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Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase

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In the present study, insulin is shown to rapidly stimulate by 8- to 12-fold the enzymatic activity of RAC-PKα, a pleckstrin homology domain containing ser/thr kinase. In contrast, activation of protein kinase C by phorbol esters had almost no effect on the enzymatic activity of RAC-PKα. Insulin activation was accompanied by a shift in molecular weight of the RAC-PKα protein, and the activated kinase was deactivated by treatment with a phosphatase, indicating that insulin activated the enzyme by stimulating its phosphorylation. This insulin-induced shift in RAC-PK was also observed in primary rat epididymal adipocytes, as well as in a muscle cell line called C2C12 cells. The insulin-stimulated increase in RAC-PKα activity was inhibited by wortmannin (an inhibitor of phosphatidylinositol 3-kinase) in a dose-dependent manner with a half-maximal inhibition of 10 nM, but not by 20 ng/ml of rapamycin. Activation of RAC-PKα activity was also observed in a variant RAC lacking the pleckstrin homology domain. These results indicate that RAC-PKα activity can be regulated by the insulin receptor. RAC-PKα may therefore play a general role in intracellular signaling mediated by receptor tyrosine kinases.

Keywords: insulin action/oncogenes/pleckstrin homology domain/ser/thr kinases/tyrosine kinases

Introduction

Three laboratories have previously independently isolated the cDNA for a ser/thr kinase of unknown function and named it either RAC-PKα (Jones *et al.*, 1991b), pkb (Coffer and Woodgett, 1991) or akt (Bellacosa *et al.*, 1991). A highly related second form of the enzyme, called RAC-PKβ, has also been identified (Jones *et al.*, 1991a). Two of these studies isolated the RAC-PKα cDNA while screening for novel ser/thr kinases (Coffer and Woodgett, 1991; Jones *et al.*, 1991b). The third study identified it as the transforming component of the AKT8 virus, an acute transforming retrovirus isolated from a rodent T cell lymphoma (Bellacosa *et al.*, 1991). RAC-PK therefore represents not only a unique ser/thr kinase, but also a novel putative proto-oncogene. Two human *RAC-PK* cellular homologs, *AKT1* and *AKT2*, have been reported to be amplified in certain carcinomas, further confirming the oncogenic potential of *RAC-PK*. *AKT1* has been shown to be amplified in a gastric adrenocarcinoma (Staal, 1987) and *AKT2* has been demonstrated to be amplified and overexpressed in some human ovarian carcinoma cell lines and primary tumors (Cheng *et al.*, 1992).

Structurally, RAC-PKα can be divided into three regions. The centrally located kinase domain shows a high degree of sequence similarity to the catalytic domains of protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) (Bellacosa *et al.*, 1991; Coffer and Woodgett, 1991; Jones *et al.*, 1991b). Whereas the additional protein sequence on the carboxyl side of the kinase domain does not show homology to any known structural protein configurations, the N-terminal protein sequence encodes a pleckstrin homology (PH) domain (Haslam *et al.*, 1993; Musacchio *et al.*, 1993; Shaw, 1993). A significant role for the PH domain in RAC-PK function has been proposed, based on the finding that this domain is conserved in a homolog found in *Drosophila* (Andjelkovic *et al.*, 1995). Moreover, the PH domain has been identified in many proteins important in signal transduction, including those that function downstream of receptor tyrosine kinases, such as phospholipase Cγ, insulin receptor substrate-1 and SOS. Presently, it is hypothesized that the PH domain functions as a modular protein domain that dictates a certain protein–protein interaction, analogous to the function of SH2 domains that bind to specific phosphotyrosine-containing sequences. Although the exact function, if any, of the PH domain has not been definitively proven, some reports suggest that the PH domain mediates an interaction with PKC. For example, pleckstrin, the protein after which the PH domain is named, is the major substrate of PKC in platelets (Tyers *et al.*, 1988) and the PH domain of the Bruton tyrosine kinase has been shown to interact with PKC *in vitro* and *in vivo* (Yao *et al.*, 1994). More recently, the PH domain of RAC-PK has also been reported to interact with PKC *in vitro* (Konishi *et al.*, 1994).

Stimulation of the intrinsic tyrosine kinase activity of the insulin receptor by ligand-binding is associated with both the direct and indirect activation of an array of ser/thr kinases that contribute towards propagating the extracellular signal within the cell (Czech *et al.*, 1988; Avruch *et al.*, 1990; Denton *et al.*, 1992). In addition, ser/thr kinase activation may play a role in the mechanism of feedback inhibition of insulin receptor signaling, either by causing ser/thr phosphorylation of the insulin receptor itself, or possibly by phosphorylating substrates of the insulin receptor tyrosine kinase (Roth *et al.*, 1994; Tanti *et al.*, 1994). An activated ser/thr kinase like RAC-PK could potentially participate in many of the biological responses initiated by the insulin receptor, such as stimulation of mitogenesis, activation of the 70 kDa S6 kinase or even activation of specific phosphatases (Cohen *et al.*, 1992; Thomas, 1992). Furthermore, many of the downstream proteins in the insulin receptor signaling cascade,

whether they possess a catalytic kinase domain or not, are characterized by having modular structural protein domains, such as SH2, SH3, or PH domains (Pawson, 1995). Since RAC-PK is a putative proto-oncogene that possesses ser/thr kinase activity and a PH domain, it bears many of the same qualities as other proteins that function downstream of the insulin receptor. We therefore wanted to determine whether RAC-PKα activity was stimulated by either insulin or activators of PKC to gain a further understanding of the role of this enzyme in signal transduction.

In the present study, we show that insulin can stimulate by 8- to 12-fold the ser/thr kinase activity of RAC-PKα, whereas activation of PKC has almost no effect on its activity. The insulin-stimulated activation of RAC-PKα is caused by an increase in ser/thr phosphorylation of this enzyme. The kinase activity is inhibited by wortmannin, indicating a role for phosphatidylinositol 3-kinase (Arkaro and Wymann, 1993; Okada *et al.*, 1994) in the activation of RAC-PKα. Since RAC-PK is present in many tissues which are targets of insulin action, including muscle, kidney and liver (Konishi *et al.*, 1994), this enzyme may play a role in mediating some of the biological responses to insulin.

