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Immunology

Tyrosine phosphorylation of protein kinase C-δ in response to the activation of the high-affinity receptor for immunoglobulin E modifies its substrate recognition

(mast cell/signaling/FcεRI)

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ABSTRACT **The δ isoform of protein kinase C is phosphorylated on tyrosine in response to antigen activation of the high-affinity receptor for immunoglobulin E. While protein kinase C-δ associates with and phosphorylates this receptor, immunoprecipitation of the receptor revealed that little, if any, tyrosine-phosphorylated protein kinase C-δ is receptor associated. *In vitro* kinase assays with immunoprecipitated tyrosine-phosphorylated protein kinase C-δ showed that the modified enzyme had diminished activity toward the receptor γ-chain peptide as a substrate but not toward histones or myelin basic protein peptide. We propose a model in which the tyrosine phosphorylation of protein kinase C-δ regulates the kinase specificity toward a given substrate. This may represent a general mechanism by which *in vivo* protein kinase activities are regulated in response to external stimuli.**

The activation of the mucosal mast cell model, rat basophilic leukemia (RBL) cells, by the interaction of antigen with cell-bound immunoglobulin E (IgE) initiates the rapid translocation and increased activity of protein kinase C (PKC) (1, 2). This PKC activation is critical to the effector function of the mast cell, namely exocytosis of mediators of the allergic response (3, 4). Protein kinase C-δ associates with and phosphorylates the high-affinity receptor for IgE FcεRI (5) on these cells, while other PKC isozymes regulate phosphoinositide hydrolysis (6) and induce protooncogene expression (7).

Recent studies have begun to suggest that PKC may regulate tyrosine kinases and is itself regulated by phosphorylation interactions with these kinases (8–10). Yao *et al.* (10) have shown a PKC-mediated inactivation of Bruton tyrosine kinase (BTK), an enzyme mutated in chromosome X-linked agammaglobulinemia patients. Moreover, BTK was found to associate with the βI isoform of PKC in mast cells. Phosphorylation of serine/threonine kinases by tyrosine kinase activity has also been described (9, 11). Denning *et al.* (9) showed that PKC-δ was tyrosine phosphorylated in cells expressing oncogenic ras. Li *et al.* (12) reported that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) treatment of PKC-δ transfectants also led to tyrosine phosphorylation of this isozyme. More recent evidence from the latter group suggests that the tyrosine phosphorylation of PKC-δ can occur in response to platelet-derived growth factor receptor stimulation in PKC-δ transfected cells (13). Collectively, the latter studies document the phosphorylation of a serine/threonine kinase by a tyrosine kinase activity in response to a stimulus, although the physiological significance of tyrosine phosphorylation of PKC-δ is unclear.

In the present study, we investigate whether tyrosine phosphorylation of PKC-δ occurs in response to FcεRI aggregation and whether the tyrosine-phosphorylated form of PKC-δ is preferentially receptor associated. We also analyze the effect of tyrosine phosphorylation on the activity of PKC-δ toward various substrates, including the FcεRI γ chain.

MATERIALS AND METHODS

Cell Cultures and Activation. The RBL-2H3 subline of RBL cells were cultured in monolayers in stationary flasks as described (14). Cells (5×10^6 cells per ml) were sensitized with dinitrophenyl-specific mouse monoclonal IgE and activated with 2,4-dinitrophenylated-bovine serum albumin (DNP-BSA) as described (5). Activation was for 1 min or for indicated times in medium or, for kinetic studies, in cell buffer without phosphatase inhibitors and EDTA (15).

Cell Fractionation and Solubilization. After stimulation, cells (2×10^7 cells per ml) were resuspended in a cell buffer (15) modified by the addition of 1 mM Na_3VO_4/5 mM $Na_4P_2O_7$/7 mM EDTA and protease inhibitors. Cells were disrupted by sonification, and 80–90% of the plasma membrane was recovered in the pellet fraction (15). Triton X-100 was added to the recovered soluble fraction to a final concentration of 0.5%. Membrane pellets were solubilized (16) by using an equal volume of 0.5% Triton X-100 containing phosphatase inhibitors, protease inhibitors, and EDTA as above to obtain a final cell equivalent concentration of 2×10^7 cells per ml. Equal volumes (0.5 ml) of membrane or cytosol lysates were used for immunoprecipitations or mixed with 2× SDS sample buffer [0.125 M Tris·HCl, pH 6.8/4% (wt/vol) SDS] for gel electrophoresis.

