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Differential Effect of 1α,25-Dihydroxyvitamin D_3 on *Hsp28* and *PKCβ* Gene Expression in the Phorbol Ester-resistant Human Myeloid HL-525 Leukemic Cells

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ABSTRACT

We investigated the effect of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on the expression of the 28-kDa heat shock protein gene (*hsp28*) and the protein kinase C beta gene (*PKCβ*) in the human myeloid HL-60 leukemic cell variant HL-525, which is resistant to phorbol ester-induced macrophage differentiation. Northern and Western blot analysis showed little or no *hsp28* gene expression in the HL-60 cell variant, HL-205, which is susceptible to such differentiation, while a relatively high basal level of *hsp28* gene expression was observed in the HL-525 cells. However, both cell lines demonstrated heat shock-induced expression of this gene. During treatment with 50-300 nM 1,25-$(OH)_2D_3$, a marked reduction of *hsp28* gene expression along with an induction of *PKCβ* gene expression was observed in HL-525 cells. A gel mobility-shift assay demonstrated that the 1,25-$(OH)_2D_3$-induced alteration of *hsp28* gene expression was not associated with heat shock transcription factor-heat shock element (HSF-HSE) binding activity. Our results suggest that the differential effect of 1,25-$(OH)_2D_3$ on *hsp28* and *PKCβ* gene expression is due to the different sequence composition of the vitamin D response element in the promoter region as well as an accessory factor for each gene or that 1,25-$(OH)_2D_3$ increases *PKCβ* gene expression, which in turn negatively regulates the expression of the *hsp28* gene, or vice versa.

INTRODUCTION

The human myeloid HL-60 leukemic cells undergo differentiation into cells with a macrophage phenotype when exposed to phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA). In contrast, some HL-60 cell variants including HL-525 (derived from an HL-60 cell line during long-term exposure to TPA) are resistant to phorbol ester-induced differentiation and display decreased protein kinase C beta (PKCβ) expression relative to the HL-60 parent cells (Homma et al., 1986; Macfarlane and Manzel, 1994). Southern blot analysis indicates that the observed reduction in PKCβ gene expression does not appear to be due to a gross deletion or rearrangement of the gene (Tonetti et al., 1992).

The involvement of PKCβ in the phorbol ester-induced macrophage differentiation of HL-60 and variant HL-525 cells has been extensively investigated. The activation of PKCβ is both necessary and sufficient for phorbol ester-induced differentiation. Restoration of PKCβ isozyme deficiency in HL-525 cells by transfecting expression vectors containing either PKCβI or PKCβII cDNA causes HL-525 cells to revert to a phenotype like that of the parental HL-60 cells, which is characterized by susceptibility to TPA-induced macrophage differentiation (Tonetti et al., 1994). PKCβ is considered one of the essential elements in the phorbol ester-induced signal transduction pathway which leads to cell differentiation (Tonetti et al., 1994). Cell adherence is also induced by the stimulation of actin polymerization, which is also dependent on PKCβ (Niu and Nachmias, 1994).

Unlike TPA, 1α,25-dihydroxyvitamin D_3 [calcitriol; 1,25-$(OH)_2D_3$] induces monocytic differentiation in HL-525 cells (Homma et al., 1986). Calcitriol also increases the expression of PKCβ mRNA in HL-60 as well as in another phorbol ester-resistant HL-60 cell variant (Macfarlane and

Manzel, 1994). These observations suggest that 1,25-$(OH)_2D_3$ alters intracellular factor(s) related to TPA resistance in HL-525 cells. Recent studies demonstrate a relationship between the 28-kDa heat shock protein (HSP28) and resistance to cytotoxic agents (Hout et al., 1991). Chinese hamster 023 cells that constitutively overexpress human HSP28 as a result of transfection with pHS2711 are resistant to antineoplastic drugs (Huot et al., 1991). Taken together, we can contemplate (a) that HL-525 cells were selected during long-term exposure to TPA, because they contain a high level of HSP28, or (b) that *hsp28* gene expression is under a negative control of PKCβ: namely, high levels of this isoenzyme cause decrease in *hsp28* gene expression and low PKCβ levels cause an increase in the *hsp28* gene expression. Our data demonstrate that when compared with phorbol ester-sensitive HL-205 cells, HL-525 cells do indeed express a low level of PKCβ but a high level of HSP28. Moreover, 1,25-$(OH)_2D_3$ decreases the level of HSP28 and increases the level of PKCβ.

MATERIALS AND METHODS

Cell culture

The human myeloid HL-60 cell variants HL-525 and HL-205 (Homma et al., 1986) were cultured in McCoy's 5a medium (Celigro). The medium was supplemented with 26 mM sodium bicarbonate and 10% iron-supplemented calf serum (HyClone). T-75 flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2.

Hyperthermic treatment

T-75 flasks containing cells were heated by total immersion in a circulating water bath (Heto) maintained within ± 0.05°C of the desired temperature.

Drug treatment

Vitamin D_3, 25-hydroxyvitamin D_3, and 1α,25-dihydroxyvitamin D_3 were obtained from Calbiochem, San Diego, CA. For drug treatment, cells were counted and added into the medium containing a drug.

