cAMP Inhibition of Akt Is Mediated by Activated and Phosphorylated Rap1b

Received for publication, February 13, 2002, and in revised form, June 27, 2002
Published, JBC Papers in Press, June 27, 2002, DOI 10.1074/jbc.M201491200

Liguang Lou‡, Julie Urbani‡, Fernando Ribeiro-Neto‡§, and Daniel L. Altschuler‡¶

From the ‡Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261 and the §Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Rap1b has been implicated in the transduction of the cAMP mitogenic signal. Rap1b is phosphorylated and activated by cAMP, and its expression in cells where cAMP is mitogenic leads to an increase in G1/S phase entry and tumor formation. The PCCL3 thyroid follicular cells represent a differentiated and physiologically relevant system that requires thyrotropin (TSH), acting via cAMP, for a full mitogenic response. In this model system, cAMP stimulation of DNA synthesis requires activation and phosphorylation of Rap1b by the cAMP-dependent protein kinase A (PKA). This scenario presents the challenge of identifying biochemical processes involved in the phosphorylation-dependent Rap1b mitogenic action. In thyroid cells, Akt has been implicated in the stimulation of cell proliferation by TSH and cAMP. However, the mechanism(s) by which cAMP regulates Akt activity remains unclear. In this study we show that in PCCL3 cells 1) TSH inhibits Akt activity via cAMP and PKA; 2) Rap1b is required for cAMP inhibition of Akt; and 3) transduction of the cAMP signal into Akt requires activation as well as phosphorylation of Rap1b by PKA.

Akt, also known as protein kinase B (PKB), the cellular homologue of the AKT8 retrovirus transforming oncogene (1), belongs to the AGC superfamily of protein kinases (homologous to PKA1 and the protein kinases G and C (PKG and PKC)). Altered Akt function has been linked to diverse processes such as diabetes, cell proliferation, differentiation, transformation, and apoptosis (2–4). Genetic (5, 6) and pharmacological (5–8) studies have shown that Akt is activated by signaling events that stimulate phosphatidylinositol 3-kinase (PI3K), which catalyzes the formation of the D3 phosphoinositides PI 3,4,5-P3 and PI 3,4-P2 (9). Binding of these membrane lipids to the pleckstrin homology domain of Akt (10–12) primes the kinase for activation by phosphorylation at two critical residues: Thr308, located in the activation loop of the catalytic domain, and Ser473 at the hydrophobic C-terminal domain (7). PDK1, a 3-phosphoinositide-dependent protein kinase, phosphorylates Thr308 (13, 14). The identity of the Ser473 kinase has not been clearly established. Whether Akt autophosphorylation (15) or other kinase(s) (16) are involved in Ser473 phosphorylation is not yet resolved. Numerous growth and survival factors that act via receptor tyrosine kinases and signaling molecules acting via G-protein-coupled receptors stimulate Akt in a PI3K-dependent manner (17). As expected for a lipid-regulated phosphoprotein, Akt is subjected to negative regulation by the lipid phosphatase PTEN (18) as well as other phosphatases (19, 20). New recently identified Akt binding partners (21–23) reinforce the notion that Akt activity is tightly regulated.

Interestingly, recent reports indicate that cAMP, an intracellular signal that (similar to Akt) has significant effects on the control of cell proliferation, differentiation, transformation, and apoptosis (24–26), modulates Akt activity either positively (27–30) or negatively (31–33).

The effects of cAMP on these processes, especially on cell proliferation, are cell type-specific. Notwithstanding the recent importance given to cAMP inhibition of cell proliferation (34), cAMP is mitogenic for numerous cell types, notably in primary cultures of epithelial cells and particularly those of endocrine origin (24, 26). The fundamental aspect of this cAMP mitogenic action is to interact synergistically with mitogens that do not signal via cAMP (35).

cAMP rapidly phosphorylates (36) and activates the G-protein Rap1b (37). Exogenous expression of Rap1b in Swiss3T3 cells is associated with an increase in cAMP-mediated G1/S phase entry and with the development of tumors when these cells are transplanted into nude mice (38). This mitogenic action of Rap1b can be recapitulated in a differentiated and physiologically relevant model, thyroid follicular cells, a system that requires the trophic factor thyrotropin (TSH), acting through cAMP for a full mitogenic response (40). Additionally, inhibition of Rap1b activity in these cells impairs TSH/cAMP mitogenic activity, implicating Rap1b as a critical player in cAMP mitogenic action (39). Thus, we have proposed that Rap1b transduces the cAMP mitogenic signal (38, 39). Likewise, Rap1b mediates cAMP inhibition of cell proliferation (41), lending strong support to the notion that Rap1b affects mitogenesis conditionally to the role that cAMP has in cell proliferation (38, 39).

