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Characterization of Tyrosine-Phosphorylated δ Isoform of Protein Kinase C Isolated from Chinese Hamster Ovary Cells[1]

Makoto Kadotani,* Teruaki Nishiuma,* Masakazu Nanahoshi,† Yosuke Tsujishita,† Kouji Ogita,* Shun-ichi Nakamura,* Ushio Kikkawa,†,[2] and Yoshinori Asaoka†

*Department of Biochemistry, Kobe University School of Medicine, Chuo-ku, Kobe, Hyogo 650; and †Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657

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Phorbol ester treatment of Chinese hamster ovary cells stably overexpressing the δ isoform of protein kinase C induced the association of the isoform with the particulate fraction and the tyrosine phosphorylation of a small portion of the δ isoform. The δ isoform without tyrosine phosphorylation was recovered as an enzyme dependent on phospholipid and diacylglycerol, whereas the tyrosine-phosphorylated δ isoform was recovered in two fractions, one dependent on, and the other independent of, phospholipid and diacylglycerol. The tyrosine-phosphorylated δ isoform independent of lipid activators might be associated with phorbol ester and phospholipids. Immunoblot analysis revealed that the δ isoform is a doublet protein of 76 and 78 kDa, and that the δ isoform fraction without tyrosine phosphorylation contained 76- and 78-kDa proteins, whereas the tyrosine-phosphorylated δ isoform contained the 78-kDa protein but not the 76-kDa protein. *In vitro* analysis showed that the 78-kDa protein of the δ isoform without tyrosine phosphorylation is an efficient substrate of tyrosine kinase only when phosphatidylserine and either diacylglycerol or phorbol ester are present; however, the 76-kDa protein can not be tyrosine-phosphorylated even in the presence of these lipid activators. The phospholipid and diacylglycerol-dependent form of the tyrosine-phosphorylated enzyme isolated from the cell line required lower concentrations of phosphatidylserine and phorbol ester for its activity *in vitro* as compared with the enzyme without tyrosine phosphorylation. These results suggest that the tyrosine-phosphorylated enzyme generated upon stimulation of the cells may associate with membranes and exert its full activity even with the lower concentrations of the lipid activators.

Key words: diacylglycerol, phorbol ester, phospholipid, protein kinase C, tyrosine phosphorylation.

Protein kinase C (PKC) comprises a large family with multiple isoforms classified into three subgroups, cPKC, nPKC, and aPKC, according to their domain structures, and the members of this enzyme family have been suggested to be activated in different ways to play distinct roles in the intracellular signaling network for the control of cellular functions (for a review, see Ref. *1*). The δ isoform, PKCδ, belongs to the nPKC subgroup that requires diacylglycerol for activation in the presence of phospholipid in a manner independent of calcium *in vitro* (*2-4*), and cells overproducing this isoform have been demonstrated to show a decreased rate of proliferation upon treatment with phorbol ester, suggesting a role of PKCδ in growth regulation (*5-8*). Despite extensive studies, the precise mechanism of activation of this isoform *in vivo* has not been fully understood (*9-15*). For example, biochemical studies have revealed that PKCδ in unstimulated cells and tissues appears as a doublet protein of 76 and 78 kDa upon SDS-PAGE (*3, 10, 11, 13, 14*), and that the 76-kDa protein shifts to the 78-kDa protein upon cell stimulation, probably by phosphorylation (*11, 13, 14*). In fact, we reported that the purified 78-kDa protein is converted to the 76-kDa protein by protein phosphatase 2A treatment, and that the autophosphorylation reaction *in vitro* does not shift the enzyme from 76 to 78 kDa (*10*), suggesting that PKCδ is phosphorylated by an undefined serine/threonine protein kinase upon stimulation of the cells. These two PKCδ proteins were indistinguishable from each other in their specific activities and response to diacylglycerol (*10*); however, the serine/threonine phosphorylation reaction of PKCδ has been suggested to be related to the regulation of the isoform (*13, 14*). On the other hand, signal-induced tyrosine phosphorylation of PKCδ has been reported using various cell types, and is accompanied with a change of

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[2] To whom correspondence should be addressed. Phone: +81-78-803-1254, Fax: +81-78-803-0994

