ACTH Promotion of p27^{Kip1} Induction in Mouse Y1 Adrenocortical Tumor Cells is Dependent on Both PKA Activation and Akt/PKB Inactivation†

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ABSTRACT: Here we report antimitogenic mechanisms activated by the adrenocorticotropic hormone (ACTH) in the mouse Y1 adrenocortical tumor cell line. ACTH receptors activate the Gαs/adenylate cyclase cAMP/PKA pathway to promote dephosphorylation of Akt/PKB enzymes, leading to induction of the cyclin-dependent kinases' (CDKs) inhibitor p27^{Kip1}. Y1 cells display high constitutive levels of phosphorylated Akt/PKB dependent on chronically elevated c-Ki-Ras·GTP and PI3K activity. Expression of the dominant negative mutant RasN17 in Y1 cells results in strong reduction of both c-Ki-Ras·GTP and phosphorylated Akt/PKB, which are restored by FGF2 treatments. Inhibitors of PI3K lead to rapid dephosphorylation of Akt/PKB and block phosphorylation of Akt/PKB promoted by FGF2. ACTH rapidly promotes dephosphorylation of Akt/PKB in Y1 adrenal cells, while constitutively high levels of c-Ki-Ras·GTP remain unchanged. ACTH and cAMP elevating agents fail to cause Akt/PKB dephosphorylation in PKA-deficient clonal mutants of Y1 cells. In addition, cholera toxin, forskolin, and 8BrcAMP all mimic ACTH, causing dephosphorylation of Akt/PKB in wild-type Y1 cells. ACTH is unable to prevent Akt/PKB phosphorylation, promoted by FGF2 in clonal lines of RasN17−Y1 transfectants displaying negligible levels of c-Ki-Ras·GTP. ACTH promotes strong p27^{Kip1} protein induction in wild-type Y1 adrenocortical cells but not in PKA-deficient Y1-clonal mutants nor in RasN17−Y1 transfectants. PI3K inhibitors induce p27^{Kip1} protein in all cells studied, i.e., wild type and transfectants. The inverse correlation between levels of phosphorylated Akt/PKB and of p27^{Kip1} protein caused by ACTH suggests a novel antimitogenic pathway activated by ACTH and mediated by cAMP/PKA in the mouse Y1 adrenocortical tumor cell line.

The extent to which the mitogenic signaling network in a mammalian cell is cell-type-specific remains unclear. In the case of adrenocortical cells, the signaling circuitry that controls cell cycle and growth has not been particularly characterized. To approach this subject, we chose to focus on characterizing mitogenic and antimitogenic signals initiated in ACTH receptors of the mouse Y1 adrenocortical tumor cell line (*1*).

In Y1 adrenocortical cells, cell-cycle-arrested at the G_0/G_1 boundary, FGF2[1] elicits a strong mitogenic response, including (a) activation of ERK1/2 (2−10 min), (b) transcription activation of the immediate early genes c-fos, c-jun, and c-myc (10−30 min), (c) induction of the cyclin D1 protein by 5 h (Schwindt, T. T., and Armelin, H. A. Unpublished results.), and (d) onset of DNA synthesis initiation by 8 h (*2*, *3*). ACTH blocks this FGF2 mitogenic response at early and middle G_1 phase, while ERK-MAPK activation and c-Fos and cyclin D1 induction remain at maximal levels (*1*−*4*). The interaction between ACTH and FGF2 is certainly a complex phenomenon. Our aim is to uncover the signaling subset, within the signaling network underlying regulation of the $G_0 \rightarrow G_1 \rightarrow S$ transition, that integrates the antagonistic signals initiated at ACTH and FGF2 receptors.

Since the early observations of Masui and Garren in 1971 (*5*), ACTH antimitogenic effects have been believed to be exerted via the cAMP/PKA pathway (*2*, *6*), but its mechanisms are still unknown. Recently (*7*, *8*), we reported that ACTH promotes dephosphorylation of the Akt/PKB enzymes and degradation of c-Myc protein, both antimitogenic effects mediated by the cAMP/PKA pathway. In this report, we show that ACTH induces the production of p27^{Kip1} protein, an inhibitor of Cdk2. This allows us to describe a complete signaling pathway initiated in ACTH receptors that can block passage through cell cycle G_1 phase in Y1 adrenocortical cells.

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[1] Abbreviations: FGF2, basic fibroblast growth factor; ACTH, adrenocorticotropin; Gαs, stimulatory G protein alpha subunit; cAMP, adenosine 3′,5′-cyclic monophosphate; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; CDK, cyclin-dependent kinase; PI3K, phosphatidylinositol 3-kinase; 8BrcAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor; FCS, fetal calf serum; MMTV, mouse mammary tumor virus; MEK1, MAP kinase kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF, insulin growth factor; G protein, GTP-binding protein; FKH, forkhead family of transcription factors; CTX, cholera toxin; PTEN/MMAC/TEP, phosphatase and tensin homologue deleted on chromosome 10; DME, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; Epac, exchange protein directly activated by cAMP; Neo, neomycin gene; G418, geneticin 418; GST, glutathione-S transferase; RBD, Raf1 binding domain.

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Akt/PKB is a mitogenic and antiapoptotic serine/threonine protein kinase that is activated by phosphorylation of Thr308 and Ser473, through a process dependent on PI3K and stimulated by growth factors (*9*). We fully document here that Y1 adrenocortical cells display high constitutive levels of phosphorylated Akt/PKB that depends on elevated levels of c-Ki-Ras·GTP and PI3K activity. In addition, we demonstrate that ACTH promotes rapid dephosphorylation of Akt/PKB in a manner entirely dependent on the Gsα/adenylate cyclase/cAMP/PKA pathway, but without changing c-Ki-Ras·GTP elevated levels. Furthermore, the pathway connecting PKA to Akt/PKB is only operative if the high constitutive levels of c-Ki-Ras·GTP, found in Y1 adrenocortical cells, are sustained. To complete our characterization of this antimitogenic signaling loop initiated in ACTH receptors, we show a strict sequential correlation between dephosphorylation of Akt/PKB and induction of the $p27^{Kip1}$ protein.