Results and discussion

RAC-PKα activity is stimulated by insulin

The human RAC-PKα cDNA was cloned using PCR. The PCR clone was sequenced, and a spontaneous mutation was identified that resulted in the conversion of Leu153 to a proline (see Materials and methods). The mutation was converted back to the wild-type sequence using single strand mutagenesis. In addition, both the wild-type and mutant RAC-PKα cDNAs were modified to encode a hemagglutinin (HA) epitope tag on the C-terminal of the proteins so that transfected protein could be detected and analyzed. The proteins expressed by these cDNAs were then characterized after expression either by transient transfection in CHO cells overexpressing the human insulin receptor (CHO.IR cells) (Ellis *et al.*, 1986), or by stable expression in the same cells. RAC-PKα was immunoprecipitated from lysates of cells expressing either the wild-type or mutant protein using a monoclonal antibody, 12CA5, directed against the HA epitope and these precipitates were analyzed by immunoblotting with a polyclonal antibody (HA.11) that also recognized the HA epitope. Only a single band of ~60 kDa was observed (this band often appeared as a tightly spaced doublet or triplet) (Figure 1A). This band was not observed in immunoprecipitates with control immunoglobulin (Figure 1A) or from lysates of non-transfected cells (data not shown). This size band is consistent with a previous study that identified RAC-PKα as a protein of M_r 59 000 using specific antisera directed against the C-terminal of the protein (Jones *et al.*, 1991b).

Insulin treatment of CHO.IR cells expressing either mutant or wild-type RAC-PKα caused the slower-migrating forms of the RAC-PKα proteins to increase in intensity, as well as causing a decrease in intensity of the faster-migrating form (Figure 1A), consistent with the hypothesis that insulin causes an increase in the phosphorylation state of RAC-PKα. The multiple bands of the RAC-PKα

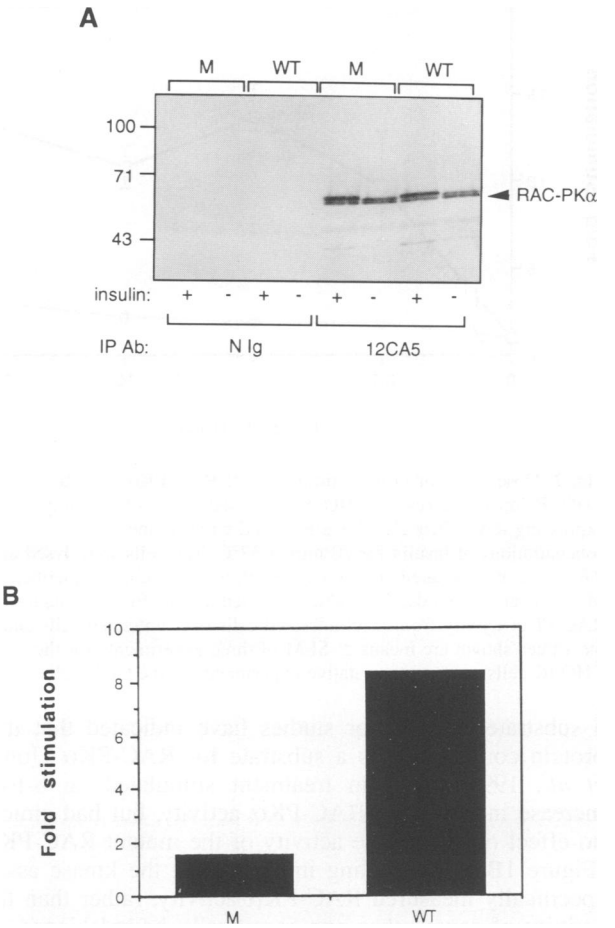

Fig. 1. Mobility pattern and kinase activity of wild-type and mutant RAC-PKα. (**A**) Mobility pattern of RAC-PKα. Stable transfectants of CHO.IR cells that express either mutant (M) or wild-type (WT) RAC-PKα were treated with (+) or without (–) 1 μM insulin for 10 min at 37°C. The cell lysates were divided in half and adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse immunoglobulin (N Ig). The immunoprecipitated proteins were separated on a 10% SDS–polyacrylamide gel and RAC-PKα was detected by Western blot using the polyclonal anti-HA antibody, HA.11. (**B**) Enzymatic activity of RAC-PKα. The immunoprecipitates were assayed for kinase activity using myelin basic protein as a substrate, as described under Materials and methods. The values depicted represent the fold stimulation of RAC-PKα activity from insulin-treated cells over control-treated cells. M, mutant; WT, wild-type.

probably indicate multiple phosphorylation states of this enzyme, only some of which may affect activity. Metabolic labeling studies indicated that RAC-PKα was phosphorylated in non-treated cells and that insulin treatment stimulated a 2.4-fold increase in ^{32}P-labeling of the RAC-PKα band (data not shown). The RAC-PKα bands were not recognized by anti-phosphotyrosine antibodies, even from insulin-treated cells, indicating that this phosphorylation was not on tyrosine residues. Moreover, phosphoamino acid analysis of *in situ* labeled RAC-PKα from insulin-treated cells did not display any phosphotyrosine (data not shown). These results indicate that insulin can stimulate the ser/thr phosphorylation of RAC-PKα.

