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The complex formation of PKCδ through its C1- and C2-like regions in H_2O_2-stimulated cells ☆

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Abstract

PKCδ was revealed to make a homologous protein complex that shows a high protein kinase activity upon H_2O_2 stimulation by expressing the enzymes having different epitope tags in COS-7 cells. The association of the endogenous PKCδ in the cells was observed by sucrose density gradients. Analysis using the mutant replacing the tyrosine phosphorylation sites showed that PKCδ is activated without tyrosine phosphorylation in the stimulated cells, and the time course of the activation was parallel with that of the complex formation. The binding sites were identified as the C1 and C2-like regions in the regulatory domain using a series of deletion mutants. The binding between the C1 and C2-like region fragments was induced by cell stimulation, whereas the association of the C1 region fragments by itself and that of the C2-like region fragments were observed even without stimulation. These results suggest that the protein complexes of PKCδ through the association between the C1 and C2-like regions by different combinations are generated in the H_2O_2-treated cells, that may show an enhanced protein kinase activity.

Keywords: PKCδ; Tyrosine phosphorylation; Complex formation; Hydrogen peroxide; Regulatory domain; C1 region; C2-like region

The protein kinase C (PKC) family is involved in the signal transduction pathways that control cell growth, differentiation, death, and stress responsiveness [1–3], and nine PKC genes are identified in human genome [4]. PKC isoforms have the regulatory domain and the serine/threonine protein kinase domain in the amino- and carboxyl-terminal halves, respectively, and are divided into three groups, cPKC, nPKC, and aPKC, based on the structural differences in their regulatory domains. The cPKC isoforms have the pseudosubstrate sequence, the C1 region containing a tandem repeat of cysteine-rich zinc finger-like sequences named C1A and C1B, and the C2 region that binds to membrane phospholipids in a Ca^{2+}-dependent manner in the regulatory domain in order from the amino- to carboxyl-terminal ends. The regulatory domain of nPKC isoforms lacks the C2 region but has the C2-like region at the amino-terminal end portion as well as the pseudosubstrate sequence and the C1 region, while the aPKC isoforms contain only the pseudosubstrate sequence and a single cysteine-rich zinc finger-like sequence in the regulatory domain. The cPKC and nPKC isoforms are activated by diacylglycerol derived from receptor-mediated hydrolysis of inositol phospholipids and are the prime targets of tumor-promoting phorbol esters, that bind to the C1 region in the regulatory domain.

On the other hand, protein kinases are generally phosphorylated, and some of them are known to be controlled by phosphorylation [5]. The PKC family members have three phosphorylation motif sites mostly conserved among the family [6,7]: a threonine residue in the activation loop of the kinase domain, and serine and threonine residues located in the carboxyl-terminal end region named the turn and hydrophobic motifs, respectively. These motif sites are constitutively phosphorylated in most of the PKC family

☆ *Abbreviations:* DO, dioleoylglycerol; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; PKC, protein kinase C; PtdSer, phosphatidylserine.

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members, and thus the modification of these residues is regarded to be a prerequisite for maturation of PKC to exhibit a protein kinase activity in the receptor-coupled signaling pathway. PKC isoforms are phosphorylated additionally at tyrosine upon stimulation of the cells, and the role of tyrosine phosphorylation has been investigated for PKCδ, a member of the nPKC group [8,9]. Thus far, Tyr52, Tyr155, Tyr187, Tyr311, Tyr332, Tyr512, and Tyr565 have been identified or proposed as the phosphorylation sites of PKCδ. It has been shown that phosphorylation at Tyr311, Tyr332, and Tyr512 is induced in the H_2O_2-stimulated cells in which a diacylglycerol-independent active form of PKCδ is generated [10,11]. Among the three residues, Tyr311 and Tyr332 are the major sites and Tyr512 is a minor site. Furthermore, in vitro phosphorylation at Tyr311 enhances the basal protein kinase activity of PKCδ in the absence of diacylglycerol, and then it has been regarded that tyrosine phosphorylation is involved in the generation of the active PKCδ [11]. To date, tyrosine phosphorylation of PKCδ has been observed in various cells, and the active enzyme is suggested to play a role in the cell regulation such as growth and apoptosis [8,9].

Subsequent analysis revealed, however, that active PKCδ is generated even without tyrosine phosphorylation, and that this enzyme makes a protein complex in the H_2O_2-stimulated cells, suggesting that the active PKCδ is produced by distinct mechanisms. Here, we report the comparison of the wild type and the mutant replacing the tyrosine phosphorylation sites as well as the analysis of the PKCδ protein complex generated in the stimulated cells.