Because both cAMP stimulation (38, 39) and inhibition (41) of mitogenesis are processes intimately linked with Rap1b activity, a question arises on whether Akt represents a target for Rap1b action downstream of cAMP. In this report, we studied the effects of TSH or cAMP on Akt activity in thyroid follicular cells. We found that cAMP signaling, in a PKA-dependent fashion, rapidly inhibits Akt activity in ways that require activation and PKA phosphorylation of Rap1b.

**EXPERIMENTAL PROCEDURES**

**Materials**—Forskolin, IBMX, and H89 were obtained from Calbiochem. Phosphorylation-specific (Ser473, Thr308) and phosphorylation

---

*This work was supported by National Institutes of Health Grant R29 CA71649, American Cancer Society Grant RFP00-291-01-TBE (to D. L. A.), and the Laboratory of Signal Transduction, NIEHS, National Institutes of Health (to F. R. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel: 412-648-9751; Fax: 412-648-1945; E-mail: altschul@server.pharm.pitt.edu.

‡The abbreviations used are: PKA, cyclic AMP-dependent kinase; PI, phosphatidylinositol; PI3K, PI 3-kinase; TSH, thyrotropin; HA, hemagglutinin; GEF, guanine nucleotide exchange factor; WT, wild type; MAPK, mitogen-activated protein kinase; IBMX, isobutyl methyl xanthine.
state-independent rabbit polyclonal anti-Akt antibodies were purchased from New England Biolabs (Beverly, MA). Antibodies against HA (HA.11) and Myc (9E10) were from Berkeley Antibody. 1γ32P]ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences. Crossstide was from Upstate Biotechnology (Lake Placid, NY). Protein G-Sepharose was from Amersham Biosciences.

Cell Culture—PCCL3 cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% fetal bovine serum and 4 hormones: TSH (1 milliunit/ml), insulin (10 μg/ml), transferring (5 μg/ml), and hydrocortisone (1 μM) at 37 °C in an atmosphere of 5% CO2 in air.

Results

In Vitro Akt Kinase Activity Assay—After treatment with the indicated agents, PCCL3 cells were lysed in a buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM EDTA, 10 mM NaPO4, 2 mM sodium orthovanadate, 100 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1% Triton X-100 for 30 min at 4 °C. The lysate was cleared by centrifugation at 15,000 × g for 10 min at 4 °C, and HA-Akt was immunoprecipitated with an anti-HA (HA.11) antibody followed by protein G-Sepharose purification. After washing the immunocomplexes samples were separated into two equal parts and used for Western blot analysis and kinase activity assay, respectively. Kinase activity was determined using crossstide as a substrate in a reaction mixture containing 50 mM Tris (pH 7.6), 10 mM MgCl2, 1 mM dithiothreitol, 50 μM ATP, 30 μM crossstide, 3 μCi [γ-32P]ATP (3000 Ci/mmol), and 50 units of heat-stable inhibitor of cAMP-dependent protein kinase. The phosphorylation reaction proceeded for 30 min at 30 °C, was terminated by spotting 25 μl of sample onto a Whatman P81 phosphocellulose paper, and was immediately immersed into 75 mM orthophosphoric acid. After several washes, the filters were rinsed in ethanol and air-dried. Radioactivity was determined by liquid scintillation. Background obtained from cells transfected with empty-vector were subtracted from all values.

Akt-T308 phosphorylation transiently, the inhibition of Ser473 displays a sustained kinetic profile.

TSH, acting via cAMP, or insulin alone has little mitogenic effect on thyroid cells (40, 42). However, TSH displays strong mitogenic effect in the presence of insulin, a potent activator of Akt (8). Therefore, we sought to determine the effect of cAMP signaling on insulin-stimulated Akt phosphorylation. Insulin strongly stimulated Akt phosphorylation reaching maximal activity within 2 min and lasting for at least 1 h (not shown). However, if cells were exposed to TSH (Fig. 2A) or forskolin (Fig. 2B) for only 5 min prior to stimulation with insulin, a marked reduction of Akt phosphorylation at Thr308 and Ser473 was observed. Results with phosphospecific antibodies were also confirmed by activity as measured in vitro in an immunocomplex kinase assay using the crossstide peptide as a substrate (43). Consistent with the results using Akt phosphospecific antibodies, TSH or forskolin inhibited both basal and insulin-
stimulated Akt activity by about 4-fold (Fig. 2C), indicating that cAMP inhibition of Akt activity is a stable process, which endures isolation of the kinase by immunoprecipitation. Interestingly, when Akt activity was maximally stimulated by a brief exposure to insulin for 5 min and subsequently exposed to forskolin for only 5 min, a marked inhibition of Akt phosphorylation was observed (Fig. 2D). These results reveal an outweighing effect of cAMP signaling on Akt as cAMP either prevents or inhibits insulin-stimulated Akt activity.

