Differential effects of bryostatin 1 and 12-O-tetradecanoylphorbol-13-acetate on the regulation and activation of RasGRP1 in mouse epidermal keratinocytes

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Abstract
The antitumor agent bryostatin 1 and the tumor-promoting phorbol esters function as structural mimetics of the second lipid messenger diacylglycerol (DAG) by binding to the C1 domain of DAG receptors. However, bryostatin 1 and the phorbol esters often differ in their cellular actions. In mouse skin, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter, whereas bryostatin 1 lacks this activity and antagonizes the tumor-promoting effects of TPA. Although protein kinase C mediates many of the effects of DAG on skin, the exact mechanisms responsible for the biology of bryostatin 1 and TPA in the epidermis have not been elucidated. We recently reported that the novel DAG receptor RasGRP1 is expressed in mouse keratinocytes and mediates TPA-induced Ras activation. This finding prompted us to examine the regulation of RasGRP1 by bryostatin 1. We found that whereas TPA induced translocation of RasGRP1 to both the plasma and internal membranes of the keratinocytes, bryostatin 1 recruited RasGRP1 only to internal membranes and the nuclear envelope. In addition, TPA led to a concentration-dependent down-regulation of RasGRP1, whereas bryostatin 1 failed to induce full RasGRP1 down-regulation. Interestingly, bryostatin 1 was less effective than TPA at activating Ras. The results presented here suggest the possibility that a differential modulation of RasGRP1 by bryostatin 1 compared with TPA could participate in the disparate responses of the epidermal cells to both DAG analogues. This result may have implications in the understanding of the antitumor effects of bryostatin 1 in the skin. [Mol Cancer Ther 2006;5(3):602–10]

Introduction
The bryostatins are a group of natural products that function as analogues of the second lipid messenger diacylglycerol (DAG). Bryostatin 1, the prototype of this group, was initially discovered based on its antiproliferative activity on murine leukemia cells (1) and is currently being tested as an anticancer agent in combination therapies for the treatment of both solid tumors and hematologic malignancies.

Like the ultrapotent analogues of DAG, the phorbol esters, bryostatin 1 also functions as a potent DAG surrogate by binding to the C1 domain of DAG receptors (2). Despite having similar affinities for binding to C1 domains, bryostatin 1 and the phorbol esters often differ in their cellular actions. One system in which bryostatin 1 and phorbol esters exert opposite effects is the mouse epidermis, where the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter on mouse skin. However, bryostatin 1 lacks this activity and antagonizes the tumor-promoting effects of TPA when coapplied on the epidermis (3).

The disparate activities of bryostatin 1 and phorbol esters have been mostly attributed to distinct regulation of the protein kinase C (PKC) family, which is the major intracellular DAG receptor (4). However, some of the biological effects of bryostatin 1 seem to be PKC independent (5). More recently, bryostatin 1 and phorbol esters have been shown to regulate new classes of DAG targets, which include the chimaerins, the Munc proteins, and the RasGRP family (6–9). The discovery of these novel targets has forced a revision of our understanding of the biology of DAG analogues.

We have recently shown that the novel DAG receptor and Ras exchange factor RasGRP1 is expressed in mouse epidermal keratinocytes and mediates activation of Ras by TPA in a PKC-independent manner (10). The purpose of the work described here was to determine if bryostatin 1 could also modulate RasGRP1 in the mouse keratinocyte system and to compare its effects with those of TPA. We found that whereas TPA induced a rapid translocation of RasGRP1 to both the plasma and internal membranes of the keratinocytes, bryostatin 1 recruited RasGRP1 only to internal membranes and the nuclear
envelope. Interestingly, bryostatin 1 was less effective than TPA at activating Ras. In addition, TPA treatment led to a concentration-dependent down-regulation of RasGRP1, whereas bryostatin 1 failed to induce complete down-regulation, although it was effective at down-regulating PKC isoforms, such as PKCa and PKCβ. Moreover, bryostatin 1 prevented RasGRP1 down-regulation by TPA in a concentration-dependent manner. Taken together, the present results suggest the possibility that a differential modulation of RasGRP1 by bryostatin 1 compared with TPA could participate in the distinct responses of the epidermis to both DAG analogues. Our findings may have implications in the understanding of the antitumor effects of bryostatin 1 and may contribute to elucidate the mechanisms that participate in tumor promotion in the skin.