Recent reports show that the c-Myc protein downregulates $p27^{Kip1}$ expression to release cell cycle progression (*10*−*13*). However, others (*14*) have not observed correlation between c-Myc protein levels and $p27^{Kip1}$ expression. These apparently contradictory results could be explained by different specificities in c-Myc regulatory pathways among different cell types. In Y1 adrenocortical cells, ACTH promotes both degradation of the c-Myc protein (*8*) and dephosphorylation of Akt/PKB, but only dephosphorylation of Akt/PKB correlates with induction of $p27^{Kip1}$ expression.

EXPERIMENTAL PROCEDURES

Cell Line Cultivation. Cells from the mouse Y1 adrenocortical tumor cell line (*15*) and Y1-transfectant clonal lines carrying, respectively, dominant negative mutant genes ras N17 and PKA-R1, were grown in 10% FCS-DME minus or plus 100 μg/mL G418. To arrest the cell cycle at G_0/G_1 boundary, we incubated cells growing exponentially in 10% FCS-DME for 48 h in SFM (serum-free medium). In SFM, Y1 cells are viable and fully responsive to both FGF2 and ACTH.

RasN17−*Y1 Clonal Lines.* Plasmid pMMrasDN is a construct containing the Neo gene marker and the rasN17 under the control of the MMTV promoter (*16*, *17*) obtained from Dr. L. Feig (Tufts University, Boston) that was transfected into Y1 cells and neutraly selected with G418 to generate clonal lines exhibiting dexamethasone-inducible RasN17 protein. Y1−Ras 1.5 and Ras 3.1 transfectant clones (see Figure 2) express RasN17 in a dexamethasone dose-dependent manner: 10^{-10}M dexamethasone is sufficient to increase RasN17 protein levels by more than 10 fold. In addition, even under dexamethasone-saturating concentrations (0.5 μM), these clonal lines remain responsive to FGF2 displaying ERK1/2 activation, c-Fos and c-Myc induction, and DNA synthesis stimulation. Therefore, the high constitutive levels of c-Ki-Ras·GTP are essentially abolished under maximally induced RasN17 protein, but the Ras pathway is still partially functioning upon FGF2 activation. Y1 ΔRas 2.1 transfectant clone is a control cell line transfected with an empty MMTV vector.

PKA-Deficient Y1 Clonal Lines. Plasmid pG324D is an expression vector carrying the neo gene marker and a mutated form of the PKA-regulatory subunit-1 (R1) gene under the control of the metalothionein promoter (*18*), which was transfected into Y1 cells and doubly selected with G418 and 8BrcAMP to obtain PKA-deficient clonal lines resistant to 8BrcAMP. Induction of PKA-deficiency was not a stable phenotype among selected clones. Thus, all Y1 PKA-R1 transfectant clonal lines displayed constitutive PKA deficiency after a few culture passages, independent of the presence of metal inducers. Y1-G1 and G4 transfectant clones show a more than 90% reduction in PKA activity when compared to Y1 wild-type cells treated cAMP derivatives or ACTH. In addition, these clones do not show steroidogenesis stimulation and are resistant to DNA synthesis inhibition by dibutyryl-cAMP and ACTH treatments. Furthermore, in Y1-G1 and G4 PKA-defective clones, residual PKA-dependent biological responses are completely eliminated by the H89 PKA inhibitor (to be detailed elsewhere).

Ras·GTP Assay by Reaction with RBD-GST Fusion Protein (Raf1 Binding Domain−*Glutathione S Transferase).* Cells were lysed in 50mM Tris-HCl pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin. Lysates were collected by centrifugation (10 000 rpm for 10 min at 4 °C) and kept frozen. Aliquots of cell lysates containing 500 μg protein, quantified by the Bradford assay, were incubated with RBD-GST beads for 60 min at 4 °C to bind Ras·GTP. Beads, carrying bound Ras·GTP and recovered by centrifugation, were washed with lysis buffer at 4 °C, suspended in SDS−PAGE sample buffer (100 mM Tris-HCl pH 7.5, 200 mM DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue), and loaded on to 12% SDS−PAGE gels. After electroblotting onto Hybond-C nitrocellulose membranes, using a semidry Bio-Rad apparatus, activated c-Ki-Ras was detected with a mouse polyclonal antibody specific for c-Ki-Ras (Santa Cruz). Each sample of activated Ras, recovered by these procedures, was compared in Western blot assays to total Ras in a aliquot (50μg protein) of the respective lysate not incubated with RBD-GST beads (*19*).

Analysis of AKT/PKB Phosphorylation. Cells were lysed in cold 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 1% w/v bromophenol blue. Lysates were sonicated for 2 min, boiled for 5 min, clarified by centrifugation (14 000 rpm, 5 min, 4 °C), and 150 μg protein aliquots were loaded on 10% SDS−PAGE gels. After electroblotting onto Hybond-C nitrocellulose membranes, using a semidry Bio-Rad apparatus, the total Akt/PKB- or Ser473-phosphorylated Akt/PKB isoforms were detected with monospecific rabbit antibodies (New England Biolabs), followed by a secondary peroxidase-conjugated antirabbit polyclonal antibody for chemiluminescent detection (ECL, Amersham-Pharmacia). A band with greater mobility than the phospho-Akt band was observed in some blots. This band is inespecific, unregulated, and not detected by all lots of polyclonal antibodies.

