0026-895X/04/6601-76–84
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:76–84, 2004

Vol. 66, No. 1
3165/1160020
Printed in U.S.A.

PKCδ Associates with and Is Involved in the Phosphorylation of RasGRP3 in Response to Phorbol Esters

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Received December 17, 2003; accepted March 18, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

RasGRP is a family of guanine nucleotide exchange factors that activate small GTPases and contain a C1 domain similar to the one present in protein kinase C (PKC). In this study, we examined the interaction of RasGRP3 and PKC in response to the phorbol ester PMA. In Chinese hamster ovary or LN-229 cells heterologously expressing RasGRP3, phorbol 12-myristate 13-acetate (PMA) induced translocation of RasGRP3 to the perinuclear region and a decrease in the electrophoretic mobility of RasGRP3. The mobility shift was associated with phosphorylation of RasGRP3 on serine residues and seemed to be PKCδ-dependent because it was blocked by the PKCδ inhibitor rottlerin as well as by a PKCδ kinase-dead mutant. Using coimmunoprecipitation, we found that PMA induced the physical association of RasGRP3 with PKCδ and, using in situ methods, we showed colocalization of PKCδ and RasGRP3 in the perinuclear region. PKCδ phosphorylated RasGRP3 in vitro. Previous studies suggest that ectopic expression of RasGRP3 increases activation of Erk1/2. We found that overexpression of either PKCδ or RasGRP3 increased the activation of Erk1/2 by PMA. In contrast, coexpression of PKCδ and RasGRP3 yielded a level of phosphorylation of Erk1/2 similar to that of control vector cells. Our results suggest that PKCδ may act as an upstream kinase associating with and phosphorylating RasGRP3 in response to PMA. The interaction between RasGRP3 and PKCδ points to the existence of complex cross-talk between various members of the phorbol ester receptors which can have important impact on major signal transduction pathways and cellular processes induced by phorbol esters or DAG

The phorbol esters, potent tumor-promoting agents for skin (Yuspa et al., 1976), were thought at one time to function exclusively through binding and activation of PKC (Castagna et al., 1982; Sharkey et al., 1984; Blumberg, 1988). Although PKC is still implicated in the majority of the effects of the phorbol esters, emerging evidence suggests the involvement of new cellular receptors in some phorbol ester responses (Ron and Kazanietz, 1999; Kazanietz, 2000). These new receptors comprise the non-PKC members of the phorbol ester receptor family and include PKCμ (Johannes et al., 1994) and PKCν (Hayashi et al., 1999), the Munc-13 proteins (Kazanietz et al., 1995; Betz et al., 1998) the chimerins (Ahmed et al., 1991; Caloca et al., 1999), DAG-kinase γ (Shindo et al., 2001) and the RasGRP proteins (Ebinu et al., 1998; Lorenzo et al., 2000). All these proteins express one or more C1 domains that bind phorbol esters and its physiological counterpart, the second messenger diacylglycerol (Brose and Rosenmund, 2002; Kazanietz, 2002).

RasGRP represents a new family of GEF proteins that regulate the dissociation of GDP from Ras GTPases to enhance the formation of the active GTP-bound form (Ebinu et al., 1998). Additional GEFs for Ras include SOS (Chardin et al., 1993) and RasGRF (Farnsworth et al., 1995). The RasGRP family consists of four members; RasGRP1 (Ebinu et al., 1998), RasGRP2 (Clyde-Smith et al., 2000), RasGRP3 (Yamashita et al., 2000), and RasGRP4 (Reuther et al., 2002; Yang et al., 2002). These proteins all share similar structure, containing the Ras GEF motif CDC25, a pair of atypical EF hands, and the C1 domain. In contrast, the various RasGRPs

This work was supported by the James S. McDonnell Foundation 21st Century Scientist Award/Brain Cancer Research (to C.B.).

ABBREVIATIONS: PKC, protein kinase C; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; KD, kinase dead; CHO, Chinese hamster ovary; MBP, maltose binding protein; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride**;** PMA, phorbol 12-myristate 13-acetate; Erk, extracellular-regulated kinase; EGF, epidermal growth factor; Gö6983, 2-[1-(3-dimethylaminopropy)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide.

have distinct specificities. RasGRP1, the first member characterized, is a GEF for Ras (Ebinu et al., 1998). RasGRP2 possesses GEF activity for N-Ras, K-Ras, and for Rap1 (Clyde-Smith et al., 2000). RasGRP3 activates both Ras and Rap1 (Yamashita et al., 2000), and RasGRP4 activates Ras (Reuther et al., 2002; Yang et al., 2002). In addition, members of the RasGRP family show distinct modes of tissue expression and biological functions (Pierret et al., 2000, 2001; Priatel et al., 2002).

In recent studies, RasGRP3 has been characterized as a high-affinity receptor for phorbol esters, and its cellular localization and its GEF activity for Ras have been shown to be modulated by phorbol ester treatment (Lorenzo et al., 2001). The ability of RasGRP3 to bind phorbol esters and DAG similarly to PKC and other phorbol ester receptors raises the question of whether various members of this extended family can translocate to the same subcellular sites and interact with each other in response to stimulation with DAG or phorbol esters. Indeed, recent studies demonstrate that members of the DAG/phorbol ester family can interact with each other (Teixeira et al., 2003; Waldron and Rozengurt, 2003).

In the current study, we examined the association of PKC and RasGRP3 in response to PMA. We found that PMA induced the colocalization and association of PKCδ and RasGRP3 and the phosphorylation of RasGRP3 by PKCδ.

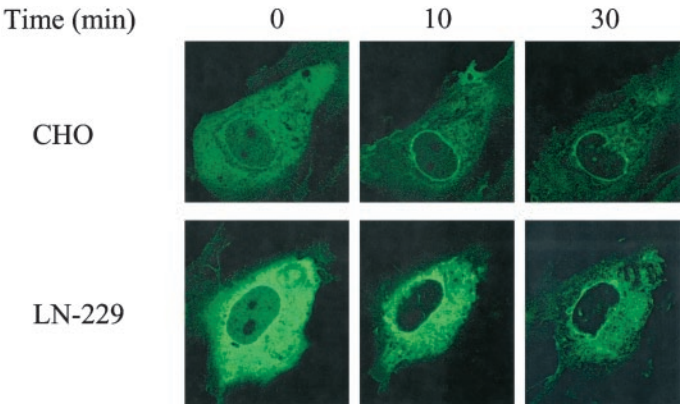

Fig. 1. Translocation of RasGRP3 in PMA-treated cells. CHO and LN-229 cells were transiently transfected with GFP-RasGRP3. After 48 h, cells were treated with PMA (100 nM), and sequential confocal images were taken every 30 s for a period of 30 min. The figures present images taken 0, 10, and 30 min after treatment. Cells shown are representative of five independent experiments.

Experimental Procedures

Materials. Anti-PKCα, -δ, and -ϵ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG antibody conjugated to peroxidase, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and sodium vanadate were obtained from Sigma Chemical Co. (St. Louis, MO). Calf alkaline phosphatase was obtained from New England Biolabs (Beverly, MA). Gö6983 was from Biomol (Plymouth Meeting, PA).

