RasGRP1 Represents a Novel Non-protein Kinase C Phorbl Ester Signaling Pathway in Mouse Epidermal Keratinocytes*

The mouse skin model of carcinogenesis has been instrumental in our appreciation of the multistage nature of carcinogenesis. In this system, tumor promotion is a critical step in the generation of tumors and is usually achieved by treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Although it is generally assumed that protein kinase C (PKC) is the sole receptor for TPA in this system, we sought to evaluate whether non-PKC pathways could also contribute to the effects of phorbol esters in skin. We documented expression of the high affinity non-PKC phorbol ester receptor and Ras activator RasGRP1 in mouse primary keratinocytes. Overexpression of RasGRP1 in keratinocytes increased the level of active GTP-loaded Ras. TPA treatment further elevated this Ras activation in a PKC-independent manner and induced the translocation and down-regulation of RasGRP1. Overexpression of RasGRP1 in keratinocytes also caused apoptosis. Finally, induction of keratinocyte differentiation by elevation of extracellular calcium suppressed expression of endogenous RasGRP1, whereas overexpression of RasGRP1 inhibited expression of the differentiation markers keratins 1 and 10 induced by high calcium in the medium. Taken together, our results demonstrate that RasGRP1 is an additional diacylglycerol/phorbol ester receptor in epidermal keratinocytes and suggest that activation of this novel receptor may contribute to some of the phorbol ester- and Ras-mediated effects in mouse epidermis.

Carcinogenesis is a multistage process that reflects the cooperation between oncogenic mutations and epigenetic mechanisms that alter cellular homeostasis. Understanding the mechanism governing this cooperation is therefore of great importance, as it may identify molecular targets that can be used for interrupting tumor development before malignant progression occurs. One of the experimental models that has been instrumental in investigating the stages of cancer formation is the induction of tumors in mouse skin. In this model, a carcinogen initiates cells by introducing a mutation in a ras gene, and the initiated population expands under treatment with phorbol esters, non-genotoxic compounds with tumor-promoting activities. Although phorbol esters are the prototypes of tumor promoters in skin, the nature of their interaction with Ras signaling in tumor promotion remains to be determined.

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is the classic mouse skin tumor promoter (1, 2). When cultures of basal proliferating epidermal keratinocytes are exposed to this agent, their growth is arrested, and they undergo a process of differentiation (3, 4). However, initiated cells that harbor a mutant ras gene are resistant to the differentiation effects of TPA (5, 6). In vivo, the consequence of heterogeneity of response is tumor promotion: the clones of the carcinogen-altered cells are selected and expanded because they resist the induction of terminal differentiation and are stimulated to proliferate (7). Historically, protein kinase C (PKC) has been considered responsible for the phorbol ester effects (8). The role of some of the PKC isoforms in the tumor-promoting actions of phorbol esters has been recently assessed using transgenic animals. PKCδ overexpression in the epidermis induces resistance to tumor promotion by TPA (9). PKCα increases cutaneous inflammation, but has no effect on tumor promotion (10). Overexpression of PKCε, which appears to be oncogenic in a variety of cell types (11, 12), causes a reduction in the papilloma burden, but enhances carcinoma formation (13). One emerging question is whether other receptors for phorbol esters contribute to tumor promotion in skin. There are at least three different families of non-PKC phorbol ester receptors: the chimerins (14), Munc13 (15), and RasGRP (16). All of these receptors can respond to phorbol esters with sensitivities similar to PKC (15, 17, 18). The chimerins are GTPase-activating proteins for Rac, a small GTPase involved in cytoskeletal rearrangements (14). Although chimerin is present in a variety of tissues (19), expression in the epidermis has not been demonstrated. The Munc13 family comprises mammalian proteins that participate in synaptic transmission and that are expressed in brain (15). Finally, RasGRP1 is a phorbol ester receptor that functions as an activator of Ras (16, 18), is transforming in rat fibroblasts (16), and is a candidate oncogene for mouse retrovirus-induced T-cell lymphomas and leukemias (20, 21). The two functional characteristics of RasGRP, the ability to bind to phorbol esters and to modulate Ras activity, make RasGRP1 an attractive candidate for an additional target of the phorbol esters in skin.