Levels of $p27^{Kip1}$ Protein. Cells were lysed in 20mM Tris-HCl pH 8.0, 135 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1mM sodium orthovanadate, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μg/mL pepstatin. After quantitation by Bradford assay, aliquots of 150 μg protein were mixed with SDS−PAGE sample buffer and loaded on to 10% SDS−PAGE gels and processed for Western blotting as described above for Akt/PKB protein, using a rabbit polyclonal antibody monospecific for mouse $p27^{Kip1}$ (Santa Cruz). Ponceau

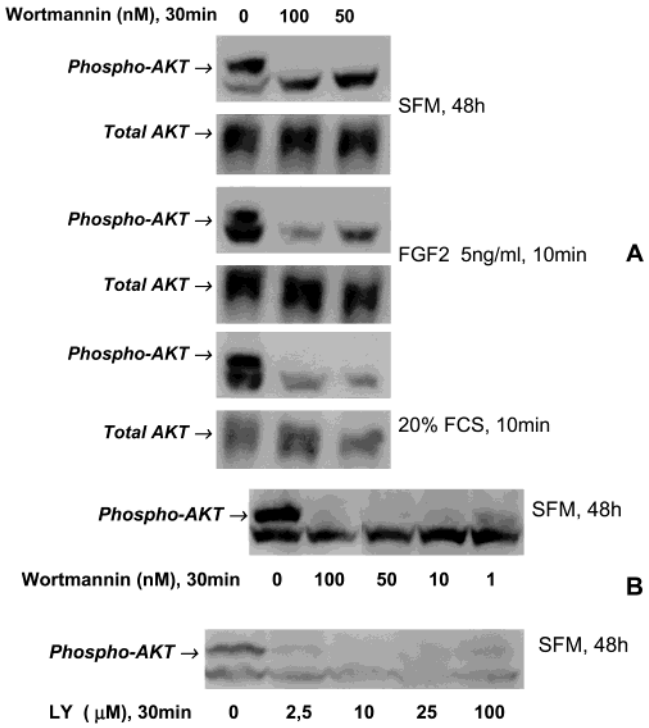

FIGURE 1: Y1 adrenocortical cells constitutively exhibit high steady-state levels of phosphorylated Akt/PKB, dependent on PI3K activity. Lysates of G_0/G_1-arrested Y1 cells that were maintained in SFM or pretreated with, respectively, FGF2 (5ng/mL; 10min) and FCS (20%; 10min) and then were treated with wortmannin (A and B) or LY294002 (B) as indicated. Western immunoblotting assays with monospecific polyclonal antibodies to, respectively, Ser-473-phosphorylated Akt/PKB and total Akt/PKB protein.

staining of electroblotted membrane was used to visually confirm efficient protein transfer.

RESULTS

Constitutively High Levels of Phosphorylated Akt Displayed by Y1 Adrenocortical Cells is Dependent on c-Ki-Ras·GTP and PI3K. Y1 cells, arrested at G_0/G_1 phase of the cell cycle by serum deprivation for 48h in serum free-medium (SFM), display high constitutive levels of Akt/PKB phosphorylated at serine 473 (Figure 1). The PI3K inhibitor wortmannin rapidly and completely abolishes phosphorylated Akt in lysates of G_0/G_1-arrested Y1 cells, irrespective of prior FGF2 or FCS treatment (Figure 1A). Under these conditions, the levels of total Akt/PKB protein remain constant (Figure 1A), indicating that the disappearance of the phosphorylated Akt/PKB form is exclusively due to dephosphorylation and not to proteolysis. These results imply that the phosphate group esterified to the serine 473 of Akt/PKB is subjected to a relatively high turnover and that in order to keep high constitutive steady-state levels of phosphorylated Akt/PKB the phosphorylation rate prevails over dephosphorylation. Dose−response curves for two PI3K inhibitors, wortmannin and LY294002, show that 30min treatment with, respectively, 10 nM and 10 μM of both inhibitors are sufficient to eliminate phosphorylated Akt/PKB (Figure 1B). On the other hand, specific inhibitors of MEK1 (PD98059) and PKC (GF109203X and Go6983) (Figure 2) and PKA (H89) (Figure 7E) have no effect on constitutive levels of phosphorylated Akt/PKB, suggesting that enzymes activities are not necessary to maintain phosphorylated Akt/PKB levels in G_0/G_1-arrested Y1 cells.

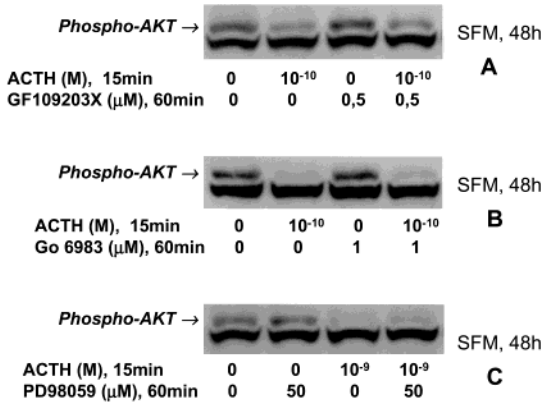

FIGURE 2: Constitutively high levels of phosphorylated Akt/PKB are independent of MEK1 and PKC inhibitors as well as the dephosphorylation of Akt/PKB promoted by ACTH. Lysates of G_0/G_1-arrested Y1 cells, treated as indicated by PKC GF109203X (A) and Go6983 (B) inhibitors and MEK1 PD98059 (C) inhibitor, were analyzed by Western blot using the monospecific rabbit Ser473-phosphorylated Akt/PKB antibody.

