Beyond regulating Rap activity, little is known regarding the regulation and function of the Rap GTPase-activating protein Rap1GAP. Tuberin and E6TP1 protein levels are tightly regulated through ubiquitin-mediated proteolysis. A role for these RapGAPs, along with SPA-1, as tumor suppressors has been demonstrated. Whether Rap1GAP performs a similar role was investigated. We now report that Rap1GAP protein levels are dynamically regulated in thyroid-stimulating hormone (TSH)-dependent thyroid cells. Upon TSH withdrawal, Rap1GAP undergoes a net increase in phosphorylation followed by proteasome-mediated degradation. Sequence analysis identified two putative destruction boxes in the Rap1GAP C-terminal domain. Glycogen synthase kinase 3β (GSK3β) phosphorylated Rap1GAP immunoprecipitated from thyroid cells, and GSK3β inhibitors prevented phosphorylation and degradation of endogenous Rap1GAP. Co-expression of GSK3β and Rap1GAP in human embryonic kidney 293 cells stimulated proteasome-dependent Rap1GAP turnover. Mutational analysis established a role for serine 525 in the regulation of Rap1GAP stability. Overexpression of Rap1GAP in thyroid cells impaired TSH/cAMP-stimulated p70S6 kinase activity and cell proliferation. These data are the first to show that Rap1GAP protein levels are tightly regulated and are the first to support a role for Rap1GAP as a tumor suppressor.

Rap1 belongs to the Ras superfamily of small G-proteins (1, 2). Unlike Ras where there is abundant evidence supporting its role in tumorigenesis, far less is known regarding the role of Rap1 in neoplastic transformation. Early studies by Altschuler and co-workers (3, 4) demonstrated that overexpression of Rap1 stimulated morphological transformation and altered growth properties, albeit in a cell type-specific fashion. Mutations in RapGEFs (5) have been identified in tumors and cancer cell lines. A subset of myeloid leukemias in BXX-2 mice contain proviral insertions in the CalDAG-GEFI gene, resulting in its activation (5). Several human cancer cell lines exhibit mutations in DOCK4, a Rap-activating protein (6). Alterations in RapGAPs have also been associated with aberrant cellular proliferation. Tuberin (7) is inactivated in the tumor predisposition syndrome, tuberous sclerosis type 2 (TSC-2). E6TP1 is targeted for ubiquitin-mediated protein turnover by the human papilloma virus, an event that appears to be required for E6-mediated transformation (8). Finally, mice lacking SPA-1 develop myelodysplastic disorders (9). Far less is known regarding the role of Rap1GAP, the first Rap1GAP to be identified with 18 U.S.C. Section 1734 solely to indicate this fact.

We report that Rap1GAP is highly expressed in TSH-dependent thyroid cells. This is striking in that thyroid cells are one of very few cellular models where cAMP stimulates cell proliferation and differentiation (reviewed in Ref. 14), effects that are mediated at least partly through Rap1 (4, 15). Moreover, Rap1GAP exhibits a number of unique features including phosphorylation by protein kinase A (16) and interaction with Ga subunits (17–19) that could be particularly pertinent to TSH signaling. These findings prompted us to examine whether TSH regulates Rap1GAP. Our results demonstrate that endogenous Rap1GAP protein levels are dynamically regulated and that TSH and GSK3β elicit opposing effects on Rap1GAP protein stability. In addition, our studies revealed striking similarities in the regulation and biological effects of Rap1GAP with those of tuberin, E6TP1, and SPA-1.