TSH induces proliferation of thyroid follicular cells via cAMP and PKA (40–42). Although PKA is a critical target for most of the biological actions of cAMP, cAMP can affect biochemical processes independently of PKA. Such is the case for a newly identified family of proteins containing a cAMP binding domain and, in addition, with guanine nucleotide exchange activity (GEFs) for Ras and Rap (44–46). *In vitro* direct binding of cAMP to these GEFs leads to the exchange of GTP for GDP independently of PKA phosphorylation (45). A possible involvement of cAMP and PKA in TSH inhibition of Akt predicts that drugs that stabilize cAMP levels should potentiate cAMP inhibition of Akt. Likewise, specific PKA inhibitors should block cAMP inhibition of Akt. For this, we tested the effects of IBMX (a cAMP phosphodiesterase inhibitor) and H89 (a specific PKA inhibitor) on forskolin inhibition of insulin-stimulated Akt activity. In the absence of IBMX, forskolin inhibited basal Akt phosphorylation while enhancing forskolin’s ability to further inhibit this process (Fig. 4A). If Rap1b activation is sufficient to mediate cAMP inhibition of Akt, then Rap1b-G12V (a constitutively active form of Rap1b) should mimic cAMP action. Expression of Rap1b-G12V greatly inhibited Akt phosphorylation in the absence of a cAMP stimulus, indicating that Rap1b-G12V behaves as an authentic mimic of cAMP signaling to Akt (Fig. 4B). The inhibition of Akt phosphorylation by Rap1b, as seen with cAMP, correlates with its effect on Akt kinase activity (Fig. 4C). These results suggest that activation of endogenous Rap1b by cAMP is sufficient to inhibit Akt activity.

If activation of endogenous Rap1b is required for cAMP inhibition of Akt, expression of a dominant-negative mutant such as Rap1b-S17N should block the inhibitory action of cAMP. Expression of Rap1b-S17N markedly reduced cAMP inhibition of basal Akt activity (Fig. 4C). Interestingly, like the PKA inhibitor H89, Rap1b-S17N stimulated Akt activity in the absence of added cAMP signaling agents, suggesting that Rap1b

---

**FIG. 2.** cAMP signaling inhibits insulin-stimulated Akt phosphorylation and its activity. Quiescent PCCL3 cells were pretreated for 5 min with either 10 μM forskolin or 1 milliunit/ml TSH followed by 5 min of 1 μg/ml insulin stimulation. A and B, an aliquot was run on SDS-PAGE and developed with Akt or anti-P-T308 (p-Akt) antibodies. C, fractions were subjected to PI kinase activity using crosstide as a substrate as described. D, forskolin treatment (5 min) was assessed before (For+Ins) or after (Ins+For) insulin stimulation. Normalized data are presented in the lower panels of A, B, and D (100% is defined as insulin stimulation).
exerts a tonic inhibitory control on Akt, similar to PKA. These results demonstrate that Rap1b is both sufficient and required for cAMP inhibition of basal Akt activity.

To address whether Rap1b is involved in cAMP inhibition of insulin-stimulated Akt, cells were co-transfected with HA-tagged Akt and Myc-tagged Rap1b-interfering constructs. The effect of cAMP on insulin-stimulated Akt activity was evaluated by forskolin dose-response curves. Forskolin inhibited insulin-stimulated HA-Akt phosphorylation in a dose-dependent manner (Fig. 5A) similar to its action on endogenous Akt (Fig. 3, A and D). This effect was completely blocked by expression of the dominant-negative Rap1b-S17N (Fig. 5, B and D). Moreover, cAMP inhibition of insulin-stimulated Akt activity was potentiated by expression of the constitutively active Rap1b-G12V construct as revealed by the marked left shift on the forskolin dose-response curve (Fig. 6, B and D). These results demonstrate that activation of Rap1b is necessary and sufficient for cAMP inhibition of insulin-stimulated Akt activity. If this is true, down-regulation of active Rap1b should block or significantly reduce cAMP inhibition of Akt. For this, the negative Rap1 regulator RapGAP was co-transfected with Akt into cells. Subsequently, these cells were serum-starved and stimulated with insulin in the presence of increasing concentrations of forskolin. Expression of Rap1GAP completely abolished the cAMP inhibition of Akt phosphorylation as revealed in the forskolin dose-response curves (Fig. 7, B and C). Thus, these data afford the conclusion that cAMP activation of Rap1b mediates the cAMP inhibition of Akt.