Immunoprecipitations. Recovered lysates were incubated with 8 μl of antisera to PKC-δ (Calbiochem; and Research & Diagnostic Antibodies, Beverly, CA) prebound to protein A-Sepharose. For control immunoprecipitates, 80 μg of rabbit IgG was used. Incubations were overnight at 4°C with mixing. In some experiments, recovered lysates were incubated with 15 μg of antibody to phosphotyrosine (PY) (PY20, ICN) and protein G-Sepharose to deplete tyrosine-phosphorylated proteins. Immunoprecipitates were washed three times in the above lysis buffer followed by an additional wash in 0.01% Triton X-100. Immunoprecipitates for kinase assays were further washed in kinase assay buffer (5). Conditions for immunoprecipitation of FcεRI were as described (5).

Gel Electrophoresis and Western Blots. Proteins were resolved on 8% or 10% polyacrylamide gels containing Tris·HCl and glycine under reducing condition unless otherwise indicated. The resolved proteins were transferred to 0.45-μm nitrocellulose membranes. The membranes were blocked and phosphotyrosine-containing proteins detected (5). A poly-

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Abbreviations: RBL, rat basophilic leukemia; DNP-BSA, 2,4-dinitrophenylated bovine serum albumin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MBP, myelin basic protein; PY, phosphotyrosine.

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clonal antiserum to PKC-δ (Research & Diagnostic Antibodies) was used at a 1:25,000 dilution. Secondary antibody, visualization, and quantitation were as described (5).

***In Vitro* Kinase Assay.** Partially purified PKC was obtained from nonactivated or activated cells by ion-exchange chromatography (Protein Kinase C Assay System, GIBCO/BRL). PKC-δ from the membrane or cytosol fraction was isolated by immunoprecipitation. Kinase reactions were carried out as described (5). Unlike the activity measured in the control IgG immunoprecipitates, activity in PKC-δ immunoprecipitates was dependent on phospholipid and phorbol 12-myristate 13-acetate (PMA) and was inhibited by a PKC-specific inhibitory peptide (5). Phosphorylated histones and γ peptide were resolved on 10% or 16% polyacrylamide gels containing Tricine, respectively. When myelin basic protein (MBP) peptide (MBP 4-14) was used as a substrate, reactions were stopped by immediate spotting on phosphocellulose filters. Quantitation of phosphorylation was by densitometric scanning of exposures as described (5) or, for some experiments, by scintillation counting of excised gel bands directly.

Generation of Stable PKC-δ Transfectant. The cDNA encoding mouse PKC-δ was inserted into a metallothionein promoter-driven eukaryotic expression vector (εMTH; ref. 17) and used to transfect RBL cells according to Alber *et al.* (18). The stable transfectants showed a 5-fold increase in PKC-δ expression, determined by Western blotting, when compared with expression in untransfected RBL cells. The relative distribution of PKC-δ in these cells (20–30% in membrane vs. 70–80% in cytosol) was found to be similar to that in the untransfected cells (4). The protein expression of transfected PKC-δ in RBL cells was not regulated by the metallothionein promoter in these cells.

RESULTS

Tyrosine Phosphorylation and Membrane Association of PKC-δ in Response to FcεRI Aggregation. Immunoprecipitation of PKC-δ from lysates prepared from antigen-activated RBL-2H3 cells led to the identification of a tyrosine-phosphorylated form of this isozyme (Fig. 1*A*). Cell fractionation showed an average increase of 2-fold for PKC-δ in the nuclear-free pelleted membrane fraction and the presence of tyrosine-phosphorylated PKC-δ with this fraction and not with the soluble fraction (Fig. 1*B*). The rate of phosphorylation was found to be rapid, with maximal phosphorylation occurring 30 sec after addition of antigen (Fig. 1*C*). PKC-δ translocation also occurred rapidly and seemed to coincide with tyrosine phosphorylation (Fig. 1*C*), suggesting a translocation-dependent phosphorylation. Phosphorylation appeared to be regulated by a phosphatase, as subsequent dephosphorylation of the tyrosine phosphorylated PKC-δ was rapid. Approximately 80% of the phosphorylated PKC-δ was no longer detected after 3 min of activation, although no apparent decrease of PKC-δ in the membrane fraction was observed. Depending on the experiment, 10–40% of membrane-associated PKC-δ was tyrosine phosphorylated (compare Table 1 and Fig. 4*A*).