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¶The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; RasGRP, Ras guanyl nucleotide-releasing protein; HA, hemagglutinin; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MOPC, mineral oil-induced plasmacytoma; MEM, Eagle’s minimal essential medium; RT, reverse transcription; PBS, phosphate-buffered saline; Ad, adenovirus; MEF, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PARP, poly(ADP-ribose)polymerase.
that could directly link phorbol ester signaling with Ras signaling and the tumorigenic process.

In this study, we report that primary mouse keratinocytes express RasGRP1 and that this receptor mediates Ras activation and can translocate and be down-regulated in response to phorbol ester treatment. Moreover, calcium-induced differentiation suppresses the levels of RasGRP1 in a concentration-dependent manner, whereas overexpression of RasGRP1 in epidermal cells inhibits the expression of markers of differentiation and causes cell death, effects that may be mediated by activated Ras. In summary, our findings indicate that RasGRP1 is an additional diacylglycerol/phorbol ester receptor in epidermal keratinocytes and suggest that activation of this novel receptor may contribute to some of the phorbol ester- and Ras-mediated effects in mouse skin.

EXPERIMENTAL PROCEDURES

Vectors and Reagents—DNAs encoding rat RasGRP1 tagged with either hemagglutinin (HA) or green fluorescent protein (GFP) tags were prepared by standard molecular biology procedures and used to generate recombinant adenoviruses with the Transpose-AD system (Qbiogene) according to the manufacturer’s instructions. Recombinant adenoviruses for expression of bacterial β-galactosidase (LacZ) or the GFP protein were obtained from Qbiogene. A plasmid utilized in transfection for expression of RasGRP1 as a GFP fusion protein was generated by subcloning the rat RasGRP1 cDNA N-terminal to GFP using the NHel site of the pQB128 plasmid (Qbiogene). The primary antibodies used in this study were as follows: anti-RasGRP1 (m199), anti-FKcα (H-7), and anti-HA (H-15) (Santa Cruz Biotechnology); anti-mouse keratin 1 and anti-mouse keratin 10 (Covance Research Products); anti-phospho-p44/42 MAPK (ERK) polyclonal, anti-phospho-p44/42 MAPK (ERK) polyclonal, and anti-poly(ADP-ribose) polymerase (PARP) (Cell Signaling); anti-actin and anti-MOPC21 IgG (Sigma); anti-Ras (clone Ras10) and anti-phospho-Sep24(Thr187)Raf-1 (Upstate Biotechnology, Inc.); and anti-RasGRP1 polyclonal (JZ-22; custom antibody).

Primary Keratinocyte Cultures and Infection—Primary cultures of normal mouse keratinocytes were prepared from newborn mice (2–3 days old) by the trypsin flotation procedure (22) with modifications. Briefly, skins were floated, dermis down, in 0.25% trypsin at 4°C overnight. Afterward, the epidermis was separated mechanically from the dermis in S-MEM-high calcium medium (Eagle’s minimal essential medium containing 8% Chexel-treated fetal bovine serum and supplemented with 1.2 mM calcium), chopped, and centrifuged. The pellet was washed twice, and the keratinocytes were resuspended in S-MEM-high calcium medium, followed by filtration through a cell strainer to remove cell debris. Cells were plated at 1.5 × 10^6 cells/80-mm Petri dish and incubated at 36°C. After the medium changed to S-MEM-low calcium medium (0.04 mM) to maintain the cells under basal proliferating conditions, keratinocytes were used within 5–6 days after plating. Adenoviral infection was done on keratinocytes plated on 60-mm dishes. The volume of the medium in the dishes was reduced to 1 ml, and adenoviruses were added to the dishes at the appropriate multiplicity of infection, followed by a 4-h incubation at 36°C. After incubation, 3 ml of S-MEM-low calcium medium was added, and the dishes were incubated for an additional 24–72-h period depending on the experiment.