Abbreviations: *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; CHO cell, Chinese hamster ovary cell; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

enzyme activity (*16-23*). Namely, Yuspa's group reported that the tyrosine phosphorylation of PKCδ in *v-ras*-transformed keratinocytes (*16*), and showed that this isoform of PKC is tyrosine-phosphorylated by mitogenic stimuli such as epidermal growth factor, transforming growth factor α, and phorbol ester in keratinocytes and BALB/c cells (*23*). The tyrosine phosphorylation of PKCδ is proposed to reduce its phorbol ester-dependent catalytic activity in these cells (*16, 23*). Pierce's group has indicated that the tyrosine phosphorylation of PKCδ is induced by phorbol ester in 32D myeloid progenitor cells and in NIH 3T3 cells that overproduce the enzyme (*17*), and by platelet-derived growth factor in cells transfected with PKCδ and the platelet-derived growth factor receptor (*18*). They suggested that the tyrosine phosphorylation of PKCδ enhances its catalytic activity (*17*). Furthermore, the tyrosine phosphorylation of the isoform has been detected in basophilic leukemia cells with immunoglobulin E receptor stimulation (*20*), salivary gland epithelial cells exposed to carbachol, substance P, and phorbol ester (*21*), and macrophages treated with phorbol ester (*22*). PKCδ has also been shown to be tyrosine-phosphorylated *in vitro*. The recombinant PKCδ produced by the baculovirus vector system is phosphorylated by tyrosine kinases such as Fyn, Src, insulin receptor, and platelet-derived growth factor receptor (*17, 23*), and PKCδ purified from pig spleen is phosphorylated by Src (*19*). However, the physiological significance of the tyrosine phosphorylation of PKCδ still remains unclear, because the tyrosine phosphorylation of the isoform is proposed to reduce its phorbol ester-dependent catalytic activity in some cells (*16, 23*), whereas the tyrosine phosphorylation appears to be positively correlated with enzyme activation in other cells (*17, 18*). Although the shift from 76 to 78 kDa protein of PKCδ seems to be important for the regulation of enzyme activity, the relation between the tyrosine phosphorylation and serine/threonine phosphorylation of the doublet protein of 76 and 78 kDa of PKCδ is not known, either. Thus, it is necessary to investigate the biochemical properties of the tyrosine-phosphorylated enzyme to elucidate the physiological role of the isoform. In the present report, we separated the tyrosine-phosphorylated and un-phosphorylated PKCδ from Chinese hamster ovary (CHO) cells stably expressing a massive amount of PKCδ, and studied the properties of the tyrosine phosphorylation reaction of the enzyme.

MATERIALS AND METHODS

Cell Lines—A CHO cell line stably overexpressing PKCδ, CHOδ-47, and a control *DHFR*$^+$ transformant, CHOd$^+$, were used (*10*). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 35 μg/ml of proline.

Purification of PKCδ—After cells reached confluency (10^5 cells/cm^2), the medium was replaced with 0.25% fetal calf serum in Dulbecco's modified Eagle's medium containing proline, and the cells were further incubated for 20 h. Cells were treated with PMA at the final concentration of 160 nM for 20 min unless otherwise indicated, rinsed twice with ice-cold phosphate-buffered saline, and scraped into the ice-cold extraction buffer containing 20 mM MES at pH 6.0, 0.5 mM EGTA, 15 mM β-glycerophosphate, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4, 100 μM *p*-APMSF, 100 μg/ml leupeptin, 5 μg/ml antipain, and 10 kU/ml aprotinin (5×10^7 cells/ml). The following procedures were carried out at 0-4°C. Cells were disrupted by sonication and centrifuged at 346,000$\times g$ for 15 min. The supernatant was used as the cytosol fraction. The precipitate was sonicated in the extraction buffer containing 1% Triton X-100 (10^8 cells/ml), then the sonicate was centrifuged at 346,000$\times g$ for 15 min, and the supernatant was employed as the particulate fraction. The cytosol fraction (8 ml from 4×10^8 cells) or the particulate fraction (4 ml from 4×10^8 cells) was applied to a HiTrap SP column (Pharmacia; 1.6×2.5 cm) equilibrated with Buffer A (20 mM MES at pH 6.0, 0.5 mM EGTA, 1 mM Na_3VO_4, 20 mM 2-mercaptoethanol, 100 μM *p*-APMSF, 10 μg/ml leupeptin, and 0.1% Triton X-100). After washing of the column with Buffer A containing 20 mM NaCl, the enzyme was eluted with 20 ml of Buffer A containing 400 mM NaCl. The eluate was brought to 1 M NaCl (pH 8.0) by adding solid NaCl and 1 M Tris base, and was applied to a TSK Phenyl-5PW column (TOSOH; 2.15×15 cm) equilibrated with Buffer B (20 mM Tris/HCl at pH 8.0, 0.5 mM EGTA, 0.1 mM Na_3VO_4, 20 mM 2-mercaptoethanol, 100 μM *p*-APMSF, 10 μg/ml leupeptin, 5% glycerol, and 0.1% Triton X-100) containing 1 M NaCl. After washing of the column with Buffer B containing 1 M NaCl, the enzyme was eluted by application of a 400-ml decreasing linear concentration gradient of NaCl (1 to 0 M) in Buffer B. Fractions collected were assayed for the enzyme activity, and active fractions were dialyzed against Buffer C (20 mM Tris/HCl at pH 8.0, 0.5 mM EGTA, 0.1 mM Na_3VO_4, 20 mM 2-mercaptoethanol, 100 μM *p*-APMSF, 10 μg/ml leupeptin, 10% glycerol, and 0.01% Triton X-100), and then applied to a Mono Q HR5/5 column (Pharmacia; 0.5×5 cm) equilibrated with Buffer C. After washing of the column with Buffer C, PKCδ was eluted by application of a 94-ml linear concentration gradient of NaCl (0 to 0.5 M) in Buffer C, and fractions (0.75 ml each) at NaCl concentrations between 100 and 400 mM were collected. PKCδ prepared from PMA-treated cells was resolved into Peaks I, II, and III as shown in Fig. 1. Where indicated, PKCδ fractions obtained by independent experiments were subjected to rechromatography on the Mono Q HR5/5 column under the conditions described above.