Materials and Methods

Vectors and Reagents

DNA encoding rat RasGRP1 tagged with either hemagglutinin (RasGRP1-HA) or green fluorescent protein (GFP; RasGRP1-GFP) was prepared by standard molecular biology procedures and used to generate recombinant adenoviruses with the Transpose-Ad system according to the manufacturer’s instructions (Qbiogene, Irvine, CA). Recombinant adenoviruses for expression of bacterial β-galactosidase (AdLacZ) were obtained from Qbiogene. The primary antibodies used in this study were anti-RasGRP1 (m199), anti-PKCα (H-7), and anti-PKCθ (C-17; Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin (Sigma-Aldrich, St. Louis, MO); and anti-Ras (clone Ras10; Upstate Biotechnology, Waltham, MA). Bryostatin 1, TPA, and GF109203X were purchased from LC Laboratories (Woburn, MA). Fetal bovine serum was from Gemini Biotechnology, Whaltman, CA; anti-PKCα (H-7), and anti-PKCθ (C-17; Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin (Sigma-Aldrich, St. Louis, MO); and anti-Ras (clone Ras10; Upstate Biotechnology, Waltham, MA). Bryostatin 1, TPA, and GF109203X were purchased from LC Laboratories (Woburn, MA). Fetal bovine serum was from Gemini Biotechnology, Whaltman, CA.

Primary Keratinocyte Cultures and Infection

Primary cultures of normal mouse keratinocytes were prepared from newborn (2- to 3-day-old) mice by the trypsin flotation procedure (11) 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 (calcium-free Eagle’s MEM containing 8% Chelex-treated fetal bovine serum and supplemented with 1.2 mmol/L 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 2 × 60 mm Petri dishes per skin in dishes coated with Coating Matrix (Cascade Biologics, Portland, OR) and incubated overnight at 36°C, after which the medium was changed to a low-calcium medium (0.04 mmol/L) to maintain the cells under basal proliferating conditions. The low-calcium medium consisted of medium 154CF (Cascade Biologies) supplemented with antibiotics/antimycotics (Invitrogen, Carlsbad, CA) and 2% Chelex-treated fetal bovine serum, 0.2% bovine pituitary extract, 5 μg/mL bovine insulin, 0.18 μg/mL hydrocortisone, 5 μg/mL bovine transferrin, and 0.2 ng/mL human epidermal growth factor (Cascade Biologies). Keratinocytes were used within 3 to 6 days after plating or were passed once before use. For adenoviral infection, the culture medium was reduced to 1 mL, and the viruses were added to the cells at a 100 multiplicities of infection. After a 4-hour incubation at 36°C, 3 mL low-calcium medium was added to the cultures and incubation continued for a further 24-hour period.

RasGTP Pull-Down Assay and Western Blots

Levels of GTP-loaded Ras (RasGTP) were measured by using the glutathione S-transferase Ras-binding domain of Raf-1 as a probe in an affinity precipitation or pull-down assay. Briefly, primary keratinocytes were serum starved overnight, treated with vehicle (DMSO), bryostatin 1, or TPA, and harvested on ice in lysis buffer containing 25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L MgCl2, 1% Igepal, 5% glycerol, and Mini Complete Roche protease inhibitors (Roche Applied Science, Indianapolis, IN). Lysates were mixed, incubated on ice for 5 minutes, and then clarified by centrifugation at 13,000 rpm for 15 minutes at 4°C. Lysate protein (500 μg) was incubated with glutathione S-transferase Ras-binding domain-Raf-1 conjugated to glutathione beads (Swell Gel Glutathione Discs, Pierce Biotechnology, Rockford, IL) for 1 hour with rotation in the cold. The affinity complexes were washed three times with lysis buffer and then resuspended in 2× Laemmli buffer, boiled, and resolved on 15% acrylamide gels. Total lysate protein (25 μg) was run in parallel as control of the “input” of total Ras in the pull-down assay. Proteins were blotted onto nitrocellulose membranes; immunostaining was done using a pan anti-Ras antibody (clone Ras10). For the down-regulation experiments, samples were harvested in lysis buffer and immediately mixed with equal parts of 2× Laemmli buffer. After boiling for 5 minutes, protein sample (25–50 μg) was resolved on an 8% SDS-PAGE gel and then blotted onto nitrocellulose membranes for immunostaining with antibodies against RasGRP1, PKCa, or PKCθ. The intensity of the Western blot bands was calculated by densitometry using UN-SCAN-IT gel software (Silk Scientific, Orem, UT).

