cAMP-dependent Oncogenic Action of Rap1b in the Thyroid Gland*

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cAMP signaling leads to activation and phosphorylation of Rap1b. Using cellular models where cAMP stimulates cell proliferation, we have demonstrated that cAMP-mediated activation, as well as phosphorylation of Rap1b, is critical for cAMP stimulation of DNA synthesis. To determine whether Rap1b stimulates mitogenesis in vivo, we have constructed a transgenic mouse where a constitutively active G12V-Rap1b, flanked by Cre recombinase LoxP sites, is followed by the dominant negative S17N mutant. Employing this novel mouse model, we have switched, in a tissue-specific (thyroid) and temporally controlled manner, the expression of Rap1b from a stimulatory to an inhibitory form. These experiments provide conclusive evidence that Rap1b is oncogenic in the thyroid in ways linked to transduction of the cAMP mitogenic signal.

Rap1 GTPases are members of the Ras superfamily of G-proteins, which regulates cell proliferation and differentiation (1). Rap1 activity can be regulated by agonists that increase the intracellular levels of cAMP (2). cAMP-dependent activation of Rap1 occurs via Epac (exchange protein activated by cAMP) (3, 4), although numerous other stimuli can activate Rap1b via a variety of Rap1-guanine nucleotide exchange factors (GEFs) (5). In addition, cAMP activation of protein kinase A results in phosphorylation of Rap1b (6) and of the tyrosine kinase c-Src, which leads to activation of the Rap-GEF C3G (7). Thus, Rap1 is an important target for cAMP signaling.

cAMP can either stimulate or inhibit cell proliferation, depending on the cell type and context (8, 9), and a striking parallel exists between the action of cAMP and Rap1b on cell proliferation. Whereas Rap1 is inhibitory in model systems where cAMP inhibits cell proliferation (10, 11), we demonstrated that Rap1b stimulates mitogenesis in specific cells where cAMP is a genuine stimulator of cell proliferation (12, 13). Moreover, we found that hormonal or cAMP stimulation of DNA synthesis required both the binding of GTP to Rap1b as well as the phosphorylation of Rap1b by protein kinase A (12).

Prior to these findings, the prevailing view held Rap1b solely as an anti-oncogene (14). This notion originated from the discovery that Rap1 reverses cellular transformation by the oncogene Ras (15) and is supported by findings that Rap1 has an effector-binding domain virtually identical to that of Ras and interacts non-productively with typical Ras targets (14, 16). Hence, we have proposed that Rap action on mitogenesis depends on cell-specific signal transduction programs such that Rap1b, like cAMP, can either stimulate or inhibit cell proliferation, i.e. that Rap can be viewed as a conditional oncogene.

Although some new indirect evidence supports the involvement of Rap1 in tumorigenesis (17–20), no direct evidence for an in vivo oncogenic effect of Rap1b exists. Thus, the merit of the conditional oncogene theory still awaits its demonstration in vivo in a physiologically relevant model system, one that requires cAMP for growth. For this we have generated a mouse model that allows the inducible switch of the expression of a stimulatory (G12V-Rap1b) to an inhibitory (S17N) form of Rap1b, in the prototypical cAMP-responsive system, i.e. the thyroid gland. We report here that thyroid expression of constitutively active (i.e. GTPase-deficient) G12V-Rap1b generates a cAMP-dependent tumorigenic phenotype; this effect is lost upon switching the expression of G12V-Rap1b to a Rap1b dominant negative mutant.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—The DNA construct contains the bovine thyroglobulin promoter (~2 kb) and a floxed G12V-Rap1b (~700 bp) unit followed by the S17N-Rap1b (~650 bp) cassette. Both Rap coding cassettes contain a rabbit b-globin intron and polyadenylation site (1.2 kb). NotI sites were engineered to release this construct free of vector sequences. Transgenic mice were obtained by pronuclear injection of the NotI fragment (~6 kb) in an FVB background (Taconic). Founder lines were analyzed by Southern blots, and littermates were genotyped by PCR (300-bp product), utilizing a sense primer derived from the thyroglobulin promoter (5’-CACATCTGCTCCTGTGCTCG-3’) and an antisense primer derived from the Rap1b sequence (5’-GAGTTTCCGCCACCCAGCTACG-3’). All procedures were performed according to the University of Pittsburgh Institutional Animal Care Committee.