Reverse Transcription (RT)-PCR— Cultures of primary keratinocytes were harvested, and total RNA was isolated using the high pure isolation kit from Roche Applied Science, treated with DNase, and used for a two-step RT-PCR using Ready-To-Go RT-PCR beads (Amersham Biosciences) according to the manufacturer’s protocol. The first reaction step was performed using 1 μg of total RNA and 0.5 μg of oligo(dT)_12-18 primer. For the amplification reaction (PCR step), specific primers for mouse RasGRP1, aag cgg ctg gcc gac gcc gaa g, and 3′-primer, gcc atc cca gcc cag ctc g, were used. In addition, the mouse ornithine decarboxylase control (5′-primer, gcc gct gaa gaa cct gaa g; 3′-primer, ccc gag cta cca tag cag cag cag cct c) were used. The PCR product was size separated from genomic DNA using gel electrophoresis. Bands of 1217 and 1237 nucleotides were excised and cloned into the pBluescript II KS+ vector. The inserts were sequenced using an Applied Biosystems 3730xl DNA Analyzer._master

Preparation and Staining of Keratinocytes—Primary keratinocytes seeded on Permanox slide chambers were fixed with methanol at ~20°C for 10 min. For staining with the m199 monoclonal antibody, blocking was performed utilizing the Vector M.O.M. kit (Vector Labs, Inc.). An 8% solution of bovine serum albumin was utilized as the blocking agent when using the J32 primary polyclonal antibody. The secondary antibodies were Alexa Fluor 594-conjugated anti-mouse (Molecular Probes, Inc.) and Cy2-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.). Staining of actin fibers was done using Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Inc.) according to the manufacturer’s protocol.

Immunoprecipitation and Immunoblotting—Primary keratinocytes were harvested in lysis buffer (50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1% IGEPA, 1 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, and one tablet of Mini-Complete protease inhibitors (Roche Applied Science)). Samples for immunoblotting were prepared in 2× Laemmli sample buffer and boiled before being subjected to SDS-PAGE. For immunoprecipitations, 2 μg of total lysate protein was incubated with 2 μg of primary antibody overnight at 4°C, followed by a 2-h incubation with a horseradish peroxidase-conjugated secondary antibody at room temperature and chemiluminescence detection using SuperSignal West (Pierce). When fractionated, cells were harvested in 50 mM Tris-HCl supplemented with protease inhibitors and then sonicated for 8 s to cause lysis. Soluble or cytosolic fractions were prepared from aliquots of 60 μl of total lysates by ultracentrifugation at 100,000 × g for 1 h at 4°C. The supernatants were collected as the “soluble” fractions.

GTP-loaded Ras Pull-down Assay—Levels of GTP-loaded Ras were measured using glutathione S-transferase/Raf-1 Ras-binding domain as a probe in an affinity precipitation or pull-down assay as described previously (23). Briefly, primary keratinocytes were serum-starved (0.1% fetal bovine serum) overnight, treated with TPA or vehicle, and harvested on ice in magnesium lysis buffer (25 mM HEPES (pH 7.5) containing 150 mM NaCl, 1% IGEPA, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate, and one tablet of Mini-Complete protease inhibitors). Lysates were rotated at 4°C and clarified by centrifugation. Lysate protein (300 μg) was incubated with glutathione S-transferase/Raf-1 Ras-binding domain conjugated to glutathione-Sepharose 4B beads (Amersham Biosciences) for 1.5 h with rotation in the cold. The affinity complexes were washed three times with lysis buffer, resuspended in 2× Laemmli buffer, boiled, and run on 15% acrylamide gels. Aliquots of the total lysates were run in parallel as a control of the “input” of total Ras in the pull-down assay. Proteins were blotted onto nitrocellulose membranes; immunostaining was performed using anti-Ras antibody (clone Ras10).

