Cloning of the Human Phospholipase C-\(\gamma\)1 Promoter and Identification of a DR6-type Vitamin D-responsive Element*

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The 5’-flanking region of the human phospholipase C-\(\gamma\)1 gene was isolated from a human P1 genomic DNA library. The S1-nuclease mapping and primer extension analysis revealed that there is a single transcriptional start site located at 135 bases upstream from the translation start codon in the human phospholipase C-\(\gamma\)1 gene. DNA sequence analysis showed that the sequence around the transcriptional start site is very GC-rich and has no TATA box. The fragment +135 to −877 in the 5’-flanking region of the human phospholipase C-\(\gamma\)1 gene was subcloned into a luciferase reporter vector. The chimeric gene produced a high level of luciferase activity and responded to 1,25-(OH)\(_2\)D\(_3\) in transiently transfected human keratinocytes. Deletion and mutation studies of the fragment +135 to −877 demonstrated a vitamin D-responsive element that contains a motif arranged as two direct repeats separated by 6 bases (DR6), AGGTCAgaccacTGGACA, located between −786 and −803 base pairs. Incubation of the oligonucleotide containing the DR6 with keratinocyte nuclear extracts produced a specific protein-DNA complex that shifted to a higher molecular weight form upon the addition of an antibody specific to the 1,25-(OH)\(_2\)D\(_3\) receptor. Therefore, the 5’-flanking region of the human phospholipase C-\(\gamma\)1 gene confers promoter activity and contains a DR6-type vitamin D-responsive element that mediates, at least in part, the enhanced expression of this gene in human keratinocytes by 1,25-(OH)\(_2\)D\(_3\).

Phospholipase C (PLC)\(^3\) is a family of isoenzymes that cleave phosphatidyl inositol bisphosphate to two second messengers, inositol triphosphate and diacylglycerol, in response to a transmembrane signal (1, 2). Diacylglycerol is the physiological activator of protein kinase C, and inositol triphosphate causes the release of calcium from the endoplasmic reticulum. PLCs can be divided into three types (PLC-\(\beta\), PLC-\(\gamma\), and PLC-\(\delta\)), and each type contains several subtypes (3, 4). PLC-\(\gamma\)1, unlike the other PLC isoenzymes, contains a src homology 2 domain through which PLC-\(\gamma\)1 interacts with various tyrosine kinase growth factor receptors (5–8). PLC-\(\gamma\)1 is overexpressed in primary human breast carcinoma (9), human colorectal cancer (10), familial adenomatous polyposis (11), and hyperproliferative epidermal diseases (12). The amount of PLC-\(\gamma\)1 protein is higher in neoplastic keratinocyte cell lines than in normal keratinocytes (13). Calcium-induced differentiating keratinocytes express over 2-fold more PLC-\(\gamma\)1 protein than undifferentiated keratinocytes (14). These observations suggest that PLC-\(\gamma\)1 might be involved in the regulation of cell proliferation and differentiation.

The differentiation of normal human keratinocytes is induced by extracellular calcium and 1,25-(OH)\(_2\)D\(_3\) (15–20). The mechanism underlying the regulation by 1,25-(OH)\(_2\)D\(_3\) is thought to include changes in intracellular calcium, PLC, and protein kinase C activation. PLC-\(\gamma\)1 is one of the major PLC isoenzymes that mediate cellular signal transduction. Treatment with 1,25-(OH)\(_2\)D\(_3\) dramatically up-regulates the protein and mRNA expression of PLC-\(\gamma\)1 (24). To understand the molecular mechanism of this regulation, we cloned the 5’-flanking region of the human PLC-\(\gamma\)1 gene that confers promoter activity and identified within the 5’-flanking region a DR6-type vitamin D-responsive element (VDRE).

MATERIALS AND METHODS

Isolation of Genomic Clone—The subclones containing phospholipase C-\(\gamma\)1 genomic DNA were obtained from a human P1 genomic DNA library using as probe a 5-kb PLC-\(\gamma\)1 cDNA (Genome Systems). To isolate the 5’-flanking region of the PLC-\(\gamma\)1 gene, the subclones were further screened by colony hybridization using the oligonucleotide (5’-CGTTGCGCTTGCTCCCGGGC-3’) from the 5’-untranslated region of PLC-\(\gamma\)1 cDNA as probe. From the selected subclone, a 1.1-kb XhoI fragment was resubcloned into a PBluescript SK(−) vector (Stratagene). The nucleotide sequence of the insert was sequenced using the dideoxy chain termination method. The sequence of each strand was confirmed by repeating the sequencing in both directions at least three times. The sequence of the GC-pressed region was confirmed using dITP instead of dGTP.

