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Cyclin C Is a Primary 1α,25-Dihydroxyvitamin D_3 Responding Gene

Patsie Polly,[1] **Carina Danielsson,**[1,2] **Magdalena Schräder,**[1] **and Carsten Carlberg**[1]*

[1]Institut für Physiologische Chemie I, Heinrich-Heine-Universität, D-40001 Düsseldorf, Germany
[2]Department of Medical Sciences, Section of Dermatology, Uppsala University, S-75185 Uppsala, Sweden

Abstract 1α,25-dihydroxyvitamin D_3 (VD) is a pleiotropic nuclear hormone that also has effects on cell cycle regulation. VD and its synthetic analogues are known inhibitors of cellular growth and inducers of apoptosis, however, the primary mediator genes of these effects largely remain unknown. In order to identify novel targets for VD, that may be involved in the regulation of the cell cycle, a differential display PCR (ddPCR) approach was applied to the MCF-7 human breast cancer cell line, which provided the gene for cyclin C as an interesting candidate. Quantitative assessment of cyclin C expression showed that the gene was significantly upregulated by VD and its analogues, EB1089 and CB1093 both on the level of mRNA expression and more so on the level of protein expression in MCF-7 cells. Upregulation of cyclin C protein expression could also be confirmed in MeWo human melanoma and in U937 human promyelocytic leukemia cells. This observation adds a new gene candidate to the list of primary VD responding genes. Cyclin C is not a typical cyclin, as it apparently modulates the activity of the RNA polymerase II complex, which provides fresh insight into the mechanisms of cell cycle and general transcriptional regulation by VD and its analogues. J. Cell. Biochem. 77:75–81, 2000.

The seco-steroid hormone 1α,25-dihydroxyvitamin D_3 (VD), is the most biologically active form of vitamin D_3 and an effective regulator of calcium homeostasis and bone metabolism [Walters, 1992]. VD has been known for some time to inhibit cell growth and to induce differentiation in several normal and malignant cell types [Abe et al., 1981; Colston et al., 1982; Frampton et al., 1982; Frampton et al., 1983]. Within recent years, VD has also been shown to induce apoptosis in human breast cancer and leukemic cell lines [Elstner et al., 1995, 1996; James et al., 1995; Danielsson et al., 1997], indicating that VD has potential as an oncostatic and even tumor-reducing drug [Colston et al., 1989]. However, a major drawback in the use of VD in cancer treatment is the requirement of high doses, which in turn may result in negative side-effects such as hypercalcemia, hypercalciuria, and soft tissue calcification [Vieth, 1990]. VD analogues were therefore synthesized in an attempt to dissociate effects on proliferation and differentiation from the effects on calcium homeostasis. Modification of VD, mainly in the side chain, has provided VD analogues with lower calcemic and more potent antiproliferative effects [Bouillon et al., 1995].

VD and its analogues are lipophilic molecules and can therefore easily pass through biological membranes to affect cellular and molecular targets. The biological effects of VD and its analogues are mainly mediated by its nuclear receptor, the vitamin D_3 receptor (VDR) [Pike, 1991]. The VDR is a ligand-activated transcription factor that belongs to the superfamily of hormone nuclear receptors [Mangelsdorf et al., 1995] and binds as a dimeric complex to a pair of hexameric core binding motifs, referred to as VD response elements (VDRE) [Carlberg, 1995]. Recently, the antiproliferative effect of the VD analogue CB1093 [Danielsson et al., 1997] on

Abbreviations used: CB1093, 20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; CDK, cyclin-dependent kinase; EB1089, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; FBS, fetal bovine serum; VD, 1α,25-dihydroxyvitamin D_3; VDR, 1α,25-dihydroxyvitamin D_3 receptor; VDRE, 1α,25-dihydroxyvitamin D_3 response element; ddPCR, differential display PCR.

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Patsie Polly and Carina Danielsson contributed equally to this work.

