THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 30, Issue of July 28, pp. 23065–23073, 2000

Printed in U.S.A.

Regulation of Phospholipid Scramblase Activity during Apoptosis and Cell Activation by Protein Kinase Cδ*

Received for publication, April 12, 2000, and in revised form, April 17, 2000
Published, JBC Papers in Press, April 18, 2000, DOI 10.1074/jbc.M003116200

S. Courtney Frasch‡, Peter M. Henson, Jenai M. Kailey, Donald A. Richter, Michael S. Janes, Valerie A. Fadok, and Donna L. Bratton

From the Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206

Phospholipid scramblase induces nonspecific bidirectional movement of phospholipids across the membrane during cell activation and has been proposed to mediate the appearance of phosphatidylserine (PS) in the plasma membrane outer leaflet during apoptosis, a cell surface change that is critical for apoptotic cell removal. We report here that protein kinase C (PKC) δ plays an important role in activated transbilayer movement of phospholipids and surface PS exposure by directly enhancing the activity of phospholipid scramblase. Specific inhibition of PKCδ by rottlerin prevented both apoptosis- and activation-induced scramblase activity. PKCδ was either selectively cleaved and activated in a caspase 3-dependent manner (during apoptosis) or translocated to the plasma membrane (in stimulated cells) and could directly phosphorylate scramblase immunoprecipitated from Jurkat cells. Furthermore, reconstitution of PKCδ and scramblase, but not scramblase or PKCδ alone in Chinese hamster ovary cells demonstrated enhanced scramblase activity.

Normal circulating blood cells exhibit an asymmetric distribution of phospholipids in the membrane where phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) reside in the inner leaflet and phosphatidylcholine (PC) and sphingomyelin are enriched on the outer leaflet (1, 2). In the resting cell, phospholipid asymmetry is relatively stable with slow exchange of phospholipids between the bilayers. Escape of PS or PE to the outer leaflet is quickly corrected by the action of an aminophospholipid translocase (APLT) (2) that selectively transports aminophospholipids such as PS, and to a lesser extent PE, from the outer leaflet back to the inner leaflet. In contrast, calcium-dependent bidirectional movement of phospholipids across the membrane, termed "scrambling," shows no selectivity for the phospholipid species or the direction of movement (3–6). A candidate scramblase was recently cloned from human erythrocytes, which exhibits such characteristics (7). Low baseline phospholipid scramblase activity is hypothesized to account for the slow exchange of phospholipids observed in resting cells. When activated, it is hypothesized that this protein induces enhanced exchange in cell activation and apoptosis.

Enhanced scramblase activity is an important feature during apoptosis and cell stimulation. Apoptosis, a fundamental process occurring in virtually all cell types, is characterized by distinct and separable biochemical and morphological changes. The most prominent feature typically occurs at the level of the nucleus and includes chromatin condensation and DNA fragmentation. Cell surface alterations are critical for apoptotic cell removal and include exposure of PS on the outer leaflet of the plasma membrane. We have previously shown that PS exposure serves as at least one important and necessary signal for their quiescent removal by phagocytes (8–11) and have recently identified a novel PS receptor that appears to mediate the recognition of apoptotic cells by phagocytes leading to their subsequent ingestion (12).

Activation of phospholipid scramblase is also a feature of activated cells and can be observed in neutrophils stimulated with fMLP or in platelets stimulated with thrombin plus collagen (3, 4, 13). Stimulation of platelets results in the externalization of PS on the outer leaflet of the plasma membrane. This externalized PS serves as a catalytic surface for the assembly of coagulation factors, therefore, initiating the coagulation cascade. Although the physiological role of the membrane randomization that occurs in activated leukocytes is less clear, it has been shown to allow release of platelet-activating factor and lyso- and oxidized phospholipids and may play a role in membrane protein function (3, 4, 14–17).

During apoptosis and with some stimuli, loss of transbilayer asymmetry appears to result from both enhanced phospholipid scramblase activity as well as a loss in APLT (18, 19). However, loss of APLT alone is not sufficient for surface exposure of PS and requires a concomitant increase in scramblase activity (18, 20). How scramblase activity is regulated in stimulated cells or during apoptosis is unknown at this time. The deduced amino acid sequence of a recently cloned candidate scramblase from human erythrocytes, however, reveals a putative protein kinase C (PKC) phosphorylation site, suggesting that phosphorylation by PKC may be one mechanism by which scramblase activity is modulated (7).

PKC isoforms belong to a large family of serine/threonine protein kinases containing at least 12 members (for review, see Ref. 21). Each member shows a high degree of homology in the catalytic region, but varies with regard to tissue distribution and activation requirements. Although the presence of intra-

* This work was supported by National Institutes of Health Grants GM48211 and HL34303 and a Great West Life Assurance fellowship (to S. C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 303-398-1282; Fax: 303-398-1381; E-mail: fraschc@njc.org.

[1] The abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; APLT, aminophospholipid translocase; fMLP, *N*-formyl-Met-Leu-Phe; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; DEVD-fmk, Asp-Glu-Val-Asp-fluoromethyl ketone; FITC, fluorescein isothiocyanate; cPAF, carbamyl-platelet-activating factor; NBD, (7-nitro-2-1,3-benzoxadiazol-4-yl); PI, propidium iodide; BSA, bovine serum albumin; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; HBS, HEPES-buffered saline; PBS, phosphate-buffered saline; PMN, polymorphonuclear cell; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

cellular PS is necessary for activation of all PKC isoforms, the requirement for additional activation cofactors can divide the family into three major groups. The conventional group, which includes α, βI, βII, and γ, is Ca^{2+}-dependent, and members are activated by phorbol esters. The novel group, which includes ϵ, δ, θ, and η, is Ca^{2+}-independent, but members are activated by phorbol esters. The atypical group includes, ζ, λ, ι, and μ, and all are insensitive to Ca^{2+} and activation by phorbol esters. The functional response elicited by PKC depends largely on the isoform and initial signal as well as the cell type.

PKCs have been suggested to play an important role in a variety of cellular functions including cell proliferation, differentiation, and activation. There are conflicting reports regarding the role of PKCs during apoptosis, complicated by the cell type used and the initial stimulus, as well as the PKC isoform investigated. Several studies have suggested a protective role for PKCs based upon experiments in which apoptosis is inhibited in cells pretreated with PMA (22). Alternatively, in some cell types, treatment with PMA induces apoptosis (23). Therefore, whether PKC will induce or protect from apoptosis may vary with cell type, stimulation conditions, and PKC activated. Recently it has been demonstrated that both PKCδ and PKCθ are cleaved during apoptosis by the interleukin-1-β-converting enzyme-like protease caspase 3 (CPP32) (24–27). Cleavage by caspase 3 generates a 45-kDa catalytic fragment and results in an increase in enzymatic activity, suggesting an active role for either PKCδ or PKCθ during apoptosis. Accordingly, we have investigated a potential role for PKCδ in the regulation of scramblase activity in stimulated cells and in those undergoing apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Endotoxin free agents and plastic dishes were used throughout this study. Jurkat cells were maintained at 1×10^6 cells/ml in RPMI 1640 plus 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Human neutrophils were isolated by the plasma Percoll method as described previously (28). Chinese hamster ovary (CHO) cells were maintained in α-minimal essential medium plus 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Reagents were purchased from the following sources. Anti-protein kinase C isoform antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphothreonine antibody was from Alexis Biochemicals (San Diego, CA). Anti-Fas IgM (CH-11) and PKC activity assay kit were from Upstate Biotechnology Inc. (Lake Placid, NY). Protein A-Sepharose was from Zymed Laboratories Inc. Rottlerin, Gö6976, phosphatase inhibitors, dioctanoylglycerol, PKCϵ peptide substrate, and recombinant PKCα, PKCδ, and PKCζ were from Calbiochem. DEVD-fmk was from Enzyme Systems Inc. Phosphatidylserine, NBD-PC, and NBD-PS were from Avanti Polar Lipids, Inc. (Alabaster, AL). FITC-annexin V was from R&D Systems (Minneapolis, MN). Rat PKCδ cloned into pTB was a generous gift from Dr. Yoshitaka Ono.

