and absence of 1,25(OH)₂D₃ and VDR. Induction of the reporter gene in response to hormone was approximately fivefold and was dependent on the cotransfection of VDR (Fig. 5). The induction was independent of p53, as 10-1 cells are p53^{-/-} (Harvey and Levine 1991) and because the DM-p21-Luc reporter lacks the p53 binding site. Thus p21^{WAF1/CIP1} appears to be a transcriptionally responsive target gene of 1,25(OH)₂D₃ and VDR.

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

	Percent	Hours				
		0	12	24	48	72
+ 1,25(OH) ₂ D ₃	G ₁	31.2	39.0	35.3	56.2	79.8
	S	64.6	53.5	41.5	30.9	9.7
	G ₂ /M	4.2	7.5	23.2	12.9	10.5
- 1,25(OH) ₂ D ₃	G ₁	31.2	35.2	35.4	35.7	34.6
	S	64.6	63.0	55.3	57.7	58.7
	G ₂ /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).

Figure 4. Effect of $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ (1×10^{-7} 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.

transducers by binding selectively to particular DNA sequences within promoters of regulated genes, thereby modulating the transcription of such genes in response to ligand. If p21 is a vitamin D responsive gene, then its promoter ought to contain one or more VDR binding sites (called VDREs). We searched the human p21 promoter–proximal 2.4 kb for putative VDREs, which characteristically are comprised of two hexameric direct repeat elements spaced by 3 bp [DR3; Fig. 6A]. One particularly good candidate VDRE was found 770 bp upstream of the start site of p21 transcription. A short oligonucleotide duplex containing the putative VDRE and some surrounding sequence (Fig. 6A) was synthesized, and used as a probe in a gel shift assay together with purified VDR and its heterodimeric partner, the retinoid X receptor (RXR) (Fig. 6A, lanes 1–10). To assess the relative affinity of the putative p21 VDRE, VDR binding to this site was compared to a second probe containing a known, high-affinity VDR recognition element, the VDRE found in the mouse osteopontin gene promoter (Noda et al. 1990) [Fig. 6A, lanes 11–20 (DR3 VDRE)]. Although VDR–RXR binding to the osteopontin VDRE is significantly stronger than it is to the p21 element, relatively low amounts of the heterodimer are required to detect a shifted p21 complex. Competition gel shifts with a nonspecific DNA indicate that VDR–RXR binding to the p21 element is selective (data not shown). In addition, a mutant probe was also made carrying the p21 sequence, where the second base pair in each half-site, normally a G/C pair, was substituted with T/A (Fig. 6B); from the known crystal structures of several nuclear receptor DNA-binding domains, this G is the target of a side-chain contact by a conserved arginine residue residing within the recognition α -helix of the DNA-binding domain (Freedman and Luisi 1993). Binding by VDR–RXR was reduced by $\sim 75\%$ with this mutated binding

site (Fig. 6B). Moreover, RXR alone could not bind to either the wild-type or mutant probe. Importantly, when a 15-bp sequence corresponding to the putative VDRE within the p21 promoter was deleted, the $1,25(\text{OH})_2\text{D}_3$ -dependent induction of the p21–Luc reporter was abolished (Fig. 6C). Thus, by several criteria, this element, centered at ~ 771 of the p21 promoter, appears to act as a functional vitamin D response element.

Ectopic expression of p21 or p27 facilitates the induction of monocyte/macrophage-specific markers in U937 cells

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 $1,25(\text{OH})_2\text{D}_3$ induces U937 cells to differentiate and that p21 is transcriptionally activated by $1,25(\text{OH})_2\text{D}_3$, 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

Figure 5. Transcriptional activation of the p21 gene by VDR. The p53-deficient cell line 10-1 (Harvey and Levine 1991) was cotransfected with WWP-p21-Luc or DM-p21-Luc and a VDR expression vector (pCMV-VDR) or its empty vector (-), together with CMV- β -gal, in the presence or absence of 1×10^{-8} M $1,25(\text{OH})_2\text{D}_3$. Luciferase activity is the average of three experiments and is expressed as arbitrary light units normalized to β -galactosidase activity.

Vitamin D₃ induction of the p21^{WAF1/CIP1} gene

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. End-labeled 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 fol-

lowing 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 cytoplasm with villous membranes and irregular nuclei, and were somewhat adherent (M. Liu, unpubl.). Amino-terminal deletions of p21 and p27

Figure 7. Induced differentiation of U937 cells by transient overexpression of p21 and/or p27 in the absence of $1,25(\text{OH})_2\text{D}_3$. U937 cells were cotransfected by electroporation with 2.0 μg of CMV- β -gal plasmid together with 10 μg of a CMV plasmid expressing either p21, p27, or amino-terminal deletion mutants of both proteins (Cp21 and Cp27) (Luo et al. 1995), or the empty vector (CMV5). Carrier DNA was added to a total of 30 μg of DNA per transfection. At 80 hr postelectroporation, cells were collected for in situ β -galactosidase assays (to normalize for transfection efficiency) and FACS analyses of the endogenous expression of the monocyte/macrophage markers CD11b and CD14. The results are expressed as the percent of transfected cells positive for CD11b or CD14 staining, normalized for transfection efficiency by in situ β -galactosidase staining. Error bars indicate the standard deviation from at least three independent experiments. As a reference, also shown is the percent of total cells positive for CD11b or CD14 staining after 80 hr of continuous $1,25(\text{OH})_2\text{D}_3$ treatment.