Y1 cells possess amplified and overexpressed c-Ki-ras proto-oncogene (*20*, *21*), exhibiting high constitutive levels of c-Ki-Ras·GTP. Attenuation of the constitutive c-Ki-Ras·GTP levels, by way of the dominant negative mutant RasN17, leads to drastic reduction of constitutive levels of phosphorylated Akt/PKB. A vector carrying RasN17 under the MMTV promoter was transfected into Y1 cells, yielding conditional transfectants that, upon dexamethasone treatment, display elevated levels of the Ha-Ras mutant (Figure 3A) and negligible levels of both c-Ki-Ras·GTP (Figure 3B) and phosphorylated Akt/PKB (Figure 3C). FGF2 partially restores the levels of c-Ki-Ras·GTP (Figure 3B) as well as of phosphorylated Akt/PKB (Figure 3C) in these dexamethasone-induced RasN17 transfectants. The signaling pathway activated by FGF2 to phosphorylate Akt/PKB is blocked by wortmannin, indicating that it is mediated by PI3K (Figure 3C). The Western blot experiment of Figure 3C was independently repeated 6 times, yielding systematically the same results. These results suggest that first, constitutive levels of phosphorylated Akt/PKB in Y1 adrenocortical tumor cells are maintained by high constitutive levels of c-Ki-Ras·GTP, via PI3K, and second, in RasN17 transfectants, FGF2 receptors transiently activate both c-Ki-Ras·GTP and AKT, mimicking normal cell function. It is not possible, however, to distinguish between the two following alternate pathways: FGF2−Rec → Ki-Ras·GTP → P13K → Akt and FGF2−Rec → P13K → Akt (see scheme depicted in Figure 9).

ACTH Receptors Activate the PKA Pathway to Rapidly Promote Dephosphorylation of Akt/PKB. Physiological concentrations of $ACTH_{39}$ rapidly promote dephosphorylation of Akt/PKB in G_0/G_1-arrested or exponentially growing Y1 cells (Figure 4A). In addition, kinetic experiments show that sustained treatment of 1 nM $ACTH_{39}$ leads to negligible levels of phosphorylated Akt/PKB in 2 min, remaining very low up to 4 h (Figure 4B).

$ACTH_{39}$ does not interfere with levels of either total c-Ki-Ras protein or c-Ki-Ras·GTP complex in G_0/G_1-arrested Y1 cells, indicating that ACTH acts downstream of c-Ki-Ras·GTP to promote dephosphorylation of Akt/PKB. In addition, inhibition of PI3K with wortmannin also does not interfere with c-Ki-Ras·GTP constitutive levels (not shown).

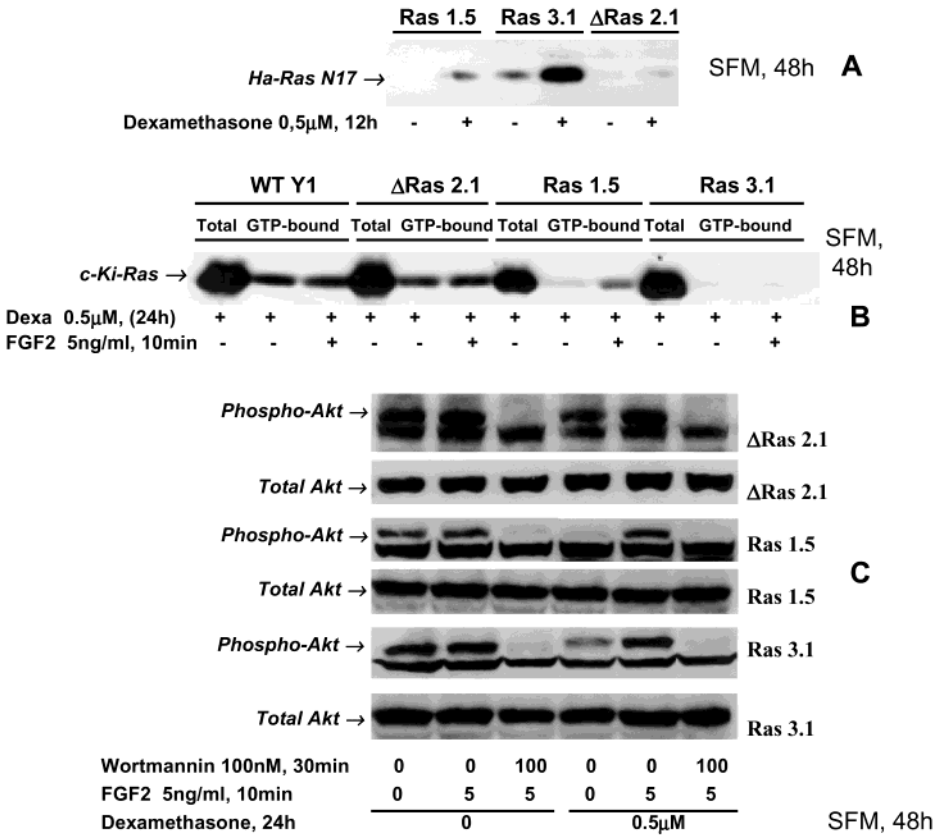

FIGURE 3: Dexamethasone-induced c-Ha-RasN17 in Y1−RasN17 transfectant clones reduces high constitutive levels of both c-Ki-Ras·GTP and phosphorylated Akt/PKB, which can be restored by FGF2 treatment. G_0/G_1-arrested Y1 cells and Y1-transfectant clones (Ras1.5, Ras3.1, and ΔRas2.1) were treated as indicated and lysed to assay for expression of c-Ha-RasN17 (A) and levels of both c-Ki-Ras·GTP (B) and phosphorylated Akt (C). A: Western blot with antibodies against the human c-Ha-RasN17 protein. B: Assays of c-Ki-Ras·GTP levels by binding to RBD-GST beads followed by Western blot with antibodies against mouse c-Ki-Ras protein as described in Methods. C: Assays for levels of phosphorylated Akt and total Akt protein.

