Differential Effects of Protein Kinase A on Ras Effector Pathways

MARSHA J. MILLER,¹ LISE RIOUX,¹ GREGORY V. PRENDERGAST,¹ SARAH CANNON,²
MICHAEL A. WHITE,³ AND JUDY L. MEINKOTH^{1*}

Department of Medicine and Therapeutics, University College Dublin, Dublin 7, Ireland²; Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235³; and Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084¹

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Ras mutants with the ability to interact with different effectors have played a critical role in the identification of Ras-dependent signaling pathways. We used two mutants, Ras^{S35} and Ras^{G37}, which differ in their ability to bind Raf-1, to examine Ras-dependent signaling in thyroid epithelial cells. Wistar rat thyroid cells are dependent upon thyrotropin (TSH) for growth. Although TSH-stimulated mitogenesis requires Ras, TSH activates protein kinase A (PKA) and downregulates signaling through Raf and the mitogen-activated protein kinase (MAPK) cascade. Cells expressing Ras^{S35}, a mutant which binds Raf, or Ras^{G37}, a mutant which binds RalGDS, exhibited TSH-independent proliferation. Ras^{S35} stimulated morphological transformation and anchorage-independent growth. Ras^{G37} stimulated proliferation but not transformation as measured by these indices. TSH exerted markedly different effects on the Ras mutants and transiently repressed MAPK phosphorylation in Ras^{S35}-expressing cells. In contrast, TSH stimulated MAPK phosphorylation and growth in cells expressing Ras^{G37}. The Ras mutants, in turn, exerted differential effects on TSH signaling. Ras^{S35} abolished TSH-stimulated changes in cell morphology and thyroglobulin expression, while Ras^{G37} had no effect on these activities. Together, the data indicate that cross talk between Ras and PKA discriminates between distinct Ras effector pathways.

Ras proteins are important signaling intermediates that convey signals initiated at the cell surface to effector pathways in the cytoplasm. Ras exerts effects on cell transformation and proliferation, the actin cytoskeleton, differentiation, and apoptosis. It is likely that these effects are mediated by multiple effectors, including Raf-1, RalGDS (2), phosphatidylinositol 3-kinase (PI3K) (48), and other Ras-binding proteins (reviewed in reference 38), as well as members of the Rho family (26, 44, 45).

The elucidation of multiple Ras effector pathways was greatly facilitated by the isolation of Ras mutants which interact with single downstream effectors (60). Ras^{S35} binds preferentially to Raf, while Ras^{G37} binds to RalGDS (49, 53, 60). Ras^{S35} stimulates mitogen-activated protein kinase (MAPK) activity and serum response element-dependent gene expression, while Ras^{G37} fails to do so. Individually, neither Ras^{S35} nor Ras^{G37} stimulated focus formation as effectively as Ras^{V12}; however, coexpression of the mutants restored this activity (60). Similarly, microinjection of a Ras^{S35} expression vector into REF52 cells failed to stimulate DNA synthesis when injected alone, although coinjection with a second mutant, Ras^{C40}, stimulated DNA synthesis (23). These findings, along with others, indicate that Ras mediates its effects through multiple pathways.

While the differential effects of Ras^{S35} and Ras^{G37} on Raf and MAPK activity are consistently observed (23, 25, 27, 46, 49, 60), other effects of these mutants vary. Both Ras^{S35} and Ras^{G37} transform some NIH 3T3 strains, inducing growth in medium with low serum levels, anchorage-independent proliferation, and tumor formation in nude mice (27). The activity of

Ras^{G37} in these assays suggests that Ras stimulates some of the same biological effects through Raf-independent pathways and that the effector pathways used by Ras vary in different cell types. Consistently, Ras^{V12} stimulates transformation in most cells, while activated forms of Raf transform fibroblasts but not epithelial cells (41). If Ras signals through multiple effectors, the selection of a particular effector pathway should be regulated. One potential way to achieve such regulation is through cross talk between Ras and other signaling pathways.

