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D609-sensitive tyrosine phosphorylation is involved in Fas-mediated phospholipase D activation

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Abbreviations: PC-PLC: phosphatidylcholine-specific phospholipase C; PKC: protein kinase C; PLD: phospholipase D; FBS: fetal bovine serum; PEt: phosphatidylethanol; TLC: thin layer chromatography; DAG: diacylglycerol; PMA: phorbol 12-myristate 13-acetate

Abstract

Both Fas and PMA can activate phospholipase D *via* activation of protein kinase Cβ in A20 cells. Phospholipase D activity was increased 4 fold in the presence of Fas and 2.5 fold in the presence of PMA. The possible involvement of tyrosine phosphorylation in Fas-induced activation of phospholipase D was investigated. In five minute after Fas cross-linking, there was a prominent increase in tyrosine phosphorylated proteins, and it was completely inhibited by D609, a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC). A tyrosine kinase inhibitor, genistein, can partially inhibit Fas-induced phospholipase D activation. There were no effects of genistein on Fas-induced activation of PC-PLC and protein kinase C. These results strongly indicate that tyrosine phosphorylation may in part account for the increase in phospholipase D activity by Fas cross-linking and D609 can block not only PC-PLC activity but also tyrosine phosphorylation involved in Fas-induced phospholipase D activation.

Keywords: Fas, phospholipase D, D609, phosphatidylcholine-specific phospholipase C, tyrosine phosphorylation, protein kinase C, A20 murine cells, genistein

Introduction

Fas known to elicit various signal transduction pathways within animal cells is ubiquitously expressed in lymphoid and nonlymphoid tissues and in many primary tumors and tumor cell lines (Wananabe-Fukunaga *et al*., 1992; Leithauser *et al.,* 1993; French *et al*., 1996). Binding of Fas ligand or anti-Fas antibodies induces Fas antigen trimerization and leads to the activation of caspase proteolytic cascade. This occurs by an association between death domains of Fas and FADD/MORT1 (Boldin *et al*., 1995; Chinnaiyan *et al*., 1995) followed by the recruitment of caspase 8 *via* interactions between death effector domains (Boldin *et al*., 1996; Muzio *et al*., 1996). Binding of caspase 8 to FADD/MORT1 induces the activation of its proteolytic activity, as well as its downstream caspases.

Earlier reports demonstrated that activation *via* the Fas antigen also stimulates phosphatidylcholine-specific phospholipase C (PC-PLC) (Cifone *et al*., 1995; Han *et al*., 1999). The sequential activations of PC-PLC and acidic sphingomyelinase, and the subsequent rise in ceramide level were presumed to be responsible for the Fas-generated apoptotic signal in lymphoid cells (Cifone *et al*., 1995). In A20 murine B lymphoma cell lines, the activation of PC-PLC by Fas ligation elicits activation of protein kinase C (PKC) and phospholipase D (PLD) (Han *et al*., 1999). The involvement of PLD activity during apoptosis has also been reported in Jurkat cells (Kasai *et al*., 1998). Our studies showed that *in vitro* PLD activity measured by using exogenous substrate in apoptotic Jurkat cell lysates was higher than that of the lysates from untreated control cells, and increased PLD activity during apoptosis was attributed to the PLD of unsaturated fatty acid dependent type.

Fas-induced apoptosis has also been previously reported to involve Fas-triggering tyrosine phosphorylation of various proteins (Eischen *et al*., 1994; Janssen *et al*., 1996; Schlottmann *et al*., 1996). The Fas-induced tyrosine phosphorylation events are generated within seconds to minutes of Fas ligation; they usually occur within 5 min and decline toward the baseline by 30 min (Eischen *et al*., 1994). Although it is still controversial whether or not tyrosine kinase activation is necessary for Fas-mediated apoptosis (Eischen *et al*., 1994; Schraven *et al*., 1995; Mollereau *et al*., 1996; Schraven *et al*., 1996), the role of tyrosine phosphorylation in Fas-induced apoptosis is supported by the observation that expression of the hematopoietic cell phosphatase is a prerequisite for Fas-induced apoptosis in several lymphoid cells (Su *et al*., 1995; Su *et al*., 1996). Recently, Simon *et al.* (1998) reported that activation of Fas signaling by anti-Fas monoclonal antibody (anti-Fas mAb) results in increased tyrosine phosphorylation of several intracellular proteins, and the tyrosine kinase inhibitors inhibit Fas-induced cell

