Phorbol Esters Modulate the Ras Exchange Factor RasGRP3

Patricia S. Lorenzo, Justin W. Kung, Drell A. Bottorff, Susan H. Garfield, James C. Stone, and Peter M. Blumberg

Laboratory of Cellular Carcinogenesis and Tumor Promotion [P. S. L., J. W. K., P. M. B.], and Laboratory of Experimental Carcinogenesis [S. H. G.], National Cancer Institute, Bethesda, Maryland 20892-4255, and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada [D. A. B., J. C. S.]

ABSTRACT

RasGRP represents the prototype of a new class of guanine nucleotide exchange factors that activate small GTPases. The guanyl nucleotide-releasing protein (GRP) family members contain catalytic domains related to CDC25, the Ras exchange factor of Saccharomyces cerevisiae. They also contain a motif resembling a pair of calcium-binding EF-hands and a C1 domain similar to the diacylglycerol interaction domain of protein kinase C. The sequence of KIAA0846, identified in a human brain cDNA library, encodes a member of the GRP family that we refer to as RasGRP3. We show here that RasGRP3 bound phorbol esters with high affinity. This binding depended on anionic phospholipids, which is characteristic of phorbol ester binding to C1 domain proteins. In addition, phorbol esters also caused activation of the RasGRP3 exchange activity in intact cells, as determined by an increase in RasGTP and phosphorylation of the extracellular-regulated kinases. Finally, both phorbol 12-myristate 13-acetate and the diacylglycerol analogue 1,2-dioctanoyl-sn-glycerol induced redistribution of RasGRP3 to the plasma membrane and/or perinuclear clear area in HEK-293 cells, as demonstrated using a green fluorescent fusion protein. We conclude that RasGRP3 serves as a PKC-independent pathway to link the tumor-promoting phorbol esters with activation of Ras GTPases.

INTRODUCTION

Phorbol esters are natural products first recognized as potent tumor-promoting agents in skin. The first described cellular target for phorbol esters was PKC, and still today, most of the actions of these compounds are considered to be mediated by PKC pathways. However, emerging evidence has revealed new cellular receptors, which respond both in vivo and in vitro with sensitivity similar to PKC. The chimerins (1) and Munc-13 proteins (2) are non-PKC members of the phorbol ester receptor family. They all have a C1 domain, the signature motif that is involved in the recognition of the phorbol ester molecule and its physiological counterpart, the second messenger diacylglycerol (3, 4). RasGRP (5, 6), which also possesses a C1 motif, represents a novel class of phorbol ester targets that functions as GEFs.

The GEFs are proteins that catalyze the dissociation of GDP from Ras GTPases, to allow the formation of the active GTP-bound conformation (7). Examples of GEFs for Ras include the well-characterized group SOS (8) and the calcium-dependent RasGRF (9). RasGRP represents the prototype of a new class of GEFs that is composed of at least three members. RasGRP was the first member characterized as a GEF for Ras (5). The related coding sequence HDC25L, described in 1997 as a potential Ras activator (10), was later shown to be a GEF for Rap1 and has been referred to as CalDAGI (11) or GRP2 (12). In a recent study, Clyde-Smith et al. (13) described an alternative spliced variant of this Rap GEF, named RasGRP2, which possesses GEF activity for N-Ras, K-Ras, and Rap1. RasGRP2 is located in chromosome 11p13, a region frequently amplified in human tumors (10, 13). Finally, the KIAA0846 sequence (14) was reported to encode a GEF (CalDAGIII or GRP3), which can activate both Ras and Rap1 (12, 15). We refer to this third member of the family as RasGRP3. Despite the differences in substrate activity, all of the GEF members share similar overall domain structure. They have the Ras GEF signature motif CDC25 (Ras GEF of Saccharomyces cerevisiae), a pair of atypical EF-hands (a calcium-binding motif), and the C1 domain.

Recent studies have revealed that RasGRP functions as a critical molecule in thymocyte differentiation and T-cell activation, linking the T-cell receptor and diacylglycerol messengers to Ras signaling (16, 17). Moreover, phorbol esters can directly interact with RasGRP with nanomolar affinity, promote the EF activity, and thereby activate Ras signaling in T cells. In this study, we present evidence that RasGRP3 is also a high-affinity receptor for phorbol esters. Not only did RasGRP3 bind phorbol esters with nanomolar affinity but also its GEF activity for Ras was increased by phorbol ester treatment in the intact cell. In addition, RasGRP3 redistributed to particulate compartments in response to phorbol ester treatment. Taken together, these findings suggest that RasGRP3 serves as a high-affinity target for the tumor-promoting phorbol esters, inducing PKC-independent activation of Ras. The widespread tissue distribution of RasGRP3 and its ability to modulate the small GTPase Ras may be relevant in the context of the phorbol ester tumor-promoting activity.