Confocal Microscopy Studies

SP-1 cells (papilloma-derived keratinocytes generated by chemical-induced carcinogenesis in SENCAR mice) were obtained from Dr. Yuspa (National Cancer Institute, Bethesda, MD) and cultured as described for the primary keratinocytes. For the microscopy studies, cells were seeded onto 40-mm circular glass coverslips coated with Coating Matrix at a density of 5 × 10^4 to 10 × 10^4 per coverslip. Two days later, the cells were infected with the AdRasGRP1-GFP construct as described above. All of the experiments were done 48 to 72 hours after infection. Confocal images were collected with a Zeiss LSM Pascal inverted scanning confocal microscope with a ×60 oil immersion objective. Excitation at 488 nm was provided by a krypton-argon laser with a 522/32 emission filter for green fluorescence. For live-cell imaging, a Bioptechs Focht...
Chamber System (Bioptechs, Butler, PA) was attached to the microscope stage with a custom stage adapter. The cells plated on the coverslip were enclosed in the chamber, connected to a temperature controller at 36°C, and perfused with low-calcium, phenol red–free medium 154CF (supplemented with 1% fetal bovine serum) using a Lambda microperfusion pump. DAG analogues were added to the medium and perfused for 5 minutes, during which time sequential images of the same cell were collected.

For the colocalization studies, SP-1 cells expressing RasGRP1-GFP were labeled with different organelle markers. For the Golgi apparatus, live keratinocytes were incubated with 36 nmol/L BODIPY 558/568–conjugated brefeldin A (Invitrogen) for 30 minutes at 36°C. Cells were then washed with culture medium, incubated with 1 µmol/L TPA or bryostatin 1 for 5 minutes, fixed with 4% paraformaldehyde at 37°C for 15 minutes, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). The endoplasmic reticulum (ER) was stained with an antibody connected to a temperature controller at 36°C, and perfused with low-calcium, phenol red–free medium 154CF (supplemented with 1% fetal bovine serum) using a Lambda microperfusion pump. DAG analogues were added to the medium and perfused for 5 minutes, during which time sequential images of the same cell were collected.

Results

Bryostatin 1 and TPA Induce Different Patterns of RasGRP1 Translocation in Mouse Epidermal Keratinocytes

We have shown previously that in mouse keratinocytes TPA treatment recruits RasGRP1 to the plasma membrane (10). To examine the ability of bryostatin 1 to cause RasGRP1 translocation, we expressed a GFP-tagged version of RasGRP1 (RasGRP1-GFP) in the keratinocyte cell line SP-1 and followed the subcellular distribution by live-cell confocal microscopy. Treatment with 1 µmol/L bryostatin 1 induced translocation of RasGRP1-GFP to internal membranes and, in some cases, to the perinuclear envelope (Fig. 1A). In contrast to 1 µmol/L TPA, which was able to translocate RasGRP1 to the plasma membrane (Fig. 1C), bryostatin 1 did not cause any apparent redistribution of RasGRP1 to the cell periphery. At a lower concentration (100 nmol/L) of bryostatin 1, translocation of RasGRP1 was partial (Fig. 1B). Moreover, about half of the cells treated with 100 nmol/L bryostatin 1 did not show any redistribution of RasGRP1 within the 5-minute treatment period. No changes in RasGRP1 localization were evident at bryostatin 1 concentrations of ≤10 nmol/L (data not shown).

The distribution of RasGRP1 to internal membranes resembled localization into Golgi and ER. To confirm this localization, we labeled cells expressing RasGRP1-GFP with markers for Golgi (brefeldin A) or ER (calreticulin). We also examined RasGRP1-GFP colocalization with red fluorescent probes specific for either mitochondria (MitoTracker) or lysosomes (LysoTracker). The experiments showed that RasGRP1-GFP translocated by bryostatin 1 colocalized with Golgi and partially with ER (Fig. 2A). The same colocalization pattern was seen for the fraction of RasGRP1 redistributed to internal membranes in response to TPA (Fig. 2B).