One of the best-studied examples of cross talk is that between Ras and protein kinase A (PKA). In many cells, cyclic AMP (cAMP) inhibits Ras signaling through Raf and the MAPK cascade (reviewed in reference 6). cAMP activates PKA, which phosphorylates Raf-1 at multiple serine residues. PKA-mediated phosphorylation reduces the affinity of Raf for Ras (17, 63) and decreases Raf kinase activity (10, 17, 57), effects which may inhibit Ras-mediated proliferation. Ras and cAMP also collaborate to produce similar effects. In PC12 cells, Ras and cAMP induce neurite extension (13, 54) and promote cell survival (64). Ras and cAMP stimulate proliferation in thyroid cells (7, 36, 39), and Ras activity is required for the mitogenic effects of thyrotropin (TSH) (32). Despite the requirement for Ras, TSH downregulates signaling through Raf and the MAPK cascade (1), suggesting that Ras mediates its effects through alternate effectors in these cells. Microinjection of a dominant negative RalA protein, RalA^{N28} (22), reduced TSH- and Ras-stimulated DNA synthesis, supporting a role for RalGDS in this signaling pathway (40).

We set out to determine if cross talk between Ras and PKA affects Ras-mediated signaling. We isolated thyroid cells stably expressing Ras^{V12} and GTPase-deficient forms of Ras^{S35} and Ras^{G37}. Ras^{V12} and the mutants conferred TSH-independent proliferation through distinct pathways. TSH increased the growth rate of Ras^{G37}-expressing cells, but not that of Ras^{S35}-expressing cells and slowed the growth of Ras^{V12} cells. TSH

* Corresponding author. Mailing address: Department of Pharmacology, University of Pennsylvania School of Medicine, 36th St. and Hamilton Walk, Philadelphia, PA 19104-6084. Phone: (215) 898-1909. Fax: (215) 573-2236. E-mail: meinkoth@pharm.med.upenn.edu.

also stimulated MAPK activity in Ras^{G37} cells and repressed it in Ras^{S35} cells. In turn, Ras^{V12} and Ras^{S35} inhibited TSH effects on thyroglobulin (Tg) expression and the actin cytoskeleton, while Ras^{G37} did not. These results demonstrate that cross talk between Ras and PKA is an important determinant of signaling pathways activated by Ras and that interplay between Ras and PKA exerts profound effects on thyroid cell biology.

MATERIALS AND METHODS

Cell culture and transformation assays. Wistar rat thyroid (WRT) cells (5) were maintained in Coon's modified Ham's F-12 medium with crude bovine TSH (10 mU/ml), insulin (10 µg/ml), transferrin (5 µg/ml), and 5% calf serum (3H medium). 2H medium, used in some studies, lacks TSH. Ras-expressing cells were isolated following Lipofectin-mediated gene transfer with pDCR plasmids encoding Ras^{V12}, Ras^{V12G37}, or Ras^{V12S35} (abbreviated as Ras^{G37} and Ras^{S35}). Transfected cells were maintained in 3H medium containing 150 µg of G418 (GIBCO) per ml.

For colony formation assays, vectors were transfected into early-passage subconfluent cells. Following recovery for 24 h in 3H medium, G418 (300 µg/ml) was added in the presence or absence of TSH for 72 h. The G418 concentration was reduced to 150 µg/ml, and 21 days later the cells were fixed and stained.

Anchorage-independent growth was evaluated by plating 5×10^3 cells in 0.33% agar in 2H medium. Cells were refed 0.5 ml of 2H medium once a week for 21 days. Colonies were stained in 2H medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml; Sigma).

Microinjection and immunostaining. For DNA synthesis measurements, cells were starved in basal medium (Coon's modified Ham's F-12 medium, 0.3% bovine serum albumin, 0.5 µg of insulin per ml) for 48 h. Glutathione *S*-transferase fusion constructs encoding Ras^{V12}, Ras^{V12G37}, and Ras^{V12S35} were expressed and the proteins were purified as described previously (40). Purified proteins were injected into the cytoplasm with an Eppendorf semiautomated injector, the cells were labeled with bromodeoxyuridine (BrdU) for 48 h, and DNA synthesis was monitored by immunostaining (34). Rabbit immunoglobulin G (IgG) (7 mg/ml) was included in the injection solutions to identify injected cells. Ras proteins were injected at concentrations ranging from 0.075 to 0.50 mg/ml. Based on an injection volume of 2×10^{-14} liter, injection of Ras at 0.5 mg/ml delivers approximately 290,000 molecules/cell, or an approximately 5- to 15-fold-larger amount than does cellular Ras (50), since approximately 30% of the purified Ras proteins are biologically active (12, 51).

Actin stress fibers were visualized by staining with rhodamine-labeled phalloidin as described by Joneson et al. (23).

Tg expression was assessed by immunostaining with an antibody raised to human Tg as described previously (33).

Western blot analysis. Ras expression was detected by Western blotting with anti-pan-Ras (1:100; Oncogene Science) and antihemagglutinin antibodies (1:300; kindly provided by J. Field) followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin antibodies (1:1,000; New England Biolabs) and detection with CDP-Star (New England Biolabs).