MATERIALS AND METHODS

Plasmids. The coding sequence of human RasGRP3 (KIAA0846) was subcloned into the vector pMAL-c2 (New England Biolabs, Beverly, MA) downstream from the MBP gene. The construct, which coded for the fusion protein MBP-RasGRP3, was used for bacterial expression and purification. To produce a construct suitable for mammalian expression and confocal studies, the cDNA of RasGRP3 was amplified by PCR and subcloned into the pQE80 vector (Quantum Biotechnologies Inc., Montreal, Canada) using the NheI site. This generated a fusion protein (RasGRP3-GFP) between the COOH terminus of RasGRP3 and the NH2 terminus of the red-shifted GFP tag. RasGRP3 was also subcloned in the retroviral vector pBabePuro (5) for generation of a RasGRP3-Puro virus.

Cell Culture. HEK-293 cells were propagated in Eagle’s MEM adjusted to contain 0.1 mM nonessential amino acids (American Type Culture Collection, Manassas, VA) and supplemented with 10% heat-inactivated horse serum (Life Technologies, Gaithersburg, MD). Cell transfections with pQBE25 or RasGRP3-pQBE25 constructs were performed using LipofectAMINE Plus (Life Technologies). For generation of stable transfectants expressing either RasGRP3-GFP or the GFP protein alone, cells were selected in the presence of 500 μg/ml of the antibiotic G-418 (Life Technologies) for 2 weeks. For generation of stable cell lines of RasGRP3 in rat2 fibroblasts, cells were infected with the RasGRP3-Puro virus and selected in the presence of puromycin.

Binding of [3H]PDBu. Binding of [3H] PDBu (777 Bq/mmol; New England Nuclear, Boston, MA) was measured as described elsewhere (18). The assay mixture contained 50 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 0.1 mM CaCl2, RasGRP3 protein, and the corresponding lipid mixture. Incubations were carried out at 18°C. Nonspecific binding was measured using an excess of PDBu (30 μM), and specific binding was done in triplicate at each ligand.
orthovanadate, 10 mM MgCl2, and protease inhibitors. After a 6-s sonication pulse, cells were washed twice with 1× Dulbecco’s PBS and resuspended in 300 µl of lysis buffer [50 mM Tris- HCI (pH 7.4), 1 mM EDTA, 10% glycerol, 1% β-mercaptoethanol, and protease inhibitors] and frozen overnight at −20°C. After thawing, cell lysis was completed by six 15-s pulses of sonication over 2 min. The lysate was centrifuged at 4°C for 20 min at 14,000 × g, and the supernatant was used as the crude extract for the subsequent purification.

The MBP-RasGRP3 fusion protein was purified using an amylose resin according to the manufacturer’s instructions (New England Biolabs). Depending on the batch, 2–10 µg of partially purified protein per tube were used for the binding assays. Lipids were transferred to 170 mM sucrose and 20 mM Tris- HCI (pH 7.4) for binding experiments. The RBD of Raf1 in a pGEX expression vector was used as a control. To determine the concentration used for each binding experiment, transferred lipids were resuspended in 1% paraformaldehyde for 10 min at room temperature. The blocking solution was removed, and cells were washed with 1× PBS for 5 min and incubated for 1 h with 8% BSA at room temperature. The blocking solution was removed, and cells were washed with 1× PBS for 5 min. Then, cells were incubated at 4°C overnight with 2.5 µg/ml Ras clone Ras10 antibody (Upstate Biotechnology) diluted in 1% BSA. After washing twice with 1× PBS for 5 min, FITC-antimouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to the coverslip for 2 h at room temperature in the dark. After a final wash with 1× PBS, cells were mounted in VectaShield with propidium iodide (Vector Laboratories, Inc., Burlingame, CA) and examined by confocal microscopy.