*Correspondence to: Carsten Carlberg, Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, Postfach 10 10 07, D-40001 Düsseldorf, Germany. E-mail: carlberg@uni-duesseldorf.de

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MCF-7 human breast cancer cells and its effect on in vivo regression of rat mammary tumors were found to be superior to those of the prominent analogue EB1089 [Colston et al., 1992b] but, most interestingly, CB1093 was found to induce apoptosis at a 10-fold lower concentration than that of EB1089. In vivo, EB1089 and CB1093 have a calcemic effect of only 50 and 27% that of VD, respectively [Danielsson et al., 1997] and are therefore considered good candidates for therapeutic use against several types of cancer.

To date, approximately 100 genes, that are diverse in function are known to be modulated in their expression or activity by VD [DeLuca et al., 1990; Walters, 1992]. However, only a minority of these genes are known as primary VD responding genes and even fewer VDREs have been identified in the promoter region of these known genes [Carlberg and Polly, 1998]. These well established VD responding genes, such as osteopontin, osteocalcin, calbindin, and carbonic anhydrase II, mainly represent genes classically involved in calcium homeostasis and bone metabolism. However, few cell cycle regulated genes, such as the proto-oncogene c-*fos* and the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$, are known to be regulated by VD [Candeliere et al., 1996; Liu et al., 1996]. Cell cycle progression from G1 to S phase is regulated by phosphorylation of proteins by cyclin/CDK complexes. CDKs are serine-threonine kinases that drive the cell cycle through phosphorylation of a number of key substrates, such as the retinoblastoma susceptibility protein (Rb). In mammalian cells, eight CDKs (CDK1–8) have been described [Sherr, 1993]. Their activity is stimulated by the association with cyclins and inhibited by the binding of CDK inhibitor proteins [Lees, 1995]. Cyclins were originally described as proteins whose abundance oscillated during the cell cycle, such as cyclins A, B, D, and E [Roberts, 1999]. Other cyclins have been identified, such as cyclins C, F, G, H, and I, but their roles in cell cycle control, if any, remain to be identified. The most interesting of these distinct cyclins appears to be cyclin C, which has been described as a component of the RNA polymerase II transcriptional machinery [Boyer et al., 1999], but no direct support in the promotion of cell cycle progression has been observed thus far [Liu et al., 1998].

This study was designed to identify novel, primary VD-regulated genes in the MCF-7 cell line, a known model cell line used to assess cellular proliferation, differentiation and apoptosis effects induced by VD-regulated gene events [Colston et al., 1992a; Welsh et al., 1995]. The gene for cyclin C was identified as a candidate using a ddPCR approach [Liang and Pardee, 1992]. Analysis of mRNA and protein expression confirmed that it acts as primary VD responding gene.

MATERIALS AND METHODS

Compounds

Comparison of the side chain structures of the VD analogue EB1089 (22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) to VD shows that EB1089 is elongated by three carbon atoms and contains two additional double bonds [Colston et al., 1992b]. The VD analogue, CB1093 (20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3), has altered stereochemistry at C_{20} (20-epi analogue) with an ethoxy substitute at carbon 22 (C_{22}) and a triple bond at C_{23}–C_{24} instead of the two double bonds present in EB1089 [Calverley et al., 1995]. The compounds were kindly provided by L. Binderup (LEO Pharmaceutical Products, Ballerup, Denmark) and dissolved in 2-propanol to provide a stock of 4 mM. Dilutions were performed in ethanol.

Cell Culture

The human cell lines, MCF-7 (breast cancer), MG63 (osteosarcoma), and HaCaT (keratinocytes) were maintained and grown in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% fetal bovine serum (FBS). The human cell lines MeWo (late-stage melanoma), WM1341 (early-stage melanoma), and U937 (promyelocytic leukemia) were maintained and grown in phenol red-free RPMI media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% FBS. The cells were cultured in phenol-red-free DMEM supplemented with 5% charcoal-treated FBS for mRNA and protein expression analysis. At approximately 70% confluence, the cells were treated with VD, EB1089, CB1093, or solvent (0.1% ethanol) for 0, 1, 2, 4, and 24 h (for RNA extraction) or for 0, 4, 8, 12, and 24 h (for protein extraction).