MAPK phosphorylation was assessed by Western blotting with a phosphospecific MAPK antibody (1 µg/ml; Promega).

RESULTS

Multiple Ras effector pathways stimulate thyroid cell proliferation. To determine if PKA influences Ras effectors in cells where Ras and PKA promote growth, we assessed the ability of Ras mutants which differ in their ability to bind Raf to stimulate colony formation in thyroid cells. Vector-transfected cells formed small colonies which failed to proliferate in the absence of TSH (Fig. 1). Transfection with constructs encoding GTPase-deficient (V12) forms of Ras, Ras^{G37}, and Ras^{S35}, as well as Ras^{G12}, stimulated the number and size of colonies formed compared to the number and size of colonies formed by vector-transfected cells (Fig. 1 and Table 1). TSH exerted differential effects on colony formation stimulated by Ras^{G12} and the mutants. Colonies formed following transfection with Ras^{S35} were larger in the absence of TSH than in its presence. In contrast, TSH increased the size of colonies formed following transfection with Ras^{G37} or Ras^{G12}. The morphology of these cells was indistinguishable from that of parental cells. Cells transfected with Ras^{V12} and Ras^{S35} were morphologically transformed, although in these assays the effects of Ras^{S35} were less pronounced than those of Ras^{V12}.

FIG. 1. Ras effector mutants stimulate colony formation in WRT cells. Cells were transfected with pDCR or pDCR encoding Ras^{G12}, Ras^{V12}, or GTPase-deficient (V12) forms of Ras^{G37} and Ras^{S35}. Twenty-four hours after transfection, TSH-supplemented (+TSH) or -deficient (-TSH) medium containing G418 was added to replicate plates, and the cells were fixed and stained 21 days later. Colony size could not be evaluated in Ras^{V12}-transfected cells since these cells were morphologically transformed and did not form compact colonies. Four experiments were performed with similar results.

These results demonstrate that both Raf-dependent (i.e., Ras^{S35}) and -independent (i.e., Ras^{G37}) pathways promote thyroid cell proliferation and suggest that these pathways may be regulated differently by PKA.

cAMP exerts differential effects on Ras^{G37}- and Ras^{S35}-mediated proliferation. To explore potential cross talk between Ras and PKA, cells stably expressing GTPase-deficient forms of Ras, Ras^{G37}, and Ras^{S35} were isolated. Western blotting revealed that all G418-resistant cells expressed abundant levels of transfected Ras (Fig. 2). Ras^{G37} and Ras^{S35} were expressed at similar levels, while Ras^{V12} was expressed at levels 7.5- to 10-fold lower than those of the effector mutants. Compared to cellular Ras, Ras^{V12} was overexpressed approximately fourfold and the effector mutants were overexpressed 30- to 40-fold (Fig. 2B). One mass population and two clonal populations of Ras^{S35}-expressing cells and two independent mass populations of Ras^{G37}-expressing cells were analyzed in our initial studies. Cells expressing either mutant exhibited TSH-independent DNA synthesis (Fig. 3), supporting the stimulatory effects on proliferation seen in the colony formation assays. To discriminate between direct effects of the mutants on proliferation and their ability to establish and/or maintain an autocrine loop, acute microinjection experiments were performed. Microinjection of purified Ras^{S35} or Ras^{G37} proteins stimulated DNA synthesis in quiescent cells (Fig. 4) (40). The effector mutants appeared less active than Ras^{V12} in these assays (Fig. 4, inset), although it is possible that differences in the activity of these proteins contribute to these results. However, the effector domain mutants are consistently reported to be less active than Ras^{V12} following transfection of expression plasmids for these constructs (27, 49, 60). Our results demonstrate that the acute effects of Ras^{G37} and Ras^{S35} can include the stimulation of DNA synthesis in some cells.

To evaluate effects of Ras^{G37} and Ras^{S35} on proliferation,

TABLE 1. Colony formation in Ras-transfected WRT cells^a

Transfected construct	No. of colonies formed	
	With TSH	Without TSH
pDCR	405	290
Ras ^{G12}	746	476
Ras ^{G37}	760	671
Ras ^{S35}	458	507

^a Only colonies larger than 0.5 mm in diameter were scored. Results shown are from the experiment depicted in Fig. 1. Three independent experiments yielded similar results.