death in both human and mouse eosinophils *in vitro*. The authors further showed that tyrosine kinase inhibitor lavendustin A prevents anti-Fas mAb-induced proteolytic cleavage of lamin B, suggesting that tyrosine kinase might amplify the proteolytic signaling cascade within interleukin-1β converting enzyme family proteases. Also, there is a report suggesting that tyrosine kinase $p59^{fyn}$ physically associates with Fas in YAC-1 cells and activates T lymphocytes from *fyn* knockout mice exhibit elevated lifespans and reduces apoptosis *in vitro* compared to their normal counterparts (Atkinson *et al*., 1996).

In this paper, we show that activation of Fas on A20 murine B lymphoma cells by anti-Fas mAb results in increased tyrosine phophorylation of several cellular proteins, and this increase in tyrosine phosphosphorylation is partly responsible for Fas-induced elevation in PLD activity. D609, a xanthogenate compound previously known as a specific inhibitor of PC-PLC (Schütze *et al*., 1992), is shown to inhibit Fas-induced tyrosine phosphorylation as well as PC-PLC activity. Hence D609 could block these two Fas-downstream signals leading to activation of PLD, resulting in a complete blockade of Fas-mediated PLD activation.

Materials and Methods

Materials

Anti-Fas mAb (Jo2) was obtained from Pharmingen (San Diego, CA). Authentic lipid standards, *Bacillus cereus* PC-PLC and D609 was from Sigma (St. Louis, MO). Genistein was from Tocris (Bristol, UK). [^{3}H]-palmitic acid, and [*methyl*-^{3}H]-choline chloride were purchased from Du Pont-New England Nuclear (Boston, MA). Fetal bovine serum (FBS), penicillin/streptomycin solution and RPMI-1640 medium were from Gibco-BRL (Gaithersburg, MD). Monoclonal anti-phosphotyrosine antibody was from Upstate Biotechnology (Waltham, MA). Polyclonal anti-protein kinase $C_β$ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemical agents were of analytical grade.

Cell culture

A20 murine B lymphoma cell line obtained from American Type Culture Collection (ATCC TIB208) was cultured at 37°C in RPMI-1640 medium supplemented with 10%(v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells grown at 37°C in a humidified CO_2-controlled (5%) incubator were then washed with RPMI-1640 medium containing 0.1% (w/v) bovine serum albumin, 100 units/ml penicillin and 100 μg/ml streptomycin (serum-free medium) and incubated in a serum-free medium at 37°C for 18 h before stimulation with anti-Fas mAb.

Determination of PLD activity

PLD activity was determined by the formation of phosphatidylethanol (PEt) as described elsewhere (Park *et al.,* 1999). Briefly, A20 cells cultured on 6-well plates were metabolically labeled with 1 μCi/ml of [^{3}H]-palmitic acid in serum free medium for 18 h. The cells were then pretreated with 1%(v/v) ethanol for 15 min before stimulation with anti-Fas mAb. After stimulation, the cells were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer (1959), and PEt was separated by a thin layer chromatography (TLC) using a solvent system of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, by vol). The regions corresponding to the authentic PEt bands were identified with 0.002% (w/v) primulin in 80%(v/v) acetone, scraped and counted using a scintillation counter.

Tyrosine phosphorylation

Serum-starved A20 cells ($1×10^6$ cells/ml) on 100 mm dishes were incubated with 200 ng/ml of anti Fas mAb for 5 min. Where indicated, D609 (50 μg/ml) and genistein (10 μM) were treated for 30 min before stimulation with anti-Fas mAb. Stimulation was stopped by the addition of 500 μl of ice-cold lysis buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM NaF, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM Na_2VO_4, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 25 mM HEPES, pH 7.4). After lysis, insoluble materials and celluar debris were excluded by centrifugation at 3,000 *g* for 5 min, and detergent soluble proteins were resolved by 10% SDS-PAGE before electrophoretic transfer to a nitrocellulose membrane. The membrane was blocked by overnight incubation with tris-buffered saline containing 0.01% Tween-20 (TTBS) with 5% (w/v) bovine serum albumin and further incubated for 1 h with diluted specific anti-phosphotyrosine antisera (1 μg/ml). The blots were then detected by using ECL western blotting detection reagents Amersham Pharmacia.

