RasGRP1 Overexpression in the Epidermis of Transgenic Mice Contributes to Tumor Progression during Multistage Skin Carcinogenesis

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Abstract

RasGRP1 is a guanine nucleotide exchange factor for Ras, activated in response to the second messenger diacylglycerol and its ultrapotent analogues, the phorbol esters. We have previously shown that RasGRP1 is expressed in mouse epidermal keratinocytes and that transgenic mice over-expressing RasGRP1 in the epidermis under the keratin 5 promoter (K5.RasGRP1) are prone to developing spontaneous papillomas and squamous cell carcinomas, suggesting a role for RasGRP1 in skin tumorigenesis. Here, we examined the response of the K5.RasGRP1 mice to multistage skin carcinogenesis, using 7,12-dimethylbenz(α)anthracene as carcinogen and 12-O-tetradecanoylphorbol-13-acetate (TPA) as tumor promoter. We found that whereas tumor multiplicity did not differ between transgenic and wild-type groups, the transgenic tumors were significantly larger than those observed in the wild-type mice (wild-type, 4.58 ± 0.25 mm; transgenic, 9.83 ± 1.05 mm). Histologic analysis further revealed that squamous cell carcinomas generated in the transgenic mice were less differentiated and more invasive than the wild-type tumors. Additionally, 30% of the transgenic mice developed tumors in the absence of initiation, suggesting that RasGRP1 overexpression could partially substitute for the initiation step induced by dimethylbenz(α)anthracene. In primary keratinocytes isolated from K5.RasGRP1 mice, TPA stimulation induced higher levels of Ras activation compared with the levels measured in the wild-type cells, indicating that constitutive overexpression of RasGRP1 in epidermal cells leads to elevated biochemical activation of endogenous Ras in response to TPA. The present data suggests that RasGRP1 participates in skin carcinogenesis via biochemical activation of endogenous wild-type Ras and predisposes to malignant progression in cooperation with Ras oncogenic signals.

Introduction

RasGRP is a family of guanine nucleotide exchange factors for Ras/Rap small GTPases and is composed of four members (RasGRP1–4) which differ in their substrate recognition (1, 2). All the RasGRP members possess a cysteine-rich domain that shares similar features as the C1 domain motif of protein kinase C (PKC), which is responsible for binding to the second messenger diacylglycerol and its ultrapotent analogues, the phorbol esters (3). Previous studies on RasGRP1 and RasGRP3 showed their high-affinity binding to diacylglycerol analogues (4–6), resulting in the activation of Ras and Ras signaling cascades, and suggesting that pathways besides PKC could transmit signals from diacylglycerol to Ras. In fact, the discovery of RasGRP1 has led to a better understanding of the link between T-cell receptor stimulation and phospholipase C activation with Ras signaling (7). In contrast to PKC, RasGRP members have limited tissue distribution. RasGRP1, for example, is expressed in T cells and at lower levels in B cells, neurons, mastocytes, and some kidney cells (8–12). Recently, we have also found RasGRP1 expression in epidermal keratinocytes, where it can mediate Ras activation in response to phorbol esters (13, 14).