FIG. 2. Ras expression in stably transfected WRT cells. Total cell lysates were prepared and protein determinations were made. (A) Western blot probed with anti-hemagglutinin antibody to detect transfected Ras. Samples from left to right are vector-transfected (V) cells, Ras^{G37} (pool 1), Ras^{S35} (pool 10b) (2 μ g each), and Ras^{V12} (clone 3, 10 μ g). The levels of Ras expression were similar in multiple pools and single-cell clones of cells transfected with Ras^{S35} and Ras^{G37}. Ras^{V12} was expressed at levels approximately 7.5- to 10-fold lower than those of the effector mutants. (B) Western blot probed with pan-Ras antibody to detect cellular Ras. Samples from left to right are WRT (100 μ g), vector-transfected (V) cells, Ras^{G37} (5 μ g), and Ras^{V12} (50 μ g). Densitometric analysis revealed that Ras^{V12} was overexpressed 4-fold and Ras^{G37} was overexpressed 40-fold relative to cellular Ras. Cellular Ras expression was not altered by transfection of Ras^{V12} or the mutants.

growth rates were determined in cells expressing these mutants. Expression of either mutant conferred TSH-independent proliferation (Table 2) on cells, as did expression of Ras^{V12}. Because the proliferation of WRT cells is TSH dependent, their growth rate is very low in its absence. Cells expressing Ras^{V12} or either mutant exhibited much shorter doubling times in the absence of TSH than did parental cells. In the presence of TSH, the growth rate of Ras^{G37} cells was markedly

FIG. 3. Ras-expressing cells exhibit TSH-independent DNA synthesis. Ras-expressing cells were incubated in TSH-deficient medium for 48 h and then labeled with BrdU for 48 h. The results shown are for Ras^{V12} (clone 3), Ras^{S35} (pool 10b), Ras^{G37} (pool 1), and vector-transfected cells (C). More than 200 cells were scored on each coverslip. Error bars indicate 95% confidence intervals based on the standard error of proportion. The absence of an error bar indicates a standard error of proportion of 1 or less. Three experiments performed in duplicate gave similar results. Three independent Ras^{S35} isolates and two independent Ras^{G37} isolates were analyzed with similar results.

FIG. 4. Microinjection of Ras^{V12}, Ras^{S35}, and Ras^{G37} proteins stimulates DNA synthesis. Quiescent cells were injected with Ras^{S35} or Ras^{G37} (0.5 mg/ml, the minimal active concentration in these cells) and labeled with BrdU for 48 h. BrdU incorporation in IgG-injected cells is shown as a negative control. (Inset) BrdU incorporation following injection of Ras^{V12}, Ras^{S35}, and Ras^{G37} at similar concentrations (0.075, 0.1, and 0.1 mg/ml, respectively). C, IgG-injected cells. Error bars indicate 95% confidence intervals based on the standard error of proportion. The absence of an error bar indicates a standard error of proportion of 1 or less. More than four independent preparations of the Ras proteins were tested with similar results.

increased (Table 2 and Fig. 5A). Ras^{G37} cells grew more rapidly than did cells expressing either Ras^{S35} or Ras^{V12} in TSH-containing medium. Similar effects were not observed in Ras^{V12} or Ras^{S35} cells, which grew more slowly, at least transiently, following TSH addition (Fig. 5B). Consistent with these results, TSH increased DNA synthesis in Ras^{G37} cells (Fig. 5C) and decreased it in Ras^{S35} cells (Fig. 5D). These results demonstrate that TSH exerts differential effects on signaling pathways stimulated by Ras^{G37} and Ras^{S35}.

Effects on MAPK phosphorylation. Ras^{S35} and Ras^{G37} differ in their ability to bind Raf and stimulate MAPK activity (23, 25, 27, 49, 60). These mutants' properties of binding to proteins in whole-cell lysates from thyroid cells were identical to those described in previous reports. Ras^{S35} interacted with Raf-1 but not RalGDS. Conversely, Ras^{G37} bound RalGDS but not Raf-1 (47).

To examine the signaling pathways activated by these mutants, we examined their effects on MAPK phosphorylation. In the absence of TSH, cells expressing Ras^{S35} or Ras^{V12} exhibited basally elevated levels of phosphorylated MAPK (Fig. 6A). MAPK phosphorylation was not elevated in cells expressing Ras^{G37} or vector-transfected cells under these conditions. TSH rapidly and transiently decreased MAPK phosphorylation

TABLE 2. Growth rates of Ras-expressing cells^a

Cell line	Doubling time (h) in medium	
	3H (+TSH)	2H (-TSH)
WRT	34.2 $\pm$ 2.4	62.7 $\pm$ 3.6
Ras ^{V12}	36.7 $\pm$ 1.9	26.6 $\pm$ 2.7
Ras ^{S35}	37.2 $\pm$ 4.1	32.0 $\pm$ 6.2
Ras ^{G37}	27.5 $\pm$ 2.3	35.7 $\pm$ 3.3

^a The *P* value for the Ras^{G37} trials was <0.025 , while the *P* value for the Ras^{V12} trials was <0.005 . The effects of TSH on growth rate in Ras^{S35} cells were not statistically significant although they were consistently observed. The results shown are representative of four independent experiments performed with similar results.