Measurement of DAG

A20 cells grown in 6-well plates were labeled with 1 μCi/ml of [^{3}H]-palmitic acid for 18 h in a serum-free medium. Following stimulation of the cells with anti-Fas monoclonal antibody, cells were quickly washed with ice-cold PBS, and then lipids were extracted using the Bligh and Dyer method (1959). The bottom layer was dried and applied to a silica gel TLC plate. The plate was developed using a solvent system of toluene/ether/ethanol/concentrated NH_4OH (50:30:20:0.2, by vol). The diacylglycerol (DAG) bands were identified with primulin, scraped and then counted using a scintillation counter.

Measurement of water-soluble products of phosphatidylcholine hydrolysis

The analysis of water-soluble products of phosphatidyl-

choline breakdown was performed according to the method of Cook and Wakelam (1989) with some modifications. A20 cells on 6-well plates were metabolically labeled with 1 μCi/ml of [*methyl*-^{3}H]-choline chloride in the serum-free medium for 18 h. Following removal of the labeling medium, the cells were washed with fresh serum-free medium and stimulated with anti-Fas monoclonal antibody. After stimulation, the cells were resuspended in 0.5 ml of ice-cold methanol and allowed to sit on ice for 10 min. Then choloroform and water were added to a final ratio of 1:1:0.9 (choloroform/methanol/water). The upper methanolic phase was diluted to 5 ml with water and applied onto Dowex-50-W column (1 ml bed volume) to achieve separation of phospho[^{3}H]-choline from [^{3}H]-choline. After washing with 4 ml of water, phosphocholine was eluted with an additional 20 ml of water and choline was eluted with 20 ml of 1 M HCl. Aliquots of each fraction were collected and liquid scintillation counting quantitated associated radioactivity.

Translocation of PKC

Serum-starved cells were incubated with 200 ng/ml anti-Fas monoclonal antibody for 30 min or 3 h, scraped in PBS and harvested by microcentrifugation. The cells were then resuspended in 1 ml of lysis buffer (50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5) and disrupted by sonication. The cytosolic fraction was separated from the particulate fraction by centrifugation at 100,000 *g* for 1 h. SDS-polyacrylamide gel electrophoresis was performed using 10% acrylamide, and proteins were transferred onto nitrocellulose membranes using a Bio-Rad semi-dry transfer system. The membranes were blocked for 1 h with TTBS with 5% (w/v) bovine serum albumin and incubated for another hour with diluted specific anti-PKC antisera (1 μg/ml). Following incubation with alkaline phosphatase conjugated-anti-rabbit IgG, the blots were developed using the Phosphatase Substrate System (Kirkegaard and Perry Laboratories).

Results and Discussion

Fas cross-linking resulted in sequential activation of PC-PLC, PKC and PLD in A20 murine B lymphoma cells (Han *et al*., 1999). D609-sensitive activation of PC-PLC and subsequent activation of PKC βI and βII are thought to be responsible for Fas-induced activation of PLD in A20 cells. As shown in Figure 1, there is more than a four-fold rise in PLD activity as determined by [^{3}H]-PEt formation in A20 cells by treatment of 200 ng/ml of anti-Fas mAb for 3 h. However, exogenous *Bacillus cereus* PC-PLC and phorbol 12-myristate 13-acetate (PMA) can only activate PLD to approximately 2 fold (Figure 1). It is