cAMP Inhibition of Akt Requires Activation and Phosphorylation of Rap1b—PKA phosphorylates Rap1b on Ser179 (36), and activated phospho-Rap1b is required for cAMP-induced G1/S entry (39). PKA activity is required for the cAMP-dependent inhibition of Akt (Fig. 3, C and D). Thus, we have explored the possibility that phospho-deficient Rap1b mutants might interfere with cAMP inhibition of Akt. Because the dominant-neg-
ative Rap1b-S17N blocks cAMP signaling into Akt, we examined whether Rap1b-S17N Ser 179 phosphorylation is at all required for its dominant-negative action. This experimental maneuver reflects an indirect assessment of the interaction of Rap1b with GEFs. If phosphorylation is required for the action of Rap1b-S17N, its phospho-deficient mutant, Rap1b-S17N-S179A, should not block the interaction between endogenous Rap1 and GEF, resulting in loss of Rap1b-S17N blockade of cAMP inhibition of Akt. However, lack of Ser179 phosphorylation did not relieve the inhibitory effect of Rap1b-S17N as indicated by the inability of cAMP to inhibit Akt (Fig. 5, C and D). This result is consistent with the observation that cAMP stimulation of Rap1-GTP binding can proceed independently of PKA (29, 45).

Alternatively, Rap1 phosphorylation might be required to transduce Rap1b-GTP-dependent inhibition of Akt. For this a S179A mutation was introduced in the context of the Rap1b-G12V background. This phosphorylation-deficient and constitutively active Rap1b mutant abolished cAMP inhibition of Akt. However, lack of Ser179 phosphorylation did not relieve the inhibitory effect of Rap1b-S17N as indicated by the inability of cAMP to inhibit Akt (Fig. 5, C and D). This result is consistent with the observation that cAMP stimulation of Rap1-GTP binding can proceed independently of PKA (29, 45).

These results indicate that transduction of the cAMP signal into Akt requires activation and phosphorylation of Rap1b.

If PKA phosphorylation of Ser179 is a co-signal for Rap1b transduction, a cAMP-independent activator of Rap1b such as ∆Epac (45) should stimulate an effector response in cells expressing Rap1b-WT but not Rap1b-S179A. Deletion of the Epac N-terminal regulatory domain converts Epac into a constitutively Rap1-GEF (∆Epac), which activates Rap1b independently of cAMP (45). We exploited the ability of ∆Epac to activate Rap1b in ways that are unrelated to cAMP by asking whether expression of ∆Epac in combination with Rap1b-WT recapitulates the cAMP inhibition of Akt and whether this response requires Rap1b-S179 phosphorylation. Results from these experiments are presented in Fig. 8. ∆Epac inhibits Akt phosphorylation only in the presence of Rap1b-WT, mimicking cAMP action. This response requires PKA because it is blocked by H89 (not shown). Moreover, in the absence of the PKA phosphorylation site Ser179 a fully activated (GTP-bound, not shown) Rap1b was unable to transduce the cAMP signal into inhibition of Akt activity. These results demonstrate that PKA phosphorylation of Rap1b-S179 is critical for Epac- and Rap1b-mediated inhibition of Akt. Thus, phosho-Ser179 functions as a critical signal for Rap1b signaling, possibly at a post-activation step.

