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Phosphatidylinositol 3<latex>$^{\prime}$</latex> Kinase: One of the Effectors of Ras [and Discussion]

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Phosphatidylinositol 3′ kinase: one of the effectors of Ras

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SUMMARY

Ras proteins are proto-oncogene products that are critical components of signalling pathways leading from cell surface receptors to control of cellular proliferation, morphology and differentiation. The ability of Ras to activate the MAP kinase pathway through interaction with the serine/threonine kinase Raf is now well established. However, recent work has shown that Ras can also interact directly with the catalytic subunit of phosphatidylinositol 3′ kinase and is involved in control of the lipid kinase in intact cells. A model is presented in which both tyrosine phosphoprotein interaction with the regulatory p85 subunit and Ras.GTP interaction with the catalytic p110 subunit is required to achieve optimal activation of phosphatidylinositol 3′ kinase in response to extracellular stimuli. The ability of Ras to regulate phosphatidylinositol 3′ kinase may be important both in Ras control of cellular morphology through the actin cytoskeleton and also in Ras control of DNA synthesis.

1. REGULATION OF PHOSPHATIDYLINOSITOL 3′ KINASE

Phosphatidylinositol 3′ kinase is stimulated in response to treatment of cells with a very wide variety of stimuli. It is subject to regulation by a number of known mechanisms, the best characterized of which is the interaction of the p85 regulatory subunit bound to p110a and b with tyrosine phosphorylated sequences in other proteins. This interaction and the subsequent modest stimulation of lipid kinase activity is described in detail in the previous article. The interaction of p85 with tyrosine phosphoproteins such as growth factor receptors may contribute to elevation of phosphatidylinositol 3′ kinase activity within the cell by a combination of translocating the enzyme to the plasma membrane where its substrate lipid is located, and also by allosteric regulation of the kinase activity, possibly by alteration of the relative orientation of the two SH2 domains.

In addition, it is now clear that there are two proline-rich motifs in p85 that are capable of binding to SH3 domains, both in other proteins ($p145^{abl}$, $p56^{lck}$, $p59^{fyn}$, $p55^{blk}$ and $p60^{src}$) and in p85 itself (Pleiman *et al.* 1993; Prasad *et al.* 1993*a*; Prasad *et al.* 1993*b*; Vogel & Fujita 1993; Kapeller *et al.* 1994). *In vitro* work suggests that the interaction of p85 with the SH3 domain of $p56^{lck}$ may result in activation of its lipid kinase activity (Pleiman *et al.* 1994). It is possible that normal regulation of phosphatidylinositol 3′ kinase involves competition between exogenous SH3 domains and p85's own SH3 domain for binding to the proline-rich motifs in p85.

Another possible regulatory mechanism for phosphatidylinositol 3′ kinase may involve the Bcr-like domain of p85. Proteins with homology to this sequence are known to act as GTPase activating proteins for members of the Rho family of Ras-related proteins, which includes Rho, Rac and CDC42 (Diekman *et al.* 1991). It has not been possible to demonstrate that the Bcr region of p85 acts as a GTPase activating protein. However, Rho does appear to be involved, either directly or indirectly, in the regulation of phosphatidylinositol 3′ kinase in platelets (Zhang *et al.* 1993*a*). Furthermore, *in vitro* evidence has been obtained for an interaction of the p85 subunit of phosphatidylinositol 3′ kinase with GTP-bound Rac and CDC42, with subsequent stimulation of lipid kinase activity (Zheng *et al.* 1994). Other groups have been unsuccessful in seeing interaction of Rho family proteins with phosphatidylinositol 3′ kinase either *in vitro* or *in vivo* (Rodriguez-Viciana *et al.* 1994), so the significance of such mechanisms in the regulation of phosphatidylinositol 3′ kinase remains uncertain.