Construction of Plasmids—The XhoI-StylI fragment was resubcloned into a pGL-3-basic vector (Promega). The PLC-\(\gamma\)1 gene was placed 2 bp upstream from the luciferase gene. Subsequent 5’ deletion constructs were made with restriction enzyme digestion. The constructs containing the fragment −748 to −825 and the fragment −786 to −803 were made by ligating the fragments to the heterologous simian virus 40 (SV40) promoter in the pGL-3-promoter vector. Correct orientation of the inserts with respect to the luciferase sequence was verified by restriction enzyme analysis.

Cell Culture—Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum free keratinocyte growth medium (Clonetics) (25). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4 °C, overnight), and primary cultures were established in keratinocyte growth medium containing 0.07 mM calcium. Second passage keratinocytes were plated in 60-mm culture dishes with keratinocyte growth medium plus 0.03 mM calcium at 20–30% confluency for the transfection experiments.

DNA Transfection and Luciferase Assay—PLC-\(\gamma\)1 luciferase chimeric plasmids were transfected into normal human keratinocytes using a polybrene method 24 h after plating cells in 60-mm culture dishes (26). Cells were co-transfected with 0.2 µg of pRSV5j-gal (27), a β-galactosidase expression vector that contains a β-galactosidase gene that is driven by a Rous sarcoma virus promoter and enhancer, which was used as an internal control to normalize for transfection efficiency. 1,25-(OH)\(_2\)D\(_3\)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U80983.

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1 The abbreviations used are: PLC, phospholipase C; VDR, vitamin D receptor; VDRE, vitamin D-responsive element; DR, direct repeat; kb, kilobase(s); bp, base pair(s).
Activities using Luciferase Assay System (Promega). The human PLC-γ1 was added to the cells 24 h after transfection at a final concentration of 10^6.

The pGL-3-basic vector, using Klenow and a [**]Xba**I restriction map of the genomic clones showing the restriction sites in the 9-kb portion using the single-letter code. The transcription start site is designated as +1.

The potential SP1 and AP2 sites, and CCAAT box are underlined. Two direct repeats are presented in bold type.

**FIG. 1.** Restriction map and sequence of 5'-flanking region of the human PLC-γ1 gene. A, restriction map of the genomic clones showing the restriction sites in the 9-kb HindIII fragment. H, HindIII; Xb, XbaI; Xh, XhoI; S, StyI. B, nucleotide sequence of the 1011-bp XhoI-StyI fragment with part of the coding region. The amino acid sequence is printed under the nucleotide sequence of the translated portion using the single-letter code. The transcription start site is designated as +1. The potential SP1 and AP2 sites, and CCAAT box are underlined and labeled respectively by SP1, AP1, and CCAAT. Two direct repeats are presented in bold type.

was added to the cells 24 h after transfection at a final concentration of 10^6/ml. The same amount of ethanol (vehicle) was added to the control plates. The total RNA and poly(A) RNA from normal human keratinocytes were isolated in the same way as that for S1-nuclease protection assay. The primer extension analysis was performed using the Primer Extension System from Promega. 2 μg of poly(A) RNA was hybridized with an end labeled primer corresponding to the region 40–60 bp downstream from the transcription start codon of the antisense strand of human PLC-γ1 cDNA. The hybridization mixture was heated at 75°C for 15 min and then incubated at 42°C for 60 min. Actinomycin D was added to the mixture at a final concentration of 75 ng/ml to inhibit secondary structure formation of the RNA. The extension products were analyzed on a denatured 6% polyacrylamide gel.