Cloning of Scramblase and Antibody to Scramblase—RNA isolated from Jurkat cells was synthesized to cDNA using a reverse transcriptase for PCR kit (CLONTECH) according to the manufacturer's instructions. cDNA was used as a template to amplify full length scramblase using the forward primer 5′-GGCAGCCAGAGAACTGTTTTA-3′ and reverse primer 5′-GCAGTTTTTCAAAGGAAGTTTCA-3′. PCR products were analyzed by agarose gel electrophoresis and cloned directly into pCR2.1 (Invitrogen). Positive colonies were analyzed by restriction enzyme digestion with *Eco*RI and sequencing to confirm the presence of the correct insert. Full-length scramblase was then subcloned into the *Eco*RI site of pcDNA3.1.

Antibodies to the amino-terminal peptide CESTGSQEQKSGVW were made by Peptide Express (Fort Collins, CO). The peptide was synthesized and linked to keyhole limpet hemocyanin and used as an immunogen to innoculate two rabbits. At 3, 6, and 9 weeks, the rabbits were bled and serum was collected and tested by Western immunoblot analysis.

Site-directed Mutagenesis of Scramblase—Human scramblase cloned into pcDNA3.1 was used as a template for site-directed mutagenesis of threonine 161 to alanine using an overlap extension PCR-based method as described (29). Basically, two fragments were generated by PCR. Fragment AB was generated using primer A (5′-GGCAGCCAGAGAACTGTTTTA-3′), which corresponds to the 5′ end of scramblase open reading frame; primer B (5′-TCCTCAAGG**C**AAAAGGTCTAG-3′), which corresponds to the PKC phosphorylation site and incorporates a threonine to alanine change at position 161 (bold letter); primer C, 5′-CTAGACCTTTT**G**CCTTGAGGA-3′, which is complementary to primer B and incorporates the threonine to alanine change at position 161 (bold letter); and primer D, 5′-GCAGTTTTTCAAAGGAAGTTTCA-3′, which corresponds to the 3′ end of scramblase. To generate full-length scramblase mutant sequence, fragments AB and CD were gel-purified and quantitated, and 20 ng of each fragment was used as a template for the full-length fragment using primers A and D. The full-length PCR product was cloned directly into pCR2.1 (CLONTECH) sequenced to verify the incorporation of the mutation and subcloned into the *Eco*RI site of pTB and pcDNA3.1 (Invitrogen).

Induction of Apoptosis and Cell Stimulation—Jurkat cells were harvested and resuspended to 10×10^6 cells/ml in RPMI 1640 plus 10% fetal bovine serum and plated in a 12-well tissue culture plate at 1 ml/well. Anti-Fas IgM was added at 400 ng/ml and incubated at 37 °C for the indicated times. Percentage of apoptosis was determined by binding of FITC-labeled annexin V or morphologically by analysis of nuclear condensation by stained cytospin preparations. For cell activation, neutrophils were resuspended at 5×10^6 cells/ml in KRPD supplemented with 0.25% lipopolysaccharide-free human serum albumin and stimulated with 100 nM fMLP at 37 °C for the indicated times. Transfected CHO cells were stimulated with 2 ng/ml PMA plus 0.5 μM calcium ionophore for 15 min.

PS Expression and Scramblase Activity—Cells bearing PS in the plasma membrane outer leaflet were identified as those binding FITC-labeled annexin V (Caltag, Burlingame, CA). The binding of FITC-labeled annexin V to phosphatidylserine on the surface of apoptotic cells correlates with the appearance of nuclear and cytoplasmic condensation by light microscopy. For staining, 10^5 cells were pelleted, then resuspended in 100 μl of HEPES-buffered saline (HBS) with 2.5 mM $CaCl_2$. The cells were transferred to a staining tube containing 400 ng of FITC-labeled annexin V and 500 ng of propidium iodide. The cells were incubated 15 min at room temperature, and then the samples were transferred to ice and the sample volume brought to 0.5 ml. The cells were examined on a Coulter XL (Miami Fl) flow cytometer, and the results analyzed with PC Lysys software (Becton Dickinson, Franklin Lakes, NJ). Annexin-positive cells were determined by setting quadrants to separate viable cells from PI-permeant cells (annexin+/PI−), and non-apoptotic cells from those staining highly for the FITC-labeled annexin V probe. Percentage of annexin-positive cells was determined from the cells staining greater than the control population threshold. Mean fluorescence of the PI impermeant cells was simultaneously determined.

Phospholipid uptake was examined to measure phospholipid scramblase activity as described previously (3, 4, 18). NBD-labeled phosphatidylcholine was prepared by drying 1 μg of 1-palmitoyl-1-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) in a glass tube. Previous studies have shown that these NBD-labeled probe lipids are readily solubilized in aqueous media containing albumin and will partition into the plasma membrane outer leaflet. Following the incubation period, 5×10^5cells were harvested, washed once, and then resuspended in 50 μl of HBS, 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4, with 1 mM $CaCl_2$. The cells were then transferred to a staining tube containing 1 ml of the lipid suspension and 5 μl of 50 μg/ml propidium iodide to check for plasma membrane integrity. These were allowed to incubate for 10 min at room temperature, after which unincorporated PC was back-extracted with 50 μl of 1% BSA in HBS for an additional 5 min. This back-extraction has been shown to remove NBD-labeled lipid from the surface of the cell membrane, leaving only that which has flipped to the interior leaflet of the membrane. The samples were then transferred to an ice bath, diluted to a final volume of 600 μl of HBS and run on a Coulter XL (Miami, FL) flow cytometer, and the results were analyzed with PC Lysys software. The mean fluorescence values of the phospholipid uptakes were determined by setting quadrants in such a manner as to separate cells staining positively for propidium iodide (dead or highly permeant cells) from viable cell populations. Mean fluorescence was determined by taking the weighted mean fluorescence of the PI-impermeant cells.

cPAF Uptake—Uptake of radiolabeled carbamyl-platelet-activating factor ([^{3}H]cPAF) was carried out as described previously (4). Basically, cells were harvested and resuspended at 10×10^6/ml in KRPD supplemented with 0.25% lipopolysaccharide-free BSA. Uptake of [^{3}H]cPAF (0.35 mCi) took place over 15 min with simultaneous stimulation with 100 nM fMLP at 37 °C. Samples were washed with two volumes of 2%

BSA in KRPD at 4 °C and sedimented at 16,000 × *g* for 1 min to remove labeled lipid present on the outer leaflet of the plasma membrane. The supernatant was removed, and the pellets were resuspended in 500 μl of 1% Triton X-100. Uptake of labeled lipid was determined by scintillation counting. To test inhibitors, cells were preincubated with the inhibitor for 30 min prior to stimulation and addition of [^{3}H]cPAF.

