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Modulation of cell cycle control by vitamin D_3 and its analogue, EB1089, in human breast cancer cells

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Examination of a panel of ER positive breast cancer cell lines showed that they were differentially growth inhibited by vitamin D_3 and its analogue EB1089. EB1089 treatment of the breast cancer cell lines MCF-7 E, BT20, T47D, and ZR75 demonstrated a correlation between a reduction in Cdk2 kinase activity towards phosphorylation of histone H1 and a decrease in DNA synthesis, while no modulation of Cdk2 activity was observed in the vitamin D_3 and EB1089 resistant cell line MCF-7 L. This was accompanied by a time dependent decrease in the percentage of S phase cells in the responsive lines. Characterization of the expression levels of Cdk2 and its related cell cycle proteins in MCF-7 E cells showed that after EB1089 treatment, there was a concentration and time dependent up-regulation of p21 as well as a decrease in cyclin A proteins. Paradoxically, cyclin E levels were increased as a function of treatment. Analysis of cyclin-Cdk2-Cdki complex formation showed that in EB1089 treated MCF-7 E cells, Cdk2, cyclin A and cyclin E immunoprecipitates contained an increased abundance of p21. In contrast to MCF-7 E cells, increases in both p21 and p27 as well as their complex formation with Cdk2 were observed in BT20 and ZR75 cells. These findings indicate that up-regulation of p21 as well as p27 in some cell types may account for the inactivation of Cdk2 activity and a G_1 block of the cell cycle following EB1089 treatment.

Keywords: breast cancer; vitamin D_3; EB1089; $p21^{WAF-1/CIP-1}$; inactivation of Cdk2 activity

Introduction

Vitamin D_3 and its synthetic analogues have generated interest as potential therapeutic agents for breast cancer. The therapeutic potential of vitamin D_3 compounds is based on their antiproliferative effects on breast cancer cell lines irrespective of their estrogen receptor status both *in vitro* and *in vivo* (Chouvet *et al*., 1986; Abe *et al*., 1991; Elstner *et al*., 1995). In addition, primary breast cancer specimens show high incidence of vitamin D_3 receptor (VDR) (Berger *et al*., 1987; Eisman *et al*., 1986; Feldman *et al*., 1980). Vitamin D_3 derivatives have been developed in an attempt to achieve higher potency of growth inhibition in the absence of the elevated calcemic effects associated with the parent compound. Analogues such as EB1089 cause a significant regression of rat mammary tumors by oral administration (Colston *et al*., 1992) An important issue arises as to the understanding of the antiproliferative mechanisms of these analogues.

Recently, molecular cloning and functional analysis of cell cycle control proteins have led to dramatic advances in the characterization of mammalian cell cycle machinery. This led us to investigate cell cycle regulatory proteins which may mediate vitamin D_3's action on growth. Progression of the mammalian cell cycle is dictated by a periodic activation and subsequent inactivation of a set of cyclin dependent serine/threonine kinases (reviewed by Sherr, 1993, 1994; Hunter and Pines, 1994). Regulation of these cyclin dependent kinases (Cdk's) involves programmed association/dissociation of specific cyclin subunits at different cell cycle stages and post-translational modifications by phosphorylation events (Hunter and Pines, 1994; Morgan, 1995). Early to mid G_1 progression and late G_1 progression leading to S phase entry are directed by cyclin $D_{1,2,3}$-$Cdk_{4,6}$ and cyclin E-Cdk2 both of which can phosphorylate the retinoblastoma protein (pRb) (Matsushime *et al*., 1992; Koff *et al*., 1992). Cyclin A which also activates Cdk2 is implicated in G_1 to S phase transition and is required for progression throughout S phase (Girard *et al*., 1991; Pagano *et al*., 1992; Zindy *et al*., 1992). The recent discovery of a group of low molecular weight cyclin-Cdk inhibitory proteins (Cdki) and their ability to form stoichiometric complexes with cyclin-Cdks revealed an important mechanism by which inactivation of Cdk occurs. At least two classes of Cdk inhibitors have been identified. The ink family members, which include p15 (Hannon and Beach, 1994), p16 (Serrano *et al*., 1993), and p18 (Guan *et al*., 1994), associate exclusively with D type cyclins and inhibit Cdk4 activity, whereas the two important members of the cip/kip family, p21 (known as sid1, waf1, cip1 and cap20) and $p27^{kip1}$ are universal inhibitors that act on a wide range of cyclin-Cdks (reviewed by Hunters and Pines, 1994).

p21 exists in a quaternary complex with cyclin, Cdk and proliferating cell nuclear antigen (PCNA) and inhibits DNA replication without disturbance of DNA repair (Xiong *et al*., 1992; Waga *et al*., 1994; Flores-Rozas *et al*., 1994). It binds and inhibits all the G_1 and S phase cyclin-Cdk complexes including cyclin D-Cdk4, cyclin E-Cdk2 and Cyclin A-Cdk2 (Gu *et al*., 1993; Harper *et al*., 1993; El-Deiry *et al*., 1993; Xiong *et al*., 1993a). Induction of p21 appears to be under transcriptional control. Multiple cis-elements including p53 (El-Deiry *et al*., 1993), TGFβ (Datto *et al*., 1995) and putative vitamin D_3 binding sites (Liu *et al*., 1996) have been identified in the p21 promoter region.