Labeling, two-dimensional polyacrylamide gel electrophoresis (PAGE), and fluorography

Cells were labeled with 20 μCi /ml [^{3}H]-leucine (sp. act. 160 Ci/mmol, Amersham) in leucine-free medium for 8 h. After labeling, cells were washed twice with cold Hanks' balanced salt solution (HBSS). For two-dimensional polyacrylamide gel electrophoresis (PAGE), samples were solubilized in sample buffer containing 8 M urea, 1.7% NP-40, and 4.3% β-mercaptoethanol. Proteins were first separated in isoelectric focusing gels (pH 3.5-10). These gels were then laid across the top of a 10-18% linear gradient sodium dodecyl sulfate (SDS) polyacrylamide gel for two-dimensional analysis (Walker, 1984). After electrophoresis, gels were fixed in 30% trichloracetic acid (TCA) for 30 min. For fluorography, gels were dehydrated by washing for 15 min in each of 25% acetic acid, 50% acetic acid, and glacial acetic acid, consecutively. After fixation, gels were placed in 125 ml PPO solution (20% (w/v) 2,5-diphenyloxazole in glacial acetic acid) for 2 h. The PPO solution was removed, and the gel was shaken gently overnight in distilled water and dried for 2.5 h at 60°C. The gel was loaded into a casette with Kodak SB-5 X-ray film and placed in a -70°C freezer. After optimum time

exposure, the fluorograph film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

One-dimensional PAGE and Western blot

Samples were mixed with 2X Laemmli lysis buffer (1X = 2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromphenol blue), and boiled for 10 min. Protein content was measured with BCA* Protein Assay Reagent (Pierce). The samples were diluted with 1X lysis buffer containing 1.28 M β-mercaptoethanol and an equal amount of protein (30 µg) was applied to a one-dimensional PAGE. Electrophoresis was carried out on a 10-18% linear gradient SDS PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting with the HSP28 monoclonal antibody (StressGen). The HSP28 antibody was diluted 1:10,000. Alkaine phosphatase-conjugated rabbit-mouse IgG (diluted 1:3,000) was used to detect the primary antibody.

Northern blot analysis

PKCβ and HSP28 mRNA levels were determined using the Northern blot technique. Total cellular RNA was extracted by the LiCl-urea method of Tushinski et al. (1977). For RNA analysis, 30 µg of total RNA was electrophoresed in a 1% agarose-formaldehyde gel (Lehrach et al., 1977). The RNA was blotted from the gels onto nitrocellulose membranes and baked at 80°C for 2 h in a vacuum oven. Membranes were prehybridized at 42°C in 50% formamide, 1X Denhardt's solution, 25 mM KPO_4 (pH 7.4), 5X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate), and 50 µg/ml denatured and fragmented salmon sperm DNA. Hybridizations were at 42°C in prehybridization solution containing 10% dextran sulphate and radiolabeled human PKCβ cDNA probes (F. Collart, Argonne

National Lab.) or human HSP28 cDNA probes (StressGen Biotechnologies Corp.) at a concentration of 1.5-4 x 10^6 cpm/ml. For posthybridization, blots were washed twice in 2X SSC for 15 min at room temperature, washed once in 0.5X SSC and 0.1% SDS for 25 min at 50°C, and washed twice in 0.2X SSC and 0.1% SDS for 1 h at 50°C. Blots were placed into a stainless steel cassette with intensifying screen and autoradiographed.

Quantitation of HSF-HSE binding activity

Conditions for the gel mobility-shift assay, a description of the ^{32}P-labeled HSE oligonucleotide, and preparation of whole-cell extracts were as published previously (Mosser et al., 1988). A double-stranded HSE oligonucleotide of the human HSP28 gene promoter (upper strand 5'-CTT AAC GAG **AGA AGG TTC CAG ATG** AGG GCT GAA-3', Hickey et al., 1986) was used. Bold nucleotides represent essential sites for HSF binding. Binding reactions with 20 µg of whole-cell extracts were performed for 15 min at 25°C in a final volume of 25 µl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) containing about 0.5 ng of radiolabeled probe, 10 µg of yeast tRNA (Boehringer Mannheim), 1 µg of *Escherichia coli* DNA (Sigma Chemical Co.), 2 µg of poly(d[I-C]) (Pharmacia LKB), 50 µg BSA (Sigma Chemical Co.). Samples were electrophoresed on a nondenaturing 4.5% polyacrylamide gel for 2.5 h at 140 V. After electrophoresis, gels were fixed with 7.5% acetic acid for 15 min and rinsed with water for 3 min. For autoradiography, gels were dried in a slab gel dryer (Model 483, Bio-Rad, Richmond, CA) for 1.5 h at 80°C and placed into a stainless steel cassette with an intensifying screen. Gels were autoradiographed on Fuji RX X-ray film. After an exposure of 2-4 days at -70°C, autoradiographic film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

RESULTS

Comparison of *hsp28* gene expression in HL-205 and HL-525 cells

The data presented in Figure 1A demonstrate no detectable level of HSP28 mRNA in unheated HL-205 cells, which are known to be sensitive to differentiation induction by phorbol esters. In contrast, a marked amount of HSP28 mRNA was detected in extracts from unheated HL-525 cells, which are resistant to such differentiation (Homma et al., 1986). The level of HSP28 mRNA increased after heat shock at 45°C for 10 min in both cell lines. The level of HSP28 mRNA in HL-525 cells was approximately twofold higher than that in HL-205 cells as determined by densitometer. Similarly, higher levels of HSP28 protein were observed by Western blots analysis (Fig. 2). The presence of HSP28 protein was detected in unheated HL-525 cells but not in HL-205 cells. The protein level increased and reached its maximum value within 6 h after heat shock at 45°C for 10 min in HL-205 cells. Unlike HL-205 cells, heat shock didn't alter the level of HSP28 protein in HL-525 cells. This may be due to the high intrinsic level of HSP28 protein in HL-525 cells.