PKC Immunoprecipitation and Activity Assay—PKCα, PKCδ, and PKCζ activities were measured in an *in vitro* kinase assay following immunoprecipitation from Jurkat cells. Following stimulation, Jurkat cells (80×10^6) were harvested and resuspended in 2 ml of lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 15 μg/ml aprotinin, 15 μg/ml leupeptin, 500 μM AEBSF) and disrupted by nitrogen cavitation at 350 p.s.i. for 8 min. Lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C to remove insoluble material and unbroken cells. For immunoprecipitation, 500 μl of 4× immunoprecipitation buffer (560 mM NaCl, 60 mM HEPES, pH 7.4, 4% Triton X-100) was added to 1.5 ml of cell lysate containing equal amounts of protein as determined by the Bradford protein assay, and 500 μl was used for immunoprecipitation. PKCα, PKCδ, and PKCζ were immunoprecipitated with 1 μg/ml amount of the appropriate antibody. For PKCα activity, protein A-Sepharose beads with bound antibody were resuspended in 50 μl of PKCα reaction mix containing 20 mM MOPS, pH 7.4, 25 mM β-glycerophosphate, 1 mM $CaCl_2$, 1 mM DTT, 100 μM PKC substrate, 5 μg of PS, 0.5 μg of dioctanoylglycerol, 100 μM ATP, 25 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP and incubated at 30 °C for 20 min. The reaction was terminated by centrifugation of the beads and spotting 25 μl of the supernatant onto P-81 phosphocellulose paper, followed by washing in 0.5% phosphoric acid. The amount of phosphorylated substrate was quantitated by scintillation counting. Laemmli sample buffer was added to the remaining reaction mix, and proteins were separated by 10% SDS-PAGE, blotted to nitrocellulose membrane, and the amount of immunoprecipitated PKC determined by Western immunoblotting. PKCδ activity was measured as for PKCα except that the reaction mix contained 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 100 μM EGTA, 1 mM DTT, 6 μg of PKCε peptide, 20 μg of PS, 2 μg of dioctanoylglycerol, 200 μM ATP, 10 μCi of [γ-^{32}P]ATP. PKCζ activity was measured as for PKCα and PKCδ, except that the reaction mix contained 25 mM Tris-HCl, pH 7.5, 500 μM EGTA, 1 mM DTT, 100 μM ATP, 10 μg of PKCα peptide, 10 μg of PS, 10 μCi of [γ-^{32}P]ATP.

Immunoprecipitation and Phosphorylation of Scramblase—Jurkat cells (20×10^6) were lysed in 500 μl of radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 7.2, 0.1% SDS, 150 mM NaCl, 0.5% DOC, 1% Triton X-100, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 15 μg/ml aprotinin, 15 μg/ml leupeptin, 500 μM AEBSF), followed by centrifugation at 12,000 × *g* for 10 min at 4 °C to remove insoluble material. Scramblase was immunoprecipitated with 5 μl of anti-scramblase rabbit serum. Protein A-Sepharose beads with bound antibody were resuspended in 40 μl of PKC reaction buffer as above except PKC peptide substrate was eliminated. Recombinant-active PKCα, PKCδ, or PKCζ were diluted 1:100, 1:10, and 1:10, respectively, in 10 mM Tris, pH 7.5, 5 mM DTT, 0.01% Triton X-100, and 2 μl of the diluted enzyme was added to the reaction mixture and incubated for the indicated times at 30 °C. The reaction was terminated by the addition of 4× Laemmli sample buffer. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylation of scramblase was visualized by autoradiography. Equal loading of scramblase was confirmed by Western immunoblotting.

Transient Transfection—Transfection of PKCδ and scramblase into CHO cells was done by using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer. CHO cells were plated at a density of 0.4×10^6 cells/well in a six-well dish 24 h prior to transfection. Transfected cells were analyzed 48 h after transfection for scramblase activity and protein expression by uptake of radiolabeled cPAF and Western immunoblotting, respectively.

^{32}P Labeling of Jurkat Cells and Phosphoamino Acid Analysis—Jurkat cells were harvested and resuspended at 20×10^6 cells/ml in phosphate-free RPMI with L-glutamine supplemented with 25 mM HEPES, pH 7.4, 0.25% BSA, 1% penicillin, and 1% streptomycin and starved for 1 h at 37 °C in a 5% CO_2 atmosphere. Cells were washed once and resuspended at 20×10^6 cells/ml in phosphate-free media as above, and 100 μ Ci of [^{32}P]orthophosphoric acid was added to each well and incubated for 2 h at 37 °C in a 5% CO_2 atmosphere, after which time anti-Fas IgM was added at 400 ng/ml. Cells were harvested at the indicated times after anti-Fas stimulation, washed five times with PBS, and lysed in 500 μl of 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholic acid, 1 mM EGTA. Insoluble material was removed by centrifugation at 14,000 rpm for 10 min. The cell lysate was precleared with 15 μl of protein A-Sepharose bead slurry for 30 min at 4 °C, and then scramblase was immunoprecipitated as above. Protein A-Sepharose beads were washed five times with PBS, resuspended in sample buffer, and heated to 100 °C for 5 min. Phosphorylated scramblase was separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and visualized by autoradiography. Equal loading of immunoprecipitated scramblase was confirmed by Western immunoblot analysis. Radioactive scramblase bands were excised and hydrolyzed in 6 N HCl at 110° for 1 h and washed three times with dH_2O in a SpeedVac centrifuge and resuspended in 5 μl of dH_2O with 1 μg each of cold Ser(P), Thr(P), and Tyr(P) as internal standards. Hydrolyzed phosphoamino acids were separated on two-dimensional electrophoresis in 2.5% formic acid, 7.8% acetic acid, pH 1.9 at 1.9 kV for 20 min in the first direction and 20 min at 1.3 kV in the second direction in 5% acetic acid, 0.5% pyridine, 0.5 mM EDTA, pH 3.5. Radioactive spots were visualized by autoradiography, and location of standards were visualized by ninhydrin staining.

Immunohistochemistry and Confocal Microscopy: PKCδ and F-Actin Staining—Human polymorphonuclear leukocytes were resuspended to 5×10^6/ml in PBS and divided into 100-μl aliquots of 5×10^5 cells. PMNs were treated with or without 100 nM fMLP for the times indicated before being fixed for 10 min at 37 °C with 2% paraformaldehyde in 15% sucrose/PBS. Following fixation, cells were permeabilized with 0.02% Tween 20/PBS for 10 min at room temperature. Cells were resuspended in 100 μl of PBS containing 0.5 μg of mouse anti-human CD16 and 0.5 μg of mouse anti-human CD32 (PharMingen) for 15 min at room temperature to block Fc receptors. Cells were then blocked in PBS plus 10% normal goat serum overnight at 4 °C, after which cells were incubated with 5 μg/ml anti-PKCδ rabbit polyclonal antibody for 90 min at 37 °C, washed five times in blocking buffer, and resuspended in a 1:500 dilution of goat anti-rabbit Cy3-F(ab)′2 (H + L-specific; Jackson Immunochemicals) for 30 min at room temperature. Before use, primary and secondary diluted antibodies were sonicated for 10 min and centrifuged at 15,000 × *g* for 15 min at 4 °C. To stain for F-actin, cells were washed five times in PBS and sedimented before resuspension in PBS containing a 1:5 dilution of FITC-phalloidin (Molecular Probes). PMNs were incubated with F-actin label for 30 min at 37 °C in the dark. Cells were washed three times in PBS and sedimented before resuspension in 10 μl of PBS. PMNs were mounted in "gel/mount" (1:4; Biomeda Corp.) and viewed with a fluorescence microscope using a 63× Zeiss water objective. Labeled PKCδ and F-actin were exposed in the Cy3 channel and the FITC channel, respectively. Confocal images were achieved using Slidebook version 2.6 (Intelligent Imaging Innovations, Inc.).

RESULTS

Scramblase Activity during Cell Activation and Apoptosis—As shown previously, phospholipid scramblase function is enhanced during both cell stimulation and apoptosis (3, 4, 18, 19). A time course of its activity was investigated in neutrophils stimulated with 100 nM fMLP, and in Jurkat cells treated with anti-Fas IgM to induce apoptosis. Although scramblase activity was induced with both treatments, the time course appeared to be quite different. Fig. 1*A* shows that, during cell activation, phospholipid scrambling peaked early at 15 min, returning to background levels at later times. In contrast, scramblase activity during apoptosis did not occur until 2 h, peaking at 3 h, and remaining persistent out to 4 h following stimulation with anti-Fas IgM. Similar results were obtained in apoptotic neutrophils stimulated with UV irradiation or in non-apoptotic Jurkat cells stimulated with calcium ionophore and PMA (data not shown), suggesting that the difference in scramblase activation is not cell type- or stimulus-dependent. These data demonstrate that activation-induced scramblase activity is induced early and is transient in contrast to the late and prolonged activation observed during apoptosis.

