Phospholipase C-γ1 Is Required for Calcium-induced Keratinocyte Differentiation*

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Phospholipase C-γ1 is the most abundant member of the phospholipase C family in keratinocytes and is induced by calcium. Phospholipase C-γ1, therefore, may be involved in the signal transduction system leading to calcium regulation of keratinocyte differentiation. To test this hypothesis, expression of phospholipase C-γ1 in human keratinocytes was blocked by transfecting cells with the antisense human phospholipase C-γ1 cDNA construct. These cells demonstrated a dramatic reduction in phospholipase C-γ1 protein level compared with the empty vector-transfected cells and a marked reduction in the mRNA and protein levels of the differentiation markers involucrin and transglutaminase following administration of calcium. Similarly, cotransfection of antisense phospholipase C-γ1 constructs with a luciferase reporter vector containing involucrin or transglutaminase promoters led to a substantial reduction in calcium-stimulated involucrin and transglutaminase promoter activities. Similar results were seen following treatment with a specific phospholipase C inhibitor U73122. To determine whether phospholipase C-γ1 regulated differentiation by controlling intracellular calcium, we examined the ability of antisense phospholipase C-γ1 to block the calcium-induced rise in intracellular calcium and found that it could. These findings indicate that phospholipase C-γ1 is a critical component of the signaling pathway mediating calcium regulation of keratinocyte differentiation via its mobilization of intracellular calcium.

A variety of extracellular stimuli exert their physiologic effects by activating phospholipase C (PLC) isoforms. PLC activation results in hydrolysis of phosphatidylinositol bisphosphate (PIP2), a cell membrane phospholipid, generating inositol trisphosphate (IP3), which releases calcium from intracellular stores, and diacylglycerol (DG), which activates protein kinase C. These second messengers subsequently activate a number of cellular reactions leading to changes in cell proliferation and differentiation (1, 2). There are at least three known types of PLC (β, γ, and δ), and each type has a number of subtypes (3, 4). The γ-type enzymes are unique in that they contain SH2 and SH3 domains and are subject to activation by a number of growth factor receptors such as receptors for epidermal growth factor, nerve growth factor, and platelet-derived growth factor (5–8).

Human keratinocytes possess a functionally active inositol lipid signaling system (9–12) that may play an essential role in keratinocyte differentiation (13–15). The addition of calcium leads to keratinocyte differentiation and the activation of several signaling pathways, one of which involves PLC-γ1, the predominant isoform of PLC in keratinocytes (13, 14, 16, 17). However, it is not clear whether PLC-γ1 plays an essential role in the regulation of keratinocyte differentiation by calcium. To address this issue, we blocked PLC-γ1 production with antisense PLC-γ1 cDNA and inhibited its activity with a specific PLC inhibitor. Our results indicate that PLC-γ1 signaling is required for calcium-induced keratinocyte differentiation.

MATERIALS AND METHODS

Cell Culture—Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as described previously (18). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4 °C, overnight), and primary cultures were established in KGM containing 0.07 mM calcium. First and second passage keratinocytes were plated with KGM containing 0.03 mM calcium and used in the experiments described.

Construction of Vectors Expressing Antisense RNA—The antisense PLC-γ1 constructs were made by inserting 4.2-kb human PLC-γ1 cDNA fragments containing the ATG start codon in a BamHI site of a pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) which expresses a neomycin (G418) resistance gene. The 3.7-kb fragment of the human involucrin promoter (gift from Dr. Lorne B. Taichman) and the 2.2-kb fragment of the human transglutaminase promoter (gift from Dr. Robert Rice) were subcloned into a pGL-3-basic vector (Promega) (19, 20), linking them to the luciferase gene. Correct orientation of the inserts with respect to the luciferase sequence was verified by restriction enzyme analysis.