Construction of PKCδ and Ras-GRP3 Plasmids. pBK-CMV-RasGRP3 was provided by James Stone (Alberta University, Canada). The full-length RasGRP3 cDNA was amplified by polymerase chain reaction using specific primers (5′-ggatccATGGGATCAAGTGGCCTTGGG-3′ and 5′-ctcgagTCAGCCATCCTCACCATC CTGTCT-3′; BamHI and XhoI sites, indicated by lower case letters, were incorporated to facilitate cloning) and subcloned into pCMV-Tag 2b (Stratagene, La Jolla, CA) generating pFLAG-RasGRP3 with an N-terminal FLAG tag. The cDNA of RasGRP3 was also subcloned into pQBI25 (Quantum Biotechnologies Inc., Canada) using the NheI site with GFP or RFP attached to the C-terminal end of RasGRP3. The integrity of the inserts was verified by DNA sequencing, which was performed by the DNA minicore, Center of Cancer Research, NCI, National Institute of Health.

cDNA encoding the murine PKCδ wild-type was fused into the N-terminal enhanced GFP vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) as described previously (Kronfeld et al., 2000). The PKCδ kinase-dead mutant (K376R) was described previously (Blass et al., 2002). PKCδ, PKCδKD, and PKCαKD were subcloned into pCMV-Tag 2b using XhoI and MluI sites.

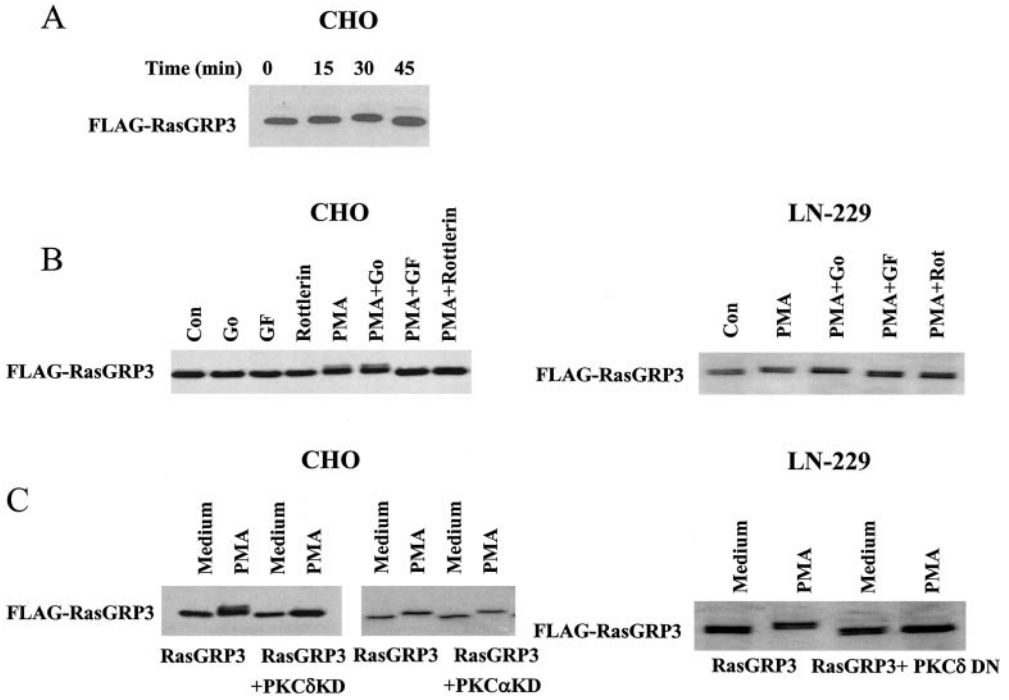

Fig. 2. PMA induces an electrophoretic shift in the mobility of RasGRP3 in a PKCδ -dependent manner. CHO transfected with FLAG-RasGRP3 were treated with PMA for various periods of time (A), or CHO and LN-229 cells overexpressing RasGRP3 were treated with PMA (100 nM) for 30 min with and without GF109203X, Gö6976, or rottlerin (B). Cells transfected with a control vector (CV), a PKCα KD mutant, or a PKCδ KD mutant were treated with PMA for 30 min (C). The cells were harvested and subjected to Western blot analysis, and the expression of RasGRP3 was detected by probing the membrane with the FLAG antibody. The results are representative of four independent experiments.

Cell Cultures and Cell Transfection. For these studies, we used CHO cells and the human glioma LN-229 cells that express endogenous RasGRP3 and PKCδ (R. Steinhart, G. Kamirsky, and C. Brodie, unpublished data). Cells were seeded on tissue culture dishes (10 cm) and were grown in medium consisting of Ham's F12 (for CHO cells) or Dulbecco's modified Eagle's medium (for LN-229 cells) containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. The cells were transiently transfected either with the empty vectors or with the RasGRP3 expression vectors using LipofectAMINE (Invitrogen, Carlsbad, CA).

For overexpression of the GFP-PKCδ fusion proteins, Cells were seeded onto 40-mm round glass coverslips at a density of 5×10^4 cells/coverslip. Twenty-four hours later, cells were transfected with the different GFP-PKCδ constructs using LipofectAMINE Plus reagent according to the manufacturer's instructions. All experiments were performed 48 h post transfection. The percentage of transfection for the LN-229 cells was about 40 to 50% and that of CHO cells was about 70%.

Preparation of Cell Homogenates. Cells were removed with a rubber policeman and sonicated in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40; 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, and 0.5 mM Na_3VO_4) for 15 s. Sample buffer (2×) was added and the samples were boiled for 5 min.

Immunoblot Analysis. Lysates (30 μg of protein) were resolved by SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in phosphate-buffered saline and subsequently stained with the primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA), and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham Bisociences, Piscataway, NJ).