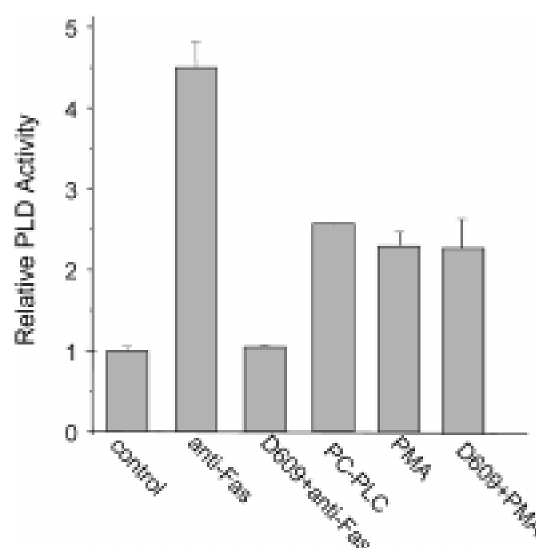

Figure 1. Fas-induced elevation in PLD activity is completely inhibited by D609 pretreatment. A20 cells were seeded onto 6-well plates at the density of 1×10^6 cells/ml and labeled with 1 μCi/ml of [^{3}H]-palmitic acid in serum free medium for 18 h. Transphosphatidylation by PLD was initiated by adding 1% (v/v) ethanol 15 min before stimulation with anti-Fas mAb or 2 h 45 min before treatment of *Bacillus cereus* PC-PLC or PMA, respectively. Control cells were treated with ethanol for 3 h and 15 min. Anti-Fas monoclonal antibody (200 ng/ml), *Bacillus cereus* PC-PLC (5U/ml) and PMA (200 nM) were treated to the cultures for 3 h, 30 min and 30 min, respectively. D609 at the concentration of 50 μg/ml was pretreated for 30 min prior to ethanol treatment. After the stimulation, lipids were extracted and [^{3}H]-PEt bands were separated by TLC as described in Materials and Methods. Radioactivity associated with [^{3}H]-PEt bands was counted using a scintillation counter, and the relative ratio of [^{3}H]-PEt counts to the total count of [^{3}H]-palmitic acid-labeled lipid was shown as PLD activity. Data are means±S.D. from triplicate experiments.

well known that PMA activates PLD *via* PKC-dependent mechanism (Exton, 1994), and PMA at the concentration of 200 nM for 30 min indeed induces activation of PKC almost to the same level as in Fas-stimulated cells (Han *et al*., 1999). Exogenous PC-PLC was also shown to be able to activate PLD, and this activation mechanism involved all the PC-PLC downstream events including generation of DAG and activation of PKC (Shin *et al*., 2000). Because of the facts that PMA and exogenous PC-PLC induced relatively slight increase in PLD activity as compared to Fas-stimulated cells and that both agents were able to activate PKC almost comparable to Fas-stimulated cells, we came to the conclusion that there might be another pathway leading to the activation of PLD initiated by Fas-ligation.

Data in Figure 1 also identify that D609, a selective inhibitor of PC-PLC, completely inhibits Fas-induced PLD activation, and there is no effect on PMA-induced activation of PLD. This had led us to postulate that PC-PLC is the enzyme solely responsible for transmitting a Fas-induced signal to PKC/PLD, and D609 selectively acts on PC-PLC and not directly on PLD. If the D609-sensitive PC-PLC-dependent pathway is the only mechanism

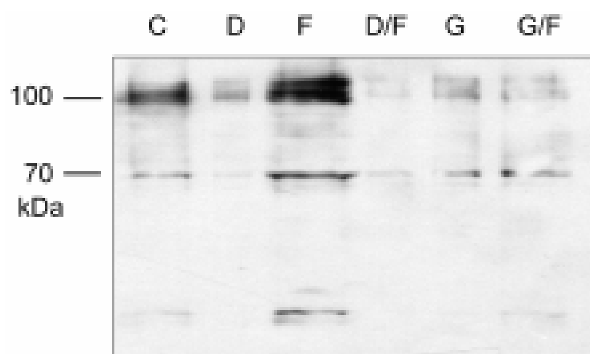

Figure 2. D609 inhibits tyrosine phosphorylation induced by anti-Fas mAb. Serum starved A20 cells were either untreated (C), or treated with D609 for 30 min (50 μg/ml, D), anti-Fas mAb for 5 min (200 ng/ml, F), D609 and anti-Fas mAb for 30 min and 5 min, respectively (D/F), genistein for 30 min (10 μM, G), or genistein and anti-Fas mAb for 30 min and 5 min, respectively (G/F). Cells were lysed and equivalent amounts of proteins were resolved on 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibody. Blots were then detected with ECL detection reagents.

responsible for Fas-induced activation of PLD, both PMA and exogenous PC-PLC should lead to the activation of PLD to almost the same level as that of Fas-stimulation. However, the data in Figure 1 show that this is not the case.