Furthermore, once APLT activity is lost in apoptosis (but not stimulation), increased scramblase activity has been proposed to be the primary mediator of surface PS exposure (18). Fig. 1*B* shows a comparison of scramblase activity and surface PS exposure in Jurkat cells undergoing Fas-mediated apoptosis. Scramblase activity, which increased at 2 h, preceded the appearance of PS on the outer leaflet of the plasma membrane (as

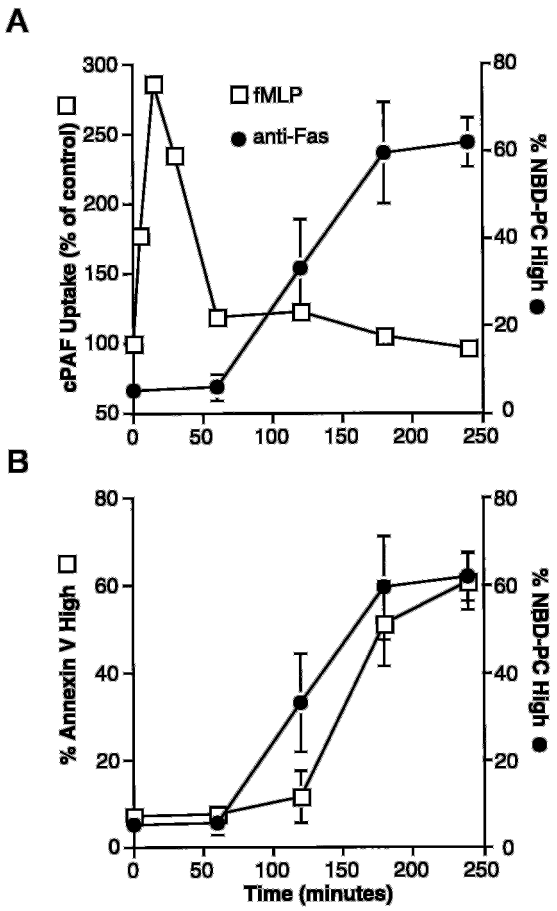

FIG. 1. **Time course of scramblase activation.** *A*, scramblase activity measured by uptake of radiolabeled cPAF in stimulated neutrophils (*squares*) or in apoptotic Jurkats by the uptake of NBD-PC (*circles*). *B*, comparison of scramblase activity in apoptotic Jurkats (*circles*) to surface PS exposure (*squares*) as measured by binding of FITC-annexin V.

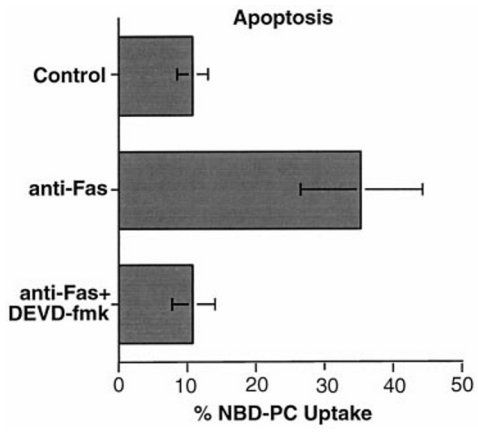

FIG. 2. **Caspase 3 is involved in apoptosis-induced scramblase activity.** Scramblase activity in apoptotic Jurkat cells in the presence or absence of the caspase 3 inhibitor DEVD-fmk.

measured by annexin V binding), which was not significantly evident until 3 h, supporting further the hypothesis that increased scramblase activity leads to surface PS exposure.

Involvement of Caspase 3 and Its Cleavage of PKCδ during Apoptosis but Not during Cell Activation—Caspase 3 has emerged as a central element in apoptosis induced by a variety of stimuli. Whether caspase 3 was necessary for apoptosis- or activation-induced scramblase activity was investigated first in Jurkat cells treated with anti-Fas IgM in the absence and presence of the caspase 3 inhibitor DEVD-fmk. Scramblase activity, as detected by the uptake of radiolabeled cPAF and scintillation counting (Fig. 2) and confirmed by the uptake of NBD-PC and flow cytometry (data not shown), showed a 3-fold increase over control values when stimulated with anti-Fas IgM. In the presence of DEVD-fmk, however, activity was inhibited to background levels, suggesting that caspase 3 was necessary for the activation of scramblase during apoptosis. The presence of DEVD-fmk also inhibited nuclear condensation and surface PS exposure (data not shown), confirming that caspase 3 is required for both nuclear and plasma membrane changes associated with apoptosis.

Several proteins have been identified as substrates for caspase 3, some of which are related to the changes observed in the nucleus, such as poly(A)DP-ribose polymerase and retinoblastoma protein (30, 31). However, several membrane, signaling and cytoskeletal-associated proteins have also been shown to be cleaved by caspase 3 and include fodrin, gelsolin, and actin. PKCδ is also cleaved by caspase 3, resulting in the removal of the regulatory subunit and consequently activating the enzyme. We investigated PKCα, PKCδ, and PKCζ activity in Jurkat cells treated with anti-Fas IgM. During apoptosis, PKCδ but not PKCα or PKCζ activity increased over time (Fig. 3*A*). This increase in activity correlated with the timing of PKCδ cleavage by caspase 3 (Fig. 3*B*) where activity and cleavage were observed at 2 h following induction of apoptosis with anti-Fas IgM. Treatment with the caspase 3 inhibitor DEVD-fmk prevented PKCδ cleavage (Fig. 3*B*) as well as its enhanced activity (Fig. 3*C*) while having no effect on either PKCα or PKCζ activity (data not shown). Neither PKCα nor PKCζ were cleaved by caspase 3 (Fig. 3*B*). These results demonstrate that during apoptosis PKCδ is activated in a caspase 3-dependent manner.

In contrast, activation of scramblase in neutrophils by 100 nM fMLP was unaffected by the presence of DEVD-fmk (Fig. 4), indicating that stimulation-induced scramblase activity is independent of caspase 3. To investigate PKCδ activation in neutrophils stimulated with fMLP, PKCδ translocation to the membrane was examined by immunofluorescence in the confocal microscope. As shown in Fig. 5, translocation of PKCδ to the membrane was observed as early as 30 s following neutrophil stimulation. Translocation was maximal between 1 and 3 min, returning to the cytosol by 10 min. These results suggest that translocation and activation of PKCδ by fMLP stimulation is transient and preceded the transient activation of scramblase activity.

