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Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors

(signal transduction/mitogenic activation/phosphatase inhibitors/phosphatidylinositol 3-kinase)

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ABSTRACT **Treatment of quiescent Swiss 3T3 fibroblasts with serum, or with the phosphatase inhibitors okadaic acid and vanadate, induced a 2- to 11-fold activation of the serine/threonine RAC protein kinase (RAC-PK). Kinase activation was accompanied by decreased mobility of RAC-PK on SDS/PAGE such that three electrophoretic species (*a* to *c*) of the kinase were detected by immunoblot analysis, indicative of differentially phosphorylated forms. Addition of vanadate to arrested cells increased the RAC-PK phosphorylation level 3- to 4-fold. Unstimulated RAC-PK was phosphorylated predominantly on serine, whereas the activated kinase was phosphorylated on both serine and threonine residues. Treatment of RAC-PK *in vitro* with protein phosphatase 2A led to kinase inactivation and an increase in electrophoretic mobility. Deletion of the N-terminal region containing the pleckstrin homology domain did not affect RAC-PK activation by okadaic acid, but it reduced vanadate-stimulated activity and also blocked the serum-induced activation. Deletion of the serine/threonine rich C-terminal region impaired both RAC-PKα basal and vanadate-stimulated activity. Studies using a kinase-deficient mutant indicated that autophosphorylation is not involved in RAC-PKα activation. Stimulation of RAC-PK activity and electrophoretic mobility changes induced by serum were sensitive to wortmannin. Taken together the results suggest that RAC-PK is a component of a signaling pathway regulated by phosphatidylinositol (PI) 3-kinase, whose action is required for RAC-PK activation by phosphorylation.**

The RAC-PKs (for *r*elated to PK*A* and *C* protein kinases; also known as PKB/Akt) represent a subfamily of second messenger-regulated serine/threonine protein kinases (1). Two human genes have been identified, termed *RACα* and *-β* that are 90% homologous (2–4). Both genes appear to be widely expressed in human tissues, implying that they play an important role in cell signaling. Mouse *RACα* (c-*akt*) is the cellular homologue of the viral oncogene v-*akt*, generated by fusion of the Gag protein from the AKT8 retrovirus to the N terminus of mouse RAC-PKα, giving rise to a 105-kDa phosphoprotein that is myristilated at its N terminus (5, 6). The mouse protein is mainly cytosolic (90%), whereas the oncoprotein is apparently equally distributed between the plasma membrane, nucleus, and cytoplasm (6). Human *RACβ* was found to be amplified in 10% of human ovarian carcinomas (4), suggesting the involvement of the RAC-PK subfamily members in regulation of cell growth. The *Drosophila* homologue (D*RAC*) shows 75% homology to the human isoforms and is ubiquitously expressed throughout the *Drosophila* life cycle (7, 8).

All characterized members of the RAC-PK subfamily have a similar domain structure: an N-terminal pleckstrin homology (PH) domain, a centrally located catalytic domain, and a serine/threonine rich C-terminal region (7). The catalytic domain exhibits a high degree of similarity to those of both protein kinase C and protein kinase A, 75% and 65%, respectively (3, 7). Furthermore, RAC-PKs possess a PH domain (9, 10) that is ≈70% similar between mammals, *Drosophila* and *Caenorhabditis elegans* (7). PH domains have been identified in more than 90 signaling and cytoskeletal molecules, many of which associate with membranes (11). The C-terminal region is rich in serine and threonine residues that are conserved between *Drosophila* and human RAC-PK (3, 7) and therefore represents a potential site of kinase regulation by phosphorylation.

We have shown previously that RAC-PK activity from *Drosophila* is developmentally regulated, being ≈8-fold higher in adult flies than in early embryos (7), and have proposed that phosphorylation may be a possible regulatory mechanism of DRAC-PK activity. Here we demonstrate that RAC-PK activity from mammalian cell lines is stimulated by serum and phosphatase inhibitors, and that reversible phosphorylation is a key mechanism of its regulation. The PH domain is required for RAC-PK activation, but is not a target for the activating phosphorylation.

MATERIALS AND METHODS

Construction of Expression Vectors. Hemagglutinin (HA) epitope-tagged constructs (HA-RAC-PKα and HA-ΔNRAC-PKα) were prepared by ligating an HA epitope encoding oligonucleotide to the RAC-PKα (2) and ΔNRAC-PKα (lacking the N-terminal 117 amino acids) cDNAs in-frame with the initiator methionine, in the mammalian expression vector pECE (12). The HA-RAC-PKαΔC and HA-ΔNRAC-PKαΔC constructs (lacking N-terminal 117 and/or C-terminal 59 amino acids, respectively) were prepared by truncation at Leu^{421} using a *Cel*II restriction site. Kinase-deficient HA-RAC-PKαK179A was created by the two-stage PCR technique. All constructs were confirmed by restriction analysis, sequencing, and expression in COS-1 cells.

