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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

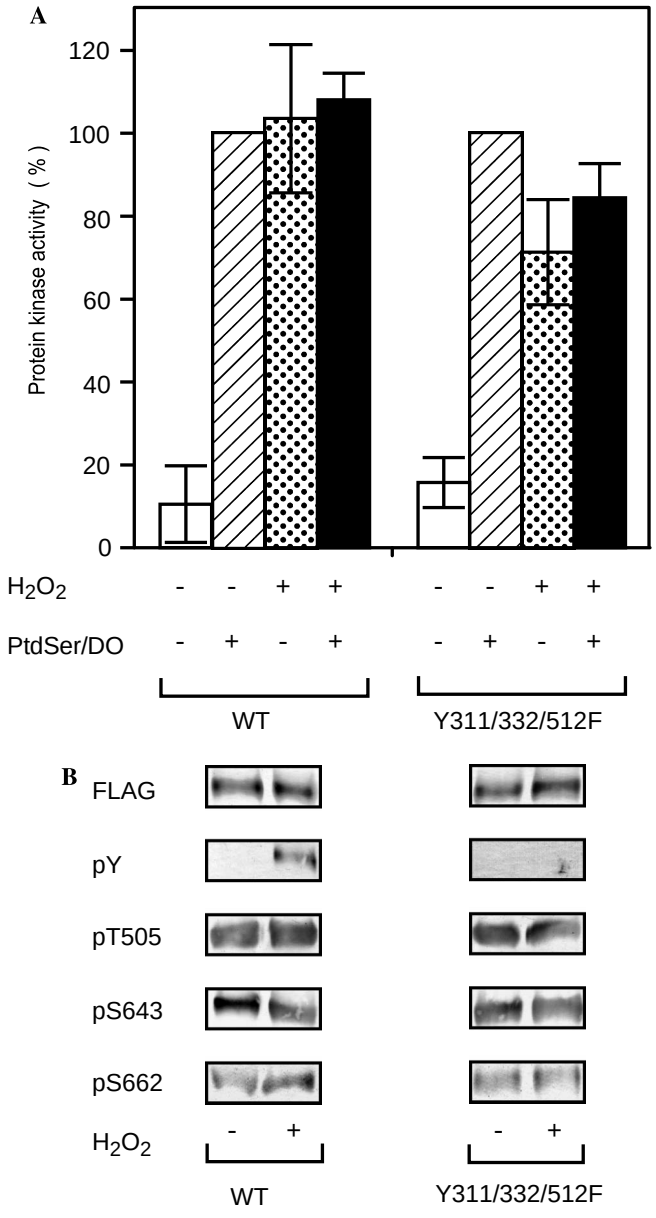

Fig. 1. Activation and phosphorylation of PKCδ and Y311/332/512F mutant. Cells transfected with FLAG-PKCδ (WT) or FLAG-Y311/332/512F (Y311/332/512F) were stimulated by H_2O_2, and the epitope-tagged proteins were immunoprecipitated by the anti-FLAG antibody. The protein kinase activity was measured in the presence or absence of PtdSer and DO (A), and immunoblot analysis was carried out using the antibodies as indicated on the left of each panel (B). The protein kinase activity recovered from the unstimulated cell in the presence of PtdSer and DO is shown as 100%. The data show means ± SD of three independent experiments.