PKCδ Is Involved in the Regulation of Scramblase Activity during Both Cell Stimulation and Apoptosis—To determine whether PKCδ may play a role in the regulation of scramblase activity, we utilized the inhibitor rottlerin, which has been reported to selectively inhibit PKCδ over other PKC isoforms (32). Fig. 6*A* is a dose response of rottlerin on PKCα, PKCδ, and PKCζ activity, demonstrating that concentrations of 10 μM or lower are specific for the inhibition of PKCδ. Fig. 6*B* shows a dose response of Gö6976, an inhibitor of cPKCs, on PKCα, PKCδ, and PKCζ activity. In contrast to rottlerin, Gö6976 had no inhibitory effect on PKCδ, whereas concentrations as low as 1 μM were completely effective at inhibiting PKCα. Jurkat or neutrophils pretreated with 10 μM rottlerin or 1 μM Gö6976 were treated with anti-Fas IgM and fMLP, respectively. Scramblase activity was measured by the uptake of NBD-PC and flow cytometry or the uptake of radiolabeled cPAF and scintillation counting. As shown in Fig. 7, scramblase activity in the presence of rottlerin was inhibited to background levels in both fMLP-stimulated cells (*panel A*) and apoptotic cells (*panel B*). In contrast, Gö6976 had no effect on either apoptosis-induced

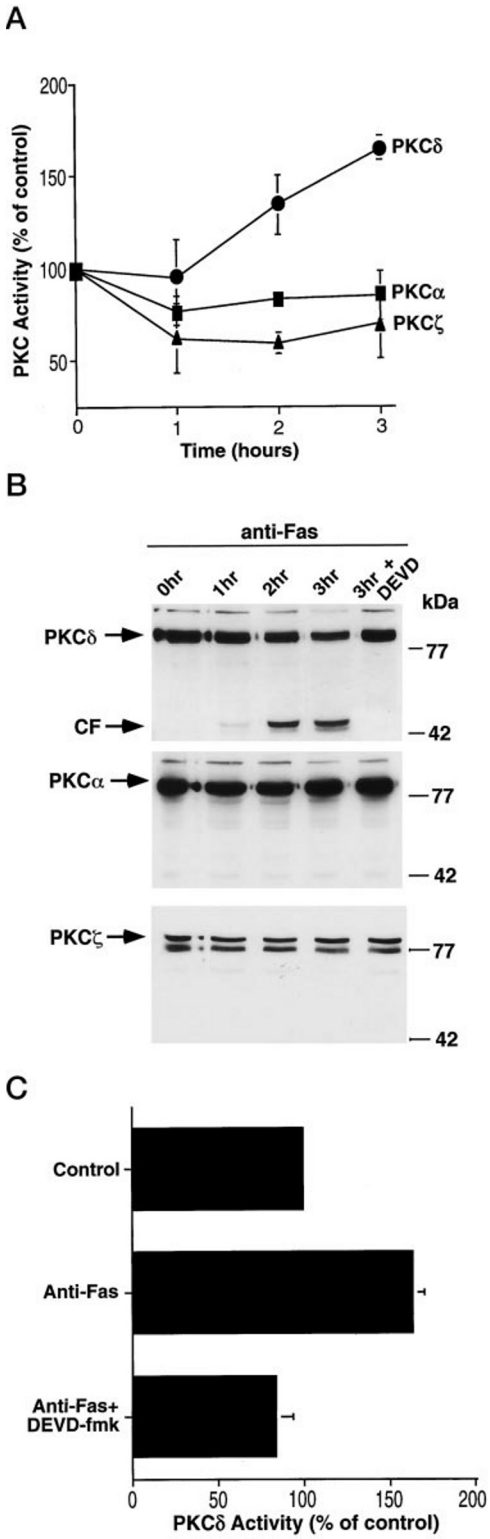

FIG. 3. **PKCδ activity is selectively cleaved and activated during apoptosis.** *A*, PKCδ, PKCα, or PKCζ activity was measured in an *in vitro* kinase assay in apoptotic Jurkat cells. *B*, Western immunoblot analysis of PKCα, PKCδ, and PKCζ during apoptosis, where cleavage of PKCδ, that generates the catalytic fragment (*CF*) is evident by 2 h. Caspase 3 inhibitor DEVD-fmk completely inhibits PKCδ cleavage. Neither PKCα nor PKCζ are cleaved. *C*, PKCδ activity is inhibited in the presence of the caspase 3 inhibitor DEVD-fmk.

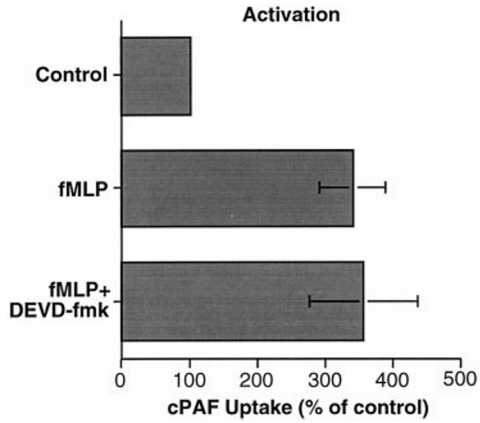

FIG. 4. **Scramblase activity in stimulated cells is independent of caspase 3.** Figure shows scramblase activity in fMLP-stimulated neutrophils in the absence or presence of the caspase 3 inhibitor DEVD-fmk.

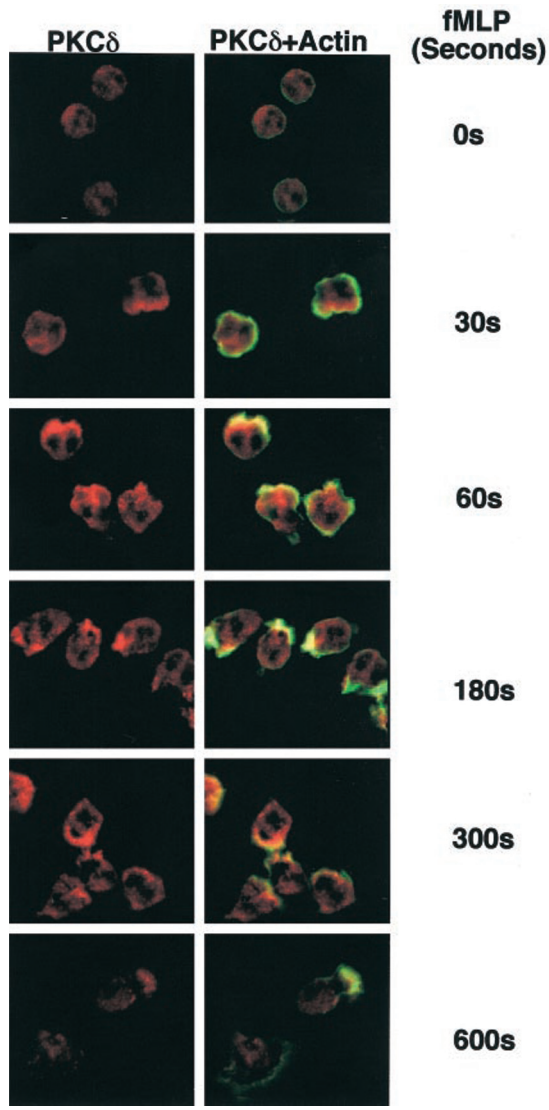

FIG. 5. **Translocation of PKCδ in fMLP-stimulated neutrophils.** PKCδ was stained either alone or with F-actin in neutrophils stimulated with fMLP for various times.

or activation-induced scramblase activity, suggesting that PKCδ but not PKCα or PKCζ is involved in the control of scramblase activity induced by apoptotic and activation stimuli.

The effect of rottlerin and Gö6976 on surface PS exposure was also investigated in apoptotic Jurkat cells treated with anti-Fas IgM. Three hours following stimulation approximately 80% of cells had PS exposed on the surface of the

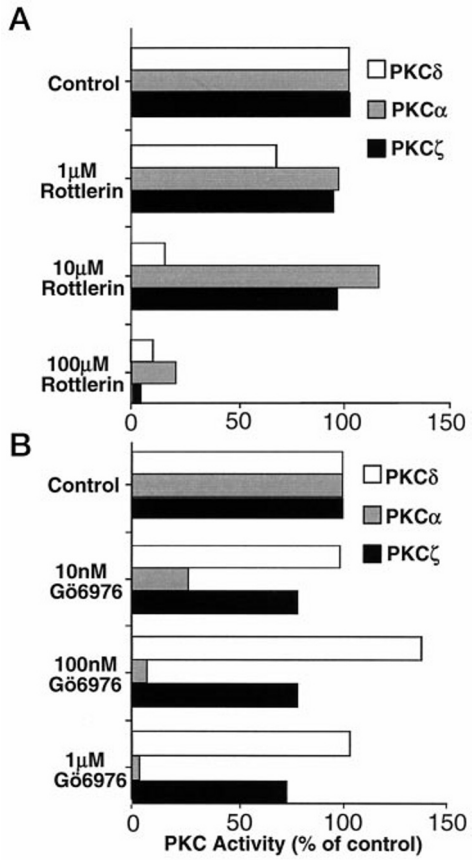

FIG. 6. **Rottlerin but not Gö6976 inhibits PKCδ activity.** *A*, dose response of rottlerin on PKC*α*, PKC*ζ*, or PKC*δ* activity. *B*, dose-response of Gö6976 on PKC*α*, PKC*ζ*, or PKC*δ* activity.

plasma membrane (data not shown). In the presence of rottlerin, the percentage of PS-expressing cells did not change, however, the mean fluorescence of the FITC-annexin V signal decreased significantly suggesting that the effect of rottlerin at the single cell level was not an "all or none" phenomenon, in that the amount of PS being expressed per cell decreased rather than the number of cells expressing PS (7C). Gö6976, on the other hand, had no effect on either the percentage of cells expressing PS or the mean fluorescence supporting further the hypothesis that PKCδ is, at least in part, responsible for the activation of scramblase activity during apoptosis.