MATERIALS AND METHODS

Reagents—pCMV2-FLAG-Rap1GAP and pMT2-HA-Rap1GAP expression vectors were kind gifts from Drs. Lawrence Quilliam (Indiana University) and Johannes L. Bos (Utrecht University), respectively. The GSK3β serine 9 to alanine mutation was generated in human Myc-tagged GSK3β in pCS2 vector (20) by site-directed mutagenesis using QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Anti-Rap1GAP serum was generously provided by Dr. Michiyuki Matsuda (Osaka University). FLAG and GSK3β (raised to rabbit GSK3β) antibodies were purchased from Sigma and Transduction Laboratories (Lexington, KY), respectively. HA and phospho-S6 antibodies were generous gifts from Drs. Jeffrey Field and Morris Birnbaum (University of Pennsylvania), respectively. Rap1, actin, and p70S6K antibodies were purchased from Sigma and Transduction Laboratories (Lexington, KY). Thyroglobulin antibody was purchased from Dako Corporation (Carpinteria, CA). Alkaline phosphatase and LAR tyrosine phosphatase were from Promega (Madison, WI). Kenpaullone and MG132 were purchased from Calbiochem.

Cell Culture—WRT cells were propagated at 37 °C in 5% CO2 in Coon’s modified Ham’s F-12 medium containing 3H (TSH, insulin, transferrin, and 5% calf serum) as described previously (15). Cells were starved in basal medium (growth factor-free Coon’s modified Ham’s F-12 medium) as described previously (15). Stable cell lines expressing FLAG-Rap1GAP were generated by co-transfection with an expression

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vector encoding G418 resistance followed by selection in 300 μg/ml and maintenance in 150 μg/ml Geneticin (Invitrogen).

**Transient Transfection**—Human embryonic kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection was carried out using LipofectAMINE 2000 (Invitrogen) according to manufacturer’s recommendations. Cells were plated in 12-well plates 18 h prior to transfection. Just prior to transfection, cells were transferred into Opti-MEM I (Invitrogen) and exposed to 100 ng of FLAG-Rap1GAP DNA, 50 ng of GSK3β99A DNA (or empty vector), and 4 μl of LipofectAMINE 2000 in 100 μl of Opti-MEM I. Transfection was for 5 h, and the cells were harvested and analyzed 24 h later.

**BrdUrd Labeling**—WRT cells plated on glass coverslips in 35-mm dishes were transfected with 2 μg of HA-Rap1GAP DNA and 3 μl of FuGENE 6 (Roche Applied Science) in 100 μl of cell extract were transferred to 3H medium overnight. Cells were then starved in basal medium for 24 h and stimulated with 1 milliunit/ml TSH, 5% bovine calf serum for 24 h. BrdUrd was added for the last 4 h. Cells were fixed and stained for incorporated BrdUrd as described previously (21).

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed according to Wang and Wilkinson (22). FLAG-Rap1GAP525 was constructed using a sense primer, 5'-GACGTCGCGGATATCTGGG-GGTCTTCTG-3', and an antisense primer, 5’-TCAACAGGACCACGAG-TCCGGAG-3’. FLAG-Rap1GAP560 was constructed using a sense primer, 5'-TTAGATGAGAATCCCTGCAGTTGGGG-3’, and an antisense primer, 5’-GACGTCGCGGATATCTGGGAGACAG-3’. All of the primers (Biosynthesis, Inc., Lewisville, TX) were 5’-phosphorylated and purified by PAGE. PCR reactions contained 150 ng of primer, 50 ng of template plasmid, 200 μM dNTPs, and 2.5 units of Pfu Turbo DNA polymerase (Stratagene). PCR products were incubated with DpnI to digest the template purified from agarose, ligated, and transformed into E. coli JM109. DNA from candidate clones was sequenced by the Nucleic Acid and Protein Resource Core Facility at Children’s Hospital of Philadelphia (Philadelphia, PA) using the primers 5’-GAATATCAGTGATTGTC-3’ and 5’-TGTGCTCTAGATGCTTC-3’.