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was first described as a potent skin tumor promoter and later identified as a diacylglycerol mimetic able to bind to PKC (15). Thus, TPA-induced skin tumor promotion has been primarily linked to the modulation of PKC in keratinocytes, although the contribution of the individual PKC isoforms to this effect is complex and still not fully understood (16). In addition to the role of PKC, differences in susceptibility to tumor promotion among mouse strains suggest that there are modifier genes that can influence the response of the epidermis to the phorbol esters. Analysis of traits associated with increased or decreased tumor promotion susceptibility revealed several genetic loci, including one locus in mouse chromosome 2 (17). Nmes1, whose role and relationship with TPA signaling is unknown, is one of the genes identified in chromosome 2 that was found elevated in the skin of susceptible mouse strains (18). Interestingly, RasGRP1 also maps near the TPA promotion susceptibility locus in chromosome 2 (17), although its participation in tumor promotion by TPA has not been explored. To investigate the potential role of RasGRP1 in skin tumorigenesis, we recently developed a transgenic mouse model for the overexpression of RasGRP1 in the epidermis (K5.RasGRP1; ref. 19). These mice are prone to developing spontaneous papillomas and squamous cell carcinomas (SCCs), implying a role for RasGRP1 in tumor initiation (19). To gain further insight into the effect of RasGRP1 during skin carcinogenesis, particularly TPA-induced tumor promotion, we subjected K5.RasGRP1 mice to the classic two-stage chemical carcinogenesis protocol using 7,12-dimethylbenz(α)anthracene (DMBA) as an initiator. We found that RasGRP1 overexpression in skin did not affect tumor formation in response to DMBA/TPA, but caused larger and more invasive tumors than those observed in the wild-type animals. These findings indicate that RasGRP1 modulation was dispensable for the TPA-induced tumor promotion of carcinogen-initiated skin, but contributed to tumor progression, suggesting that elevated biochemical activation of Ras through increased expression of RasGRP1 could cooperate with Ras oncogenic signals towards malignancy.
Materials and Methods

Animals and skin carcinogenesis experiments. The K5.RasGRP1 mice were generated as previously described on the FVB/N background strain (19). For the skin carcinogenesis experiments, the back of 6- to 8-week-old mice, both males and females, were shaved with electric clippers 2 days before the beginning of the protocol. The mice in each group, transgenic and wild-type, were divided into two cohorts of 16 animals each of mixed gender to be treated with either DMBA plus TPA or acetone plus TPA. The DMBA treatment consisted of a single topical application of 26 mg of DMBA in 200 µL of acetone on the shaved dorsal skin; TPA treatment was initiated 2 weeks after DMBA initiation by topical application of 2 µg of TPA in 200 µL of acetone twice a week for 20 weeks. Animals were monitored at the time of TPA applications, and tumors were counted and measured with a caliper at least once a week. After the TPA treatment was ended, mice were followed for an additional 8 weeks and then euthanized by CO2 asphyxiation. Tumor samples were collected and fixed for histology.

Epidermal hyperplasia. TPA-induced acute hyperplasia was evaluated in both K5.RasGRP1 and wild-type mice of 6 to 8 weeks of age after 48 h treatment with 3 µg of TPA in 200 µL of acetone applied to the dorsal skin. The skin area to be treated was shaved with electric clippers 2 days before TPA treatment. Control, acetone treatments, were also done in place of TPA. The epidermal thickness was determined by microscopic examination of H&E-stained skin samples. Microphotographs were taken with a CoolSnap CDD camera (Roper Scientific) at ×400 magnification and the thickness of the epidermis was measured in micrometers using MetaMorph (Molecular Devices Corporation). The grid of a hemacytometer was used for calibration of the MetaMorph software. Each sample was measured at three different locations before calculating the average thickness within each treatment group.

Histopathology and immunohistochemistry. Skin tumors were fixed in 4% paraformaldehyde for 24 h and maintained in 70% ethanol until paraffin-embedded. H&E-stained slides were used for descriptive histopathology. Immunohistochemical localization of the transgenic RasGRP1-HA protein was performed as previously described (19). Briefly, deparaffinized sections were subjected to heat-induced epitope retrieval. After blocking, tissues were incubated with anti-HA antibody (Santa Cruz Biotechnology) followed by HRP-conjugated AffinityPure donkey anti-rabbit F(ab')2 fragment–specific antibody (Jackson ImmunoResearch). 3,3'-Diaminobenzidine was used as a substrate (Dako). Tissues were counterstained with Mayer's hematoxylin (InnoGenex).