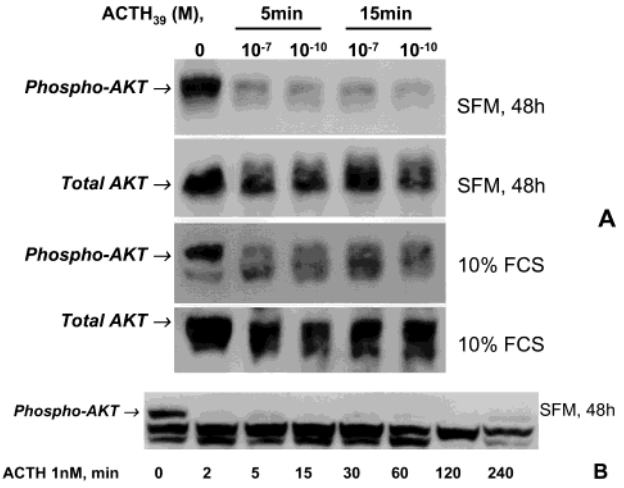

FIGURE 4: ACTH promotes dephosphorylation of Akt/PKB in Y1 adrenocortical cells. A: Exponentially growing (10% FCS) and G_0/G_1-arrested (SFM, 48h) Y1 adrenocortical cells were treated as indicated, lysed, and assayed for, respectively, phosphorylated Akt and Akt protein by Western blot. B: Kinetics of Akt/PKB dephosphorylation promoted by 1nM ACTH in G_0/G_1-arrested Y1 adrenocortical cells assayed by Western Blot.

In RasN17-expressing clones of Y1 cells, displaying negligible levels of both c-Ki-Ras·GTP and phosphorylated Akt/PKB, FGF2 (5 ng/mL, 10min) increases both c-Ki-Ras·GTP and phosphorylated Akt/PKB (Figure 3). This activation of Akt/PKB by FGF2 is blocked by wortmannin, due to inhibition of PI3K. However, addition of $ACTH_{39}$ (10^{-10}–10^{-7} M) together with FGF2 (5ng/mL, 10min) does not prevent upregulation of phospho-Akt/PKB (Figure 5). Thus, the ability of ACTH to promote dephosphorylation of Akt depends on the constitutively high levels of c-Ki-Ras·GTP found in the Y1 adrenocortical tumor cell line.

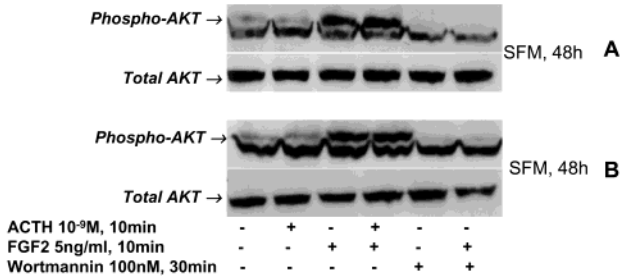

FIGURE 5: Concomitant addition of ACTH and FGF2 does not prevent phosphorylation of AKT stimulated by FGF2 in transfectant clones of Y1 cells expressing high levels of dominant negative RasN17. Prior treatment with wortmannin blocks AKT phosphorylation by FGF2. G_0/G_1-arrested Ras 1.5 (A) and 3.1 (B) transfectant clones, treated with FGF2, ACTH, and wortmannin as indicated, were lysed to assay for phosphorylated Akt/PKB by Western blot.

Several lines of evidence indicate that ACTH receptors activate the Gαs/adenylate cyclase/cAMP/PKA pathway to promote dephosphorylation of Akt/PKB in Y1 cells. First, the inactive peptide $ACTH_{7-38}$ antagonizes $ACTH_{39}$ activity via competition for the binding site in ACTH receptors (*22*). Dephosphorylation of Akt/PKB promoted by 0.1nM $ACTH_{39}$ is completely blocked by a 10^4-fold higher concentration of