**RESULTS**

**Phorbel Ester Binding to RasGRP3.** The schematic structure of RasGRP3 is shown in Fig. 1A. The other two members of the family, RasGRP and RasGRP2, are also included for comparison. All of the members have a RasGEF domain belonging to the CDC25 family of GEFs and a RasGEF-associated NH2-terminal Ras exchange motif (REM). They also possess a pair of EF-hand calcium-binding domains, and a potential diacylglycerol/phorbol ester binding motif C1. The presence in RasGRP3 of a C1 domain that appears to have the structural features appropriate for diacylglycerol/phorbol ester binding prompted us to investigate the modulation of this protein by phorbol esters. Alignment studies showed high sequence similarities between the RasGRP3 C1 domain and those present in other diacylglycerol receptors, such as PKCε and PKCδ (Fig. 1B). As expected
RasGRP3 GEF Activity Is Increased by PMA in Vivo. The ERKs (ERK1/ERK2) are typically activated in response to growth promoting factors through Ras-dependent pathways. In fact, RasGRP3 expression has been shown to induce biological activities attributable to Ras activation, including ERK stimulation (15). We tested whether RasGRP3 activation of Ras pathways could be affected by phorbol esters. ERK1/ERK2 is activated enzymatically through tyrosine and threonine phosphorylation; therefore, we measured activation by determining the level of phosphothreonine 202 and phosphotyrosine 204 of ERK1/ERK2. For the experiments, we used a pool of stable transfected cells expressing modest levels of RasGRP3 to reduce the basal activation of ERK1/ERK2 in the absence of PMA treatment. To eliminate the PKC-mediated contribution on ERK1/ERK2 activation, the PKC inhibitor GF109203X was used before and during PMA treatment. When cells that were transinfected with RasGRP3 were treated with PMA for 15 min, ERK1/ERK2 was activated in a concentration-dependent manner (Fig. 3). The densitometric analysis revealed a maximal RasGRP3-dependent, PKC-independent activation at 100 nM PMA, with a higher concentration diminishing the response. The level of total ERK1/ERK2 remained constant for the time points and concentrations tested. Neither the HEK-293 untransfected cells nor the empty vector transfected pools (pQBI25) showed any PKC-independent ERK1/ERK2 phosphorylation induced by PMA treatment (Fig. 3, A and B).

To confirm the ability of RasGRP3 to promote guanine nucleotide exchange on Ras in response to phorbol esters, we used an assay based on the differential affinity of the RBD of Raf1 for the active GTP-loaded Ras (RasGTP). Expression of RasGRP3 in HEK-293 cells induced a weak activation of endogenous Ras under low serum

from this similarity, RasGRP3 bound phorbol esters with high affinity. Fig. 1C shows the saturation binding curve and Scatchard plot (inset) obtained by using recombinant RasGRP3 protein and [3H]PDBu as a ligand. The dissociation constant (K_d) for this binding was 1.53 ± 0.33 nM (n = 4).

High-affinity binding of phorbol esters to C1 domain proteins requires phospholipid as a cofactor. The Scatchard plot shown in Fig. 1C was obtained in the presence of 100 μg/ml phosphatidylyserine. To determine the sensitivity of RasGRP3 binding to the phospholipid composition, we examined two different vesicle mixtures: POPA/POPC and POPS/POPC. Previous findings have shown that these mixtures induced efficient phorbol ester binding for the related family member RasGRP (20). The effect of increasing concentrations of lipid vesicles (10–3000 μM) is shown in Fig. 2. RasGRP3 displayed an almost identical pattern of phorbol ester binding for both anionic phospholipids, with strong dependence on the mole percentage content of anionic phospholipid. Weak binding levels were observed at 5 mol % anionic phospholipid, with EC_{so} values approximately 30-fold higher than the EC_{so} values at 20 mol % (EC_{so} POPA at 5 mol % = 6300 ± 1100 μM; POPA at 20 mol % = 235 ± 15 μM; POPS at 5 mol % = 6100 ± 1000 μM, POPS at 20 mol % = 273 ± 18 μM). In contrast to the previous results observed for RasGRP (20), RasGRP3 required a greater mole fraction of phosphatidylyserine or phosphatidic acid to reach maximal binding to phorbol esters. This dependence is more similar to what has been found for some PKC isoforms, such as PKCα (20).
conditions (Fig. 4). This activation was further increased by treatment with the phorbol ester PMA. The activation was independent of PKC because it was not abolished or reduced by preincubation with 5 μM GF109203X. Under the same concentrations, PMA did not significantly affect the level of RasGTP in HEK-293 cells transfected with the pQBI25 vector alone and assayed in the presence of GF109203X (Fig. 4).

