RasGRP1 Is Essential for Ras Activation by the Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate in Epidermal Keratinocytes

Amrish Sharma, Courtney T. Luke, Nancy A. Dower, James C. Stone, and Patricia S. Lorenzo

From the Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, Hawaii 96813 and the Departments of Pediatrics and Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

RasGRP1 is a guanine nucleotide exchange factor for Ras that binds with high affinity to diacylglycerol analogs like the phorbol esters. Recently, we demonstrated a role for RasGRP1 in skin carcinogenesis and suggested its participation in the action of tumor-promoting phorbol esters like 12-O-tetradecanoylphorbol-13-acetate (TPA) on Ras pathways in epidermal cells. Given the importance of Ras in carcinogenesis, we sought to discern whether RasGRP1 was a critical pathway in Ras activation, using a RasGRP1 knockout (KO) mouse model to examine the response of keratinocytes to TPA. In contrast to the effect seen in wild type keratinocytes, RasGRP1 knockout cells were barely detected in RasGRP1 KO cells even after 60 min of exposure to phorbol esters. The lack of response was rescued by enforced expression of RasGRP1. Furthermore, small hairpin RNA-induced silencing of RasGRP1 abrogated the effect of TPA on Ras. Analysis of Ras isoforms showed that both H-Ras and N-Ras depended on RasGRP1 for activation by TPA, whereas activation of K-Ras could not be detected. Although RasGRP1 was dispensable for ERK activation in response to TPA, JNK activation was reduced in the KO keratinocytes. Notably, TPA-induced phosphorylation of JNK2, but not JNK1, was reduced by RasGRP1 depletion. These data identify RasGRP1 as a critical molecule in the activation of Ras by TPA in primary mouse keratinocytes and suggest JNK2 as one of the relevant downstream targets. Given the role of TPA as a skin tumor promoter, our findings provide additional support for a role for RasGRP1 in skin carcinogenesis.

The action of phorbol esters like 12-O-tetradecanoylphorbol-13-acetate (TPA) in skin, both in proliferation and differentiation of keratinocytes as well as in hyperplasia and tumor promotion, has been historically attributed to its ability to activate protein kinase C (PKC) isoforms (1, 2). Although there is an undisputable role of PKC in skin biology (3, 4), the fact that TPA can signal via non-PKC targets in other tissues (5, 6) suggests that this could also be the case in the epidermis. In fact, there is already evidence of expression and, in some cases of function, of non-PKC receptors in the epidermis, such as protein kinase D1 (PKD1)/PKCζ (7), diacylglycerol kinase (8), and RasGRP1 (9).

RasGRP1 is one of the members of the RasGRP family, composed of a total of four isomers (RasGRP1 to 4) that have different tissue distribution and specificity for Ras-like members (10–14). RasGRP1 functions as a guanine nucleotide exchange factor for Ras small GTPases, promoting Ras binding to GTP and thus Ras activation (15). It is abundantly expressed in T-cells but is also present in other hematopoietic cells and in the brain (14). Recently, we found that keratinocytes derived from the skin also express RasGRP1 (9), where it can mediate Ras activation in response to diacylglycerol analogs caused by the presence of a C1-binding domain similar to that of the classic and novel PKC isoforms (9, 16, 17). Interestingly, Ras activation in response to the ultrapotent analog TPA (also known as phorbol 12-myristate 13-acetate, PMA) proceeds in a PKC-independent manner and is substantially increased by enforced expression of RasGRP1 (9, 16). However, the relative contribution of RasGRP1 to Ras signals in keratinocytes in absence of overexpression remained to be defined. Here we utilized primary mouse keratinocytes derived from a RasGRP1 knockout (RasGRP1 KO) mouse model as well as an shRNA approach to demonstrate that RasGRP1 is essential for Ras activation in response to TPA. Moreover, deletion of RasGRP1 reduced the activation of JNK2 induced by TPA, whereas ERK phosphorylation was not affected.