**Pulse-Chase Analysis**—Pulse-chase was performed essentially as described previously (23). WRT cells were incubated in cysteine- and methionine-free RPMI 1640 medium supplemented with TSH (1 milliunit/ml) for 30 min and then labeled with [35S]methionine/cysteine (160 μCi/ml) for 30 min. Following treatment, cell lysates were analyzed by SDS-PAGE. Gels were fixed, soaked in Amplify solution (Amersham) for 5 min, and then soaked in Amplify solution for 5 min. To verify the activity of LAR tyrosine phosphorylation was abolished.2 To verify the activity of LAR tyrosine phosphorylation was abolished.2 To verify the activity of LAR tyrosine phosphorylation was abolished. WRT cells were lysed in 50 mM Tris, pH 8.0, 0.1% Triton X-100, and 2 mM MgCl2, 60 μg of cell extract were incubated with 30 units of alkaline or tyrosine phosphatase for 1 h at 4 °C followed by protein A-agarose for 1 h at 4 °C. Immune complexes were washed with RIP buffer, heated to 95 °C in Laemmli sample buffer, and resolved by SDS-PAGE. Gels were fixed, soaked in Amplify solution (Amersham Biosciences), dried, and subjected to autoradiography at −80 °C. All of the data were analyzed on a PhosphorImager Storm 840 (Molecular Dynamics, Amersham Biosciences) using ImageQuant software.

**Phosphatase Treatment**—For alkaline phosphatase experiments, conditions were derived where TSH-induced serine/threonine phosphorylation was abolished.2 To verify the activity of LAR tyrosine phosphatase, conditions were derived where insulin-induced tyrosine phosphorylation was abolished. WRT cells were lysed in 50 mM Tris, pH 8.0, 0.1% Triton X-100, and 2 mM MgCl2, 60 μg of cell extract were incubated with 30 units of alkaline or tyrosine phosphatase for 1 h according to the manufacturer’s recommendations. The reaction was terminated by the addition of 6× Laemmli sample buffer, and samples were heated at 95 °C for 5 min and resolved on 8% SDS-PAGE.

**GSK3β Assays**—GSK3β assays were performed as described previously (24, 25). Rap1GAP was immunoprecipitated from WRT cells, and the immune complexes were washed with RIPA and then kinase (20 mM Tris, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol) buffers. Kinase reactions were carried out in 30 μl of kinase buffer containing 0.2 μl of rabbit GSK3β (500,000 units/ml, New England Biolabs, Inc., Beverly, MA) and 5 μCi of [γ-32P]ATP for 25 min at 30 °C and stopped by the addition of Laemmli sample buffer and heating for 5 min at 95 °C. Proteins were separated on 8% PAGE, transferred to nitrocellulose membrane, exposed to film, and blotted with anti-Rap1GAP serum.

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*O. M. Tygankova and J. L. Meinkoth, unpublished observations.*

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**RESULTS**

**TSH Regulates Rap1GAP Protein Stability**—Immunoblotting using a peptide-directed Rap1GAP serum demonstrated a prominent 95-kDa band and a spectrum of faster migrating protein species in lysates prepared from growing WRT cells (Fig. 1A, 3H). A similar pattern has been reported previously in other cells (10, 11, 26, 27). When lysates prepared from cells transferred to growth factor-free basal medium were analyzed, the Rap1GAP serum detected reduced levels of a discrete upshifted protein species (Fig. 1A, basal). Remarkably, the inclusion of either TSH or forskolin in basal medium prevented the upshift and decrease in Rap1GAP expression.

A time course experiment was performed to examine the kinetics over which these changes took place. Cells growing in 3H were transferred to basal medium for times ranging from 15 min to 24 h, and Rap1GAP expression was assessed by Western blotting. One of five experiments that yielded similar results is shown. B, Rap1GAP expression was examined in lysates prepared from cells growing in 3H and in lysates from cells transferred to basal medium for the indicated times (in hours). Western blotting with an actin antibody was used to document protein loading. Three time course experiments were performed with the same results. C, lysates prepared from cells starved in basal medium (B) or growing in 3H medium (3H) were subjected to alkaline and in vitro kinase (20 mM Tris, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol) buffer. Kinase reactions were carried out in 30 μl of kinase buffer containing 0.2 μl of rabbit GSK3β (500,000 units/ml, New England Biolabs, Inc., Beverly, MA) and 5 μCi of [γ-32P]ATP for 25 min at 30 °C and stopped by the addition of Laemmli sample buffer and heating for 5 min at 95 °C. Proteins were separated on 8% PAGE, transferred to nitrocellulose membrane, exposed to film, and blotted with anti-Rap1GAP serum.

