Protein Kinase B and Rac Are Activated in Parallel within a Phosphatidylinositide 30H-kinase-controlled Signaling Pathway*

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The GTPase Rac and the protein kinase (PKB) are downstream targets of phosphatidylinositide 3OH-kinase in platelet-derived growth factor-stimulated signaling pathways. We have generated PAE cell lines inductively expressing mutants of Rac. Use of these cell lines suggests that Rac is involved in both platelet-derived growth factor-stimulated membrane ruffling and the activation of p70S6K but not in the activation of PKB. Furthermore, expression of constitutively active alleles of PKB in PAE cells suggests that PKB is able to regulate the activity of p70S6K but not the cytoskeletal changes underlying membrane ruffling. Thus, our results indicate that Rac and PKB are on separate pathways downstream of phosphatidylinositide 3OH-kinase in these cells but that both of these pathways are involved in the regulation of p70S6K.

The PI3-kinase1 signaling pathway is widely used by receptors for growth factors, inflammatory stimuli, and antigens (1, 2). In the last few years, a number of PI3-kinase inhibition strategies have been used that have identified a family of downstream responses that are thought to be regulated by this pathway, including complex actions such as cell growth, differentiation, and movement (2). One of the current aims of research in this area is to understand the molecular mechanisms by which PI3-kinases engage the regulatory pathways associated with these responses.

The protein kinase PKB has previously been shown to be a ubiquitous target of PI3-kinase regulation in a variety of different contexts (3, 4). Very recently, good evidence has been provided that it is a direct molecular target for phosphatidylinositide 3OH-kinase (PI3-kinase) (5). In addition, the PI3-kinase signaling system operates in the absence of both PKB and Rac (6). However, whether PKB and Rac are activated in some form of hierarchy or as independent pathways has not been established explicitly. We have attempted to address this question in PDGF-stimulated PAE cell lines, where we have previously established that PI3-kinase-dependent membrane ruffling is dependent upon activation of Rac (16, 17).

EXPERIMENTAL PROCEDURES

Antibodies—Mouse mAb anti-HA (12CA5) was from Babco. Rabbit polyclonal anti-PKB serum was used as described (Burgering and Coff (4)). Mouse mAb anti-Myc was a gift from Nick Kitistakis (Babraham Institute, UK). Mouse mAb anti-EE and rabbit polyclonal anti-Rac serum were obtained from ONXY Pharmaceuticals. Rabbit polyclonal anti-p70S6K antibody and the 32-mer p70S6K peptide substrate were gifts from Neil Anderson (University of Manchester, UK). Mouse mAb anti-RacI was from Transduction Laboratories (Lexington, KY).

Cell Culture—Parental PAE cells expressing the PDGF receptor (Ref. 17; designated Wt-PAE cells in this study) were grown in F12 nutrient mixture (Ham F12; Life Technologies, Inc.) containing 10% heat-inactivated FBS (HI-FBS) at 37 °C in a 6% CO2 humidified atmosphere. Inducible Rac PAE cell lines were maintained in F12 nutrient mixture, 10% HI-FBS, 0.3 μg/ml puromycin and 60 μg/ml hygromycin.