Goitrogen Treatment—Goitrogen treatment consisted of methimazole (0.5g/liter) and sodium percholate (5g/liter) in drinking water ad libitum, changed once weekly.

Recombination—4-OH tamoxifen was suspended in sunflower oil and injected daily on 5 consecutive days (intraperitoneally, 1 mg/200 µl/mouse). Cre-mediated recombination at the DNA level was evaluated by PCR. For the recombinated product (~350 bp) a sense primer derived from the thyroglobulin promoter (5’-ACTGGCCAAGTGGTCTCCTC-3’) and an antisense primer derived from the His6-tagged-S17N cassette (5’-CACGTTGTACGGGAGGATT-3’) were utilized. For the non-recombined product (~530 bp) a primer derived from the rabbit b-globin intron and polyadenylation site from the G12V cassette (5’-GTCCTCTGAGGAACGCGATG-3’) and the same antisense primer described above were utilized. To assess recombination at the protein level, we exploited the fact that only the S17N cassette contains a His6 tag. Thyroid extracts were prepared (pool of three mice/condition) in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet

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¶ The abbreviations used are: GEF, guanine nucleotide exchange factor; BrdUrd, bromodeoxyuridine; GAP, GTPase activating protein; ER, estrogen receptor; HA, hemagglutinin; TSH, thyroid stimulating hormone; Tx, tamoxifen; WT, wild-type; H&E, hematoxylin and eosin; T4, thyroxine; NTA, nitrolotriacetic acid.
stimulation of Cre by tamoxifen. As shown on the upper panels blotting with anti-HA antibodies and normalized with anti-Akt antibodies (Rap1bV12-LoxP-N17/CRE-ER-Tx model *T* asterisk) (Fig. 1B) follicles (Rap1bV12-LoxP-N17 mouse line (*T*)) in which HA-G12V-Rap1b has been flanked by LoxP sites and followed by the His6-HA-S17N-Rap1b to generate the thyroglobulin promoter (*BTG*). In this construct, “floxed” HA-tagged con- the enrichment of HA-tagged G12V-Rap1b either by gene dos- PCR (not shown) and HA-Western blots, thus demonstrating the predicted Mendelian distribution for both males and females, indicating that targeted expression of G12V-Rap1b to the thyroid, a gland critical for normal embryonic development (22), had no deleterious effect on viability. Mice expressing HA-G12V-Rap1b showed no signs of hypo- or hyperthyroidism and exhibited normal secondary sexual characteristics and growth profiles (Fig. 1C, lower panel), indicating normal physiological levels of thyroidal, growth, and gonadal hormones. Analysis of young (<2 months) and old (>1 year) transgenic and wild-type (WT) sibling mice showed no significant differences in thyroid hormone levels (total T4 (*T*4 = 4.33 ± 0.38 μg/dl), thyroid stimulating hormone (TSH = 2.65 ± 0.15 ng/ml), or thyroid histology (Fig. 1C, upper panel). Thus, under basal low thyroidal cAMP signaling, expression of G12V-Rap1b, unlike...
Ras-G12V, does not interfere with differentiation of the gland and does not produce any mitogenic advantage in either heterozygous or homozygous mice. These results are consistent with in vitro findings demonstrating that G12V-Rap1b by itself is not mitogenic but enhances the mitogenic response to cAMP while maintaining differentiation (12).