Immunoprecipitation—The sample (approximately 1 mg/ml, 1 ml) was incubated at 4°C for 12 h with 4 μg of the mouse monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology), and further incubated for 2 h after the addition of 100 μl of protein A-Sepharose beads slurry (50 μl of packed beads, Pharmacia). The immune-complex was collected, washed with ice-cold phosphate-buffered saline three times, and subjected to immunoblot analysis as described below.

Anti-Phosphotyrosine Agarose Column Chromatography—The sample was applied to an anti-phosphotyrosine agarose column (Transduction Laboratories; 1×0.3 cm) preequilibrated with Buffer C. After washing of the column with 2 ml of Buffer C, the sample was eluted with 1 ml of Buffer C containing 1 M NaCl.

Assay of PKCδ Activity—PKC was assayed by measuring the incorporation of ^{32}P into myelin basic protein from [γ-^{32}P]ATP in the presence of 32 μg/ml phosphatidylserine and 3.2 μg/ml diolein as described earlier (*10*).

In Vitro Tyrosine Phosphorylation of PKCδ—Tyrosine

kinase was purified partially from CHOd$^+$ cells. The cytosol fraction of CHOd$^+$ cells (2 ml from 10^8 cells) extracted with the buffer containing 20 mM Tris/HCl at pH 8.0, 0.5 mM EGTA, 15 mM β-glycerophosphate, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4, 100 μM *p*-APMSF, 10 μg/ml leupeptin, 5 μg/ml antipain, and 10 kU/ml aprotinin, was directly applied to a Mono Q HR5/5 column equilibrated with Buffer C. After washing of the column with 20 ml of Buffer C, the tyrosine kinase activity was eluted by application of a 100-ml linear concentration gradient of NaCl (0 to 0.5 M) in Buffer C. Tyrosine phosphorylation of PKCδ was carried out at 30°C in the standard reaction mixture containing 10 mM HEPES at pH 7.5, 50 μM ATP, 5 mM $MgCl_2$, 0.1 μg of PKCδ, and the tyrosine kinase fraction of CHOd$^+$ cells, in the presence or absence of 32 μg/ml phosphatidylserine and 3.2 μg/ml diolein. Where indicated, c-Src (1.5 μU/assay, Upstate Biotechnology) was employed instead of the tyrosine kinase fraction prepared from CHOd$^+$ cells.

Immunoblot Analysis—Samples were subjected to SDS-PAGE using 7% gel and transferred to an Immobilon filter (Millipore), and immunoblotting was carried out as described (*10*). A polyclonal antibody, CKpV3δ-a, which recognizes PKCδ selectively (*2*) and the mouse monoclonal anti-phosphotyrosine antibody (clone 4G10) were employed as first antibodies.

RESULTS

Phorbol Ester-Induced Tyrosine Phosphorylation of PKCδ in CHOδ-47 Cells—In various tissues and cell types, PKCδ is distributed in both the cytosol and particulate fractions, and the enzyme in the cultured cells is recovered in the particulate fraction upon stimulation (*10, 12, 14*). The subcellular distribution and the tyrosine phosphorylation of PKCδ were studied by using CHOδ-47 cells (Fig. 1). In CHOδ-47 cells, the 76- and 78-kDa proteins were both detected in the cytosol and particulate fractions, while PKCδ was recovered mostly in the particulate fraction and a small amount was detected in the cytosol fraction when the cells were treated with PMA for 30 min (Fig. 1A). The enzyme recovered in the particulate fraction from the phorbol ester-treated cells contained predominantly the 78-kDa protein, and the immunoprecipitation procedure using the antibody specific to phosphotyrosine indicated that the 78-kDa protein contained tyrosine-phosphorylated enzyme (Fig. 1B).