Flow Cytometry—Primary keratinocytes were trypsinized for 5 min, centrifuged, washed twice with cold phosphate-buffered saline (PBS) containing 0.1% fetal bovine serum, and then fixed in 5 ml of ice-cold 70% ethanol. After incubation on ice for 30 min, cells were centrifuged, washed twice with PBS, and rehydrated in the last wash with PBS by incubation at room temperature for 15 min. After centrifugation, the keratinocytes were incubated for 30 min at 37°C in 200 μl of PBS containing 1 μg/ml RNase A, followed by an additional 15-min incubation at room temperature with 1 ml of a 3 μM propidium iodide solution containing 100 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2, and 0.1% IGEPA. Cells were then analyzed by flow cytometry using a FACScan apparatus equipped with a 488-nm Argon ion laser.

Results

Expression of RasGRP1 in Primary Keratinocytes—To study the expression of RasGRP1 in skin, we used primary cultures of mouse keratinocytes. Mouse keratinocytes are the target cells for inactivating the reverse transcriptase prior to initiating the RT reaction. RT-PCR with the OCD primers also served as a control for DNA contamination. The PCR product used for genomic DNA purification should be 1237 nucleotides versus the 530-nucleotide product that originated from mRNA.
in chemically induced skin carcinogenesis. Cells were cultured for 3–6 days, and total cellular RNA was isolated, treated with DNase to eliminate genomic DNA contamination, and then utilized for RT-PCR using specific primers for mouse RasGRP1. As shown in Fig. 1A, the results revealed a transcript of ~600 bp (expected size of 578 bp). The product was excised from the gel, purified, and sequenced to confirm identity to the published sequence for mouse RasGRP1.

To confirm expression of RasGRP1 at the protein level, we performed immunoprecipitations followed by immunoblotting using a monoclonal antibody that has reactivity toward mouse RasGRP1 (m199). This antibody recognized two bands with an apparent molecular mass of ~90 kDa, which most likely reflect post-translational modifications. Similar doublets were also observed in lysates from T-cells of mouse origin (24). These bands were not detected when immunoprecipitation was performed with an irrelevant mouse isotype IgG1 (Fig. 1B, second lane). Immunofluorescence studies using both monoclonal and polyclonal antibodies against RasGRP1 yielded a consistent image for the subcellular distribution of this protein in keratinocytes, which corresponded to cytoplasmic distribution with strong perinuclear localization (Fig. 2). As expected, cells ectopically expressing the GFP fusion protein of RasGRP1 (Ras-GRP1-GFP) showed a localization pattern for the GRP protein similar to that for the native protein (Fig. 2).

**Gam**

**Fig. 1. Expression of RasGRP1 in mouse keratinocytes.** A, shown is a representative gel of the RT-PCR amplification of the 3’ coding region of mouse RasGRP1 mRNA from DNA-free total RNA isolated from primary keratinocytes from newborn mice. The amplification products were separated on a 1.2% agarose gel and visualized by ethidium bromide. The 600-bp band in lane 1 indicates the presence of the mouse RasGRP1 transcript in the cells. Lane 2 corresponds to a control for genomic DNA contamination (inactivated reverse transcriptase). The band in lane 3 is a control RT-PCR using the ornithine decarboxylase housekeeping (ODC) gene. B, lysates of primary mouse keratinocytes were immunoprecipitated (IP) with an anti-RasGRP1 monoclonal antibody (m199) (first lane) or with an irrelevant mouse IgG1 isotype antibody (MOPC21; second lane). The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with the m199 antibody. Results are from a representative experiment. Four additional experiments yielded similar results.

**Gam**

**Fig. 2. Localization of RasGRP1 in keratinocytes.** Primary mouse keratinocytes were grown on Permanox slides, fixed, and then labeled with a set of antibodies against RasGRP1 (a and c) or the corresponding irrelevant IgGs (b and d) as described under “Experimental Procedures.” a, m199 antibody; b, control IgG1 (MOPC21); c, J32 antibody; d, normal rabbit IgG; e and f, distribution of ectopically expressed RasGRP1-GFP and GFP alone, respectively.