G12V-Rap1b Enhances cAMP-stimulated G1/S Entry in Vivo—In agreement with the in vitro work, chronic elevation of thyroidal cAMP by increasing circulating levels of TSH produced a switch to mitogenic action in G12V-Rap1b heterozygous mice. This was achieved using a standard goitrogenic protocol (methimazole/sodium perchlorate) (23), which disrupts the negative feedback regulation exerted by thyroid hormone (24). In 14 days, the serum levels of T4 decreased from ~4 to <0.5 μg/dl and those for TSH increased from ~3 to ~100 ng/ml and then remained constant during the goitrogenic regimen with no apparent differences between transgenic and WT mice. After 2 months of this treatment and despite no apparent histological differences (Fig. 2A, top), clusters of cells entering the cell cycle were seen only in the transgenic mice on goitrogen. B, gross anatomy illustrating the advantage imparted by G12V-Rap1b in TSH/cAMP-induced goiter. C–E, H&E stain showing focal nodular lesions in the Rap1bV12-LoxP-N17 thyroids (C) correlating with HA-G12V-Rap1b expression (D), despite similar pituitary hyperplasia induced by the goitrogen protocol (E). F, a time course for up to 8 months of goitrogen action is shown. G, the focal nodular lesions were reversed upon a 2-month goitrogen-free regimen. H, the thyroid enlargement in transgenic mice correlated with an increased BrdUrd (BrdU) labeling index, illustrated here after 4 months on goitrogen.

The hyperplastic foci, consistent with a diagnosis of adenoma. Diffuse follicular cell hyperplasia was also observed elsewhere in the gland, similar to the thyroids of WT mice. As expected, no significant differences in goitrogen-induced thyrotrhopic hyperplasia were observed in their pituitary glands. (Fig. 2F). The enlargement of the G12V-Rap1b thyroids was dependent on the duration of goitrogen treatment (Fig. 2F) as reflected by an approximate 50% increase in thyroid cross-section area and correlated with a nearly 2-fold increase in BrdUrd labeling index (Fig. 2H) but not with a potential suppression of apoptosis induced by G12V-Rap1b (not shown). These results are consistent with in vitro studies showing that the mitogenic action of Rap1b is linked to the ability of Rap to promote S-phase entry (12, 13). Moreover, the increased propensity of transgenic mice thyrocytes to enter S-phase only under persistently elevated thyroidal cAMP represents original evidence in vivo that cAMP must elicit signaling events that endow G12V-Rap1b with mitogenic action.

The hyperplastic and nodular phenotype observed in the G12V-Rap1b mice thyroids after 6 months of sustained elevation of thyroidal cAMP was completely reversed after removal of the goitrogenic stimulus for 2 months (Fig. 2G). Under these conditions, serum TSH returned to normal levels (~3.5 ± 1.1 ng/ml) coupled to an euthyroid state (T4 ~4.9 ± 0.7 μg/dl), and typical follicular structures started to be reestablished, albeit with a colloid-filled lumen and surrounded by a single line of flattened thyrocytes, typical of hypofunction. However, no significant structural or functional differences were observed between the thyroids of G12V-Rap1b and WT mice. Thus, the Rap1b-linked phenotype seen after 6 months of goitrogen treatment does not have the characteristics of an autonomous adenoma; instead, it is entirely dependent on persistently elevated thyroidal cAMP. This reversibility is not unexpected because high TSH levels under goitrogen treatment likely preclude the selection of mutations that lead to a TSH-independent phenotype. Experimentally induced adenomas and follicular carci-
mas were reported to be TSH-dependent upon $^{131}$I-labeled goitrogen treatment (27).