FIG. 5. TSH exerts differential effects on Ras^{S35} and Ras^{G37}-mediated proliferation. Ni, cell number at day N; No, number of cells plated on day 1. (A) Cells were plated in 3H medium overnight and transferred to 2H medium, and cell number was assessed at days 2 and 4 (open symbols). On day 4, replicate plates were fed 3H (filled symbols) or 2H (open symbols) medium, and the cell number was determined 2 days later. Results shown are the means $\pm$ standard deviations from three experiments performed in duplicate with similar results. The absence of an error bar indicates a standard deviation of 1 or less. Two independent isolates of Ras^{G37}-expressing cells were analyzed with similar results. (B) Experimental conditions were the same as for panel A with Ras^{S35} cells. Three experiments with three independent Ras^{S35} isolates were performed in duplicate with similar results. (C) Ras^{G37} cells were incubated in 2H medium (-TSH) or 3H medium (+TSH), and BrdU incorporation was measured over 24 h. WRT cells were included for comparison. Error bars indicate 95% confidence intervals. Three experiments performed in duplicate gave similar results. (D) BrdU incorporation in Ras^{S35} cells with or without TSH. Cells were incubated in basal medium for 48 h and subsequently stimulated with the same medium (-TSH) or TSH-supplemented medium (+TSH) containing BrdU for 24 h. Error bars indicate 95% confidence intervals. Four experiments yielded similar results.

in both Ras^{V12} and Ras^{S35} cells (Fig. 6A), an effect that was mimicked by cAMP-elevating agents (Fig. 6B). Maximal repression was observed 2 to 5 min after TSH addition, after which MAPK phosphorylation returned to levels observed in untreated cells. In Ras^{S35} cells, both basal levels of MAPK phosphorylation (data not shown) and the return to these levels following TSH stimulation were MEK dependent (Fig. 6C).

In contrast to its effects in Ras^{S35} cells, TSH stimulated a marked increase in MAPK phosphorylation in Ras^{G37} cells (Fig. 6A). TSH increased MAPK phosphorylation within 2 to 5 min, and this increase was sustained for at least 60 min. The effects of TSH on MAPK phosphorylation were mimicked by cAMP-elevating agents, demonstrating that they were PKA mediated (Fig. 6B). Interestingly, in Ras^{G37} cells, pretreatment with the MEK inhibitor PD98059 abolished the effects of TSH on MAPK phosphorylation, supporting a role for MEK1 in

TSH-mediated regulation of MAPK (Fig. 6C). The differential effects of cAMP on MAPK phosphorylation provide additional evidence that Ras^{S35} and Ras^{G37} activate distinct signaling pathways, although these pathways may converge at MEK or MAPK.

Ras^{S35}, but not Ras^{G37}, inhibits thyroglobulin expression. Acute or chronic expression of Ras^{V12} decreased the expression of Tg, a marker of the differentiated phenotype, in WRT cells (33). To explore the pathways which mediate this effect, the effects of Ras^{G37} and Ras^{S35} on Tg expression were examined. Because Tg expression is stimulated by TSH, cells were incubated in TSH-deficient medium for 6 days to reduce Tg expression. Basal levels of Tg expression, as measured by immunostaining with a Tg-specific antibody, were undetectable under these conditions (data not shown). TSH stimulated Tg expression in Ras^{G37}-expressing cells to levels similar to those achieved in parental and vector-transfected cells (Fig. 7). In