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**FIG. 1.** Rap1GAP is differentially regulated in the absence and presence of TSH. A, cells growing in 3H were transferred to basal medium in the absence (−) or presence of TSH (T) or forskolin (F) for 4 h, and Rap1GAP expression was assessed by Western blotting. One of five experiments that yielded similar results is shown. B, Rap1GAP expression was examined in lysates prepared from cells growing in 3H and in lysates from cells transferred to basal medium for the indicated times (in hours). Western blotting with an actin antibody was used to document protein loading. Three time course experiments were performed with the same results. C, lysates prepared from cells starved in basal medium (B) or growing in 3H medium (3H) were subjected to alkaline and in vitro kinase (20 mM Tris, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol) buffer. Kinase reactions were carried out in 30 μl of kinase buffer containing 0.2 μl of rabbit GSK3β (500,000 units/ml, New England Biolabs, Inc., Beverly, MA) and 5 μCi of [γ-32P]ATP for 25 min at 30 °C and stopped by the addition of Laemmli sample buffer and heating for 5 min at 95 °C. Proteins were separated on 8% PAGE, transferred to nitrocellulose membrane, exposed to film, and blotted with anti-Rap1GAP serum.
tase treatment. Phosphatase treatment (Fig. 1C, lane 5) converted the upshifted Rap1GAP species observed in starved cells (lane 2) to the diffuse species typical of growing cells (lane 1). In contrast, exposure to a protein tyrosine phosphatase had no effect (compare lanes 3 and 6). These results suggest that TSH withdrawal results in a net increase in serine/threonine phosphorylation of Rap1GAP followed by a decline in Rap1GAP expression. Similar results were obtained in two other rat thyroid cell lines, PC-Cl3 and FRTL-5, indicating that regulation of Rap1GAP is a conserved feature in thyroid cells.

To explore the mechanism through which TSH regulates Rap1GAP expression, pulse-chase experiments were performed. Cells were labeled with [35S]methionine/cysteine in TSH-supplemented medium for 2 h and then chased in the presence or absence of TSH for various times. Rap1GAP was immunoprecipitated, resolved by SDS-PAGE, and subjected to autoradiography. Rap1GAP levels decreased by 50% within 5 h of TSH withdrawal (Fig. 2, open circles). In the presence of TSH, Rap1GAP levels decreased by only 10% over the same time period (closed squares). Not only did TSH stabilize Rap1GAP, it also prevented the upshift in the migration of Rap1GAP (Fig. 2). Together, these results suggest that TSH regulates the phosphorylation status of Rap1GAP, resulting in the stabilization of Rap1GAP protein levels. As phosphorylation triggers the proteasomal degradation of many proteins (reviewed in Refs. 28 and 29), experiments to determine whether Rap1GAP is a target for proteasome-mediated turn-over were performed.

Rap1GAP Is a Target for Proteasomal Degradation—Proteasome inhibitors were used in pulse-chase experiments to assess whether inhibition of the proteasome stabilized Rap1GAP. Cells were pulse-labeled for 2 h in TSH-supplemented basal medium in the absence or presence of MG132 (30, 31) and then chased in basal medium without TSH in the presence or absence of proteasome inhibitor. As seen before (Fig. 1B), Rap1GAP was upshifted within 2 h following TSH withdrawal and its abundance declined over the next 4 h (Fig. 3A). Although inclusion of MG132 did not prevent the Rap1GAP upshift, it markedly delayed the decline in Rap1GAP (Fig. 3B, open circles). Two additional proteasome inhibitors, MG115 and lactacystin, also increased Rap1GAP protein levels. These results support the idea that Rap1GAP is a substrate for proteasome-mediated degradation.