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Several SP1 sites have been additionally detected that are probably involved in transcriptional induction of p21 by differentiation inducing agents such as phorbol ester and okadaic acid (Biggs *et al*., 1996). Ectopic expression of p21 suppresses proliferation of both normal human fibroblasts and malignant cells (Harper *et al*., 1993; El-Deiry *et al*., 1993). In addition p21 null embryonic fibroblasts in mice are deficient in their G_1 check point control (Deng *et al*., 1995). In contrast to normal cells, most transformed cells lack or show low levels of p21 complex formation with the cyclin-Cdks (Xiong *et al*., 1993b). Thus, p21 appears to be a key regulator of cell growth as well as oncogenesis and links multiple extracellular or intracellular responses.

p27 relates to p21 in that it shares homologous amino-terminal regions which are essential in the association and inhibition of multiple cyclin-Cdk complexes (Polyak *et al*., 1994b). However, it differs from p21 in that it does not bind to PCNA (Luo *et al*., 1995). p27 has been shown to accumulate following serum starvation (Toyoshima *et al*., 1994) as well as during cell-cell contact inhibition (Polyak *et al*., 1994a,b) and decreases following mitogen induced re-entry of quiescent cells into S phase (Coats *et al*., 1996). The activity of p27 is also induced by various antimitogenic agents (Nourse *et al*., 1994; Polyak *et al*., 1994; Kato *et al*., 1994). The induction appears to be under translational regulation (Hengst and Reed, 1996). Accumulation of p27 inactivates G_1 cyclin-Cdk complexes and causes cell cycle arrest at G_1, which is reversed by antisense p27 overexpression (Rivard *et al*., 1996). The functional similarity of p21 and p27 reflects their complementary roles in cell cycle control.

Previous reports in T47D breast cancer cells (Eisman *et al*., 1989) as well as in other systems (Abe *et al*., 1986; Rigby *et al*., 1985), document that vitamin D_3 causes a cell cycle block in G_1. The breast cancer cell lines examined in this study showed a similar G_1 block by the vitamin D_3 analogue EB1089. In addition, EB1089 induced a reduction in Cdk2 activity, which is essential to G_1 to S phase transition and S phase progression (Ohtsubo *et al*., 1995; Pagano *et al*., 1992). We therefore investigated modulation of p21 and p27 expression and their complex formation with Cdk2 by EB1089 which may account for the decreased Cdk2 activity.

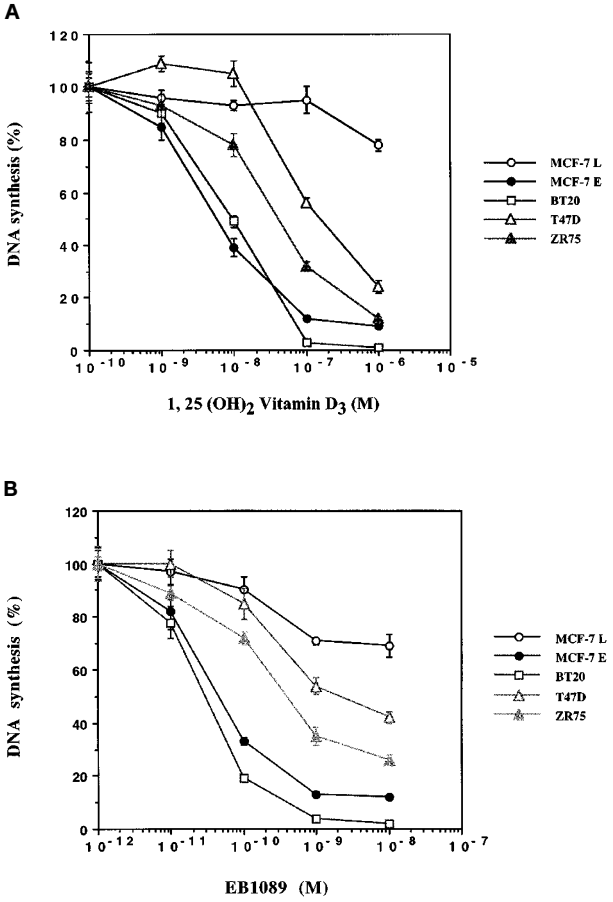

Figure 1 Concentration dependent inhibition of human breast cancer cells by 1, 25 $(OH)_2$ vitamin D_3 and EB1089. Breast cancer cell lines were plated in 24-well tissue culture plates at a density of 1.5×10^4 cells/well, followed by addition of different concentrations of 1, 25 $(OH)_2$ vitamin D_3 or EB1089, as indicated. Cells were cultured for 96 h and [^{3}H]-thymidine incorporation into DNA was determined and expressed as percentage of control in the absence of compounds. Each point is the mean $\pm$ s.e. of four replicates

Results

Inhibition of DNA synthesis by 1, 25 $(OH)_2$ vitamin D_3 and EB1089

Effects of vitamin D_3 and EB1089 on cell proliferation of a panel of ER positive breast cancer cells were analysed by measurement of [^{3}H]thymidine incorporation. 1.5×10^4 cells were plated into 24-well tissue culture plates in the presence of various concentrations of 1, 25 $(OH)_2$ vitamin D_3 or EB1089, allowed to grow for 96 h and subjected to DNA synthesis analysis. The breast cancer cell lines showed differential concentration dependent responses to the compounds (Figure 1a and b). MCF-7 early passage cells (MCF-7 E) and BT20 were the most responsive lines, displaying IC_{50} values of approximately 7×10^{-9} M and 4×10^{-11} M for 1, 25 $(OH)_2$ vitamin D_3 and EB1089, respectively. ZR75 and T47D demonstrated a weaker response with a shift to IC_{50} values of 10–15-fold higher. Late passage MCF-7 cells (MCF-7 L) were relatively resistant to the compounds and, therefore, used as a negative control in subsequent experiments.

Modulation of Cdk2 kinase activity by EB1089

Since Cdk2 activity plays an essential role in late G_1 progression, G_1-S phase transition and S phase progression, the possible effects of the vitamin D_3 compounds on modulation of its kinase activity were evaluated. Treatment of the cell lines with 10^{-8} M EB1089 for 48 h generated 60–65% inhibition of DNA synthesis for MCF-7 E and BT20, approximately 20% for T47D and 40% for ZR75 (Figure 2a). Protein lysates were prepared from both treated and untreated cells in parallel and Cdk2 associated immune complexes were analysed for their activity to phosphorylate histone H1 (Figure 2b). MCF-7 E and BT20 showed a dramatic decrease in Cdk2 kinase activity. The decrease in Cdk2 activity was not as prevalent in ZR75 and even weaker in T47D. No