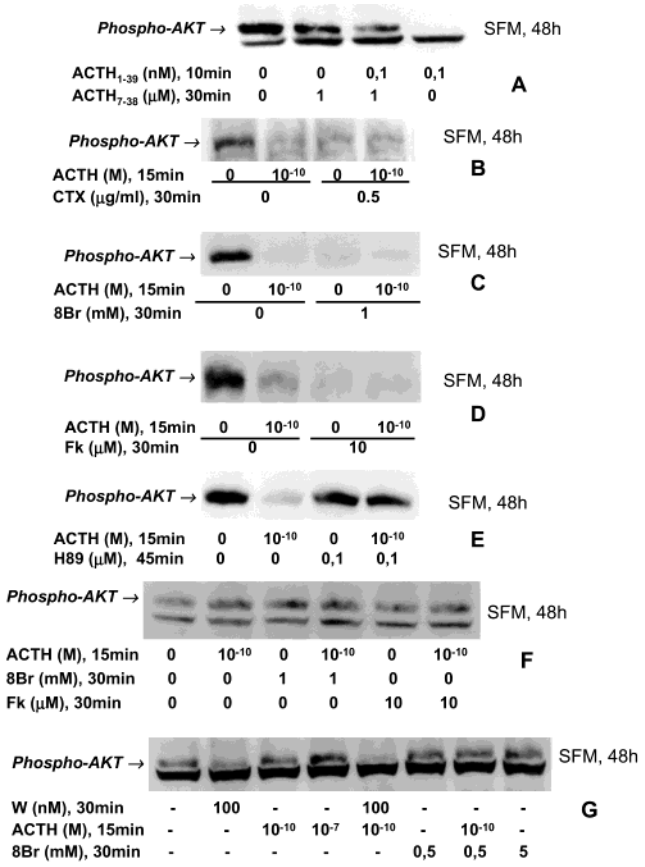

FIGURE 6: Effects of inhibitors and activators of the ACTH-receptor/Gs proteins/adenylate cyclase/cAMP/PKA pathway on dephosphorylation of AKT promoted by $ACTH_{39}$. G_0/G_1-arrested wild-type Y1 adrenocortical cells and PKA-deficient Y1 transfectant clones were treated with $ACTH_{39}$, inhibitors, and activators as indicated, lysed, and assayed for phosphorylated AKT by Western blot. A−E, wild-type Y1 cells; F and G, Y1 PKA-deficient transfectant clones Y1-G1 and Y1-G4, respectively. A, $ACTH_{7-38}$, competitive inhibitor for binding to ACTH receptors; B, cholera toxin (CTX), activator of Gα; C, 8BrcAMP (8Br), activator of PKA; D, forskolin (Fk), activator of adenylate cyclase; E, H89, inhibitor of PKA; F, 8BrcAMP (8Br) and forskolin (Fk); G, wortmannin (W) and 8BrcAMP (8Br).

$ACTH_{7-38}$ (Figure 6A), implying that this dephosphorylation effect of $ACTH_{39}$ is specifically mediated by ACTH receptors. Second, cholera toxin (Figure 6B), forskolin (Figure 6D), and 8Br-cAMP (Figure 6C) activators of Gαs, adenylate cyclase, and PKA, respectively, all mimic ACTH causing downregulation of phosphorylated Akt/PKB in G_0/G_1-arrested Y1 cells. Third, in parental Y1 cells, the PKA inhibitor H89 blocks dephosphorylation of Akt/PKB promoted by $ACTH_{39}$ (Figure 6E), whereas in clonal mutants of Y1 cells deficient in PKA, $ACTH_{39}$, forskolin, and 8Br-cAMP fail to cause dephosphorylation of Akt/PKB (Figure 6F,G). Finally, inhibitors of PKC and MEK/ERK pathways, respectively, GF109203X, Go6983, and PD98059, do not block dephosphorylation of Akt/PKB promoted by $ACTH_{39}$ (Figure 2), indicating that this hormone promotes dephosphorylation of Akt/PKB independently of MEK/ERK and PKC pathways.

ACTH Sequentially Promotes Akt/PKB Dephosphorylation and p27Kip1 Induction. As expected for mitogens, in G_0/G_1-arrested Y1 cells, sustained treatment with FGF2 or FCS causes an accentuated decrease in $p27^{Kip1}$ steady-state levels by 5 h, whereas 1nM $ACTH_{39}$ leads to strong elevation in $p27^{Kip1}$ within 2 h (Figure 7A). In combinations of $ACTH_{39}$ and FGF2 or FCS, $ACTH_{39}$ prevails, leading to high levels of $p27^{Kip1}$ expression (Figure 7A). Under the same conditions, wortmannin also causes a strong increase in $p27^{Kip1}$ levels independent of the presence of $ACTH_{39}$ (Figure 7A). On the other hand, in RasN17, transfectants displaying negligible levels of both c-Ki-Ras·GTP and phosphorylated Akt, in which ACTH is unable to prevent transient phosphorylation of Akt stimulated by FGF2 (see Figure 5). ACTH per se does not induce $p27^{Kip1}$ and does not block downregulation of $p27^{Kip1}$ induced by FGF2 and FCS (Figure 7B).

In PKA-defective Y1 mutants, wortmannin down-regulates phosphorylated Akt/PKB, leading to a $p27^{Kip1}$ induction over the steady-state levels, whereas $ACTH_{39}$ does not down-regulate phosphorylated Akt/PKB (see, respectively, panels F and G of Figure 6) and does not induce $p27^{Kip1}$ expression, independent of treatment time, or the presence of FGF2 or FCS (Figure 8). These $p27^{Kip1}$ Western blot experiments were repeated 3 times, systematically yielding identical results. We conclude that there is a strict correlation between the ability of ACTH to promote dephosphorylation of Akt and to induce production of $p27^{Kip1}$. This suggests that induction of $p27^{Kip1}$ by ACTH is mediated by dephosphorylation of Akt.

DISCUSSION

Y1 adrenocortical cells display a remarkably tight control of the $G_0 \rightarrow G_1 \rightarrow S$ transition of the cell cycle, despite carrying an amplification of the c-Ki-ras proto-oncogene (*20*), necessary to maintain their tumorigenic phenotype (*8*, *21*). In addition, ACTH blocks the Y1 cell cycle at early and middle G_1 phase, antagonizing the mitogenic effect of FGF2 (*1*, *2*, *23*). Therefore, elucidation of the modes of integrating

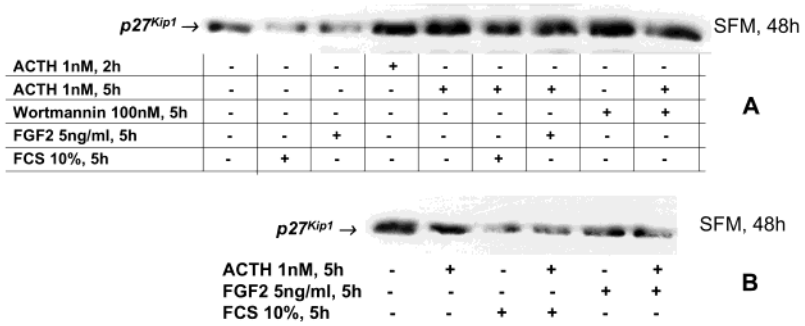

FIGURE 7: ACTH induces $p27^{Kip1}$ expression, irrespective of the FGF2 or FCS presence, in Y1 adrenocortical cells but not in Y1−RasN17 transfectants. G_0/G_1-arrested cells, treated as indicated, were lysed and assayed for $p27^{Kip1}$ by Western blot. A: Wild-type Y1 cells. B: Y1−RasN17 clone Ras 3.1. These results are representative of three independent experiments repeated in identical conditions.