An analysis of the primary structure of Rap1GAP revealed 56 potential serine/threonine phosphorylation sites. Within these sites, there were two DSGXXS destruction box motifs analogous to one found in β-catenin (Fig. 1A) (32). Phosphorylation of serine residues within this motif by GSK3β triggers the recognition of β-catenin by the F-box protein βTrCP, leading to its ubiquitination and degradation (reviewed in Ref. 33). To determine whether Rap1GAP was a GSK3β substrate, Rap1GAP was immunoprecipitated from growing WRT cells and used as a substrate for recombinant GSK3β in an immune complex kinase assay. The data shown in Fig. 4B demonstrate that GSK3β phosphorylates Rap1GAP in vitro.

To determine whether GSK3β phosphorylates endogenous Rap1GAP, WRT cells were transferred to basal medium in the presence or absence of LiCl, a well characterized inhibitor of GSK3β activity (24). Inclusion of 5–40 mM LiCl prevented the upshift and attenuated the loss of faster migrating Rap1GAP species (Fig. 4C). This was not due to changes in osmolarity, because the transfer to NaCl-supplemented basal medium did not alter Rap1GAP mobility or expression. Furthermore, the effects of LiCl on Rap1GAP were reproduced by kenpaullone, an alternative GSK3β inhibitor (Fig. 4D) (34). When used independently, these inhibitors are highly specific for GSK3β (35). Therefore, these data provide strong support for the role of GSK3β in targeting Rap1GAP for proteasome-mediated degradation.

GSK3β Regulates Rap1GAP Protein Levels—To prove that GSK3β can regulate Rap1GAP stability, transient overexpression studies were performed in human embryonic kidney 293T cells, which exhibit significantly higher transfection efficiencies than thyroid cells. FLAG-tagged Rap1GAP was co-expressed with GSK3β and Rap1GAP protein levels assessed by Western blotting 24 h post-transfection. We used human GSK3βS9A, which contains a serine to alanine substitution at position 9 to prevent the inactivating phosphorylation of GSK3β at this site (36, 37) that occurs constitutively in 293T cells. Rap1GAP levels were decreased by co-expression of

3 P. S. Klein, unpublished observations.
GSK3β/S9A but not by empty vector (Fig. 5A). Mutation of a serine residue within the destruction box (S33Y) stabilizes β-catenin protein by preventing GSK3β-mediated phosphorylation (38–41). Therefore, Rap1GAP mutants were constructed in which the first serine in each destruction box motif was changed to isoleucine, N-terminal Rap1GAPS525I (N-Rap1GAP) and C-terminal Rap1GAPS606I (C-Rap1GAP). A double mutant, N- and C-terminal Rap1GAPS525/606I (NC-Rap1GAP), was also constructed. Unlike wild-type Rap1GAP, N-Rap1GAP was stable in the presence of GSK3β/S9A. Additionally, N-Rap1GAP was consistently expressed at higher levels than C-Rap1GAP, whose expression was decreased by GSK3β/S9A. As expected, the mutation of both serine residues abolished down-regulation of Rap1GAP by GSK3β/S9A. The ability of GSK3β/S9A to decrease Rap1GAP expression was impaired by the proteasome inhibitor, MG132 (Fig. 5B). Collectively, these findings demonstrate that Rap1GAP can be targeted for proteasomal degradation by phosphorylation of serine 525 by GSK3β.