Translocation Is Required for Tyrosine Phosphorylation of PKC-δ but Not Vice Versa. Various agents that promote or inhibit PKC activity by binding to PKC were tested for their ability to translocate PKC-δ or to induce tyrosine phosphorylation of this isozyme. As can be seen in Table 1, agents that promoted translocation also induced tyrosine phosphorylation. One inhibitor of PKC, calphostin C, did not cause translocation and failed to induce tyrosine phosphorylation. In contrast, all agents which effectively caused translocation of PKC-δ, although some of these inhibit PKC activity, led to tyrosine phosphorylation of approximately 10% of the membrane-associated isozyme.

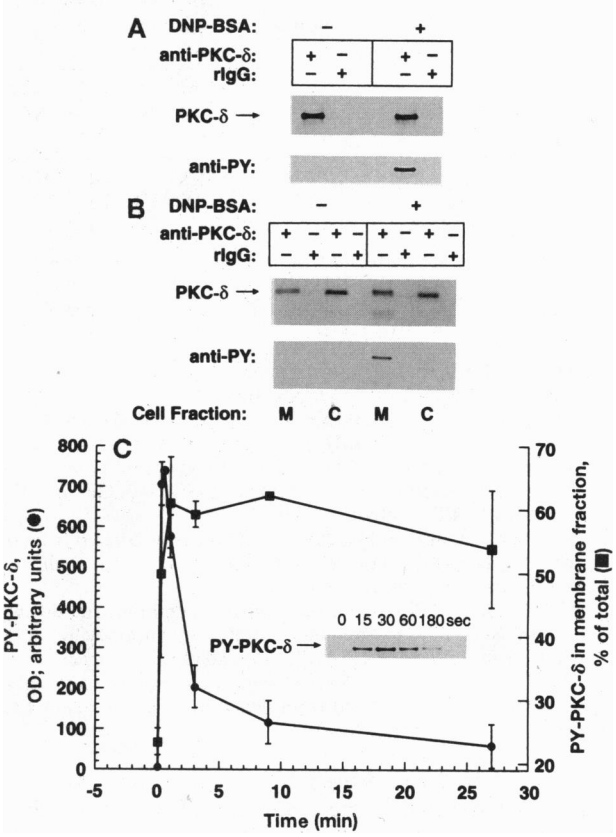

FIG. 1. PKC-δ is tyrosine phosphorylated and membrane associated. (*A*) Immunoprecipitation of PKC-δ from Triton X-100 lysates of nonactivated or activated cells. Nitrocellulose membranes were first probed with anti-PY antibodies, then stripped of this antibody as described (5). After being extensively washed, membranes were blocked as described (15) and probed with antibody to PKC-δ. One representative of three experiments is shown. rIgG, rabbit IgG. (*B*) Immunoprecipitation of PKC-δ from the membrane (M) or cytosol (C) fractions. Cell fractions were obtained as described in *Materials and Methods*. Immunoblots were probed as described above. One representative of seven experiments is shown. (*C*) Kinetics of tyrosine phosphorylation (●) and translocation (■) of PKC-δ. Data were compiled from three experiments for tyrosine phosphorylation and two experiments for translocation. (*Inset*) One representative experiment of the kinetics of tyrosine phosphorylation is shown.