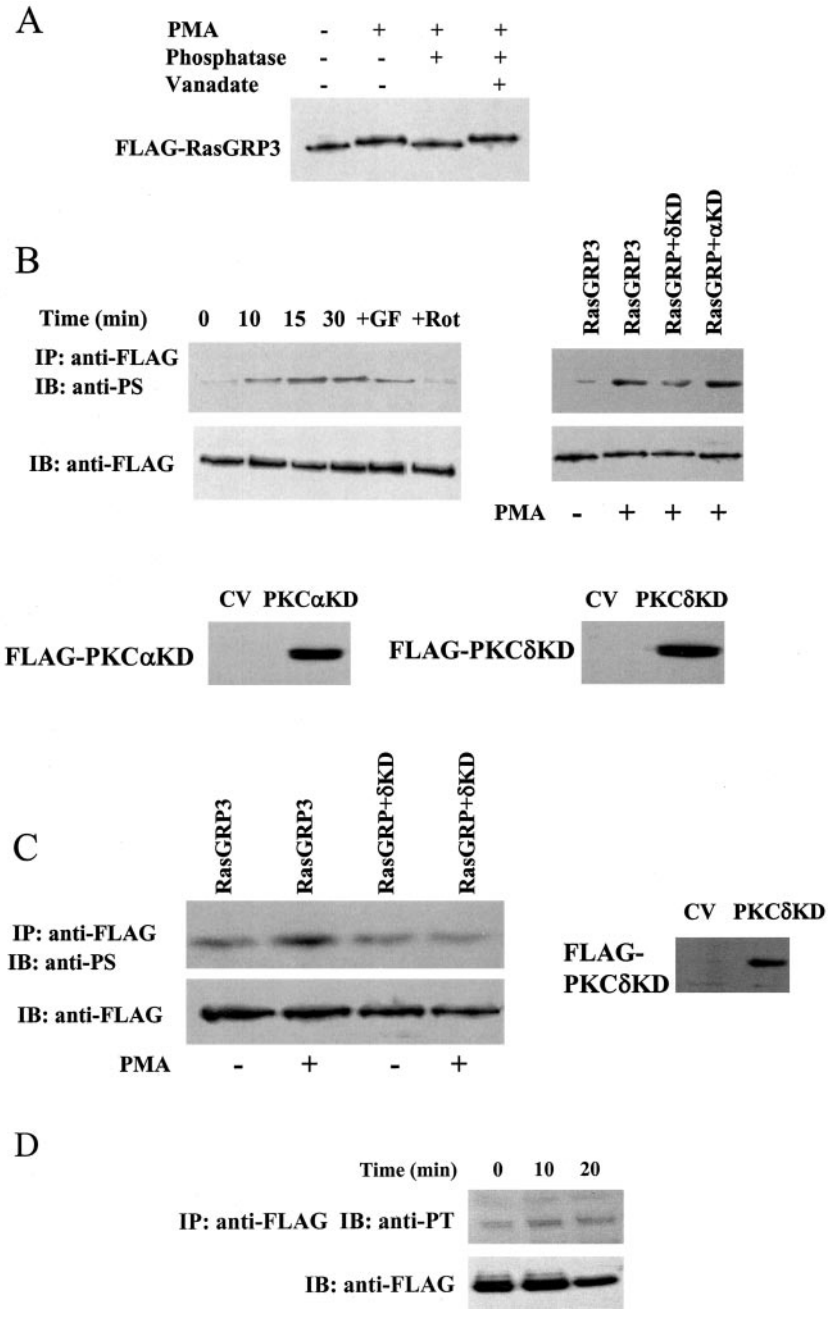

Fig. 3. PMA induces phosphorylation of RasGRP3. CHO cells overexpressing RasGRP3 were treated with PMA (100 nM) for 20 min; RasGRP3 was immunoprecipitated and the immunoprecipitates were treated with alkaline phosphatase for 30 min (A). CHO cells transfected with RasGRP3 were treated with PMA (100 nM) for various times in the presence of GF109203X and rottlerin. RasGRP3 was immunoprecipitated, proteins subjected to Western blot analysis, and membranes were probed with anti-serine (B) and anti-threonine (D) antibodies. CHO (B) and LN-229 (C) cells cotransfected with RasGRP3, PKCα KD, or PKCδ KD were stimulated with PMA for 30 min. RasGRP3 was immunoprecipitated and after Western blot, membrane were probed with anti-serine antibody. Overexpression of PKCαKD (B) and of PKCδKD mutants (B and C) was examined using an anti-FLAG antibody. The results are representative of four independent experiments.

Immunoprecipitation. Immunoprecipitation was performed as described previously (Kronfeld et al., 2000). In brief, CHO or LN-229 cells transfected with RasGRP3 were treated for different periods of time with PMA (100 nM). The samples were preabsorbed with 25 μl of protein A/G-Sepharose (50%) for 10 min and immunoprecipitation was performed using 4 μg/ml antibody for 1 h at 4°C and then incubated with 30 μl of A/G-Sepharose for an additional hour. After washes, the pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. The entire supernatant was subjected to Western blotting. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed and visualized by the enhanced chemiluminescence system.

In Vitro Phosphatase Treatment. Cells overexpressing RasGRP3 were treated with PMA for 20 min and RasGRP3 was immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were incubated with calf intestine alakaline phosphatase (New England Biolabs) in the presence and absence of sodium orthovanadate (0.5 mM) for 20 min at 37°C. Proteins were separated on 7.5% gel and were immunoblotted with anti-FLAG antibody.

Phosphorylation of Maltose Binding Protein Tagged Purified Ras-GRP3 (MBP-Ras-GRP3). MBP-Ras-GRP3 (50–100 μg/ml; 125.3 kDa) or MBP (34–50 μg/ml; 42.7 kDa) (exact amounts are indicated in the figure legends) was incubated with the specified protein kinases (all from BIOMOL Research Laboratories, Plymouth Meeting, PA) in a 50-μl reaction mixture. The purity of the kinases was > 95% according to BIOMOL, and we verified that they showed only a single band upon SDS gel electrophoresis and staining for protein. Activities for the PKC isoforms were determined on histone H1 substrate; for PKA, which has different substrate specificity, activity was determined on histone HIIA substrate. Based on these measurements, the activities of the kinases included in the total incubation mix for the assays of RasGRP3 phosphorylation were 21 to 30 pmol of phosphate transferred/min (Fig. 6, A and B). In Fig. 6, C and D, which used a different lot of PKCδ, activity was determined with the use of the PKCε pseudosubstrate peptide substrate (Calbiochem, La Jolla, CA). The activity included in those experiments was approximately 40 pmol of phosphate transferred/min. The exact values are listed in the figure legends. The mixture contained the following: for PKCα and PKCβ 1, 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 200 μg/ml phosphatidylserine, 20 μg/ml 1,2-dioctanoyl-*sn*-glycerol, 100 μM [γ-^{33}P]ATP, and 100 μM $CaCl_2$; for PKCδ, PKCε, and PKCμ: 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 200 μg/ml phosphatidylserine, 20 μg/ml DAG, 100 μM γ ^{33}P-ATP and 100 μM EGTA; for PKA, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 100 μM [γ-^{33}P]ATP. Chemicals were from Sigma, except [γ-^{33}P]ATP, which was from ICN (Costa Mesa, CA). Where indicated, the assays with PKCδ included 0.03% Triton X-100 and 5% dimethyl sulfoxide. The reactions were initiated by adding the radioactive substrates. After incubation for 60 to 240 min as indicated at 37°C, 10 μl of the reaction mixtures was added to 10 μl of 2× SDS sample buffer and boiled for 5 min. The proteins were then subjected to polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained and dried. The stained, dried gels were then subjected to autoradiography.

Results

PMA Induced Translocation of RasGRP3 in CHO Cells. Translocation of RasGRP3 in response to PMA was studied using a RasGRP3-GFP fusion protein. CHO cells or the human glioma LN-229 cells were transiently transfected with the plasmid, and translocation was assessed in live cells using confocal microscopy. PMA induced translocation of RasGRP3 to the perinuclear region and the nuclear membrane in both cell types. Translocation was first observed after 10 min of treatment, reached plateau levels after 20 to 30 min of treatment (Fig. 1), and persisted up to 60 min (data not shown). Similar results were observed in the human neuroblastoma SH-SY5Y cells (data not shown).