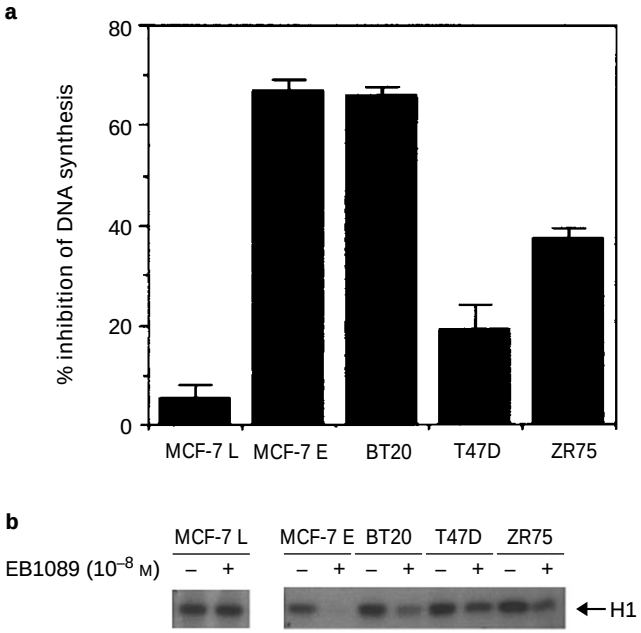

Figure 2 Reduction of Cdk2 kinase activity in cell lines inhibited by EB1089. Cells were plated at a density of 1.5×10^4 cells/well in 24-well plates for DNA determination and 7.8×10^6 cells/100 mm^2 dish for protein analysis. After attaining exponential growth (2 days after plating), cells were treated with 10^{-8} M EB1089 for 48 h. (**a**) [^{3}H]thymidine incorporation into DNA was measured in cultures grown in 24-well plates and the results were depicted as in Figure 1. (**b**) Protein lysates were prepared and Cdk2 associated histone kinase was analysed as described in Materials and methods. Radioactive phosphorylated histone H1 bands were visualized by autoradiography

change in the kinase activity was observed for MCF-7 L. Thus the decrease in Cdk2 kinase activity was correlated with inhibition of DNA synthesis in these breast cancer cell lines.

EB1089 blocks the cell cycle in G_1

To determine the effect of EB1089 on cell cycle phase distribution, MCF-7 E and MCF-7 L cells were treated with 10^{-8} M EB1089 for the indicated time periods and harvested for flow cytometry analysis (Figure 3). A time dependent decrease in the percentage of cells in S phase was observed in EB1089 treated MCF-7 E cells, reaching 50% reduction after 72 h (Figure 3a). The decrease in S phase cells resulting from EB1089 treatment was complemented by an increase in the G_0-G_1 population. The percentage of cells in the G_2-M compartment was relatively unaffected. In contrast, EB1089 treatment did not cause any change in these parameters in the MCF-7 L cell line (Figure 3b). Reduction of S phase cells by levels commensurate with the decrease in Cdk2 activity was also observed in the other three breast cancer cell lines (data not shown). These results showed that EB1089 arrested cell cycle progression in G_1.

Modulation of levels of Cdk2 and its related cell cycle proteins in MCF-7 E cells by EB1089

To sort out the specific cell cycle regulatory proteins responsible for the reduced Cdk2 activity following

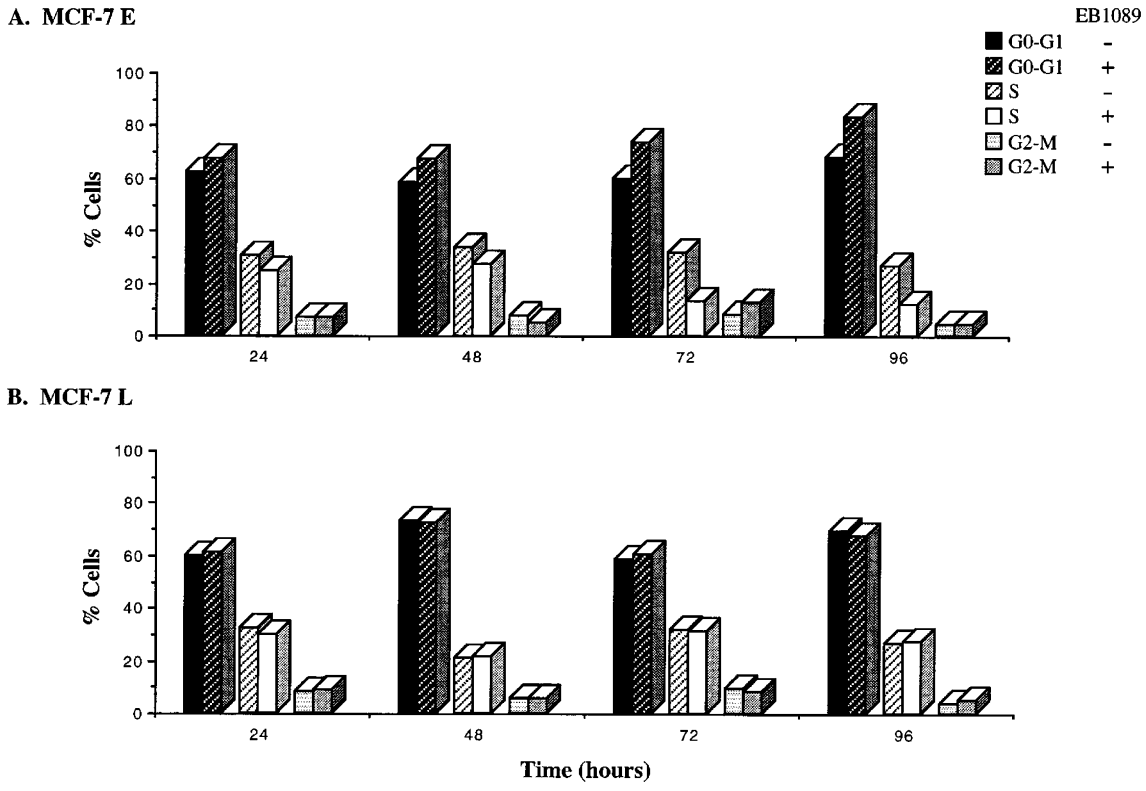

Figure 3 Flow cytometry analysis of blockade of the cell cycle in G_1 by EB1089. Exponential phase MCF-7 E (**a**) and MCF-7 L (**b**) cells were treated with 10^{-8} M EB1089 for the indicated periods and harvested for flow cytometry analysis. Cell cycle parameters were determined at each point as described in Materials and methods