Isolation of Tyrosine-Phosphorylated PKCδ—As the α, δ, and ζ isoforms of cPKC, nPKC, and aPKC subgroups, respectively, are expressed and other isoforms are hardly detected in CHO cells (*5*), the nPKC activity in this cell line, that is dependent on diacylglycerol and phosphatidylserine and independent of calcium, is concluded to be PKCδ free from other nPKC isoforms. Thus, PKCδ extracted from the particulate fraction of CHOδ-47 cells was separated from other PKC isoforms by two steps of column

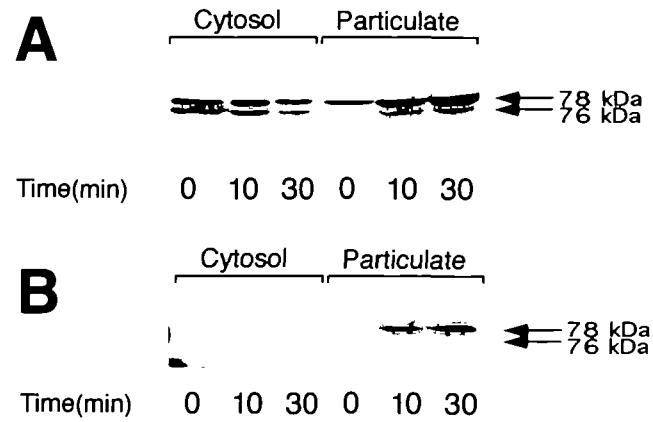

Fig. 1. **Subcellular localization and tyrosine phosphorylation of PKCδ in CHOδ-47 cells.** Cells were treated with 160 nM PMA for the indicated periods, and the cytosol and particulate fractions were prepared. A: Aliquots of the cytosol and particulate fractions (each fraction corresponds to 10^5 cells) were subjected to immunoblot analysis using CKpV3δ-a. B: The cytosol and particulate fractions (each fraction corresponds to 10^7 cells) were immunoprecipitated with the anti-phosphotyrosine antibody, and aliquots of the immunoprecipitates were subjected to immunoblot analysis using CKpV3δ-a. Positions of 76- and 78-kDa proteins are indicated by arrows.

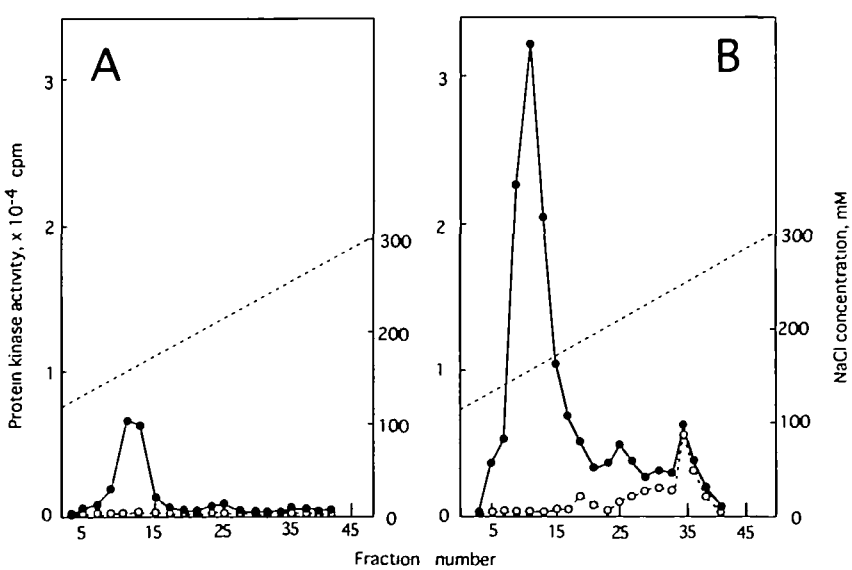

Fig. 2. **Separation of PKCδ prepared from CHOδ-47 cells.** PKCδ was partially purified from the particulate fraction of CHOδ-47 cells, and subjected to Mono Q column chromatography. A: Unstimulated cells. B: PMA-stimulated cells. Protein kinase activity was assayed in the presence (●) or absence (○) of phosphatidylserine and diolein. Broken lines represent NaCl concentrations in the elution buffer.