Effect of 1,25-$(OH)_2D_3$ on the expression of *PKCβ* and *hsp28* genes

Northern blots in Figure 3 illustrate the effect of various concentrations (1-50 nM in Fig. 3A and 50-300 nM in Fig. 3B) of 1,25-$(OH)_2D_3$ treatment for 3 days on the levels of PKCβ and HSP28 mRNA in HL-525 cells. The level of PKCβ mRNA increased during 1,25-$(OH)_2D_3$ treatment, whereas the level of HSP28 mRNA decreased during this treatment. The level of alteration was dependent upon 1,25-$(OH)_2D_3$ concentration. The significant alterations occurred at the drug concentrations equal to or above 50 nM. These results are consistent with those of Obeid et al. (1990) and correspond to concentrations of 1,25-$(OH)_2D_3$ that induce maximal differentiation of HL-60 cells. Data from

Western blot analysis also clearly demonstrated that treatment with 50-300 nM 1,25-$(OH)_2D_3$ markedly suppressed the level of HSP28 in HL-525 cells (Fig. 4). Similar results were obtained in the experiments for the kinetics of treatment with a single dose (50 nM) of 1,25-$(CH)_2D_3$ (Figs. 5 and 6). Figure 5 shows that the level of HSP28 mRNA markedly decreased within 1 day and reached minimal value within 2 days during the treatment with the drug in HL-525 cells. In contrast, the level of PKCβ increased rapidly and reached maximal value within 2 days during the treatment with the drug. Unlike HSP28 mRNA, the level of HSP28 protein decreased gradually during 3 days of the drug treatment (Fig. 6). This discrepancy is probably due to differences in the half-life of HSP28 mRNA and its protein, short half-life of HSP28 mRNA vs. long half-life of HSP28 protein.

Comparison between the effect of vitamin D_3 and that of its metabolites on the level of HSP28

To examine whether vitamin D_3 and its metabolites have a similar effect on the level of HSP28, we chose vitamin D_3, and its metabolites, 25-hydroxyvitamin D_3 (25-(OH) D_3) and 1,25-$(OH)_2D_3$. Figure 7 shows that various concentrations of vitamin D_3 (1 nM-100 μM) and 25-(OH) D_3 (1 nM-1 μM) did not markedly affect the level of HSP28, whereas 1,25-$(OH)_2D_3$ (1 nM-300 nM) reduced the level of HSP28 in HL-525 cells. The level of reduction was dependent upon the drug concentrations. Higher concentrations of 1,25-$(OH)_2D_3$ (50-300 nM) significantly reduced the level of HSP28.

Effect of 1,25-$(OH)_2D_3$ on HSF-HSE binding activity

Our previous results (Figs. 3 and 5) indicate that the effect of 1,25-$(OH)_2D_3$ on *hsp28* gene expression occurs at the transcriptional level. To determine whether the suppression of *hsp28* gene expression was due to the alteration of upstream regulation of transcription, heat shock transcription

factor (HSF)-heat shock element (HSE) binding activity was measured by gel mobility-shift assay. It is known that the binding of HSF to HSE is necessary for transcriptional activation of eukaryotic heat shock genes (Pelham, 1982; Sorger et al., 1987; Kingston et al., 1987). Gel mobility-shift analysis of whole cell extracts from heated cells showed the formation of HSF-HSE complex: activated HSF binds to HSE (H in Fig. 8). Although the levels of HSP28 mRNA and its protein were prominent in HL-525 cells (Figs. 1-7), gel mobility-shift assay failed to detect the formation of HSF-HSE complex. Moreover, no HSF binding activity was detected in extracts from cells treated with various concentrations of 1,25-$(OH)_2D_3$ (1-50 nM).

DISCUSSION

Several conclusions can be drawn from the data presented. Unlike HL-205 cells, phorbol ester-resistant HL-525 cells contain a relatively high level of HSP28 mRNA and protein. While *hsp28* gene expression was suppressed during treatment with 1,25-$(OH)_2D_3$ in HL-525 cells, *PKCβ* gene expression was stimulated. These results are consistent with previous reports that demonstrate the induction of PKCβ gene expression by treatment with 1,25-$(OH)_2D_3$ (Obeid et al., 1990; Macfarlane and Manzel, 1994; Gamard et al., 1994). The alteration of *hsp28* gene expression resulted from treatment with 1,25-$(OH)_2D_3$, the most active natural metabolite of vitamin D_3, but not with 25-(OH) D_3 or vitamin D_3 itself. This alteration was not related to the formation of the HSF-HSE complex at the promoter region of the *hsp28* gene.

The low molecular weight heat shock protein HSP28 consists of several isoforms, including unphosphorylated (HSP28a in Fig. 6) and phosphorylated (HSP28b in Fig. 6) protein. It can be

phosphorylated following stimulation with heat shock, as well as treatment with the Ca^{2+} ionophore, addition of serum to medium, sodium arsenite, cycloheximide, or cytokines (Kim et al., 1984; Welch, 1985; Hepburn et al., 1988; Kaur and Saklatvala, 1988; Crete and Landry, 1990; Freshney et al., 1994). Data from Fig. 6 show little or no presence of phosphorylated HSP28b with or without 1,25-$(OH)_2D_3$ treatment. HSP28 is known to be involved in the development of thermotolerance (Landry et al., 1989) and drug-resistance (Huot et al., 1991). This protein can function as a molecular chaperone (Jakob et al., 1993). Data from Fig. 1 show the presence of HSP28 in HL-525 cells but not in HL-205 cells. Taken together, these observations suggest that expression of *hsp28* gene is associated with phorbol ester resistance in HL-525 cells.

It is well established that the transcriptional induction of heat shock genes in eukaryotes is mediated by the heat shock transcription factor (HSF) (Parker and Topol, 1984; Wu, 1985; Wu et al., 1987; Kingston et al., 1987; Sorger et al., 1987; Wiederrecht et al., 1987; Sorger and Pelham, 1987, 1988). This protein can be activated upon heat shock (Zimarino and Wu, 1987; Kingston et al., 1987; Sorger et al., 1987; Nieto-Sotelo et al., 1990). The activated HSF binds to the promoters which contain the heat shock element (HSE) (Pelham, 1982) and then stimulates transcription (Zimarino and Wu, 1987; Kingston et al., 1987; Sorger et al., 1987). Our data in Figs. 1 and 8 confirm that *hsp28* gene expression was accompanied by the binding of HSF to HSE. However, our data also present that constitutive expression of *hsp28* gene occurred without the formation of the HSF-HSE complex in HL-525 cells. These results suggest that another transcription factor(s) is(are) also involved in the regulation of *hsp28* gene expression in HL-525 cells.