Development of an Inducible Expression System—We generated an inducible CMV promoter following the original development of IPTG-inducible SV40 promoters (18–20). This involved the insertion of lac repressor (LacI) binding sequences into the CMV promoter in a fashion whereby they did not significantly attenuate the efficacy of the promoter in the absence of LacI but were able to severely inhibit its action in its presence. Previous work with SV40 promoters indicated that would probably involve the insertion of multiple copies of LacI-binding sites near the TATA box. We used a convenient SacI site in the 2.1-kilobase pair CMV promoter to achieve this (Fig. 1A; there is another SacI site in the first intron of this promoter which we also exploited to insert further LacI-binding sites). We first constructed a simple transcription expression vector, pCMV1-Luc, where expression of luciferase was under the control of the lac repressor (LacI) binding sequences into the CMV promoter in a fashion whereby they did not significantly attenuate the efficacy of the promoter in the absence of LacI but were able to severely inhibit its action in its presence. Previous work with SV40 promoters indicated that would probably involve the insertion of multiple copies of LacI-binding sites near the TATA box. We used a convenient SacI site in the 2.1-kilobase pair CMV promoter to achieve this (Fig. 1A; there is another SacI site in the first intron of this promoter which we also exploited to insert further LacI-binding sites). We first constructed a simple transcription expression vector, pCMV1-Luc, where expression of luciferase was under the control of the lac repressor (LacI) binding sequences into the CMV promoter in a fashion whereby they did not significantly attenuate the efficacy of the promoter in the absence of LacI but were able to severely inhibit its action in its presence. Previous work with SV40 promoters indicated that would probably involve the insertion of multiple copies of LacI-binding sites near the TATA box. We used a convenient SacI site in the 2.1-kilobase pair CMV promoter to achieve this (Fig. 1A; there is another SacI site in the first intron of this promoter which we also exploited to insert further LacI-binding sites). We first constructed a simple transcription expression vector, pCMV1-Luc, where expression of luciferase was under the control of the lac repressor (LacI) binding sequences into the CMV promoter in a fashion whereby they did not significantly attenuate the efficacy of the promoter in the absence of LacI but were able to severely inhibit its action in its presence. Previous work with SV40 promoters indicated that would probably involve the insertion of multiple copies of LacI-binding sites near the TATA box. We used a convenient SacI site in the 2.1-kilobase pair CMV promoter to achieve this (Fig. 1A; there is anotherSacI site in the first intron of this promoter which we also exploited to insert further LacI-binding sites). We first constructed a simple transcription expression vector, pCMV1-Luc, where expression of luciferase was under the control of the lac repressor (LacI) binding sequences into the CMV promoter in a fashion whereby they did not significantly attenuate the efficacy of the promoter in the absence of LacI but were able to severely inhibit its action in its presence.
expression of luciferase is shown in Fig. 1A. We then added three further LacI-binding sites by ligating the SV40-derived intron from pOPT3CAT (Stratagene) downstream of the CMV3 promoter (now denoted CMV3R, this followed advice from D. Wzborski, Stratagene, suggesting that, while not very effective in transient expression assays, this “R” provided good repression of the Rous sarcoma virus promoter in stable cell lines).

LacI Constructs—The F9-1 promoter driving expression of LacI in p3′SS (Stratagene) was replaced with the CMV1 promoter to increase expression of nucleus-localized LacI (23) pCMVLacI, available from Stratagene. pCMVLacI has a cassette conferring hygromycin resistance, as a construction of the cell lines described below, this was deleted from the plasmid by using a puromycin resistance cassette (derived from pBSpac (24) to make pCMVLacPuuro).

Generation of Stable PAE Cell Lines Inducibly Expressing Rac Mutants—Clonal stable cell lines expressing nuclear-localized LacI were selected with puromycin (0.5 μg/ml) over a period of 8–10 days post-transfection with pCMVLacPuuro and isolated by ring cloning (while puromycin was continuously present in the medium). Clones expressing LacI protein were identified by immuno-cytostaining with a rabbit polyclonal anti-LacI serum (Stratagene) and expanded. One clone showing strong, nuclear-localized LacI expression (P23 PAE) was chosen for further use. All inducible lines described in this work were obtained from P23 PAE by a further round of transfection with pCMV3RlacI (see above), selection (hygromycin, 60 μg/ml) and screening. Cloning for inducible expression of Rac mutants was done largely by Western blotting using the polyclonal rabbit anti-Rac serum, although in some experiments expression of the EE-tagged Rac mutants was quantified by anti-EE immunoprecipitations from [32]Smethionine-labeled cells and SDS-PAGE (Fig. 1B) or by Western blot using a mouse mAb anti-Rac1 (Fig. 1C). Between 4 and 12 individual clones for each Rac mutant were finally selected for expansion and storage. For a number of the experiments presented here, more than one Rac mutant was investigated of each type, with essentially identical results.