However, upon a long term goitrogenic treatment, i.e. 1 year of sustained elevated thyroidal cAMP, all G12V-Rap1b transgenic but not WT mice developed very large multilobular and hyperemic glands (Fig. 3A). These glands exhibited signs of thyroid follicular cell carcinoma characterized by the presence of invasion of the thyroid capsule, surrounding tissues, and blood vessels. Evidence for the formation of a new capsule around the invasive cells (I). C. H&E stain of a classical example of vascular invasion seen in the Rap1bV12-LoxP-N17 mice. D, that the invading cells are indeed HA-G12V-Rap1b-expressing cells and lodged within a blood vessel is shown by Factor VIII immunohistochemistry to illustrate the endothelial cells lining the vessel. Note that the infiltrated cells shown by H&E scored positive for HA staining. E, extracapsular invasion in one of the Rap1bV12-LoxP-N17 mice at low (inset) and high magnification on H&E sections. Note the highly proliferating index (BrDU) of HA-positive cells (HA) invading the parenchyma (neck muscle).

**Fig. 3.** Progression into follicular carcinoma of the Rap1bV12-LoxP-N17 thyroids upon sustained long term goitrogen treatment. A, Rap1bV12-LoxP-N17 and non-transgenic mice were placed under goitrogen treatment for 12 months, after which their thyroids were dissected, fixed, and processed. Examples of gross anatomy of four giant multilobulated transgenic thyroids are shown. B, capsular invasion of follicular carcinoma cells (C) upon 1 year of goitrogen treatment in Rap1bV12-LoxP-N17 mice. Note the formation of a new capsule (arrow) around the invasive cells (I). C. H&E stain of a classical example of vascular invasion seen in the Rap1bV12-LoxP-N17 mice. D, that the invading cells are indeed HA-G12V-Rap1b-expressing cells and lodged within a blood vessel is shown by Factor VIII immunohistochemistry to illustrate the endothelial cells lining the vessel. Note that the infiltrated cells shown by H&E scored positive for HA staining. E, extracapsular invasion in one of the Rap1bV12-LoxP-N17 mice at low (inset) and high magnification on H&E sections. Note the highly proliferating index (BrDU) of HA-positive cells (HA) invading the parenchyma (neck muscle).

Induction of Cre-dependent Recombination Leads to Excision of the G12V-Rap1b Transcription Unit and Expression of S17N-Rap1b—We investigated whether the enhanced cAMP-mediated tumorigenesis seen in thyroids of the Rap1bV12-LoxP-N17 mice was causally linked to the expression of G12V-Rap1b by using the G-protein activity-state switch mouse model Rap1bV12-LoxP-N17/CRE-ER-Tx (Fig. 1A). This model offers the unique opportunity of allowing cAMP tumorigenesis to proceed while controlling the timing of inactivation of G12V-
Rap1b expression. In these mice, tamoxifen-dependent stimulation of Cre activity leads to the expression of S17N-Rap1b upon the excision of the G12V-Rap1b transcription unit. Cre-dependent recombination was assessed both at the DNA and protein expression level (Fig. 4A). First, PCR was used to assess genomic DNA prepared from mice treated for 5 consecutive days with tamoxifen (1 mg/0.2 ml/mouse/day) or vehicle control (oil). The expected recombination product (0.35 kb) was observed only in tamoxifen-treated mice as early as 1 day after injection, increasing linearly up to day 3, and reaching a plateau by days 4–5 (Fig. 4A, top). The appearance of the recombined fragment was proportional and concomitant with a decrease of the non-recombined product (0.5 kb). In contrast, no recombination occurred in tamoxifen-treated Rap1bV12-LoxP-N17 mice (not shown), confirming Cre-mediated recombination at the DNA level. Cre-mediated expression of the expected protein (His-HA-N17-Rap1b) was verified after treatment of mice with tamoxifen for 5 days. Thyroid extracts were prepared, and His-HA-N17-Rap1b was affinity-purified on nitrilotriacetic acid-agarose and analyzed by Western blot with anti-HA antibodies. Only the Rap1bV12-LoxP-N17/CRE-ER-Tx mice expressed the expected tamoxifen-dependent HA-Rap1b immunoreactivity (Fig. 4A, bottom). These results demonstrate the success of the strategy used to generate the G-protein activity-state switch mouse model.