We investigated the possibility that translocation of PKC-δ requires tyrosine phosphorylation. Cells were incubated in the presence of 300 μM genistein, a concentration previously shown to effectively inhibit tyrosine phosphorylation in RBL-2H3 cells (21). As shown in Table 2, tyrosine phosphorylation of total cellular proteins in response to antigen activation was effectively inhibited (69% ± 7%) in the presence of genistein, as was the tyrosine phosphorylation of PKC-δ in response to antigen activation (53% ± 6%). Treatment of RBL-2H3 cells with PMA also induced tyrosine phosphorylation of PKC-δ (Table 1), and this phosphorylation was inhibited by 68% ± 11% (Table 2) in the presence of genistein. However, translocation of PKC-δ induced by either stimulus was not inhibited (7% ± 9%; 6% ± 5%) by the inhibition of PKC-δ tyrosine phosphorylation.

Tyrosine-Phosphorylated PKC-δ Does Not Preferentially Associate with Receptor. Since PKC-δ was found to associate with FcεRI (5), we investigated the possibility that tyrosine-phosphorylated PKC-δ is found exclusively with the receptor. In these experiments, the noncovalent association of receptor subunits ($\alpha\beta\gamma_2$) was maintained as described (5), and the intact receptor was depleted from antigen-activated membrane fractions by immunoprecipitation with antibody to mouse IgE. Fig. 2 shows that these conditions led to greater than 90% depletion

Table 1. Tyrosine phosphorylation of PKC-δ is induced by various agents that promote translocation

Agent	Membrane-associated PKC-δ, %*	Membrane-associated, PY-PKC-δ to PKC-δ %†
IgE	24.4 ± 10	0
IgE-Agn	48.6 ± 12	10.8 ± 1.3
PMA	88.3 ± 3	10.5 ± 2.0
Bryostatin 1	71.3 ± 3	10.8 ± 2.2
Ro 31-7549	51.2 ± 14	15.4 ± 4.3
GF 109203X	70.9 ± 14	6.8 ± 3.8
Calphostin C	19.7 ± 10	0

For translocation experiments incubation with DNP-BSA (Agn) was as described in *Materials and Methods*. Incubation with other agents was for 1 min at 37°C: PMA (100 nM), bryostatin 1 (1 μM), Ro 31-7549 (10 μM), GF 102903X (10 μM), and calphostin C (10 μM). These concentrations have previously been shown to be optimal for promoting (PMA and bryostatin 1) or inhibiting (Ro 31-7549, GF 102903X, and calphostin C) PKC activity (3, 19, 20).

*The percent of membrane-associated PKC-δ was calculated from the sum of PKC-δ in immunoblots from the cytosol and membrane fractions. Data are from five experiments.

†Immunoprecipitated PKC-δ derived from the membrane fraction was resolved by SDS/PAGE, immunoblotted with antibody to either phosphotyrosine (PY) or PKC-δ (PKC-δ) and visualized by enhanced chemiluminescence (ECL). Ratios were determined from mean values (arbitrary units) derived from densitometric analysis of ECL films from three experiments (5).

of the receptor, as measured by the amount of tyrosine-phosphorylated β chain (lane 1 vs. lane 3). While the receptor was effectively removed from the membrane fraction, little, if any, tyrosine-phosphorylated PKC-δ was depleted by immunoprecipitation with anti-mouse IgE (Fig. 2, lane 3 vs. lane 4). Moreover, no detectable tyrosine phosphorylation was present at the molecular weight of PKC-δ in the receptor immunoprecipitates (Fig. 2, lane 1), although PKC-δ was present in these immunoprecipitates (Fig. 2). We cannot exclude the possibility that small amounts of tyrosine-phosphorylated PKC-δ, not detectable with anti-phosphotyrosine antibodies, might associate with the receptor. Nevertheless, the majority of the tyrosine-phosphorylated PKC-δ is not coimmunoprecipitated with the receptor (Fig. 2, lane 3). Similar results were obtained when receptor immunoprecipitation was done with antibody directed to the FcεRI β chain (data not shown).