In summary, our studies provide the first evidence for a critical role for RasGRP1 in the activation of Ras in epidermal cells, and establish a clear link between TPA and Ras independently of PKC in this system. Considering that TPA is a potent skin tumor promoter and that JNK pathways are involved in tumorgenic responses in skin (18, 19), our findings may provide insights into the mechanisms of action of tumor promotion and keratinocyte transformation in response to phorbol esters.

EXPERIMENTAL PROCEDURES

Mice—The RasGRP1 KO mice, produced by inserting the Escherichia coli β-galactosidase gene and a neomycin cassette in exon 2 of Rasgrp1 (20), were originally obtained in 129/J-B6 mixed background and backcrossed for >10 generations to the FVB/N background. Wild type mice of FVB/N background were bred in house. The mice were housed and utilized accord-
ing to Institutional Animal Care and Use Committee guidelines at the University of Hawaii Animal Facility.

**Primary Keratinocyte Culture**—Mouse keratinocytes were isolated from wild type or RasGRP1 KO newborn mice by the trypsin flotation method (21) with modifications as described before (16, 22). The cells were plated on 60-mm dishes coated with collagen I (Coating Matrix; Invitrogen) and incubated overnight in Eagle’s minimum essential medium containing 1.2 mM CaCl₂, antibiotics, antimycotics, and 8% fetal bovine serum. Afterward, the cells were washed with Dulbecco’s phosphate buffered saline and cultured in 154 medium (Invitrogen) supplemented with 50 μg/ml CaCl₂, antibiotics, antimycotics, 2% calcium-free fetal bovine serum, and a human epidermal growth factor supplement containing epidermal growth factor, pituitary extract, insulin, transferrin, and hydrocortisone (Invitrogen). Keratinocytes were used within 5–6 days after plating.

**Adenoviral Vectors**—Recombinant adenoviral vectors encoding rat RasGRP1 were generated with the Transpose-Ad system (Qbiogene, Irvine, CA) as described elsewhere (9). Adenoviral vectors for expression of bacterial β-galactosidase (LacZ) were purchased from Qbiogene. Infection with the adenoviral vectors was performed as described before (16). The cells were utilized 48–72 h post-infection. For the shRNA approach, an RNAi-Ready pSiren shuttle shRNA-RasGRP1 vector was generated using a 19-mer sequence corresponding to base pairs 1584–1602 of mouse Rasgr1 (accession number NM_011246). The adenoviral shRNA constructs were prepared with the pSiren-compatible Adeno-X System according to the manufacturer’s instructions (Clontech). A nontargeting shRNA adenoviral vector (irrelevant shRNA) was utilized as control for nonspecific effects in the silencing experiments. Purification of the recombinant adenoviruses was done using the AdEasy virus purification kit (Stratagene, La Jolla, CA). Keratinocyte infections were done following similar protocols described before for the Transpose-based recombinant adenoviruses (see above).