**DISCUSSION**

**cAMP Signaling into Akt**—Similar to the actions of cAMP on many biological processes (24–26), there appears to be a puzzling duality in the effects of cAMP on Akt; cAMP modulates Akt activity either positively or negatively (27, 28, 30–33). cAMP inhibits the mitogenic activity in certain cell types including most transformed cell lines (25) while increasing mitogenicity for most cell types, particularly of endocrine origin (24, 26). As is the case with the effects of cAMP on cell proliferation, cAMP actions on Akt appear to be cell type-specific. In Wistar
Fig. 7. Inhibition of endogenous activation of Rap1b by Rap-GAP blocks cAMP inhibition of Akt. Forskolin dose responses were performed as described in the legends to Figs. 3, 5, and 6, on cells co-transfected with HA-Akt and HA-vector (A) or HA-RapGAP (B). Akt phosphorylation was analyzed as described, after immunoprecipitation with HA and immunoblotting with HA (HA-Akt) or anti-P-T308 (pHA-Akt). An aliquot of the lysate blotted with HA antibody is shown to check RapGAP expression level. Normalized data are presented in C (100% is defined as insulin stimulation).

rat thyroid cells (29, 30, 47) and primary cultures of rat ovarian granulosa cells primed with estradiol (27), cell types in which cAMP is a positive regulator of cell growth, cAMP signaling agents stimulated Akt activity, albeit with very slow kinetics uncharacteristic for an acute bona fide cAMP signaling event. In contrast, but consistent with the positive-negative duality of cAMP signaling event.

Regardless, it is clear that stimulation of Akt activity is not sufficient to explain cAMP mitogenesis; insulin (or serum) maximally stimulates Akt, but these agents by themselves have little or no effect on G1/S entry (35). To gain new insight about the mitogenic synergism between cAMP and insulin, identification of early signaling events of cAMP and how they are transduced into mitogenic activity is needed. The small G-protein Rap1b is a convergent target for apparent divergent processes emanating from the cAMP signal, which rapidly activates and phosphorylates Rap1b via the nucleotide exchange protein Epac, a small G-protein GEF, and is inhibited by Akt via Phospho-Rap1b. Cells were co-transfected with HA-Akt, full-length Myc-Epac (WT), or constitutively active Myc-Epac (Δ) and either Myc-Rap1b-WT (WT) or Myc-Rap1b-S179A (S179A). Akt phosphorylation was analyzed as described, after immunoprecipitation with HA and immunoblotting with HA (HA-Akt) or anti-P-T308 (pHA-Akt). An aliquot of the lysate blotted with Myc antibody is shown to check Rap1b and Epac expression levels.

First, activation of Rap1 mediates cAMP activation of Akt in Wistar rat thyroid cells appears to be dependent on P13K activity, in 293-EBNA cells cAMP stimulation of Akt is unaffected by inhibition of P13K (49). This suggests that cAMP may act on a post-Akt activation step. According to this interpretation, cAMP action on Akt should not occur exclusively under cAMP signaling pretreatment schemes as it has generally been investigated, but it also must occur in a post-insulin treatment once a significant pool of Akt has already been phosphorylated and activated. Consistent with this, we found that forskolin used as pretreatment or in post-treatment protocols is equally effective in blocking insulin-stimulated Akt phosphorylation (Fig. 2D). Although this finding does not formally exclude an effect of cAMP on the upstream activation of Akt, it is consistent with our interpretation that cAMP signaling does affect a post-activation event. Interestingly, other Akt inhibitors such as ceramide (20, 50, 51) and hyperosmolarity (52) also act at a post-activation step, most likely via the action of phosphatases.

Regardless, it is clear that stimulation of Akt activity is not sufficient to explain cAMP mitogenesis; insulin (or serum) maximally stimulates Akt, but these agents by themselves have little or no effect on G1/S entry (35). To gain new insight about the mitogenic synergism between cAMP and insulin, identification of early signaling events of cAMP and how they are transduced into mitogenic activity is needed. The small G-protein Rap1b is a convergent target for apparent divergent processes emanating from the cAMP signal, which rapidly activates and phosphorylates Rap1b via the nucleotide exchange protein Epac, and PKA, respectively. Thus, Rap1b is an attractive candidate target for the transduction of the cAMP signal into Akt.

Rap1 Signaling into Akt—Three patterns for the transduction of the cAMP signal into Akt by Rap1 can be identified. First, activation of Rap1 mediates cAMP activation of Akt in the Wistar rat thyroid cells, characterized by a PKA-independent effect with slow kinetics and small magnitude (30). Because cAMP is a well characterized mitogen in those cells, a correlative scheme as it has generally been investigated, but it also must occur in a post-insulin treatment once a significant pool of Akt has already been phosphorylated and activated. Consistent with this, we found that forskolin used as pretreatment or in post-treatment protocols is equally effective in blocking insulin-stimulated Akt phosphorylation (Fig. 2D). Although this finding does not formally exclude an effect of cAMP on the upstream activation of Akt, it is consistent with our interpretation that cAMP signaling does affect a post-activation event. Interestingly, other Akt inhibitors such as ceramide (20, 50, 51) and hyperosmolarity (52) also act at a post-activation step, most likely via the action of phosphatases.