Table 2. Effect of genistein on tyrosine phosphorylation and translocation of PKC-δ

Protein	Stimulus	% inhibition* PY-phosphorylation	% inhibition* Translocation
Total cell proteins	DNP-BSA	69 ± 7	ND
PKC-δ	DNP-BSA	53 ± 6	7 ± 9
PKC-δ	PMA	68 ± 11	6 ± 5

Cells (5×10^6/ml) were sensitized with dinitrophenyl-specific mouse monoclonal IgE and incubated in the presence or absence of 300 μM genistein for 20 min at 37°C prior to the addition of DNP-BSA (1 μg/ml) or PMA (100 nM) for an additional 1 min. Cells were sonicated and fractionated as described in *Materials and Methods*. PKC-δ in membrane and cytosol fractions was detected as described in legend to Fig. 1. Effect of genistein on tyrosine phosphorylation of PKC-δ was determined by immunoprecipitation of PKC-δ and immunoblotting with antibody to PY or PKC-δ. Genistein had no effect on PKC-δ from nonstimulated cells.

*Percent inhibition of PY phosphorylation was calculated from the observed phosphorylation in the presence or absence of genistein and normalized to protein content. Inhibition of translocation was determined from the amount of PKC-δ present in the cytosol or membrane fractions in the presence or absence of genistein and normalized to total protein. ND indicates experiment was not done. Data compiled from three experiments.

Collectively, these experiments suggest that a significant amount (57% ± 8%) of the activated receptor is associated with PKC-δ since the depletion of PKC-δ from the membrane fraction by immunoprecipitation resulted in the coprecipitation of large amounts of phosphorylated β chain (Fig. 2, lane 4). These findings provide further evidence for the association of PKC-δ with the activated receptor and suggest that the tyrosine-phosphorylated PKC-δ is not exclusively associated with receptor.

Tyrosine Phosphorylation Modifies the Substrate Recognition of PKC-δ. In these experiments, we utilized an RBL cell line stably transfected with PKC-δ to increase the amount of tyrosine-phosphorylated PKC-δ present in immunoprecipitates. The amount of tyrosine-phosphorylated PKC-δ in this transfectant was ≈3-fold more than that in untransfected RBL cells. This correlated with a 3- to 5-fold increase of PKC-δ associated with the membrane fraction of the transfectant, although the relative amount of PKC-δ translocated in response to antigen activation did not differ significantly from that of untransfected RBL cells (48% vs. 51% respectively; compare Fig. 1*B* and Fig. 3*B*). Partial purification of total PKC from nonactivated and activated cells revealed an increased activity (1.3- to 1.5-fold) toward MBP peptide due to cell activation (Fig. 3*A*). Likewise, a 2.2-fold increase in the activity of PKC-δ toward MBP peptide was found in the membrane fraction after cell activation (Fig. 3*C*). This was accompanied by a concomitant depletion of activity from the cytosol and therefore is likely to be a reflection of the 2- to 2.5-fold increase in membrane-associated PKC-δ (Fig. 3*B*). When histone type IIIs was used as a substrate, the PKC-δ from the membrane fraction of activated cells also exhibited an increased activity toward this substrate (Fig. 3*D*). In contrast, when a peptide encoding the entire cytoplasmic domain of the FcεRI γ chain (5) was used as a substrate, most of the PKC-δ activity was found in the membrane fraction from nonactivated cells (Fig. 3*E*). Activation of the cells resulted in greater than 60% inhibition of the PKC-δ activity towards the γ peptide.