**Ras Activation Assay and Western Blots**—Levels of active, GTP-loaded Ras (RasGTP) were measured using the glutathione S-transferase-tagged Ras-binding domain of Raf-1 as a probe in a Ras affinity precipitation or pulldown assay. For the assay, keratinocytes were harvested on ice in lysis buffer containing 15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1% Igepal, 5% glycerol, and protease inhibitors (Mini Complete with EDTA; Roche Applied Science). The lysates were vortexed and incubated on ice for 5 min followed by centrifugation at 13,000 rpm for 5 min at 4°C. Five hundred μg of protein from the supernatant was then incubated with glutathione S-transferase-tagged Ras-binding domain conjugated to glutathione-Sepharose 4B beads (GE Healthcare) for 1 h with rotation in the cold. The affinity complexes were washed thrice with lysis buffer and then resuspended in 2× Laemmli buffer, boiled, and resolved on 15% acrylamide gels. Fifty μg of the total lystate protein was run in parallel as measurement of input of total Ras in the assay. The proteins were blotted onto nitrocellulose membranes, and immunostaining was done using the pan anti-Ras clone RAS10 antibody (Calbiochem, San Diego, CA) or antibodies specific for either H-Ras (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or N-Ras (Calbiochem). Samples to evaluate RasGRP1 levels were run on 8% acrylamide gels, blotted, and immunostained with a monoclonal anti-RasGRP1 antibody (m199; Santa Cruz Biotechnology). Analysis of ERK and JNK forms was done on 12% acrylamide gels, followed by blotting on nitrocellulose membranes and immunostaining with one of the following antibodies: p44/42 MAPK (ERK), phospho-p44/42 MAPK Thr202/Tyr204 ERK1, Thr183/Tyr185 ERK2 (p-ERK), p56/44-SAPK/JNK (JNK), and phospho-p56/44 SAPK/JNK Thr183/Tyr185 (p-JNK) (Cell Signaling Technology, Danvers, MA). All of the primary antibody incubations were done overnight at 4°C; incubations with the secondary antibody conjugated to horseradish peroxidase were performed for 1 h at room temperature. Chemiluminescence was generated by using ECL Advance (GE Healthcare).

**Data Analysis**—Semi-quantitative densitometry was done on scanned films using Image J 1.42q. The data were analyzed with Prism 5 (GraphPad Software Inc., La Jolla, CA), and the selection of the statistical test for analysis of significance was done according to the experimental design. Briefly, Student’s unpaired t test was employed to compare the means between two independent groups. One-way ANOVA was used to compare the means of three independent groups. When two independent variables (genotype and time) needed to be compared between two independent groups, two-way ANOVA was the test performed. If a significant difference was detected by ANOVA, Tukey’s or Bonferroni’s post-test was applied to specifically find which means were different from each other. The statistical significance level was set at p values of <0.05.

**RESULTS**

**RasGRP1 Knockout Cells Show Impaired Ras Activation in Response to TPA**—To investigate the extent of RasGRP1 contribution to Ras activation by TPA in keratinocytes, we analyzed the response of keratinocytes derived from RasGRP1 KO mouse skin. The chief phenotypic trait of the RasGRP1 KO mice is defective T-cell development (20), leading to late onset lymphoproliferation and autoimmunity. No other alterations have been described, and the gross anatomy of the skin and skin appendages looks unremarkable. However, when primary RasGRP1 KO keratinocytes were treated with TPA, the levels of active GTP-loaded Ras (RasGTP) were barely detectable even after 60 min of treatment, in clear contrast to the activation observed in wild type cells that showed a rapid stimulation of Ras declining after 30 min of treatment (Fig. 1, A and B).

Enforced expression of RasGRP1 in the KO keratinocytes significantly rescued the stimulation of Ras by TPA, suggesting that the effect on Ras was specific to RasGRP1 (Fig. 1C). As an independent test of a RasGRP1-specific effect and to rule out any indirect developmental effects that could arise from early loss of RasGRP1 in the mouse mutant, we used a shRNA approach to silence RasGRP1 in wild type primary keratinocytes. This approach resulted in >90% depletion of RasGRP1 from the cells and led to a significant reduction in the ability of TPA to stimulate Ras, similar to the one observed in the KO keratinocytes (Fig. 1D). Keratinocytes infected with a nontargeting shRNA vector (irrelevant
shRNA) did not differ from uninfected, control epidermal cells in the activation of Ras in response to TPA (Fig. 1D).