Rac Constructs—The cDNAs encoding N-terminally EE-tagged WT-Rac, N17Rac (12), and V12Rac (12) were obtained from ONYX Pharmaceuticals. Standard cloning procedures were used to ligate these cDNAs under the CMV3R promoter (see above) in a vector containing an expression cassette conferring hygromycin resistance (p4T4d; a gift from A. Venkitaraman, LMB, Cambridge).

PKB Constructs—The cDNA encoding bovine αPKB (from Boudewijn M. T. Burgelder and Paul J. Coffer, Utrecht, The Netherlands) was ligated into a pCMV transient expression vector with either N-terminal EE (MEEEMFMPMEF) or Myc (MEQKLISEEDLEF) tags (generated by standard polymerase chain reaction-based strategies). The single cDNAs under the CMV3R promoter with either N-terminal EE (MEEEEFMPMEF) or Myc (MEQKLISEEDLEF) tags (generated by standard polymerase chain reaction-generated errors. In some experiments, HA-tagged Wt-PAE cells or N17Rac-PAE cells were transfected with pCMV3RlacI downstream of the CMV3 promoter (now denoted CMV3R, this followed advice from D. Wzborski, Stratagene, suggesting that, while not very effective in transient expression assays, this “R” provided good repression of the Rous sarcoma virus promoter in stable cell lines). They were then pretreated or not with 100 nM wortmannin for 10 min and stimulated with the indicated concentrations of PDGF for 5 min at 37 °C. Activation was stopped by aspirating the medium and immediate addition of 4% paraformaldehyde in 100 mM Pipes, pH 7.2, 2 mM EGTA, 2 mM MgCl₂ for 15 min at room temperature. The cells were washed three times in 150 mM Tris, pH 7.2, 1 mM NaCl, 0.05% Triton X-100, 10 mM dithiothreitol, 20 mM para-nitrophenol phosphate). The samples were prewarmed to 30 °C for 3 min in 50 μl of p70S6K buffer containing 2.5 μM cyclic AMP-dependent protein kinase inhibitor and 3.5 μg of 32-mer p70S6K substrate peptide. The reaction was started by addition of 10 mM ATP solution per sample (80 mM MgCl₂, 200 μM MgATP, and 1 μg of [γ-32P]ATP). Incubation was carried out at 30 °C for 15 min and stopped by the addition of orthophosphoric acid to a final concentration of 200 mM. Samples were spotted onto Whatman P81 paper, washed 6 × 5 min in 175 mM orthophosphoric acid, and quantified by scintillation β-counting.

Immunofluorescence Microscopy—Cells for immunofluorescence microscopy were grown on glass coverslips and serum-starved as described. For phalloidin staining, cells were fixed with 4% paraformaldehyde in 100 mM Pipes, pH 7.2, 2 mM EGTA, 2 mM MgCl₂ for 15 min at room temperature. The cells were washed three times in 150 mM Tris, pH 7.2, 1 mM NaCl, 0.05% Triton X-100, 10 mM dithiothreitol, 20 mM para-nitrophenol phosphate). The samples were prewarmed to 30 °C for 3 min in 50 μl of p70S6K buffer containing 2.5 μM cyclic AMP-dependent protein kinase inhibitor and 3.5 μg of 32-mer p70S6K substrate peptide. The reaction was started by addition of 10 mM ATP solution per sample (80 mM MgCl₂, 200 μM MgATP, and 1 μg of [γ-32P]ATP). Incubation was carried out at 30 °C for 15 min and stopped by the addition of orthophosphoric acid to a final concentration of 200 mM. Samples were spotted onto Whatman P81 paper, washed 6 × 5 min in 175 mM orthophosphoric acid, and quantified by scintillation β-counting.