**Gam**

**Fig. 3. Immunoprecipitation of RasGRP1 in keratinocytes.** Lysates of primary mouse keratinocytes were immunoprecipitated (IP) with an anti-RasGRP1 monoclonal antibody (m199) or with an irrelevant mouse IgG1 isotype antibody (MOPC21). The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with the m199 antibody. Results are from a representative experiment. Four additional experiments yielded similar results. **A**, Western blot.
Fig. 2, RasGRP1 showed a cytosolic distribution and perinuclear localization in resting keratinocytes. A 15-min treatment with TPA induced a dose-dependent decrease in the RasGRP1 levels in the soluble (cytosolic) fraction (Fig. 4A), suggesting translocation of RasGRP1 to particulate compartments of the cell. To determine the localization of the translocated RasGRP1, we examined its cellular distribution in response to TPA by confocal microscopy in live mouse keratinocytes expressing the RasGRP1-GFP fusion protein. As shown in Fig. 4B, 1 μM TPA translocated RasGRP1-GFP to the plasma membrane of the keratinocytes during a 15-min treatment. This result confirms that RasGRP1 is able to redistribute in response to phorbol esters in mouse keratinocytes and indicates translocation to the membrane upon short-term exposure to TPA. Additionally, when mouse epidermal cells were exposed to TPA for 24 h, the level of endogenous RasGRP1 was reduced in a concentration-dependent manner (Fig. 4C), suggesting that RasGRP1, like some members of the PKC family such as PKCδ and PKCα, is down-regulated in this system in response to phorbol ester treatment.

RasGRP1 Overexpression Induces Apoptosis—To gain insight into the role of RasGRP1 in keratinocyte biology, we used recombinant AdRasGRP1-HA to overexpress C-terminally HA epitope-tagged rat RasGRP1. With this approach, >90% of the cells were infected. Fig. 5A shows the levels of expression of the recombinant protein after a 48-h infection at different multiplicities of infection. The tagged rat RasGRP1 protein migrated slightly slower than the endogenous mouse RasGRP1 protein, probably because of the presence of the HA tag. We noticed that the levels of endogenous RasGRP1 were lower in infected cells compared with control keratinocytes. This was evident not only for the RasGRP1-HA overexpressor, but also in keratinocytes infected with the AdLacZ control vector, although to a much lesser extent.

Although normal mouse keratinocytes in low Ca²⁺ medium (0.04 mM) showed characteristics of basal cell morphology, in-
Infection with AdRasGRP1 caused significant rounding and detachment of the cells (Fig. 5B). When mouse keratinocytes were analyzed by staining with phalloidin to visualize the actin cytoskeleton, we observed that ectopic expression of RasGPR1 caused the disappearance of actin stress fibers (Fig. 5C). To assess whether these changes were causing apoptosis, we monitored three parameters that are associated with the apoptotic process: cleavage of the caspase substrate PARP, externalization of phosphatidylserine (annexin V binding), and DNA fragmentation (hypodiploid DNA or sub-G1 peak). As shown in Fig. 5A, RasGRP1 overexpression resulted in increased cleavage of PARP, indicating caspase activation. Furthermore, by labeling with annexin V and propidium iodide, we observed an increase in both early apoptosis (binding of annexin V to the plasma membrane with no propidium iodide staining of the nucleus) and late apoptosis (annexin V and propidium iodide staining) after 48 h of infection with AdRasGRP1 (Fig. 5D). In contrast, cell cultures infected with the control adenovirus (AdLacZ) displayed a considerably lower number of cells staining positive for annexin V and/or propidium iodide (Fig. 5D). DNA content analysis of the cell population attached to the dishes at 48 h post-infection showed that the hypodiploid peak (sub-G1), which corresponds to cells with fragmented DNA due to apoptotic degradation, was significantly higher in cells overexpressing RasGRP1 (15.29 ± 2.12%, n = 3) compared with control (LacZ) cells (0.35 ± 0.19%, n = 3) (Fig. 5E). Taken together, these results confirm that RasGRP1 overexpression induces apoptosis in primary keratinocytes.