Although we could not control for off target effects of our RNA interference approach, the fact that the shRNA data paralleled what we observed in the KO keratinocytes supports a RasGRP1-specific effect. Taken together, these results strongly suggest that RasGRP1 is essential to TPA-induced Ras stimulation in epidermal keratinocytes. **Both N- and H-Ras Are Dependent on RasGRP1 for TPA-induced Activation**—Previous studies have described RasGRP1 as a guanine nucleotide exchange factor for the classic Ras proteins H-, N-, and K-Ras (10, 15, 23, 24). We therefore sought to determine whether TPA showed a similar activation of these Ras isoforms in keratinocytes, or a preferential activation of one isoform over the others. We performed the studies using a 15-min TPA treatment, which produced Ras stimulation within the maximal levels observed for phorbol ester treatment (Fig. 1B). The three Ras isoforms could be detected in keratinocytes lysates, although the K-Ras total levels were lower than those of H- and N-Ras (data not shown). Under these conditions, activation of K-Ras could not be detected, whereas N- and H-Ras displayed a ~2-fold activation in response to TPA in the wild type keratinocytes (Fig. 2). In contrast, TPA treatment had a negligible effect on the activation of either isoform in the RasGRP1 KO keratinocytes (Fig. 2, A and B), consistent with a dependence on RasGRP1 for stimulation. We conclude that RasGRP1 mediates both H- and N-Ras activation in response to phorbol esters in keratinocytes.

**ERK-induced Phosphorylation by TPA Proceeds Independently of RasGRP1**—Ras has multiple downstream effectors, and one of them is the MAPK ERK (25), also a downstream response element of TPA in various cell types, including keratinocytes (26). We have previously reported that overexpression of RasGRP1 in keratinocytes is associated with increased ERK phosphorylation; however, further ERK stimulation with TPA could be entirely blocked by the PKC inhibitor GF103209X (9). Whereas the data clearly suggest a PKC-dependent effect of TPA on ERK activation, they do not rule out a role for RasGRP1 downstream of PKC. To assess the participation of RasGRP1, we compared levels of ERK activation in response to TPA treatment between wild type and RasGRP1 KO keratinocytes. We

**FIGURE 1.** Depletion of RasGRP1 abrogates TPA-induced activation of Ras in mouse primary keratinocytes. A, mouse primary keratinocytes isolated from the epidermis of either wild type (Wt) or RasGRP1 KO mice, were serum-starved overnight and treated with 1 μM TPA for the times indicated. Ras^GTP^ levels were pulled down from 500 μg of total lysate protein, as described under “Experimental Procedures.” Total Ras and RasGRP1 were measured in a 50-μg aliquot of the total lysate used in the pulldown assay. The results shown are from a representative experiment of at least five independent experiments. B, densitometry analysis of Ras^GTP^ levels normalized by the total amount of Ras in each lane and plotted as the means ± S.E. White circles, Wt keratinocytes; black circles, KO keratinocytes. **, p < 0.01; ***, p < 0.001, between Wt and KO values, two-way ANOVA followed by Bonferroni test (n = 5–7). C, KO keratinocytes were infected for 48 h with adenoviral vectors encoding for either RasGRP1 (AdRGRP1) or the irrelevant protein LacZ (AdLacZ), serum-starved overnight, and treated for 15 min with 1 μM TPA (+) or control vehicle (DMSO; −). Ras^GTP^ was precipitated by pulldown assay and run along with aliquots of the input to measure total Ras and RasGRP1 as indicated above. The results shown are representative of three independent experiments. D, mouse primary keratinocytes isolated from the epidermis of wild type mice were transduced with adenoviral vectors containing the short hairpin RNA sequence targeting mouse Rasgrp1 (shRNA-RGRP1) or a nontargeting, irrelevant sequence (shRNA-Irel). Uninfected keratinocytes (control) were also included for comparison. 48 h later, the cells were serum-starved overnight and treated for 15 min with 1 μM TPA (+) or control vehicle (DMSO; −). Ras^GTP^, total Ras, and RasGRP1 levels were measured as described above. The results shown are representative of three independent experiments.
exploited the fact that activation of ERK requires its phosphorylation and evaluated levels of phosphothreonine/phosphoryrosine ERK by using specific antibodies. TPA rapidly induced ERK phosphorylation within 5 min of treatment in both wild type and RasGRP1 KO keratinocytes, with no differences in response between the two cell genotypes during the 60-min time course performed (Fig. 3). The total amount of ERK in the cells remained unchanged during TPA treatment (Fig. 3A). Together, the data suggest a RasGRP1-independent mechanism for ERK activation by TPA in mouse keratinocytes.