Membrane Ruffling in PAE Cells Stably Expressing Rac Mutants—To investigate the role of Rac in the PI3-kinase signaling pathway, we have generated

RESULTS

Lamellipodia Formation and Membrane Ruffling in PAE Cells Stably Expressing Rac Mutants—
stable PAE cell lines that inducibly express Wt-Rac (CL-25), constitutively active V12Rac (CL-84), or dominant negative N17Rac (CL-23), using a strategy that is detailed under “Experimental Procedures” (Fig. 1). These cell lines expressed low basal levels of the exogenous Rac mutants (≤10% of endogenous Rac) but, in all cases, the expression increased dramatically upon addition of increasing concentrations of IPTG (Fig. 1, B and C). The sensitivity of induction to IPTG varied between individual clones, but the maximal level of expression of heterologous Rac was between approximately 5-fold (CL-23 N17Rac line) and approximately 20-fold (CL-25 Wt-Rac line) above endogenous Rac levels (Fig. 1C).

Wt-PAE cells respond to PDGF treatment with dramatic formation of lamellipodia (protrusions of the cell that look like large flat pseudopods) and membrane ruffles (areas at the edges of lamellipodia where the plasma membrane detaches from the support and rolls up upon itself). This “ruffling response” is dependent on a wortmannin-sensitive PI3-kinase (16). In parental Wt-PAE cells, the ruffling response was unaffected by addition of up to 15 mM IPTG for 15 h (Fig. 2; a typical lamellipodium with membrane ruffles is marked with an arrowhead). When N17Rac expression was induced with 15 mM IPTG in the cell line CL-23, PDGF-stimulated lamellipodia formation and membrane ruffling were severely attenuated (Fig. 2; CL-23 cells clearly ruffle in response to PDGF in the absence of IPTG). These results are in close agreement with previously published work using microinjection or transient expression of Rac mutants (12) showing that Rac activity is necessary for the ruffling response. However, whereas the PI3-kinase inhibitor wortmannin completely reversed any apparent effect of PDGF on the actin cytoskeleton, N17Rac expression blocked lamellipodia formation and membrane ruffling, but the cells still exhibited clear PDGF-stimulated “boundary actin” polymerization and microspike formation (Fig. 2; a typical microspike is marked with an arrow). These results suggest that Rac may not be the only mediator of PI3-kinase-controlled effects on the actin cytoskeleton.

IPTG-induced Wt-Rac or V12Rac expression in the CL-25 and CL-84 cell lines, respectively, caused a dramatic formation of lamellipodia and membrane ruffles around the entire periphery of the cells (Fig. 2). This response was seen even in the absence of PDGF, showing that Rac activation is a sufficient signal to cause these rearrangements of the actin cytoskeleton. Moreover, Wt-Rac or V12Rac-induced lamellipodia formation and membrane ruffling were not inhibited by wortmannin, confirming previous findings that Rac is situated downstream of PI3-kinase.
Overexpression of Wt-Rac has been suggested to activate Rac-dependent pathways by escaping the normal regulatory constraint of binding to guanine nucleotide dissociation inhibitor (17), and may be a less extreme way of Rac activation than expressing V12Rac. Indeed, we observed that a large number of inducible V12Rac-PAE-cell clones exhibited some basal lamellipodia formation in the absence of IPTG and detectable V12Rac expression on Western blots (Figs. 1C and 2), suggesting that V12Rac is a very potent activator of Rac-dependent responses.