As described in the article by M. D. Waterfield, the family of phosphatidylinositol 3′ kinases consists of many members in addition to those such as p110α and β that bind to p85 regulatory subunits. Other members of the family are likely to be regulated in very different ways since they are not associated with subunits containing SH2, SH3, SH3-binding and Bcr-like domains. The best characterized control mechanism operating independently of p85 is the regulation of certain phosphatidylinositol 3′ kinases by the bg subunits of heterotrimeric GTP binding proteins (Stephens *et al.* 1994*a*), although this may also be capable of acting on some p85 associated phosphatidylinositol 3′ kinases (Thomason *et al.* 1994). It therefore appears that several different regulatory mechanisms can act simultaneously on these lipid kinases to control their activity in response to extracellular signals. In this article I will consider in more detail whether the small

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GTP-binding protein Ras is also involved directly in the control of phosphatidylinositol 3′ kinase and what the significance of such an interaction might be in the function of Ras in the control of cellular behaviour.

2. RAS PROTEINS AND THEIR EFFECTORS

The three proto-oncogenes, H-, K- and N-*ras* encode very closely related 21 000 molecular weight monomeric GTP binding proteins. They are the prototypic members of the large Ras superfamily of low molecular weight GTPases (Lowy & Willumsen 1993). The superfamily is made up of a number of families, the best characterized of which are: (i) the Ras family, whose members play a role in the control of cellular proliferation; (ii) the Rho family, whose members are involved in regulation of the cytoskeleton among other functions; and (iii) the Rab family, which is involved in the regulation of intracellular membrane trafficking. All of these proteins bind to GTP and catalyse its hydrolysis to GDP. When in the GTP-bound state they are biologically active, while in the GDP-bound conformation they are inactive. The nucleotide binding of Ras proteins is controlled by two sets of proteins, the GTPase activating proteins (GAPs) which stimulate the rate of hydrolysis of GTP bound to Ras, thus switching off the Ras proteins, and the guanine nucleotide exchange factors, which stimulate the rate at which nucleotide exchanges on and off Ras. Because the predominant guanine nucleotide in the cytosol is GTP, exchange factors act within the cell to activate Ras proteins.

The activation state of Ras itself is regulated in response to a very wide variety of extracellular stimuli. Most factors that interact with cell surface receptors of the tyrosine kinase or seven membrane spanning families have been shown to be capable of activating Ras in at least some cell types. In addition, a great many hematological cytokine activate Ras. The list of factors that will activate Ras is remarkably similar to those that will activate phosphatidylinositol 3′ kinase (Stephens *et al.* 1993). Over the past few years enormous advances have been made in understanding the mechanisms involved in the regulation of Ras, particularly the formation of complexes of tyrosine phosphorylated proteins, such as the epidermal growth factor receptor or Shc, with SH2 and SH3 domain containing adaptor proteins, such as Grb2, and guanine nucleotide exchange factors, such as Sos (McCormick 1993).

Once in the active, GTP-bound state, Ras influences cell behaviour by interacting with target proteins, generally known as effectors. The best characterized effectors for Ras are the serine/threonine kinases of the Raf family, A-Raf, B-Raf and c-Raf-1 (Rapp 1991). These interact with Ras.GTP, but not Ras.GDP, both *in vitro* (Moodie *et al.* 1993; Vojtek *et al.* 1993; Warne *et al.* 1993; Zhang *et al.* 1993*b*), in the yeast two-hybrid system (Van Aelst *et al.* 1993; Vojtek *et al.* 1993; Zhang *et al.* 1993*b*) and in intact cells (Finney *et al.* 1993; Hallberg *et al.* 1994). The interaction is through the regulatory CR1 region in the amino-terminal part of Raf. As a result of this interaction, Raf is brought to the plasma membrane where Ras is localized because of its post-translational modification with farnesyl and palmityl moieties (Leevers *et al.* 1994; Stokoe *et al.* 1994). Whereas the interaction of Ras.GTP with Raf does not appear to cause activation of its kinase activity directly, it appears to be responsible for presenting Raf to another signalling component, probably localized in the plasma membrane, which is able to further modify it resulting in increased kinase activity. Once Raf has been activated, it phosphorylates and activates MEK, the MAP kinase kinase, which in turn phosphorylates and activates MAP kinase. By this kinase cascade, Ras is able to activate the MAP kinase pathway leading to phosphorylation of a number of transcription factors and hence stimulation of the expression of immediate early genes (Egan & Weinberg 1993).