1,25-$(OH)_2D_3$ has several cellular functions. The drug produces a stimulation of calcium influx through activation of voltage-gated Ca^{+2} channels and an involvement of cyclic AMP-dependent PKA

and PKC (Marinissen et al., 1994). The drug also has an important role in cell growth and differentiation. 1,25-$(OH)_2D_3$ affects cell differentiation by direct interaction with the cell membrane and by the transduction of the signal to the genome. The direct effects of the drug on the cell membrane may be mediated by stimulation of phosphoinositide metabolism (Wali et al., 1995) and activation of PKCβ (Obeid et al., 1990). 1,25-$(OH)_2D_3$ receptor (VDR), which is known as a 55-kDa nucleoprotein, may play a principal role in the signal transduction (Studzinski et al., 1993). The VDR becomes phosphorylated upon treatment with 1,25-$(OH)_2D_3$ (Darwish et al., 1993). Activated Raf-1 and MAP kinase may be involved in this phosphorylation (Kharbanda et al., 1994). The phsophorylation plays a central role in the transcriptional activity of the VDR (Darwish et al., 1993). The VDR interacts with the vitamin D response elements (VDRE) in the promoter region of a number of target genes, either as a homodimer or a heterodimer with retinoid or orphan receptors. The binding of the VDR to the VDRE either stimulates the expression of the target genes such as calbindin-D 9-kDa (Strom et al., 1992; Darwish and DeLuca, 1992), osteocalcin (Demay et al., 1992a) and 24-hydroxylase (Chen et al., 1993) or suppresses transcription of the genes encoding Id (Kawaguchi et al., 1992) and parathyroid hormone (Demay et al., 1992b). The binding of the receptor to the response element does not require the ligand but does require a protein termed an accessory factor which has a molecular weight of 59-64 kDa (Ross et al., 1992). Recent studies show that differential effects of 1,25-$(OH)_2D_3$ on various target genes may be due to differences in VDRE sequence composition and the requirements for particular cellular factors rather than VDR itself (Demay et al., 1992b). What remains unknown is how 1,25-$(OH)_2D_3$ differentially alters *hsp28* gene and *PKCβ* gene expression in HL-525 cells. At the present time, only speculations can be made concerning mechanisms. Since 1,25-$(OH)_2D_3$ has up- and down-regulatory effects by binding to VDRE, it suggests that the sequence composition of VDRE in the promoter region of the *hsp28* gene as well as the accessory factor differ from that for the *PKCβ* gene. However, other possibilities such

as indirect effect(s) of the 1,25-$(OH)_2D_3$ on the regulation of either *hsp28* or *PKCβ* gene expression should be considered. A most plausible indirect effect may involve the up-regulation of *PKCβ* gene expression, which in turn negatively regulates the expression of the *hsp28* gene, or vice versa. Obviously, further studies at the cellular and molecular levels are necessary to understand the differential mechanism involved in the regulation of these genes by 1,25-$(OH)_2$ D_3 in HL-525 cells.

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FIGURE LEGENDS

Figure 1.

Northern blot analysis of HSP28 mRNA from heated HL-205 or HL-525 cells. Cells were heated at 45°C for 10 min and incubated at 37°C for the times (2-8 h) indicated at the bottom of each lane. Cells were harvested and RNA was isolated. An equal amount of RNA (30 µg) was loaded onto each lane of an agarose-formaldehyde gel for separation (A panel). After separation, RNA was blotted onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled probe for HSP28 mRNA (B panel). C= unheated control cells. Arrowheads indicate the position of the 28S and 18S rRNAs.

Figure 2.

Western blots with an anti-HSP28 antibody. HL-205 or HL-525 cells were heated at 45°C for 10 min and incubated at 37°C for the times (2-8 h) indicated at the bottom of each lane. Equal amounts of protein (30 µg) from cell lysates were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and processed for immunoblotting with HSP28 antibody. C= unheated control cells.

Figure 3.

Northern blot analysis of PKCβ and HSP28 mRNA from 1,25-$(OH)_2D_3$ treated HL-525 cells. Cells were treated for 3 days with various concentrations of 1,25-$(OH)_2D_3$ indicated at the bottom of each lane. Cells were harvested and RNA was isolated and separated as described in Fig. 1. After separation, RNA was blotted onto a nitrocellulose membrane and hybridized with ^{32}P-labeled probes for PKCβ, HSP28, and GAPDH mRNA. A panel: 0-50 nM treated cells. B panel: 0-300 nM treated cells. GAPDH: GAPDH probe was used to verify the equivalent amounts and integrity of RNAs

loaded in each lane. The apparent reduction of GAPDH mRNA in lane 0 in B panel was the result of underloading of the sample. Autoradiograms were from the same blot, which was stripped and rehybridized with different probes.

Figure 4.

Western blots with an anti-HSP28 antibody. HL-525 cells were treated for 3 days with 0-300 nM 1,25-$(OH)_2D_3$, as indicated at the bottom of each lane. Western blot analysis was performed as described in Fig. 2.

Figure 5.

Northern blot analysis of PKCβ and HSP28 mRNA from 1,25-$(OH)_2D_3$ treated HL-525 cells. Cells were treated with 50 nM for 0-3 days, as indicated at the bottom of each lane. Northern blot analysis was performed as described in Fig. 1. GAPDH: GAPDH probe hybridized to the same blot to verify RNA uniformity.

Figure 6.

Two-dimensional SDS-polyacrylamide gel electrophoretic analysis of proteins. HL-525 cells were treated with 50 nM 1,25-$(OH)_2D_3$ for 0-3 days, as indicated at the top of each panel. Cells were then labeled with 20 μCi/ml [^{3}H]-leucine for 8 h in leucine-free medium. Lysates from cells were analyzed and [^{3}H]-labeled proteins were detected by fluorography. Only a section of the fluorograph is shown. The locations of HSP28a (a), HSP28b (b), and actin (A) are identified. The numbers in the panels indicate the length of the drug treatment in days.