Differential Display PCR

Cells were collected and total RNA was isolated using Trizol (Life Technologies, Eggenstein, Germany) and genomic DNA contaminants were removed by DNase I (Promega, Mannhein, Germany) treatment. Synthesis of cDNA was performed with 12 different downstream primers dT_{11} with the 3′-sequences CA, CG, CT, CC, GA, GG, GT, GC, AA, AG, AT, and AC, respectively [Liang and Pardee, 1992]. Reverse transcription was performed with total RNA (2 µg) in the presence of 2.5 µM downstream primer, 0.5 mM dNTPs, 5 mM DTT, 40 U RNasin (Promega), 200 U MMLV-RT (Life Technologies), and first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) for 60 min at 37°C. The cDNA was then precipitated, diluted (1:10) and used as a template for a polymerase chain reaction (PCR) reaction containing 2.5 µM downstream primer, 0.5 µM upstream primer [according to Bauer et al., 1993], 2 µM dNTPs, 1 µCi [α^{33}P]-dCTP, 1.8 mM $MgCl_2$, and 1 U *Taq* polymerase (Life Technologies) in a total volume of 20 µl. After initial denaturation for 5 min at 94°C, the PCR profile was 30 s at 94°C, 1 min at 42°C, and 1 min at 72°C for 40 cycles. The PCR reaction products were vacuum dried and resuspended in 3 µl gel loading buffer (20 mM EDTA, 95% deionized formamide, 0.3% xylene cyanol FF, 0.3% bromophenol blue) and separated on a 6% nondenaturing polyacrylamide gel in TBE-buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 2 mM EDTA) at 50 W. Gels were transferred to filter paper and dried under vacuum at 80°C and exposed to x-ray film (X-OMAT, Kodak, Stuttgart, Germany) overnight with intensifying screens. Candidate bands that demonstrated varying intensity of expression patterns in comparison to control lanes were excised and eluted in 100 µl H_2O by heating at 85°C for 10 min. Eluted DNA was reamplified in the presence of 0.2 µM downstream primer, 0.2 µM upstream primer, 50 µM dNTPs, 1.5 mM $MgCl_2$, and 1 U *Taq* polymerase in a total volume of 50 µl with a PCR profile of 30 s at 94°C, 1 min at 40°C, and 1 min at 72°C for 40 cycles. The amplified cDNA was subcloned into pCR-Script SK(+) (Stratagene, Heidelberg, Germany) and sequenced. The cDNA sequences were compared with sequence information present in GeneBank and EMBL databases.

Semiquantitative RT-PCR

First-strand cDNA synthesis was performed on total RNA (2 µg) using MMLV reverse transcriptase (RT) (Life Technologies). This cDNA (2.5% of total) served as a template in a PCR reaction with a profile of 1 min at 94°C, 2 min at 58°C, and 30 s at 72°C for 4 cycles and then 10 s at 94°C, 1 min at 58°C, and 30 s at 72°C for 26 (cyclin C), 30 (24-hydroxylase), or 22 (β_2-microglobulin) cycles, respectively. PCR reactions were performed with 1 µM of gene-specific primer pairs with the sequence listed below, that were 5′-end-labeled using [γ^{32}P]-ATP and T_4 polynucleotide kinase (Promega):

Cyclin C^+	TGCGAGCAATTCTTCTGGAT
Cyclin C^-	CAGCCAGGACACAATAGTCA
24-Hydroxylase$^+$	CTGCTGCAGATTCTCTGGAA
24-Hydroxylase$^-$	ATGATGAAGTTCACAGCTTC
β_2-Microglobulin$^+$	CCCCCACTGAAAAAGATGA-GTATGCCTG
β_2-Microglobulin$^-$	CCTGTGGAGCAACCTGCTC-AGATACATC