We also used the cell line rat2 to corroborate the effect of phorbol esters on the activation of Ras by RasGRP3. In rat2 cells expressing RasGRP3, PMA induced both a significant increase in the amount of RasGTP and an increase in ERK1/ERK2 activation compared with the control, vector-transfected cells (Fig. 5). In an independent experiment, we found that treatment of these cells with a calcium ionophore neither activated Ras nor augmented PMA-induced Ras activation (data not shown).

**RasGRP3 Redistributes in Vivo in Response to Phorbol Esters.** Ras proteins must be localized to the inner leaflet of the plasma membrane to be biologically active. Thus, RasGRP3 is expected to localize to the plasma membrane to activate Ras. We confirmed by immunofluorescence that endogenous Ras localized to the plasma membrane in HEK-293 cells (Fig. 6). There was also a punctate pattern of Ras distribution, possibly indicative of localization in the Golgi apparatus and endosomes, which has been described by others as part of the trafficking pool of Ras to the plasma membrane (21). By the use of the GFP fusion protein with RasGRP3 (RasGRP3-GFP), we looked at the subcellular distribution of RasGRP3 in live HEK-293 cells. As shown on Fig. 7, RasGRP3 localized mainly in the cytosol with some perinuclear concentration. We investigated the ability of phorbol esters to recruit RasGRP3 to the plasma membrane by following RasGRP3 redistribution in individual cells. As a control, we performed translocation studies using the GFP protein alone (pQBI25-transfected cells). The control GFP protein distributed evenly throughout the cells, including the nucleus. At the highest concentration of PMA tested (1 μM), no changes in the subcellular localization of the GFP protein were observed (Fig. 7A, lower panel). In contrast, PMA changed the distribution of RasGRP3-GFP in the HEK-293 cells in a concentration-dependent manner. For concentrations up to 10 nM, no changes in localization were evident (Fig. 7A). PMA (100 nM) caused RasGRP3 to localize preferentially to the plasma membrane after 15 min (Fig. 7A). At 1 μM, PMA induced not only some plasma membrane localization but also perinuclear distribution and nuclear membrane localization (Fig. 7A). To determine whether the perinuclear distribution observed at 1 μM PMA corresponded to localization in the Golgi apparatus, we visualized Golgi with Bodipy-Ceramide (red) and followed the pattern of translocation of RasGRP3 (green) by confocal microscopy. After 15-min exposure to PMA (1 μM), images showed colocalization of RasGRP3 in green and Bodipy-Ceramide in red, suggesting a redistribution of RasGRP3 to the Golgi apparatus (Fig. 7C). We also looked at the translocation pattern of RasGRP3 in response to the membrane-permeable analogue of diacylglycerol, DOG. At the two concentrations tested, 10 and 100 μM, DOG induced redistribution of RasGRP3 to the perinuclear area and nuclear membrane with some plasma membrane localization after 15-min treatment (Fig. 7B).

**Chromosomal Mapping of RasGRP3.** RasGRP3 has been found in several human tissues, including brain, heart, lung, and kidney (15). Using a human Northern blot, we also observed expression in skeletal muscle, liver, and placenta (data not shown). To determine the chromosomal location of the human RASGRP3 gene, we performed FISH with human metaphase chromosomes from normal lymphocytes. The analysis revealed one genomic locus in chromosome 2p23 (data not shown). No significant background was observed in any other chromosome. To confirm the localization, we took advantage of the relative ease of linkage analysis in the mouse and the well-known tendency of related human and mouse orthologues to be found in regions of conserved synteny. Using a probe derived from the cloned mouse gene, we observed a 7.2-kb EcoRI band that hybridizes strongly in the genomic DNA of C57Bl/6J strain mice. In Mus spretus, an alternative 6.6-kb band was evident. Both species exhibited a 1.9-kb band. Using this assay, we typed the gene structure in a panel of 89 animals derived from a (C57Bl/6J/M. spretus) F1 × C57Bl/6J...
backcross. No recombinants were observed between our gene and the chromosome marker D17Hun17 on distal chromosome 17 (95% confidence limit, 3.8 cM). The sequence, to which we here assign the name Rasgrp3, is located at a position _44 cM from the centromere. Many mouse sequences in this region have homologous human relatives in chromosome 2p2, as expected.