Many of the effects of Ras on the cell may be mediated through the MAP kinase pathway. However, it is clear that the induction of cellular proliferation caused by expression of activated mutants of MEK in fibroblasts is distinct from that caused by expression of activated mutants of Ras (Cowley *et al.* 1994). The MAP kinase pathway may be capable of causing many of the transcriptional effects of Ras and its stimulation of DNA synthesis, but it does not induce the profound changes in cell morphology that Ras does. This raises the likelihood that there are other effectors of Ras in addition to Raf. It has long been considered a possibility that the GTPase activating proteins $p120^{GAP}$ and neurofibromin might act as Ras effectors because they interact only with the GTP- and not the GDP-bound form of Ras. Some evidence exists to support this hypothesis for $p120^{GAP}$ in the case of Ras-induced germinal vesicle breakdown in *Xenopus* oocytes (Duchesne *et al.* 1993): however, the fact that fibroblasts from mouse embryos which lack neurofibromin or $p120^{GAP}$ because of homologous recombination are able to grow apparently normally in culture argues against a major role for these proteins downstream of Ras. In contrast, blocking Ras function in most cell types by the use of dominant negative mutants of Ras or anti-Ras antibodies results in arrest of cell growth.

Another family of proteins that may have Ras effector function was identified in the yeast two-hybrid screen (Albright *et al.* 1993). This consists of two closely related members that interact with Ras.GTP but not Ras.GDP. One of these proteins was identical to a previously characterized protein known as RalGDS: this protein was identified originally as a guanine nucleotide exchange factor (GDS stands for GDP dissociation stimulator) for the Ras family member Ral. The function of Ral is not known. Despite having about 50% identity to Ras, it is not able to transform cells. RalGDS does not have exchange activity towards Ras; in fact, Ras does not interact with the exchange factor domain (CDC25-like) of RalGDS, but rather with the amino terminal regulatory region. One might imagine that activated Ras controls the guanine nucleotide exchange rate on Ral through RalGDS: however, such regulation has not been possible to demonstrate a yet. The role of RalGDS and RalGDS-related protein in the effects of Ras on cellular function are entirely unknown at this time.

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The other family of proteins that are candidate effectors for Ras are the phosphatidylinositol 3′ kinases. The evidence for believing that Ras signals at least in part through phosphatidylinositol 3′ kinase is discussed below.

3. EVIDENCE THAT RAS REGULATES PHOSPHATIDYLINOSITOL 3′ KINASE

The first indication that Ras might interact with phosphatidylinositol 3′ kinase came from the observation that some phosphatidylinositol 3′ kinase activity could be found in immunoprecipitates of Ras from Ras transformed cells (Sjölander *et al.* 1991). At the time the significance of this observation was questioned because the monoclonal antibody used to immunoprecipitate Ras was Y13-259, which blocks the biological activity of Ras by binding close to the region that undergoes a conformational change on GTP hydrolysis and is implicated as the site of interaction with biological targets from mutational analysis (Lowy & Willumsen 1993). It would therefore not be expected to allow co-immunoprecipitation of Ras with an effector.

These observations prompted study in my laboratory of the possible interaction of Ras with phosphatidylinositol 3′ kinase (Rodriguez-Viciana *et al.* 1994). Ras proteins were expressed by using a baculovirus system and purified to homogeneity. Phosphatidylinositol 3′ kinase p85α or β and p110α subunits were expressed in a similar manner. Both untagged p85/p110 was purified and a form in which the p110 subunit was GST tagged to allow easier purification. It was found that purified Ras immobilized on agarose beads would bind to p85/p110 when it was GTP-bound but not when it was GDP-bound; this interaction could be observed either by measurement of lipid kinase activity or immunoblotting for p85. The interaction could be shown to be direct and not to involve other proteins. This indicated that phosphatidylinositol 3′ kinase might act as a direct effector of Ras, a hypothesis that was further supported by the observations that mutations in the 'effector loop' of Ras, which are known to destroy its biological activity, prevented it from interacting with p85/p110. Furthermore, a peptide derived from the sequence of the Ras effector loop, which is known to block the association of Ras with its effector Raf (Warne *et al.* 1993), was also able to interfere with the interaction of Ras with phosphatidylinositol 3′ kinase. The interaction between Ras and phosphatidylinositol 3′ kinase was found to be very heavily, though possibly not completely, inhibited by the antibody Y13-259, but not the non-neutralizing antibody Y13-238. It is possible that the original observation of phosphatidylinositol 3′ kinase activity in Y13-259 immunoprecipitates was caused by heterogeneity in the preparations of antibody used in different laboratories.