To account for this discrepancy, tyrosine phosphorylation elicited by Fas cross-linking was chosen as another possible candidate for transmitting Fas-induced, D609-sensitive signal to the activation of PLD. As shown in Figure 2, Fas-ligation resulted in a prompt and prominent increase in tyrosine phosphorylated proteins in 5 min. When the cells were pretreated with D609 at the concentration of 50 μg/ml for 30 min, the Fas-induced increase in tyrosine phosphorylation was completely diminished. The same inhibitory effect on tyrosine phoshorylation was also evident in genistein, a tyrosine kinase inhibitor, treated cells. To our knowledge, this is the first report regarding an inhibitory effect of tyrosine kinase by D609. The effect of D609 on other protein kinases was reported by Warlo and Rosenthal (1997), where the authors showed *in vitro* inhibitory effect of D609 on PKC and no effect on casein kinase 1 and cAMP-dependent protein kinase. However, in our previous report (Han *et al*., 1999), D609 cannot inhibit PKC directly to prevent PMA-induced PKC translocation *in vivo.*

Convinced that D609-sensitive tyrosine phosphorylation occurred by Fas-ligation, we sought to investigate a possible involvement of tyrosine phosphorylation in Fas-induced PLD activation. There is some evidence of a role played by tyrosine phosphorylation in the activation of PLD. Bourgoin and Grinstein (1992) reported that treatment of permeabilized HL-60 cells with vanadyl hydroperoxide induced accumulation of tyrosine phosphorylated proteins and concomitant activation of PLD. Inhibitory effect of tyrosine kinase inhibitors on agonist-induced PLD activation was shown in neutrophils (Uings *et al*., 1992) and in RBL cells (Kumada *et al*., 1993). Furthermore, the v-Src activated cells showed an increase in transphosphatidylation activity, indicating the activation of PLD (Song *et al*., 1991).

In Figure 3A, the effect of tyrosine kinase inhibitor on Fas-induced PLD activity is shown. A tyrosine kinase inhibitor genistein, at the concentration of 10 μM for 30 min, partially reduced Fas-induced PLD activity to almost the same level of PLD activity in PMA-treated cells. However, there was no significant effect of genistein on PMA-induced PLD activity, confirming that the stimulatory effect of PMA on PLD is only mediated by PKC. These data suggest that Fas-induced activation of PLD might occur through two mutually independent pathways, genistein-sensitive pathway involving tyrosine phosphorylation and genistein-insensitive pathway that occurs via activation of PC-PLC and PKC. Both PMA and Fas seem to share PKC as a common mediator in stimulating PLD activity, and Fas might have an additional stimulatory effect on PLD *via* tyrosine phosphorylation.

Next, it was necessary to examine the effect of genistein on PC-PLC activity, DAG level and PKC activity since there still remains a possibility that tyrosine kinase might intervene in PC-PLC/PKC pathway to mediate Fas-induced signal. Genistein at the concentration sufficient to perturb tyrosine phosphorylation (10 μM), failed to elicit any changes in PC-PLC activation by Fas-ligation (Figure 3B). Data in Figure 3C also shows that Fas-induced elevation in DAG level after 30 min of anti-Fas treatment was not changed by pretreatment of genistein. However, after Fas-ligation for 3 h, there was some inhibitory effect of genistein on Fas-induced elevation of DAG level. As we reported previously (Han *et al*., 1999), the initial surge in Fas-induced DAG level resulted from the activation of PC-PLC, and the later sustained increase in DAG resulted from PLD and subsequent activation of phosphatidic acid phosphohydrolase. Therefore, the reduced level of DAG in 3 h after Fas-stimulation in genistein pretreated cells is the result of the inhibitory effect of genistein on PLD, not on PC-PLC.