Wild-type and mutant RAC-PKα immunoprecipitated from either the transiently transfected cells or the stable cell lines derived from CHO.IR cells were also tested for *in vitro* kinase activity. Myelin basic protein was used as

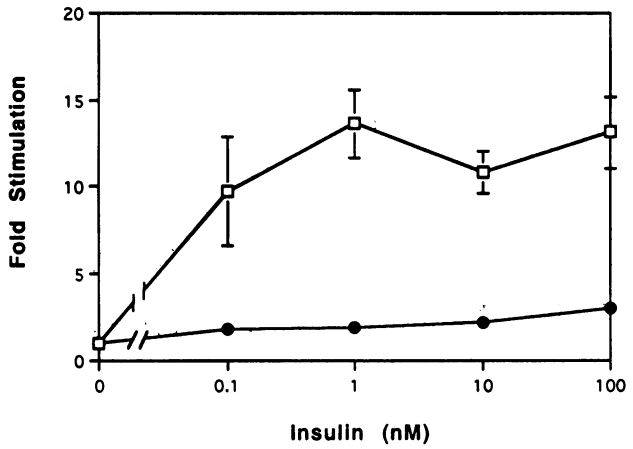

Fig. 2. Dose curve of insulin stimulation of RAC-PKα activity. CHO.IR (open squares) or CHO cells (closed circles) transiently expressing RAC-PKα cDNA were treated with the indicated concentrations of insulin for 20 min at 37°C. The cells were lysed and RAC-PKα was isolated and its kinase activity assayed, as described in Materials and methods. The values depicted are the fold stimulation of RAC-PKα activity from insulin-treated cells over untreated cells and the values shown are means ± SEM of three experiments for the CHO.IR cells and a representative experiment for the CHO cells.

a substrate, since prior studies have indicated that this protein could serve as a substrate for RAC-PKα (Jones *et al.*, 1991b). Insulin treatment stimulated an 8-fold increase in wild-type RAC-PKα activity, but had almost no effect on the kinase activity of the mutant RAC-PKα (Figure 1B). This finding indicates that the kinase assay specifically measured RAC-PKα activity, rather than the activity of some other non-specifically bound kinase. In addition, control immunoprecipitates from the parental CHO.IR cells did not exhibit kinase activity either.

RAC-PKα activity was stimulated by insulin in a dose-dependent manner. RAC-PKα transiently expressed in CHO.IR cells was stimulated ~10-fold over basal levels by as little as 100 pM insulin (Figure 2). Higher concentrations of insulin caused RAC-PKα activity to plateau at ~12-fold over basal levels. When transiently transfected into CHO cells that do not overexpress the insulin receptor but do contain a low endogenous level of insulin and insulin-like growth factor I receptors (Ellis *et al.*, 1986), RAC-PKα activity was only maximally stimulated 3-fold over basal levels, and this effect required higher insulin concentrations (Figure 2). The greater stimulation of RAC-PKα activity in cells overexpressing the insulin receptor argues that the insulin receptor is responsible for the activation of RAC-PKα. Moreover, the finding that even in CHO cells insulin was capable of activating RAC-PKα indicates that even low levels of the insulin receptor are sufficient for this activation.

To see whether insulin could stimulate RAC-PKα in non-transfected cells, primary rat adipocytes and the non-transfected muscle cell line C2C12 were treated with insulin. The endogenous RAC-PK was analyzed by immunoblotting (Figure 3). A shift in migration of this protein was detected after insulin treatment in both of these cell types.

Insulin increases RAC-PKα activity by stimulating its phosphorylation

Since insulin was observed both to induce the RAC-PKα protein to shift to a higher molecular weight and to

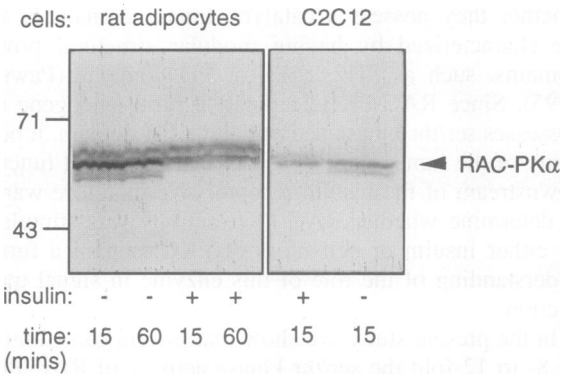

Fig. 3. Insulin-induced shift of RAC-PKα in primary rat adipocytes and a muscle cell line. Rat adipocytes were incubated with 10 nM insulin or buffer for either 15 or 60 min, as indicated. C2C12 muscle cells were incubated with 1 μM insulin or buffer for 15 min as indicated. Cells were lysed and the lysates were electrophoresed and blotted with a polyclonal antibody to RAC-PKα.

increase its enzymatic activity (Figures 1–3), we tested the hypothesis that this increase in the enzymatic activity of RAC-PKα was due to an increase in its phosphorylation. Transiently expressed HA epitope-tagged RAC-PKα protein was immunoprecipitated from control- and insulin-treated CHO.IR cell lysates. Before using the immunoprecipitates in a kinase assay, antibody-bound RAC-PKα protein was either mock-treated with buffer alone, dephosphorylated using alkaline phosphatase, or treated with phosphatase that had been pre-mixed with a mixture of phosphatase inhibitors. In this experiment, the activity of the mock-treated RAC-PKα from cells that had previously been exposed to insulin was stimulated 14-fold compared with the protein from non-treated cells. In contrast, the RAC-PKα protein from insulin-stimulated cells that had been treated with phosphatase showed kinase activity that was almost equal to that of RAC-PKα immunoprecipitated from non-stimulated cells (Figure 4). The presence of phosphatase inhibitors during the dephosphorylation reaction blocked this conversion of RAC-PKα to its inactive form (Figure 4). The dephosphorylation of the RAC-PKα from insulin-treated cells was also associated with its reversion to the faster-migrating form (data not shown). These results argue that insulin treatment of cells stimulate the activity of RAC-PKα by increasing its level of phosphorylation.