Whereas Ras interacted well with phosphatidylinositol 3′ kinase, with an affinity of roughly 300 nM compared with 50 nM for the Ras-Raf interaction, we could find no evidence for association of any Rho family proteins with p85/p110. However, the closely related members of the Ras family Rap1a, Rap1b, R-Ras and TC21 all interacted with phosphatidylinositol 3′ kinase, although Rap2 and Ral did not. A similar pattern of binding is observed for Raf, although the significance of these Ras-related proteins is not well

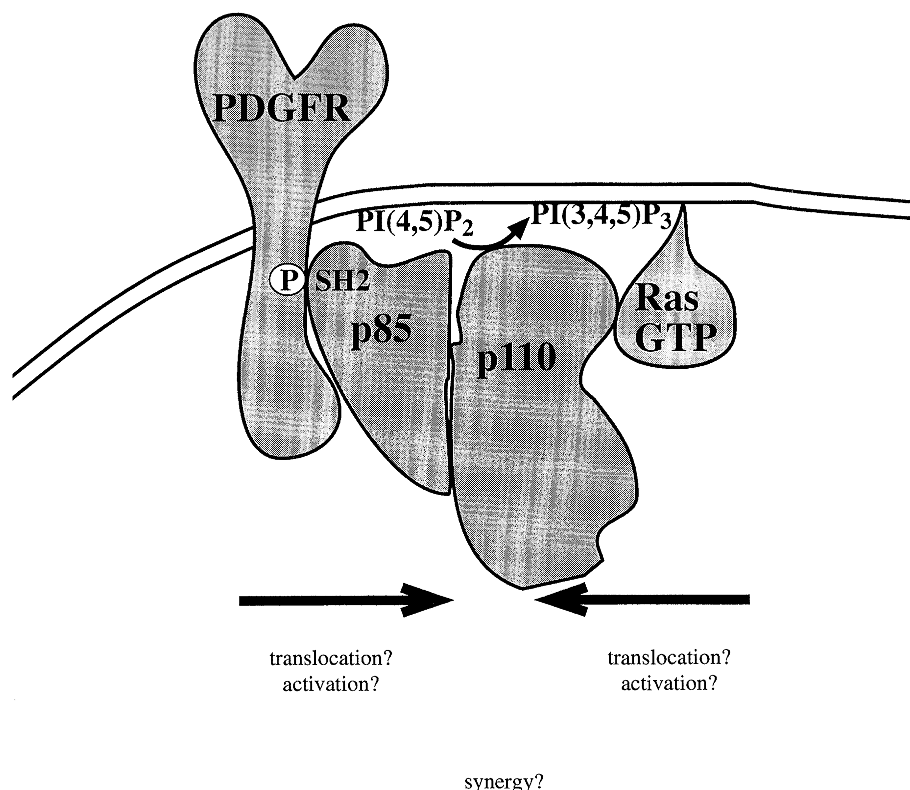

Figure 1. Contribution of Ras.GTP to activation of PI 3-kinase by PDGF. (See text for details).

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understood. The fact that only Ras and not Rho proteins were interacting with phosphatidylinositol 3′ kinase suggested that the interaction was not through the Bcr-like domain in p85. When binding of Ras to p85 or p110 was assessed separately, it was found that Ras interacted with p110, the catalytic subunit, but not with p85, the regulatory subunit. It therefore seems likely that possible regulation of phosphatidylinositol 3′ kinase by Ras would be through a different subunit to regulation by tyrosine phosphoproteins.