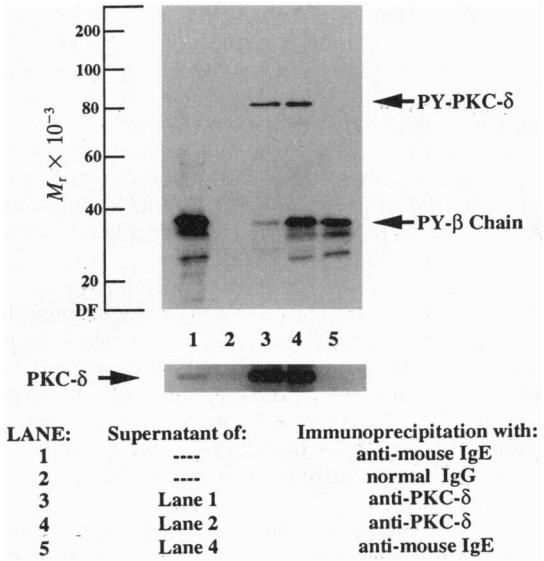

FIG. 2. Tyrosine-phosphorylated PKC-δ does not coimmunoprecipitate with FcεRI. The FcεRI was immunoprecipitated from DNP-BSA-activated cells (lane 1) with goat anti-mouse IgE and immunoblotted with antibody to PY. A control immunoprecipitate with nonimmune goat IgG is also shown (lane 2). The respective supernatants of these immunoprecipitates were sequentially treated with antibody to PKC δ (lanes 3 and 4). The resulting supernatant from the immunoprecipitation of PKC δ in lane 4 was further treated with antibody to mouse IgE (lane 5). The bottom panel shows the presence of PKC δ in the respective immunoprecipitates. One representative of three experiments is shown. DF, dye front.