Regardless, it is clear that stimulation of Akt activity is not sufficient to explain cAMP mitogenesis; insulin (or serum) maximally stimulates Akt, but these agents by themselves have little or no effect on G1/S entry (35). To gain new insight about the mitogenic synergism between cAMP and insulin, identification of early signaling events of cAMP and how they are transduced into mitogenic activity is needed. The small G-protein Rap1b is a convergent target for apparent divergent processes emanating from the cAMP signal, which rapidly activates and phosphorylates Rap1b via the nucleotide exchange protein Epac, and PKA, respectively. Thus, Rap1b is an attractive candidate target for the transduction of the cAMP signal into Akt.

Rap1 Signaling into Akt—Three patterns for the transduction of the cAMP signal into Akt by Rap1 can be identified. First, activation of Rap1 mediates cAMP activation of Akt in the Wistar rat thyroid cells, characterized by a PKA-independent effect with slow kinetics and small magnitude (30). Because cAMP is a well characterized mitogen in those cells, a correlative scheme as it has generally been investigated, but it also must occur in a post-insulin treatment once a significant pool of Akt has already been phosphorylated and activated. Consistent with this, we found that forskolin used as pretreatment or in post-treatment protocols is equally effective in blocking insulin-stimulated Akt phosphorylation (Fig. 2D). Although this finding does not formally exclude an effect of cAMP on the upstream activation of Akt, it is consistent with our interpretation that cAMP signaling does affect a post-activation event. Interestingly, other Akt inhibitors such as ceramide (20, 50, 51) and hyperosmolarity (52) also act at a post-activation step, most likely via the action of phosphatases.

Regardless, it is clear that stimulation of Akt activity is not sufficient to explain cAMP mitogenesis; insulin (or serum) maximally stimulates Akt, but these agents by themselves have little or no effect on G1/S entry (35). To gain new insight about the mitogenic synergism between cAMP and insulin, identification of early signaling events of cAMP and how they are transduced into mitogenic activity is needed. The small G-protein Rap1b is a convergent target for apparent divergent processes emanating from the cAMP signal, which rapidly activates and phosphorylates Rap1b via the nucleotide exchange protein Epac, and PKA, respectively. Thus, Rap1b is an attractive candidate target for the transduction of the cAMP signal into Akt.
late has been postulated despite that in these cells Rap1 activation has been linked to differentiation rather than proliferation. Second, in C6 glioma cells where cAMP is anti-mitogenic, cAMP-dependent inhibition of Rap1b mediates an inhibition of Akt with slow kinetics that is unrelated to PKA or Epac (33). This scenario contrasts with solid evidence showing that cAMP is a strong activator of Rap1 in cells where cAMP stimulates or inhibits mitogenesis (29, 37, 44, 45, 53, 54) and that the anti-mitogenic effects of cAMP in NIH3T3 cells are mediated by Rap1 activation in a PKA-dependent manner (41, 55). Third, the studies described here in the PCCL3 thyroid cell line, characterized by a robust and rapid inhibition of Akt by cAMP, require activation of both PKA and Rap1b. In this model system, cAMP stimulation of G,S entry has been critically linked to a relay signal originated from activated and phosphorylated Rap1b (39). Evidently, other scenarios are likely to be found, given the diversity of signals transduced by Rap1 and the multiple processes related to the relay of the Rap1 signal (56).