(called C-p21 and C-p27) that remove both proteins' Cdk inhibitory activity (Luo et al. 1995) fail to induce CD11b and CD14 when these constructs were transiently expressed (Fig. 7), suggesting that an intact Cdk inhibitory domain is required for cell cycle withdrawal and differentiation. Taken together, these results indicate that overexpression of p21 and/or p27 facilitate U937 cells to differentiate along a monocyte/macrophage pathway, most likely by inhibiting Cdk.

Discussion

The role of Cdk inhibitors in differentiation

Hormonal ligands such as $1,25(\text{OH})_2\text{D}_3$ and retinoids are well-known growth inhibitors and inducers of differen-

tiation. We have shown here that one transcriptionally activated target gene of the vitamin D_3 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 $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ induce genes that mediate G_1 arrest to initiate an induction leading to terminal differentiation. Simply arresting these cells in G_1 with p21 and p27 is apparently sufficient to activate the differentiation pathway. Consistent with this, several groups have reported that p21^{WAF1}, CIP1 expression is up-regulated during the differentiation of a number of cell types in vitro (Jiang et al. 1994; Steinman et al. 1994; Halevy et al. 1995; Macleod et al. 1995; Missero 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 (Halevy 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 $1,25(\text{OH})_2\text{D}_3$ (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 G_1 , 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 (Liyokawa et al. 1994). Alternatively, other genes induced by $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ -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 addition to U937 cells occurred very rapidly (within 2 hr for p21 and 4 hr for p27) and was resistant to cycloheximide (Figs. 2 and 3A), suggesting that the response was transcriptional. This was confirmed for p21 by demonstrating that the p21 promoter conferred 1,25(OH)₂D₃ inducibility to a reporter gene and that this induction was selectively lost when a short sequence that bound a VDR–RXR heterodimer *in vitro* was deleted from the promoter (Figs. 5 and 6). However, the time course for cell cycle inhibition and differentiation occurs well after the early p21/p27 response. The levels of CD14 mRNA increased quantitatively following

1,25(OH)₂D₃ treatment (Fig. 3A), implying that asynchronized 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 embryo 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 0.5×10^6 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 μ g/ml of cycloheximide, 200 mM 4-thiouridine, and 2.5 mCi/ml of [³H]uridine 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 II SK⁺ plasmid cDNA library (Stratagene), and portions of the library were used to transform epicurian *E. 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 μ g/ml of poly(A), and 100 μ g/ml of poly(C), with a final probe concentration of 2.0×10^6 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 (1.0×10^5 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 $\pm$ cDNA probes as in the primary screen. Candidates after the secondary screen were then isolated for Northern analysis. Total RNAs untreated and treated for 4 hr in the presence or absence of cycloheximide were subjected to electrophoresis on a 1% formaldehyde-agarose gel. Inserts of interested clones were prepared by PCR with T3 and T7 primers and then ³²P-labeled by random priming. Hybridization was carried out in 50% formamide at 42°C for 24 hr, followed by washes in 0.1× SSC/0.1% SDS at 50°C.

RNA and protein analysis

1,25(OH)₂D₃-Treated cell extracts from each time point (hours after addition of ligand) were prepared for Northern analysis as

described above, and for protein, by lysis in 50 mM Tris-Cl (pH 7.4), 200 mM NaCl, 2 mM EDTA, 0.5% NP-40, 0.3 mM Na-orthovanadate, 50 mM NaF, 0.5 mM DTT, and protease inhibitors. Equal amounts of the lysate were boiled in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with either p21 (Pharmingen) or p27 antibodies (Polyak et al. 1994b). 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 H1 kinase activity as described previously (Koff et al. 1993).

Transient reporter transfections, protein-DNA binding, and oligonucleotide-directed mutagenesis

p53^{-/-} mouse embryonic fibroblast 10-1 cells (kindly provided by Dr. A. Levine, Princeton University, NJ) were transfected by the calcium phosphate/DNA coprecipitation method using 2.0 μ g of either WWP-p21-Luc or DM-p21-Luc reporters (provided by B. Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD) and 0.5 μ g 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 (Cheski 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 (Cheski 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 VDRE (-778 to -765) [5'-GGGAAACAGAAGAAT-TGGACATACACCCTAACATCACCTGAAC-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 6×10^6 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 1.0×10^6 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 (2×10^6) were washed in ice-cold $1 \times$ PBS (-Mg/Ca, 1% BSA) and permeabilized with 70% ethanol at 4°C for ≥1 hour. After centrifugation, cell pellets were resuspended in 1×10^6 /ml of $1 \times$ 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 FACScan (Beckton Dickinson) were analyzed using the Multicycle program from Phoenix Flow System.

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