EB1089 treatment, Western analysis using antibodies specific to cyclin A, cyclin E, Cdk2 and to the inhibitors p21 and p27 was performed (Figure 4a and c). After 48 h treatment, MCF-7 E cells demonstrated an approximate twofold increase in p21 protein levels which was maintained up to 96 h. In contrast, p27 levels remained relatively unchanged (Figure 4a). Up-regulation of p21 was accompanied by a reduction in cyclin A, whereas no significant change in Cdk2 levels was detected. Longer periods of treatment (72 and 96 h) also generated an increase in cyclin E proteins, which normally accumulates at late G_1. The increased cycin E levels may be reflective of a block at the G_1-S boundary (Wang *et al*., 1996). A quantitative representation of Figure 4a is seen in Figure 4b. The modulation in these levels of protein expression were also concentration dependent (Figure 4c). A quantitative representation of Figure 4c is seen in Figure 4d.

Modulation of complex formation by Cdk2 related proteins in MCF-7 E cells

Alterations in the expression profiles of cell cycle proteins described above indicated that mechanisms by which Cdk2 kinase activity was decreased in EB1089 treated MCF-7 E cells was complicated. To gain further insights, cyclin A and cyclin E associated histone H1 kinase activity were analysed after treatment of MCF-7 E cells with EB1089 (Figure 5a, top panel). The reduction in Cdk2 associated kinase activity by EB1089 was reflected by a reduction in both cyclin A and cyclin E associated histone kinase activity. Analysis of complex formation of Cdk2 and cyclins with inhibitors revealed that after EB1089 treatment, an increase in p21 complexing to Cdk2, cyclin A and cyclin E had occurred (Figure 5a, bottom panel). No increase of p27 complexing to any of these components was noted (data not shown). In contrast,

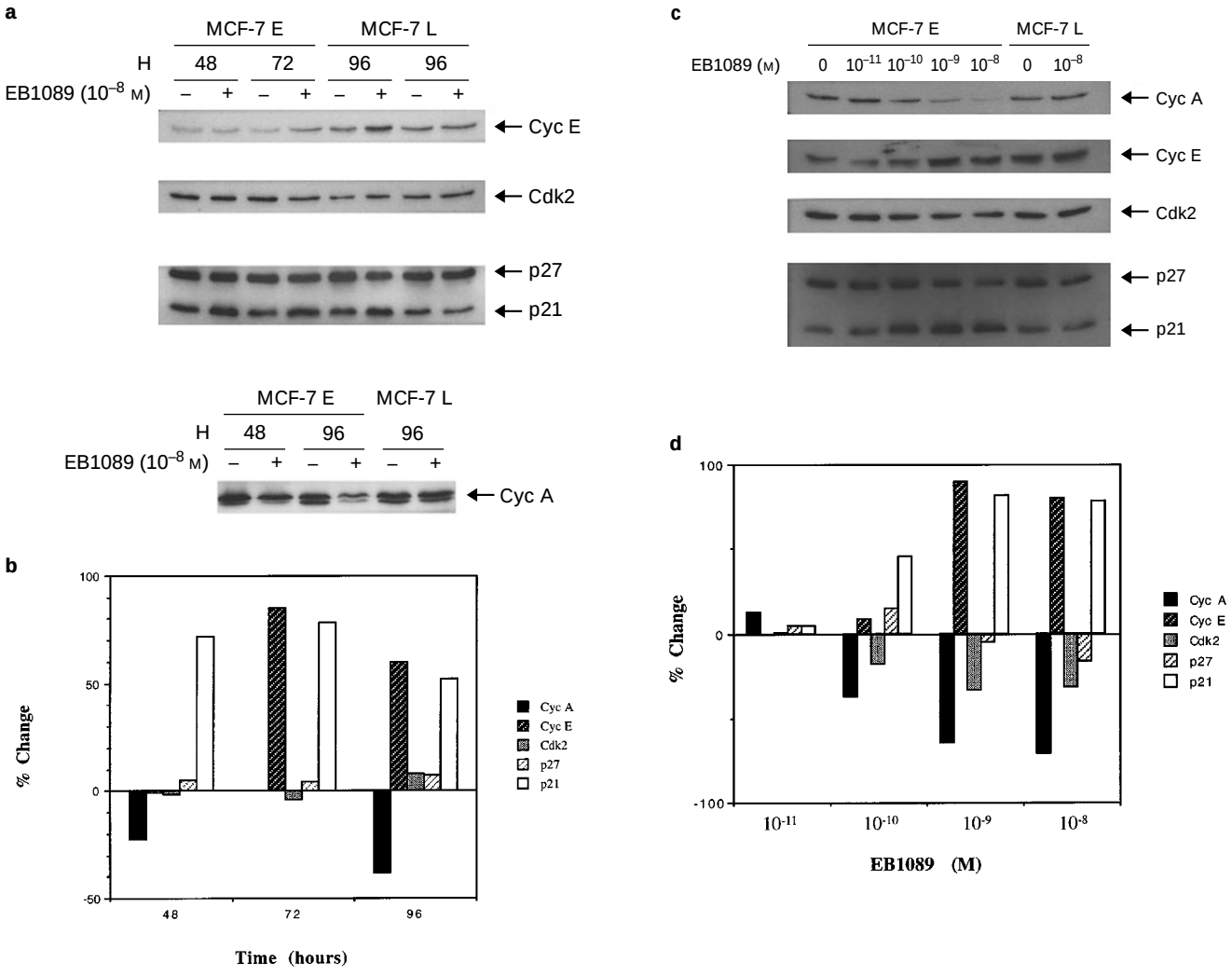

Figure 4 Determination of cell cycle proteins involved in complex formation with Cdk2 by EB1089 treatment. (**a**) Kinetics: Exponential phase MCF-7 E and MCF-7 L cells were treated with 10^{-8} M EB1089 for the indicated time periods. Total cell lysates were prepared and subjected to Western analysis, as described in Materials and methods. Detection of the specific proteins was performed by the ECL method. (**b**) Densitometric scanning of specific bands in MCF-7 E cells were analysed using an Ambis system. Graph represents quantitative % change after treatment. (**c**) Concentration dependence Exponential MCF-7 E and MCF-7 L cells were treated for 96 h with increasing concentrations of EB1089 as indicated. Western analysis was performed as in (**a**) except that cyclin A sc-751 antibody was used instead of cyclin A sc-239 antibody. (**d**) Densitometric scanning of changes in the respective protein levels in MCF-7 E cells was performed as in (**b**). Graph represents quantitative % change after treatment