Rap1-GTP Is Required to Maintain TSH- and cAMP-mediated Hyperplasia—Finally, we tested whether the enhanced cAMP-mediated tumorigenesis seen in the thyroids of Rap1bV12-LoxP-N17 mice was causally linked to G12V-Rap1b expression. Two-month-old Rap1bV12-LoxP-N17/CRE-ER-Tx mice were treated with goitrogen and a 5-day tamoxifen/oil regimen and then tested either at the beginning (establishment) or during (maintenance) the goitrogen protocol. After 4 and 6 months, animals were sacrificed, and their thyroids were analyzed. The thyroids of tamoxifen-treated mice were significantly smaller than those of controls (Fig. 4B). Thyroid cross-section areas were ~50% decreased in mice subjected to the tamoxifen regimen both at establishment and during maintenance of the hyperplastic state (Fig. 4B), indicating that Rap1-GTP is required to maintain this state. Moreover, the Ki67 (Fig. 4C) and BrdUrd (not shown) labeling indices as well as the total fraction of cycling thyrocytes measured by proliferating cell nuclear antigen staining (not shown) were

![Diagram A](image1.png)

**Fig. 4.** cAMP-dependent hyperplasia in vivo requires Rap1b-GTP. A, Rap1bV12-LoxP-N17/CRE-ER-Tx mice on goitrogen (G) were injected with oil or tamoxifen for 5 consecutive days (†). At the indicated times (†), thyroids were extracted and recombination assessed at the DNA level by PCR (top) or at the protein level by Western blot using total lysates (bottom) or upon affinity chromatography on a NTA column (Ni column). The recombined product (His-HA-N17-Rap1b, see Fig. 1) occurred only upon tamoxifen treatment. B, Rap1bV12-LoxP-N17/CRE-ER-Tx mice were subjected to a goitrogen treatment for 4 (upper panels) or 6 months (lower panels). A consecutive 5-day regimen of oil or tamoxifen injection was performed at the beginning (upper panels, Wk#1) or at 3 months after goitrogen stimulation (lower panels, Wk#13). Thyroids were analyzed by gross anatomy (upper right), by H&E stains (lower right), and by cross-section measurement areas (left). C, thyroid proliferative index as assessed by Ki67 staining. Shown is the analysis of mice thyroids under 6 months of goitrogen treatment in which Cre-dependent recombination was induced at week 13. * Insets show intestine samples from the same animals as controls.
markedly reduced upon induction of the tamoxifen switch. The partial tamoxifen-dependent inhibition correlated well with the partial penetrance of the recombination event. None of these changes were seen in Rap1bV12-LoxP-N17 mice (not shown), indicating that the effects of tamoxifen were all Cre-dependent. In addition, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling and DNA ladder analysis failed to reveal any differences in the apoptotic indices of tamoxifen-treated animals whether these were the Rap1bV12-LoxP-N17 (tamoxifen-insensitive) or the Rap1bV12-LoxP-N17/CRE-ER-Tx mice, which are sensitive to tamoxifen. In fact the number of apoptotic cells was remarkably low under goitrogen, varying from 14 to 19 per cross-section slide (not shown). Also careful examination of the H&E stains showed no evidence for significant apoptosis, such as extra- and intracellular apoptotic bodies (Fig. 4B). These findings suggest that the growth-inhibitory effect caused by the G12V to S17N expression switch does not appear to be related to induction of apoptosis by tamoxifen but to suppression of the propensity of thyrocytes to enter G1/S. Thus, TSH activation of Rap1b is a critical component in the transduction of cAMP-mediated cell cycle progression in vivo. The effect of tamoxifen induction on the long term goitrogen-dependent malignant phenotype is currently under investigation.