Cell Culture. Quiescent Swiss 3T3 cells (13) and transfected COS-1 cells (7) were serum-starved for 24 hr, followed by stimulation with 10% fetal calf serum (FCS), 1 μM okadaic acid, or 0.1 mM vanadate prepared with 0.2 mM H_2O_2 (14). Pretreatment of quiescent Swiss 3T3 cells with 200 nM wortmannin and 5 nM rapamycin before serum stimulation was carried out as described (15).

Immunoprecipitation and *in Vitro* Kinase Assay. Swiss 3T3 and COS-1 extracts were prepared by lysing cells in a buffer

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Abbreviations: RAC-PK, RAC protein kinase; PH, pleckstrin homology; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; HA, hemagglutinin; PI, phosphatidylinositol.

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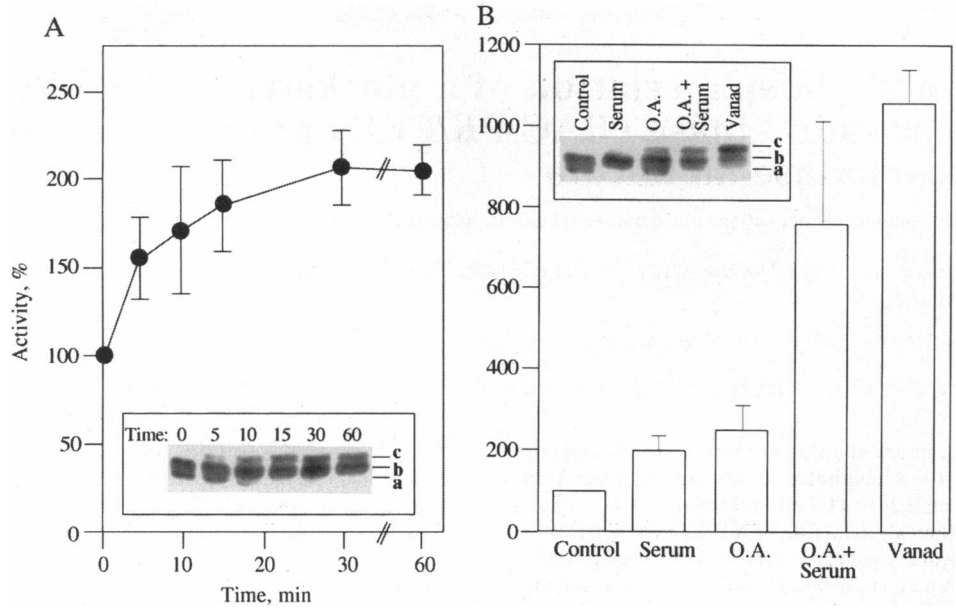

FIG. 1. Effects of serum and phosphatase inhibitors on RAC-PK from Swiss 3T3 cells. (*A*) Time course of RAC-PK activation following serum stimulation. Kinase activity is the average (±SD) of three experiments. Activity of RAC-PK immunoprecipitated from quiescent cells was taken as 100%. (*Inset*) Immunoblot analysis of RAC-PK. (*B*) Effect of phosphatase inhibitor treatment on RAC-PK activity. Quiescent cells (Control) were treated for 120 min with either 10% serum, 1 μM okadaic acid, 1 μM okadaic acid/10% serum, or with 0.1 mM vanadate for 15 min. Kinase activity is the average (±SD) of two experiments with duplicate immunoprecipitates. (*Inset*) Representative immunoblot showing RAC-PK migration following the above treatments.

containing 50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/120 mM NaCl/1 mM EDTA/50 mM NaF/40 mM β-glycerophosphate/0.1 mM sodium vanadate/2 μg/ml leupeptin/2 μg/ml aprotinin/1 mM benzamidine/0.5 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 15 min at 12,000 × *g* at 4°C. Human and mouse RAC-PKα were immunoprecipitated using a rabbit polyclonal antibody specific for the conserved C-terminus (anti-RAC$^{469-480}$; ref. 2). These antisera also recognize the β isoform, as its C-terminus differs from that of RAC-PKα only in the last three amino acids (4). HA-tagged versions of RAC-PKα were immunoprecipitated using the 12CA5 monoclonal antibody. Immune complexes were collected using protein A-Sepharose beads. RAC-PK activity was assayed as described with myelin basic protein as substrate (2). Immunoprecipitation and assay of p70^{s6k} were as reported (15).