Rac activates p70S6K but Not PKB—Earlier reports have shown that both PKB and p70S6K are downstream targets of PI3-kinase (3, 4, 29), and studies using transient transfection of fibroblasts with N17Rac and p70S6K have indicated that Rac mediates the activation of p70S6K by PDGF (30). We used the inducible Rac-PAE cell lines to study the effects of Wt-Rac, V12Rac, and N17Rac expression on the PDGF-stimulated activation of PKB and p70S6K in PAE cells (Fig. 3). In parental PAE cells, PDGF-stimulated PKB and p70S6K activation was about 10- and 2.5-fold over basal, respectively, and the activation of both enzymes was wortmannin-sensitive.
The level of PDGF-stimulated p70S6K activation in CL-23 and CL-84 cells was only similar to that in parental cells as long as the cells were not treated with IPTG (Fig. 3A); IPTG-induced N17Rac expression significantly inhibited PDGF-stimulated p70S6K activation, whereas V12Rac expression led to p70S6K activation even in the absence of PDGF (Fig. 3A). These results confirm those obtained earlier in fibroblasts that Rac has a regulatory input into the PDGF-stimulated activation of p70S6K (30). However, while wortmannin treatment of PAE cells inhibited p70S6K activation completely, N17Rac expression reproducibly led to only a 60% inhibition (Fig. 3A; basal activity = 37.5 ± 1.1 (arbitrary units); PDGF-stimulated activity in the absence of IPTG = 93.0 ± 0.7 and in the presence of IPTG = 60.9 ± 0.7). This level of inhibition was identical at submaximal doses of PDGF and at varying doses of IPTG (Fig. 3A and data not shown), suggesting that the partial inhibition was not due to insufficient expression of N17Rac. Therefore, it seems likely that Rac is not the sole mediator of PDGF-stimulated p70S6K activation. Furthermore, p70S6K activation is not required for PDGF-stimulated lamellipodia formation and membrane ruffling, as rapamycin, a p70S6K inhibitor, has no effect on this response (data not shown).

The level of PDGF-stimulated PKB activation in the cell lines CL-23, CL-25, and CL-84 was similar to that in parental cells (Fig. 3B).
cells, regardless of whether the expression of N17Rac, Wt-Rac, or V12Rac had been induced with IPTG or not (Fig. 3). Therefore, Rac does not seem to mediate the PDGF-stimulated activation of PKB in PAE cells.

PKB Is Activated via Its PH Domain and by Phosphorylation—Recent work has suggested that PKB may be a direct target of PtdIns(3,4,5)P3 via the binding of the lipid to its PH domain (3, 31). The activation of PKB by PtdIns(3,4,5)P3 is complex, however, and also involves PI3-kinase-dependent phosphorylation of at least two sites, Thr-308 and Ser-473 (32). A Thr-308-directed PKB kinase has been recently characterized (6–8) that may be directly regulated by PtdIns(3,4,5)P3 (8) and is probably at least partially responsible for PDGF-stimulated phosphorylation of this site in vivo. To situate PKB in the PDGF signaling pathway in PAE cells, we looked next at the PDGF-stimulated activation of a range of phosphorylation site and PH domain mutants of PKB transiently transfected into Wt-PAE cells (Fig. 4, A and B).

The use of the phosphorylation site mutants T308A and S473A demonstrated an absolute dependence on Thr-308 phosphorylation and a partial dependence on Ser-473 phosphorylation for PDGF-stimulated PKB activation (Fig. 4A). These results are in close agreement with previous work that implicates PtdIns(3,4,5)P3-dependent kinases in PKB activation (32). Ser-473 phosphorylation-dependent PKB activation could be mimicked by a S473D mutation, whereas the PKB activity could not be recovered with a T308D mutant (Fig. 4A). This is in apparent contrast to previous observations (32) and prevented us from using a S473D/T308D mutant as a constitutively active version of PKB (see below).

The PH domain point mutants of PKB, R25C and W99L, and the mutant PLCδPH-PKB (where the PH domain of PKB had been exchanged for that of phosphoinositidase Cδ) had low basal activity and could not be activated by PDGF (Fig. 4B). These results show that an intact PH domain is required for normal PDGF-stimulated activation of PKB in PAE cells. In contrast, removing the PH domain completely from PKB generated a constitutively active enzyme which could not be significantly activated by PDGF. This could reflect the ability of the ΔPH mutant to act as a substrate for the upstream PKB kinase in the absence of PtdIns(3,4,5)P3 (6). However, in other experiments the ΔPH mutant had a lower basal activity than that presented in Fig. 4 and did exhibit some activation by PDGF. We suspect that this variability was due to the degree of serum starvation of the cells (data not shown). This may explain some of the discrepancies in the literature over whether the PH domain of PKB is (3) or is not (33) critical for the activation of PKB. Taken together, our results indicate that PKB is activated by PDGF in PAE cells in a PH domain and