In order to determine the effect of genistein on PKC activation by Fas-ligation and PMA, the genistein-pretreated cells were stimulated either with anti-Fas mAb or PMA and assayed for PKC activation (Figure 3D). Since PKC is redistributed from cytosol to membrane after activation, we have tested activation of PKC by measuring PKC translocation. Pretreatment of genistein failed to block the translocation of PKC βI and βII from cytosol to membrane upon anti-Fas mAb and PMA treatment, suggesting that PKC activity stimulated by anti-Fas mAb or PMA was not changed by interfering with tyrosine phosphorylation.

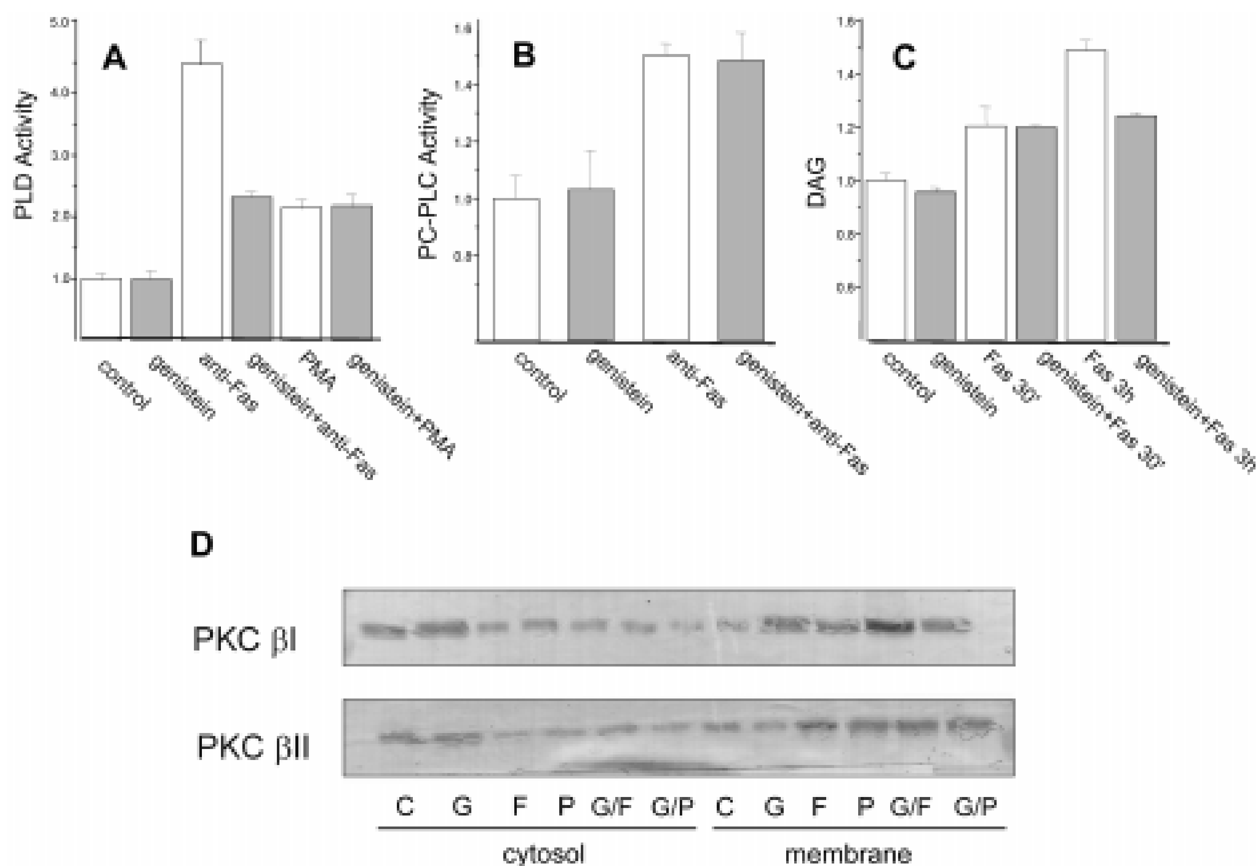

Figure 3. Effect of genistein on Fas-induced PLD activity (A), PC-PLC activity (B), DAG level (C) and PKC translocation (D). (A), A20 cells were labeled with 1 μCi/ml of [^{3}H]-palmitic acid in serum free medium for 18 h. Transphosphatidylation by PLD was initiated by adding 1% (v/v) ethanol either 15 min or 2 h 45 min before stimulation with anti-Fas mAb or PMA, respectively. For the inhibition of tyrosine kinase, 10 μM of genistein was pretreated 30 min prior to ethanol treatment. After the stimulation, lipids were extracted and [^{3}H]-PEt bands were separated by TLC as described in Materials and Methods. Radioactivity associated with [^{3}H]-PEt bands was counted using a scintillation counter, and the relative ratio of [^{3}H]-PEt counts to the total count of [^{3}H]-palmitic acid-labeled lipid was shown as PLD activity. Data are means±S.D. from triplicate experiments. (B), A20 cells labeled with 1 μCi/ml of [*methyl*-^{3}H]-choline chloride in serum free medium for 18 h were treated with genistein (10 μM) for 30 min, or anti-Fas mAb (200 ng/ml) for indicated times. Intracellular levels of choline phosphates were determined as described in Materials and Methods. Data are means±S.D. from triplicate experiments. (C), Cells on 6-well plates (1×10^6 cells/ml) labeled with 1 μCi/ml of [^{3}H]-palmitic acid for 18 h were treated with genistein (10 μM) for 30 min or anti-Fas mAb (200 ng/ml) for 30 min. Lipids were then extracted and intracellular content of DAG was monitored by TLC analysis as described in Materials and Methods. Data are means±S.D. from triplicate experiments. (D), Cells