PKCδ Phosphorylates Scramblase Directly—We have demonstrated above by inhibitor studies that PKCδ plays a role in the regulation of scramblase activity in both cell stimulation and apoptosis, presumably exerting its effects by phosphorylation. This hypothesis was examined directly by using immunoprecipitated scramblase as a substrate for recombinant active PKCδ. Fig. 8*A* demonstrates a time-dependent phosphorylation of scramblase in the presence of PKCδ, where maximal phosphorylation was reached by 20 min. To determine whether scramblase was phosphorylated *in vivo*, Jurkat cells were metabolically labeled with [^{32}P]orthophosphoric acid and stimulated with anti-Fas IgM. Scramblase was immunoprecipitated and subjected to phosphoamino acid analysis. Fig. 8*B* demonstrates that in unstimulated cells, there was base-line phosphorylation of scramblase on serine residues. Following the induction of apoptosis, however, phosphorylation of serine residues decreased and it increased on threonine, consistent with the predicted PKC phosphorylation site at Thr-161. These results were also confirmed by Western immunoblot analysis using an antibody directed against phosphothreonine where, following stimulation with anti-Fas IgM, increased threonine

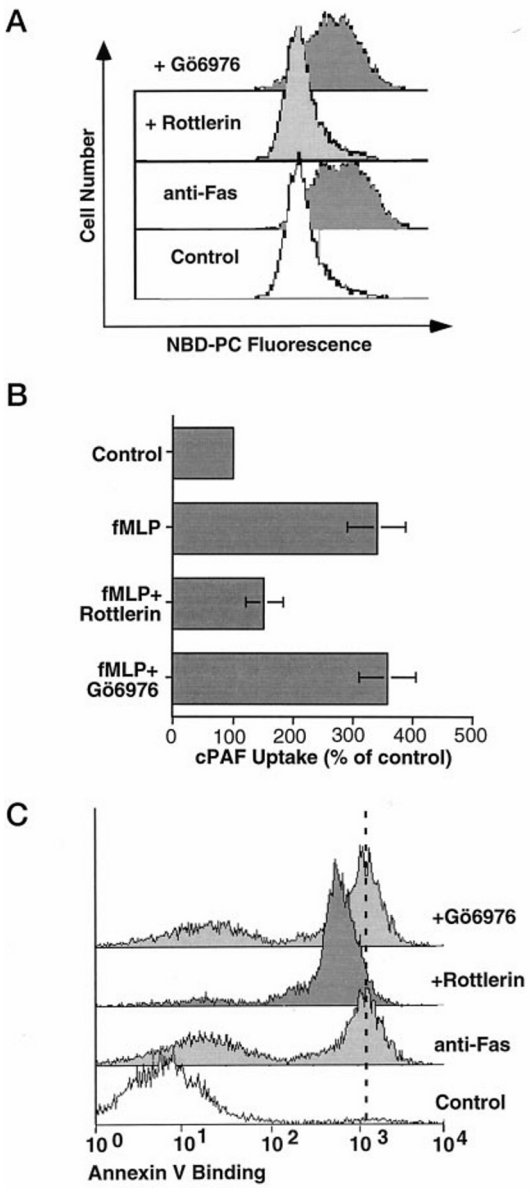

FIG. 7. **Rottlerin but not Gö6976 inhibits scramblase activity during both apoptosis and stimulation.** *A*, representative histogram of scramblase activity measured in apoptotic Jurkat cells by the uptake of NBD-PC in the absence or presence of rottlerin or Gö6976. *B*, scramblase activity measured in fMLP-stimulated neutrophils by the uptake of cPAF in the absence or presence of rottlerin or Gö6976. *C*, mean fluorescence of surface PS exposed in apoptotic Jurkat cells as measured by the binding of FITC-annexin V.

phosphorylation was observed (Fig. 8*C*).

Transfection of CHO Cells with Scramblase and PKCδ, but Not Scramblase or PKCδ Alone, Increased Scramblase Activity—To better determine the requirement of PKCδ on the activation of scramblase, we cotransfected CHO cells, which lack both scramblase and PKCδ by Western immunoblot analysis and activity assays (data not shown) with PKCδ, scramblase, and both together. Scramblase activity was measured by the uptake of cPAF following stimulation with PMA plus calcium ionophore. Fig. 9*A* demonstrates scramblase activity in cells transfected with vector, scramblase, or PKCδ alone, and those transfected with both PKCδ and scramblase. Notably, increased scramblase activity was significantly enhanced only in cells transfected with both PKCδ and scramblase ($p = <0.0001$). Little or no scramblase activity was present in vector control, scramblase-, or PKCδ-transfected cells, suggesting

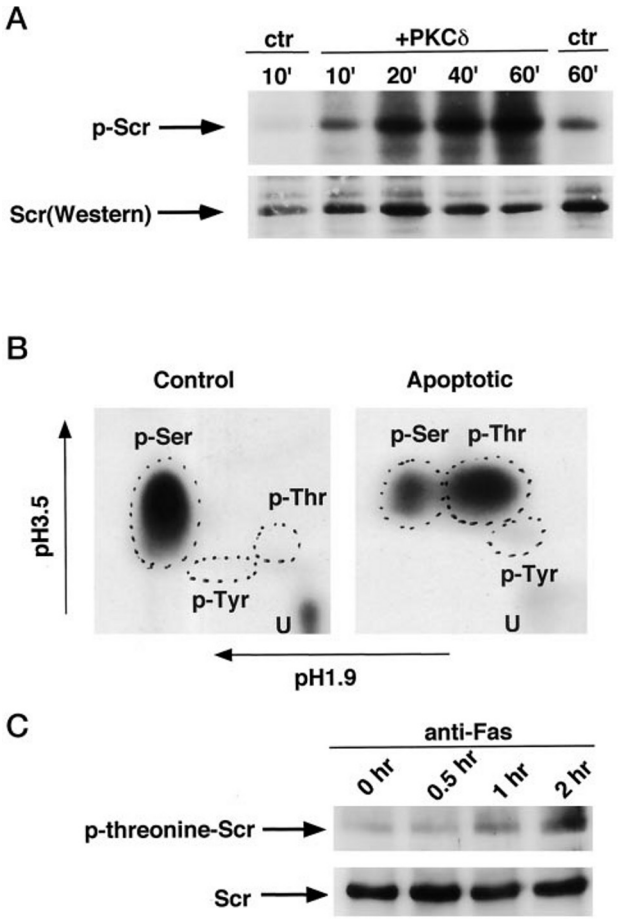

FIG. 8. **Scramblase is phosphorylated by PKCδ and *in vivo* on serine and threonine residues.** *A*, phosphorylation of scramblase by incubation with recombinant-active PKCδ. *B*, phosphoamino acid analysis of scramblase immunoprecipitated from ^{32}P-labeled control and anti-Fas-stimulated Jurkat cells. *p-Ser*, phosphoserine; *p-Thr*, phosphothreonine; *p-Tyr*, phosphotyrosine; *U*, undigested or partially digested amino acids. *C*, Western blot analysis of immunoprecipitated scramblase from apoptotic Jurkat cells probed with an antibody to phosphothreonine.