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**Fig. 4.** Phosphorylation site mutants, PH domain mutants, and constitutively active mutants of PKB. Wt-PAE cells were transiently transfected with EE-tagged phosphorylation site mutants (A), PH domain mutants (B), or constitutively active mutants (C and D) of PKB. The cells were grown for 12 h in F12, 10% HI-FBS, serum-starved for 10 h in F12, 0.1% fatty acid-free BSA, and then incubated in the presence or absence of 5 ng/ml PDGF for 5 min at 37 °C. A–C, PKB activity was determined and corrected for variations in expression levels between the various mutants as detailed under “Experimental Procedures.” Data are means ± S.D. for n = 2 from one representative experiment out of 1–4. An arbitrary value of 100 was assigned to the data derived from 5 ng/ml PDGF-stimulated Wt-PKB. D, p70S6K activity was determined and corrected for variations in expression levels between the PKB mutants as detailed under “Experimental Procedures.” Data are means ± S.D. for n = 4 from one experiment. An arbitrary value of 100 was assigned to the data derived from cells transfected with kinase-dead PKB. □, −PDGF; ■, +PDGF.

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phosphorylation-dependent manner similar to the mechanisms recently described in other systems.

Constitutively Active Mutants of PKB—The data shown in Fig. 3 have suggested that Rac is not involved in the activation of PKB in PDGF-stimulated PAE cells, and those shown in Fig. 4, A and B, are consistent with the idea that PKB is an immediate target of PI3-kinase in this pathway. This leaves two possibilities, PKB could either be situated upstream of Rac or parallel to Rac. To study whether PKB controls Rac, we constructed three PKB cDNAs that might encode constitutively active PKB alleles, DPH-PKB (described in Fig. 4B), Gag-PKB (the oncogenic viral version (34)) and myrist-PKB which carries the membrane targeting myristoylation/palmitoylation consensus of Yes fused to its N terminus (35). Gag-PKB and myrist-PKB have been shown to act as active PKBs in recent studies, supposedly because both are myristoylated and consequently membrane-targeted (33, 34, 36).

Each of the constitutively active mutants exhibited substantially increased PKB activity in the absence of PDGF stimulation when immunoprecipitated from transiently transfected Wt-PAE cells and assayed in vitro with an exogenous substrate (myelin basic protein) (Fig. 4C, note that the scale of the y axis has changed compared with Fig. 4, A and B). Western blots showed that the expression of the full-length Gag-PKB was very low (most of the exogenous protein was expressed as a truncated version, possibly generated from an alternative internal initiation site). Therefore, the specific activity of the full-length Gag-PKB is probably much higher than that shown in Fig. 4C. Expression of each of the active forms led to increased p70S6K activity, indicating that PKB activates p70S6K (4, 37). Therefore, these constitutively active mutants of PKB may be useful tools in defining signaling pathways downstream of PKB.

PKB Does Not Induce Lamellipodia Formation and Membrane Ruffling—We used the constitutively active PKB mutants to study their effect on the Rac-mediated response of lamellipodia formation and membrane ruffling. We transiently transfected Wt-PAE cells with Wt-PKB, the constitutively active PKB mutants, or kinase-dead PKB, stimulated them with PDGF, and analyzed the effects on lamellipodia formation and membrane ruffling by immunofluorescence microscopy after double staining for the exogenous PKB proteins and filamentous actin (Fig. 5).

In resting cells, exogenous Wt-PKB was mostly localized in the nucleus (excluding the nucleoli), and the remainder was in the cytoplasm. This localization was confirmed by confocal microscopy (data not shown). In response to PDGF stimulation, some of the Wt-PKB translocated to the plasma membrane.

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Figure 6. Effect of Rac mutants on p70^{S6K} in PAE cells transfected with constitutively active PKB. N17Rac (CL-23) cells were transiently transfected with kinase-dead PKB (K179A, K180E) or Gag-PKB. After 7 h in F12, 10% HI-FBS, they were serum-starved for 15 h in F12, 1% FBS in the presence or absence of 15 mM IPTG. p70^{S6K} activity was determined as detailed under "Experimental Procedures." Data are means ± S.D. for n = 4 from one experiment. The value obtained for kinase-dead PKB, 0 mM IPTG was arbitrarily standardized to the basal p70^{S6K} activity in CL-23 cells in Fig. 3. □, 0 mM IPTG; ■, 15 mM IPTG.