Amplified PCR products were separated from unincorporated primers on 5% nondenaturing polyacrylamide gels in 0.5× TBE buffer. Specificity of PCR products was confirmed by restriction analysis. The gels were dried and exposed to a Fuji MP2040S imaging screen overnight and PCR products and unincorporated primers were detected on a Fuji FLA2000 reader (Fuji, Tokio, Japan) using Image Gauge Software (Raytest, Sprockhövel, Germany). The ratio of PCR products and unincorporated primers was normalized to the respective β_2-microglobulin housekeeping gene PCR product/primer ratio, thus providing a value for relative cyclin C and 24-hydroxylase mRNA expression, respectively. The statistical significance of mRNA upregulation was analyzed by Student's *t*-test.

Western Blotting Analysis

Cells were harvested, lysed, and disrupted by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 0.1 µg/ml leupeptin, 0.5 mM PMSF). Equal volumes of total cellular extracts (25–30 µg) were separated through a 12% SDS-polyacrylamide gel and electroblotted at 15 V for 30 min at room temperature (Trans-Blot SD, Semi-Dry Transfer Cell, BioRad, München, Germany) in transfer buffer (25 mM Tris-HCl, pH 8.5, 0.2 M glycine, 20% methanol) to PVDF Hybond-P membranes (Amersham Pharmacia, Freiburg,

Germany). Membranes were blocked in PBST buffer (1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20) containing 10% low-fat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with either anti-cyclin C (diluted 1:100 in PBST containing 10% low-fat milk) or anti-actin polyclonal antibodies (diluted 1:1,000 in PBST containing 10% low-fat milk), respectively (both antibodies were purchased from Santa Cruz, Heidelberg, Germany). After extensive washing with PBST, the immunoblots were further incubated for 1 h at room temperature with either a secondary fluorescein linked anti-rabbit IgG antibody (diluted 1:600 in PBST containing 10% low-fat milk, Amersham Life Sciences) or a secondary anti-goat IgG (diluted 1:1,000 in PBST containing 10% low-fat milk, Santa Cruz). Subsequent ECF fluorodetection was performed according to the manufacturer's recommendations (Amersham Pharmacia). Levels of protein expression were quantified by the use of a Fuji FLA2000 reader using Image Gauge Software (Raytest). Equivalent loading of protein samples was confirmed by immunodetection with an anti-actin polyclonal antibody (diluted 1:1,000 in PBST containing 10% low-fat milk, Santa Cruz) and additional Coomassie blue staining of respective blots and replicate gels. The statistical significance of protein upregulation was analyzed by Student's *t*-test.

RESULTS

Identification of New Candidates of Primary VD Responding Genes by ddPCR

Use of the ddPCR approach permitted identification of the gene for cyclin C as a primary VD responding gene candidate (Fig. 1). In total, 39 different combinations of upstream and downstream primers were used to analyze total RNA preparations that were generated and isolated from MCF-7 cells treated in a time course with VD. The entire experimental series provided 176 candidate bands, from which PCR products were isolated, reamplified, subcloned and sequenced. Further screening narrowed the number of candidates down to 34 positive clones, where two candidates proved to be genes that are known, such as cyclin C and DCIS-1, a marker for breast cancer [Holt et al., 1994]; the remaining candidates are as yet unidentified. Only very limited information is presently available for DCIS-1, therefore further analysis, such as mRNA and protein expression, was only continued with cyclin C.