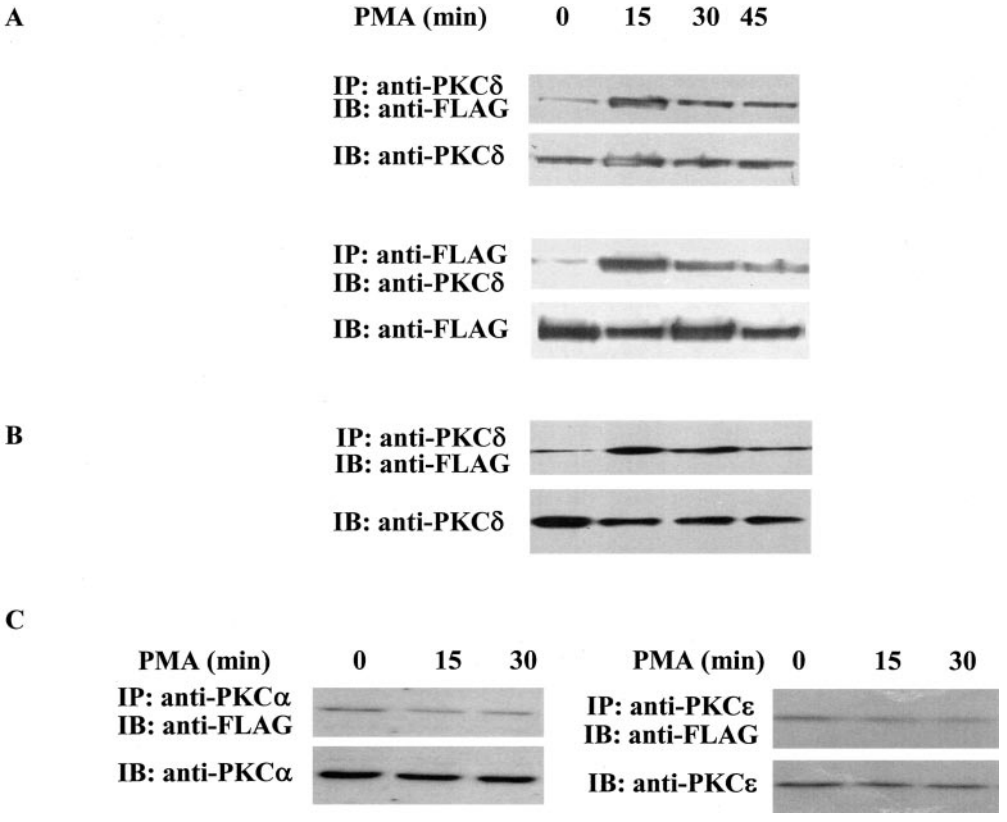

Fig. 4. PMA induces the association of RasGRP3 with PKCδ. CHO (A) and LN-229 (B) cells were cotransfected with FLAG-RasGRP3, PKCδ, or PKCα. Cells were then harvested, and immunoprecipitation (IP) of PKCδ (A and B), PKCα, or PKCε (C) was performed using anti-PKCδ, PKCα, or PKCε antibodies, respectively, and that of RasGRP3 was performed with anti-FLAG antibodies as described under *Materials and Methods*. After SDS-PAGE, membranes were stained with anti-FLAG or with anti-PKCδ antibodies. The results are from one representative experiment of four separate experiments. IB, immunoblot.

PMA Induced a Decrease in the Electrophoretic Mobility of RasGRP3. To further examine the effect of PMA on RasGRP3 function, we treated CHO or LN-229 cells overexpressing RasGRP3 with PMA (100 nM) for various periods of time. Treatment of the CHO cells with PMA for 15 min decreased the electrophoretic mobility of RasGRP3 (Fig. 2A). This effect was abolished after 45 min. Similar results were observed with the LN-229 cells (data not shown). To explore the role of specific PKC isoforms in the electrophoretic mobility shift of RasGRP3, we used the PKC inhibitors Gö6976 (5 μM), GF109203X (2 μM), and rottlerin (5 μM). Cells were treated with the PKC inhibitors 30 min before the addition of PMA. As presented in Fig. 2B, GF109203X, an inhibitor of both classic and novel PKCs, and rottlerin, an inhibitor of PKCδ, reduced the mobility shift of RasGRP3 in CHO and LN-299 cells, whereas Gö6976, an inhibitor of the classic PKCs, had no significant effect. Because the specificity of rottlerin for PKCδ has been questioned, we also examined the effect of a PKCδ kinase-dead mutant (PKCδ KD, K376R) and found that PMA did not induce an electrophoretic mobility shift in cells coexpressing RasGRP3 and the PKCδ kinase-dead mutant (Fig. 2C). These results suggest a role of PKCδ in the mobility shift of RasGRP3. In contrast, a PKCα KD (K368R) mutant did not affect the mobility shift of RasGRP3. Similar changes in the electrophoretic mobility of RasGRP3 were obtained in the LN-229 cells overexpressing RasGRP3 (Fig. 2C).

PMA Induces Serine Phosphorylation of RasGRP3. Because phosphorylation often shifts the mobility of proteins subjected to electrophoresis on SDS polyacrylamide gels, we examined whether PMA induced phosphorylation of RasGRP3. We first examined the effect of a phosphatase treatment on the migration of RasGRP3 in PMA-treated cells. Immunoprecipitates from control and PMA-treated cells were treated with alkaline phosphatase for 30 min in the presence and absence of the phosphatase inhibitor sodium orthovanadate. The results of this experiment demonstrate that the phosphatase treatment reversed the decrease in the mobility shift of RasGRP3 induced by PMA, whereas it did not affect that of the control cells. The decrease in the mobility shift of RasGRP3 induced by the phosphatase treatment was abolished in the presence of sodium vanadate (Fig. 3A). To further characterize the phosphorylation of RasGRP3 by PKCδ, we treated CHO and LN-220 cells overexpressing RasGRP3 with PMA, immunoprecipitated RasGRP3, and evaluated phosphorylation on serine, threonine, and tyrosine residues using phospho-amino acid specific antibodies. As presented in Fig. 3B, PMA induced an increase in the serine phosphorylation of RasGRP3 that was evident after 10 min of PMA treatment and reached plateau levels after 15 min. Rottlerin, GF109203X, and a PKCδ KD mutant decreased the serine phosphorylation of RasGRP3, whereas a PKCα KD mutant did not affect the serine phosphorylation of RasGRP3 (Fig. 3B). Likewise, a PKCδ KD mutant decreased the serine phosphorylation of RasGRP3 in PMA-treated LN-229 cells (Fig. 3C). PMA also induced a small increase in the threonine phosphorylation of RasGRP3 (Fig. 3D). In contrast, no change in the tyrosine phosphorylation of RasGRP3 was observed in PMA-treated cells (data not shown).

PKCδ Associates with RasGRP3. The association of PKCδ and RasGRP3 was examined using coimmunoprecipitation. For these studies, CHO and LN-229 cells transfected with FLAG-tagged RasGRP3 were treated with PMA for various times, and immunoprecipitation was performed using either anti-FLAG antibody or anti-PKCδ. A small degree of association between RasGRP3 and PKCδ was observed in both untreated CHO and LN-229 cells. Treatment of the cells with PMA increased this association, which was maximal at 15 min for CHO cells (Fig. 4A) and for LN-220 cells (Fig. 4B). The association partially declined by 30 to 45 min (Fig. 4, A and B). Similar results were obtained with the reciprocal immunoprecipitation (Fig. 4A). The association of PKCδ with RasGRP3 in response to PMA was specific. Using coimmunoprecipitation, we found that PKCα (Fig. 4C) and ϵ (Fig. 4C) exhibited a low degree of association with RasGRP3 and no increase in this association was observed in PMA-treated cells.