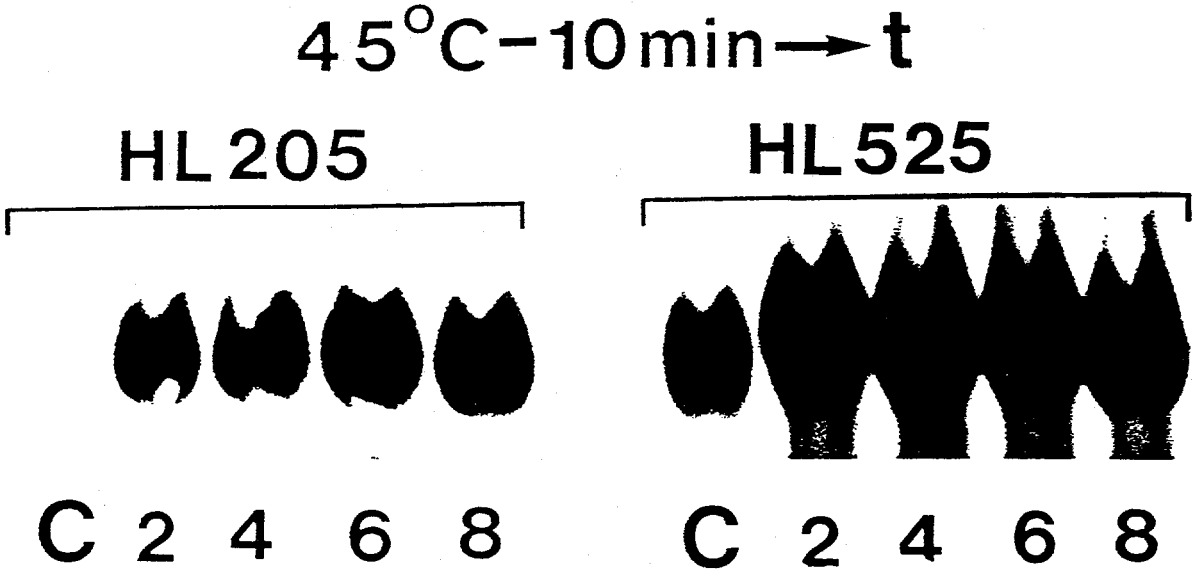
Figure 7.

Western blots with an anti-HSP28 antibody. HL-525 cells were treated for 3 days with the concentrations (nM) of vitamin D_3, 25-hydroxyvitamin D_3 [OH D_3], or 1α,25-dihydroxyvitamin D_3 [$(OH)_2D_3$] indicated at the bottom of each lane. Western blot analysis was performed as described in Fig. 2.

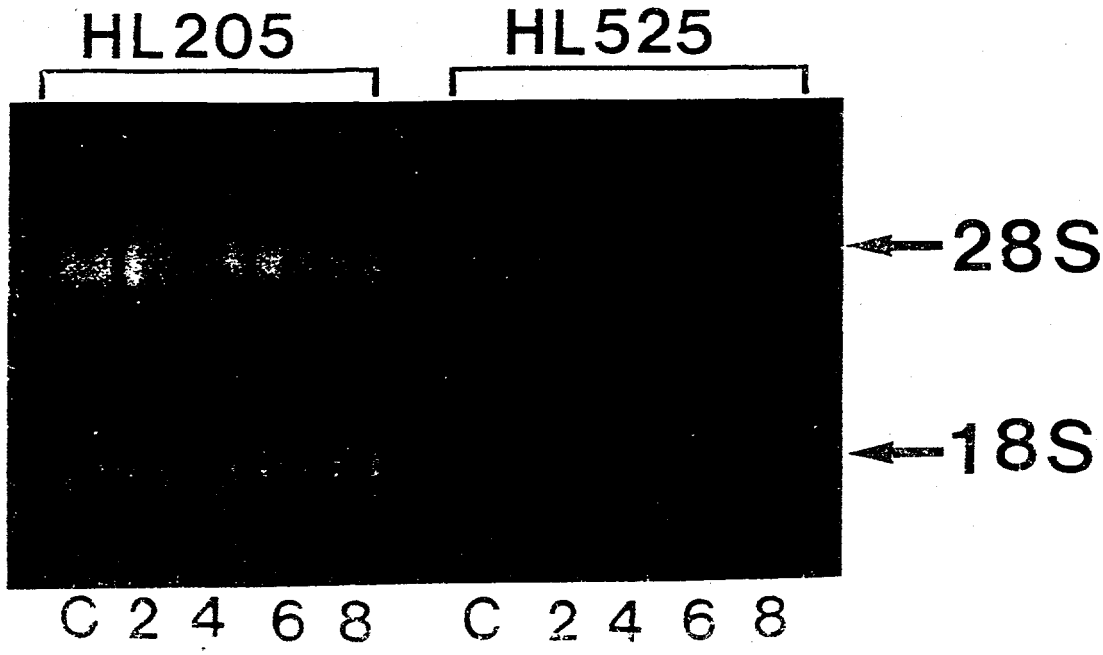
Figure 8.

Detection of a heat shock element (HSE)-binding factor (HSF) in HL-525 cells. The gel mobility-shift assay was performed with a ^{32}P-labeled HSP28 HSE and whole-cell extracts (20 µg protein) prepared from untreated control cells, 1,25-$(OH)_2D_3$ treated cells, or heated cells. C: untreated unheated control cells. 1,25-$(OH)_2D_3$: cells were treated for 3 days with various concentrations of 1,25-$(OH)_2D_3$ indicated at the top of each lane. H: cells were heated at 45°C for 15 min. H + HSE: competition assays were performed by adding a 200-fold molar excess of a nonlabeled HSE oligonucleotide. Closed arrow indicates the position of the HSF-HSE complex. Closed arrowhead indicates a nonheatspecific interaction. Open arrow indicates a free ^{32}P-labeled oligonucleotide fragment (FREE).