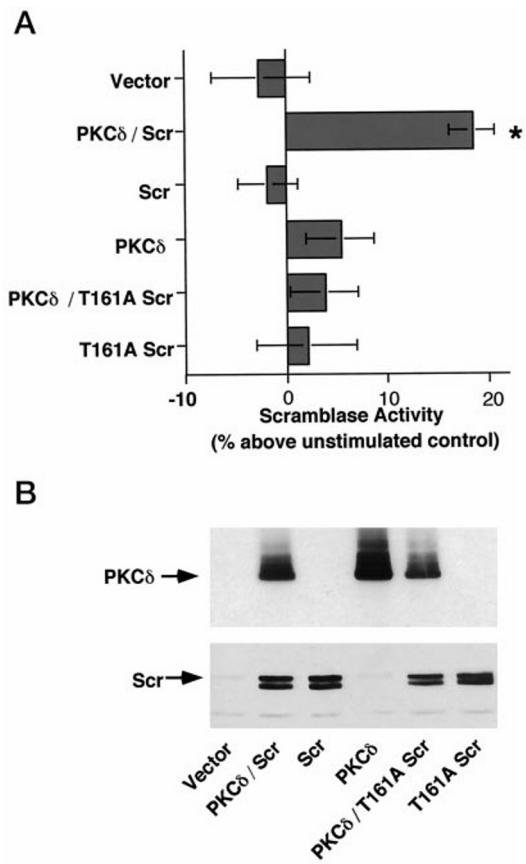

FIG. 9. **Increased scramblase activity requires activation of PKCδ and phosphorylation of Thr-161.** *A*, scramblase activity as measured by the uptake of radiolabeled cPAF in CHO cells transfected with vector, PKCδ plus scramblase, scramblase alone or PKCδ alone, PKCδ plus T161A scramblase, or T161A scramblase alone. *, $p = <0.0001$. *B*, Western immunoblot analysis of transfected CHO cells.

that PKCδ is necessary for activation of scramblase activity.

As stated above, the deduced amino acid sequence of scramblase reveals a putative PKC phosphorylation site at position Thr-161, and we have shown that the phosphorylation of scramblase follows induction of apoptosis. To determine whether Thr-161 was important for scramblase function, Thr-161 was changed to an Ala residue by site-directed mutagenesis. CHO cells were co-transfected with PKCδ and T161A scramblase, and scramblase activity was measured as described above. As shown in Fig. 9*A*, scramblase activity in the scramblase mutant-transfected CHO cells showed no increase following stimulation with PMA plus calcium ionophore, suggesting that phosphorylation of Thr-161 by PKCδ is important for scramblase function. Mutation of Thr-161 to Ala had no effect on protein expression as shown by Western immunoblot analysis in Fig. 9*B*.

DISCUSSION

In this report, we demonstrate that PKCδ regulates phospholipid scramblase activity during both cell stimulation and apoptosis. We have shown that scramblase activity during activation is transient in contrast to the sustained activation observed during apoptosis. Because apoptosis is an irreversible event and APLT activity is also inhibited, sustained activation of scramblase would allow for maximal PS externalization, which has been shown to serve as at least one signal for the recognition and quiescent removal of apoptotic cells by phagocytes (9). Clearance of apoptotic cells before they lyse their toxic contents into the surrounding tissue represents an important mechanism for limiting tissue injury; therefore, it is critical to ensure the timely generation of this recognition signal. In addition, we demonstrate that scramblase activity precedes annexin V binding, supporting further the hypothesis that enhanced scramblase activity is required for surface PS exposure.

Cell stimulation, on the other hand, is a relatively rapid response, and, because the APLT is still active, sustained PS exposure is not a general feature observed in these cells even though scramblase is activated. Under these circumstances, scramblase activity is transient. The function of the membrane phospholipid randomization observed during cell stimulation remains unclear but may relate to altered protein function. Neutrophils play a primary role in the inflammatory response, being one of the first cells recruited to the site of injury. Enhanced scramblase activity during cell stimulation may be important for the uptake and release of important lipid mediators. For instance it has been demonstrated that this may be the primary mechanism by which platelet-activating factor is internalized as well as released by neutrophils (3, 4). It has also been suggested that changes in membrane organization of stimulated cells may contribute to subsequent events important for the inflammatory response (4, 33, 34).

PKC activation has been classically associated with transient translocation to the plasma membrane that is mediated by the regulatory subunit where the diacylglycerol and PS

binding sites are located (35, 36). This translocation event also appears to be the mechanism for terminating PKC activity, possibly by signaling proteolytic degradation (ubiquitination) and therefore down-regulation (37). During apoptosis, however, cleavage of PKCδ by caspase 3 at a single site in the hinge region removes the regulatory subunit, thereby eliminating the membrane translocation requirement rendering the catalytic subunit constitutively active. Whether this cleavage event also signals down-regulation of PKCδ remains unclear. In contrast, cleavage of PKCζ in HeLa cells following UV irradiation inactivates the protein, suggesting that PKCζ is a pro-survival kinase (38). However, we observed sustained activation of PKCδ but not PKCα or PKCζ during apoptosis and propose that constitutive activation of PKCδ may be the mechanism by which scramblase activity is prolonged. Clearly, multiple mechanisms exist for the activation and down-regulation of PKCδ and are under current investigation.

The involvement of PKCδ in the activation of scramblase was also demonstrated by the use of the inhibitors rottlerin and Gö6976, which inhibit PKCδ and PKCα/β, respectively. Because we were able to demonstrate isoform specificity at defined concentrations, these inhibitors served as useful tools for the identification of PKCδ as a regulator of scramblase activity. Although we have demonstrated by inhibitor studies that PKCδ plays a primary role in the regulation of scramblase activity during apoptosis and cell stimulation, it is possible that other PKC isoforms can perform the same function since the PKC phosphorylation site on scramblase does not provide PKC isoform specificity. PKCθ also serves as a substrate for caspase 3 and has been reported to be activated during apoptosis. Because there are no specific inhibitors for PKCθ, involvement of this isoform in the regulation of scramblase activation cannot be ruled out at this time. Our data support, however, the involvement of PKCδ in scramblase activation since inhibition with rottlerin completely inhibits scramblase activity while having no effect on PKCθ activity (32).

The involvement of PKCδ in the regulation of scramblase activity was demonstrated by cotransfection of both scramblase and PKCδ in CHO cells. Although only moderate scramblase activity was induced in this system, this may relate to transfection efficiency. However, it is also reasonable to assume that regulation of scramblase activity is more complicated than a two-component system involving only PKCδ. We hypothesize that other components are likely to be necessary for full activation of scramblase. Several proteins have been described to be involved in the transbilayer movement of phospholipids and include the multidrug-resistant proteins and ATP-binding cassette-1 (39–41). Whether these proteins are directly involved in scramblase activity or function upstream of scramblase is under current investigation.

Phosphorylation as a mechanism of regulation has been described in a number of signaling pathways. Phosphorylation/dephosphorylation cascades allow for immediate modulation of enzymatic activity and represent a very efficient and tightly controlled mechanism of regulation. Our data support the observation that phosphorylation of phospholipid scramblase by PKCδ increases scramblase activity during apoptosis and cell stimulation, resulting in surface PS exposure in the case of apoptosis. Importantly, we have also demonstrated that mutation of Thr-161 to Ala in scramblase completely inhibited the ability of scramblase to be activated by PKCδ, suggesting further that phosphorylation, particularly phosphorylation by PKC, is an important mechanism for regulating scramblase function.