Ras(GTP) pull-down assay and Western blots. Levels of GTP-loaded Ras (Ras(GTP)) were measured by using the GST-RBD domain of Raf-1 as a probe in an affinity precipitation or pull-down assay. Briefly, primary keratinocytes were first isolated from K5.RasGRP1 or wild-type mice as described elsewhere (14). Cells were serum starved overnight, treated with vehicle (Me2SO) or 1 µmol/L of TPA for 15 min, and harvested on ice in lysis buffer containing 25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L of NaCl,

Figure 1. Skin tumor development in K5.RasGRP1 transgenic mice subjected to two-stage carcinogenesis. A, tumor multiplicity (average number of tumors per mouse ± SE) and (B) incidence (percentage of mice with tumors) in wild-type (○) and K5.RasGRP1 transgenic (●) mice treated with TPA following initiation with DMBA. C, wild-type (Wt) and K5.RasGRP1 transgenic (Tg) mice bearing tumors. Pictures were taken at the end of the protocol. D, tumor size (diameter in millimeters) at 17 and 28 wk after initiation with DMBA in both wild-type (Wt) and K5.RasGRP1 transgenic (Tg) mice. Values represent the mean ± SE of all the tumors in each group (n). *, P < 0.05; ***, P < 0.0001 (Student’s t test).
Figure 2. Tumor distribution by size and histologic type. A, size distribution of tumors at the end of the protocol in wild-type (empty columns) and K5.RasGRP1 transgenic (filled columns) mice. Columns, means of tumors in 12 to 15 mice; bars, SE; **, P < 0.009; ***, P < 0.0001, compared with the wild-type values (Student’s t test). B, tumors and dysplasias were histologically assessed and classified into four types: papilloma (Pap), keratoacanthoma (Ker), focal epidermal dysplasia (FED), and squamous cell carcinoma (SCC). Percentages of the total number of tumors/dysplasias in wild-type (empty columns) or K5.RasGRP1 transgenic (filled columns) mice. Number of tumors/dysplasias of a defined type versus the total number in the group assessed in parentheses. C, SCCs were evaluated on the basis of invasiveness and differentiation level. Left, SCCs were classified as either microinvasive (micro, one or few clusters of cells invading the dermis) or extensive invasion (extensive, advancing fronts of invading cells into the dermis and subcutaneous tissue). Number of SCCs of a defined type in wild-type (empty columns) or K5.RasGRP1 transgenic (filled columns) mice. Percentages in parentheses. **, P < 0.0015 (Fisher’s exact test). Right, SCCs were classified as well-differentiated (Well), moderately differentiated (Moderate), or poorly differentiated (Poor). Percentage of the total number of SCCs in wild-type (empty columns, 40 tumors) or K5.RasGRP1 transgenic (filled columns, 25 tumors) mice. D, histologic appearance of tumors in wild-type (Wt) and K5.RasGRP1 transgenic (Tg) mice. Insets, close-up views of each microphotograph.
5 mmol/L of MgCl₂, 1 mmol/L of NaF, 1 mmol/L of sodium orthovanadate, 1% IGEPAL, 5% glycerol, and Mini Complete Roche-protease inhibitors (Roche Applied Science). Lysates were mixed, incubated on ice for 5 min and then clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Five hundred micrograms of lysate protein were incubated with GST-RBD-Raf-1 conjugated to glutathione beads (Swell Gel Glutathione Discs; Pierce Biotechnology) for 1 h with rotation in the cold. The affinity complexes were washed thrice with lysis buffer and then resuspended in 2/C² Laemmli buffer, boiled, and resolved on 15% acrylamide gels. Twenty-five micrograms of the total lysate protein were run in parallel as measurement of input of total Ras in the assay. Proteins were blotted onto nitrocellulose membranes and immunostaining was done using the pan anti-Ras clone Ras10 antibody (Millipore-Upstate). RasGRP1 levels were evaluated by immunostaining using a monoclonal anti-RasGRP1 antibody (m199; Santa Cruz Biotechnology). This antibody detected both endogenous mouse RasGRP1 and the transgenic rat RasGRP1-HA protein.