Materials and methods

Expression plasmids. FLAG- and hemagglutinin (HA)-epitope-tagged expression plasmids of rat PKCδ (amino acids 1–673) were constructed in pcDNA3 as described previously [10,11], and the mutant fragment named Y311/332/512F replacing Tyr311, Tyr332, and Tyr512 of PKCδ with phenylalanine [11] was cloned into the epitope-tagged plasmids. The protein products were designated as FLAG-PKCδ, HA-PKCδ, FLAG-Y311/332/512F, and HA-Y311/332/512F, respectively. The expression plasmid of the green fluorescent protein (GFP)-fusion protein of PKCδ in pTB701 (GFP-PKCδ) was prepared as described [12]. The fragments encoding the regulatory domain (amino acids 1–286), the kinase domain (amino acids 287–673), the C2-like region (amino acids 1–141), and the C1 region (amino acids 156–286) were amplified by PCR to make GFP-fused deletion mutant molecules designated GFP-RD, GFP-KD, GFP-C2-like, and GFP-C1, respectively. The expression plasmids of the glutathione *S*-transferase (GST)-fusion proteins of PKCδ, the C2-like region, the pseudosubstrate sequence (amino acids 142–155), the C1 region, the C1A region (amino acids 156–226), and the C1B region (amino acids 227–285) were constructed using pEBG [13], and designated as GST-PKCδ, GST-C2-like, GST-PS, GST-C1, GST-C1A, and GST-C1B, respectively. The DNA sequences of these constructs were confirmed by the dideoxy chain-termination method with a DNA sequencing system Model 3100 Avant (Applied Biosystems).

Cell culture and transfection. COS-7 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 incubator. NIH3T3 cells were cultured in the presence of calf serum. The cells were transfected by the lipofection method using lipofectamine (Invitrogen) according to the manufacturer's protocol and cultured in the medium for 24 h. The cells were further cultured in the serum-free medium containing 20 mM Hepes at pH 7.4 and 0.1% bovine serum albumin for 24 h, and then stimulated by 10 mM H_2O_2 for 10 min unless otherwise indicated. Where indicated, the cells were treated with 200 μM of genistein for 10 min before stimulation.

Immunoprecipitation and affinity purification. The following procedures were carried out at 0–4 °C essentially as described [10,14]. Briefly, the cells were washed with phosphate-buffered saline and lysed in 20 mM Tris–HCl at pH 7.5 containing 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4, 1.5 μg/ml aprotinin, and 50 μg/ml phenylmethylsulfonyl fluoride. The lysates were centrifuged for 10 min at 18,000*g*, and the supernatant (500–600 μg of protein) was incubated for 1 h with an anti-FLAG monoclonal antibody (M2, Sigma), an anti-HA monoclonal antibody (3F10, Roche), or an anti-GFP antibody (Molecular Probes). Then, rProtein A-Sepharose Fast Flow (Amersham Bioscience) was added to the mixture and incubated for 30 min. For the experiments of GST-fusion proteins, the supernatant was incubated with glutathione–Sepharose 4B (Amersham Bioscience) for 1 h. The precipitates were collected by centrifugation and washed with 20 mM Tris–HCl at pH 7.5 containing 150 mM NaCl and 1% Triton X-100.

Immunoblot analysis. The samples were boiled in SDS-sample buffer, and proteins were separated by SDS–PAGE and transferred to an Immobilon P membrane (Millipore). Immunoblot analysis was carried out with the use of an anti-FLAG antibody (M5, Sigma), the anti-HA antibody, the anti-GFP antibody, an anti-GST antibody (Z-5, Santa Cruz), a monoclonal anti-phosphotyrosine (pY) antibody (4G10, Upstate Biotechnology), an anti-PKCδ antibody (C-20, Santa Cruz), or antibodies directed against phosphorylated Thr505, Ser643, and Ser662 of PKCδ (pT505, pS643, and pS662, respectively) (Cell Signaling). The alkaline phosphatase-conjugated anti-mouse, anti-rabbit, or anti-rat antibody (Chemicon) was used as the secondary antibody. The color reaction was carried out with the use of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates. Prestained protein markers (broad range, New England Biolabs) were employed as molecular size markers.

Protein kinase assay. The PKC activity was assayed by measuring the incorporation of ^{32}Pi into calf thymus H1 histone from [γ-^{32}P]ATP in the reaction mixture containing 20 mM Tris–HCl at pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, 15–50 kBq of [γ-^{32}P]ATP, and 200 μg/ml H1 histone [10]. The incubation was carried out for 5 min at 30 °C in the presence or absence of 8 μg/ml phosphatidylserine (PtdSer), 0.8 μg/ml dioleoylglycerol (DO), and the radioactivity was quantitated by a liquid scintillation counter Model LS6500 (Beckman).

Sucrose density gradient analysis. The cell extracts (1.5 ml) were loaded onto linear sucrose gradients (5–25%) in 20 mM Tris–HCl at pH 7.5 containing 1 mM EDTA, 1% Triton X-100, and 150 mM NaCl prepared in 25 × 89-mm centrifugation tubes (Beckman) made with a Gradient Master (BioComp Instruments) and were immediately subjected to centrifugation at 28,000 rpm for 24 h at 4 °C with an SW28 rotor (Beckman). The gradients were collected in 28 serial fractions from the top of the gradients using a Piston Gradient Fractionator (BioComp Instruments), and aliquots of each fraction were subjected to SDS–PAGE followed by immunoblot analysis. Marker proteins (Amersham Bioscience) such as transferrin (80 kDa), aldolase (158 kDa), and catalase (232 kDa) were subjected to sucrose gradients under the conditions described above as the marker proteins and detected by Coomassie staining after SDS–PAGE.

Results

The generation of the active form of PKCδ was studied using COS-7 cells expressing the wild type enzyme and the mutant Y311/332/512F replacing the three tyrosine residues phosphorylated in the stimulated cells (Fig. 1). As previously reported [10,11], the wild type PKCδ isolated from the H_2O_2-stimulated cells had a protein kinase activity almost independent of diacylglycerol, whereas the enzyme obtained from the control cells was activated by