Bryostatin 1 Is Less Effective than TPA at Inducing Ras Activation

To assess if the distinct pattern of RasGRP1 translocation in response to bryostatin 1 and TPA had any functional significance, we measured activation of the immediate downstream target of RasGRP1, the small GTPase Ras. For these experiments, we used primary mouse keratinocytes instead of the SP-1 cell line, because the latter possesses a mutant form of H-Ras that is in the GTP-loaded active state and is unresponsive to exchange factors like RasGRP1.

When serum-starved mouse primary keratinocytes were treated with 1 µmol/L TPA for 5 minutes, RasGTP levels increased 4.5-fold above the basal levels of active Ras in untreated cells. In contrast, 1 µmol/L bryostatin 1 only produced a 2.7-fold increase in Ras activation (Fig. 3A). Bryostatin 1 was less effective than TPA in activating Ras at all the concentrations tested (Fig. 3B).

Overexpression of RasGRP1 by infection of the keratinocytes with a replication-deficient adenovirus encoding rat RasGRP1-HA (AdRasGRP1) was associated with elevated RasGTP levels despite the lack of serum or growth factors in the medium (Fig. 4A). The magnitude of this Ras activation (6-fold compared with RasGTP levels in control cells) was higher than that caused by 5-minute treatment with 1 µmol/L TPA (compare Figs. 3 and 4A). Under these conditions, treatment with either 1 µmol/L TPA or 1 µmol/L bryostatin 1 caused a minimal increase in RasGTP levels above the already elevated levels of Ras.
active Ras (Fig. 4B). Nevertheless, both TPA and bryostatin 1 induced an 8-fold total increase in Ras activation when compared with the basal levels of RasGTP in control, noninfected keratinocytes.

To rule out any potential effect of the infection procedure, we did pull-down experiments with keratinocytes infected with a replication-deficient adenovirus encoding an irrelevant protein (LacZ). Under basal condition, Ras GTP levels were scarcely detectable in AdLacZ-infected cells (Fig. 4C). As in the noninfected keratinocytes, TPA (1 μmol/L) increased Ras activation by 4.7-fold, whereas 1 μmol/L bryostatin 1 treatment led to a 2.2-fold increase in RasGTP in the AdLacZ-infected cells (compare Figs. 3 and 4C).

We have reported previously that in mouse keratinocytes activation of Ras by TPA was independent of PKC (10). To investigate the relative contribution of PKC in the activation of Ras by bryostatin 1, we preincubated the mouse keratinocytes with the PKC inhibitor GF109203X at a 10 μmol/L final concentration, which is sufficient to inhibit PKC activity in these cells (12). Ras activation by 1 μmol/L bryostatin 1 was insensitive to the PKC inhibitor (Fig. 5A). Similarly, 10 μmol/L GF109203X did not have any effect on Ras activation induced by 5-minute treatment with 1 μmol/L TPA. Nevertheless, as we have reported previously for TPA (10), acute activation of extracellular signal-regulated kinase (ERK) by bryostatin 1 was sensitive to PKC inhibition (data not shown).

In cells overexpressing RasGRP1, the effect of neither 1 μmol/L bryostatin 1 nor 1 μmol/L TPA was inhibited by GF109203X pretreatment (Fig. 5B). We observed, however, that the effect of TPA on Ras tended to become sensitive to GF103209X pretreatment in AdLacZ-infected keratinocytes, although it did not reach statistical significance (Fig. 5C).

**Differential Down-Regulation of RasGRP1 by Bryostatin 1 and TPA**

Prolonged exposure to DAG analogues like bryostatin 1 and TPA can cause degradation or down-regulation of the target receptor. Previously, we showed that TPA is able to down-regulate RasGRP1 in primary mouse keratinocytes (10). To analyze if the effect of bryostatin 1 differed from that of TPA on RasGRP1 degradation, we treated primary cells with increasing concentrations of bryostatin 1 for 24 hours and then measured the levels of RasGRP1 in whole-cell lysates. Whereas RasGRP1 underwent full down-regulation by 24-hour TPA treatment, with an EC50 of 2.2 μmol/L, a similar treatment with bryostatin...
1 degraded only 40% to 50% of RasGRP1 (Fig. 6A). For comparison, we also measured the down-regulation of PKCα and PKCθ in response to bryostatin 1 and TPA and found that it followed concentration-response curves that were similar to those reported in the past by another group (13). In this regard, PKCα levels were reduced by both TPA and bryostatin 1 in a concentration-dependent manner, with bryostatin 1 being ~1 order of magnitude more potent than TPA (Fig. 6B). On the other hand, the down-regulation of PKCθ by bryostatin 1 followed a biphasic concentration-response curve, whereas TPA down-regulated PKCθ completely, with an ED50 in the low nanomolar range (Fig. 6B).