FIG. 6. MAPK phosphorylation in Ras-expressing cells is differentially regulated by cAMP. (A) Cells maintained in basal medium for 24 h were stimulated with TSH (1 mU/ml) for various times (given in minutes), and MAPK phosphorylation was assessed. TSH diminished MAPK phosphorylation in Ras^{S35} (pool 10b) and Ras^{V12} (clone 3) cells. TSH increased MAPK phosphorylation in Ras^{G37} (pool 1) cells. TSH stimulated a small increase in MAPK phosphorylation in vector (V)-transfected cells, as it did in parental cells (data not shown). The results shown are from one of two experiments performed with these clones which yielded similar findings. A third experiment with independent isolates of Ras^{G37} (pool 2) and Ras^{S35} (pool C) gave similar results. (B) Ras^{G37} (pool 1) and Ras^{S35} (pool 10b) cells were treated as for panel A except that they were stimulated with 8Br (1 mM) for various times (given in minutes). (C) Pretreatment with PD98059 (25 μ M) for 24 h abolished MAPK phosphorylation 30 min after TSH treatment in Ras^{S35} cells and TSH-stimulated phosphorylation (at 30 min) in Ras^{G37} cells. Two experiments were performed with similar results.

contrast, TSH failed to stimulate Tg expression in cells expressing Ras^{S35}, similarly to the effects previously reported for cells expressing Ras^{V12} (33). Because dedifferentiation frequently accompanies transformation of thyroid cells, the effects of Ras^{S35} and Ras^{G37} on morphological transformation and anchorage-independent growth were examined.

Ras^{S35}, but not Ras^{G37}, stimulates morphological transformation and anchorage-independent growth. Ras^{V12} and Ras^{S35} cells, but not those expressing Ras^{G37}, were morphologically transformed (Fig. 8, top). Ras^{G37} cells exhibited an epithelial morphology indistinguishable from that of parental cells and proliferated as small, well-defined colonies. Cells expressing Ras^{V12} or Ras^{S35} exhibited a fibroblast-like morphology characterized by highly refractile, elongated, spindle-shaped cells which were scattered across the culture dishes. Ras-mediated alterations in cell morphology were accompanied by profound changes in the actin cytoskeleton. In the absence of TSH, Ras^{G37}-expressing cells, like parental cells, displayed abundant longitudinal actin stress fibers (Fig. 8, middle). Ras^{V12} and Ras^{S35} cells exhibited a marked decrease in stress fibers. The most striking feature of these cells was the presence of abundant large membrane extensions with prominent ruffles.

TSH and cAMP analogs exerted dramatic effects on the

FIG. 7. Ras^{S35}, but not Ras^{G37}, inhibits Tg expression. WRT cells (A), pDCR-transfected controls (B), and Ras^{G37} (pool 1) (C) and Ras^{S35} (pool 10b) (D) cells were stimulated with TSH (1 mU/ml) and stained for Tg expression 48 h later. Photomicrographs were taken at identical exposure times. Three independent experiments with these isolates yielded similar results. Three independent isolates of Ras^{S35}-expressing cells and two independent isolates of Ras^{G37}-expressing cells were analyzed.

cytoskeleton, including cytoplasmic retraction, the dissolution of actin stress fibers, increased peripheral actin staining, and extensive membrane ruffling at sites of cell-cell contact (Fig. 8, bottom). These effects were preserved in cells expressing Ras^{G37} but lost in Ras^{V12} and Ras^{S35} cells, which retained membrane extensions in the presence of TSH.

Because actin rearrangements are often associated with changes in cell-substrate attachment, the ability of these cells to proliferate in soft agar was examined. Both Ras^{V12} and Ras^{S35} cells exhibited anchorage-independent proliferation in the absence of TSH, while parental cells and vector-transfected cells (data not shown) were unable to form colonies under these conditions (Fig. 9). Ras^{G37} cells occasionally formed small colonies in either the presence or absence of TSH; however, these results were inconsistent. Together, these results demonstrate that Ras^{V12} and Ras^{S35} stimulate several markers of transformation: dedifferentiation, cytoskeletal alterations, and anchorage-independent growth. In contrast, Ras^{G37} stimulates proliferation but not transformation.

DISCUSSION

Two Ras effector mutants which differ in their ability to bind Raf (60) conferred hormone-independent proliferation on thyroid cells through signaling pathways differentially regulated by PKA. cAMP is a mitogen in WRT cells, which are TSH dependent for growth. TSH stimulates proliferation through PKA and Ras (32), but not through Raf-1 (1). Ras^{G37}, a mutant that binds RalGDS (61), increased the growth rate of cells in the presence of TSH. Interestingly, this effect was not observed in cells expressing Ras^{V12} or Ras^{S35}, proteins competent to bind Raf. These cells grew more slowly than cells expressing Ras^{G37} in the presence of TSH. These results suggest that growth rates in TSH may reflect the affinities of these proteins for Raf-1, an interaction which is inhibited by PKA (reviewed in reference 6). In support of this idea, the rank