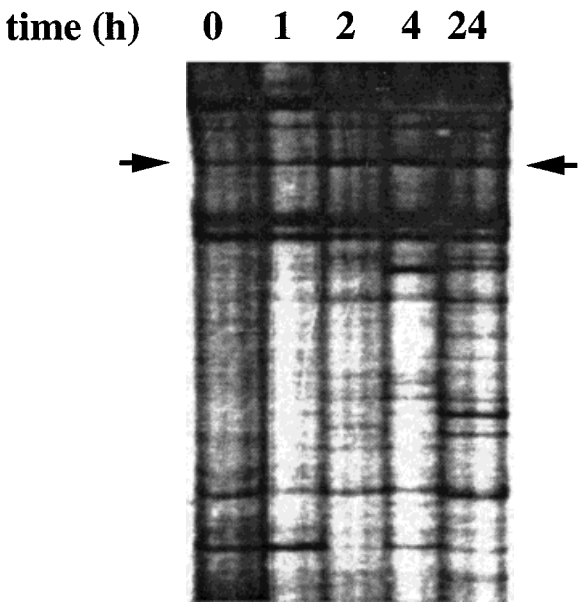

Fig. 1. ddPCR analysis in MCF-7 cells. MCF-7 cells were used as a model to identify candidate genes that are regulated by VD. The cells were treated for 1, 2, 4, and 24 h with 100 nM VD and were compared with nontreated cells (0 h). Total RNA was isolated, and reverse transcription was performed with 12 different dT11NN (downstream) primers. ddPCR was performed with the respective downstream primers and up to 20 different random decameric (upstream) primers on these 12 cDNA pools. From these 240 possible combinations, 39 proved effective. Polymerase chain reaction (PCR) was carried out in the presence of [^{33}P]-dCTP and the reaction products were resolved on a 6% nondenaturing polyacrylamide gel and visualized by autoradiography. A representative section of such a gel is shown. PCR was performed in duplicate. The arrowheads indicate a 150-bp fragment of the cyclin C gene that was upregulated by VD in a time-dependent fashion. The band was excised and eluted and the identity of the DNA fragment determined by subcloning and sequencing.

Analysis of mRNA Expression by RT-PCR

MCF-7 cells were treated for 1, 2, 4, and 24 h with saturating concentrations of VD and its analogues, EB1089 and CB1093 (100 nM each). Total RNA was extracted and reverse-transcribed into cDNA. PCR was performed on the same cDNA batch with specific primer pairs for the test gene cyclin C, the control gene 24-hydroxylase (as positive control for VD signalling) and the "housekeeping" gene β_2-microglobulin (for normalization), respectively. Cyclin C mRNA expression appeared to be slightly upregulated by VD and EB1089 and more prominently upregulated by CB1093 (2.4-fold, Fig. 2A). 24-Hydroxylase mRNA was found to be upregulated to 15-fold with VD and EB1089

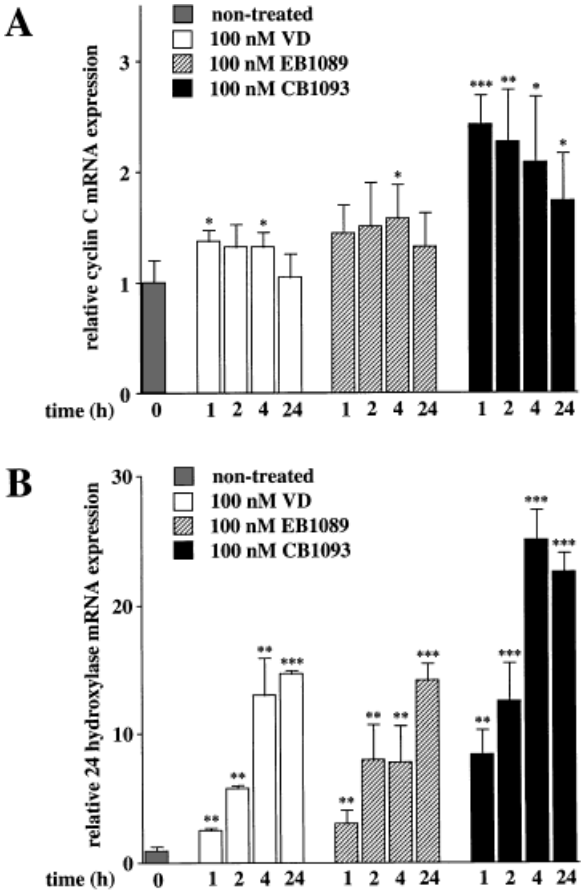

Fig. 2. Time course of cyclin C and 24-hydroxylase mRNA expression in response to VD and its analogues. MCF-7 cells were treated for 1, 2, 4, and 24 h with VD, EB1089, and CB1093 (100 nM each) and were compared with nontreated cells (0 h). Total RNA was isolated and reverse transcription-polymerase chain reaction (RT-PCR) was performed with primer pairs that were specific for the cyclin C (**A**) and 24-hydroxylase (**B**) gene. The mRNA expression was normalized for β_2-microglobulin ("housekeeping") gene mRNA expression. Columns, mean values of three separate RNA preparations (PCR reactions were performed in duplicate); bars, standard deviations.