Analysis of ras mutations. A mutation-specific PCR assay developed by Nelson et al. (20) was employed to determine the presence of Ha-ras mutations in codon 61 in the tumors. Briefly, DNA was extracted from a minimum of two 10-µm sections of paraffin-embedded tumors using the QiAamp DNA Micro kit (Qiagen) according to the manufacturer's instructions. Deparaffinization was done following standard histology procedures, and proteinase K treatment of the deparaffinized samples was done overnight. One hundred nanograms of DNA were used for the PCR reaction with the following primers: upstream ras primer, 5'-CTG TGG TGG TGG AGG AC-3'; downstream mutant ras primer, 5'-CAT GGC ACT ATA CTC TTC TA-3'. This primer combination produced a 110-bp band. Wild-type Ha-ras was also amplified as a control (downstream wild-type ras primer: 5'-CAT GGC ACT CTA CTC TTC TT-3'), and also generated a 110-bp PCR product.

Chemicals. DMBA was purchased from Sigma-Aldrich; TPA was from LC Laboratories.

Results

Response of K5.RasGRP1 mice to two-stage carcinogenesis. To address the role of RasGRP1 in TPA-induced tumor promotion in skin, we subjected K5.RasGRP1 transgenic and wild-type control mice to the classic two-stage chemical carcinogenesis protocol, using DMBA as the initiator. DMBA causes mutations in the ras proto-oncogene, mainly in Ha-ras (21).

K5.RasGRP1 mice treated with DMBA/TPA developed a similar number of tumors as the wild-type counterparts (Fig. 1A), although there was a slight decrease in tumor latency in the transgenic population (Fig. 1B). Notably, tumors generated in the transgenic mice were considerably larger than those from the wild-type group (Fig. 1C). This difference was evident not only at the end of the protocol, but also during the exponential growth of the tumors. At week no. 17 post-initiation, the transgenic tumors
were on average, 36% larger than the wild-type ones (Fig. 1D, left). After 28 weeks of initiation, the average tumor size in the transgenic population was 2.2 times larger than in the wild-type animals (Fig. 1D, right). Of note, 30% of the transgenic animals had to be euthanized before the termination of the carcinogenesis protocol due to the large size of some tumors. Thus, some of the largest transgenic-derived tumors could not be accounted for in the final size analysis.

The majority of the tumors which developed in the K5.RasGRP1 mice were 5 mm in diameter or larger, in contrast to the size distribution observed in the wild-type population (Fig. 2A). In particular, tumors of size >10 mm in diameter represented 30% of the tumor population in transgenic mice versus only 3% in the wild-type group. This difference in size suggested a high rate of tumor expansion in the K5.RasGRP1 transgenic mice. To investigate if there was an association between this increased growth rate and tumor progression, we histologically examined a sample of tumors from each group. In the wild-type population, 14.6% of the analyzed tumors were benign papillomas compared with only 3.7% of papillomas in the K5.RasGRP1 group (Fig. 2B). The remaining tumors were SCCs, except for a non-tumor growth (focal epidermal dysplasia) in the wild-type group and one keratoacanthoma in the transgenic animals. The high percentage of SCCs observed in both transgenic and wild-type mice was not surprising considering that the FVB/N mouse strain is highly susceptible to tumor progression (22). Nevertheless, despite the seemingly equal incidence of malignant tumors in both groups, further analysis showed that although most of the SCCs from the wild-type population were well-differentiated tumors with only one or a few small clusters of cells invading the dermis (microinvasive), 60% of the transgenic-derived SCCs were moderately or poorly differentiated, with extensive fronts of cells invading the dermis and subcutaneous tissues (Fig. 2C and D). These findings suggested that the K5.RasGRP1 mice were highly susceptible to tumor progression in the two-stage carcinogenesis protocol.