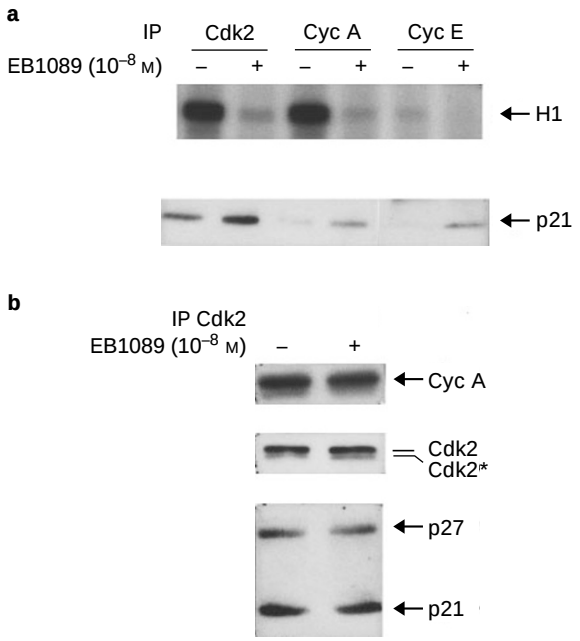

Figure 5 (**a**) Determination of cyclin A and cyclin E associated kinase activity and p21 levels in MCF-7 E cells. Top panel, cell lysates were prepared from exponential growing MCF-7 E cells treated with or without 10^{-8} M EB1089 for 72 h. Triplicate samples from control (−) and EB1089 treated (+) cells were immunoprecipitated with Cdk2, cyclin A and cyclin E and analysed for their histone kinase activity. Bottom panel, in parallel with the IP's described in the top panel, the immunoprecipitates were analysed for complexed p21 levels by Western blot analysis. (**b**) Determination of Cdk2 associated p21 and p27 levels in MCF-7 L. Exponential phase MCF-7 L cells were treated with 10^{-8} M EB1089 for 72 h and subjected to immunoprecipitation with Cdk2 antibody followed by Western blot analysis of Cdk2 and bound cyclin A, p21 and p27. *indicates active phosphorylated form of Cdk2. Results shown are representative of three independent experiments

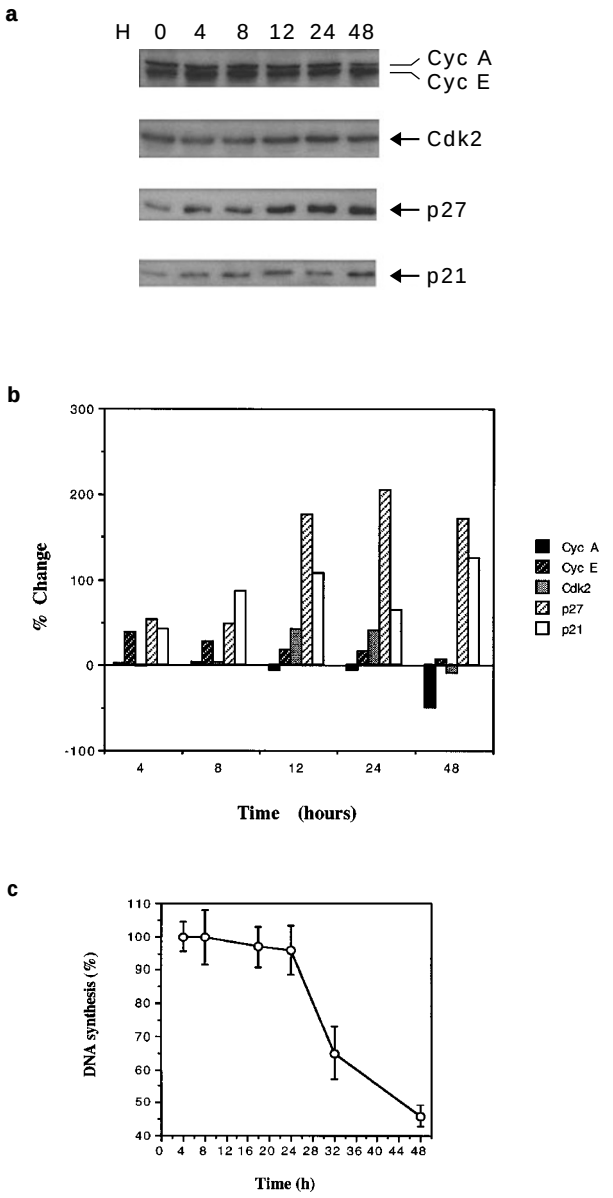

Figure 6 Kinetics of the effects of EB1089 on modulation of Cdk2 related proteins in BT20. (**a**) Exponential BT20 cells were treated with 10^{-8} M EB1089 for the indicated times and Western blot analyses were carried out for the indicated proteins. (**b**) The individual bands were quantitated by densitometric scanning as in 4b. Graph represents quantitative % change after treatment. (**c**) Determination of [^{3}H]thymidine incorporation into DNA was performed at each time point and depicted as in Figure 1

levels of inhibitors (p21 and p27) associated with Cdk2 did not change in the resistant cell line MCF-7 L (Figure 5b).