FIG. 8. Morphological changes in cells expressing Ras^{V12} and Ras^{S35}. (Top) Phase-contrast photomicrographs of WRT, Ras^{G37} (pool 1), Ras^{S35} (pool 10b), and Ras^{V12} (clone 3) cells. (Middle) Actin staining in the same cells maintained in basal medium (i.e., without TSH) for 48 h. (Bottom) Actin staining in cells acutely stimulated with TSH (1 mU/ml) for 30 min. Results shown are representative of three independent experiments with these isolates. Two independent pools of cells expressing Ras^{G37} and Ras^{S35} were examined.

order activity of the Ras proteins was reversed when the cells were grown in the absence of TSH. Under these conditions, cells expressing Ras^{V12} and Ras^{S35} grew more rapidly than did cells expressing Ras^{G37}. The apparent reduction in growth rate observed in Ras^{V12} and Ras^{S35} cells in the presence of TSH could be a consequence of increased apoptosis (25). Because TSH inhibits Ras-Raf signaling, expression of Ras proteins which bind Raf may constitute an inappropriate signal for proliferation in the presence of TSH-stimulated acute cAMP elevation. We have not directly examined apoptosis in these cells; however, cells injected with these Ras proteins are recovered in the expected numbers in the presence of TSH. This indicates that if apoptosis occurs, it does so in a small fraction of cells or with such asynchrony that it is not detected in these assays.

Our results provide the first demonstration that the acute effects of Ras^{S35} or Ras^{G37} include the stimulation of DNA synthesis. These results differ from those of Joneson et al., who reported that microinjection of a vector encoding Ras^{S35} failed to stimulate DNA synthesis in REF52 cells (23). Whether these differences are technical or a consequence of different cellular environments is unclear. Consistent with our findings, Ras^{S35} and Ras^{G37} enhanced the growth rate of NIH 3T3 cells in low serum levels (27).

The effects of TSH on Ras-stimulated proliferation were reflected in effects on MAPK activity. In the absence of TSH,

basal levels of MAPK phosphorylation were elevated in Ras^{V12} and Ras^{S35} cells, but not in Ras^{G37} cells. These results are consistent with previous reports that Ras^{S35}, but not Ras^{G37}, stimulates MAPK activity (23, 25, 27, 49, 60). TSH transiently repressed MAPK phosphorylation in Ras^{V12} and Ras^{S35} cells. This effect most likely reflects the inhibitory action of PKA on Ras-Raf signaling, since it was mimicked by cell-permeative cAMP analogs. The transient nature of this inhibition may reflect downregulation of PKA activity or the stimulation of other pathways that contribute to MAPK activation, since 8-bromo-cAMP-stimulated DNA synthesis is Ras dependent in these cells (32). In Ras^{V12}-expressing cells, MAPK inhibition by TSH was accompanied by a decrease in growth rate. The effects of TSH on Ras^{S35}-mediated growth were less pronounced, possibly as a consequence of the lower affinity of Ras^{S35} than of Ras^{V12} for Raf, or perhaps due to the higher levels of overexpression of Ras^{S35} than of Ras^{V12}. While basal levels of MAPK phosphorylation were low in Ras^{G37} cells, TSH stimulated a dramatic increase in MAPK phosphorylation. This was unexpected, since Ras^{G37} has not been reported to stimulate MAPK activity either alone or in cooperation with Raf-1 (61), and TSH decreased serum- as well as insulin-like growth factor 1-stimulated MAPK activity in parental WRT cells (40). TSH was also reported not to stimulate MAPK activity in primary canine thyroid cells (35). Nonetheless, WRT cells expressing Ras^{G37} grew faster than cells expressing

FIG. 9. Ras^{V12} and Ras^{S35} stimulate anchorage-independent proliferation. Cells were plated in agar and 2H medium as described in Materials and Methods. Three independent experiments were performed in duplicate with similar results.

Ras^{V12} or Ras^{S35} in the presence of TSH, suggesting that Ras^{G37} collaborates with pathways activated by TSH to stimulate growth.