In the absence of initiation, ~30% of the transgenic mice developed an average of 1.5 tumors in response to TPA treatment (Fig. 3A and B), primarily well-differentiated SCCs (Fig. 3C). No tumors formed in the wild-type animals. A sample of the transgenic tumors was analyzed for mutations in codon 61 of Ha-ras. As previously observed with the spontaneous K5.RasGRP1-derived tumors (19), no mutations were detected (Fig. 3D), suggesting that RasGRP1 overexpression could be responsible for initiation in the absence of mutations in the ras proto-oncogene.

Expression of the RasGRP1 transgenic protein was readily detected in the K5.RasGRP1-derived tumors originated by either DMBA/TPA or TPA treatment alone (Fig. 4). Endogenous RasGRP1 levels were evaluated using either a polyclonal or a monoclonal anti-RasGRP1 antibody; however, we observed diffuse nuclear staining accompanying cytoplasmic localization in wild-type and transgenic-derived tumor (data not shown), raising concerns about the specificity of the signal detected with those antibodies.

**Effect of TPA in keratinocytes and epidermis from K5.RasGRP1 transgenic mice.** We have previously found that transient overexpression of RasGRP1 in primary keratinocytes in vitro causes Ras activation and that TPA further increases it (13, 14). To test whether keratinocytes derived from K5.RasGRP1 mice were also more sensitive to the TPA-mediated activation of Ras than the wild-type cells, we measured the levels of active, GTP-bound Ras using a pull-down assay in keratinocytes derived from both groups. Ras activation in response to 1 μmol/L of TPA was significantly higher in the transgenic keratinocytes than in the wild-type cells (Fig. 5A). As we previously reported (19), K5.RasGRP1-derived keratinocytes also exhibited elevated constitutive levels of RasGTP compared with that of the wild-type cells, even when cultured under low serum concentrations (Fig. 5A).
Ras is known to signal proliferative responses in the epidermis in vivo (23), and the activation of RasGRP1 in the skin could mediate proliferation. This led us to analyze if overexpression of RasGRP1 in the epidermis could sensitize the skin to the acute hyperplastic effects of TPA. As shown in Fig. 5B, both K5.RasGRP1 and wild-type mice developed comparable epidermal thickening when exposed to a single topical application of TPA for 48 h, indicating that RasGRP1 overexpression did not significantly affect TPA-induced acute hyperplasia.

Discussion

In the classic multistage skin carcinogenesis model, initiated cells carrying activating ras mutations induced by the carcinogen DMBA are clonally expanded to form tumors in response to the phorbol ester TPA. These two stages of initiation and tumor promotion have been extensively studied to identify pathways relevant to tumorigenesis. In the present study, we show that overexpression of the phorbol ester receptor and Ras-guanine nucleotide exchange factor RasGRP1 in basal keratinocytes by a transgenic approach did not affect the response of the epidermis to TPA-induced tumor promotion but resulted in tumors of larger size and which were more invasive than the tumors generated in wild-type skin. These data indicate that the K5.RasGRP1 transgenic mice are more susceptible to tumor progression, a third stage in the multistage skin carcinogenesis model that involves the malignant progression of tumors.

We have previously reported that the K5.RasGRP1 mice are prone to developing spontaneous squamous cell papillomas and carcinomas of the skin (19), which seems to arise from wounding sites, suggesting that elevated expression of RasGRP1—through biochemical Ras activation—confers an initiation status which
makes keratinocytes susceptible to endogenous tumor promoters released during epidermal regeneration. In the DMBA/TPA protocol, initiation is induced by mutations in the ras proto-

oncogene (21). The strong Ras activation signaling conferred by oncogenic Ras is clearly sufficient to cooperate with TPA in tumor formation; in this scenario, one could argue that oncogenetic Ras and biochemical Ras activation by RasGRP1 are functionally redundant pathways; thus, DMBA-induced mutations obscure the participation of RasGRP1 overexpression during tumor promotion.