Immunoblot Analysis. Cell extracts and immunoprecipitates were resolved by SDS/7.5% PAGE, transferred to Immobilon P membranes, and incubated either with the anti-RAC$^{469-480}$ antibody, or with the 12CA5 antibody. Detection was performed using alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibody.

***In Vivo* Labeling and Phosphoamino Acid Analysis.** Swiss 3T3 cells arrested in phosphate-free DMEM/FCS (13) and transfected COS-1 cells were serum-starved in phosphate-free DMEM for 16 hr prior to labeling with [^{32}P]orthophosphate for 6–10 hr (2 mCi per 15-cm dish or 1 mCi per 10-cm dish; 1 Ci = 37 GBq). Stimulation was performed with 10% FCS or 0.1 mM vanadate. Phosphoamino acid analysis was carried out according to Boyle *et al.* (16). Quantification of phosphorylation was performed using a phosphorimager and ImageQuant software (Molecular Dynamics).

Protein Phosphatase 2A (PP2A) Treatment. Immunoprecipitated RAC-PK was incubated with 0.3 unit/ml of porcine muscle PP2Ac or 1.7 units/ml of rabbit muscle PP2A$_2$ in 30 μl buffer containing 50 mM Tris·HCl, pH 7.5/1% 2-mercaptoethanol/1 mM $MnCl_2$/1 mM benzamidine/0.5 mM phenylmethylsulfonyl fluoride at 30°C for 60 min (one unit is defined as 1 nmol of Pi released from phosphorylase *a* per min). The reactions were stopped by addition of 50 nM of calyculin A or 1 μM of okadaic acid. The immune complexes were washed with 50 mM Tris·HCl, pH 7.5/1 mM benzamidine/0.5 mM phenylmethylsulfonyl fluoride/50 nM calyculin A (or 1 μM okadaic acid) and RAC-PK was assayed as described above.

RESULTS

Mitogenic Stimulation and Phosphorylation of RAC-PK. The Swiss 3T3 cell line was used to investigate the possible involvement of RAC-PK in growth factor signaling. Immunoprecipitated RAC-PK activity was found to be 2- to 4-fold higher in serum-stimulated versus quiescent cells (Fig. 1*A* and see also Fig. 5*A*). Activation occurred within 5 min, kinase activity remained elevated for at least 60 min (Fig. 1 *A* and *B*) and coincided with decreased mobility of RAC-PK on SDS/PAGE. At least three different mobility forms could be detected by immunoblot analysis, termed **a**, **b**, and **c** (Fig. 1 *A* and *B insets*). The kinase from quiescent cells migrated as a doublet of the **a** and **b** forms, whereas during stimulation a slower migrating form **c** appeared, followed by disappearance of form **a** (see Fig. 1*A inset*). These results suggested that RAC-PK activity was modulated by reversible phosphorylation. To test this possibility we examined the *in vivo* effects of the phosphatase inhibitors, okadaic acid and vanadate, on RAC-PK from Swiss 3T3 cells. Treatment of cells with 1 μM of okadaic acid, a specific inhibitor of PP2A and protein phosphatase 1 (PP1) (17), induced an ~3-fold increase in RAC-PK activity and decreased electrophoretic mobility [Fig. 1*B*, lane 3 (O.A.)]. Simultaneous treatment with 1 μM of okadaic acid and 10% serum caused a 5.5- to 10-fold activation (the observed variation was apparently due to different batches of okadaic acid) and a larger alteration of the electrophoretic mobility [Fig. 1*B*, lane 4 (O.A.+ serum)]. Surprisingly, an 11-fold activation was observed following treatment with 0.1 mM vanadate, which converted the major part of the protein into the slowest-migrating form **c** [Fig. 1*B*, lane 5 (Vanad)]. Simultaneous addition of serum and vanadate to cells did not lead to any increase in the extent of RAC-PK activation (data not shown).

To confirm that multiple electrophoretic mobility forms reflect different phosphorylation states of the kinase,

RAC-PK was immunoprecipitated from ^{32}P-labeled quiescent and vanadate-stimulated Swiss 3T3 cells. Vanadate treatment led to a 3- to 4-fold increase in phosphorylation, demonstrating that the mobility forms **b** and **c** represent phosphorylated RAC-PK (Fig. 2*A*). Phosphoamino acid analysis revealed that the kinase from arrested cells was phosphorylated mainly on serine residues, and at a low level on threonine (Fig. 2*B*, *left*). Vanadate stimulation led to an increase in phosphoserine and in particular in phosphothreonine content (1.3- and 5.1-fold, respectively; Fig. 2*B*, *right*). Phosphotyrosine was not detected after vanadate stimulation, either by phosphoamino acid analysis, or by immunoblot analysis using an anti-phosphotyrosine antibody (data not shown). Treatment of quiescent Swiss 3T3 cells with serum led to an ~1.3-fold increase in RAC-PK phosphorylation, with phosphoserine and low levels of phosphothreonine detected by phosphoamino acid analysis (data not shown). These results show that RAC-PK activation by vanadate and serum involves phosphorylation on serine and threonine residues. Furthermore, we conclude that RAC-PK activation mediated by vanadate is probably indirect (see *Discussion*).