This can be seen by the more sheetlike appearance of the labeling, which is of uniform intensity right into the very edges of the cell and on a plane of focus that seems to hover over the nuclear labeling (which appears in consequence more blurred). Overexpression of Wt-PKB had no effect on PDGF-stimulated lamellipodia formation and membrane ruffling (Fig. 5).

Kinase-dead PKB showed the same subcellular localization as Wt-PKB and also translocated to the plasma membrane in response to PDGF, showing that PKB activity is not required for membrane translocation. Expression of kinase-dead PKB did not inhibit PDGF-stimulated lamellipodia formation and membrane ruffling (Fig. 5).

ΔPH-PKB also showed a similar localization in resting cells but did not translocate to the plasma membrane in response to PDGF, which was expected, since the PH domain is the lipid-binding part of the enzyme (31). However, constitutively active ΔPH-PKB did not induce lamellipodia formation or membrane ruffling in resting cells.

Constitutively active Gag-PKB (data not shown) and myrist.-PKB (Fig. 5) were localized at the plasma membrane in both resting and activated cells, as expected. Their expression caused a dramatic change in phenotype; the cells were large and flat and were characterized by the long parallel actin bundles. However, lamellipodia formation or membrane ruffling were not induced in these cells (the cell visible in the lower right corner of the photography is dead, not ruffled) although, in response to PDGF, they could clearly "edge-ruffle."

These results demonstrate that lamellipodia formation and membrane ruffling are not induced by expression of active PKB alleles which suggests that PKB is not situated upstream of, but rather parallel to, Rac in the PDGF-stimulated PI3-kinase-pathways in these cells. PKB Activates p70^{S6K} Independently of Rac—We have shown above that both Rac and PKB can activate p70^{S6K} (Figs. 3A and 4D). To investigate further the relationship between these three enzymes within the PDGF pathway, we used the N17Rac line CL-23 to test whether PKB-mediated activation of p70^{S6K} is dependent or independent of Rac activity. For this, CL-23 cells were transiently transfected with kinase-dead PKB or the constitutively active Gag-PKB. Gag-PKB induced a 2–3-fold activation of p70^{S6K} (Fig. 6). Induction of dominant negative N17Rac with IPTG did not make a significant difference to this response, showing that PKB-mediated activation of p70^{S6K} is independent of Rac activity.

DISCUSSION

We studied here the relationship between Rac and PKB in the PDGF-stimulated PI3-kinase signaling pathway in PAE cells. Inducible expression of Rac mutants had no effect on PDGF-stimulated activation of PKB, suggesting that PKB is positioned upstream of or parallel to Rac in this pathway. Active PKB mutants were not sufficient to cause Rac-dependent lamellipodia formation and membrane ruffling, suggesting that Rac and PKB are activated in parallel.

The generation of cell lines that stably and inducibly express constitutively active or dominant negative forms of Rac has allowed us to show that Rac activity is both necessary and sufficient for lamellipodia formation/membrane ruffling and for the activation of p70^{S6K}. As Rac is situated downstream of PI3-kinase in the PDGF signaling pathway, wortmannin would not be predicted to inhibit the activation of p70^{S6K} by expression of V12Rac. Interestingly, Chou and Blenis (30) and we (data not shown) have found, however, that wortmannin does partially inhibit the activation of V12Rac to activate p70^{S6K}. We do not understand the mechanism of this effect, but the use of wortmannin as a tool to inhibit PI3-kinases is widespread and generally accepted to be quite specific, with relatively few alternative targets in the nanomolar range. This suggests that PI3-kinases or related proteins that are wortmannin-sensitive are involved in the activation of p70^{S6K} by Rac (one possible mechanism is the involvement of an autocrine loop in these cells, dependent upon Rac).