In papillomas produced by DMBA/TPA treatment, the ras oncogene mutation is heterozygous (21). Tumor conversion to SCCs is usually accompanied by loss of the wild-type ras allele and/or amplification of the mutant allele (24, 25). It has been proposed that nonstimulated wild-type Ras could have a tumor suppressor or anti-transforming role in several biological scenarios (26); taken together, this evidence supports the concept that the dosage of active Ras is critical in malignant progression. However, activation of Ras by mutation, or deletion of the wild-type allele, may not be the only mechanism in place during tumor progression. For example, double transgenic mice for v-Ha-Ras and transforming growth factor-α are predisposed to malignant conversion via activation of wild-type c-Ha-Ras by transforming growth factor-α signals (27). Thus, biochemical activation of wild-type Ras, like the one induced by high levels of growth factors, could also contribute to Ras activation status and malignant progression. Similarly, amplification of upstream Ras activators may also participate in progression; the susceptibility of the K5.RasGRP1 mice to transformation could result from biochemical activation of endogenous wild-type Ras in response to the overexpression of RasGRP1.

The tumorigenic response of the K5.RasGRP1 mice to TPA treatment alone, albeit a weak one, suggests that RasGRP1 overexpression could act as an initiation event that, together with TPA, induces papilloma formation in the absence of Ha-ras mutations in codon 61. Although other mutations in the ras proto-

oncogenes were not evaluated here, it is unlikely that promotion by TPA alone could be responsible for mutations in ras in the K5.RasGRP1 transgenic mice. To our knowledge, only the highly susceptible SENCAR mice have been shown to develop tumors that display Ha-ras mutations in response to TPA treatment alone (28, 29). The more likely scenario under our experimental conditions is that RasGRP1 overexpression acts as an initiation event by activating endogenous Ras. It should be noted that the tumor incidence in the TPA-treated group did not differ significantly from the incidence of spontaneous tumors which we previously observed in this transgenic model (19). Thus, we cannot exclude the possibility that some of the tumors generated in K5.RasGRP1 mice treated with TPA alone were spontaneous tumors. However, whereas wounding seems to be the trigger to spontaneous tumor formation in the K5.RasGRP1 mice, we did not detect any skin injuries that could be associated with tumorigenesis in the TPA-treated transgenic mice.

The susceptibility of the K5.RasGRP1 mice to wounding, as well as to phorbol esters, suggests the possibility that regenerative hyperplasia resulting from epidermal repair or elicited by TPA treatment (30, 31) might further activate the RasGRP1-Ras axis in the keratinocytes and contribute to carcinogenesis. Thus, the fact that we did not find increased sensitivity of the epidermis of K5.RasGRP1 mice to the acute hyperplastic effects of TPA was unexpected. It is possible that a single exposure to TPA does not reflect the response of the K5.RasGRP1 mice to the repetitive, chronic TPA treatments used during skin carcinogenesis. In this regard, a transgenic mouse model for overexpression of the activated v-Ha-ras oncogene (Tg.AC mice), which responds to both wounding and TPA by developing papillomas, was also indistinguishable from the wild-type control in its response to TPA-induced hyperplasia until the 4th week of treatment, during which the Tg.AC animals started to show areas of marked epidermal thickening compared with the wild-type mice (32). These findings could be interpreted as an indication that Ras does not play a significant role in the acute inflammatory effects of a single TPA treatment on skin.

In conclusion, our data show that RasGRP1 plays a role in malignant progression in the two-stage mouse skin carcinogenesis model. The relevance of these findings to human skin carcinogenesis is unknown at this point. It is important to note that whereas Ras is activated in most human SCCs, not all tumors possess Ras oncogenic mutations (33–35); therefore, amplification and/or biochemical activation of Ras must account for the elevated RasGTP levels observed in the tumors with no mutations in ras proto-oncogenes. We speculate that RasGRP1 could represent a link between growth factor signals and Ras activation in human keratinocytes during carcinogenesis.

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