Because bryostatin 1 exerts antagonist effects on many of the TPA-mediated actions in the epidermis, we tested whether it could counteract the TPA-induced down-regulation of RasGRP1 in the keratinocytes. For the cotreatment with bryostatin 1, we selected a high concentration of TPA (1 μmol/L) that produced maximal degradation of RasGRP1. As shown in Fig. 6C, bryostatin 1 prevented RasGRP1 down-regulation by TPA in a concentration-dependent manner. This finding suggests that bryostatin 1 has a predominance over the effect of TPA on RasGRP1 degradation, which resembles the protection of PKCθ from TPA-induced down-regulation in mouse fibroblasts and keratinocytes (13, 14).

**Discussion**

RasGRP1 is an exchange factor for the Ras small GTPases and activates all of the three classic Ras proteins both *in vitro* and *in vivo* (15, 16). It belongs to a family composed of four members, which differ in their target G-protein specificity and tissue distribution (17–19). RasGRP1 is activated in response to the second messenger DAG, which binds to the DAG-C1 binding domain at the COOH terminus of the protein (15). This exchange factor has a limited tissue distribution and has been reported to be expressed in cells of the T-cell lineage and in some neuronal types (15, 16, 20). Recently, we showed expression of RasGRP1 in epidermal keratinocytes (10), which are the target cells in nonmelanoma skin cancer and in the chemical-induced carcinogenesis in mouse skin, where the phorbol ester TPA is used as a tumor promoter. We have shown that the epidermal RasGRP1 mediates activation of

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**Figure 2.** Subcellular localization of RasGRP1 in response to bryostatin 1 and TPA. SP-1 cells expressing RasGRP1-GFP were treated with either 1 μmol/L bryostatin 1 (Bryo; A) or 1 μmol/L TPA (B) and labeled with different organelle markers for staining of Golgi (brefeldin A), ER (calreticulin), mitochondria (Mito; MitoTracker), and lysosomes (Lyso; LysoTracker) as described in Materials and Methods. Green, RasGRP1-GFP protein (left); red, organelle markers (middle). Colocalization of RasGRP1-GFP with the organelle markers (yellow) in the merged images (right). The fluorescence was detected using a confocal microscope. Similar results were observed in two to three independent experiments.
Ras in response to TPA treatment. Our present findings show that bryostatin 1 also modulates RasGRP1 in mouse epidermal keratinocytes, although it differs from the effects of TPA.

Our previous structure-activity analysis revealed that RasGRP1 is a high-affinity target not only for phorbol esters but also for daphnane derivatives, indole alkaloids, and bryostatins (8). As such, bryostatin 1 was expected to modulate RasGRP1 in the mouse keratinocytes. However, we found that despite similar potencies in vitro, bryostatin 1 was less effective than the phorbol ester TPA at inducing Ras activation in the keratinocytes. In fact, similar in vitro potencies do not necessarily predict similar in vivo activities, because factors like pharmacokinetics can influence the in vivo potency of a DAG analogue. In this regard, recent elegant studies from Dr. Blumberg’s laboratory have shown that the lipophilicity of a phorbol ester is an important determinant in its uptake kinetics and persistence in membranes, affecting the translocation kinetics of DAG receptors, such as PKCα, PKCε, and RasGRP3 (21). Ultimately, the subcellular distribution that a receptor adopts should determine access to its substrates, having profound implications in downstream signaling. In the context of Ras signaling, the plasma membrane was considered until recently the sole location for Ras activation and signaling. However, recent work has shown active pools of Ras in the Golgi apparatus and the ER (22). Moreover, activation of Ras in Golgi by RasGRP1 has also been postulated in T cells (23) and in COS-1 cells ectopically expressing the exchange factor (24), suggesting that Ras can be directly activated in internal membranes. Our findings that TPA and bryostatin 1 induced a different pattern of translocation of RasGRP1 in the mouse keratinocytes. Primary mouse keratinocytes were serum starved overnight and then treated for 5 min with either DMSO (vehicle), TPA, or bryostatin 1 (Bryo). RasGTP was precipitated by the pull-down technique as described in Materials and Methods. Intensities of the Western blot bands were determined by densitometry, and results for RasGTP were normalized by the total amount of Ras in each lane (relative RasGTP levels). Levels of total Ras and RasGRP1 were measured from aliquots of the total lysate used in the pull-down experiment.