Activation of RAC-PKα by different agents

As discussed in the Introduction, RAC-PKα contains a pleckstrin homology domain at its N-terminus, and this domain has been suggested to mediate an association with PKC. Since CHO.IR cells contain PKCα and PKCγ and these forms of PKC can be activated by the phorbol ester PMA (Chin *et al.*, 1993), we examined whether PMA stimulates RAC-PKα activity. Stable transfectants of CHO.IR cells overexpressing epitope-tagged RAC-PKα were treated with control vehicle alone or with 1 μM PMA for different amounts of time. RAC-PKα was then immunoprecipitated and its kinase activity measured. A barely detectable increase in RAC-PKα activity was observed with PMA treatment at all times measured (Figure 5A). In comparison, insulin treatment was observed to cause a much larger stimulation of RAC-PKα

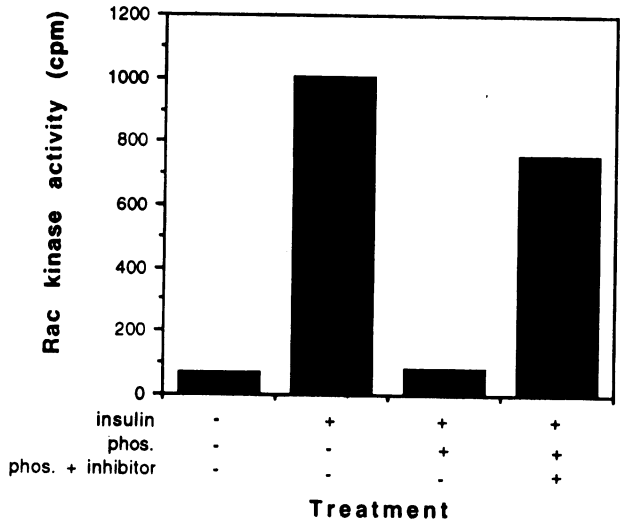

Fig. 4. Effect of phosphatase treatment on RAC-PKα activity. CHO.IR cells transiently expressing RAC-PKα cDNA were treated without or with 1 μM insulin for 20 min at 37°C. The cell lysates were divided in half and adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse immunoglobulin. The precipitates were then incubated with either buffer, 10 U alkaline phosphatase (phos.), or 10 U phosphatase and a mixture of phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, 30 mM $NaPP_i$) (phos. + inhibitor). RAC-PKα activity was measured after dephosphorylation using myelin basic protein as a substrate, as described in Materials and methods.

protein activity even after 1 min, and by 10 min the increase in insulin stimulation approached 10-fold (Figure 5A). PMA added together with insulin also did not affect the stimulation of RAC-PKα activity over that obtained with insulin alone (data not shown). Furthermore, whereas insulin-treatment of these cells was associated with an increase in the slower migrating form of RAC-PKα when using SDS–PAGE, PMA treatment did not change the mobility pattern of RAC-PKα (Figure 5B). Since PMA had a minor effect on RAC-PKα activity, particularly in comparison with the effect of insulin, we conclude that at least PKCα and PKCγ do not play a significant role in regulating RAC-PKα activity.

To test whether another tyrosine kinase could also activate RAC-PKα, epitope tagged RAC-PKα was transiently transfected into A431 cells, a cell line with high levels of the epidermal growth factor receptor (Fabricant *et al.*, 1977). In these cells, epidermal growth factor was found to stimulate RAC-PKα activity by ~5-fold.

The insulin stimulation of RAC-PKα activity is inhibited by wortmannin

One of the earliest known actions of the insulin receptor tyrosine kinase is the activation of phosphatidylinositol 3-kinase (PI3K), predominantly mediated via the binding of tyrosine phosphorylated insulin receptor substrate-1 to PI3K (Backer *et al.*, 1992). To test whether RAC-PKα activation occurs downstream of PI3K, we utilized wortmannin, a relatively specific inhibitor of PI3K (Arkaro and Wymann, 1993; Okada *et al.*, 1994). CHO.IR cells transiently transfected with RAC-PKα cDNA were pretreated with increasing concentrations of wortmannin for 10 min and then stimulated with insulin for 5 min. RAC-PKα was immunoprecipitated from these cell lysates and the kinase activity was measured. Wortmannin inhibited

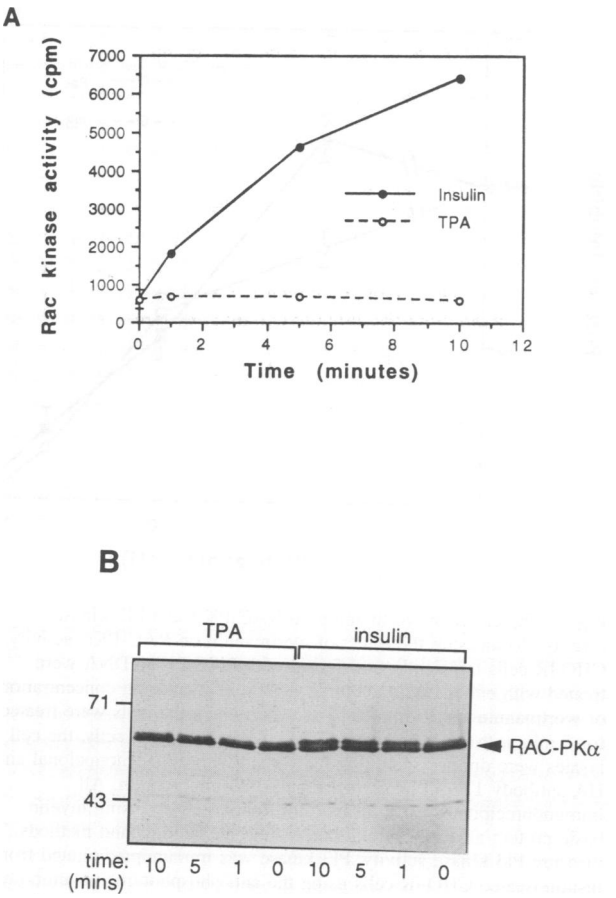

Fig. 5. Comparison of the effect over time of PMA versus insulin stimulation on RAC-PKα activity. Stable transfectants of CHO.IR cells expressing RAC-PKα cDNA were treated with either control vehicle, 1 μM insulin, or 1 μM PMA for the indicated times at 37°C. After the cells were lysed, the cell lysates were divided in half and adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse immunoglobulin. All immunoprecipitates were assayed for kinase activity using myelin basic protein as a substrate, as described in Materials and methods. RAC-PKα activity was measured (**A**) and RAC-PKα protein was detected (**B**) as described in the legend to Figure 1. In (A), RAC-PKα activity measured at the zero time point is an average ± SEM of each of the zero time point samples included in the insulin and PMA series.

in a dose-dependent manner the insulin-stimulated increase in RAC-PKα activity (Figure 6). This inhibition of RAC-PKα activation paralleled the inhibition of PI3K in anti-phosphotyrosine precipitates (Figure 6). The IC_{50} of both effects was observed at ~10 nM wortmannin, a value close to that previously observed for the inhibition of PI3K (Arkaro and Wymann, 1993; Okada *et al.*, 1994). Controls using immunoprecipitated activated RAC-PKα demonstrated that this kinase was unaffected by 0.1 μM wortmannin added directly to the kinase mixture *in vitro* (data not shown), indicating that wortmannin does not directly inhibit RAC-PKα activity. These results suggest that activation of RAC-PKα by insulin is likely to occur downstream of PI3K activation.