On the Requirement for Rap1b Activation and Phosphorylation for cAMP Inhibitory Signaling to Akt—The striking finding in this report is that activation of Rap1b alone is not sufficient for the transduction of the cAMP signal into Akt. It is evident from our results that an additional signal, phosphorylation of Rap1b-S179, is required for Rap1 inhibition of Akt. This constitutes a phosphorylation-dependent step on a G-protein itself, Rap1, critical for its action on a biochemical process. We have shown recently an obligatory role for activation and phosphorylation of Rap1b in a biological response, i.e. cAMP-mediated G,S entry (39). Although solid evidence exists that in NIH3T3 cells cAMP inhibition of mitogenesis is mediated by Rap1b and PKA (41), it is not known yet whether phosphorylation of Rap1b is critically involved in this biological response. The experiments described here, the use of double Rap1b mutants and an Epac construct that is not regulated by cAMP (ΔEpac), unequivocally implicate an obligatory role for activated and phosphorylated Rap1b in relaying the inhibitory signal into Akt. Although the constitutively active Rap1b mutant markedly inhibited Akt, its phospho-deficient version eliminated cAMP inhibition of Akt (Figs. 6 and 8), despite being fully active (GTP binding, data not shown). ΔEpac, which activates Rap1b independently of cAMP, inhibited Akt activity when co-expressed with Rap1b-WT but not when co-expressed with its phospho-deficient version, Rap1b-S179A. In this way, the cAMP-insensitive ΔEpac mimicked the cAMP inhibition of Akt in a manner that is dependent on another cAMP action, PKA phosphorylation of Rap1b-S179. The dissociation of Rap1 activation and phosphorylation under these conditions most likely reflects the different relative affinities of cAMP for Epac versus PKA (57). Thus, although Rap1b-WT and ΔEpac are fully active in the ability to inhibit Akt in the absence of a cAMP stimulus, the fully activated Rap1b (GTP-bound) in the absence of its Ser phoshorylation is unable to transduce the cAMP signal into inhibition of Akt activity, implicating PKA phosphorylation of Rap1b-S179 as a critical co-signal for Rap1b signaling.

These findings have important consequences that need to be further discussed. First, what is the role of the phospho-Ser phosphorylation signal on an activated Rap1b? The double mutant Rap1b-S179A does a similar inhibitory effect on cAMP inhibition of Akt as does S17N, suggesting that phosphorylation of residue Ser does not play a major role in the activation of endogenous Rap1b. This is consistent with the finding that Rap1 phosphorylation is not required for Epac-dependent Rap1 activation (45). Additionally we find that the subcellular localization of Rap1b does not appear to be altered by its phosphorylation state (39), suggesting that the phospho-S179A signal is not involved in the subcellular targeting of Rap1b. An attractive hypothesis is that the signal emanating from phospho-S179-Rap1b is related to the discrimination of effectors, most likely as a reflection of differential binding affinities. Kataoka and co-workers (58) reported that PKA phosphorylation of Rap1 reduces its affinity for the protein kinase c-Raf, blocking the ability of Rap to inhibit Ras-dependent Raf activation. Conversely, phosphorylation of Rap1b seems to be necessary for activation of B-Raf (59). These kinases are critical for the activation of the MAPK signaling cascade. Although cAMP stimulation of mitogenesis in thyroid appears to be unrelated to MAPK activation (60, 61), we have recently uncovered that in the PCCL3 system cAMP-mediated G,S entry depends on activation of MAPK. Moreover, the cAMP/Epac-dependent inhibition of Akt follows a time course identical to the activation MAPK. In this system, cAMP is a genuine mitogen (40, 42, 48), but in an apparent paradox it inhibits Akt activity. A likely scenario arises from the observation that Akt phosphorylates and inhibits the MAPK stimulator c-Raf on Ser (62); Rap1b may play a gating function in the cross-talk Akt-Raf-MAPK and in this way actively participate in the biological synergism between cAMP and growth factors like insulin/IGF-1. Although Akt activation is regarded as an anti-apoptotic and transforming signal, it is tempting to speculate that cAMP/phospho-Rap1b inhibition of its activity may represent a physiological event in the multi-factor process of cAMP stimulation of mitogenesis. The mechanism(s) involved in the phospho-Rap1b-GTP inhibition of Akt are unknown at this time. However, given that our finding suggests that cAMP inhibition of Akt affects a post-activation step, it is tempting to propose that cAMP/phospho-Rap1b stimulates a phosphatase that dephosphorylates and inhibits Akt.

Acknowledgments—We thank Drs. J. L. Bos (The Netherlands) for providing the Epac and RapGAP plasmids and A. Toker (Harvard Medical School) for the Akt plasmids.