diacylglycerol (Fig. 1A). The active wild type enzyme accompanied tyrosine phosphorylation, but the modification was not detected in the Y311/332/512F mutant in H_2O_2-stimulated cells as judged by immunoblot analysis using the phosphotyrosine-specific antibody (Fig. 1B). The comparison of the Y311/332/512F mutant and the wild type enzyme indicated that they showed apparently the same catalytic activities, i.e., the mutant protein obtained from the control cells was dependent on, and that isolated from the stimulated cells was, rather unexpectedly, independent of diacylglycerol (Fig. 1A). As the Y311/332/512F mutant was recovered as an active form without tyrosine phosphorylation, the modification of other residues, i.e., at the phosphorylation motif sites [6,7], was analyzed by using the phospho-specific antibodies (Fig. 1B). In the wild type PKCδ, phosphorylation at Thr505, Ser643, and Ser662, in the activation loop, the turn motif, and the hydrophobic motif, respectively, was detected comparably in both of the resting and H_2O_2-stimulated cells as previously reported [11]. Similar results were obtained for the Y311/332/512F mutant: the mutant is phosphorylated at these three motif sites and the H_2O_2 stimulation did not change the phosphorylation at the motif sites. These results indicate that the active form of PKCδ can be generated in the H_2O_2-stimulated cells by some mechanism(s) distinct from the phosphorylation reaction at tyrosine, serine, and threonine.

Further analysis revealed the association between PKCδ molecules in the H_2O_2-stimulated cells (Fig. 2). When the cells expressing FLAG-PKCδ and HA-PKCδ, the wild type enzymes with different epitope tags, were stimulated, FLAG-PKCδ was detected in the anti-HA antibody immunoprecipitates and HA-PKCδ was found in the immunoprecipitates by the anti-FLAG antibody (Fig. 2, left panels). Genistein, a potent tyrosine kinase inhibitor, was employed to examine the relationship between the association and tyrosine phosphorylation of PKCδ. The enzymes isolated from the H_2O_2-stimulated cells were significantly phosphorylated at tyrosine (Fig. 2, left panels), and the pretreatment of the cells with genistein suppressed considerably the tyrosine phosphorylation of the enzymes, but did not affect the association between FLAG-PKCδ and HA-PKCδ (Fig. 2, middle panels). H_2O_2-induced activation of the wild type PKCδ was observed in the cells pretreated with genistein (data not shown). The H_2O_2-induced association was also observed between the Y311/332/512F mutants having FLAG- and HA-epitope tags (Fig. 2, right

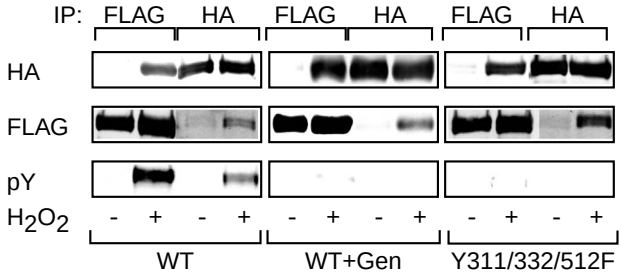

Fig. 2. Co-immunoprecipitation of PKCδ. Cells transfected with FLAG-PKCδ and HA-PKCδ (WT) or FLAG-Y311/332/512F and HA-Y311/332/512F (Y311/332/512F) were stimulated by H_2O_2, and the epitope-tagged proteins were immunoprecipitated (IP) by either the anti-FLAG or anti-HA antibody. Immunoblot analysis was carried out using the antibodies as indicated on the left of each panel. Where indicated, the cells transfected with FLAG-PKCδ and HA-PKCδ were treated with genistein (Gen) before stimulation. The results are representative of three reproducible experiments.