RAC-PK could not be activated by exposing Swiss 3T3 cells to heat-shock (30 min at 42°C), or UV light (36 J/m^2 for 5, 30, and 60 min), as judged by kinase assays and lack of electrophoretic mobility changes.

Inactivation of RAC-PK by PP2A *in Vitro*. To confirm that RAC-PK was regulated by phosphorylation we investigated the effects of PP2A treatment on the kinase immunoprecipitated from quiescent and vanadate-stimulated Swiss 3T3 cells. As treatment of cells with 1 μM okadaic acid for 2 hr preferentially inactivates PP2A rather than PP1 (B. Favre, P. Turowski, B. A. Hemmings, unpublished), RAC-PK was incubated either with the purified PP2A catalytic subunit (PP2Ac), or PP2A dimer consisting of the catalytic and regulatory PR65 subunit (PP2A$_2$). Dephosphorylation of the activated RAC-PK *in vitro* by PP2Ac resulted in an 87% reduction of kinase activity and concomitant change in electrophoretic mobility, converting it from form **c** to **b**, whereas PP2A$_2$ treatment led to a 92% reduction of activity and restored the protein mobility on SDS/PAGE to the **a**/**b** doublet (Fig. 3). Inhibition of PP2Ac *in vitro* by 10 nM okadaic acid prevented RAC-PK inactivation and increase in electrophoretic mobility (data not shown). These results confirm that the activity changes observed are achieved by a reversible phosphorylation mechanism, and also argue that PP2A may negatively regulate RAC-PK activity *in vivo*.

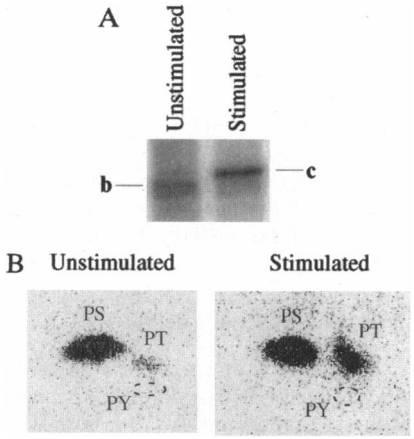

FIG. 2. Effect of vanadate on the phosphorylation state of RAC-PK from Swiss 3T3 cells. (*A*) Phosphorylation state of RAC-PK immunoprecipitated from *in vivo* ^{32}P-labeled quiescent and 0.1 mM vanadate-stimulated cells (15 min). (*B*) Phosphoamino acid analysis of ^{32}P-labeled RAC-PK obtained from (*A*). PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

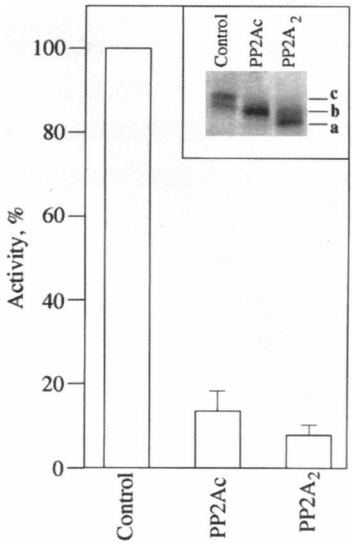

FIG. 3. PP2A inactivates RAC-PK from vanadate-stimulated Swiss 3T3 cells. RAC-PK immunoprecipitated from cells stimulated with 0.1 mM vanadate (15 min) was incubated for 1 hr with either PP2Ac, PP2A$_2$, or buffer. Kinase activity is the average (±SD) of two experiments with duplicate immunoprecipitates. Activity of stimulated RAC-PK was taken as 100%. (*Inset*) Immunoblot analysis of RAC-PK following phosphatase treatment.