Activation of the Raf/MEK/ERK Cascade in Response to Ras-GRP1 and TPA—Activation of Ras in the epidermis selectively activates the Raf/MEK/ERK cascade (27, 28). We have shown that RasGRP1 overexpression caused a significant increase in the levels of active Ras in primary keratinocytes (Fig. 3, C and D). As expected, the level of active phospho-Thr202/Tyr204 ERK was significantly elevated in primary keratinocytes infected with AdRasGPR1 (Fig. 6A). No changes were observed in the total amount of ERK protein (Fig. 6A), indicating that the increment in phospho-ERK was the result of phosphorylation and not of up-regulation of ERK levels in the cells. When cells overexpressing RasGRP1 were treated with 1 μM TPA for a short period of time (15 min), a further and robust increase in ERK phosphorylation was observed (Fig. 6B). However, this increase was sensitive to PKC inhibition by GF109203X treatment. To investigate this, we examined the effect of TPA on
Raf-1, the first step in the cascade downstream of Ras activation. Raf-1 was analyzed by measuring the level of phospho-Ser338, a phosphorylation that is absolutely required for activation of Raf-1 and that closely coincides with Raf-1 kinase activity (29, 30). In serum-starved cells, the addition of TPA caused an increase in the amount of phospho-Ser338 Raf-1 (Fig. 6B). However, as was observed with ERK phosphorylation, inhibition of PKC by GF109203X treatment completely abrogated Raf-1 phosphorylation (Fig. 6B). These results indicate that, although TPA increases GTP-loaded Ras levels in a PKC-independent manner, PKC is required for TPA-mediated ERK phosphorylation through activation of Raf-1.

RasGRP1 and Keratinocyte Differentiation—Phorbol esters like TPA have been shown to induce morphological changes in cultured keratinocytes that resemble some aspects of keratinocyte differentiation (4, 31). Because differentiation is typically controlled by the calcium concentration in the media, we investigated whether RasGRP1 levels could be also modulated by changes in extracellular calcium. As shown in Fig. 7A, cells exposed to calcium showed morphological changes indicative of keratinocyte differentiation, characterized by cell flattening and shedding from the plates. Although the level of RasGRP1 was similar in cells cultured at an extracellular calcium concentration of 0.12 mM compared with 0.04 mM, there was a decrease of 80% in the level of endogenous RasGRP1 in cells cultured for 24 h in medium at 1.2 mM calcium (Fig. 7A). These results suggest that RasGRP1 levels, as has been observed for some of the PKC isoforms, may be regulated in vitro by the status of differentiation of the keratinocytes. To further examine the impact of RasGRP1 activation on differentiation, we
RasGRP1 is a novel phorbol ester receptor in keratinocytes

The mouse epidermis is a tissue responsive to phorbol esters like TPA, which is the classic tumor-promoting agent utilized in the two-stage carcinogenesis protocol in skin. The PKC family members are the best known receptors for phorbol esters, and at least five isoforms (33) have been identified in keratinocytes, the target cells in skin carcinogenesis. In this report, we have shown that RasGRP1, a non-PKC phorbol ester receptor, is also expressed in mouse epidermal keratinocytes and can mediate phorbol ester effects independently of PKC.

RasGRP1 is a family of guanyl nucleotide exchange factors for the Ras-like GTPases, and it is composed of at least four members: RasGRP1 (16, 18), RasGRP2 (34, 35), RasGRP3 (23, 36), and RasGRP4 (37). Expression of the RasGRP proteins is high in brain and hematopoietic tissues (16, 25, 34), although RasGRP transcripts have been detected also in lung, heart, and kidney by RT-PCR (36). Using a multiple-tissue panel of human normalized first-strand cDNA, we were able to detect RasGRP1 transcripts not only in thymus, primary leukocytes, spleen, and brain, but also in testis, placenta and kidney, although expression of RasGRP1 at the protein level in those tissues still needs to be confirmed. The RasGRP1 protein extracted from primary keratinocyte cultures from newborn mice migrated as a doublet on SDS gels, a finding that is consistent with previous observations of RasGRP1 from T-cells of mouse origin (24).