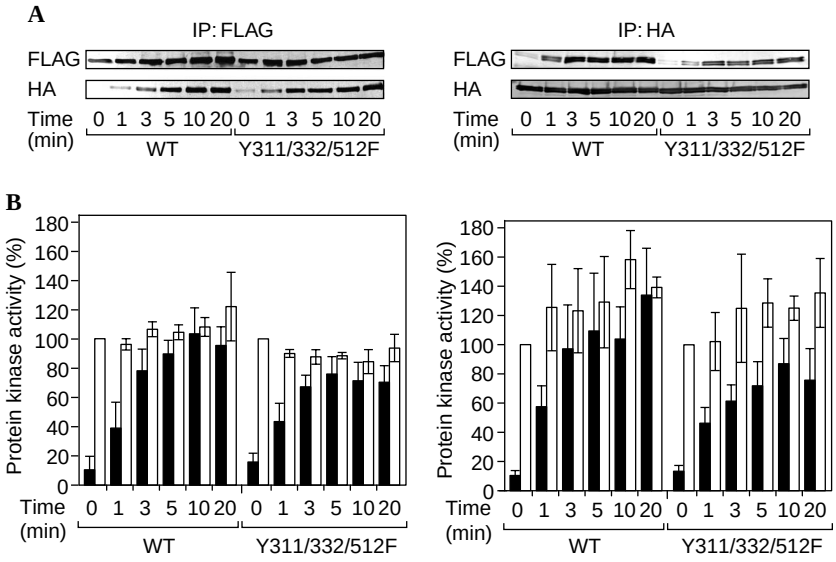

Fig. 3. Time-dependent association and activation of PKCδ. (A) Cells transfected with FLAG-PKCδ and HA-PKCδ (WT) or FLAG-Y311/332/512F and HA-Y311/332/512F (Y311/332/512F) were stimulated by H_2O_2 for indicated time. The proteins immunoprecipitated (IP) by the anti-FLAG antibody (left panels) and by the anti-HA antibody (right panels) were subjected to immunoblot analysis using the antibodies as indicated on the left of each panel. The results are representative of three reproducible experiments. (B) Cells transfected with FLAG-PKCδ or Y311/332/512F (left panel) or HA-PKCδ or Y311/332/512F (right panel) were stimulated by H_2O_2 for indicated time. The epitope-tagged protein was immunoprecipitated by the anti-FLAG and anti-HA antibodies, respectively, and subjected to protein kinase assay in the presence (white bar) or absence (black bar) of PtdSer and DO. The protein kinase activity recovered from the unstimulated cell in the presence of PtdSer and DO is shown as 100%. The data show means ± SD of three independent experiments.

panels). These results indicate that the association between PKCδ molecules is provoked in the H_2O_2-stimulated cells in a manner independent of tyrosine phosphorylation of the enzyme.

The relationship of the association of PKCδ with the generation of the active form was studied by observing the time courses (Fig. 3). The cells expressing both FLAG-PKCδ and HA-PKCδ were stimulated by H_2O_2 for indicated time, and HA-PKCδ associated with FLAG-PKCδ and vice versa FLAG-PKCδ coupled with HA-PKCδ were detected (Fig. 3A). The association was found 1 min after the stimulation and reached the maximal level within 10 min. The generation of the active form was monitored by measuring the protein kinase activity of the immunoprecipitates by the anti-FLAG or anti-HA antibody from the cells expressing each epitope-tagged PKCδ (Fig. 3B). The diacylglycerol-independent protein kinase activity increased in a time-dependent manner analogous to that of the association. Similar results were obtained for the association of the Y311/332/512F mutants (Fig. 3A) and the generation of the active mutant enzyme (Fig. 3B).

PKCδ endogenously expressed in the cells was next studied by subjecting the cell extracts to sucrose density gradient analysis (Fig. 4). PKCδ obtained from the control cells appeared broadly in the fractions 5–12 of the density gradient. The most abundant fractions 7 and 8 corresponded to the position of the 80-kDa marker protein. As the deduced molecular mass of PKCδ is 76 kDa [15], the major part of the endogenous PKCδ seems to exist as a monomer

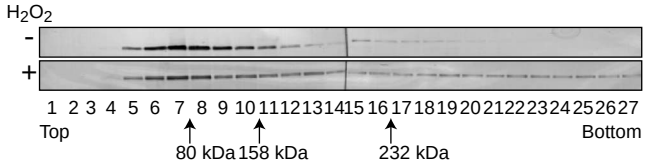

Fig. 4. Sucrose density gradient analysis of the endogenously expressed PKCδ. Cell lysates prepared from the cells stimulated by H_2O_2 (+) and the control cells (−) were subjected to sucrose density gradients, and aliquots of the serial fractions from the top of the gradients were subjected to immunoblot analysis using the anti-PKCδ antibody. Arrows indicate the position of the marker proteins. The results are representative of three reproducible experiments.