Colocalization of PKCδ and RasGRP3 in PMA-Treated Cells. To further explore the association of PKCδ with RasGRP3, we examined their localization in response to

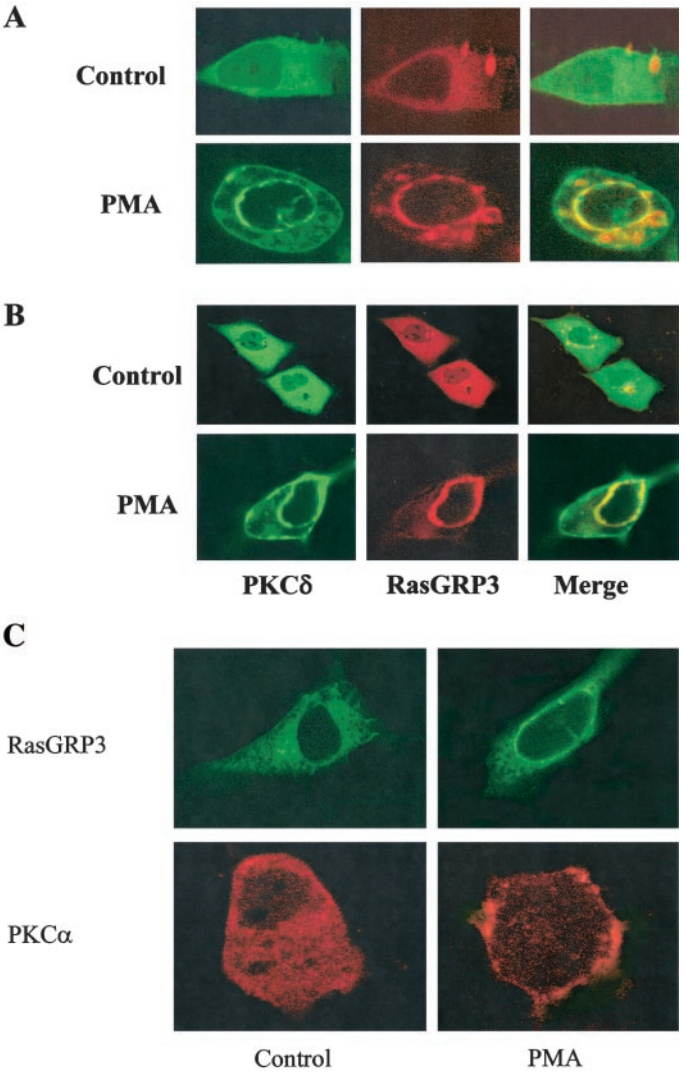

Fig. 5. PMA induces the colocalization of RasGRP3 and PKCδ in the perinuclear region. CHO (A) and LN-229 (B) cells were cotransfected with DsRed1-RasGRP3 and GFP-PKCδ (A) or with GFP-RasGRP3 and DsRed1-PKCα (CHO cells, C). After 48 h, the cells were treated with PMA (100 nM) for 30 min. The cells were fixed and viewed using confocal microscopy. Images shown are representative of three independent experiments.

PMA treatment in intact cells using confocal microscopy. For these experiments, we cotransfected CHO and LN-229 cells with RasGRP3 fused to RFP and PKCδ fused to GFP, and the response of the cells to PMA was monitored over a period of 30 min.

At first, PKCδ was distributed throughout both CHO and LN-229 cells, whereas RasGRP3 was localized to the cytoplasm. Treatment of the cells with PMA induced translocation of PKCδ to the plasma membrane followed by partial translocation to the perinuclear membrane as described previously (Wang et al., 1999). In contrast, PMA induced translocation of RasGRP3 only to the perinuclear region. After 15 min of PMA treatment, PKCδ and RasGRP3 were colocalized in the perinuclear region (Fig. 5A), and this colocalization persisted up to 60 min after PMA administration (data not shown). Similar results were observed with LN-229 cells (Fig. 5B). PKCα (Fig. 5C) and PKCε (data not shown) rapidly translocated to the plasma membrane in response to PMA and no localization in the peri-nuclear region was observed for either of these isoforms (Fig. 5C and data not shown).

PKCδ Phosphorylates RasGRP3 in Vitro. The ability of PKC isoforms to phosphorylate RasGRP3 was evaluated in vitro. Purified MBP-RasGRP3 or MBP (tag, control) were subjected to phosphorylation by PKA or various PKC isoforms (α, β1, δ, and ε). For quantitative analysis, 10 μl of reaction mixture was separated by polyacrylamide electrophoresis after 240 min of reaction. The gel was stained with Coomassie Brilliant Blue (Fig. 6A) and was subjected to autoradiography (Fig. 6B). As shown, equimolar amounts of MBP-RasGRP3 and MBP (tag, control) were loaded (Fig. 6A).