**DISCUSSION**

In this study, we present evidence that RasGRP3 has the capacity to bind phorbol esters and be modulated by them. The presence of a C1 domain in the protein and the sensitivity of RasGRP3 to phorbol esters and DAG analogues suggest that RasGRP3 is a high-affinity target for the second messenger diacylglycerol. In addition to its high affinity for phorbol esters, RasGRP3 shares another characteristic of the phorbol ester-binding proteins: its dependence on anionic phospholipids. The requirement for phospholipid has been extensively demonstrated for the classic phorbol ester receptors, the PKCs (22, 23). However, the molecular basis of the lipid interaction is still under investigation. It seems to involve not only the C1 but also the pseudosubstrate region, and the calcium-binding motif, or C2 domain. Although RasGRP3 possesses a calcium-binding site, it is not a C2 domain but a pair of EF-hands. RasGRP also shows dependence on phospholipids despite the absence of a C2 domain (20). Interestingly, RasGRP3 showed a higher requirement for phosphatidylserine and phosphatidic acid than did RasGRP (present results and Ref. 20). In this regard, although the studies on RasGRP3 were done on the whole recombinant protein, we performed the RasGRP experiments using a truncated version of the protein consisting of the EF-hand and C1 domain plus a cluster of basic residues found at the COOH-terminal side of the C1 domain in all of the three GRP family members. Presumably, domains other than these could contribute to the phospholipid recognition by these GEFs.

Phosphatidylserine accounts for most of the anionic phospholipid present in the inner side of the plasma membrane and endosomes, and it is one of the main contributors to the electrostatic attraction of proteins to membrane compartments (24). The requirement of RasGRP3 for anionic phospholipids suggests the possibility of RasGRP3 redistribution, or translocation, by phorbol ester treatment in intact cells. Our experiments confirmed that RasGRP3 did translocate...
in response to the phorbol ester PMA. We found two different patterns of translocation in live cells, depending on the concentration of PMA. At 100 nM PMA, we observed predominant plasma membrane localization of RasGRP3, whereas higher concentrations induced preferential perinuclear and nuclear membrane distribution. A membrane-permeable analogue of DAG, DOG, also induced RasGRP3 redistribution to the perinuclear region and, to a lesser extent, to the plasma membrane. The localization to the plasma membrane may be relevant for the activation of Ras, because it is in the plasma membrane where Ras localizes (25). There are several examples of EFs for Ras that are recruited to the plasma membrane with activation, such as SOS (26), RasGRF2 (27), and RasGRP (5, 6). The significance of the perinuclear distribution of RasGRP3, however, remains to be investigated. Another target of RasGRP3 resides mainly in the Golgi apparatus and endosomes: the small G protein Rap1 (28, 29). Preliminary studies indicate that Rap1, like Ras, is activated by phorbol esters.
esters through RasGRP3.\(^3\) On the basis of the differential pattern of distribution, it is tempting to speculate that RasGRP3 would preferentially activate Rap1 over Ras at high concentrations of PMA. In a physiological context, this could provide a mechanism for differential modulation of Ras and Rap1 depending on the site and extend of the DAG generation in the cell. Additional studies are needed to examine this hypothesis.

One of the downstream targets of Ras is the Raf1/MEK/ERK cascade (30). As expected from Ras stimulation, we found that RasGRP3 induced ERK activation in a PMA-dependent manner. It should be noted that the PMA-concentration profile for ERK activation did not correlate quantitatively with the translocation pattern of RasGRP3. Although 10 nM PMA induced almost maximal activation of ERK, redistribution of RasGRP3 was predominantly at high ligand concentrations. This shift in concentration-response curves would be consistent with the concept of “spare receptors” (31), in which activation of a fraction of the receptors is sufficient for saturation of the downstream response.

In conclusion, our results demonstrate that RasGRP3 can serve as a phorbol ester-activated signaling pathway that independently of PKC modulates Ras. RasGRP3 and the other members of the GRP family represent particularly interesting targets of the phorbol ester tumor promoters. This is because the GRP proteins directly modulate PKC modulates Ras. RasGRP3 and the other members of the GRP sequence. Proc. Natl. Acad. Sci. USA, 95: 13278–13283, 1998.


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