Modulation of Cdk2 inhibitors in BT20 and ZR75 by EB1089

To test whether similar mechanisms occur in other responsive cell lines, Western analyses were performed on cell cycle proteins in BT20 and ZR75 cells as a function of time following EB1089 treatment. p21 levels increased in both cell lines in conjunction with the onset of inhibition of DNA synthesis (Figure 6a and c, 7a and c). However, unlike MCF-7 E cells, these two cell lines also demonstrated an increase in p27 levels (Figure 6a and 7a). The induction was 3–4-fold after 48 h treatment with EB1089, which generated a 40–50% inhibition of DNA synthesis. Compared to ZR75 cells, the induction of p27 in BT20 cells (which were more sensitive to EB1089 growth inhibition) was approximately 12 h earlier. A quantitative representation of Figure 6a and 7a is seen in Figure 6b and 7b, respectively. To determine complex formation of the two Cdki's with Cdk2, ZR75 cells were treated with 10^{-8} M of EB1089 for 48 h and subjected to immunoprecipitation with a Cdk2 antibody. Both p21 and p27 were found to be increased in the EB1089 treated Cdk2 immunoprecipitates (Figure 8a and b), suggesting that the enhanced complex formation of the two inhibitors with Cdk2 may account for the decreased Cdk2 activity.

Discussion

Growth inhibitory mechanisms of vitamin D_3 analogues in human breast cancer cells are not well understood. A set of periodically activated proteins have been characterized as key players in cell cycle control and are often found to be deregulated in oncogenesis. Modifications of the expression levels of

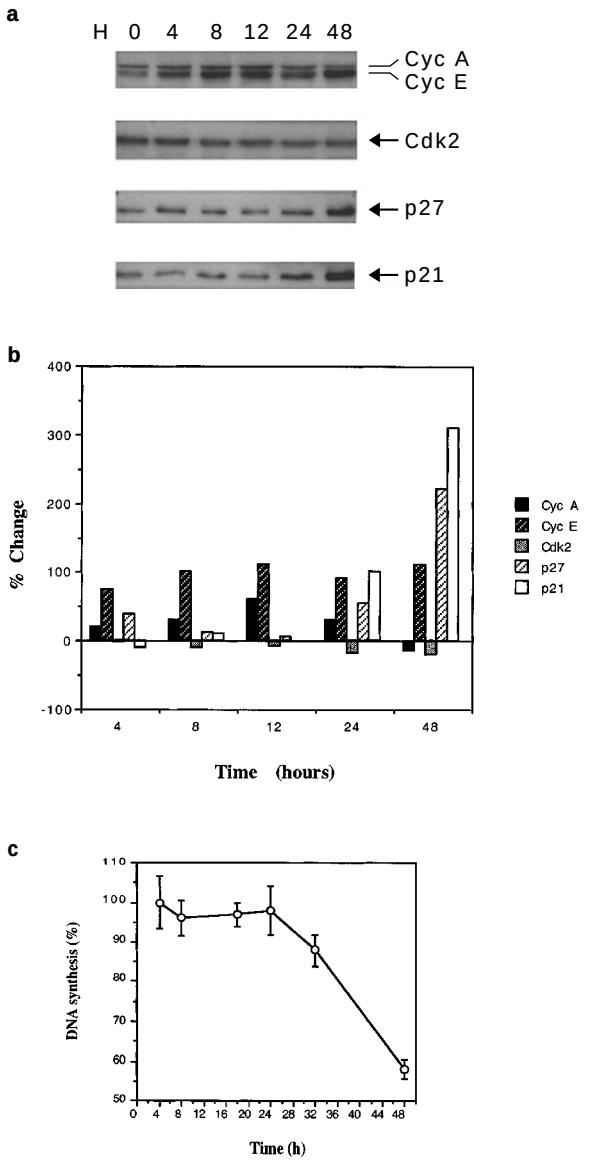

Figure 7 Kinetics of the effects of EB1089 on modulation of Cdk2 related proteins in ZR75. (**a**) Exponential ZR75 cells were treated with EB1089 and Western blot analyses were performed as in Figure 6a. (**b**) Densitometric scanning was performed as in Figure 6b. (**c**) Determination of DNA synthesis was performed and depicted as in Figure 6c

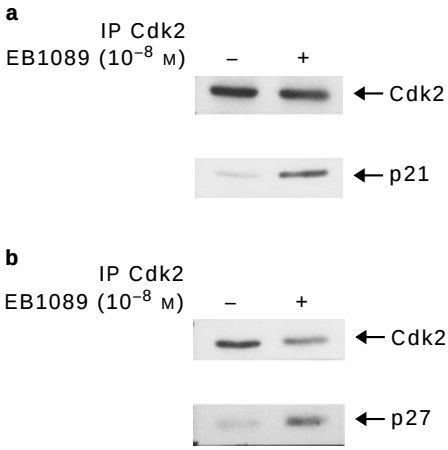

Figure 8 Analysis of EB1089 modulation of p21 and p27 complex formation with Cdk2 in ZR75. Exponential ZR75 cells were treated with 10^{-8} M EB1089 for 48 h. (**a**) Cell lysates were immunoprecipitated with Cdk2 and subsequently analysed for the associated p21 levels by Western blot analysis. Cdk2 levels in the immunoprecipitates were also analysed and used as a control for the immunoprecipitation. (**b**) Cell lysates were immunoprecipitated with Cdk2 and subsequently analysed for the associated p27 levels by Western blot analysis

these cell cycle regulatory proteins as well as their functionality by vitamin D_3 compounds may provide an important mechanism underlying inhibition of uncontrolled growth. We demonstrated that in response to EB1089, an up-regulation of the Cdk inhibitor p21, and in some cellular contexts p27, correlated with a decrease in DNA synthesis and a reduction in Cdk2 kinase activity, paralleling a G_1 arrest. Moreover, direct evidence was obtained that EB1089 caused increased association of these inhibitors with Cdk2/cyclin A and Cdk2/cyclin E complexes.