Deletion of the PH-Domain and C-Terminal Region Affects RAC-PKα Activity. To assess the role of the PH (amino acids 1–117) and C-terminal (amino acids 421–480) domains in RAC-PKα regulation, we expressed a series of HA-tagged deletion constructs in COS-1 cells: HA-RAC-PKα, HA-ΔNRAC-PKα, HA-RAC-PKαΔC, and HA-ΔNRAC-PKαΔC (Fig. 4*A*). Expression and migration of the proteins were confirmed by immunoblot analysis with an antibody specific for the HA-tag (Fig. 4*B*). It was then established that overexpressed wild-type HA-tagged RAC-PKα responded to serum and phosphatase inhibitor treatment in a manner similar to the endogenous protein from Swiss 3T3 cells. Vanadate was the most potent activator, whereas okadaic acid or serum were less efficient in activating RAC-PKα (Fig. 4*C*). Synergism between okadaic acid and serum was also observed in this system (data not shown). The activation of HA-RAC-PKα by okadaic acid and vanadate was followed by a decrease in the SDS/PAGE mobility (Fig. 4*B*, lanes 1–3). Deletion of the N-terminal PH domain did not affect basal activity of the kinase, but it reduced vanadate-stimulated activity by ≈70%, and almost completely blocked serum-induced activation, without affecting its electrophoretic mobility (Fig. 4 *B* and *C*). In contrast, okadaic acid stimulation led to an efficient activation and concomitant electrophoretic mobility changes of HA-ΔNRAC-PKα (Fig. 4 *B* and *C*). Vanadate-, serum-, and okadaic acid-induced increase of HA-RAC-PKα and HA-ΔNRAC-PKα activity was sensitive to *in vitro* PP2A treatment (data not shown). Surprisingly, deletion of either the C- or N- and C-terminal domains apparently abrogated both basal and vanadate-stimulated RAC-PK activity (data not shown). Furthermore, vanadate treatment did not alter the migration of HA-RAC-PKαΔC and HA-ΔNRAC-PKαΔC on SDS/PAGE (Fig. 4*B*, lanes 7–10). From these experiments it was clear that maximal RAC-PK activation by vanadate and serum, and therefore phosphorylation, requires both the PH and C-terminal domains. The fact that HA-ΔNRAC-PKα could still be partially activated by vanadate and even more efficiently by okadaic acid implies that the activating phosphorylation site(s) resides C-terminal to the PH domain.

To obtain further information on the activation mechanism, the kinase-deficient mutant HA-RAC-PKαK179A was ex-