**Rap1GAP Impairs Cell Proliferation and p70S6 Kinase**—The ability of TSH to stabilize Rap1GAP implied an important role for Rap1GAP in mediating TSH effects in thyroid cells. To explore this possibility, WRT cells stably overexpressing Rap1GAP were isolated. Of 12 cell lines screened, only two overexpressed Rap1GAP compared with parental cells and the level of overexpression was modest (Fig. 6A). Nonetheless, modest overexpression of Rap1GAP was sufficient to impair Rap1 activation by TSH (Fig. 6B) and forskolin. Rap1GAP expression was regulated similarly in the overexpressing cells as in parental cells. Upon removal of TSH, Rap1GAP was upshifted and its expression decreased (Fig. 6A, basal or B). Intriguingly, Rap1GAP-overexpressing cells grew more slowly than parental cells (Fig. 6C). To ensure that this was not attributed to secondary changes associated with the isolation of stable cell lines, the effects of transient overexpression of Rap1GAP on DNA synthesis were investigated. Both basal and TSH/serum-stimulated DNA synthesis were impaired in Rap1GAP-transfected cells (Fig. 6D). These results are strikingly similar to those reported for tuberin where overexpression slowed cell proliferation (42, 43). Indeed, as for tuberin (44, 45), overexpression of Rap1GAP impaired activation of p70S6K kinase in WRT cells. However, in this case, inhibition was specific for cAMP-elevating agents. Fig. 7 demonstrates that...
the ability of TSH and forskolin to stimulate the phosphorylation of ribosomal S6 protein, a substrate of p70S6K, was markedly impaired in Rap1GAP-overexpressing cells. p70S6K activity is regulated by multi-site phosphorylation including phosphorylation on threonine 389 by mTOR (46, 47). Consistent with its effects on S6 phosphorylation, Rap1GAP overexpression attenuated the effects of TSH and forskolin on mTOR-dependent phosphorylation of p70S6K (Fig. 7, lower panel). Insulin- and serum-stimulated p70S6K activities assessed by S6 or p70S6K protein phosphorylation were only modestly decreased by Rap1GAP overexpression (Fig. 7). Because p70S6K activity is required for thyroid cell proliferation (48, 49), the slower growth rate of the Rap1GAP-overexpressing cells could be a consequence of impaired p70S6K activity. Alterations in Rap1GAP expression have been reported in myeloid cells (13). Immature bone marrow cells express high levels of SPA-1 but not Rap1GAP. Upon maturation, the expression of SPA-1 decreases and Rap1GAP expression is increased (9). As TSH is the primary regulator of differentiated gene expression in thyroid cells, we assessed whether increased Rap1GAP expression induced changes in differentiated gene expression. Overexpression of Rap1GAP markedly enhanced the effects of TSH and forskolin on thyroglobulin expression, a marker of thyroid differentiation (Fig. 8). The effects of TSH on Rap1GAP stability, together with the alterations in growth and differentiated gene expression in Rap1GAP-overexpressing cells, support an important role for Rap1GAP in glycoprotein hormone action.

**DISCUSSION**

Prior to this report, little was known regarding the regulation or function of Rap1GAP. We now present a model for the dynamic regulation of Rap1GAP and new insight into potential roles for Rap1GAP in the regulation of thyroid cell proliferation and differentiation.

Rap1GAP exists as multiple phosphorylated protein species in thyroid cells growing in the presence of TSH. Upon TSH withdrawal, Rap1GAP undergoes a phosphatase-sensitive upshift followed by a rapid decline in its expression. Treatment with cAMP-elevating agents including TSH or with GSK3β inhibitors prevented both the upshift and decline in Rap1GAP protein levels. On the other hand, proteasome inhibitors stabilized Rap1GAP but had no effect on the Rap1GAP upshift. Together, these data support a model wherein TSH withdrawal enhances phosphorylation of Rap1GAP by GSK3β, an event that triggers the proteasomal degradation of Rap1GAP. In support of this model, we identified a destruction box in Rap1GAP similar to the motif in β-catenin (33). Co-expression of Rap1GAP with GSK3β in 293 cells decreased Rap1GAP levels in a proteasome-dependent manner, and mutation of serine 526 in the N-terminal destruction box (Rap1GAPSS526) rendered Rap1GAP insensitive to GSK3β. These studies clearly reveal the potential for regulation of Rap1GAP by GSK3β. Three lines of evidence support the similar regulation of endogenous Rap1GAP by GSK3β in thyroid cells. First, cellular Rap1GAP was stabilized by inclusion of lithium or kenpaullone. Because the inhibitory profiles of lithium and kenpaullone on other protein kinases do not overlap (35), this provides compelling evidence that cellular Rap1GAP is sensitive to GSK3β activity. Second, treatment with three different proteasome inhibitors stabilized endogenous Rap1GAP levels. Third, Rap1GAP immunoprecipitated from thyroid cells was a substrate for GSK3β.