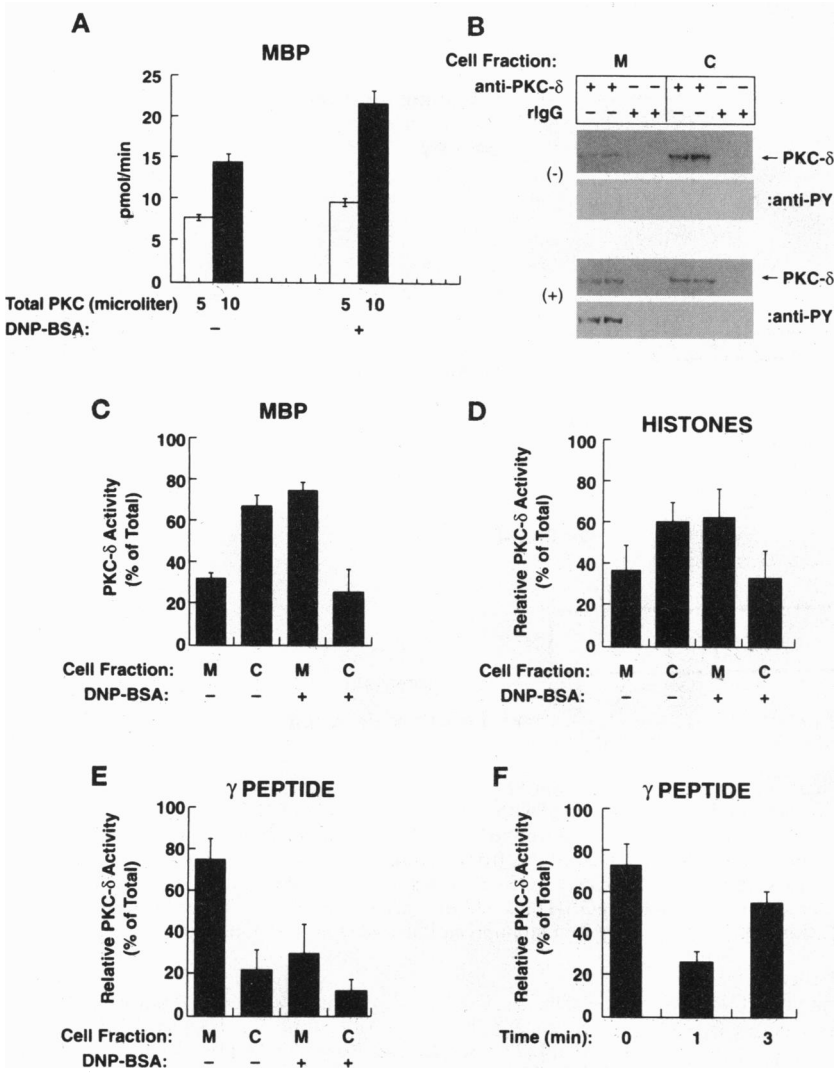

FIG. 3. Substrate phosphorylation by PKC-δ derived from PKC-δ transfectant: effect of tyrosine phosphorylation. (*A*) Activity of partially purified total PKC from nonactivated (−) or DNP-BSA-activated (+) cells was measured in the linear range by using MBP as a substrate. (*B*) Immunoprecipitation of PKC-δ from nonactivated (−) or DNP-BSA-activated (+) cells. Immunoblots were first probed with antibody to PY and then by antibody to PKC-δ, as described in legend to Fig. 1. Duplicate samples as shown were used for *in vitro* kinase assays. Controls were duplicate immunoprecipitates with nonspecific rabbit IgG (rIgG). One representative of five experiments is shown. (*C*) *In vitro* kinase assay with immunoprecipitated PKC-δ derived from membrane (M) or cytosol (C) fractions of nonactivated (−) or DNP-BSA-activated (+) cells. MBP peptide was used as substrate. Data are from two experiments with triplicate samples. (*D*) Same as *C*, except that histone type IIIs was used as substrate. (*E*) Same as *C*, except that FcεRI γ chain peptide was used as substrate. Data are from five experiments. (*F*) Immunoprecipitated PKC-δ from the membrane fraction of cells activated for the indicated times. Data are from two experiments with triplicate samples. The percent of total activity is a net value and was determined from the sum of activities in the membrane (M) and cytosol (C) fractions prior to antigen activation. Relative activity reflects quantitation of activity by autoradiography and densitometric analysis.

Similar results were obtained with the untransfected RBL-2H3 cells although the extent of inhibition was less (18–49%; $n = 5$). The activity of membrane-associated PKC-δ toward the γ peptide at various times is shown in Fig. 3*F*. The inhibition observed at 1 min of activation is partially reversed after 3 min of activation. This coincides with the loss of approximately 80% of the tyrosine-phosphorylated PKC-δ (Fig. 1*C*).

To determine if the presence of tyrosine-phosphorylated PKC-δ in immunoprecipitates of PKC-δ was responsible for the inhibitory effect toward γ-peptide phosphorylation, tyrosine-phosphorylated PKC-δ was depleted from membrane lysates of activated cells, and the kinase activity of the remaining PKC-δ was compared with the total membrane-associated PKC-δ activity. Fig. 4*A* shows that in this representative experiment 36% ± 7% of the PKC-δ is tyrosine phosphorylated. While depletion of the tyrosine phosphorylated PKC-δ was not complete (73% ± 6%), the activity, normalized for the amount of PKC-δ present in the immunoprecipitates (Fig. 4*B*), was found to be approximately 3-fold higher in the immunoprecipitate depleted of tyrosine-phosphorylated PKC-δ (Fig. 4*C*). The activity toward histones was decreased in the immunodepleted samples (data not shown).

DISCUSSION

The principal findings of this study are as follows: (*i*) Tyrosine phosphorylation of PKC-δ is a rapid consequence of activation of FcεRI. (*ii*) Translocation is a requirement for tyrosine phosphorylation of PKC-δ but not vice versa, suggesting that the tyrosine kinase that is responsible for this phosphorylation is membrane localized. (*iii*) While PKC-δ is associated with FcεRI, no detectable amount of tyrosine-phosphorylated PKC-δ was found associated with the receptor. (*iv*) The tyrosine-phosphorylated PKC-δ from the membrane fraction of activated cells showed a decreased activity toward the FcεRI γ peptide but an increased activity toward MBP peptide and histone type IIIs.

The difference in the activity of tyrosine-phosphorylated PKC-δ observed by Denning *et al.* (9) and Li *et al.* (12) could be explained, on the basis of our results, by a difference in the substrates used to measure the activity of the modified PKC-δ. While translocation of PKC to the membrane fraction has traditionally been accepted as an indicator of PKC activation, our study suggests that substrate specificity is paramount in determining whether a translocated PKC isozyme is in fact active or inactive. Therefore, measurements of activity by utilizing nonspecific substrates may only serve as a measure of translocation and may not accurately reflect the activity of a given isozyme toward a specific substrate.