**Figure 3.** Activation of Ras by bryostatin 1 and TPA in mouse keratinocytes. Primary mouse keratinocytes were serum starved overnight and then treated for 5 min with either DMSO (vehicle), TPA, or bryostatin 1 (Bryo). RasGTP was precipitated by the pull-down technique as described in Materials and Methods. Intensities of the Western blot bands were determined by densitometry, and results for RasGTP were normalized by the total amount of Ras in each lane (relative RasGTP levels). Levels of total Ras and RasGRP1 were measured from aliquots of the total lysate used in the pull-down experiment. A, effect of 1 μM bryostatin 1 and 1 μM TPA. Western blot results from a representative experiment. Columns, mean of four independent experiments; bars, SE. *, P < 0.05 versus DMSO; #, P < 0.05 versus TPA. B, concentration-response curves for Ras activation on bryostatin 1 and TPA treatment. Columns, mean of three to four independent experiments; bars, SE. **, P < 0.01 versus DMSO; *, P < 0.05 versus DMSO.

**Figure 4.** Effect of RasGRP1 overexpression on Ras activation in mouse keratinocytes. A, primary mouse keratinocytes were infected for 24 h with either AdRasGRP1 or AdLacZ. RasGTP was precipitated by the pull-down technique as described in Materials and Methods. Intensities of the Western blot bands for noninfected (NI), AdRasGRP1-infected (AdRasGRP1), and AdLacZ-infected (AdLacZ) keratinocytes were determined by densitometry, and results for RasGTP were normalized by the total amount of Ras in each lane and expressed as fold increase of the relative levels of RasGTP in noninfected cells. Columns, mean of at least five independent experiments; bars, SE. **, P < 0.01 versus noninfected. B and C, after overnight serum starvation, cells infected with either AdRasGRP1 (B) or AdLacZ (C) were treated for 5 min with either DMSO, 1 μM TPA, or 1 μM bryostatin 1 (Bryo) for 5 min. Intensities of the Western blot bands were determined by densitometry, and results for RasGTP were normalized by the total amount of Ras in each lane (relative RasGTP levels). Levels of total Ras and RasGRP1 were measured from aliquots of the total lysate used in the pull-down experiment. Western blot results are from a representative experiment. Columns, mean of at least five independent experiments; bars, SE. **, P < 0.01 versus noninfected (B); ***, P < 0.01 versus DMSO; *, P < 0.05 versus DMSO; #, P < 0.01 versus TPA (C).
Figure 5. Effect of PKC inhibition on Ras activation by bryostatin 1 and TPA in mouse keratinocytes. Primary mouse keratinocytes, either control (A) or infected for 24 h with AdRasGRP1 (B) or AdLacZ (C), were serum starved overnight followed by a 5-min treatment with either DMSO (vehicle), 1 μmol/L TPA, or 1 μmol/L bryostatin 1 (Bryo). When indicated, cells were preincubated for 30 min with 10 μmol/L GF109203X (GF). RasGTP was precipitated by the pull-down technique as described in Materials and Methods. Intensities of the RasGTP bands were determined by densitometry and then normalized by the total amount of Ras in each lane (relative RasGTP levels). Columns, mean of at least three independent experiments; bars, SE.

Figure 6. Down-regulation of RasGRP1, PKCα, and PKCβ in response to bryostatin 1 and TPA in mouse keratinocytes. Primary mouse keratinocytes were incubated with increasing concentrations of either TPA, bryostatin 1 (Bryo), or both compounds for 24 h. A, levels of RasGRP1 were detected by Western blot analysis of total cell lysates. Intensities of the RasGRP1 bands were measured by densitometry and expressed as percentage of RasGRP1 in the control, untreated cells. Points, mean of at least four independent experiments; bars, SE. Curve analysis for TPA-induced down-regulation was done using a sigmoidal-logistic fit. B, Western blot analysis of PKCα and PKCβ in total lysates from keratinocytes treated with either bryostatin 1 or TPA. Representative of three independent experiments. C, primary keratinocytes were treated with DMSO or 1 μmol/L TPA and the indicated concentrations of bryostatin 1 for 24 h. Total samples were prepared and RasGRP1 was detected by Western blot. Representative of three independent experiments. Actin is included as a loading control.