chromatography as described (*10*), and further subjected to Mono Q column chromatography (Fig. 2). PKCδ obtained from the untreated cells appeared as a single peak (Fig. 2A), whereas the enzyme isolated from PMA-treated cells was resolved into three peaks (Fig. 2B). Peak I (Fractions 7 to 17), that corresponds to PKCδ from the untreated cells, was followed by two smaller peaks, Peak II (Fractions 23 to 27) and Peak III (Fractions 33 to 35). Peak I and Peak II showed enzyme activities dependent on phosphatidylserine and diacylglycerol, like the enzyme obtained from untreated cells, and Peak III exerted its activity without these lipid activators. Peak II and Peak III, which were found only in the PMA-treated cells, were subjected to immunoblot analysis together with Peak I using the polyclonal antibody against PKCδ (Fig. 3). Peak II and Peak III both contained the 78-kDa protein, but not the 76-kDa protein. On the other hand, Peak I was composed mainly of the 78-kDa protein, and the 76-kDa protein is also present in earlier fractions of Peak I. The 78-kDa protein in Peak II and Peak III was recognized by the anti-phosphotyrosine antibody, although neither the 76-kDa protein nor 78-kDa protein in Peak I was efficiently detected by the anti-phosphotyrosine antibody. Anti-phosphotyrosine agarose column chromatography showed that PKCδ in Peak II and Peak III bound to the resin, whereas most of the enzyme in Peak I was not adsorbed on the column (Table I). These results strongly suggest that Peak II and Peak III are composed of the tyrosine-phosphorylated enzyme, and Peak I contains mostly the enzyme that is not tyrosine-phosphorylated. Peak II and Peak III are both tyrosine-phosphorylated stoichiometrically as they bind to the anti-phosphotyrosine agarose column; however, Peak II was recognized more efficiently than Peak III by the anti-phosphotyrosine antibody (Fig. 3B). PKCδ has been reported to be phosphorylated at more than one tyrosine residue, such as Tyr-52 (*24*) and Tyr-187 (*25*), and it also seems to be phosphorylated at more than one tyrosine residue in CHOδ-47 cells. Peak III may correspond to the tyrosine-phosphorylated PKCδ that is enzymatically unresponsive to activation by phorbol ester, observed in *v-ras*-transformed keratinocytes (*16*). By comparison of the protein kinase activities of the three peaks, approximately 10% of PKCδ was calculated to be tyrosine-phosphorylated in PMA-treated CHOδ-47 cells.

To distinguish the tyrosine-phosphorylated PKCδ of

TABLE I. **Anti-phosphotyrosine agarose column chromatography of tyrosine-phosphorylated and non-phosphorylated PKCδ.** PKCδ samples (0.75 ml each) corresponding to fractions 11, 25, and 35 of Mono Q column chromatography in Fig. 2B, obtained by an independent experiment, were employed as Peaks I, II, and III, respectively. Each enzyme fraction was applied to the anti-phosphotyrosine agarose column, and the enzyme activities of the pass-through and eluate fractions were assayed in the presence of phosphatidylserine and diolein. The values in parentheses indicate the enzyme activity in the absence of phosphatidylserine and diolein.

Enzyme fraction	Enzyme activity (cpm)	
	Pass-through	Eluate
Peak I	12,000 (100)	760 (120)
Peak II	300 (150)	2,240 (190)
Peak III	150 (220)	2,880 (2,140)

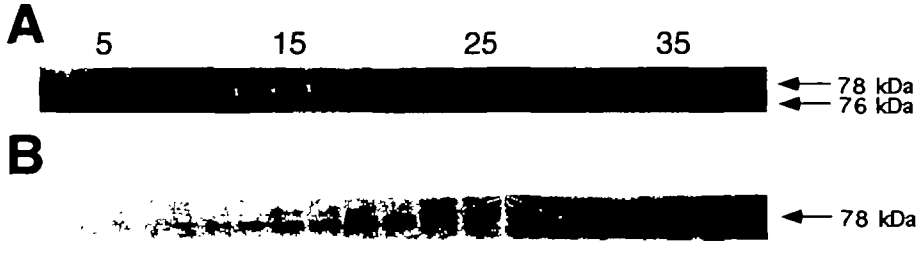

Fig. 3. **Immunoblot analysis of PKCδ prepared from CHOδ-47 cells.** Each fraction obtained from the particulate fraction of PMA-stimulated CHOδ-47 cells (Fig. 2B) was subjected to immunoblot analysis using CKpV3δ-a (A) and the anti-phosphotyrosine antibody (B). Numbers indicate the fraction numbers of Mono Q column chromatography in Fig. 2B. Positions of 76- and 78-kDa proteins are indicated by arrows.