The convincing interaction of Ras with the catalytic subunit of phosphatidylinositol 3′ kinase in a strictly GTP-dependent manner *in vitro* suggested that Ras might be controlling phosphatidylinositol 3′ kinase activity in intact cells. To investigate this possibility, expression plasmids coding for activated mutants of Ras were transfected into COS cells. The levels of 3′ phosphorylated phosphoinositides were measured in the cells 48 hours after transfection. Activated Ras was found to cause a large increase in the amount of phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate in the cells. A similar increase was observed when the catalytic and regulatory subunits of phosphatidylinositol 3′ kinase were transfected in to the cells. When Ras and p85a/p110a were transfected into COS cells together, there was a synergistic increase in phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate levels. Effector mutants of Ras were inactive. Interestingly, activated mutants of Raf were also inactive in this assay, suggesting that the effect of Ras on phosphatidylinositol 3′ kinase activity was the result of a bifurcation in Ras effector pathways. Rho family proteins were inactive in this assay.

It appears therefore that Ras is capable of stimulating phosphatidylinositol 3′ kinase activity in intact cells, at least when it is overexpressed. However, how important is the function of endogenous Ras in the activation of phosphatidylinositol 3′ kinase in response to growth factor treatment of cells. To investigate this, PC12 rat pheochromocytoma cells were created that expressed a dominant negative mutant of Ras, N17. Unlike most untransformed cells in culture, PC12 do not require Ras function for continued proliferation, but instead differentiate into neuron-like cells in response to activated Ras. Normally, treatment of PC12 cells with nerve growth factor (NGF) or epidermal growth factor (EGF) result in strong activation of phosphatidylinositol 3′ kinase activity as can be seen from measuring the levels of the 3′ phosphorylated phosphoinositides (Carter & Downes 1992). When PC12 are expressing dominant negative Ras, the ability of NGF and EGF to elevate levels of these lipids is greatly reduced, to about 25% of the levels achieved in the absence of N17 Ras. These data indicate that Ras contributes significantly to the activation of phosphatidylinositol 3′ kinase activity in response to growth factor activation of receptor tyrosine kinases. It is likely that interaction of Ras.GTP with the catalytic subunit cooperates with tyrosine phosphoprotein interaction with regulatory subunit to give optimal activation of phosphatidylinositol 3′ kinase activity.

What other data exists to support the hypothesis that phosphatidylinositol 3′ kinase acts as an effector of Ras? It has been known for some time that the amount of phosphatidylinositol 3′ kinase found in anti-phophotyrosine immunoprecipitates following cellular stimulation does not always correlate well with the level of phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate in the cells. For example, when mast cell were incubated with a range of cytokines including interleukins-3, -4 and -5, granulocyte-macrophage colony stimulating factor and Steel factor, it was found that interleukin-4 (IL-4) caused much the weakest increase in 3′ phosphorylated lipid levels, but the largest increase in phosphatidylinositol 3′ kinase activity associated with anti-phosphotyrosine immunoprecipitates, indicating that the *in vitro* assay is a poor reflection of the *in vivo* activation state of the lipid kinase (Gold *et al.* 1994). This is similar to what was found in the N17 Ras expressing PC12 cells, where 3′ phosphorylated lipid levels were greatly reduced without effecting the amount of phosphatidylinositol 3′ kinase activity in anti-phosphotyrosine immunoprecipitates. Interestingly, IL-4 is incapable of activating Ras in mast cells (Duronio *et al.* 1992), whereas the other cytokines do, suggesting that the reason that IL-4 is a poor activator of *in vivo* phosphatidylinositol 3′ kinase activity is that it only activates one of the two pathways leading to phosphatidylinositol 3′ kinase, namely tyrosine phosphoprotein interaction with p85.