The mechanism through which TSH stabilizes Rap1GAP is not yet clear. Several kinases activated by TSH including protein kinase A, Akt, and p70S6K inhibit GSK3β activity via phosphorylation on serine 9 (36, 50–54). However, inhibition of these kinases had no effect on the ability of TSH to stabilize Rap1GAP protein levels.2 Treatment with okadaic acid stimulated a Rap1GAP upshift when growing cells were transferred to basal or TSH-supplemented basal medium.2 Cumulatively, these findings suggest that TSH stabilizes Rap1GAP through effects on an okadaic acid-sensitive phosphatase, possibly protein phosphatase 2A.

Rap1GAP is not the first example of a Rap1GAP targeted for proteasomal turnover. E6TP1 interacts with the high risk human papillomavirus E6 oncoprotein, an interaction that targets E6TP1 for proteasomal degradation (8). Tuberin, the product of the tsc2 locus (55), is stabilized through its interaction...
with the TSC1 gene product, hamartin (56). Phosphorylation of tuberin by Akt destabilizes this complex and results in the ubiquitination and proteasome-dependent degradation of tuberin (44, 57). Although the mechanisms through which these RapGAPs are targeted for degradation differ from that for Rap1GAP in thyroid cells, the finding that their protein levels are dynamically regulated supports important regulatory roles for these proteins. Tuberin functions as a tumor suppressor and, when overexpressed, impairs cell proliferation (42, 58). Growth inhibition is mediated through the ability of the tuberin-hamartin complex to inhibit p70S6K activity by blocking mTOR-mediated phosphorylation of threonine 389 (44, 45). Even when modestly overexpressed, Rap1GAP impaired mTOR-dependent phosphorylation of p70S6K, an important mediator of proliferation in thyroid cells (48, 49), and slowed thyroid cell proliferation. Collectively, these data reveal an interesting duality in the regulation of Rap1GAP and tuberin protein levels as well as in their ability to inhibit cell proliferation. The ability of Rap1GAP to impair CAMP-dependent proliferation in thyroid cells is intriguing in that thyroid cells are one of very few cellular models where CAMP stimulates proliferation (reviewed in Refs. 14 and 59) and where Rap1 has been proposed to function as an oncogene (4).

The stabilizing effects of TSH on Rap1GAP, a putative growth suppressor, would seem counter-intuitive as TSH is strictly required for thyroid cell proliferation. However, thyroid cell proliferation is regulated by the cooperative action of TSH, insulin, and serum (14). These factors may act in concert to dynamically regulate the stability and/or growth-suppressive activity of Rap1GAP. A primary role of TSH is to regulate the expression of genes involved in thyroid hormone biosynthesis including thyroglobulin. Overexpression of Rap1GAP enhanced the effects of TSH on thyroglobulin expression, suggesting that Rap1GAP plays a role in TSH-stimulated differentiation.

A critically important issue that remains to be addressed is whether Rap1GAP elicits effects distinct from the negative regulation of Rap1 activity. Although overexpression of either Rap1GAP or Rap1A17N impaired Rap1 activity, their other effects in thyroid cells were distinct. Rap1A17N-expressing cells exhibited an enhanced growth rate and impaired differentiated gene expression (15), effects opposite to those induced by Rap1GAP. Rap1GAP impaired TSH/CAMP-stimulated p70S6K activity, whereas dominant negative Rap1A had no effect. Although confounded by issues associated with stable overexpression, these results raise the intriguing possibility that the effects of Rap1GAP may not be limited to impaired Rap1 activity. Stable complexes among Gz, Rap1, and Rap1GAP have been reported previously (19), raising the interesting possibility that multi-protein complexes containing Rap1GAP1 elicit activities of their own.

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