Our findings are most consistent with a model in which the threonine phosphorylation of the FcεRI γ chain occurs in the absence of activation (5, 22), although activation of the receptor causes a 2- to 3-fold increase in threonine phosphorylation of the γ chain (5, 22). This implies an active form of PKC-δ that is sequestered in the membrane prior to cell activation. Activation of the receptor results in translocation of up to 40% of the cytosolic PKC-δ to the membrane (ref. 4 and this study). This translocation is a requirement for tyrosine phosphoryla-

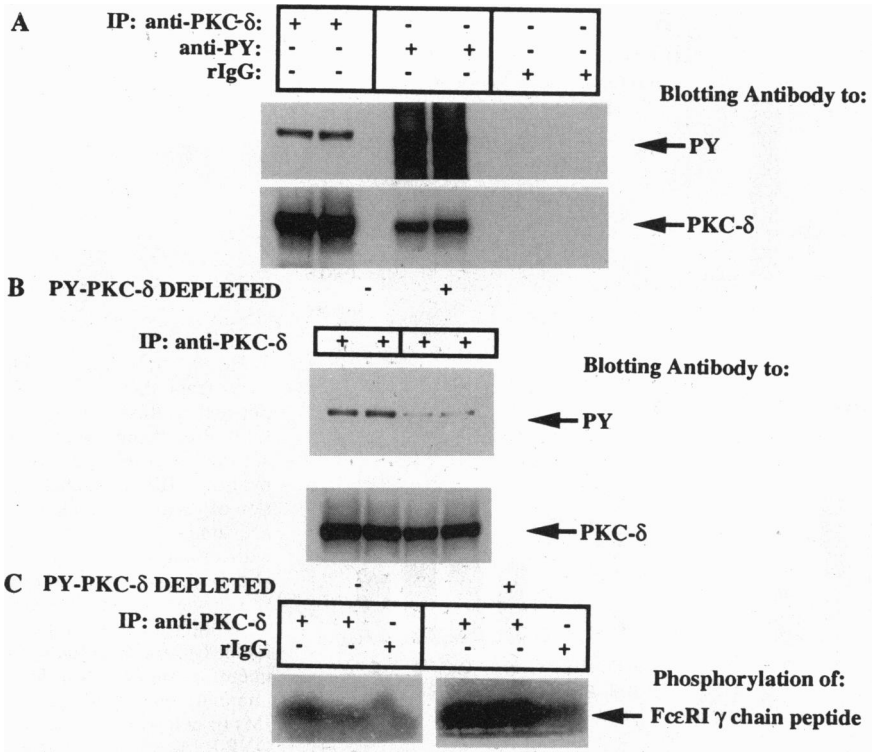

FIG. 4. Depletion of tyrosine-phosphorylated PKC-δ leads to increased activity toward FcεRI γ-chain peptide. (*A*) Quantitation and depletion of tyrosine-phosphorylated PKC-δ. Membrane lysates from activated cells were treated with antibodies to PKC-δ, PY, or rabbit IgG, followed by protein A-Sepharose. Immunoprecipitates (IPs) were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with anti-PY. Blots were reprobed with anti-PKC-δ. (*B*) Depletion of tyrosine-phosphorylated PKC-δ. Supernatants from *A* were treated with antibody to PKC-δ and then with protein A-Sepharose. The resulting immunoprecipitates were resolved by SDS/PAGE and transferred to nitrocellulose. Western blots were as in *A*. PKC-δ immunoprecipitates of supernatants from anti-PY or rabbit IgG (*A*) are shown. (*C*) *In vitro* kinase assay of γ peptide with tyrosine-phosphorylated depleted or nondepleted PKC-δ. Control rabbit IgG (rIgG) immunoprecipitates were as in *A*. One representative of three experiments is shown.

tion of PKC-δ suggesting: (*i*) that translocation brings PKC-δ in juxtaposition with a tyrosine kinase that phosphorylates it and (*ii*) that this tyrosine kinase must be active, localized in the membrane, and regulated since translocation and tyrosine phosphorylation of PKC-δ can occur in the absence of increased tyrosine phosphorylation of cellular substrate—i.e., in the presence of PMA. The tyrosine-phosphorylated PKC-δ does not actively phosphorylate the FcεRI γ chain although our studies suggest that interaction does occur (Fig. 4). Nevertheless, the tyrosine-phosphorylated PKC-δ is active as determined by its ability to phosphorylate MBP peptide and histones, and therefore may target a substrate previously not recognized by the non-tyrosine-phosphorylated form of PKC-δ. Our results advance the intriguing possibility that regulation of PKC isozyme substrate specificity *in vivo* is mediated by crosstalk with enzymes of the tyrosine kinase families.

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