Regulation of RasGRP1 by Bryostatin and Phorbol Esters

keratinocytes suggest that these DAG analogues could cause the activation of different pools of Ras in the cells. In contrast to TPA, bryostatin 1 did not show any apparent translocation of RasGRP1 to the plasma membrane. One could argue that TPA may activate a plasma membrane pool of Ras in the mouse keratinocytes that is inaccessible to bryostatin 1–activated RasGRP1. Among the classic Ras proteins, only KRas escapes localization in the Golgi and seems to signal exclusively from the plasma membrane (25, 26). Thus, bryostatin 1 could be less efficient at activating Ras than TPA due to the inability to stimulate KRas in the keratinocytes. We are currently examining this hypothesis.

Our findings in mouse keratinocytes suggest that PKC is mainly dispensable for Ras activation in response to TPA and bryostatin 1, although it is required for activation of the downstream extracellular signal-regulated kinase (ERK) signaling (10). Recent studies in T cells have identified a PKC-RasGRP1 mechanism for Ras and ERK activation in response to phorbol esters and antigen receptor stimulation, which involves RasGRP1 phosphorylation by the novel PKC isoform θ (27). In the study, the PKC inhibitor Rottlerin (as well as RO 31-8220) abrogates both Ras stimulation and extracellular signal-regulated kinase phosphorylation in response to TPA treatment. These data and our findings suggest that the mechanisms by which DAG analogues activate Ras may be distinct in different tissues. Alternatively, the differences may be in part the result of the pharmacologic agents used to inhibit PKC. In our studies, we employed the bisindolylmaleimide GF109203X, which inhibits both conventional and novel PKC isoforms (28), whereas Rottlerin has been initially defined as a specific PKCθ inhibitor (29), although it also inhibits other novel isoforms. On the other hand, RO 31-8220 is another bisindolylmaleimide that inhibits conventional and novel isoforms (30). Given the nonspecific activities of some of these compounds (31, 32), alternative studies may be required to define the mechanisms of PKC participation in Ras activation by DAG analogues.

DAG analogues, such as TPA and bryostatin 1, are slowly metabolized in the cell and usually cause degradation and inactivation of the receptor by proteolysis, a phenomenon...
known as down-regulation. We have reported previously, and also shown in this study, that TPA causes a concentration-dependent down-regulation of RasGRP1 in primary mouse keratinocytes (10). In contrast, bryostatin 1 only partially down-regulated RasGRP1. This is an interesting finding, because our results suggest that whereas TPA is more effective than bryostatin 1 at activating the RasGRP1-Ras pathway in acute conditions, TPA may have a long-term antagonistic effect on RasGRP1 compared with bryostatin 1. Our results also show that bryostatin 1 prevented TPA from inducing maximal down-regulation of RasGRP1. This adds to the list of biological effects in mouse keratinocytes in which bryostatin 1 exerts a predominant effect over that of TPA.

Our findings on RasGRP1 down-regulation by bryostatin 1 and TPA are apparently at odds with work done on T cells showing that neither DAG analogue causes degradation of RasGRP1 (27, 33). However, TPA-induced down-regulation of RasGRP1 has been reported in the murine B cells WEHI-231 with kinetics similar to those of PKCδ degradation (34). It is possible that rather than being conflicting reports, these findings reflect cell-based differences in regulation of RasGRP1, like it has been documented for the PKC isoforms.

In summary, our results show that RasGRP1 is differentially regulated by bryostatin 1 and TPA in the mouse keratinocytes, and this regulation can contribute to biological differences like those seen on Ras activation. Although PKC is still the major receptor for both bryostatin 1 and TPA in skin and is responsible for many of the biological effects of DAG analogues in the epidermis, our present results support the hypothesis that some of the divergent effects of TPA and bryostatin 1 could result from targeting non-PKC receptors, like RasGRP1.

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