In contrast, 20 ng/ml of rapamycin, an inhibitor of the insulin-stimulated increase in ribosomal protein 70 kDa S6 kinase (Kuo *et al.*, 1992; Price *et al.*, 1992), did not effect the insulin-stimulated increase in RAC-PKα activity, although it did almost completely inhibit the insulin-stimulated increase in S6 kinase activity in the same cells

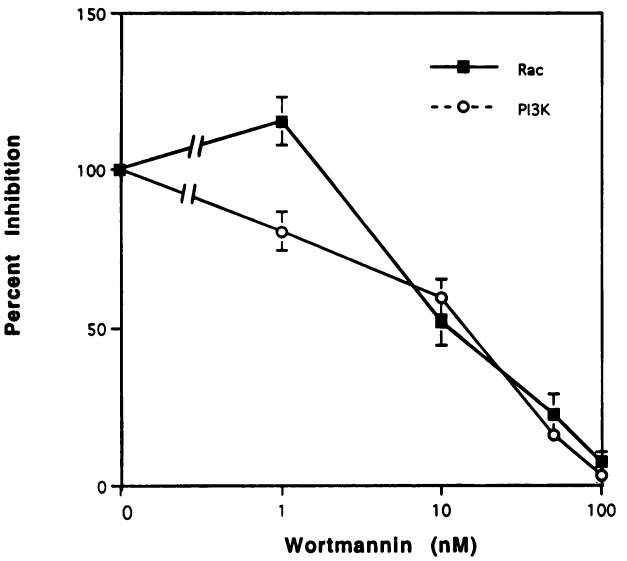

Fig. 6. The effect of wortmannin on RAC-PKα and PI3 kinase activity. To measure the effect of wortmannin on RAC-PKα activity, CHO.IR cells transiently transfected with RAC-PKα cDNA were treated with either DMSO control vehicle or increasing concentrations of wortmannin for 10 min at 37°C, after which the cells were treated for 5 min with 1 μM insulin at 37°C. After lysing the cells, the cell lysates were divided in half and adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse immunoglobulin. All immunoprecipitates were assayed for kinase activity using myelin basic protein as a substrate, as described in Materials and methods. To measure PI3 kinase activity, PI3 kinase was immunoprecipitated from insulin-treated CHO.IR cells using the anti-phosphotyrosine antibody PY20. PI3 kinase activity was measured as described in Materials and methods in the presence of either DMSO control vehicle or increasing concentrations of wortmannin added *in vitro*.

(data not shown). These results indicate that RAC-PKα does not function downstream of the 70 kDa S6 kinase.

Insulin-stimulated activation of the enzymatic activity of a variant RAC-PKα lacking its PH domain

RAC-PKα contains a PH domain whose function is not known (Haslam *et al.*, 1993; Musacchio *et al.*, 1993; Shaw, 1993). To test whether this domain is required in the activation process, a cDNA was constructed which encodes a variant RAC-PKα lacking its entire PH domain (residues 4–129) and containing the HA epitope. This mutant protein was readily expressed in transiently transfected CHO and CHO.IR cells and migrated on SDS gels at a position consistent with a molecular weight of ~45 kDa. In five experiments, the expressed levels of this mutant protein were ~5-fold higher than the comparably transfected wild-type protein. Insulin treatment of either of these cells clearly stimulated the kinase activity of the PH⁻ RAC-PKα (Figure 7), indicating that the PH domain was not required for the activation process.

Ability of overexpressed mutant RAC-PKα to inhibit activation of native RAC-PKα

To test whether overexpression of the inactive RAC-PKα could inhibit the insulin-stimulated increase in enzymatic activity of the wild-type enzyme, CHO-IR cells were transiently transfected with cDNAs encoding both forms of the enzyme. To ensure an increased level of the inactive enzyme, the cDNA encoding the inactive enzyme was present at a 5-fold higher level than the wild-type enzyme. To selectively measure the kinase activity of the wild-type enzyme, only this cDNA encoded an enzyme with the HA epitope. When cells were transfected with cDNAs encoding both the inactive and active RAC-PKα, the insulin-stimulated increase in enzymatic activity of the HA-tagged wild-type enzyme was inhibited by ~80% (Figure 8). In contrast, the amount of the wild-type enzyme protein precipitated by the HA antibody was the same whether or not the mutant was expressed (data not shown). Overexpression of the PH⁻ variant as well as the native RAC-PKα was also found to inhibit the insulin-stimulated increase in activity of the HA-tagged wild-type enzyme. These results indicate that some factor crucial for the activation of RAC-PKα is limiting in these cells. Moreover, since the variant RAC-PKα which lacks the PH domain is still capable of inhibiting the activation of wild-type enzyme, these results support the conclusion that the PH domain is not required for the insulin-stimulated activation.