Transduction of the cAMP Mitogenic Signal by Rap1b—We have put forward the notion that Rap1 transduces the cAMP signal based largely on results from in vitro studies (2, 6) and, in the specific case of cAMP-mediated mitogenesis, that Rap1 collaborates with Ras signals to elicit a full mitogenic response (12, 13). Since that time, it has become evident that Rap1b can transduce cAMP signaling in vivo as targeted expression of a dominant negative form of Rap1b to the mouse forebrain impaired cAMP-dependent synaptic plasticity (34). Moreover, that the actions of Rap may not be restricted to Ras antagonism (13, 35) has recently been recapitulated in vivo because expression of G12V-Rap1 in mouse T lymphocytes enhanced T cell receptor-mediated responses (36) without antagonizing activation of the T cell receptor and Ras, consistent with the notion that activation of Rap1 by several extracellular signals does not interfere with Ras signaling (4). However, an intriguing aspect of the mitogenic action of Rap1b recapitulated in vivo by the present studies is the observation that the activating G12V mutation is insufficient to promote G0/S entry in vitro (12); G12V-Rap1b imparted a growth advantage only upon a sustained increase in thyroidal cAMP. The goitrogen-dependent phenotype observed in the heterozygous mice is not simply because of Rap1b gene dosage effects. Similar expression levels of HA-G12V-Rap1b were observed in homozygous mice (Fig. 1B, upper right) in the absence of goitrogen without any mitogenic manifestation. This suggests that cAMP elicits additional signaling events that act on or in cooperation with G12V-Rap1b. One signal might be the protein kinase A-dependent phosphorylation of G12V-Rap1b S179 without which cAMP-Rap1b. One signal might be the protein kinase A-dependent phosphorylation of G12V-Rap1b S179 without which cAMP- and Rap-dependent stimulation of DNA synthesis in vitro does not occur (12). Preliminary evidence suggests that Rap1 phosphorylation occurs after goitrogen treatment of transgenic mice (not shown). However, whether Rap1 phosphorylation is required for its mitogenic and oncogenic properties in vivo and whether phosphorylated Rap-GTP is sufficient to mediate cAMP action remain to be demonstrated in an appropriate mouse model.

The involvement of the cAMP pathway in thyroid oncogenesis has been demonstrated in several mouse models. Expression of the A2 adenosine receptor, which acts via Gs/adenyl cyclase, results in elevated levels of thyroidal cAMP leading to diffuse thyroid hyperplasia (37); a similar phenotype was observed with expression of an activated form of Goalpha (R201H) (38). Moreover, the onset of diffuse hyperplasia observed in mice thyroids expressing the cholera toxin A1 subunit, which activates endogenous Goalpha, correlated rather well with increases in the basal levels of cAMP (39). In all of these models, evidence for malignancy was only observed in older mice, either directly by observation of invasion or indirectly via the identification of DNA aneuploidy and a large number of mitotic figures (40). As expected for an effector of the cAMP mitogenic signal, thyroid expression of G12V-Rap1b recapitulates some of these findings; however, whereas all these models carry a built-in sustained cAMP signal, the model described here depends on goitrogen action to fulfill the same goal. The results obtained from these earlier models demonstrate that persistent activation of the cAMP cascade carries a mitogenic advantage. The Rap1b activity-state switch mouse model described here affords the conclusion that the activation of Rap1b is a component of the transduction of the cAMP mitogenic signal.

The long latency and focal nature of the lesions seen in models expressing elements of the cAMP pathway suggest that persistent elevation of thyroidal cAMP alone is not sufficient to trigger a malignant phenotype. Presumably, sustained activation of the cAMP pathway causes thyrocyte proliferation, thus increasing the likelihood of other genetic or epigenetic events that are required for full progression into a malignant condition. For example, the progression to malignancy was accelerated when a mutant alphag adrenergic receptor (Lys-288, His-290, Leu-293), which constitutively activates both the adenyl cyclase and the phospholipase C pathways, was expressed in mouse thyroid (41); increased numbers of malignant lesions as well as shorter onset times were observed when compared with the A2 adenosine receptor (transgenic line), which only activates the cAMP pathway. The identification of mutations in the TSH receptor and Goalpha in human samples of hyperfunctioning adenomas as well as differentiated follicular carcinomas (42) suggests that mutations in elements of the cAMP pathway carry an oncogenic potential. The relevance of the Rap1 mouse model described here for human cancer awaits the identification of amplification and/or mutations in either Rap1, its regulators (GEFs, Rap-GAPs), or Rap1b effectors in human thyroid tumor samples. Nonetheless, the Rap1b activity-state transgenic line will clearly provide a valuable tool to determine the Rap1 mechanism of action in a physiological in vivo context.