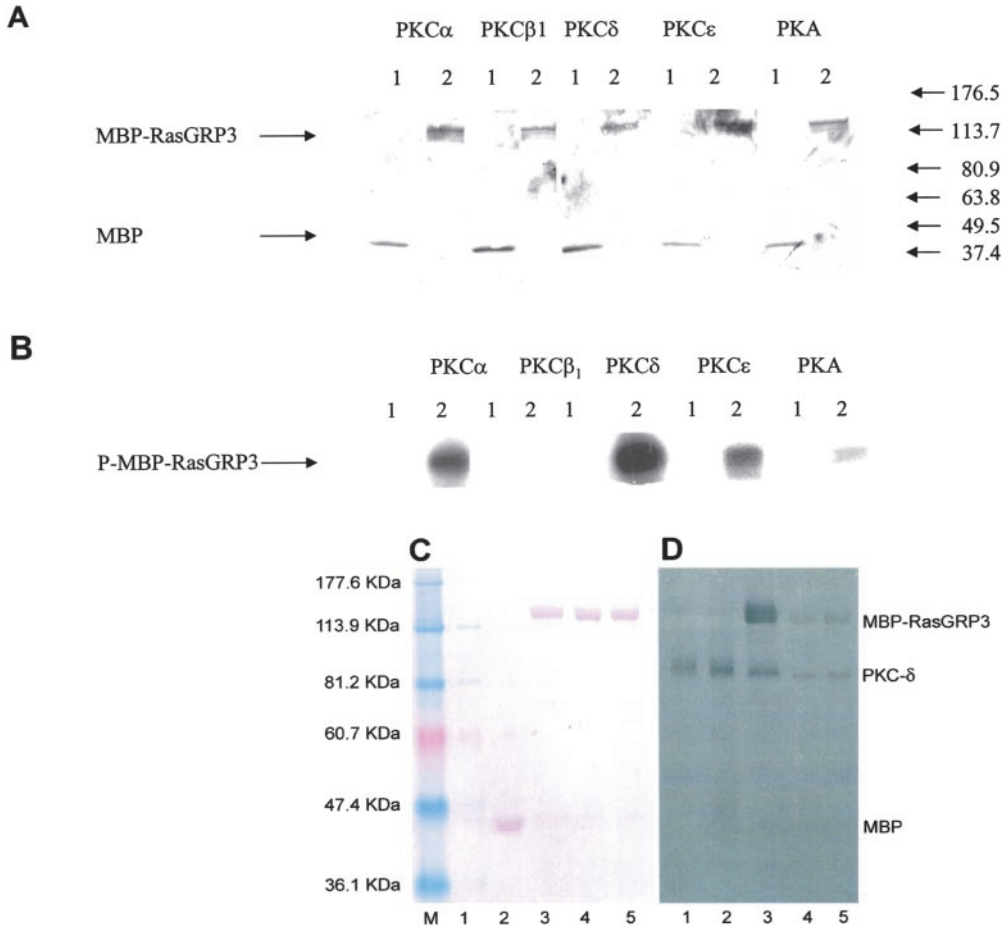

Fig. 6. In vitro phosphorylation of MBP-RasGRP3. A and B, equimolar amounts of MBP-RasGRP3 and MBP were subjected to in vitro phosphorylation. To detect the phosphorylation of MBP-RasGRP3 by various kinases (indicated), 10 μl of phosphorylation reaction mixture (containing about 8 pmol of substrates) was subjected to SDS-PAGE after a 240-min reaction. To confirm the relative amounts of proteins, the gels were stained by Coomassie Brilliant blue (A) before autoradiography (B). In A and B, lane 1 indicates MBP and lane 2 indicates MBP-RasGRP3. Experiments were done three times with similar results. The activities of the PKC isoforms on histone H1 were (per 10 μl of reaction mix) PKCα, 4.2 pmol/min; PKCβ1, 6.0 pmol/min; PKCδ, 5.4 pmol/min; and PKCε, 4.2 pmol/min. The activity of PKA on histone IIA was 4.6 pmol/min. The amounts of protein per 10 μl of reaction mix (based on the protein values provided by BIOMOL) were PKCα, 20 ng; PKCβ1, 80 ng; PKCδ, 34 ng; PKCε, 25 ng; and PKA, 90 ng. C and D, MBP-RasGRP3 or MBP (50 μg/ml) were incubated with PKCδ in a 50-μl reaction as described under *Materials and Methods*. In these experiments, using an independent lot of PKCδ different from those used above, we measured the activity of the PKCδ, using ε pseudosubstrate (Calbiochem) as the substrate, as 8.13 ± 0.51 pmol/min (per 10-μl reaction mix; mean ± S.E.M., $n = 3$). This value is similar to the activity specified by BIOMOL of 12.7 pmol/min for the ε pseudosubstrate peptide under these conditions. The amount of PKCδ per 10 μl of reaction mix (based on the protein value provided by BIOMOL) was 10 ng. The incubation mixture included 0.03% Triton X-100 and 5% DMSO. Inhibition was assessed in the presence of 50 nM or 25 nM Gö6983, a nonisoform-selective PKC inhibitor. After 60 min at 30°C, 10 μl of the reaction mixtures were mixed with 2× SDS sample buffer, boiled for 5 min, and subjected to SDS polyacrylamide gel electrophoresis on 8% gels. They were then transferred to polyvinylidene difluoride membranes (Millipore), stained with the Reversible protein detection kit (Sigma), dried, and subjected to autoradiography. C, stained gel; D, autoradiograph. Lanes: M, prestained protein ladder; 1, PKCδ; 2, PKCδ and MBP; 3, PKCδ and MBP-RasGRP3; 4, PKCδ, MBP-RasGRP3 + 50 nM Gö6983; 5, PKCδ, MBP-RasGRP3 + 25 nM Gö6983. Five additional experiments gave similar results

In case of the MBP, no phosphorylation was detected under these circumstances (data not shown). In contrast, phosphate incorporation was detected in case of the MBP-RasGRP3 with all of the PKC isoforms. To evaluate the possibility that the phosphorylation reflected a contaminating kinase, we repeated the experiment with PKCδ in the presence or absence of the PKC inhibitor Gö6983, which is not isotype selective. Phosphorylation of MBP-RasGRP3 was inhibited in parallel with inhibition of the activity of PKCδ itself, as reflected in the reduction in PKCδ autophosphorylation (Fig. 6, C and D). We conclude that RasGRP3 is a substrate for PKCs, including PKCδ, and that only modest selectivity among PKC isoforms was observed in vitro.

Activation of Erk1/2 by RasGRP3 and PKCδ. Previous studies showed that overexpression of RasGRP3 increased activation of Erk1/2 (Lorenzo et al., 2001). To explore the effects of the association between PKCδ and RasGRP3 on Erk activation, we examined the effects on Erk1/2 activation by PMA when RasGRP3 and PKCδ were overexpressed either individually or in combination (Fig. 7). All experiments were done on cells overexpressing similar levels of PKCδ and RasGRP3. Cells overexpressing control vector exhibited an increase in Erk1/2 activation after 5 min of PMA treatment, and this effect was sustained up to 120 min of treatment (data not shown), thus showing somewhat more persistence than the phosphorylation of RasGRP3. Overexpression of either PKCδ or RasGRP3 by itself increased the activation of Erk1/2 by PMA, whereas cells overexpressing a PKCδ KD mutant exhibited a decreased level of Erk1/2 activation by PMA (Fig. 7). In contrast, coexpression of PKCδ and RasGRP3 resulted in phosphorylation of Erk1/2 similar to the levels observed in CV cells. This result emphasizes the complicated coregulation of Erk1/2 by PKCδ and RasGRP3 and suggests that their interactions can be inhibitory.

Discussion

RasGRP3 is a guanine nucleotide exchange factor that contains a C1 domain that enables it to bind DAG and phorbol esters with high affinity (Wang et al., 1999; Yamashita et al., 2000; Lorenzo et al., 2001). In recent studies, RasGRP have been implicated as a mediator of PMA effects that are PKC-independent (Lorenzo et al., 2001). In this study, we examined the interaction between the phorbol ester receptors PKCδ and RasGRP3 in response to PMA.

We found that PMA induced translocation of RasGRP3 to the perinuclear region. RasGRP3 has been reported to bind phorbol esters (Rong et al., 2002) and to translocate in response to phorbol ester treatment to distinct subcellular sites (Lorenzo et al., 2001). In a recent study, the translocation of RasGRP3 in human embryonic kidney cells was reported to be dose-dependent; lower concentrations of PMA induced translocation to the membrane, whereas higher concentrations induced translocation to the Golgi (Lorenzo et al., 2001). We did not find plasma membrane translocation of RasGRP3 in response to PMA, and the differences in the translocation of RasGRP3 in the two systems are probably a result of the use of different cellular systems. The translocation of RasGRP3 to the perinuclear region may be relevant to the activation of Rap1, which is localized to the perinuclear region (Mochizuki et al., 2001) or to the activation of Ras localized in the Golgi or endoplasmic reticulum (Chiu et al., 2002; Bivona and Philips, 2003; Caloca et al., 2003). Indeed, recent reports indicate that Ras localizes to and transmits signals from the Golgi and endoplasmic reticulum (Chiu et al., 2002; Bivona and Philips, 2003) and that members of the RasGRP family mediate the activation of Ras in the Golgi (Caloca et al., 2003).

In addition to translocation of RasGRP3 to the perinuclear region, PMA also induced a decrease in the electrophoretic mobility of RasGRP3. This effect of PMA was caused by phosphorylation, in that it was inhibited by alkaline phosphatase, and was PKC-dependent in that it was inhibited by PKC inhibitors. A candidate isoform of PKC for the PMA effects in this system was PKCδ because the electrophoretic mobility shift of RasGRP3 was inhibited by the general PKC inhibitor GF109203X, by the specific PKCδ inhibitor rottlerin, and by a PKCδ KD mutant. We found that the mobility shift was associated with phosphorylation of RasGRP3 on serine residues, which was also inhibited by a PKCδ KD mutant and not by a PKCα KD mutant. Phosphorylation of other Ras GEFs has already been reported. For example, both EGF and PMA induced the phosphorylation of human SOS in a mitogen-activated protein kinase-dependent manner (Porfiri and McCormick, 1996).