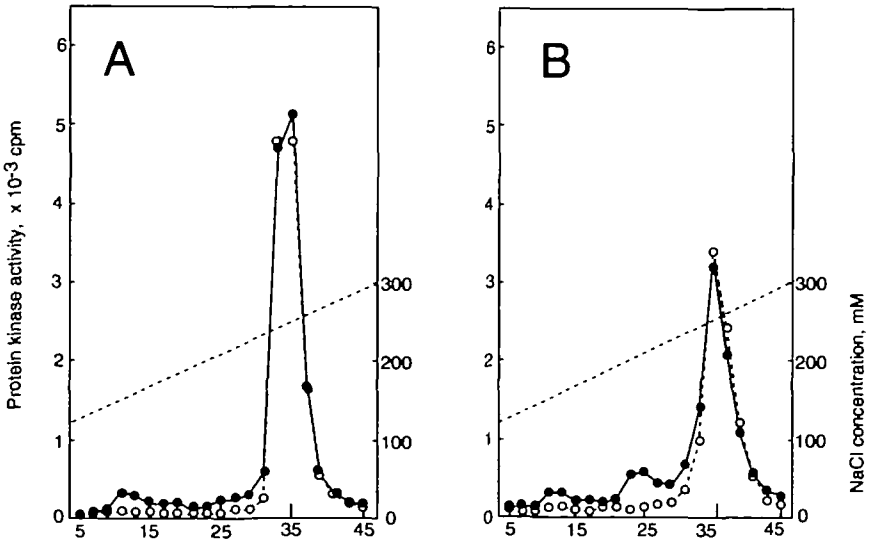

Fig. 4. **Release of the activator-dependent PKCδ from the activator-independent PKCδ.** A PKCδ sample (5.2 ml) corresponding to fractions 33-39 of Mono Q column chromatography in Fig. 2B, obtained by an independent experiment, was treated with 5% Triton X-100 by sonication on ice for 15 s 6 times with 5-min intervals, and rechromatographed on the Mono Q column after dialysis against Buffer C at 0-4°C. A: Untreated sample. B: Triton-treated sample. Protein kinase activity was assayed in the presence (●) or absence (○) of phosphatidylserine and diolein. Broken lines represent NaCl concentrations in the elution buffer.

Peak II and Peak III, that show catalytic activities dependent on and independent of phosphatidylserine and diacylglycerol, respectively, Peak III was treated with Triton X-100 and rechromatographed on the Mono Q column (Fig. 4). In the sample treated with Triton X-100, a small enzyme activity dependent on phosphatidylserine and diacylglycerol appeared at the position of Peak II in fractions 23 to 27 of Fig. 4B. PKCδ newly appeared at the position of Peak II seems to be derived from Peak III, because no significant enzyme activity was found in such fractions in the control experiment (Fig. 4A). Therefore, it seems possible that Peak III contains the enzyme associated with PMA and probably membrane phospholipids to show catalytic activity independent of lipid activators, whereas Peak II is the enzyme free from such PKC activators. It is still possible that the tyrosine phosphorylation causes PKCδ to show such high basal activity without association with lipid activators, as only a small portion of the enzyme appeared at the position of Peak II in Fig. 4B. Peak III might contain a small amount of the enzyme associated with lipid activators and a large fraction of the enzyme independent of such activators, both of which are tyrosine-phosphorylated, presumably at different ratios.

In Vitro Tyrosine Phosphorylation of PKCδ—As Peak II and Peak III recovered from phorbol ester-treated cells contain different amounts of phosphotyrosine and show distinct response to lipid activators, PKCδ was phosphorylated by the tyrosine kinases *in vitro* to study the effects of tyrosine phosphorylation on this PKC isoform (Fig. 5). When the 78-kDa PKCδ (Fraction 13 in Fig. 2B) was incubated with the tyrosine kinase fraction partially purified from CHOd$^+$ cells, the time-dependent tyrosine phosphorylation of the 78-kDa protein was observed in the simultaneous presence of phosphatidylserine and diacylglycerol (Fig. 5B). The 78-kDa protein was not phosphorylated efficiently in the absence of phosphatidylserine or diacylglycerol. The tyrosine kinase fraction employed contained a small amount of the 76-kDa PKCδ, but no tyrosine-phosphorylated protein around 76 to 78 kDa was found, either before or after the incubation (Fig. 5A). In addition, when the fraction containing the 76-kDa PKCδ (Fraction 5 in Fig. 2B) was employed, tyrosine phosphorylation of either the 76-kDa or 78-kDa protein was not detected (Fig. 5C). As the 76-kDa protein is not a substrate of the tyrosine kinase *in vitro*, it is not likely that the 76-kDa PKCδ could be phosphorylated on its tyrosine residues to afford the 78-kDa protein. Phorbol ester can substitute for diacylglycerol in this tyrosine phosphorylation reaction. c-Src phosphorylated the 78-kDa protein in a manner similar to that of the tyrosine kinase fraction prepared from CHOd$^+$ cells (Fig. 5D). When PKCδ of Peak I was subjected to rechromatography on the Mono Q column after *in vitro* tyrosine phosphorylation in the presence of phosphatidylserine and diacylglycerol, the enzyme activity was recovered in the fractions that roughly correspond to the position of Peak III as a mostly activator-independent enzyme, and no enzyme activity dependent on lipid activators appeared at the position of Peak II (data not shown). Thus, the *in vitro* tyrosine phosphorylation of PKCδ by the tyrosine kinases generated the enzyme corresponding to Peak III, but not Peak II among the tyrosine-phosphorylated PKCδ produced in the PMA-treated cells.