in the control cells. Although PKCδ from the H_2O_2-stimulated cells was still rich in the fractions 7 and 8, the enzyme shifted to the high-density fractions. No prominent peak was observed in the high molecular weight fractions, but PKCδ was detected in the fractions 10 and 11 corresponding to the position of the 158-kDa marker protein, that is close to the calculated molecular size of a dimeric PKCδ, 152 kDa, and also the enzyme appeared widely in the bottom-side fractions. It is difficult to analyze the nature of the enzyme appearing in the high molecular weight fractions, but the endogenous PKCδ seems to make different protein complexes upon stimulation with H_2O_2.

Based on the results of the endogenous as well as recombinant proteins, it is most plausible that the protein complex of PKCδ includes the oligomeric forms of the enzyme. Thus, the interacting site between the PKCδ molecules was analyzed by using a series of deletion mutants

(Fig. 5A). When the cells co-expressing FLAG-PKCδ with each GFP-fusion protein were stimulated, FLAG-PKCδ was detected in the anti-GFP antibody immunoprecipitates obtained from the cells expressing the full-length molecule and the regulatory domain, but was not found in that from the extract prepared from the cells expressing the kinase domain (Fig. 5B). Therefore, the kinase domain is not included, but the regulatory domain is responsible for the association of PKCδ. The interacting site was further analyzed expressing the GST-fusion proteins of the different regions of the regulatory domain in the cells (Fig. 5C). The binding of FLAG-PKCδ was observed with the fragments of the C1 and C2-like regions but not that of the pseudosubstrate sequence purified using glutathione–Sepharose. The results indicate that the C1 and C2-like regions can bind to the whole PKCδ molecule, independently. Then, the interaction between these two regions was studied. The binding of GFP-C2-like to GST-C1 was induced by the stimulation (Fig. 5D, upper half) and consistently the binding of GFP-C1 to GST-C2-like was provoked by the cell stimulation. In addition, the binding of GFP-C1 to GST-C1 was found in the cells expressing these two fusion proteins even without stimulation, and the association was enhanced by the H_2O_2 treatment (Fig. 5D, upper half). The constitutive binding of GFP-C2-like to GST-C2-like was also observed in the resting cells, but was not increased efficiently by the H_2O_2 treatment (Fig. 5D, lower half). The results indicate that the binding between these two regions is induced by the H_2O_2 stimulation, and that each of the C1 and C2-like fragments can make a complex by itself in the cells.