Current mutagenesis, structural and functional analyses have not generated an unified scheme concerning the stoichiometry of the interactions among p21, Cdks and cyclins. Inhibition of Cdk2 may result from an increase in overall p21 levels, leading to an increased interaction with the Cdk2-cyclin complex (Zhang *et al*., 1994). In the untreated MCF-7 E cells, there was a substantial amount of Cdk2 kinase activity. After EB1089 treatment, the activity was completely abolished (Figure 5a, top panel). This reduction of kinase activity was correlated with increased p21 complex formation with Cdk2 (Figure 5a, bottom panel). Distinct cyclin and Cdk binding sites have been located on the p21 molecule and optimal inhibition occurs only when p21 associates with both components of the Cdk-cyclin complex (Fotedar *et al*., 1996; Chen *et al*., 1996). Chen *et al*. demonstrated that p21 contains one Cdk binding site and two cyclin binding sites which have different affinities. Using deletion mutagenesis analysis, they showed that *in vitro* p21 deletion mutants containing only the Cdk binding site in the absence of the cyclin binding sites failed to inhibit Cdk2 kinase activity, while mutants containing only the cyclin binding sites allowed a partial block. Therefore, their model proposes that in order for p21 to inactivate Cdk2, cyclins first bind to the cyclin binding sites on p21, which then affect yet unknown intermediate substrates, stabilizing or promoting the association of Cdk2 with the Cdk binding site on p21 thereby leading to maximal inhibition. EB1089 treatment in MCF-7 E cells induced a twofold increase in p21 proteins and a reduction in cyclin A proteins (Figure 4a,c). The decrease in total cyclin A did not cause a reduction in its complex formation with Cdk2 (data not shown), indicating that reduction of total cyclin A was not a cause for inactivation of Cdk2. However, the increased complex formation of p21 with both cyclin A and cyclin E suggests a possible cyclin-mediated inhibition of Cdk2 activity. Further detailed stoichiometric and catalytic studies would be required to uncover the exact order of events that lead to inactivation of the Cdk2 catalytic subunit.

Multiple anti-tumor agents have been shown to utilize p21 to mediate their effects on inhibiting growth of breast cancer cells. Tumor necrosis factor-α inhibited growth of MCF-7 cells by blocking the cell cycle in G_1 (Jeoung *et al*., 1995). The G_1 arrest was associated with increased p21 levels and decreased phosphorylation of retinoblastoma protein. Prostaglandin A_2 also inhibited growth of MCF-7 cells by induction of p21, which suppressed Cdk2 activity (Gorospe *et al*., 1996). However, these agents are not components of our daily dietary intake. In contrast, the active metabolite of vitamin D_3, an essential dietary component in maintaining calcium homeostasis, possesses similar activity and thus may serve as a potential chemopreventive and therapeutic agent against breast cancer. Unfortunately, the effective dose of active vitamin D_3 to elicit these responses would cause hypercalcemia leading to symptoms such as malaise, fatigue, polyuria and nephrolithiasis. To overcome these hypercalcemic effects analogues such as EB1089 have been developed which have increased inhibitory potency as well as reduced hypercalcemic effects. We have demonstrated here that the active metabolite of vitamin D_3 was capable of inhibiting DNA synthesis in a panel of ER^+ breast cancer cells; however, EB1089 had similar effects at lower concentrations. In addition EB1089 treatment induced p21 protein along with increased complexing to Cdk2, inhibited Cdk2 associated histone H1 kinase activity and induced G_1 arrest in the breast cancer cell line MCF-7 E. The increased potency and reduced hypercalcemic effects relative to vitamin D_3 make EB1089 and similar analogues an attractive potential chemopreventive and therapeutic agents for breast cancer.

Mechanisms associated with the cell cycle response to vitamin D_3 analogues may involve other growth regulators. Transforming growth factor β (TGFβ), a potent growth inhibitor for breast cancer cells, has been shown to directly enhance p21 expression via a TGFβ responsive element in the p21 promoter (Datto *et al*., 1995). TGFβ upregulated p21 in ovarian adenocarcinoma cells (Elbendary *et al*., 1994) and increased p21 nuclear localization in TGFβ responsive human breast cancer MCF-7 cells in a TGFβ type II receptor (RII) dependent manner (Mazars *et al*., 1995). TGFβ receptors were found to be repressed in many malignancies including estrogen receptor positive breast cancer cell lines (Brattain *et al*., 1996; Kalkhoven *et al*., 1995) and reconstitution of a functional receptor system led to reduced malignancy (Sun *et al*., 1994). Modulation of p21 and its complex formation with cyclin/Cdk2 by both TGFβ and vitamin D_3 therefore reveals a common downstream effector in their signaling. This may lead to important information in understanding the mechanisms of interaction between these growth modulators and thus facilitate design or selection of new treatment approaches for breast cancer. However, it should be noted that in contrast to MCF-7 E, ZR-75 and BT-20 lack cell surface receptors for TGFβ (Brattain *et al*., 1996). Therefore, these cells are resistant to TGFβ and the mechanism underlying vitamin D_3 induction of p21 in these cells is not related to TGFβ responsiveness.

In addition to an induction of p21, p27 was increased by EB1089 treatment in BT20 and ZR75 breast cancer cells (Figure 6a and 7a), similar to the responses observed in vitamin D_3 induced differentiation of the myeloid leukemia cell line U937 (Liu *et al*., 1996). These responses seem to be mediated by VDR, since all the breast cancer cell lines examined express VDR (data not shown). Induction of these inhibitors preceded the onset of inhibition of DNA synthesis which was not apparent until 48 h after treatment with EB1089 (Figure 6c and 7c). Thus depending on the particular cell line, p21 can either act independently or in conjunction with p27 to inhibit Cdk2 kinase activity. The lack of an overall response in MCF-7 L cells indicates that certain common factor(s) involved in VDR signaling are deficient in this cell line. Taken together, our results indicate that in EB1089 growth inhibited breast cancer cells, induction of p21 and increased p21 complex formation with Cdk2 is a universal response.