Rap1b and Oncogenesis—Indirect evidence that Rap1 may be endowed with mitogenic and oncogenic potential in vivo has recently become available by studies utilizing activators and negative modulators of Rap activity (43). A Rap1-specific exchange factor (CAL-DAG-GEF1) has been implicated in the development of myeloid leukemias (17), and the degradation of the Rap deactivator Rap-GAP E6TP1 has been associated with the development of cancers linked with human papillomavirus infection (18). Likewise, targeted disruption of the SPA-1 gene, which encodes a Rap-GAP, results in the development of a myeloid proliferative phenotype (20). These results are consistent with an earlier finding that somatic inactivation of the tuberin gene, which also encodes a Rap1-GAP, is associated with the development of tumors (19). However, these earlier studies did not directly implicate Rap1 in tumorogenesis; Rap-GEFs might have novel functions independent of Rap activation (44), and the lack of GAP activity in tuberous sclerosis and SPA-1+/- mice may not be restricted only to Rap (45). Targeted expression of G12V-Rap1b to the thyroid promoted the development of cAMP-dependent malignant signs, thus providing a direct demonstration that Rap1b is endowed with oncogenic action.

Although, collectively, these studies support the original no-
tion that Rap1 is endowed with oncogenic potential (12, 13), the predominant view that Rap1 is mainly an anti-oncogenic protein still remains. Consistent with this view, targeted expression of G12V-Rap1 to mouse astrocytes produced no growth advantage and decreased astrocyte proliferation in mice expressing G12V-Ras (46). In addition, inactivation in mouse osteosarcoma cells of a Rap1 activator, DOCK4, leads to loss of contact inhibition, growth in soft agar, and invasive tumors in nude mice (47). This contrasts with findings that activation of a Rap1-GEF enhances growth and causes morphological transformation in Rat2 and Swiss 3T3 fibroblasts as well as in myeloid cells (17) and that overexpression of Rap1b in Swiss 3T3 fibroblasts leads to an increased susceptibility to enter S-phase and to formation of tumors in nude mice (13). Although seemingly contradictory, these data point to an intriguing regulatory duality in the action of Rap1 on mitogenesis and oncogenesis which might depend on cell-specific signal transduction programs upon which Rap can either stimulate or inhibit cell proliferation. It will be of great importance to determine the molecular basis that conditions Rap to have either positive or negative actions on mitogenesis.

Rap1 regulates a variety of cellular processes that might be relevant to its action on mitogenesis, such as G1/S-phase entry (13, 48), secretion (49, 50), integrin (29, 30) and E-cadherin (51) activation, and the strength of adherens junctions (47, 52). Which of these functions is directly involved in Rap-mediated tumorigenesis is for the moment unknown. Our results using both cellular (12, 13) and mouse models (Figs. 2 and 4) have implicated G1/S entry as a key process for the mitogenic and oncogenic action of Rap1b. In vitro, cAMP/Rap1b-mediated G1/S appears to be unrelated to the secretion of soluble auto- crine factors (12, 13). To what extent the effects of Rap1b on integrin-mediated cell-extracellular matrix adhesion or E-cadherin- and/or adherens junction-mediated cell-cell communication contribute to cAMP-dependent tumorigenesis remains to be determined. Our studies raise the interesting question of how Rap1b-mediated augmentation of cAMP-stimulated DNA synthesis relates to tumorigenesis.