A


45°C-10min→t
HL205
HL525
C 2 4 6 8
C 2 4 6 8

B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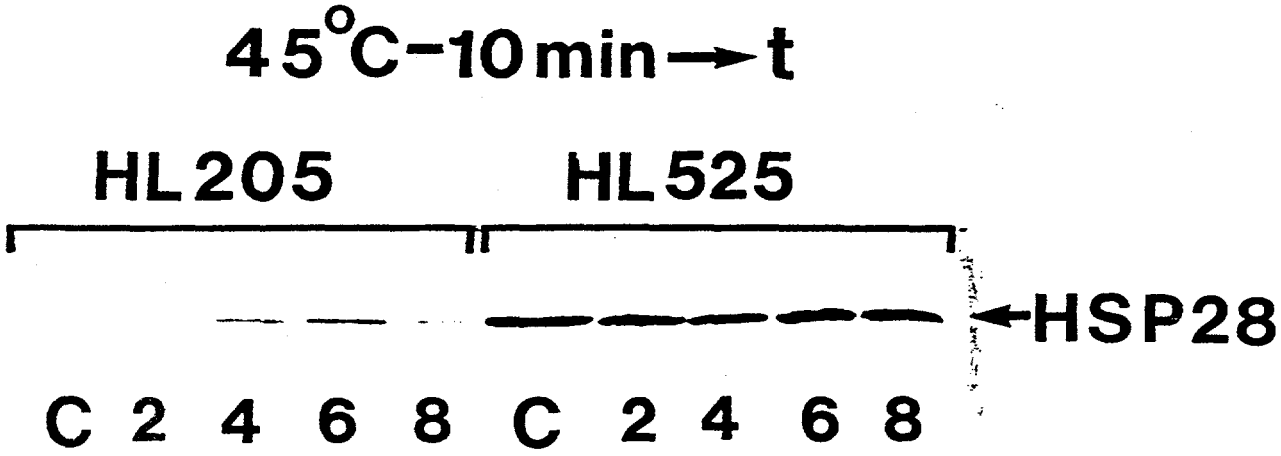
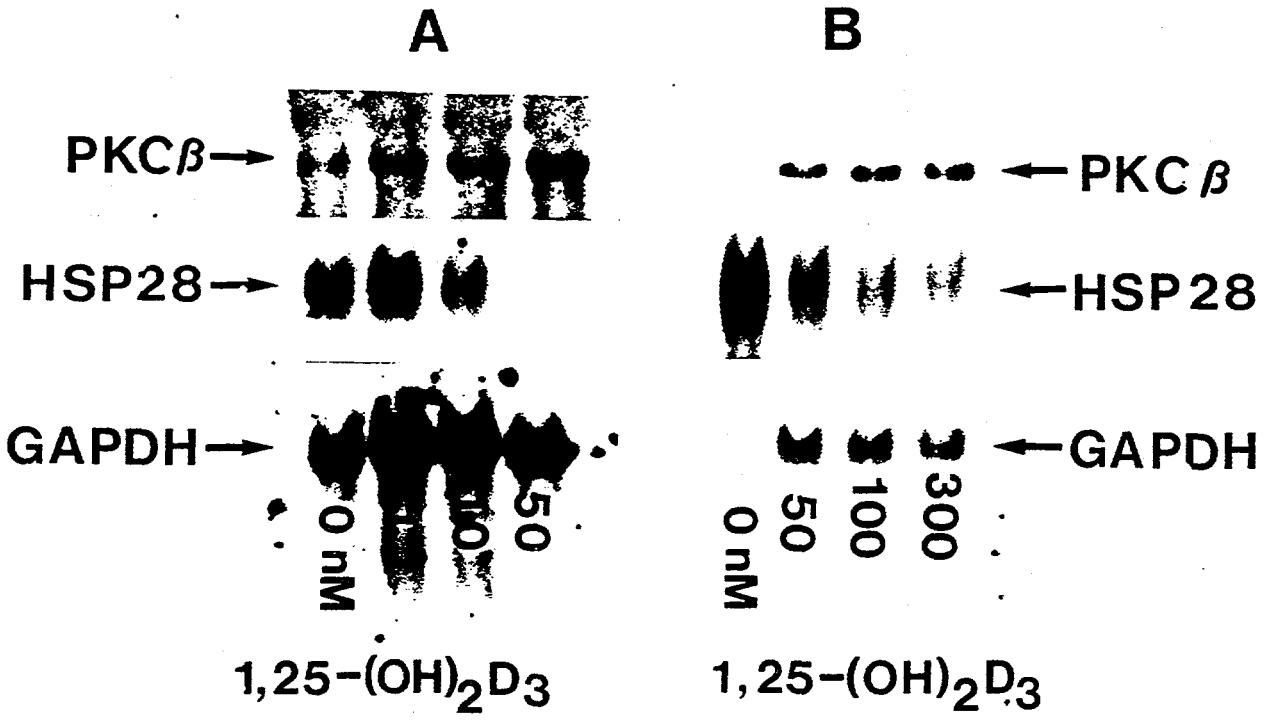
HL525
28S
18S
C 2 4 6 8 C 2 4 6 8