**FIG. 2.** Determination of the transcriptional start site. A, in a nuclease protection assay, an antisense probe spanning 212 bp upstream from the translation start codon in the human PLC-γ1 genomic DNA was hybridized with human keratinocyte poly(A) RNA and then digested by S1 nuclease. The size of the protected fragment was 135 bp. Lanes G, A, T, and C are sequence reactions of the antisense probe used as a size marker. B, a primer extension experiment was carried out using as primer a 5'-labeled oligonucleotide corresponding to the antisense strand of the region 40–60 bp downstream from the translation start codon in the human PLC-γ1 gene and human keratinocyte poly(A) RNA as template. The size of the extension fragment was 195 bp, confirming the transcription start site as 135 bp upstream of the translation start codon. M, size marker.

was accomplished by use of the antisense GL primer2 primer that bound the downstream sense GL primer2 primer in the vector such that the synthesized probe spanned the insert. The probe was coprecipitated with 1 μg of the normal human keratinocyte poly(A) RNA. Hybridization was performed by dissolving the precipitate in 10 μl of hybridization buffer at 42°C overnight. Unprotected DNA was digested with S1 nuclease at 37°C for 30 min. The resulting fragment was recovered by ethanol precipitation, denatured, and analyzed on an 8% sequencing gel with a sequencing ladder as a standard. The sequencing reaction was performed using the dye deoxy chain termination method.

**Primer Extension Analysis**—The total RNA and poly(A) RNA from normal human keratinocytes were isolated in the same way as that for S1-nuclease protection assay. The primer extension analysis was performed using the Primer Extension System from Promega. 2 μg of poly(A) RNA was hybridized with an end labeled primer corresponding to the region 40–60 bp downstream from the translation start codon of the antisense strand of human PLC-γ1 cDNA. The hybridization mixture was heated at 75°C for 15 min and then incubated at 42°C for 60 min. Actinomycin D was added to the mixture at a final concentration of 75 ng/ml to inhibit secondary structure formation of the RNA. The extension products were analyzed on a denatured 6% polyacrylamide gel.

**DNA Mobility Shift Assay**—The nuclear extracts were made from normal human keratinocytes according to the method described by Abmayr and Workman (28). The recombinant vitamin D receptor was from Affinity Bioreagents Inc. Synthetic oligonucleotides used for the DNA mobility shift assay were end-labeled by T4 polynucleotide kinase. The DNA-protein reactions were performed in a total of 17 μl; nuclear extracts (12 μg of protein) were incubated with 2 μg of poly(dI-dC) (Pharmacia Biotech Inc.) and 10,000 cpm of 32P-labeled probe in 10 ml of binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.5 mM dithiothreitol) at 30°C for 25 min. Unlabeled competitors were added at the preincubation step. In the super gel shift reaction, a polyclonal anti-vitamin D receptor antibody (3 μl from the original stock, Affinity Bioreagents Inc.) was added to the DNA-protein reaction and incubated for an additional 25 min. Protein-DNA complexes were electrophoresed in a 6% nondenaturing polyacrylamide gel in 1 × gel shift running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5).

**RESULTS**

Three positive clones were obtained from the human P1 genomic DNA library screening. One of the positive clones was digested by HindIII, and the random fragments were subcloned into a pZErO vector (Invitrogen). Three independent subclones that contain the 5'-flanking region of the human PLC-γ1 gene
were isolated from the random subclones by screening 96 subcultures using an oligonucleotide from the untranslated region of the human PLC-γ1 cDNA (see "Materials and Methods"). Restriction analysis indicated that all three positive subclones contained a 9-kb HindIII insert spanning more than 8 kb upstream from the translation start codon in the human PLC-γ1 gene. The restriction map for the 9-kb fragment is shown in Fig. 1A. The HindIII-StylI (9 kb), XhoI-StylI (2.5 kb), and XhoI-StylII (1 kb) fragments were individually subcloned in a pGL-3-basic vector and transfected into human keratinocytes. The results showed that the 1-kb XhoI-StylI fragment construct expressed the highest luciferase activity (data not shown). Therefore, we focused on the 1-kb XhoI-StylII fragment in the subsequent experiments. The sequence analysis revealed that the 1-kb XhoI-StylI fragment in the 5′-flanking region of the human PLC-γ1 gene was very GC-rich. 16 putative SP1 sites and 8 putative AP2 sites were clustered in the 1-kb XhoI-StylI fragment. No TATA box was found in this fragment. There was a putative CCAAT box located between −581 and −585 bp upstream from the transcriptional start site (Fig. 1B).