Under our experimental conditions, the RasGRP1 doublet was observed only when using the monoclonal antibody raised against full-length rat RasGRP1 (m199) and not with a polyclonal antibody raised against the N terminus of RasGRP1 (data not shown), suggesting that the RasGRP1 doublets may represent a post-translational modification or proteolysis involving the N-terminal region of RasGRP1. It should be noted, however, that the recombinant rat RasGRP1-HA protein showed only one band on blots stained with the m199 antibody. In any case, the C-terminally tagged RasGRP1 proteins (HA and GFP-tagged) were functional, as shown in this study as well as in a previous report in which the C-terminally epitope-tagged RasGRP proteins were successfully utilized for in vitro studies (23). In the study, we found that RasGRP1 was able to respond as a phorbol ester receptor in mouse keratinocytes, as evidenced by our findings on TPA-induced translocation and down-regulation and the direct activation of Ras. The ability of RasGRP1 to respond to phorbol esters depends on the presence of the C1 domain, which has high homology to the C1 domain present in PKC (18). Our earlier studies demonstrated that the C1 domain of RasGRP1 recognizes different diacylglycerol analogs with affinities in the nanomolar range, similar to those shown by some of the PKC isoforms (18), and that ectopically expressed RasGRP1 is able to translocate in response to phorbol esters (16). In mouse keratinocytes, endogenous RasGRP1 also translocated in response to TPA. Using GFP-tagged RasGRP1, we observed that the translocation occurred mainly to the plasma membrane during the first 15 min of exposure to TPA. A similar pattern of translocation has been shown for mouse RasGRP1 ectopically expressed in NIH3T3 cells (25). Interestingly, Ras proteins can signal not only from the plasma membrane, but also from endomembrane compartments (38); and in recent studies, RasGRP1 has been shown to mediate Ras activation in the Golgi (39, 40). In this context, it is interesting to note that our previous studies on another member of the RasGRP family, RasGRP3, demonstrate different patterns of translocation to the plasma membrane and the Golgi apparatus at different concentrations of TPA (23), suggesting that the pattern of redistribution may be concentration- and/or time-dependent. We are currently investigating whether this is also the case for RasGRP1 redistribution in response to TPA in keratinocytes.

In the case of the PKC isoforms, activation by phorbol esters is usually accompanied by subsequent down-regulation, typically between 3 and 24 h after phorbol ester treatment. We

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**Fig. 6. Activation of ERK in mouse keratinocytes overexpressing RasGRP1.** A, keratinocytes were infected with AdLacZ or AdRasGRP1 (HA epitope-tagged) for 48 h and then harvested, and the lysates were subjected to SDS-PAGE, followed by immunoblotting for detection of levels of RasGRP1, cleaved PARP, phospho-ERK (pERK), total ERK (TERK), and actin. B, GTP-loaded Ras (RasGTP) levels in keratinocytes expressing either LacZ or RasGRP1 were affinity-precipitated as described under “Experimental Procedures.” Total lysates used in the experiment were also subjected to SDS-PAGE, followed by blotting for staining with anti-phospho-ERK, anti-phospho-Ser<sup>338</sup> Raf-1 (pS338-Raf-1), and anti-Ras antibodies. Results are from a representative experiment. Two additional experiments yielded similar results. MOI, multiplicity of infection.

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*P. S. Lorenzo, unpublished data.*
have observed that this is also the case for endogenous RasGRP1 in keratinocytes. It should be noted, however, that a previous study has shown that RasGRP1 is not down-regulated by TPA treatment in a murine T-cell clone (24). Divergent responses in different biological systems to phorbol ester-induced translocation and down-regulation have been extensively documented in the literature for the PKC isoforms. For example, although TPA causes PKCα and PKCβI down-regulation in glioma cells, the same treatment of melanoma cells does not lead to changes in the levels of PKC (41). Therefore, the possibility remains that, as with PKC, the differences in RasGRP1 down-regulation in response to phorbol esters may be the result of the different cell types examined.