Conclusions

In the present studies, the ser/thr kinase activity of the PH domain-containing kinase RAC-PKα was found to be stimulated 8- to 12-fold by insulin treatment. This stimulation was observed after expression of RAC-PKα by either transient or stable transfection in CHO cells overexpressing the human insulin receptor. Surprisingly, phorbol esters were unable to activate RAC-PKα expressed in CHO.IR cells, despite data indicating an interaction between PH domains and PKC. Moreover, no coprecipitation of RAC-PKα and PKC was observed in the present studies, even when immunoprecipitated RAC-PKα was incubated *in vitro* with extracts of cells overexpressing PKCα (data not shown). Although it is still possible that a particular PKC isoenzyme which is not present in CHO cells could interact with RAC-PKα, or that activation of RAC-PKα could occur via a non-conventional PKC which is not activated by PMA, these results suggest that PKC is not a major activator of RAC-PKα.

The insulin-mediated activation of RAC-PKα appeared to be mediated via ser/thr phosphorylation of the enzyme, since activation could be reversed by phosphatase treatment of the protein and since activation was correlated with a shift on SDS gel electrophoresis of the RAC-PKα band to a position of higher molecular weight. The presence of multiple tightly spaced bands for the enzyme (only some of which appear to be shifted by insulin treatment) suggest a complex regulation of this enzyme by phosphorylation. The insulin stimulated increase in ser/thr phosphorylation of RAC-PKα did not appear to be mediated via an autophosphorylation reaction, since a mutant enzyme which lacked kinase activity appeared to be shifted like the native one in response to insulin. The requirement for phosphorylation to activate the enzyme is consistent with the hyperphosphorylated state of the oncogenic form of the enzyme and the previously reported low enzymatic activity of the native enzyme which had not been hyperphosphorylated (Bellacosa *et al.*, 1991). The ability of even low levels of insulin receptors in the parental CHO cells to activate RAC-PKα, albeit to only a partial extent, the high levels of activation of this enzyme

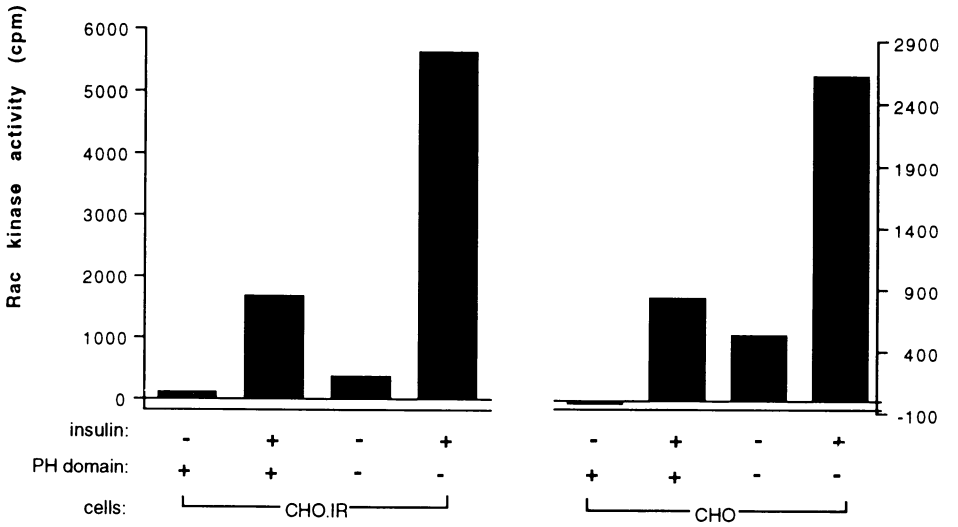

Fig. 7. Insulin-stimulated activation of a variant RAC-PKα lacking the PH domain. CHO or CHO.IR cells were transfected with a cDNA encoding either the epitope-tagged native RAC-PKα or a variant which lacked the PH domain. The two molecules were immunoprecipitated from insulin or control treated cells with the antibody to the HA-epitope and the kinase activity of the precipitates was measured. The levels of the expressed enzymes immunoprecipitated from both cell types were concurrently measured by immunoblotting.

in both transient and stable transfectants of CHO cells expressing high levels of the receptor, the rapid activation of this enzyme and the presence of RAC-PKα in many target tissues for insulin action (i.e. muscle, liver and kidney) all support the hypothesis that RAC-PKα would be activated under physiological conditions in response to insulin. This hypothesis is further supported by the finding that insulin can induce the shift to higher molecular weight of RAC-PKα in primary rat adipocytes as well as in a non-transfected muscle cell line (the C2C12 cells). The finding that RAC-PKα can also be activated via the epidermal growth factor receptor would indicate that this enzyme may play a general role in signal transduction via tyrosine kinases. Consistent with this hypothesis is the recent report of Franke *et al.* (1995) that PDGF can also activate this enzyme and that this activation also requires the PI3-kinase activity.

The mechanism for activation of RAC-PKα appears to be via an insulin-induced ser/thr phosphorylation of this enzyme. This process does not appear to require the presence of the PH domain. Moreover, it appears to be inhibited by overexpression of either native or mutant forms of the enzyme. These results indicate that a factor (possibly the ser/thr kinase responsible for the phosphorylation of the enzyme) required for the insulin-stimulated activation of RAC-PKα may be present in limited amounts. It may therefore be possible to test further the role of this enzyme in mediating various biological processes by stably overexpressing inactive forms of the enzyme.

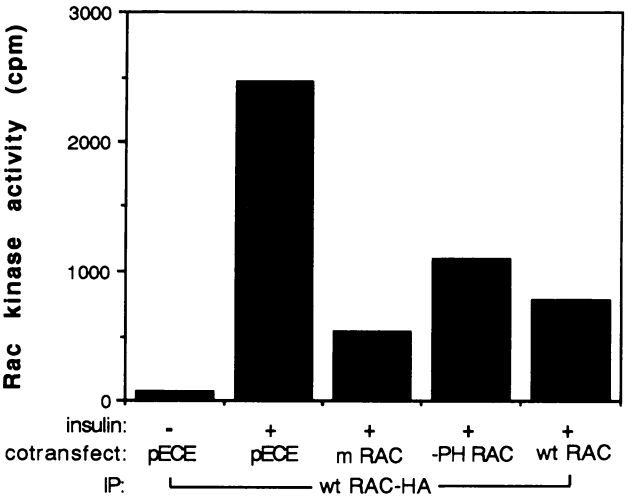

Fig. 8. Overexpression of mutant and wild-type RAC-PKα inhibits activation of the epitope tagged enzyme. CHO.IR cells were cotransfected with 2 μg of plasmid encoding the native HA-tagged RAC-PKα and 10 μg of either the empty expression vector (pECE), the expression vector encoding the mutant enzyme which lacks kinase activity (m RAC), a variant of the enzyme which lacks the PH domain (–PH RAC) or the native enzyme (wt RAC). Cells were treated with or without insulin, lysed and the HA-tagged RAC-PKα was specifically immunoprecipitated and assayed for kinase activity. The amount of immunoprecipitated HA-tagged enzyme was concurrently measured by immunoblotting.