on 100 mm dishes at the density of 1×10^6 cells/ml were serum-starved for 18 h and stimulated with either 200 ng/ml of anti-Fas monoclonal antibody for 3 h or 200 nM of PMA for 30 min. For the inhibition of tyrosine kinase, 10 μM of genistein was pretreated 30 min before stimulation with agonists. Subcellular fractionation of proteins and subsequent western blots were performed as described in Materials and Methods. C, control; G, genistein; F, anti-Fas mAb; P, PMA; G/F, genistein plus anti-Fas mAb; G/P, genistein plus PMA.

Taken together, these data suggest that stimulation with anti-Fas mAb activates two independent pathways, PC-PLC/PKC pathway and tyrosine kinase pathway, both leading to the activation of PLD. Also, our additional novel finding is that D609, previously known as a specific inhibitor of PC-PLC, can inhibit both PC-PLC and tyrosine kinase in Fas-induced signal transduction, respectively. We still could not completely exclude the possibility that tyrosine kinase might be located somewhere downstream of PC-PLC. But it is highly unlikely that tyrosine kinase acts downstream of PC-PLC in transmitting Fas-induced signal, since tyrosine phosphorylation by Fas-ligation occurs very rapidly (within minutes) and maximum activation of PC-PLC is achieved in 30 min after Fas-stimulation (Han *et al*., 1999).

Although there is mounting evidence indicating that PLD might be regulated by tyrosine kinases (Eischen *et al*., 1994; Atkinson *et al*., 1996; Janssen *et al*., 1996; Schlottmann *et al*., 1996; Simon *et al*., 1998), the exact mechanism is yet to be elucidated. Because the Fas antigen does not contain intrinsic kinase activity (Itoh *et al*., 1991), the possible Fas downstream tyrosine kinase responsible for transmitting Fas-induced signal is currently under investigation. Recently, tyrosine kinase Lyn (Simon *et al*., 1998) and Fyn (Atkinson *et al*., 1996) are suggested as possible mediators of Fas-induced signaling. Clearly, the identification of a physiologically relevant kinase associated with the Fas antigen and elucidation of

mechanisms by which tyrosine phosphorylation modulates PLD activity should be further elucidated in the future.

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