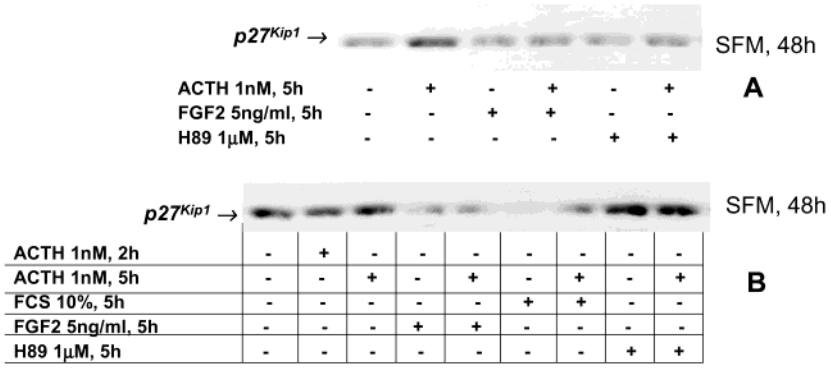

FIGURE 8: ACTH fails to induce p27^{Kip1} protein expression in PKA-deficient Y1 transfectant clones. G_0/G_1-arrested PKA-deficient Y1-G1 (A) and Y1-G4 (B) were treated as indicated, lysed, and assayed for p27^{Kip1} protein by Western blot. The PKA-specific inhibitor, H89, was used to warrant total inhibition of PKA. These results are representative of three independent experiments repeated in identical conditions.

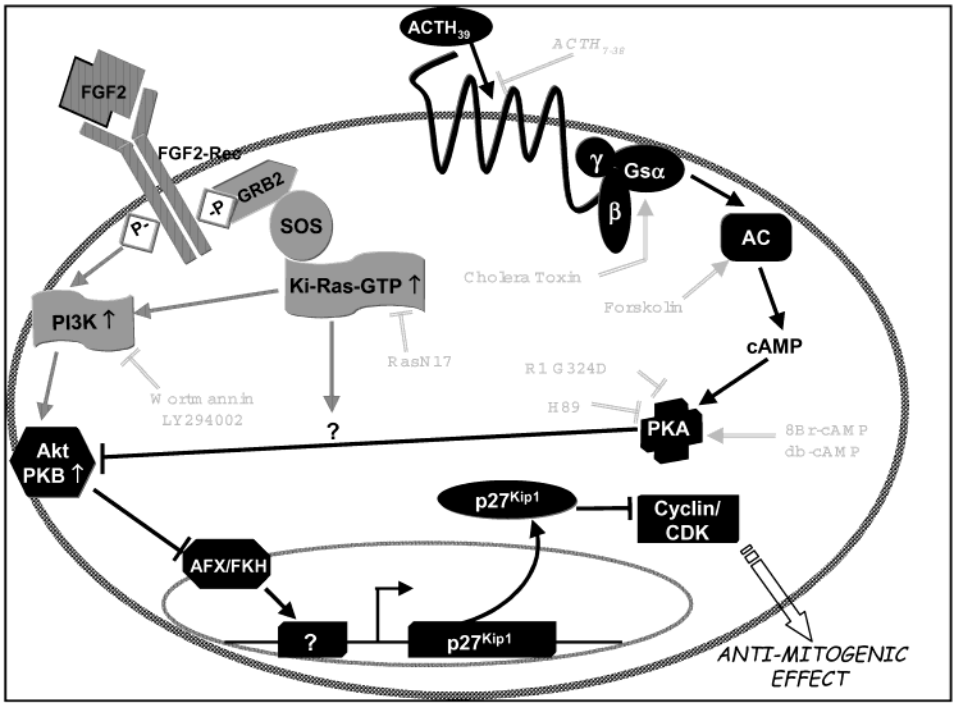

FIGURE 9: Scheme of the proposed antimitogenic pathway activated by ACTH receptors in the mouse Y1 adrenocortical cell line.

signals initiated in receptors of FGF2 and ACTH is likely to uncover regulatory mechanisms underlying the $G_0 \rightarrow G_1 \rightarrow S$ transition. In this report, we show that (a) the high levels of phosphorylated Akt/PKB constitutively displayed by Y1 cells are dependent on chronically elevated c-Ki-Ras·GTP and PI3K activity, (b) FGF2 restores high levels of both c-Ki-Ras·GTP and phosphorylated Akt/PKB in Y1 cells expressing the dominant negative rasN17 gene, (c) ACTH activates the Gαs/adenylate cyclase/PKA pathway to promote, downstream of both c-Ki-Ras·GTP and PI3K, dephosphorylation/deactivation of Akt/PKB, allowing induction of the p27^{Kip1} protein, and (d) ACTH ability to promote dephosphorylation/deactivation of Akt/PKB depends on sustained c-Ki-Ras·GTP high levels found in the Y1 adrenocortical cell line.

Reports from the last years (see ref *9* for a review) have shown that Akt/PKB is a target of PI3K, an enzyme located downstream of p21Ras, in a pathway potently activated by growth factor Tyr-kinase receptors (*24*). These findings critically advanced the elucidation of the PI3K pathway, generating great interest in the biological consequences of Akt/PKB activation (*9*). However, the links between Akt/PKB and the G-protein-coupled receptors are still poorly understood, despite extensive studies on biological effects and modes of activation of Akt/PKB, under way in a number of laboratories (*9*, *25*). For instance, in 3T3 fibroblasts, expression of PI3Kβ confers ability to respond to GPCR agonists with rapid Akt/PKB activation (*26*). In primary rat hepatocytes, endogenous Akt/PKB is rapidly activated through the cAMP/PKA pathway (*27*, *28*). On the other hand, the first report of inhibition of Akt/PKB by activators of the cAMP/PKA pathway, involving opposing effects of Epac and PKA on Akt/PKB activation in HEK-293 cells, was recently published (*29*).