Materials and methods

Materials

Restriction and modifying enzymes were purchased from New England Biolabs or Gibco-BRL; Sequenase and DNA sequencing kit from United States Biochemical; pET 17b expression vector from Novagen; Takara DNA ligation kit from Panvera; protein G–Sepharose, dNTPs and DEAE–dextran from Pharmacia; chloroquine, DMSO, myelin basic protein, protein kinase A inhibitor peptide, wortmannin and ATP from Sigma; [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham and [^{32}P]orthophosphate (9000 Ci/mmol) from NEN Dupont; calf intestinal alkaline phosphatase and 12CA5 monoclonal antibody from Boehringer Mannheim; HA.11 polyclonal antibody from Babco; Rac-CT, a polyclonal antibody directed against a C-terminal peptide of human RAC-PK, from Upstate Biotechnology; porcine insulin from Elanco; PMA from Calbiochem; thin-layer silica gel plates (0.2 mm thickness) from E.Merck; protein A–Sepharose from Repligen; polyclonal antibodies to the 70 kDa S6 kinase were a gift from Dr John Blenis and Py20 was a gift from Dr John Glenney.

Cloning of RAC-PKα and RAC-PKα constructs

Human RAC-PKα was cloned from HeLa cDNA using PCR. The primer pair 5′-ATGAGCGACGTGGCTATTGTGAAG-3′ and 5′-CGTGGGT-CTGGAAAGAGTACTTC-3′ was used to amplify a 661 bp fragment

that encodes the N-terminal half of RAC-PKα. The primer pair 5′-CTTCCTCACAGCCCTGAAGTACT-3′ and 5′-CTCAGGCCGTGC-TGCTGG-3′ was used to isolate an overlapping 820 bp fragment that encodes the C-terminus of RAC-PKα. The fragments were joined using a *ScaI* restriction site present in the overlapping portions of both fragments and they were subcloned into the PCR1000 vector of the TA cloning kit (Invitrogen). The intact RAC-PKα cDNA was subcloned into pBS-KS+ using *Eco*RI and *Not*I in the multiple cloning site.

The hemaglutinin (HA) epitope tag was added to the C-terminus of RAC-PKα in pBS-KS + by single strand mutagenesis using the method of Kunkel (Kunkel *et al.*, 1987; Sambrook *et al.*, 1989), except that Sequenase was used in the polymerizing reaction. The sequence of the priming oligonucleotide was 5′-GCCGCGGTGAGAACCGTTTCACA-GTCCAGGTCCCAGACTGGCGTAGTCGGGGACGTCGTAAGGAT-AAGCCATGGCCGTGCTGCTGGCCGA-3′. The PCR-derived clone was sequenced and a spontaneous mutation was uncovered at nucleotide 656 (using the numbering of Jones *et al.*, 1991b) that converted a T to a C which, in turn, converted the leucine at amino acid position 153 to a proline. To convert the RAC-PKα clone back to the wild-type sequence, a 369 bp *PstI* fragment was subcloned into pBS-KS. The clone was converted back to the wild-type sequence using single strand mutagenesis. The mutagenic primer was 5′-AGCAGCTTCAGGTACTCAA-3′. The entire *PstI* fragment was sequenced and confirmed to be consistent with the wild-type sequence. The *PstI* fragment containing the mutation was replaced by the fragment corresponding to the wild-type sequence within the RAC-PKα clone. The modified RAC-PKα with the HA epitope was subcloned into the mammalian expression vector, pECE (Ellis *et al.*, 1986), using *Eco*RI and *SstI* in the multiple cloning site.

To construct (–PH)RAC-PKαHA-pECE, a *StuI* site was created at base pair 208 using single strand mutagenesis. The unique *AlwNI* site within RAC-PKα was blunted using T4 DNA polymerase and ligated directly to the *StuI* site. This resulted in the conversion of Asp3 to Glu3 and the deletion of amino acids 4–129, which encompasses the entire PH domain (amino acid residues 7–106) (Shaw, 1993).

Transient and stable expression of RAC-PKα

CHO.IR cells were maintained in F12 Ham's media containing 10% newborn calf serum and 1% penicillin–streptomycin in 5% CO_2. CHO.IR cells were transiently transfected with RAC-PKαHA-pECE, essentially as previously described (Conklin *et al.*, 1992). Briefly, 100 mm plates of CHO.IR cells that were 85% confluent were washed once with PBS and then incubated for 2.5 h in complete media in the presence of DEAE–dextran (250 μg/ml), chloroquine (100 mM), and 4 μg plasmid DNA, except for the cotransfection experiments, in which a total of 12 μg of plasmid DNA were used. The cells were then shocked for 1 min with 10% DMSO and washed twice with PBS before adding fresh complete media. Following transfection, the cells were incubated for 24–36 h before being used in subsequent experiments.

Stable transformants of CHO.IR cells expressing RAC-PKα with the HA epitope were established using calcium phosphate precipitation in the presence of chloroquine (Sambrook *et al.*, 1989). Briefly, two 60 mm plates of CHO.IR cells, each 85% confluent, were cotransfected with 4 μg RAC-PKαHA-pECE and 0.1 μg of plasmid conferring puromycin (de la Luna *et al.*, 1988) resistance by calcium phosphate precipitation. Following a 3 h incubation in the presence of 100 mM chloroquine, the cells were washed twice with PBS. After the cells were incubated for 24 h in complete media, the two plates were combined and split 1:5 in complete media containing 10 μg/ml puromycin. Approximately 2 weeks later individual colonies were picked and screened by electrophoresing total cell lysates on 10% SDS–polyacrylamide gels, transferring, and immunoblotting with the polyclonal anti-HA antibody HA.11 to detect any expressed RAC-PKα. By Western blotting with a polyclonal antibody to the human enzyme, the levels of the expressed RAC-PKα were found to be comparable with the levels of endogenous enzyme.