REFERENCES

1. Bellacosa, A., Testa, J. R., Staal, S. F., and Tsichlis, P. N. (1991) Science 254, 274–277.
2. Testa, J. R., and Bellacosa, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10985–10989.
3. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) Annu. Rev. Biochem. 68, 965–1014.
4. Brazil, D. P., and Hemmings, B. A. (2001) Trends Biochem. Sci. 26, 657–664.
5. Kataoka, T., and Bellacosa, A. (1999) Genes Dev. 13, 599–602.
6. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kaalauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736.
7. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) EMBO J. 15, 6541–6551.
8. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295.
9. Toker, A., and Cantley, L. C. (1997) Nature 387, 673–676.
10. Andjelkovic, M., Jakubcewicz, T., Cron, P., Ming, X. F., Han, J. W., and Hemmings, B. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5659–5704.
11. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668.
12. Freech, M., Andjelkovic, M., Ingleby, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 8474–8481.
13. Alessi, D. R., James, S. R., Dawes, C. P., Holme, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) EMBO J. 16, 2691–2699.
14. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570.
15. Toker, A., and Newton, A. C. (2000) J. Biol. Chem. 275, 8271–8274.
16. Delommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11211–11216.
17. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2956–2927.
18. Masahama, T., and Dixon, J. E. (1999) Trends Cell Biol. 9, 125–128.
19. Sato, S., Fujita, N., and Tsuruo, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837.
20. Schubert, K. M., Scheid, M. P., and Duronio, V. (2000) J. Biol. Chem. 275, 13330–13335.
21. Laine, J., Kunste, G., Obata, T., Sha, M., and Noguchi, M. (2000) Mol. Cell 6, 395–407.
22. Maira, S. M., Galetic, I., Brazil, D. P., Kaeche, S., Ingleby, E., Thelen, M., and Hemmings, B. A. (2001) Science 294, 374–380.

‡ Lou, L., Ribeiro-Neto, F., and Altschuler, D. L., manuscript in preparation.
cAMP Inhibits Akt via Phospho-Rap1b

23. Pekarsky, Y., Koval, A., Hallas, C., Bichi, R., Tresini, M., Malstrom, S., Russo, G., Tsichlis, P., and Croce, C. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3628–3633
24. Dumont, J. E., Jauniaux, J. C., and Roger, P. P. (1989) *Trends Biochem. Sci.* **14**, 67–71
25. Boynton, A. L., and Whitfield, J. F. (1983) *Adv. Cyclic Nucleotide Res.* **15**, 193–294
26. Richards, J. S. (2001) *Mol. Endocrinol.* **15**, 209–218
27. Gonzalez-Robayna, I. J., Falender, A. E., Ochsner, S., Firestone, G. L., and Richards, J. S. (2000) *Mol. Endocrinol.* **14**, 1283–1300
28. Filippa, N., Sable, C. L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999) *Mol. Cell. Biol.* **19**, 4899–5000
29. Tsygankova, O. M., Saaavedra, A., Rebhun, J. F., Quilliam, L. A., and Meinkoth, J. L. (2001) *Mol. Cell. Biol.* **21**, 1921–1929
30. Cass, L. A., Summers, S. A., Prendergast, G. V., Backer, J. M., Birnbaum, M. J., and Meinkoth, J. L. (1999) *Mol. Cell. Biol.* **19**, 5882–5891
31. Forti, F. L., and Armelin, H. A. (2000) *Endocr. Res.* **26**, 911–914
32. Kim, S., Lee, K., Kim, D., Koh, H., and Chung, J. (2001) *Trends Endocrinol. Metab.* **12**, 386–390
33. Wang, L., Liu, F., and Adamo, M. L. (2001) *J. Biol. Chem.* **276**, 37242–37249
34. Graves, L., and Lawrence, J., Jr. (1996) *Trends Endocrinol. Metab.* **7**, 43–50
35. Rozengurt, E. (1986) *Science* **234**, 161–166
36. Altschuler, D., and Lapetina, E. G. (1993) *J. Biol. Chem.* **268**, 7527–7531
37. Altschuler, D. L., Peterson, S. N., Ostrowski, M. C., and Lapetina, E. G. (1995) *J. Biol. Chem.* **270**, 10373–10376
38. Altschuler, D. L., and Ribeiro-Neto, F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7475–7479
39. Ribeiro-Neto, F., Urban, J., Leme, N., Loh, L., and Altschuler, D. L. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 5418–5423
40. Medina, D. L., and Santisteban, P. (2000) *Eur. J. Endocrinol.* **143**, 161–178
41. Schmitt, J. M., and Stork, P. J. (2001) *Mol. Cell. Biol.* **21**, 3671–3683
42. Kimura, T., Dumont, J. E., Fusco, A., and Golstein, J. (1999) *Nature* **396**, 785–789
43. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Houseman, D. E., and Graybiel, A. M. (1998) *Science* **282**, 2275–2279
44. de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittighofer, A., and Bos, J. L. (1998) *Nature* **396**, 474–477
45. De Blasi, A. (2001) *Curr. Biol.* **11**, 140, 2275–2279
46. Kimura, T., Dumont, J. E., Fusco, A., and Golstein, J. (1999) *Eur. J. Endocrinol.* **140**, 94–103