Here we show that Y1 cells display high constitutive levels of phosphorylated Akt/PKB, whose maintenance is completely dependent on PI3K activity (Figure 1). Inhibition of PI3K causes very rapid dephosphorylation of Akt/PKB, implying that Y1 cells possess high steady-state levels of Akt/PKB-phosphatase and the two forms of Akt/PKB, phosphorylated and unphosphorylated, are in a balance of elevated turn over. In addition, the high levels of phospho-

rylated Akt/PKB seem to be a direct consequence of chronic high levels of c-Ki-Ras·GTP found in Y1 cells (Figure 3). This last conclusion is based on judicious experimental exploration of a inducible form of the dominant negative mutant RasN17 to attenuate the activity of endogenous c-Ki-Ras, without complete blockage of the Ras pathway (Figure 3).

ACTH receptors rapidly promote dephosphorylation of Akt/PKB (Figure 4) via activation of the Gαs/adenylate cyclase/cAMP/PKA pathway (Figure 6). However, this effect of PKA (deactivation of Akt/PKB) is dependent on sustained high levels of c-Ki-Ras·GTP found in the Y1 adrenocortical tumor cell line. However, the mechanisms of PKA action, in this case, are still undefined, though we can state that PKA acts on reactions downstream of both c-Ki-Ras·GTP and PI3K. First, ACTH has no effect on the steadystate levels of c-Ki-Ras·GTP (not shown). Second, circumstantial evidence indicates that ACTH does not interfere with PI3K activity: (a) FGF2 induces the cyclin D1 protein in G_0/G_1-arrested Y1 cells, by a process that is abolished by inhibitors of PI3K; (b) $ACTH_{39}$, which antagonizes the mitogenic effect of FGF2 in G_0/G_1-arrested Y1 cells, does not block induction of cyclin D1 *(1*; Schwindt, T. T., and Armelin, H. A. Unpublished results.). Therefore, we are left with three possible explanations for how PKA promotes reduction in Akt/PKB phosphorylation levels: (a) activation of PTEN/MMAC/TEP (*30*); (b) activation of Akt/PKB phosphatases (*31*, *32*); (c) inhibition of PDK1, phosphoinositide-dependent kinase 1 (*14*). None of these three possibilities can presently be ruled out. PTEN, Akt/PKB phosphatases and PDK1 are not direct substrates of PKA, so this PKA effect must be indirect. These results and interpretations are timely and deserve attention, given the importance of the PI3K/PTEN/Akt pathway (*30*) and the recently demonstrated central role played by the proto-oncogene c-Ki-ras in initiation and maintenance of mouse lung tumors (*33*).

Recent reports (*34*, *35*) have shown that activated Akt/PKB phosphorylates forkhead transcription factors (AFX, FKHR and FKHR-L1), keeping them inactive in the cytoplasm (*36*). On the other hand, inactivation of Akt/PKB leads to dephosphorylation of these forkhead transcription factors, allowing migration to the nucleus and transcription activation of some genes, particularly the gene encoding the $p27^{Kip1}$ protein. Therefore, the PI3K/Akt/forkhead factors/$p27^{Kip1}$-gene pathway plays a central role in the control of cell cycle and mitogenesis. Mitogens that are ligands of Tyr-kinase receptors (FGF, PDGF, EGF, IGF, etc.) activate the PI3K/Akt pathway, down-regulating forkhead factors and $p27^{Kip1}$-gene transcription (*37*). Antimitogens promote dephosphorylation of Akt/PKB, liberating forkhead factors to activate transcription of the $p27^{Kip1}$gene (*38*). In Y1 cells, we have seen that high constitutive levels of c-Ki-Ras·GTP, due to amplification and overexpression of the c-Ki-ras proto-oncogene (*17*, *18*), lead to constitutive activation of Akt/PKB and consequently permanent downregulation of $p27^{Kip1}$ expression. On the other hand, we have also shown that ACTH promotes rapid dephosphorylation of Akt/PKB (Figure 4), via the PKA pathway (Figure 6), followed by induction of the $p27^{Kip1}$protein (Figure 7). Although we have not measured phosphorylation of forkhead transcription factors in Y1 cells, these factors are strong candidates to be mediators in the activation of transcription of the $p27^{Kip1}$-gene stimulated by ACTH, as depicted in Figure 9. In fact, the proposed link between ACTH-receptors/cAMP/PKA to Akt/PKB deactivation/$p27^{Kip1}$ induction characterizes a pathway that contributes to suppress the oncogenic activity of the c-Ki-ras oncogene. This proposed ACTH antimitogenic mechanism is not restricted to cell lines with elevated c-Ki-Ras·GTP levels (*39*, *40*); however, high levels of phosphorylated Akt/PKB may be observed by elevating c-ki-ras expression in either normal or tumor cells (*41*, *42*).

We have previously shown that ACTH promotes degradation of the c-Myc protein blocking G_1 progression in the cell cycle of Y1 adrenocortical cells (8*)*, by still undefined mechanisms. Recent reports implicate the c-Myc protein in down-regulation of $p27^{Kip1}$ expression to release cell cycle progression and vice-versa (*10*−*13*). However, others (*14*) have not observed any correlation between c-Myc protein levels and $p27^{Kip1}$ expression, suggesting that c-Myc regulatory pathways are cell-type specific. In Y1 adrenocortical cells, ACTH promotes in a concerted manner both degradation of the c-Myc protein and dephosphorylation of Akt/PKB though only dephosphorylation of Akt/PKB correlates with induction of $p27^{Kip1}$ expression.

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