**TPA Depends on RasGRP1 for Phosphorylation of JNK2—JNK** is another member of the MAPK family that can be activated in response to Ras and phorbol esters and has been implicated in a myriad of effects on skin, from inflammatory responses to proliferation and skin cancer (27–29). Therefore, we examined the effect of RasGRP1 on JNK activation by TPA in primary mouse keratinocytes. Like ERK, JNK activation also requires phosphorylation at threonine and tyrosine residues. Therefore, we evaluated phospho-JNK levels as a measurement of its activation. In contrast to the effect of TPA on ERK, JNK activation was altered in keratinocytes lacking RasGRP1 (Fig. 4A). Interestingly, phosphorylation of JNK1 and JNK2, the two isoforms present in keratinocytes, was differentially affected by depletion of RasGRP1. Specifically, JNK2 phosphorylation was reduced in the RasGRP1 KO keratinocytes compared with the wild type counterparts, whereas levels of phospho-JNK1 did not significantly differ between the groups (Fig. 4). Ectopic expression of RasGRP1 in the KO keratinocytes rescued the response of JNK2 to TPA (Fig. 5), further demonstrating a RasGRP1-specific effect. Taken together, these results position JNK2 as a potential downstream target of RasGRP1 in epidermal cells.

**DISCUSSION**

Our previous studies have demonstrated expression of RasGRP1 in epidermal keratinocytes and suggested an important role for this exchange factor in mediating effects of ultrapotent diacylglycerol analogs like TPA in a PKC-independent manner (9, 16). Our current study provides the critical data that define RasGRP1 as an essential component in the pathway to Ras activation in response to TPA in these cells.

The dependence on RasGRP1 for Ras activation by TPA in keratinocytes was initially reminiscent of the effect seen in T-cells (20, 30). However, the effect in T-cells involves PKC through phosphorylation of RasGRP1 (31), a mechanism that we have not found in keratinocytes, at least when probing PKC participation with specific inhibitors. Moreover, whereas RasGRP1 induces ERK phosphorylation when overexpressed in keratinocytes (9), ERK activation by
TPA is a RasGRP1-independent event, in contrast with the requirements observed in T-cells. Although our results were initially surprising, given the established link between Ras and ERK (32–34), there are several examples in the literature of Ras-independent mechanisms of ERK activation (32, 35–37) suggesting that pathways that utilize ERK can substantially differ depending on cell type and stimuli.

Interestingly, another member of the MAPK family, JNK, required RasGRP1 for activation by phorbol esters in keratinocytes. Similarly, natural killer cells show reduced TPA-mediated activation of JNK when RasGRP1 was silenced by RNA interference approach (38). However, as in the case of ERK, the effect of RasGRP1 on JNK cannot be generalized. For example, thymocytes from RasGRP1 KO mice did not show any defect in JNK activation (30), and although mutant Jurkat cells depleted from RasGRP1 display a reduction in JNK phosphorylation upon TPA treatment, the defect in activation could not be rescued by RasGRP1 replenishment (31). One should note that there are differences in the concentration of TPA and time course employed among these studies. Thus, the discrepancies in response to RasGRP1 depletion may not only depend on the cell type but also on the experimental conditions utilized for the studies.