45°C-10min→t
HL205
HL525
HSP28
C 2 4 6 8 C 2 4 6 8

HL 525


A
B
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HSP28
GAPDH
PKC β
HSP28
GAPDH
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50
100
300
0 nM
50
100
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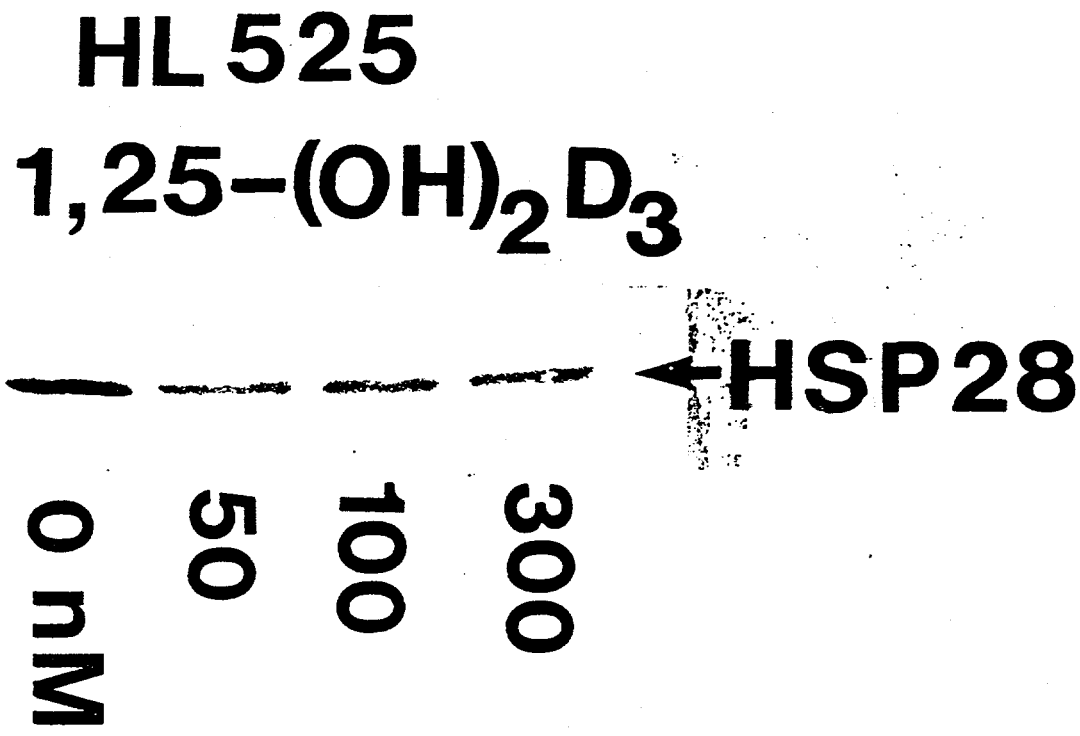
1,25-(OH)2D3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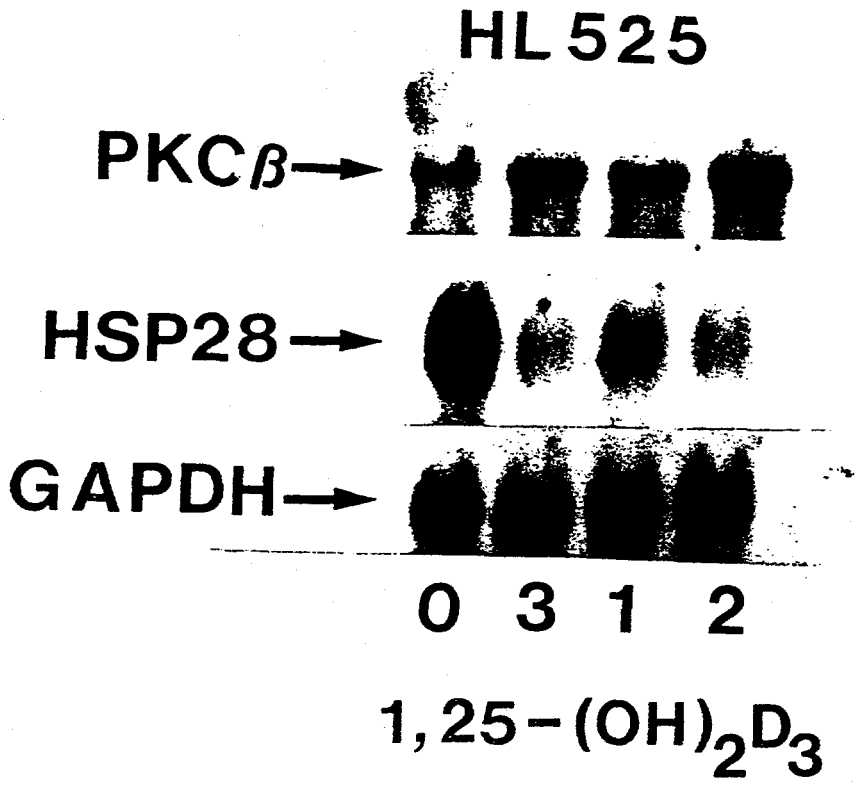
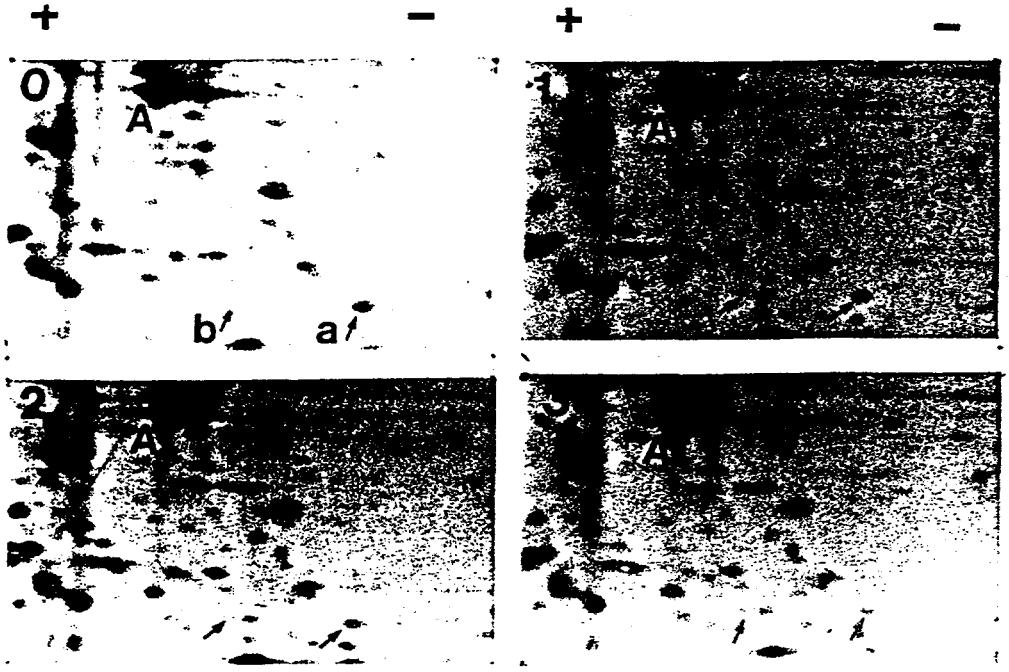
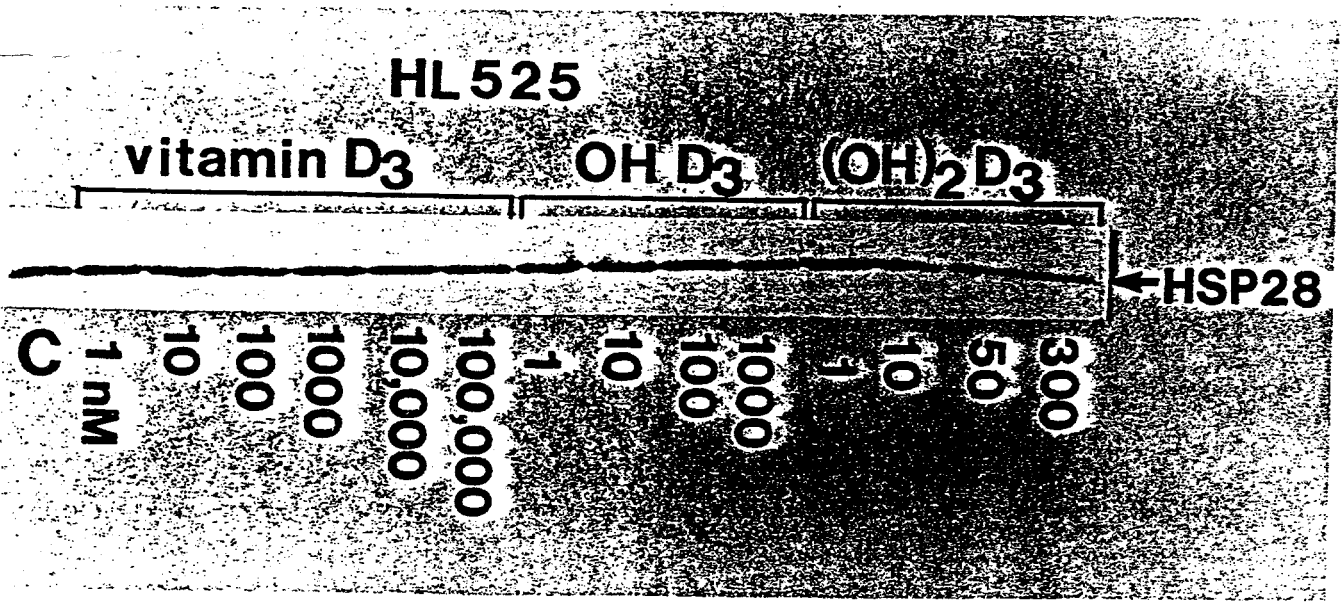
HSP28
0 nM
50
100
300