Transfection, Selection, and Luciferase Assay—First passage keratinocytes were transfected in suspension with the antisense PLC-γ1 (A) or pcDNA3.1(+) vector (V) using a Polybrene/glycerol method (21) and incubated in KGM with 0.07 mM calcium. The transfected cells were selected by a 4-day incubation in 300 μg/ml G418 starting 2 days after transfection. 1.2 mM calcium was added to the medium 2 days after selection, and cells were harvested 2 days later. The cellular protein was isolated, and the protein levels for PLC-γ1, PLC-β1, PLC-δ1, involucrin, and transglutaminase were quantitated by Western analysis. The cellular RNA was isolated, and the mRNA levels for involucrin and transglutaminase were quantitated by Northern analysis. In other experiments, second passage keratinocytes plated in 60-mm culture dishes were co-transfected with antisense PLC-γ1 (A) or pcDNA3.1(+) vector (V), involucrin, or transglutaminase-luciferase chimeric plasmid promoter constructs and with 0.2 μg of pRSVβ-gal (22) using a Polybrene method (20). pRSVβ-gal is 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. Calcium chloride was added to the cells 24 h after transfection at a final concentration of 1.2 mM. The cells were lysed 24 h later, and the cell extracts were assayed for luciferase activity using the Luciferase Assay System (Promega) and β-galactosidase activity using the Galacto-LightTM kit (Tropix Inc., Bedford, MA). A pGL-3-control vector (Promega) containing an SV40 promoter and SV40 enhancer, shown to be unresponsive to calcium, was included as an internal control to normalize for transfection efficiency.

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1 The abbreviations used are: PLC, phospholipase C; PIP2, phosphatidylinositol; IP3, inositol trisphosphate; KGM, keratinocyte growth medium; DG, diacylglycerol; kb, kilobase.

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in each transfection experiment as a control. Every experiment was done in triplicate and was repeated at least three times.

**Northern Analysis—**Total RNA was isolated from the keratinocytes using the STAT-60 kit™ (Tel-Test “B”, Inc., Friendswood, TX), according to the procedures recommended by the manufacturer. The isolated RNA was electrophoresed through a 0.8% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) using PosiBlot 30–30 Pressure Blotter (Stratagene), and immobilized by baking the membrane at 80 °C for 2 h. The human PLC-γ1 cDNA probe (gift from Dr. John Imboden) was labeled with 32P-dCTP (Amersham Pharmacia Biotech) by Random Primed DNA Labeling Kit (Stratagene), and purified by NucleoTrap Pro. Purification Columns (Stratagene). The membrane was prehybridized and hybridized in 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 20 μg/ml salmon sperm DNA with the 32P-labeled human PLC-γ1 cDNA. Following hybridization at 65 °C overnight, the membrane was washed in solutions with decreasing ionic strength and increasing temperature to a final stringency of 0.1× SSC, 0.1% SDS, at 65 °C. The 32P-cDNA–mRNA hybrids were visualized by exposing to x-ray film and quantitated by densitometry using the program NIH Image. The human PLC-γ1 mRNA was normalized to the levels of 18S ribosomal RNA in the same RNA blots as determined by rehybridization of the filter with a 32P-labeled cDNA for 18 S RNA.