RAC-PKα kinase assay

CHO.IR cells expressing RAC-PKαHA were washed once with PBS and then starved for 4 h at 37°C by adding 5 ml serum-free F12 Ham's media containing 20 mM HEPES pH 7.4 and 1% penicillin–streptomycin to each plate of cells. At the end of the incubation, 1 mg/ml BSA was added and the cells were treated as indicated in the figure legends. The plates were washed on ice once with HEPES-buffered saline (HBS pH 7.4) and the cells were lysed in 820 μl of lysis buffer (50 mM HEPES pH 7.6, 1% Triton X-100, 1 mg/ml bacitracin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 30 mM $NaPP_i$, 150 mM NaCl, 1 mM EDTA). After 10 min at 0°C, the lysates were centrifuged in a microfuge at 4°C for 5 min. Immunoprecipitates were prepared by preadsorbing for 3 h at 4°C 5 μg of either control normal mouse immunoglobulin or 12CA5 anti-HA monoclonal antibody to 10 μl of a 50:50 slurry of protein A–Sepharose. The beads were washed twice with 1 ml of cold HBS pH 7.4 before dividing the supernatant of each cell lysate in half and adding 400 μl to the preadsorbed antibodies. The immunoprecipitates were incubated for 3 h at 4°C and then washed three times with wash buffer (25 mM HEPES pH 7.9, 0.1% BSA, 10% glycerol, 1% Triton X-100, 1 M NaCl) and twice with kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). The beads were resuspended in 50 μl of kinase mixture (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 μM ATP, 1 μM protein kinase A inhibitor peptide, 25 μg myelin basic protein and 2 μCi [γ-^{32}P]ATP) and incubated at 30°C for 30 min. The kinase buffer was then transferred to an Eppendorf tube containing 10 μl 3× Laemmli sample buffer, after which the samples were boiled for 4 min at 100°C and electrophoresed on 15% SDS–polyacrylamide gels. The gels were stained with Coomassie Blue, dried, and autoradiographed. The band corresponding to myelin basic protein was cut out and counted. All samples were corrected by subtraction of a background value from an identically treated normal mouse immunoglobulin precipitate, a value that was usually <10% of the insulin-stimulated value. 3× Laemmli sample buffer (40 μl) was also added to the remaining beads. The bound protein was eluted by incubating for 4 min at 100°C and these samples were electrophoresed on 10% SDS–polyacrylamide gels. The gels were transferred and immunoblotted using the polyclonal anti-HA antibody HA.11 to detect RAC-PKα.

For the non-transfected cells, the effect of insulin on the migration of the endogenous RAC-PKα was measured by immunoblotting since these antibodies could not efficiently immunoprecipitate the enzyme. For the rat epididymal adipocytes, cells were prepared as previously described (Stagsted *et al.*, 1993). The C2C12 cells were grown as described (Rando *et al.*, 1994). After insulin treatment, the cells were lysed as above and the lysates were directly analyzed by SDS gel electrophoresis and immunoblotting.

Alkaline phosphatase treatment of RAC-PKα

CHO.IR cells transiently transfected with RAC-PKαHA-pECE were lysed and RAC-PKα was immunoprecipitated, as described above. After the cell lysates were incubated with the preabsorbed antibody, the beads were washed three times with 25 mM HEPES pH 7.6, 0.1% BSA, 10% glycerol, 0.1% Triton X-100 and then twice with dephosphorylation buffer (100 mM Tris pH 8, 1 mM $MgCl_2$, 1 mM DTT). The beads were then incubated with shaking at 37°C for 1 h in phosphatase buffer with or without 10 U of calf intestinal alkaline phosphatase. Where noted, the alkaline phosphatase was premixed with phosphatase inhibitor buffer (1 mM Na_3VO_4, 10 mM NaF, 30 mM $NaPP_i$) before it was added to the immunoprecipitated samples. The reactions were terminated by adding phosphatase inhibitor buffer. The beads were then washed once with 25 mM HEPES pH 7.6, 0.1% BSA, 10% glycerol, 0.1% Triton X-100 and twice with kinase buffer prior to the kinase assay.

Phosphatidylinositol 3-kinase assay

CHO.IR cells were washed once with PBS and then serum-starved for 4 h at 37°C by adding 5 ml serum-free F12 Ham's media containing 20 mM HEPES pH 7.4 and 1% penicillin–streptomycin to each plate of cells. At the end of the incubation, 1 mg/ml BSA and 10^{-6} M insulin was added to each plate and the plates were incubated for 5 min at 37°C. Cells were lysed, the lysates were immunoprecipitated with anti-phosphotyrosine antibodies and a PI3-kinase assay was performed as previously described (Chin *et al.*, 1993), except that the indicated concentrations of wortmannin were included in the PI3-kinase assay buffer.

^{32}P-labeling of RAC-PKα

Stable CHO.IR transformants expressing RAC-PKα were labeled with [^{32}P]orthophosphate as previously described (Kovacina *et al.*, 1990). The immunoprecipitated RAC-PKα was electrophoresed and the band was excised and counted and phosphoamino acid analysis was performed as described (Cooper *et al.*, 1983).

Immunoblotting of RAC-PKα

Total cell lysates or immunoprecipitates of RAC-PKα were boiled in 3× Laemmli buffer for 4 min at 100°C, electrophoresed on 10% SDS–polyacrylamide gels and transferred to nitrocellulose. The blots were probed overnight with primary antibody, as indicated in the figure legends. Bound antibody was detected using alkaline phosphatase-conjugated anti-immunoglobulin (Promega).

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