Our data show that overexpression of RasGRP1 by itself caused activation of Ras in mouse keratinocytes. TPA treatment further increased this level of Ras activation in a PKC-independent manner. However, the same PKC inhibitory treatment (GF109203X) that did not abrogate TPA-induced
Ras activation was effective in reducing TPA-mediated Raf-1 and ERK phosphorylation. This result suggests that, although RasGRP1 can directly activate Ras in response to phorbol esters, PKC is still required for the downstream stimulation of the Raf/MEK/ERK pathway. It also indicates that the point of PKC contribution in the cascade is at the level of Raf-1 because PKC inhibition abrogated TPA-induced phosphorylation of Raf-1 at Ser338, a phosphorylation that is indispensable for Raf-1 activity (29). An important emerging concept is that the different families of diacylglycerol/phorbol ester receptors often function together, providing opportunities for synergy or antagonism. Thus, RasGRP1 phosphorylates by PKC coincident with Ras activation after stimulation in Ramos B cells (42). Conversely, diacylglycerol both activates RasGRP1 and causes the activation and translocation of diacylglycerol kinase, which abrogates the activation of the GRP protein (43, 44). Our findings with RasGRP1 in keratinocytes provide another striking example in which both the RasGRP and PKC cascades feed into the same signaling pathway.

In mouse keratinocytes, RasGRP1 overexpression led to apoptosis. This effect appears to be a consequence of increased keratinocyte detachment from the substratum. Consistent with alterations in cell attachment, we observed the disappearance of actin stress fibers in keratinocytes overexpressing RasGRP1. Keratinocyte cell death is a mechanism of the GRP protein (43, 44). Our findings with RasGRP1 in Ramos B cells (42). Conversely, diacylglycerol both inhibits for synergy or antagonism. Thus, RasGRP3 is phosphorylated at Ser338, a phosphorylation that is indispensable for Raf-1 activity (29). An important emerging concept is that the different families of diacylglycerol/phorbol ester receptors often function together, providing opportunities for synergy or antagonism. Thus, RasGRP1 phosphorylates by PKC coincident with Ras activation after stimulation in Ramos B cells (42). Conversely, diacylglycerol both activates RasGRP1 and causes the activation and translocation of diacylglycerol kinase, which abrogates the activation of the GRP protein (43, 44). Our findings with RasGRP1 in keratinocytes provide another striking example in which both the RasGRP and PKC cascades feed into the same signaling pathway.

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40. Caloca, M. J., Zagaza, J. L., and Bustelo, X. R. (2003) *J. Biol. Chem.* 278, 33465–33473
41. Szaniawska, B., Maternicka, K., Kowalczyk, D., Miloszewaska, J., and Janik, P. (1996) *Cancer Lett.* 107, 205–209
42. Teixeira, C., Stang, S. L., Zheng, Y., Beswick, N. S., and Stone, J. C. (2003) *Blood* 102, 1414–1420
43. Sanjuan, M. A., Pradet-Balade, B., Jones, D. R., Martinez, A. C., Stone, J. C., Garcia-Sanz, J. A., and Merida, I. (2003) *J. Immunol.* 170, 2877–2883
44. Topham, M. K., and Prescott, S. M. (2001) *J. Cell Biol.* 152, 1135–1143
45. Lin, A. W., and Lowe, S. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 5025–5030
46. Roper, E., Weinberg, W., Watt, F. M., and Land, H. (2001) *EMBO Rep.* 2, 145–150
47. Cheng, C., Kilkenny, A. E., Roop, D., and Yuspa, S. H. (1990) *Mol. Carcinog.* 3, 363–373
48. Karen, J., Wang, Y., Javaherian, A., Vaccariello, M., Fusenig, N. E., and Garlick, J. A. (1999) *Cancer Res.* 59, 474–481
49. Dower, N. A., Stang, S. L., Botorff, D. A., Ehnu, J. O., Dickie, P., Ostergaard, H. L., and Stone, J. C. (2000) *Nat. Immunol.* 1, 317–321