Materials and methods

Cell cultures, compounds and antibodies

MCF-7 early passage cells (MCF-7 E, passage number 150) were obtained from Drs J Pauley and HD Soule from the Michigan Cancer Foundation. MCF-7 late passage cells (MCF-7 L) were from ATCC and used at a passage number greater than 500. BT20, T47D and ZR75 cell lines were all originally from ATCC. These cell lines were maintained in McCoy's 5A medium supplemented with ten per cent fetal bovine serum, vitamins, amino acids and antibiotics. 1, 25 $(OH)_2$ vitamin D_3 and its analogue EB1089 were provided by Dr Lise Binderup of LEO Pharmaceutical Products (Ballerup, Denmark). Rabbit polyclonal antibodies: p21 (sc-397), p27(sc-528), Cdk2 (sc-163), cyclin E (sc-198), cyclin A (sc-751) and mouse monoclonal cyclin A antibody (sc-239) were from Santa Cruz Biotechnology while mouse monoclonal cyclin E antibody (CC05-2) was from Amersham Corp.

Assay of DNA synthesis by [^{3}H]thymidine incorporation

DNA synthesis was analysed by quantification of [^{3}H]thymidine incorporation into trichloroacetic acid (TCA) precipitable material, as described previously (Sun *et al*., 1994). Briefly, the breast cancer cells were grown in 24 well tissue culture plates to exponential phase, pulsed with [^{3}H]thymidine (7 μCi; 46 Ci/mmol, Amersham) for 1 h, washed with phosphate buffered saline (PBS) once and precipitated with ice cold 10% TCA for 10 min. The cells were subsequently lysed with 0.5 ml of 0.2 N sodium hydroxide containing 40 ng/ml sperm DNA and the radioactivity was measured by liquid scintillation counting.

Flow cytometry analysis

$2\text{-}3 \times 10^6$ cells grown in 100 mm^2 tissue culture plates were trypsinized at 37°C until the cells were detached and then washed twice with 8 ml cold PBS. The cell pellets were collected by centrifugation at 1500 r.p.m. for 5 min at 20°C and resuspended in 0.4–0.6 ml of low salt propidium iodide stain [0.03 g/ml polyethylene glycol, 0.05 mg/ml propidium iodide (Sigma), 4 mM sodium citrate, 0.1% Triton X-100]. The suspensions were then transferred to eppendorf microfuge tubes with 10 μl RNase A (2 mg/ml, DNAase free), vortexed gently for less than 2 s and incubated at 37°C for 20 min. An equal volume of high salt stain (same as above except 400 mM sodium chloride was used instead of 4 mM sodium citrate) was added after the incubation. The mixture was vortexed gently for less

than 2 s and stored at 4°C overnight before proceeding to analysis of DNA content. The cell cycle phase distribution was determined by a FACScan flow cytometer (Becton Dickinson) and cell cycle parameters were obtained using a ModFit LT program, (Verity Software House Inc.).

Preparation of cell lysates and Western analysis

Monolayers of cells were washed with PBS at room temperature and harvested in cold lysis buffer [50 mM Tris-Hcl (pH 7.4), 150 mM sodium chloride, 0.5% Nonidet P-40, 50 mM sodium fluoride, 1 mM sodium metavanadate, 1 mM phenylmethyl sulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, 25 μg/ml leupeptin, 1 mM DTT, and 20 mM β-glycerol phosphate]. Crude lysates were passed through a 21 gauge needle 4× to sheer DNA and cleared by centrifugation at 15 000 r.p.m. for 20 min at 4°C. Equal volume of 2× sample buffer containing 2% β-mercaptoethanol, was added to total proteins (50 μg), heat denatured at 95°C and then separated by 12% SDS–PAGE. Proteins were transferred to nitrocellulose membranes (Amersham) using a Bio Rad electroblotting apparatus for 1 h at 100 volts. Equal loading and transfer was determined by staining of the membrane with ponceau S dye (Sigma, St Louis). Membranes were incubated with blocking buffer (Tris-buffered saline with 5% non-fat dried milk) for 1 h and then subjected to incubation (overnight at 4°C or 2 h at room temperature) with 1 μg/ml of the following antibodies: cyclin A, cycin E, Cdk2, p21 and p27. Secondary antibodies (anti-mouse or anti-rabbit horseradish peroxidase conjugated IgG's, Jackson Immuno Research Lab. Inc.) were added at 1 : 5000 dilution and allowed to react with primary antibodies for 1 h. Proteins were visualized using the enhanced chemiluminescence (ECL) method (Amersham). To determine the specificity of the antibodies used, antibodies were preincubated in the presence or absence of a control peptide specific for that antibody, followed by Western blot analysis as described above. No specific detectable bands were noted with the control peptide.

Immunoprecipitation and histone kinase assay

Rabbit polyclonal antibody (1 μg) specific to Cdk2 (sc-163), cyclin A (sc-751), cyclin E (sc-198), p21 (sc-397) or p27 (sc-528) was added to 100 μg of total protein from cell lysates which was diluted to a final volume of 300 μl with NP40 lysis buffer. The mixture was placed on a rotator overnight at 4°C (2–3 h for kinase assay), followed by co-precipitation with 25 μl (15 μl for kinase assay) slurry of protein A sepharose beads for an additional 2 h. The beads were then washed three times with 0.5 ml of NP40 lysis buffer, microfuged for 30 s and resuspended in sample loading buffer with 1% β-mercaptoethanol. Samples were then processed for Western analysis as described above. For histone kinase assays, the beads were washed three times with 0.33 ml of NP40 lysis buffer, three times with 0.33 ml of histone kinase buffer (20 mM Tris at pH 7.5 and 4 mM $MgCl_2$), collected by centrifugation and then resuspended in 10 μl of kinase buffer containing 2 μCi gamma ^{32}P ATP (10 Ci/mmol, NEN), 50 mM cold ATP and 2.4 μg histone H1 (Sigma). The reaction was incubated at 37°C for 30 min and terminated by adding 10 μl of 2× sample loading buffer and boiling for 5 min. Proteins were then separated by 10% SDS–PAGE and the phosphorylated histone H1 was visualized by autoradiography.

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