The association of JNK phosphorylation with Ras activation has been documented in various cell types, including keratinocytes (39–41). Recent evidence suggest that Ras-induced JNK activation distinctively occurs in endomembranes such as endoplasmic reticulum and the Golgi apparatus (42, 43). This is particularly interesting if one considers that RasGRP1 preferentially activates Ras in endomembranes (43–45). We have previously shown that TPA is able to translocate RasGRP1 to both plasma and internal membranes in keratinocytes and that the fraction redistributed to internal membranes colocalizes with Golgi partially with endoplasmic reticulum (8). Together, the data generated in the keratinocytes support a model for RasGRP1 activation of Ras in endomembranes leading to JNK phosphorylation. However, the precise molecular pathway involved in this activation remains to be elucidated.

The fact that RasGRP1 preferentially signals to Ras activation in internal membranes also suggests that not all Ras GTPases can be substrates for RasGRP1 in vivo. For the classic Ras isoforms, this means that only H- and N-Ras, but not K-Ras (46, 47), could be activated by RasGRP1, despite the fact that in vitro they all work as RasGRP1 substrates (10, 15, 24). Because TPA can translocate RasGRP1 to the plasma membrane in keratinocytes (9, 16), we cannot rule out the stimulation of plasma membrane-bound Ras isoforms under our experimental conditions. However, we were unable to detect any activation of K-Ras in response to TPA in keratinocytes, which supports the idea that the main site of activation of Ras by RasGRP1 is in endomembranes or that the localization of the active Ras-GRP1 pool in plasma membrane differs from that of K-Ras. Alternatively, our results on K-Ras may just be a reflection of poor antibody sensitivity on Western blot, a situation that could only be resolved if new antibodies become available. Nevertheless, the results with JNK activation independently argue in favor of a TPA-RasGRP1 pathway for Ras activation in endomembranes.

Previous studies from our laboratory demonstrated that transgenic animals overexpressing RasGRP1 in the epidermis are prone to develop skin tumors derived from keratinocytes and also respond to multistage carcinogenesis protocols in which TPA is used as a tumor promoter, with
FIGURE 5. Enforced expression of RasGRP1 rescues JNK2 phosphorylation induced by TPA in RasGRP1 KO mouse keratinocytes. A, primary keratinocytes derived from RasGRP1 KO mouse skin were infected for 48 h with adenoviral vectors encoding for either RasGRP1 (AdRGRP1) or the irrelevant protein LacZ (AdLacZ), then serum-starved overnight, and treated for 15 min with 1 μM TPA (+) or control vehicle (DMSO, −). The results shown are representative of four independent experiments. B, densitometry analysis of pJNK2 normalized by the total JNK2 levels and plotted as the means ± S.E. White bars, DMSO control; black bars, TPA treatment. *, p < 0.05; **, p < 0.01, one-way ANOVA followed by Tukey’s test (n = 4).

generation of larger and more aggressive carcinomas than the wild type mice (22, 48). Together, the studies suggest that RasGRP1 is required during carcinogenesis in the skin, through its role as Ras activator. Both ERK and JNK have been linked to the effects of oncogenic Ras, and thus the participation of a RasGRP1-JNK kinase pathway in keratinocyte transformation is plausible. ERK is an essential pathway in Ras-induced keratinocytes transformation (49, 50). The role of JNK is less evident, because this kinase has been associated with both pro- and anti-tumorigenic effects in various tissues, including skin (29, 51–54). However, recent carcinogenesis studies using genetically engineered mice have shed light onto the action of JNK in skin cancer, revealing opposite functions of the two isoforms expressed in keratinocytes, JNK1 and JNK2. Specifically, deletion of JNK1 enhances tumor promotion (18), whereas JNK2 knockout suppresses epidermal carcinogenesis (19), revealing a pro-tumorigenic role of JNK2 in the skin. These findings are particular relevant to our present observations because we have found that TPA-induced activation of JNK2, but not JNK1, is susceptible to RasGRP1 depletion in the mouse epidermal cells. We speculate that RasGRP1 may represent a link between TPA and JNK2 activation in the skin, with the consequent implications for tumorigenesis.

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