Both S1 nuclease protection assay and primer extension analysis were performed to determine the transcriptional start site for the human PLC-γ1 gene. The S1 nuclease protection assay was performed using an antisense probe spanning 212 bp upstream from the translation start codon. This probe hybridized to the poly(A) RNA isolated from human keratinocytes. After S1 nuclease digestion, a single protected fragment of 135 bp was detected (Fig. 2A). The result suggested that the transcriptional start site is 135 bp upstream from the translation start codon. The primer extension analysis showed a 195-bp single extension fragment whose 5′ end is 135 bp upstream from the translation start codon (Fig. 2B), confirming the result obtained with the S1 nuclease protection assay.

In an attempt to delineate the sequences essential for human PLC-γ1 gene transcription, nine deletional fragments spanning from +135 to −877 bp in the 5′-flanking region were fused with the coding region of the luciferase gene in the luciferase vector and transfected into normal human keratinocytes (Fig. 3A). The construct containing the +135 to −877 fragment construct expressed luciferase activity 50-fold higher than that from the vector alone (Fig. 3B). The data clearly indicated that the 5′-flanking region of the human PLC-γ1 gene contains a sequence that confers promoter activity. Deletional analysis to −200 bp showed little loss in basal activity. When the 5′ deletions reached −39 bp, the luciferase activities were greatly reduced. The fragment +13 to +135, which did not contain the transcriptional start site, lost all activity (Fig. 3B). The data suggested that the most proximal 200 bp of the 5′-flanking region of the human PLC-γ1 gene are essential for transcriptional initiation.

To determine if the human PLC-γ1 gene transcriptionally responds to 1,25-(OH)_{2}D_{3}, the nine deletional constructs were transfected into human keratinocytes in the presence or the absence of 1,25-(OH)_{2}D_{3}. The results showed that the construct containing fragment +135 to −877 was responsive to 1,25-(OH)_{2}D_{3} stimulation. The luciferase activity was increased over 3-fold after 24 h of exposure to 1,25-(OH)_{2}D_{3}. 5′ deletion to −748 bp totally abolished the responsiveness to 1,25-(OH)_{2}D_{3} vector showed the vector background. C, a similar experiment was performed with constructs containing −748 to −828, −786 to −803, and a mutant construct in which two random sequences replaced AGGTCA and TGGACA. The results are normalized to β-galactosidase activity. A construct containing the vitamin D-responsive region at −143 to −293 in the human 24-hydroxylase gene (24-hydroxylase) was used as a positive control. The activity obtained from the pGL-3 promoter vector showed the vector background.
type fragment keratinocytes by 1,25-(OH)2D3 (Fig. 3). The promoter activity was induced over 2-fold in human
DNA fragments containing a VDRE, this fragment was subcloned into the pGL-3-promoter vector. Transfection experiments showed
that the sequence in the human PLC-1 gene that confers promoter activity when transiently trans-
fected into human keratinocytes, an 80-bp synthetic oligonucleotide (named W1) representing the vitamin D-responsive region
-748 to -828 bp was evaluated using the DNA mobility shift assay. Incubation of the oligonucleotide W1 with the nuclear extracts from the human keratinocytes yielded two specific DNA-protein binding complexes (Fig. 4A). The specificity of the binding was verified by competition with the same or mutant unlabeled oligonucleotides at 100 molar excess. The results showed that the two binding complexes were reduced by W1 but not by a mutant fragment (named M1) containing MPDR6 instead of PDR6 (Fig. 4A). These data suggest that the two
bands are specific complexes of the sequence PDR6 with the nuclear factors in the human keratinocytes. The bands are not
SP1 complexes because binding was not blocked by an SP1 consensus oligonucleotide even though there is a putative SP1 site in this region (Fig. 4A). However, the upper band was competed out by a 21-bp unlabeled oligonucleotide (named H) containing a DR3-type VDRE (AGGTGAgcgAGGGCG) found in the human 24-hydroxylase gene, suggesting that the upper band was a VDR-VDRE complex (Fig. 4A). To narrow down the vitamin D binding region, we repeated the experiment but used a 38-bp oligonucleotide (named W2) containing the sequence PDR6 with 10 flanking bases on each side. The results showed that a single main complex formed after the incubation of the fragment W2 with the nuclear extracts from the human keratinocytes (Fig. 4B). The binding complex was reduced by the unlabeled oligonucleotides W2 and H but not by a mutant fragment (named M2) containing MPDR6 instead of PDR6. The binding complex was shifted to a higher molecular weight form upon the addition of an antibody specific to the VDR. Incubation of the labeled fragment W2 with the recombinant vitamin D receptor yielded two shifted bands that were blocked by an unlabeled oligonucleotide W2 (Fig. 4B). The results indicate that the sequence PDR6 in the region -786 to -803 binds to the VDR in human keratinocytes.