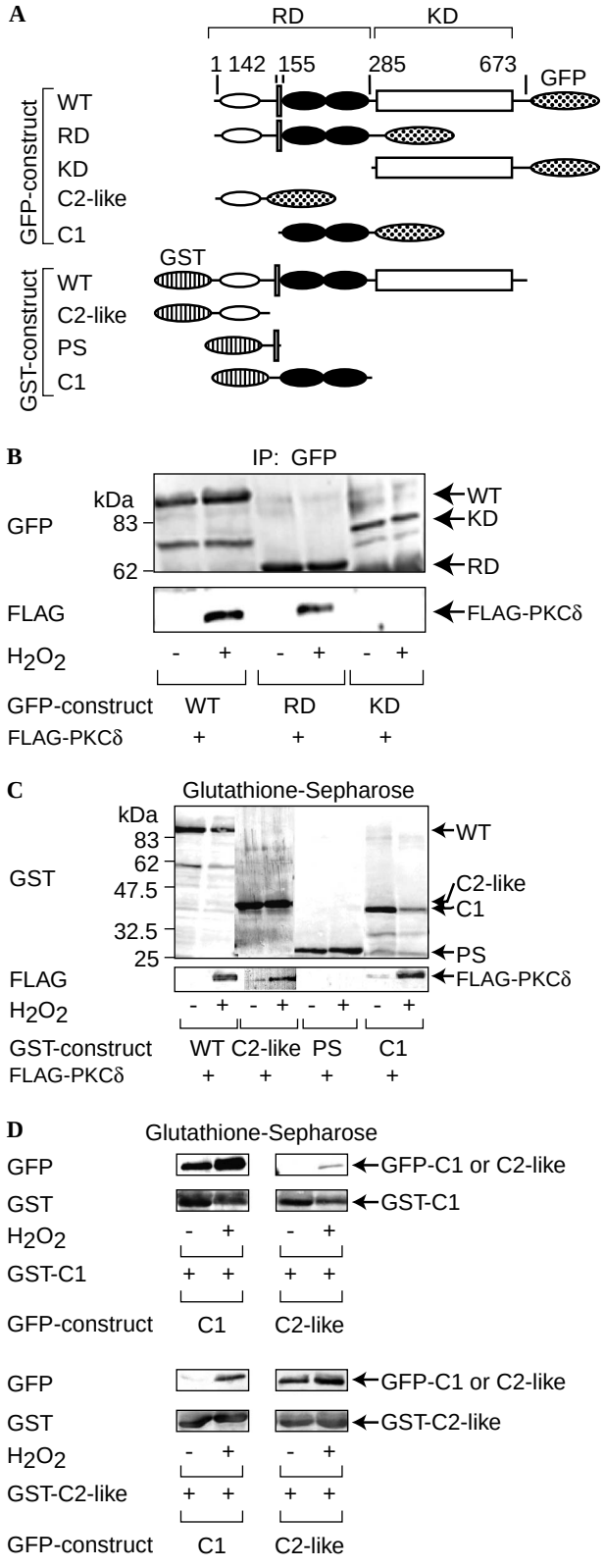

Fig. 5. Binding sites in PKCδ. (A) Schematic presentation of the GFP- and GST-fusion proteins employed. Amino acid residue numbers are indicated at the top. (B) Cells transfected with FLAG-PKCδ in the company of either GFP-PKCδ (WT), GFP-RD, or GFP-KD were stimulated by H_2O_2, and the GFP-fusion proteins were immunoprecipitated (IP) by the anti-GFP antibody. (C) Cells transfected with FLAG-PKCδ in the company of either GST-PKCδ (WT), GST-C2-like, GST-PS, or GST-C1 were stimulated by H_2O_2, and the GST-fusion proteins were purified using glutathione–Sepharose. (D) Cells transfected with GST-C1 (upper half) and GST-C2-like (lower half) in the company of either GFP-C1 or GFP-C2-like were stimulated by H_2O_2, and the GST-fusion proteins were purified using glutathione–Sepharose. Immunoblot analysis was carried out by using the antibodies as indicated on the left of each panel (B–D). The positions of the marker proteins are indicated in kDa (B,C). The results are representative of three reproducible experiments.

Discussion

Although the three-dimensional structure of the whole PKCδ molecule is not yet available, it is assumed that PKCδ holds an inactive conformation under the resting conditions, in which the catalytic activity of the kinase domain is suppressed by the interaction with the regulatory domain. The phosphorylation at the three motif sites constitutively modified seems to be a prerequisite for the catalytic activity. The binding of diacylglycerol and phorbol ester to the regulatory domain may bring about a structural change in the enzyme to gain access to the substrate proteins. Consistent with this model, an active catalytic fragment is generated by the caspase-mediated cleavage

of PKCδ at specific caspase-recognition site in the hinge region between the regulatory and kinase domains [16]. On the other hand, PKCδ recovered from the H_2O_2-stimulated cells is constitutively active accompanying tyrosine phosphorylation at Tyr311, Tyr332, and Tyr512 [11]. As PKCδ phosphorylated at Tyr311 in vitro showed an enhanced enzymatic activity, tyrosine phosphorylation in the hinge region at Tyr311 as well as Tyr332 is regarded to be involved in the generation of the active enzyme, presumably introducing a conformational change in PKCδ.