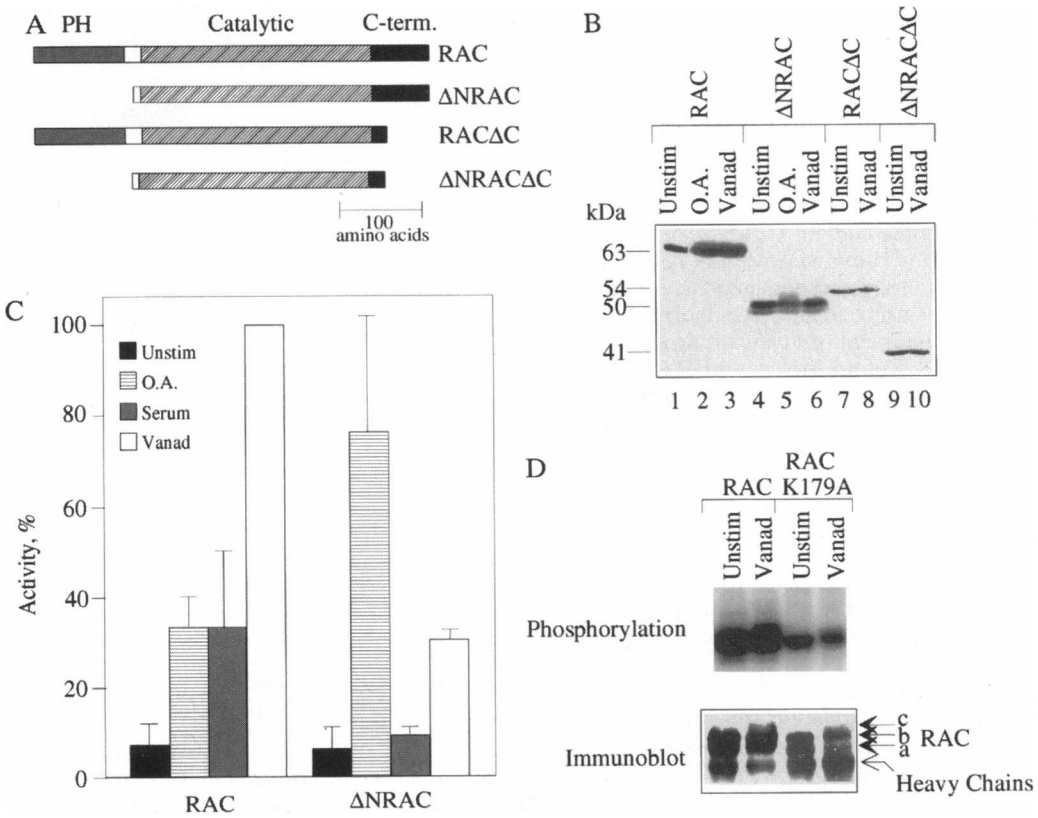

FIG. 4. Effect of deletion of the PH domain and C-terminal region on RAC-PKα activity. (*A*) Constructs expressed in COS-1 cells: HA-RAC-PKα, HA-ΔNRAC-PKα, HA-RAC-PKαΔC, and HA-ΔNRAC-PKαΔC. (*B*) Immunoblot showing expression and migration of the full-length and truncated HA-RAC-PKα proteins from COS-1 treated with 1 μM okadaic acid (120 min) or 0.1 mM vanadate (15 min) or unstimulated. The apparent M_r is indicated. (*C*) The expressed proteins were immunoprecipitated with an anti-HA-tag antibody from the cells treated as described above, and kinase activity determined. Stimulation with 10% serum was for 15 min. Kinase activity is the average (±SD) of two experiments with duplicate immunoprecipitates. Activity of HA-RAC-PKα from vanadate stimulated cells was taken as 100%. (*D*) Phosphorylation (top panel) and immunoblot analysis (bottom panel) of HA-RAC-PKα and kinase deficient HA-RAC-PKαK179A immunoprecipitated with an antibody specific for the HA-tag from serum-starved and 0.1 mM vanadate-stimulated (15 min) ^{32}P-labeled COS-1 cells.

pressed in COS-1 cells. Both active and inactive kinase were phosphorylated *in vivo* in serum-starved cells, but the labeling of HA-RAC-PKαK179A was reduced by 75% (Fig. 4*D*, top panel). Following vanadate treatment, both active and inactive proteins displayed decreased electrophoretic mobility (Fig. 4*D*, bottom panel). In contrast with the earlier experiments with the endogenous enzyme from Swiss 3T3 cells (see Fig. 2), it was not possible to observe a large increase in the phosphorylation of the overexpressed protein (Fig. 4*D*, top panel), suggesting that an upstream kinase was limiting and therefore unable to fully activate the overexpressed RAC-PK. If autophosphorylation was responsible for RAC-PK activation it would be reasonable to expect complete activation. Consistently, only a limited amount of overexpressed protein was activated in transfected cells. Taken together, our data demonstrate that the autophosphorylation mechanism contributes to RAC-PK phosphorylation in arrested cells, but is probably not responsible for further phosphorylation and activation.

RAC-PKα Mobility Changes and Activation Are Sensitive to Wortmannin but Not to Rapamycin. Our results indicated that the PH domain is required for the activation of RAC-PKα by serum and vanadate. It was reported that PH domains can bind PI (4,5)-bisphosphate (19) and recent results from our laboratory demonstrated that the PH domain of RAC-PKα also binds PI (3,4,5)-trisphosphate at submicromolar concentrations (M. Frech and B.A.H., unpublished results). Initial experiments demonstrated that the activation of RAC-PK by serum was sensitive to wortmannin, suggesting that RAC-PK may be regulated by PI 3-kinase. Wortmannin is also known to prevent p70^{s6k} activation affecting the same set of phosphorylation sites as the immunosuppressant rapamycin (15). Inhibition of p70^{s6k} activation by these agents was therefore used as a positive control in our experiments. Stimulation of quiescent Swiss 3T3 fibroblasts led to an ≈4-fold induction of RAC-PK activity, whereas wortmannin treatment preceding serum stimulation almost completely blocked the activation (Fig. 5*A*). On the other hand, rapamycin pretreatment did not exert any significant effect on RAC-PK activation (Fig. 5*A*). In the same experiment, both wortmannin and rapamycin pretreatment abolished a 3-fold p70^{s6k} activation (Fig. 5*A*). Interestingly, wortmannin also reduced RAC-PK activation by vanadate (30–40%) and prevented the emergence of the slowest migrating form on SDS/PAGE (data not shown). Wortmannin, but not rapamycin, also blocked insulin-induced activation of HA-RAC-PKα overexpressed in 293 cells (data not shown). These results suggest that RAC-PK activation by serum, insulin, and at least partially by vanadate is dependent on PI 3-kinase activity.