Several mechanisms can be envisioned for the effects of TSH on MAPK activity in Ras^{G37} cells. Ras^{G37} binds RalGDS or a related molecule in thyroid cell extracts (47). Microinjection of RalA^{N28} (22), a mutant protein which binds RalGDS, decreases Ras- and TSH-stimulated DNA synthesis, supporting a role for RalGDS in growth stimulation (40). Consistent with such a role, RalGDS is phosphorylated by PKA in vitro, although phosphorylation reportedly had no effect on exchange factor activity (29). It is conceivable that PKA relocalizes RalGDS to a membrane compartment where it binds Ras, is activated, and stimulates pathways leading to MAPK phosphorylation. We failed to detect any consistent effects of stable expression of RalGDS on cell proliferation; however, we were unable to drive RalGDS expression in thyroid cells to high levels. In addition to binding Ras (20, 28, 52, 60), RalGDS binds Rap1A (18, 21, 43, 56, 62), originally identified as K-rev1 (30). PKA activates Rap1B (3), and microinjection of Rap1B stimulates DNA synthesis in cAMP-responsive Swiss 3T3 cells (65), suggesting a role for Rap in cAMP-mediated proliferation. cAMP activates MAPK through Rap1 and B-Raf in PC12 cells (59), providing one potential mechanism through which TSH could stimulate MAPK activity in Ras^{G37} cells. However, in PC12 cells, the effects of cAMP on MAPK were Ras independent. In Swiss 3T3 cells, prolonged platelet-derived growth factor-stimulated MAPK activation requires protein kinase C and PI3K activity (16). The mitogenic activity of TSH requires PI3K and p70S6k activity (9a). It is possible that PI3K contributes to the effects of TSH on MAPK activity observed in Ras^{G37} cells in a manner that does not invoke PI3K binding to Ras^{G37}. Rho family members cooperate with Raf to activate MAPK (14), as does p21-activated kinase (55). Whether TSH signals through Rho family members is not yet clear, although it is interesting that Ral binding proteins with cdc42 GTPase-activating protein-like domains have been identified (8, 24, 42).

Cross talk between Ras and PKA is bidirectional. While TSH differentially affects Ras-mediated growth, Ras mutants exert distinct effects on TSH signaling. Ras^{V12} and Ras^{S35} stimulate morphological transformation, anchorage-independent growth, and dedifferentiation. In contrast with earlier reports (23, 49), thyroid cells expressing Ras^{S35} exhibited dra-

matic membrane ruffles, an effect that was mimicked in cells expressing Ras^{V12} but not Ras^{G37}. Whether this is a direct effect of Ras^{S35} or is secondary to autocrine secretion is not yet clear. As reported previously, NIH 3T3 cells expressing Ras^{V12} or Ras^{S35} but not Ras^{G37} also exhibited a decrease in the number of actin stress fibers (27). In WRT cells, TSH stimulates rapid morphological changes including cytoplasmic retraction and extensive ruffling at sites of cell-cell contact. These effects of TSH were abolished in Ras^{V12} and Ras^{S35} cells but retained in Ras^{G37} cells. This provides another molecular marker of differential cross talk between PKA and Ras effectors. In this case, the effects of Ras predominate over those of TSH.

Morphological transformation elicited by Ras^{V12} and Ras^{S35} was accompanied by a new ability of these cells to form colonies in soft agar in the absence of TSH. WRT cells form small colonies in the presence, but not in the absence, of TSH. Expression of Ras^{V12} or Ras^{S35} conferred TSH-independent growth in agar, while expression of Ras^{G37} did not. These effects differ somewhat from those reported for NIH 3T3 cells, where both mutants promoted anchorage-independent growth.

TSH is required for maintenance of the differentiated phenotype (reviewed in references 11 and 58) by affecting the expression of thyroid-specific gene products, including the TSH receptor (31), the Na-I symporter (reviewed in reference 9), thyroid peroxidase (37), and Tg (19). Ras decreases the expression of some thyroid-specific genes (4, 15). In WRT cells, acute or chronic expression of Ras^{V12} abolished TSH-stimulated Tg expression (33). This effect appears to be Raf dependent, since Ras^{S35} exhibited similar effects while Ras^{G37} did not. As for the actin cytoskeleton, the effects of Ras^{V12} and Ras^{S35} on differentiation were dominant over the effects of TSH. In 10T1/2 cells, Ras^{V12} and Ras^{S35} also interfered with a marker of differentiation, MyoD-induced myogenesis (46).

Substantial progress in understanding the mechanisms through which Ras exerts its biological effects has been made. There are multiple effector pathways activated by Ras, and those pathways can vary in different cells. The challenge is to understand how Ras-mediated signals are directed at distinct effector pathways. Our results indicate that cross talk between signaling pathways, i.e., Ras and PKA, influences the effectors through which Ras conveys its signals. In turn, discrete Ras effector pathways differentially affect the cAMP/PKA pathway. Interplay between these signaling pathways may allow cells to respond appropriately to changing cues in their cellular environment.

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