**DISCUSSION**

We have cloned the 5'-flanking region of the human PLC-1 gene that confers promoter activity when transiently trans-
fected into human keratinocytes. The sequence in the human PLC-1 flanking region is GC-rich and has no TATA box, similar
to many genes that are important in the control of cell proliferation and differentiation such as transforming growth factor-β1 (32), epidermal growth factor receptor (33), and nerve growth factor (34). The region between -200 and -39 bp contains several putative SP1 and AP2 binding sites and appears to contain the promoter because the deletion from -280 to -39 bp dramatically reduced promoter activity (Fig. 3B). The transcription initiation of the human PLC-1 gene could be similar to other GC-rich genes in which SP1 binding to the GC-boxes, rather than a TFIID-TATA complex, is able to activate gene transcription (35).

The pentanucleotide CCAAT, which is usually found within -50 to -100 bp upstream from the transcriptional start site in mammalian genes where it appears to have a role in mediating promoter function (36), was located between -581 bp and -585 bp in the human PLC-1 gene. However, this putative CCAAT
box has no clear function because the deletion from −613 to −551 bp did not remarkably reduce basal promoter activity. Therefore, basal transcription of the human PLC-γ1 gene does not appear to require a CCAAT-binding protein.

A DR-6 type VDRE, AGGTCAgaccacTGGACA, has been precisely localized within the 5'-flanking region of the human PLC-γ1 gene. The transfection experiments showed that the DR6 sequence was completely silent in the human PLC-γ1 gene in the absence of 1,25-(OH)2D3 but was activated by the addition of 1,25-(OH)2D3. In the DNA mobility shift assays, the DR6 specifically bound to the vitamin D receptor in the human keratinocytes, as recognized by the vitamin D receptor antibody. Substitution mutation of the 6-base repeats in the DR6 sequence totally abolished the response to vitamin D as well as the DNA binding ability, indicating that 1,25-(OH)2D3 activates the human PLC-γ1 gene through its vitamin D receptor interacting with the DR6-type VDRE localized in the 5'-flanking region. The sequences of the two repeats share some homology with the known DR6-type VDREs in the human osteocalcin gene (30) and rat 24-hydroxylase gene (31) (Fig. 5). Alignment of these DR6-type VDREs showed that one-third of the nucleotides are identical between each repeat. It seems that the second and the sixth nucleotides within each repeat are always G and A, respectively, suggesting that these bases are critical for DNA-receptor binding and confer transactivation upon vitamin D stimulation. Single base mutations will be required to define the precise nucleotides that are essential to mediate the responsiveness to 1,25-(OH)2D3.

Vitamin D receptor and nonreceptor transcriptional factors binding to distinct sites in a promoter or enhancer region is one mechanism by which the profound alteration in gene expression can occur from small changes in the concentration of trans-acting factors (37). A typical vitamin D receptor and nonreceptor transcriptional factor interaction model was reported in the human osteocalcin VDRE, which contains an AP1 site; AP1 binding proteins were shown to regulate VDRE function (38–40). Although no AP1 site was found in the human PLC-γ1 gene, SP1 has also been reported to interact with the vitamin D receptor by independently binding to a different motif (37). We found 16 putative SP1 sites clustered downstream of the DR6 sequence in the 5'-flanking region of the human PLC-γ1 gene. However, the isolated human PLC-γ1 VDRE ligated to a heterologous SV40 promoter did not appear to differ in the degree of response to 1,25-(OH)2D3 as the VDRE within its own gene context. The data suggest that the SP1 sites are not involved in the vitamin D-induced human PLC-γ1 transcription.

DR6-type VDREs of the human osteocalcin gene (41) and the rat 24-hydroxylase gene have been shown to bind VDR-RAR heterodimers, as well as VDR-VDR homodimers (31). In this report, we found that recombinant VDR was able to bind to the human PLC-γ1 VDRE, as shown by DNA mobility shift assay, implying that VDR might be binding to the human PLC-γ1 VDRE as a homodimer or monomer. However, the keratinocyte nuclear extracts give a different pattern of binding to the DR6 than the recombinant VDR, suggesting that other factors are also involved in VDR-VDRE binding. Further experiments are needed to identify these additional factors.

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