We have used a range of PKB mutants to show that an intact PH domain and the capacity to phosphorylate Thr-308 and Ser-473 are required for PDGF-stimulated activation of PKB in PAE cells, confirming reports in other systems (3, 32, 33). We then created a range of constitutively active PKBs (i.e. the ΔPH mutant and the membrane-targeted mutants Gag-PKB and myrist.-PKB) and used these to show that PKB activity is sufficient for the activation of p70^{S6K} but not for lamellipodia formation and membrane ruffling. A mechanism of action for these constitutively active PKB mutants has been suggested by others (35), namely membrane targeting followed by increased phosphorylation and consequent activation. In agreement with this, we have found that Thr-308 mutants of myrist.-PKB are inactive (data not shown). We have also found that Thr-308 mutants of ΔPH-PKB are inactive (data not shown). It is surprising that the deletion of the PH domain should lead to increased phosphorylation of Thr-308 and consequent PKB activation. However, these observations are consistent with a model whereby the phosphorylation of PKB is regulated by PH domain-mediated translocation to the membrane and also a PH domain-controlled inhibition of Thr-308 phosphorylation (6).

As mentioned above, the use of the constitutively active mutants has demonstrated that PKB activity is not sufficient for lamellipodia formation and membrane ruffling, i.e. further targets of PI3-kinase are involved in this response, beyond those dedicated to the activation of PKB. We cannot, however, rule out a contributory role for PKB in this pathway. This would require an effective PKB inhibition strategy. The literature contains reports of the use of kinase-dead PKB as a dominant negative allele to block the effect of endogenous PKB on cell survival pathways (38); unfortunately, our use of this construct results in the reduction in expression of reporter constructs (e.g. heterologously expressed PKB or p70^{S6K}) or has no measurable change in the PDGF-stimulated activity of the relevant endogenous proteins (we have selected populations of cells transiently expressing kinase-dead PKB by co-transfection with green fluorescent protein expression vectors and fluorescence-activated cell sorting; data not shown).

When we transfected PKB mutants into PAE cells, we found
that, in the starved and resting cells, Wt-PKB, ΔPH-PKB, and kinase-dead PKB were mostly localized in the nucleus and only partially in the cytoplasm, whereas earlier reports have indicated that although the oncogenic viral form or Gag-PKB is nuclear, non-transforming versions are cytoplasmic (36, 39). Recent work has also shown that, in 293 cells, overexpressed HA-tagged Wt-PKB is cytosolic in resting cells and translocates into the nucleus upon cell stimulation with insulin-like growth factor-1 (40). We cannot, at present, explain the differences between our results and these studies. The nuclear localization that we observed was not simply due to pCMV-driven overexpression, since we observed the same localization when expressing lower amounts of heterologous PKB (using pSG5-driven HA-tagged PKB; cells stained with mAb anti-HA), and when we labeled endogenous PKB with rabbit polyclonal anti-PKB serum (data not shown). Furthermore, the predominantly nuclear localization of heterologously expressed PKB was not restricted to PAE cells, as we observed it equally in COS, HepG2, or Chinese hamster ovary cells transfected with Myc-tagged PKB with various techniques (DEAE-dextran, liposomes, or calcium phosphate; data not shown). These observations suggest that PKB might have a role as a nuclear signaling enzyme, in addition to its putative role at the plasma membrane.

Our data have shown that PKB and Rac are activated separately in a PI3-kinase-controlled pathway. Although PKB seems to be a direct target of PtdIns(3,4,5)P₃, it is very likely that further direct targets are involved in the activation of Rac. Other analogous second messenger-generating signaling systems (e.g. cAMP or inositol 1,4,5-trisphosphate) operate through a very small number of primary targets for the messenger molecule. It is possible, however, that PtdIns(3,4,5)P₃ may act via its interaction with membrane-targeting PH domains (41) to regulate a larger number of proteins in parallel, reflecting an increased emphasis on positional information as a major regulatory function of the messenger molecule.

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