In conclusion, we have developed a mouse model that allowed us to directly test the hypothesis that Rap1 is endowed with oncogenic action. These tests relied on our ability to control the expression of activity-state forms of Rap1b in a physiologically relevant organism, one that exhibits a strict dependence on cAMP for growth (8). Although a limited number of reports have shown that TSH may also signal through other G-proteins to activate other signaling cascades, e.g. phospholipase C (53), to date none of the biological effects of TSH has been causally linked to activation of phospholipase C. Ever since Sutherland and co-workers (54) discovered that TSH stimulated thyroid hormone synthesis of cAMP and the original finding (55), that cAMP stimulated mitogenesis, it became evident that TSH-mediated nodular hyperplasia in mice and goiters in humans were mediated by cAMP as a result of increased DNA synthesis by the thyrocytes (8). Mediators of this response were elusive for half a century. The G-protein activity-state mouse model described here allowed the switch of expression from an active to a dominant negative form of Rap1b with a concomitant reduction of cAMP-stimulated DNA synthesis, demonstrating for the first time that Rap1b is a critical component in the transduction of the cAMP mitogenic signal in vivo.

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REFERENCES

1. Takai, Y., Sasaki, T., and Matozaki, T. (2001) Physiol. Rev. 81, 153–208
2. Altschuler, D. L., Peterson, S. N., Ostrowski, M. C., and Lapetina, E. G. (1995) J. Biol. Chem. 270, 10373–10376
3. Kawasaki, H., Springer, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) Science 282, 2275–2279
4. de Ruysser, J., Zwaal, R. F., and Kieffer, L. J. (1997) Blood 89, 2554–2563
5. Quinlan, A. J., Kühn, M., Gusev, Y., Westra, W. H., Takiyama, Y., Dooley, W. C., Kohn, L. D., and Levine, M. A. (1997) J. Biol. Chem. 272, 278, 33738–33746
6. Xiao, G. H., Shorainicag, F., Jin, F., Golemis, E. A., and Yeung, R. S. (1997) J. Biol. Chem. 272, 6097–6100
7. Apicelli, A. J., Chinnan, E. J., Baldwin, R. L., Ding, H., Nagy, A., Guth, K., and Gutmann, D. H. (2003) Glia 42, 225–234
8. Yajnik, V., Paulding, C., Sordella, R., McClatchey, A. I., Saito, M., Wahrer, D. C., Reynolds, P., Bell, D. W., Lake, R., van den Heuvel, S., Settleman, J., and Haber, D. A. (2003) Cell 112, 673–684
48. Yoshida, Y., Kawata, M., Miura, Y., Musha, T., Sasaki, T., Kikuchi, A., and Takai, Y. (1992) Mol. Cell. Biol. 12, 3407–3414
49. Maillet, M., Robert, S. J., Casquevel, M., Gastineau, M., Vivien, D., Bertoglio, J., Zugaza, J. L., Fischmeister, R., and Lezoualch, F. (2003) Nat. Cell Biol. 5, 633–639
50. Kang, G., Joseph, J. W., Chepurny, O. G., Monaco, M., Wheeler, M. B., Bos, J. L., Schwede, F., Genieser, H. G., and Holz, G. G. (2003) J. Biol. Chem. 278, 8279–8285
51. Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G., and Bos, J. L. (2004) J. Biol. Chem.
52. Knox, A. L., and Brown, N. H. (2002) Science 295, 1285–1288
53. Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J. E., and Roger, P. P. (2001) Endocr. Rev. 22, 631–656
54. Klainer, L. M., Chi, Y. M., Freidberg, S. L., Rall, T. W., and Sutherland, E. W. (1962) J. Biol. Chem. 237, 1239–1243
55. Selye, H., Veilleux, R., and Cantin, M. (1961) Science 133, 44–45

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