DISCUSSION

The data presented here demonstrate that RAC-PK activity is regulated by reversible phosphorylation. We envisage that the following events are required for the rapid and efficient activation. Growth factors binding to their cognate receptors stimulate PI 3-kinase activity, thereby generating 3-phosphorylated phosphoinositides leading to the activation of an upstream kinase (RAC-PKK). After the recruitment of RAC-PK to the membrane via its PH domain it becomes phosphorylated and activated by the RAC-PKK. In this scheme PP2A would

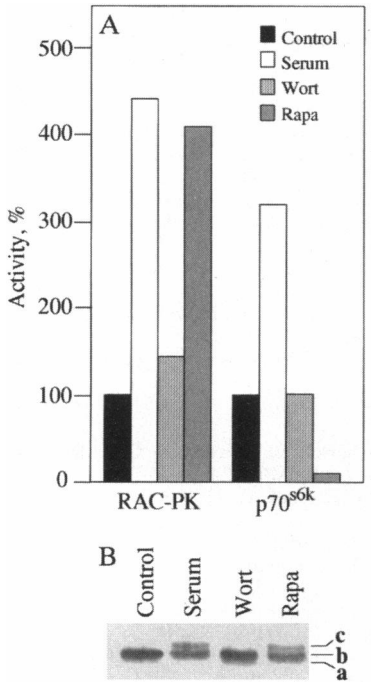

FIG. 5. Effects of wortmannin and rapamycin on RAC-PK and $p70^{s6k}$ activation by serum. Quiescent Swiss 3T3 fibroblasts were pretreated for 30 min with 200 nM wortmannin or 5 nM rapamycin prior to a 15 min serum stimulation. (*A*) RAC-PK and $p70^{s6k}$ activity determined in immunoprecipitates. Activity from quiescent cells was taken as 100%. (*B*) Corresponding immunoblot demonstrating the effects of wortmannin and rapamycin on RAC-PK migration on SDS/PAGE. Confluent cells were not serum-starved before stimulation which explains the slower mobility of the **a** form. This did not affect either basal or stimulated RAC-PK activity.

act as a negative regulator of the activating phosphorylation. Therefore, the balance between RAC-PKK and PP2A activity determines the activation (phosphorylation) state of RAC-PK. Our current model is summarized in Fig. 6, and the results supporting this model are discussed below.

Serum and vanadate apparently activate RAC-PK through a common mechanism. They are both able to induce similar electrophoretic mobility changes of RAC-PK. Their effects are not synergistic suggesting that they both act at the level of receptor tyrosine kinases. Due to the fact that no phosphotyrosine could be detected in RAC-PK we conclude that vanadate, a broad spectrum phosphatase inhibitor, promotes kinase activation by affecting upstream tyrosine phosphorylation events, such as receptor tyrosine kinase activation. The activation induced by serum and vanadate was sensitive to wortmannin, implying that RAC-PK is a cellular target of receptor-activated PI 3-kinase. This is consistent with the data of Franke *et al.* (20) who showed the involvement of PI 3-kinase in RAC-PK activation using PDGF receptor point mutants. A similar finding was published by Burgering and Coffer (21) while our manuscript was in review. Furthermore, our data show that insulin activates RAC-PK through a wortmannin sensitive mechanism. During the review of this manuscript Kohn *et al.* (22) published similar results (see below).

The activation of RAC-PK induced by okadaic acid appears to be independent of receptor tyrosine kinase mediated PI3-kinase activation, because it is unaffected by 200 nM wortmannin (M.A., unpublished results). Moreover, okadaic acid treatment acts synergistically with serum, supporting the concept that this inhibitor influences a later step in the activation process. We therefore conclude that inhibition of PP2A in cells leads to the accumulation of phosphorylated, active RAC-PK, suggesting that a modest amount of the kinase is shuttling

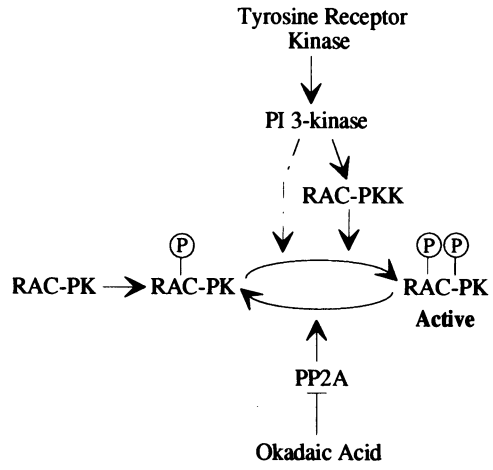

FIG. 6. Hypothetical model for the activation of RAC-PK. Stimulation of tyrosine kinase receptors by growth factors and vanadate lead to PI 3-kinase activation, which in turn activates RAC-PK through an intermediate kinase (RAC-PKK), or maybe by direct phosphorylation (dashed lines). The balance between RAC-PKK and PP2A activity determines the RAC-PK activation state. Okadaic acid shifts this equilibrium toward RAC-PK activation by inhibiting PP2A.

between the active and less active state (see Fig. 6). The data obtained by *in vitro* phosphatase treatment are consistent with PP2A being a primary negative regulator of RAC-PK activity.