A role of PKCδ in the phosphorylation of RasGRP3 is

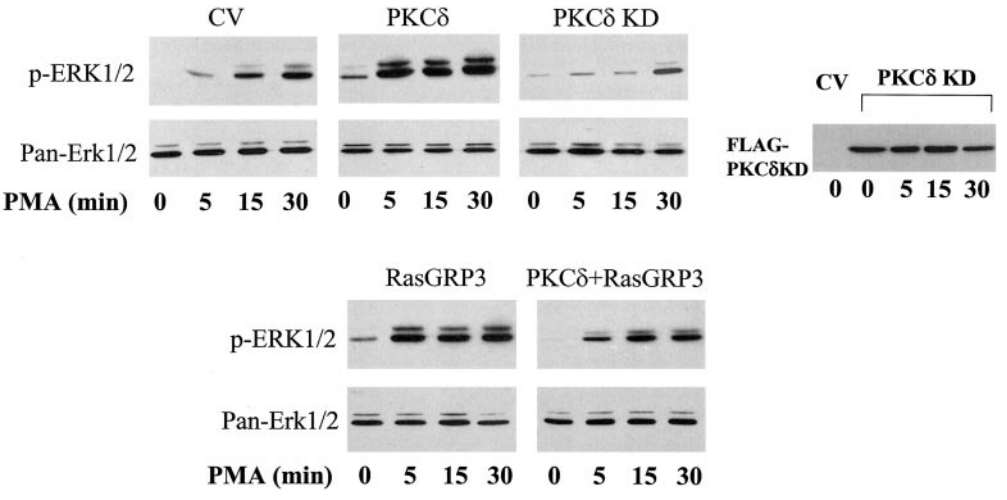

Fig. 7. Phosphorylation of Erk1/2 by RasGRP3 and PKCδ. Cells overexpressing FLAG-RasGRP3 and PKCδ either individually or in combination or overexpressing PKCδ KD mutant were treated with PMA for various periods of time. The cells were harvested and subjected to Western blot analysis and the membranes were probes with anti-phospho Erk1/2 and with pan-Erk1/2. Overexpression of the PKCδKD mutant was examined using an anti-FLAG antibody. The results are representative of three independent experiments.

supported by evidence for the association of PKCδ and RasGRP3. First, we demonstrated by confocal analysis that PKCδ partially colocalized with RasGRP3, which is a prerequisite for functional interaction of these two proteins. Colocalization of PKCδ and RasGRP3 occurred in the perinuclear membrane. Moreover, we found that PKCδ coimmunoprecipitated with RasGRP3 in PMA-treated cells, arguing for the physical association of these two proteins. These results suggest that PKCδ lies functionally upstream of RasGRP3 and phosphorylates it in response to PMA.

The evidence for the in vivo phosphorylation of RasGRP3 by PKCδ was supported by results of in vitro phosphorylation. Using different recombinant protein kinases, we confirmed in vitro phosphorylation of RasGRP3 by PKCδ, as well as other PKC isoforms. In the case of PKCδ, we further demonstrated that the phosphorylation of RasGRP3 was inhibited by the PKC inhibitor Gö6983, arguing that the phosphorylation was in fact caused by the PKCδ rather than by an adventitious contaminating kinase in the purified enzyme preparation. The identity of the phosphorylation sites in RasGRP3 is currently being studied. Although phosphorylation of RasGRP3 was observed for PKCα, -β1, and -ε, as well as for PKCδ, we can exclude the involvement of these isoforms in the phosphorylation of RasGRP3 in CHO cells based on the lack of association of RasGRP3 with PKCα, -β1, and -ε in the PMA-treated cells as demonstrated by coimmunoprecipitation assays, by the lack of colocalization of RasGRP3 with these PKC isoforms, by the inhibition of phosphorylation by the kinase dead mutant of PKCδ, and, for PKCα at least, by the lack of effect of the kinase-dead mutant of PKCα.

The importance of the phosphorylation of RasGRP3 by PKCδ for the function of RasGRP3 was examined with regard to its effects on the activation of Erk1/2, which represents one of the downstream targets of Ras (Marshall, 1995). Previous studies demonstrated that overexpression of RasGRP3 increased Erk1/2 phosphorylation by PMA (Lorenzo et al., 2001). Similar to these findings, we also found that overexpression of RasGRP3 in CHO cells increased the phosphorylation of Erk1/2. In addition, overexpression of PKCδ similarly increased the phosphorylation of Erk1/2. It is interesting that coexpression of both RasGRP3 and PKCδ resulted in low level phosphorylation of Erk1/2 similar to that observed in control cells. These results suggest that the interaction of PKCδ and RasGRP3 in this system may provide a negative signal for the activation of Erk1/2 when PKCδ is expressed in high levels. The mechanisms of this inhibition are unclear, but one possibility is that the interaction of RasGRP3 and PKCδ or the phosphorylation of RasGRP3 may interfere with its ability to activate the Erk pathway. For example, the phosphorylation of RasGRP3 may inhibits its GEF activity toward Ras or increase its activation of Rap1, which in turn can interfere with the Ras-mediated Erk activation induced by PKCδ (Cook et al., 1993). In other systems, PMA has been reported to affect the specificity of Ras signaling by differentially modulating the activation of various Ras effector proteins (Rusanescu et al., 2001). Likewise, PMA and EGF phosphorylate hSOS1 and decrease the ability of EGF to induce Ras activation (Porfiri and McCormick, 1996). RasGRP1 and GTP-H-Ras were recently reported to be localized in the perinuclear region in PMA-treated cells (Caloca et al., 2003). The importance of the subcellular localization of Ras and the RasGRP proteins to the signals transmitted by Ras are currently not understood. Likewise, further studies are needed to understand the role of the interaction of PKCδ and RasGRP3 in the ability of Ras to activate downstream signaling pathways such as Erk1/2.

It has been shown recently that PKCμ/PKD, another member of the extended phorbol-ester family (Johannes et al., 1994), is activated via PKC-dependent pathways (Waldron and Rozengurt, 2003). For example, PKD has been reported to associate, to colocalize with, and to be phosphorylated by PKCη (Waldron and Rozengurt, 2003) and to regulate the Jun kinase pathway via PKCε (Brandlin et al., 2002). Likewise, our results of functional interaction of PKCδ with RasGRP3 point to the existence of complex cross-talk between various members of the phorbol-ester receptor family with potential impact for major signaling pathways activated by PKCs and RasGRP3, whether in response to the phorbol esters or to their endogenous analogs, the diacylglycerols.

References

Ahmed S, Kozma R, Lee J, Monfries C, Harden N and Lim L (1991) The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc. Evidence for the involvement of a zinc-dependent structure in phorbol ester binding. *Biochem J* **280:**233–241.

Betz A, Ashery U, Rickmann M, Augustin I, Neher E, Sudhof TC, Rettig J, and Brose N (1998) Munc13–1 Is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21:**123–136.

Bivona TG and Philips MR (2003) Ras Pathway signaling on endomembranes. *Curr Opin Cell Biol* **15:**136–142.