**Western Analysis—**Keratinocytes were washed twice with phosphate-buffered saline and then incubated in RIPA lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin) for 5 min. Cells were scrapped into microfuge tubes, incubated on ice for 15 min, and pelleted by centrifugation. The supernatant was collected. The protein concentration of the lysate was measured by the BCA Protein Assay Kit (Pierce). Equal amounts of protein were then electrophoresed through 7.5% polyacrylamide gels at 200 V for 30 min and electroblotted onto polyvinylidene fluoride microporous membranes (Immobilon-P, 0.45 μm, Millipore) in an electroblotting buffer (25 mM Tris, 192 mM glycine, 5% methanol) at 130 V for 2 h. Following incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% non-fat milk, and 0.5% Tween 20), the blot was incubated with the appropriate antibodies overnight at 4 °C. Involucrin protein was detected with a polyclonal mouse anti-human involucrin antibody (Sigma) at a dilution of 1:2000 in blocking buffer. Transglutaminase protein was detected with a monoclonal mouse anti-human transglutaminase antibody (gift from Dr. Robert Rice) at a dilution of 1:200 in the blocking buffer. PLC-γ1, -γ2, and -γ1 were detected with a polyclonal rabbit anti-human PLC-γ1 antibody (Santa Cruz Biotechnology) at a dilution of 1:200, a polyclonal rabbit anti-human PLC-β1 antibody (Santa Cruz Biotechnology) at a dilution of 1:200, and a polyclonal goat anti-human PLC-δ1 antibody (Santa Cruz Biotechnology) at a dilution of 1:200. The blots were incubated in KGM with 0.03 mM calcium. The transfected cells were then alternately illuminated with 200-ms flashes of 340 and 380 nm light every 10 s, monitoring the emission wavelength of 510 nm. Calcium levels of keratinocytes attached to glass coverslips were measured using a Dual-wavelength Fluorescence Imaging System (Intracellular Imaging Inc., Cincinnati, OH). Briefly, the cells were loaded with 1 μM Fura-2 (Molecular Probes, Eugene, OR) in 0.1% Pluronic F127 (Molecular Probes, Eugene, OR) in 0.1% Pluronic F127 (Molecular Probes, Eugene, OR) in buffer A (20 mM HEPES buffer, pH 7.4, containing 120 mM sodium chloride, 5 mM potassium chloride, 0.5 mM magnesium chloride, 1 mM MgCl2, 1 mM/ ml pyruvate, 1 mM/ml glucose, and 0.03 mM calcium chloride) at room temperature for 30 min followed by a 30-min rinse in buffer B. The cells were then alternately illuminated with 200-ms flashes of 340 and 380 nm light every 10 s, monitoring the emission wavelength of 510 nm. Intracellular calcium concentration was calculated based on the ratio of emission at the two excitation wavelengths based on the formula developed by Grynkiewicz et al. (23).

**RESULTS**

To examine the role played by PLC-γ1 in keratinocyte differentiation, we examined the ability of the antisense PLC-γ1 construct to block calcium induction of the differentiation markers involucrin and transglutaminase. Keratinocytes were transfected with the full-length cDNA for PLC-γ1 in the antisense orientation to suppress PLC-γ1 expression. The transfectants were selected with a neomycin analogue G418 dissolved in KGM containing 0.03 mM calcium for 4 days to enrich transfected cells. The cells were cultured in 0.03 mM calcium for 5 days and then in 1.2 mM calcium for an additional 2 days. The results showed that 1.2 mM calcium increased the levels of involucrin and transglutaminase in cells transfected with the vector. However, the stimulation of the involucrin and transglutaminase protein by calcium was markedly reduced by the antisense PLC-γ1 cDNA construct (Fig. 1a). The calcium-induced increase in involucrin and transglutaminase mRNA was also inhibited by antisense PLC-γ1 cDNA (Fig. 1b). Basal involucrin and transglutaminase mRNA and protein levels at 0.03 mM calcium were not affected by antisense PLC-γ1 cDNA constructs (Fig. 1, a and b).