During studies on the role of tyrosine phosphorylation, we found that the mutant Y311/332/512F replacing these three tyrosine residues is recovered as an active form as the wild type PKCδ from the H_2O_2-stimulated COS-7 cells. Similar results were observed in NIH3T3 and HEK293 cells (data not shown). The results do not necessarily eliminate the involvement of tyrosine phosphorylation in the regulation of PKCδ, but strongly suggest that this protein kinase is activated by another mechanism independent of tyrosine phosphorylation in the H_2O_2-stimulated cells. Opposing effects, activation and inhibition, have been proposed to occur on PKC in the cells exposed to oxidative stress by measuring the protein kinase activity in the crude supernatant and particulate fractions prepared from the cells [17,18]. It was thus hypothesized to explain these contradictory results that the oxidative modification in the regulatory domain stimulates PKC, whereas oxidation of the cysteine residues in the kinase domain decreases the kinase activity [2]. In this study, the association of PKCδ molecules with different epitope tags was found in the H_2O_2-stimulated cells and the same results were obtained using the mutant replacing the three tyrosine residues as well as in the cells pretreated with the tyrosine kinase inhibitor. Importantly, the endogenous PKCδ was recovered mostly as a monomeric form from the resting cells, and shifted to high molecular weight fractions upon stimulation with H_2O_2 on sucrose gradients. When the cells co-expressing FLAG-PKCδ and GST-PKCδ were stimulated by H_2O_2 and each protein was purified by respective affinity column chromatography, FLAG-PKCδ and GST-PKCδ were stained but no major co-purified protein band was observed by silver staining of SDS–PAGE gels (data not shown). Taken together, PKCδ makes protein complexes in the H_2O_2-stimulated cells, which are plausibly composed of the PKCδ oligomers. As the time course for the complex formation well agrees with that of generation of the diacylglycerol-independent active enzyme, it is reasonable to suppose that the complex formation is an integral part for the production of the active PKCδ.

Concerning the mechanism for the association, it was rather unexpected but the C1 and C2-like regions in the regulatory domain were identified for the binding sites. The C1 region contains a tandem repeat of cysteine-rich zinc finger-like sequences named C1A and C1B, that are the recognition sites for diacylglycerol and phorbol ester [1,3], and each cysteine-rich sequence was sufficient for the binding with the full-length PKCδ (data not shown). The crystal structure of the C1B region of PKCδ is determined, which has a pocket between two pulled-apart β sheets at the tip of the region for the binding with phorbol ester [19]. The structure model of the C1B region is accommodated to the two-dimensional crystal structure of the regulatory domain of PKCδ [20], suggesting that the region is located on the surface of the whole molecule. On the other hand, the C2 region is found in various proteins, that bind to membrane phospholipids in a Ca^{2+}-dependent manner [21]. The crystal structure of the C2-like region of PKCδ has the C2 fold but lacks sequences required for Ca^{2+} coordination [22] and is shown to serve as protein–protein interaction domain [23]. Recently, the C2-like region of PKCδ has been proposed to be a novel phosphotyrosine-binding domain [24], but the association observed in this study was observed in the proteins without tyrosine phosphorylation.