Properties of Tyrosine-Phosphorylated PKCδ—The affinities for the activators of PKCδ were studied using Peak I and Peak II obtained from the PMA-treated cells, since the enzyme tyrosine-phosphorylated *in vitro* was generated efficiently only in the presence of phosphatidylserine and diacylglycerol (Fig. 5). When Peak I and Peak II in Fig. 2B were assayed in the presence of various concentrations of PMA and phosphatidylserine, the tyrosine-phosphorylated enzyme showed affinities to these activators approximately threefold higher than those of the non-phosphorylated enzyme (Fig. 6). Similar results were obtained using the enzymes affinity-purified by anti-phosphotyrosine agarose column chromatography. The tyrosine-phosphorylated and non-phosphorylated PKCδ showed similar V_{max} values, when assayed in the presence of saturating concentrations of PMA and phosphatidylserine.

DISCUSSION

The tyrosine phosphorylation of PKCδ has been described

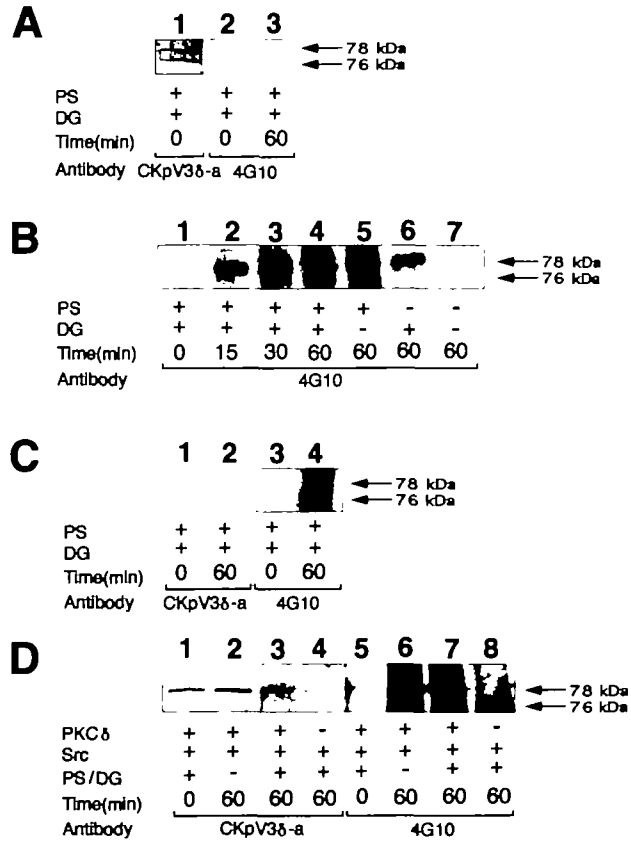

Fig. 5. ***In vitro* tyrosine phosphorylation of PKCδ.** Peak I of Mono Q column chromatography (Fig. 2B) was phosphorylated for the indicated time by tyrosine kinase partially purified from CHOd$^+$ cells (A, B, and C) or by c-Src (D) in the presence and absence of phosphatidylserine and diolein, and subjected to immunoblot analysis. A: Without Peak I PKCδ. B: With Peak I PKCδ (Fraction 13 in Fig. 2B). C: With Peak I PKCδ (Fraction 5 in Fig. 2B). D: With Peak I PKCδ (Fraction 13 in Fig. 2B) by c-Src. Immunoblot analysis was carried out using CKpV3δ-a for lane 1 in (A), lanes 1 and 2 in (C), and lanes 1-4 in (D), and the anti-phosphotyrosine antibody (4G10) for lanes 2 and 3 in (A), lanes 1-7 in (B), lanes 3 and 4 in (C), and lanes 5-8 in (D). Positions of the 76- and 78-kDa proteins are indicated by arrows. PS and DG represent phosphatidylserine and diolein, respectively.