The specificity of the antisense construct for PLC-γ1 was assayed by Western analysis using PLC-γ1, -γ2, -β1, and -δ1 antibodies. As shown in Fig. 1a, calcium increased the protein levels of PLC-γ1, -β1, and -δ1. PLC-γ2 was not detected at either calcium concentration (data not shown). However, the PLC-γ1 protein level in the human keratinocytes transfected by antisense PLC-γ1 construct in the presence of 0.03 or 1.2 mM calcium was reduced dramatically compared with that in the human keratinocytes transfected by the vector alone. In contrast, the PLC-β1 and -δ1 protein levels were not reduced by the antisense PLC-γ1 construct (Fig. 1a). These data demon-
The aim of this work was to study the role of PLC-γ1 in mediating the regulation of keratinocyte differentiation by calcium. Antisense cDNA methodology is useful in the analysis of specific gene functions. The antisense transcript has a sequence complementary to the target mRNA and anneals with the mRNA to disrupt normal processing or translation (24). In this study, the antisense PLC-γ1 cDNA was used to selectively inhibit the expression of PLC-γ1 in normal human keratinocytes treated with calcium to determine whether PLC-γ1 is involved in calcium-induced human keratinocyte differentiation. The 4.2-kb antisense PLC-γ1 mRNA specifically blocked PLC-γ1 expression without affecting PLC-β1 and -δ1 expression, the other major PLC isozymes in human keratinocytes. Involucrin and transglutaminase, a substrate and enzyme required for cornified envelope formation (25–27), are two markers for keratinocyte differentiation (28). The blockage of PLC-γ1 production results in inhibition of calcium-induced involucrin and transglutaminase expression, indicating that PLC-γ1 is required for keratinocyte differentiation.

To confirm these results, we used inhibitor of PLC. The compound U73122 is capable of inhibiting several PLC-dependent processes (29–31). It has been suggested that this compound inhibits PLC activity by binding to a calcium-binding site on the PLC which must be occupied by calcium for PLC activity to occur. We used this agent to block PLC activity in keratinocytes as a second means of testing the role of PLC in calcium stimulation of keratinocyte differentiation. In agreement with the results obtained from the antisense transfection experiments, calcium-induced involucrin and transglutami-
nase expression was blocked by the PLC inhibitor. Thus, PLC is required for calcium-induced differentiation. Several mechanisms for this effect need to be considered.

In murine keratinocytes, calcium-induced keratinocyte differentiation is associated with enhanced PLC-γ1 protein expression but not increased mRNA level (14, 17). Our current data show that in human keratinocytes, both PLC-γ1 protein and mRNA levels are induced by calcium. The shift to differentiation associated with increasing the calcium level of the medium from 0.03 to 1.2 mM is quite rapid. Within hours of the calcium switch, morphological changes related to differentiation described previously (25) are apparent under phase-contrast microscope in the cells transfected with vector and the cells treated with vehicle or the inactive analogue of the PLC inhibitor. The cells transfected with antisense PLC-γ1 construct or treated with the PLC inhibitor did not show the morphological changes. These data are consistent with the observations of the decreased expression of the two differentiation markers involucrin and transglutaminase in the cells transfected with antisense PLC-γ1 construct and the cells treated with PLC inhibitor.

PLC-γ1 hydrolyzes PIP_{2} into two second messengers, IP_{3} and DG, which are likely to play important roles in the differentiation process. DG production increases PKC activity which activates AP1 factors. Microinjection of PLC-γ1 antibody into fibroblasts was reported to block platelet-derived growth factor induction of c-fos, a transcriptional factor binding to AP1 sites (32). Our laboratory has identified the calcium-responsive element in the involucrin gene and shown that it contains an AP1 site that is critical for its function (33). Our laboratory has also shown that transglutaminase promoter constructs containing AP1 sites (34) when transfected into human keratinocytes were responsive to calcium although we have not yet precisely localized the calcium-responsive element.2 IP_{3}, on the other hand, increases the release of calcium from intracellular stores which can also stimulate PKC activity. Previous studies have shown that calcium-induced keratinocyte differentiation requires an increase in intracellular calcium (35–39). Our study determined that PLC-γ1 is required for the increase in intracellular calcium mobilization following the calcium switch. Thus, calcium requires PLC-γ1 to induce differentiation in keratinocytes, perhaps because of the ability of PLC-γ1 to generate the second messengers, IP_{3}, DG, and calcium, which trigger the differentiation response.

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