Blass M, Kronfeld I, Kazimirsky G, Blumberg PM, and Brodie C (2002) Tyrosine phosphorylation of protein kinase Cdelta is essential for its apoptotic effect in response to etoposide. *Mol Cell Biol* **22:**182–195.

Blumberg PM (1988) Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. *Cancer Res* **48:**1–8.

Brandlin I, Eiseler T, Salowsky R, and Johannes FJ (2002) Protein kinase Cμ regulation of the JNK pathway is triggered via phosphoinositide-dependent kinase 1 and protein kinase Cε. *J Biol Chem* **277:**45451–45457.

Brose N and Rosenmund C (2002) Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J Cell Sci* **115:**4399–4411.

Caloca MJ, Garcia-Bermejo ML, Blumberg PM, Lewin NE, Kremmer E, Mischak H, Wang S, Nacro K, Bienfait B, Marquez VE, et al. (1999) Beta2-chimaerin is a novel target for diacylglycerol: binding properties and changes in subcellular localization mediated by ligand binding to its C1 domain. *Proc Natl Acad Sci USA* **96:**11854–11859.

Caloca MJ, Zugaza JL, and Bustelo XR (2003) Exchange factors of the RasGRP family mediate Ras activation in the Golgi. *J Biol Chem.* **278:**33465–33473.

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, and Nishizuka Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257:**7847–7851.

Chardin P, Camonis JH, Gale NW, van Aelst L, Schlessinger J, Wigler MH, and Bar-Sagi D (1993) Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. *Science (Wash DC)* **260:**1338–1343.

Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, Johnson RL 2nd, Cox AD, and Philips MR (2002) Ras signalling on the endoplasmic reticulum and the Golgi. *Nat Cell Biol* **4:**343–350.

Clyde-Smith J, Silins G, Gartside M, Grimmond S, Etheridge M, Apolloni A, Hayward N, and Hancock JF (2000) Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. *J Biol Chem* **275:**32260–32267.

Cook SJ, Rubinfeld B, Albert I, and McCormick F (1993) RapV12 Antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO (Eur Mol Biol Organ) J* **12:**3475–3485.

Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, and Stone JC (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science (Wash DC)* **280:**1082–1086.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, and Feig LA (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature (Lond)* **376:**524–527.

Hayashi A, Seki N, Hattori A, Kozuma S, and Saito T (1999) PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. *Biochim Biophys Acta* **1450:**99–106.

Johannes FJ, Prestle J, Eis S, Oberhagemann P, and Pfizenmaier K (1994) PKCμ is a novel, atypical member of the protein kinase C family. *J Biol Chem* **269:**6140–6148.

Kazanietz MG (2000) Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. *Mol Carcinog* **28:**5–11.

Kazanietz MG (2002) Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol Pharmacol* **61:**759–767.

Kazanietz MG, Lewin NE, Bruns JD, and Blumberg PM (1995) Characterization of the cysteine-rich region of the *Caenorhabditis elegans* protein Unc-13 as a high affinity phorbol ester receptor. Analysis of ligand-binding interactions, lipid cofactor requirements and inhibitor sensitivity. *J Biol Chem* **270:**10777–10783.

Kronfeld I, Kazimirsky G, Lorenzo PS, Garfield SH, Blumberg PM, and Brodie C

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(2000) Phosphorylation of PKCδ on distinct tyrosine residues regulates specific cellular functions. *J Biol Chem* **275:**35491–35498.
Lorenzo PS, Beheshti M, Pettit GR, Stone JC, and Blumberg PM (2000) The guanine nucleotide exchange factor RasGRP is a high-affinity target for diacylglycerol and phorbol esters. *Mol Pharmacol* **57:**840–846.
Lorenzo PS, Kung JW, Bottorff DA, Garfield SH, Stone JC, and Blumberg PM (2001) Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res* **61:**943–949.
Marshall MS (1995) Ras target proteins in eukaryotic cells. *FASEB J* **9:**1311–1318.
Mochizuki N, Yamashita S, Kurokawa K, Ohba Y, Nagai T, Miyawaki A, and Matsuda M (2001) Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature (Lond)* **411:**1065–1068.
Pierret P, Dunn RJ, Djordjevic B, Stone JC, and Richardson PM (2000) Distribution of Ras guanyl releasing protein (RasGRP) mRNA in the adult rat central nervous system. *J Neurocytol* **29:**485–497.
Pierret P, Vallee A, Mechawar N, Dower NA, Stone JC, Richardson PM, and Dunn RJ (2001) Cellular and subcellular localization of Ras guanyl nucleotide-releasing protein in the rat hippocampus. *Neuroscience* **108:**381–390.
Porfiri E and McCormick F (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the Ras exchange factor HSOS1. *J Biol Chem* **271:**5871–5877.
Priatel JJ, Teh SJ, Dower NA, Stone JC, and Teh HS (2002) RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis and differentiation. *Immunity* **17:**617–627.
Reuther GW, Lambert QT, Rebhun JF, Caligiuri MA, Quilliam LA, and Der CJ (2002) RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia. *J Biol Chem* **277:**30508–30514.
Ron D and Kazanietz MG (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* **13:**1658–1676.
Rong SB, Enyedy IJ, Qiao L, Zhao L, Ma D, Pearce LL, Lorenzo PS, Stone JC, Blumberg PM, Wang S, et al. (2002) Structural basis of RasGRP binding to high-affinity PKC ligands. *J Med Chem* **45:**853–860.
Rusanescu G, Gotoh T, Tian X, and Feig LA (2001) Regulation of Ras signaling specificity by protein kinase C. *Mol Cell Biol* **21:**2650–2658.
Sharkey NA, Leach KL, and Blumberg PM (1984) Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc Natl Acad Sci USA* **81:**607–610.
Shindo M, Irie K, Ohigashi H, Kuriyama M, and Saito N (2001) Diacylglycerol kinase gamma is one of the specific receptors of tumor-promoting phorbol esters. *Biochem Biophys Res Commun* **289:**451–456.
Teixeira C, Stang SL, Zheng Y, Beswick NS, and Stone JC (2003) Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3. *Blood* **102:**1414–1420.
Waldron RT and Rozengurt E (2003) Protein kinase C phosphorylates protein kinase δ activation loop Ser744 and Ser748 and releases autoinhibition by the pleckstrin homology domain. *J Biol Chem* **278:**154–163.
Wang QM, Bhattacharyya D, Garfield S, Marquez VE, and Blumberg PM (1999) Differential localization of protein kinase C-δ by phorbol esters and related compounds using a fusion protein with green fluorescent protein. *J Biol Chem* **274:**37233–37239.
Yamashita S, Mochizuki N, Ohba Y, Tobiume M, Okada Y, Sawa H, Nagashima K, and Matsuda M (2000) CalDAG-GEFIII activation of Ras, R-Ras, and Rap1. *J Biol Chem* **275:**25488–25493.
Yang Y, Li L, Wong GW, Krilis SA, Madhusudhan MS, Sali A, and Stevens RL (2002) RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J Biol Chem* **277:**25756–25774.
Yuspa SH, Lichti U, Ben T, Patterson E, Hennings H, Slaga TJ, Colburn N, and Kelsey W (1976) Phorbol esters stimulate DNA synthesis and ornithine decarboxylase activity in mouse epidermal cell cultures. *Nature (Lond)* **262:**402–404.

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