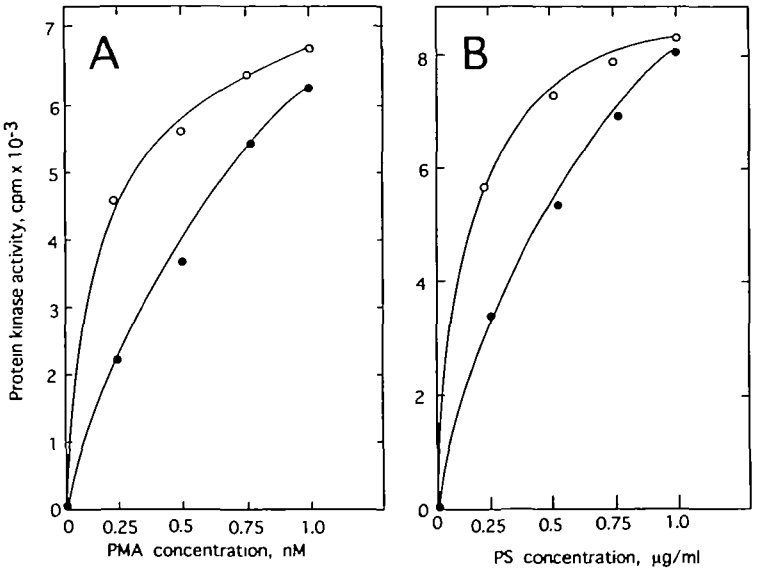

Fig. 6. **Requirements of phorbol ester and phosphatidylserine for activation of PKCδ.** Tyrosine-phosphorylated PKCδ (Fraction 25 in Fig. 2B) and non-phosphorylated PKCδ (Fraction 11 in Fig. 2B) were assayed in the presence of phosphatidylserine (0.5 μg/ml) and various concentrations of PMA (A), and in the presence of PMA (5 nM) and various concentrations of phosphatidylserine (B). (●) Non-phosphorylated PKCδ. (○) Tyrosine-phosphorylated PKCδ. PS represents phosphatidylserine.

in various cultured cells, and different effects of the post-translational modification reaction on this PKC isoform have been reported (*15-23*). In this study, we showed that a small portion of PKCδ is tyrosine-phosphorylated when the CHOδ-47 cells are stimulated by phorbol ester. The tyrosine-phosphorylated PKCδ showed the molecular mass of 78 kDa on SDS-PAGE, and the enzyme without the modification contained the 76- and 78-kDa proteins. The 78-kDa protein without tyrosine phosphorylation was a good substrate of the tyrosine kinases, whereas the 76-kDa protein was not tyrosine-phosphorylated *in vitro*. Because the 78-kDa protein is a phosphorylated form at serine/threonine residue(s), PKCδ seems to be phosphorylated sequentially at serine/threonine residue(s) and then at tyrosine residue(s) upon stimulation of the cells. The protein kinase that catalyzes the serine/threonine phosphorylation of the 76-kDa protein to produce the 78-kDa protein and the enzyme responsible for the tyrosine phosphorylation of PKCδ *in vivo* remain to be clarified. Tyr-52 and Tyr-187 of PKCδ have been identified as tyrosine phosphorylation sites (*24, 25*), and PKCδ was suggested to be tyrosine-phosphorylated on different sites in this study. It is necessary to identify the enzymes that phosphorylate PKCδ to reveal the mechanisms of regulation of the enzyme.

The tyrosine-phosphorylated PKCδ was recovered in two forms, *i.e.*, dependent on and independent of phosphatidylserine and diacylglycerol. The tyrosine phosphorylation has been proposed to have different effects on the catalytic activity of PKCδ. The tyrosine phosphorylation reduces the activity in keratinocytes (*16, 23*) and enhances it in murine myeloid progenitor cells (*17, 18*). It is not clear why the tyrosine phosphorylation should have such opposite effects on catalytic activity, though it might be possible that activation and inhibition of the tyrosine-phosphorylated enzyme were observed using enzyme fractions containing different ratios of the two forms of PKCδ that are dependent on and independent of the lipid activators. In particular, the tyrosine-phosphorylated PKCδ, that has reduced activity and is enzymatically unresponsive to activation by phorbol ester observed in *v-ras*-transformed keratinocytes (*16*), may reflect the enzyme identified as Peak III in this study. Enzymological analysis revealed that the tyrosine-phosphorylated PKCδ that is dependent on phosphatidylserine and diacylglycerol shows reduced requirements for phosphatidylserine and phorbol ester for its activation, compared to the enzyme that is not tyrosine-phosphorylated. It is attractive to surmise that the tyrosine phosphorylation of PKCδ may modulate its interaction with the lipid activators in the membranes to regulate the roles of the enzyme in signal transduction. PKCδ seems to be tyrosine-phosphorylated at more than one site *in vivo*, and it is necessary to study the effects of tyrosine phosphorylation at different residues. On the other hand, the tyrosine phosphorylation of PKCδ was found in the cells transformed by *v-ras* (*16*). The precise relationship between *ras* and PKC signal transduction pathways is still unclear, but there are at least two possibilities for the mechanism of the tyrosine phosphorylation of PKCδ in *v-ras*-transformed cells. Namely, PKCδ is phosphorylated by the tyrosine kinase activated in the transformed cells, or PKCδ activated downstream of *ras* is a target of tyrosine phosphorylation. When the cells are treated with phorbol ester, it seems possible that the activated PKCδ may be preferentially phosphorylated by tyrosine kinase, because the 76-kDa protein is first serine/threonine-phosphorylated to become the 78-kDa protein in activated cells, and then phosphorylated on tyrosine residues. Furthermore, the tyrosine phosphorylation reaction of PKCδ requires phosphatidylserine and diacylglycerol, at least *in vitro*. If activation of PKCδ is a prerequisite for its tyrosine phosphorylation, the modification reaction on the tyrosine residues might be important for the regulation of the functions of PKCδ, such as the recognition of substrate proteins, the interaction with associating proteins, and the maintenance of catalytic activity. Further studies are necessary to elucidate of the physiological role of the tyrosine phosphorylation of PKCδ.

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