Little is known about the biological function of PKCδ. PKCδ has been implicated in the regulation of cell growth and differentiation as well as apoptosis and tumor progression (for review, see Ref. 42). For instance, overexpression of PKCδ in a variety of cell types resulted in growth inhibition whereas expression in the myeloid 32D cell line mediated macrophage differentiation following treatment with either PMA or platelet-derived growth factor. Activation of PKCδ during apoptosis appears to be a common event occurring in a variety of cell types (26, 27, 43–45). Little is known, however, about PKCδ substrates that contribute to the apoptotic phenotype. Recently, the PKCδ catalytic subunit has been reported to interact with the DNA-dependent protein kinase, a kinase responsible for the repair of double-stranded DNA breaks, in U937 monoblastic leukemia cells (46). This interaction causes inactivation of DNA-dependent protein kinase presumably by direct phosphorylation, possibly contributing to DNA degradation observed during apoptosis. In our system, inhibition of PKCδ by rottlerin had no effect on the nuclear morphology. One explanation could be that, in our system, PKCδ was inhibited directly by rottlerin, thereby eliminating the possibility of altered substrate phosphorylation that may occur in an overexpression system.

For the first time, we report the direct phosphorylation of scramblase by PKCδ during apoptosis and cell stimulation, resulting in increased scramblase activity. As outlined above, enhanced scramblase activity has significant functional consequences and tight regulation of this activity is necessary. The results presented here provide evidence for a specific substrate and function for PKCδ during both cell stimulation and apoptosis in the regulation of scramblase activity.

REFERENCES

1. Bretscher, M. S. (1972) *Nat. New Biol.* **236,** 11–12
2. Zachowski, A., Favre, E., Cribier, S., Herve, P., and Devaux, P. F. (1986) *Biochemistry* **25,** 2585–2590
3. Bratton, D. L. (1993) *J. Biol. Chem.* **268,** 3364–3373
4. Bratton, D. L., Dreyer, E., Kailey, J. M., Fadok, V. A., Clay, K. L., and Henson, P. M. (1992) *J. Immunol.* **148,** 514–523
5. Comfurius, P., Sneden, J. M. G., Tilly, R. H. H., Shroit, A. J., Bevers, E. M., and Zwaal, R. F. (1990) *Biochim. Biophys. Acta* **1026,** 153–160
6. Comfurius, P., Williamson, P., Smeets, E. F., Schlegel, R. A., Bevers, E. M., and Zwaal, R. F. (1996) *Biochemistry* **35,** 7631–7634
7. Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., and Sims, P. J. (1997) *J. Biol. Chem.* **272,** 18240–18244
8. Fadok, V. A., Bratton, D. L., Frasch, S. C., Warner, M. L., and Henson, P. M. (1998) *Cell Death Diff.* **5,** 551–562
9. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) *J. Immunol.* **148,** 2207–2216
10. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998) *J. Clin. Invest.* **101,** 890–898
11. Fadok, V. A., Warner, M. L., Bratton, D. L., and Henson, P. M. (1998) *J. Immunol.* **161,** 6250–6257
12. Fadok, V., Bratton, D., Rose, D. M., Pearson, A., Ezekewitz, R. A. B., and Henson, P. M. (2000) *Nature* **405,** 85–90
13. Hemker, H. C., van Rijn, J. L., Rosing, J., van Dieijen, G., Bevers, E. M., and Zwaal, R. F. (1983) *Blood Cells* **9,** 303–317
14. Bratton, D. L., Kailey, J. M., Clay, K. L., and Henson, P. M. (1991) *Biochim. Biophys. Acta* **1062,** 24–34
15. Bratton, D. L. (1994) *J. Biol. Chem.* **269,** 22517–22523
16. Bratton, D. L., Clay, K. L., Kailey, J. M., Dreyer, E., Fadok, V. A., and Henson, P. M. (1994) *J. Lipid Mediators* **10,** 43–45
17. Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., and Henson, P. M. (1997) *J. Biol. Chem.* **272,** 26159–26165
18. Verhoven, B., Krahling, S., Schlegel, R. A., and Williamson, P. (1999) *Cell Death Diff.* **6,** 262–270
19. Middelkoop, E., Lubin, B. H., Bevers, E. M., Op den Kamp, J. A. F., Comfurius, P., Chiu, D. T.-Y., Zwaal, R. F. A., van Deenen, L. L. M., and Roelofsen, B. (1988) *Biochim. Biophys. Acta* **937,** 281–288
20. Liu, W. S., and Heckman, C. A. (1998) *Cell. Signal.* **10,** 529–542
21. Rodriquez-Tarducy, G., and Lopez-Rivas, A. (1989) *Biochem. Biophys. Res. Commun.* **164,** 1069–1075
22. Iseki, R., Mukai, M., and Iwata, M. (1991) *J. Immunol.* **147,** 4286–4282
23. Reyland, M. E., Anderson, S. M., Matassa, A. A., Barzen, K. A., and Quissell, D. O. (1999) *J. Biol. Chem.* **274,** 19115–19123.
24. Pongracz, J., Webb, P., Want, K., Deacon, E., Lunn, O. J., and Lord, J. M. (1999) *J. Biol. Chem.* **274,** 37329–37334
25. Emoto, Y., Kisaki, H., Manome, Y., Kharbanda, S., and Kufe, D. (1996) *Blood* **87,** 1990–1996
26. Datta, R., Kojima, H., Yoshida, K., and Kufe, D. (1997) *J. Biol. Chem.* **272,** 20317–20320
27. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* **371,** 346–347

28. An, B., and Cou, Q. P. (1996) *Cancer Res.* **56,** 438–442
29. Gschwendt, M., Müller, H-J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., and Marks, F. (1994) *Biochem. Biophys. Res. Commun.* **199,** 93–98
30. McEvoy, L., Worthen, G. S., Williamson, P., Henson, P. M., and Schlegel, R. A. (1986) *FASEB J.* **130,** 504
31. Schlegel, R. A., and Williamson, P. (1987) *J. Cell. Physiol.* **132,** 381
32. Emoto, Y., Manome, Y. Meinhardt, G., Kisaki, H., Kharbanda, S., Roberson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R., and Kufe, D. (1995) *EMBO J.* **14,** 6148–6156
33. Ghayur, T., Hugunin, M., Talanian, R. V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) *J. Exp. Med.* **184,** 2399–2404
34. Newton, A. C. (1995) *J. Biol. Chem.* **270,** 28495–28498
35. Newton, A. C. (1997) *Curr. Opin. Cell Biol.* **9,** 161–167
36. Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M., and Foster, D. A. (1998) *Mol. Cell. Biol.* **18,** 839–845
37. Frutos, S., Moscat, J., and Diaz-Meco, M. T. (1999) *J. Biol. Chem.* **274,** 10765–10770
38. Ruetz, S., and Gros, P. (1994) *Cell* **77,** 1071–1081
39. Smit, J. J., Schinke, A. H., Oude, Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N. M., and van Roon, M. A. (1993) *Cell* **75,** 451–462
40. Marguet, D., Luciani, M. F., Moynault, A., Williamson, P., and Chimini, G. (1999) *Nat. Cell Biol.* **1,** 454–456
41. Gschwendt, M. (1999) *Eur. J. Biochem.* **259,** 555–564
42. Khwaja, A., and Tatton, L. (1999) *Blood* **94,** 291–301
43. Bharti, A., Kraeft, S.-K., Gounder, M., Pandey, P., Jin, S., Yuan, Z.-M., Lees-Miller, S. P., Weichselbaum, R., Weaver, D., Chen, L. B., Kufe, D., and Kharbanda, S. (1998) *Mol. Cell. Biol.* **18,** 6719–6728
44. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. B., Jr., and Henson P. M. (1985) *Am. J. Pathol.* **119,** 101–110
45. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59