HL525
PKCβ
HSP28
GAPDH
0 3 1 2
1,25-(OH)2D3

HL525

$1,25-(OH)_2D_3$




HL525
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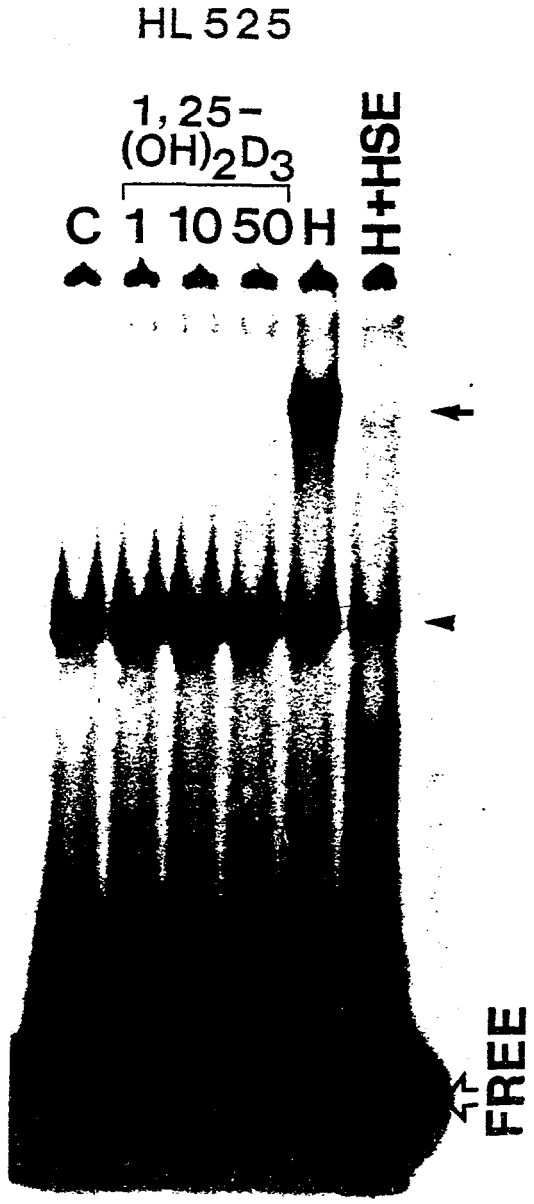
OH D3
(OH)2D3
HSP28
C
1 nM
10
100
1000
10,000
100,000
1
10
100
1000
1
10
50
300


HL 525
1,25-
(OH)2D3
C 1 10 50 H
H+HSE
FREE