and up to 25-fold with CB1093 (Fig. 2B) and thus served as a positive control for the inducibility of MCF-7 cells.

Analysis of Protein Expression by Western Blotting and ECF™ Immunodetection

Cyclin C protein expression was analyzed by Western blotting and ECF™ immunodetection of total cell extracts from MCF-7 cells that were treated with both VD and CB1093 (100 nM) for 4, 6, 8, and 12 h. Cyclin C protein expression was maximally stimulated 17-fold (with VD for 6 h) (Fig. 3), demonstrating that VD was slightly more effective than CB1093 on the protein level. Moreover, cyclin C protein expression was examined in several representative VDR expressing

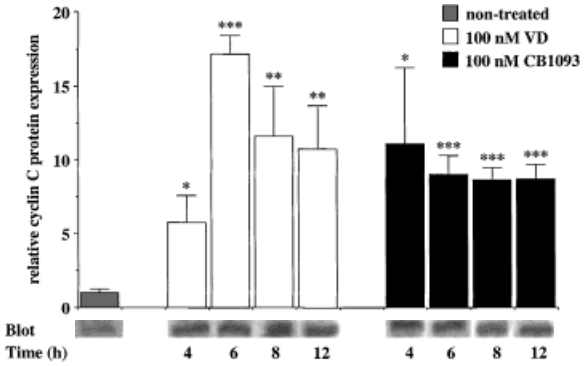

Fig. 3. Time course of cyclin C protein expression in response to VD and CB1093. MCF-7 cells were treated for 4, 6, 8, and 12 h with VD and CB1093 (each at 100 nM) and were compared with nontreated cells (0 h). Equal amounts of total cellular extracts were separated through a 12% sodium dodecyl sulfate polyacrylamide gel and electroblotted to a PVDF membrane. Cyclin C protein expression was assessed with a primary anti-cyclin C polyclonal antibody (Santa Cruz) and subsequent ECF™ immunodetection (Amersham Pharmacia). Representative Western blots are shown below the columns. The protein expression was quantified by fluoroimaging analysis using a Fuji FLA2000 reader and Image Gauge software and normalized to actin ("housekeeping") protein expression. Values obtained for 4-, 6-, 8-, and 12-h treatments were expressed as fold induction of control treated samples. Columns, mean values of triplicate Western blots; bars, standard deviations.

human cell lines, including MeWo (late-stage melanoma), WM1341 (early-stage melanoma), MG63 (osteosarcoma), HaCaT (keratinocyte), and U937 (promyelocytic leukemia) cells treated with VD (100 nM) for 24 h. A significant upregulation of cyclin C protein expression could only be determined in MeWo and U937 cells (Fig. 4).

DISCUSSION

The control of cell growth is a tightly regulated process that must respond to a variety of internal and external signals. However, inhibitors of CDKs provide a major mechanism of negative regulation on cell cycle progression [Hunter and Pines, 1994]. Several studies have investigated the effect of VD and its analogues on the proliferation of cancer cell lines, but findings indicating that the CDK inhibitor $p21^{WAF1/CIP1}$ is upregulated by VD in the p53-U937 cell line [Liu et al., 1996], were considered as a first insight into the molecular mechanisms of how VD mediates growth arrest in cancer cells. However, it appears that $p21^{WAF1/CIP1}$ does not act as a primary VD responding gene in all cell types; in particular in MCF-7 cells ($p53^+$), primary effects of VD on $p21^{WAF1/CIP1}$ expression could not be observed (M. Schräder and C. Carlberg, unpublished results).