In this study, the binding between the fragments of the C1 and C2-like regions was detected only in the H_2O_2-treated, but not in unstimulated cells. In contrast, the binding of GFP-C1 with GST-C1 was found even in the unstimulated cells and was enhanced by the H_2O_2 stimulation. The binding ability of the C1 region is, however, hidden in the whole PKCδ molecule, because the full-length molecule as well as the regulatory domain do not show any binding activity in the unstimulated cells. The binding ability of the C1 region may be uncovered by the conformational change of PKCδ in the H_2O_2-treated cells. In addition, it has been reported that H_2O_2 induces a structural change in the C1 region to release zinc from the cysteine-rich zinc finger-like sequence [25], and thus it seems to be possible that such a structural alteration triggers the association and enhances the binding ability of the C1 region in stimulated cells. The association between GFP-C2-like and GST-C2-like was also found in the resting cells, but was not enhanced by cell stimulation. It is plausible that the binding ability of the C2-like region hidden in the intact cells is also uncovered upon H_2O_2 stimulation. These results support the possibility that the C1 region is modified in the stimulated cells, and it is interesting to assume that the binding activity of the C1 and C2-like regions is uncovered by the modification of the C1 region and the conformational alteration of PKCδ induced by other mechanisms to make the protein complexes in the H_2O_2-treated cells. The binding between different combinations of these regions, C1 to C1, C2-like to C2-like, and C1 to C2-like among the PKCδ molecules, may produce the complexes recovered in diverse high molecular weight fractions of sucrose gradients. Presumably, PKCδ in the protein complexes shows a diacylglycerol-independent activity, but further studies are required to clarify the precise composition of the protein complexes and the relationship with tyrosine phosphorylation. In PKCδ-null mice, it was found that vascular smooth muscle cells of the vein grafts transplanted in carotid arteries proliferate unlimitedly to make arteriosclerotic region [26]. The results of mice deficient in PKCδ suggest that the enzyme activated by

oxidative stress under the high oxygen concentrations in arteries plays a role to prevent abnormal growth of smooth muscle cells presumably inducing apoptosis. It is attractive to assume that PKCδ activated by the complex formation and/or tyrosine phosphorylation contributes to the maintenance of homeostasis of the cells. On the other hand, PKCδ phosphorylated on Tyr332 is shown to bind the Src homology 2 domain of the adaptor protein Shc to attenuate the antigen-induced degranulation from mast cells [27]. The expression of the Y311/332/512F mutant in the COS-7 cells, however, did not show apparent effect on their shape and growth. It is necessary to study further the role of the tyrosine phosphorylation especially on Tyr332 of PKCδ in the specific cellular functions.

The PKC family members have been shown to contribute various signal transduction pathways. Studies of the roles of PKCδ and other family members regulated by the multiple activation mechanisms will be important to understand signaling processes under the normal and pathological conditions.