Analysis of the role of the PH domain in RAC-PK activation by growth factor receptors indicates that it probably functions as an adaptor. In contrast, Kohn *et al.* (22) reported that the PH domain is not required for RAC-PK activation by insulin in Chinese hamster ovary cells overexpressing insulin receptors. These data are reminiscent of the insulin receptor substrate-1 whose PH domain is required for signaling from the endogenous receptor, but the defect is overcome by insulin receptor overexpression (23).

It was reported that RAC-PK molecules dimerize through the PH domain (24), and that the PH domain is required for RAC-PK activation by platelet-derived growth factor (20, 24). This led to a model (25) in which RAC-PK dimers are activated by autophosphorylation following elevation of 3-phosphorylated phosphoinositides produced by PI 3-kinase. However, our studies with the overexpressed wild-type and kinase-deficient RAC-PKα showed that the kinase is extensively autophosphorylated *in vivo* in serum-starved cells, but only has basal activity. We conclude that the phosphorylation responsible for the activation occurs through the action of another, upstream kinase, based on several lines of evidence: (*i*) both wild-type and kinase-deficient RAC-PKα undergo similar electrophoretic mobility changes following vanadate stimulation, (*ii*) only a limited amount of overexpressed RAC-PKα is activated following stimulation of cell, (*iii*) the increase in RAC-PK activity and (activating) phosphorylation induced by serum and vanadate are sensitive to wortmannin implying that they occur through a PI 3-kinase dependent mechanism, whereas basal activity and phosphorylation are unaffected by this drug. This is supported by data from Burgering and Coffer (21) showing that wortmannin pretreatment blocked the appearance of platelet-derived growth factor-induced RAC-PK phosphorylation, without affecting its basal phosphorylation. In this context the role of the PH domain, which binds PI 3-kinase generated phospholipids, would be to ensure RAC-PK translocation or attachment to the membranes. The structure of the PH domain of phospholipase C-1 δ1 bound to inositol (1,4,5)-trisphosphate revealed that residues involved in the high-affinity interactions are conserved among different PH domains. This suggests that phosphoinositides binding through electrostatic interactions may be a common mecha-

nism for providing membrane localization of many PH domain containing proteins (26). In our model it is necessary to speculate that the activating kinase is also located at the membrane and regulated by phospholipids. PI 3-kinase itself is also a possible candidate for the RAC-PK kinase, as it has been demonstrated that lipid kinases may function as protein kinases (27).

Identification of the phosphorylation sites associated with RAC-PK regulation is of considerable importance for a detailed understanding of the activating mechanism and delineation of the signaling pathway. The available data indicate that these sites reside in the catalytic domain and/or C-terminal region. Based on comparison with the structures of protein kinase A, CDK2, and $p42^{mapk}$ (28), Thr^{308} of RAC-PKα can be predicted as a potential phosphorylation site involved in the regulation of its kinase activity. Furthermore, the C-terminal domain of RAC-PK contains two conserved motifs that were shown to be involved in the regulation of other kinases by phosphorylation. First, Thr^{450} in RAC-PKα corresponds to a similar site (TPXD) in PKCβ_1 involved in maturation and activation of the enzyme (29). Second, the sequence context of Ser^{473} in RAC-PKα is homologous to the principal rapamycin-sensitive phosphorylation site in $p70^{s6k}$ (30). Significantly, all of these sites are conserved in the β-isoform and in DRAC-PK (7).

The only downstream target of RAC-PK identified thus far is glycogen synthase kinase-3, which can be phosphorylated and inactivated by RAC-PK following insulin stimulation (18). Recently, Burgering and Coffer found that overexpression of the oncogenic form of RAC-PK was able to stimulate $p70^{s6k}$ activity. Significantly, we could show that overexpression of wild-type RAC-PK activates $p70^{s6k}$ and also enhances insulin-stimulated $p70^{s6k}$ activity (X.-F.M. and M.A., unpublished results). Taken together the results suggest that RAC-PK plays a central role in insulin and growth factor signaling. The identification of the upstream components between PI 3-kinase and RAC-PK, and other downstream targets will be crucial for understanding this signaling pathway.

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