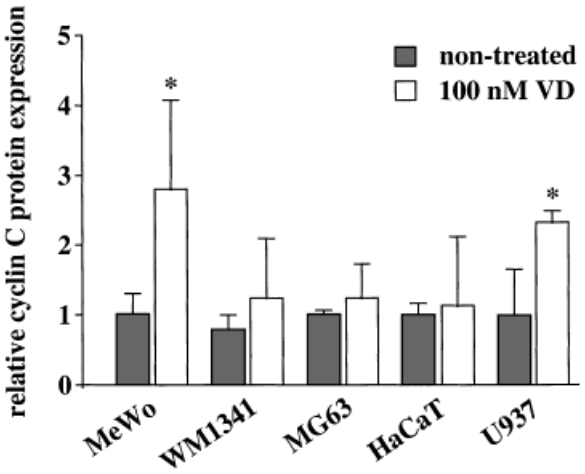

Fig. 4. Cyclin C protein expression in representative VDR cellular targets. MeWo (late-stage melanoma), WM1341 (early-stage melanoma), MG63 (osteosarcoma), HaCaT (keratinocyte), and U937 (promyelocytic) cells were treated for 24 h with VD (each at 100 nM) and were compared with nontreated cells (control). Equal amounts of total cellular extracts were separated through a 12% sodium dodecyl sulfate polyacrylamide gel and electroblotted to a PVDF membrane. Cyclin C protein expression was assessed with a primary anti-cyclin C polyclonal antibody (Santa Cruz) and subsequent ECF™ immunodetection (Amersham Pharmacia). Protein expression was quantified by fluoroimaging analysis using a Fuji FLA2000 reader and Image Gauge software and normalized to actin ("housekeeping") protein expression. Values were expressed as fold induction of their respective controls. Columns, mean values of triplicate Western blots; bars, standard deviations.

Cyclin C is a widely expressed protein [Rickert et al., 1996] associated with CDK8 [Tassan et al., 1995]. Interestingly, cyclin C is closely related to the yeast protein SRB11, which has been shown to be a component of the basal transcriptional machinery [Liao et al., 1995; Leclerc et al., 1996]. Recently, cyclin C has been shown to be a component of the Srb/Mediator complex that bridges the transcription factor E1A and the basal transcriptional machinery [Boyer et al., 1999]. Several of such bridging multi-subunit complexes have been described within the past year, referred to as human and mouse Mediator, NAT, CRSP and TRAP/DRIP/ARP/SMCC, that share some of their subunits [Kingston, 1999]. Some of these mediators, such as TRAP/DRIP [Yuan et al., 1998; Rachez et al., 1999], were shown to interact with nuclear receptors, such as the VDR. This finding suggests that the primary VD responding gene, cyclin C, may be involved in enhancing the activity of other VD responding genes.

In general, cyclins are considered positive regulators of the activity of CDKs. The described upregulation of cyclin C in this study by the antiproliferative hormone, VD, therefore appears puzzling. However, it is known that cyclins play a role not only during progression of the cell cycle, but during differentiation and apoptosis as well [Gao and Zelenka, 1997]. It can therefore be assumed that an upregulation of cyclin C results in an enhanced transcriptional activity of RNA polymerase II, i.e. in an increased transcription of a broad range of genes. This in turn may result in reduced proliferation and onset of differentiation or apoptosis.

The promoter of cyclin C has not yet been cloned, but the identification of this gene as a primary VD responding gene leads to the assumption that it contains a VDRE. It is interesting to note that the potent VD analogue, CB1093, was found to be more potent than the natural hormone, VD, on upregulation of cyclin C at the mRNA level but not at the protein level. This observation thus suggests that findings that describe transcription of VD responding genes cannot automatically be extrapolated to the expression of their gene products.

In conclusion, this study adds cyclin C as a new candidate to the list of primary VD responding genes that will provide an interesting perspective both to the cell regulatory and general transcriptional function of VD and its analogues.

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