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References

[1] Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular responses, FASEB J. 9 (1995) 484–496.
[2] R. Gopalakrishna, S. Jaken, Protein kinase C signaling and oxidative stress, Free Radic. Biol. Med. 28 (2000) 1349–1361.
[3] S. Ohno, Y. Nishizuka, Protein kinase C isotypes and their specific functions: prologue, J. Biochem. 132 (2002) 509–511.
[4] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human genome, Science 298 (2002) 1912–1934.
[5] L.N. Johnson, M.E. Noble, D.J. Owen, Active and inactive protein kinases: structural basis for regulation, Cell 85 (1996) 149–158.
[6] D.B. Parekh, W. Ziegler, P.J. Parker, Multiple pathways control protein kinase C phosphorylation, EMBO J. 19 (2000) 496–503.
[7] A.C. Newton, Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm, Biochem. J. 370 (2003) 361–371.
[8] U. Kikkawa, H. Matsuzaki, T. Yamamoto, Protein kinase Cδ (PKCδ): activation mechanisms and functions, J. Biochem. 132 (2002) 831–839.
[9] S.F. Steinberg, Distinctive activation mechanisms and functions for protein kinase Cδ, Biochem. J. 384 (2004) 449–459.
[10] H. Konishi, M. Tanaka, Y. Takemura, H. Matsuzaki, Y. Ono, U. Kikkawa, Y. Nishizuka, Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2, Proc. Natl. Acad. Sci. USA 94 (1997) 11233–11237.
[11] H. Konishi, E. Yamauchi, H. Taniguchi, T. Yamamoto, H. Matsuzaki, Y. Takemura, K. Ohmae, U. Kikkawa, Y. Nishizuka, Phosphorylation sites of protein kinase C δ in H_2O_2-treated cells and its activation by tyrosine kinase in vitro, Proc. Natl. Acad. Sci. USA 98 (2001) 6587–6592.
[12] S. Ohmori, Y. Shirai, N. Sakai, M. Fujii, H. Konishi, U. Kikkawa, N. Saito, Three distinct mechanisms for translocation and activation of the δ subspecies of protein kinase C, Mol. Cell. Biol. 18 (1998) 5263–5271.
[13] M. Tanaka, R. Gupta, B.J. Mayer, Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins, Mol. Cell. Biol. 15 (1995) 6829–6837.
[14] H. Konishi, H. Matsuzaki, H. Takaishi, T. Yamamoto, M. Fukunaga, Y. Ono, U. Kikkawa, Opposing effects of protein kinase C δ and protein kinase B α on H_2O_2-induced apoptosis in CHO cells, Biochem. Biophys. Res. Commun. 264 (1999) 840–846.
[15] Y. Ono, T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, Y. Nishizuka, The structure, expression, and properties of additional members of the protein kinase C family, J. Biol. Chem. 263 (1988) 6927–6932.
[16] Y. Emoto, Y. Manome, G. Meinhardt, H. Kisaki, S. Kharbanda, M. Robertson, T. Ghayur, W.W. Wong, R. Kamen, R. Weichselbaum, D. Kufe, Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells, EMBO J. 14 (1995) 6148–6156.
[17] R. Gopalakrishna, W.B. Anderson, Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain, Proc. Natl. Acad. Sci. USA 86 (1989) 6758–6762.
[18] R. Gopalakrishna, W.B. Anderson, Reversible oxidative activation and inactivation of protein kinase C by the mitogen/tumor promoter periodate, Arch. Biochem. Biophys. 285 (1991) 382–387.
[19] G. Zhang, M.G. Kazanietz, P.M. Blumberg, J.H. Hurley, Crystal structure of the cys2 activator-binding domain of protein kinase Cδ in complex with phorbol ester, Cell 81 (1995) 917–924.
[20] A.S. Solodukhin, H.L. Caldwell, J.J. Sando, R.H. Kretsinger, Two-dimensional crystal structures of protein kinase C-δ, its regulatory domain, and the enzyme complexed with myelin basic protein, Biophys. J. 82 (2002) 2700–2708.
[21] E.A. Nalefski, J.J. Falke, The C2 domain calcium-binding motif: structural and functional diversity, Protein Sci. 5 (1996) 2375–2390.
[22] H. Pappa, J. Murray-Rust, L.V. Dekker, P.J. Parker, N.Q. McDonald, Crystal structure of the C2 domain from protein kinase C-δ, Structure 6 (1998) 885–894.
[23] L.V. Dekker, P.J. Parker, Regulated binding of the protein kinase C substrate GAP-43 to the V0/C2 region of protein kinase C-δ, J. Biol. Chem. 272 (1997) 12747–12753.
[24] C.H. Benes, N. Wu, A.E. Elia, T. Dharia, L.C. Cantley, S.P. Soltoff, The C2 domain of PKCδ is a phosphotyrosine binding domain, Cell 121 (2005) 158–160.
[25] I. Korichneva, B. Hoyos, R. Chua, E. Levi, U. Hammerling, Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen, J. Biol. Chem. 277 (2002) 44327–44331.
[26] M. Leitges, M. Mayr, U. Braun, U. Mayr, C. Li, G. Pfister, N. Ghaffari-Tabrizi, G. Baier, Y. Hu, Q. Xu, Exacerbated vein graft arteriosclerosis in protein kinase Cδ-null mice, J. Clin. Invest. 108 (2001) 1505–1512.
[27] M. Leitges, K. Gimborn, W. Elis, J. Kalesnikoff, M.R. Hughes, G. Krystal, M. Huber, Protein kinase C-δ is a negative regulator of antigen-induced mast cell degranulation, Mol. Cell. Biol. 22 (2002) 3970–3980.