Another case which is related to this is that of polyoma virus middle-T antigen, which is phosphorylated at multiple tyrosine residues by $p60^{c\text{-}src}$. A non-transforming mutant of middle-T exists that does not bind to Shc/Grb2 and therefore does not activate guanine nucleotide exchange on Ras (Campbell *et al.* 1994; Dilworth *et al.* 1994). This mutant binds the same amount of phosphatidylinositol 3′ kinase through its tyrosine phosphorylated p85 binding site: however, it does not cause elevation of 3′ phosphorylated phosphoinositides in whole cells (Ling *et al.* 1992). This may be because of failure to activate both phosphotyrosine mediated p85 binding and Ras.GTP interaction with p110 at the same time. Somewhat similar observation have also been made with Trk, the high affinity receptor for nerve growth factor (R. Baxter and P. Downes, personal communication; J.D., unpublished observations). In this case, mutation of tyrosine 490, a Shc binding site presumably involved in Ras activation, blocks the ability of NGF to stimulate phosphatidylinositol 3′ kinase *in vivo*, although lipid kinase activity in anti-phosphotyrosine immunoprecipitates also goes down in this case. Phosphatidylinositol 3′ kinase does not bind directly to Trk, but rather to a 110 kDa protein that is phosphorylated in response to NGF (Stephens *et al.* 1994*b*). There is no evidence that Shc binds to p85.

One other system has been reported in which there is good evidence that Ras controls the activity of phosphatidylinositol 3′ kinase. This is an artificially created strain of the yeast *Schizosaccharomyces pombe* which expresses mammalian p110 on an inducible promoter. Normally yeast only contain phosphatidylinositol (3) phosphate, but not more heavily phos-

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phorylated 3′ phosphoinositides. On expression of p110, phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate are found in the cells and their growth is profoundly inhibited (Kodaki *et al.* 1994). When p85 is also expressed in the same cells, the amount of phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate is greatly reduced and the inhibition of cell growth is relieved. This suggests that the basal function of p85 is to inhibit the activity of p110; presumably binding to tyrosine phosphoproteins normally overcomes this inhibition. When activated mutant Ras, but not effector mutants, are put into yeast expressing p85 and p110, growth is once again inhibited and cellular levels of phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate elevated. Thus activated Ras opposes the effect of unstimulated p85 on the activity of the catalytic subunit of phosphatidylinositol 3′ kinase.

4. POSSIBLE ROLES OF PHOSPHATIDYLINOSITOL 3′ KINASE IN MEDIATING RAS EFFECTS ON THE CELL

As discussed above, there is good evidence to suggest that the interaction of Ras.GTP with p110 is one of a number of pathways involved in regulating the activity of phosphatidylinositol 3′ kinase. It is likely that this pathway synergizes with the interaction of tyrosine phosphoproteins with p85, and that both inputs are required to achieve good activation of lipid kinase activity in response to growth factors and other stimuli. As discussed in the article by M. D. Waterfield, several forms of phosphatidylinositol 3′ kinase exist that do not interact with p85 regulatory subunits. Some of these enzymes do, however, interact with Ras.GTP (J. D. and R. Wetzker, unpublished observations); in these cases Ras may synergize with other signalling pathways. The question remains, however, of how important is phosphatidylinositol 3′ kinase activity to the effects of Ras on cellular function. Whereas little is definitively known on this topic at present, in the final section of this review some speculation on this will be entered into.

The importance of the Raf/MAP kinase pathway in mediating the effects of Ras on activation of gene transcription is now well established. However, is the activation of this pathway alone sufficient to give cellular proliferation, or, in cases of constitutive activation, transformation? Clearly, activated Raf mutants are potent oncogenes (Rapp 1991) and activated mutants of MEK are able to induce proliferation of NIH 3T3 fibroblasts (Cowley *et al.* 1994). However, it is possible that some of these effects are secondary to the activation of expression of genes encoding autocrine growth factors, such as TGFα. In the case of activated MEK mutants, morphological transformation is inhibited by neutralizing anti-Ras antibodies, suggesting that these cytoskeletal changes are caused by factors secreted by the cells which act through receptors coupled to Ras along pathways distinct from the Raf/MAP kinase pathway. Of the possible alternative effectors for Ras in addition to Raf, phosphatidylinositol 3′ kinase is probably the best candidate to be involved in inducing cytoskeletal changes.

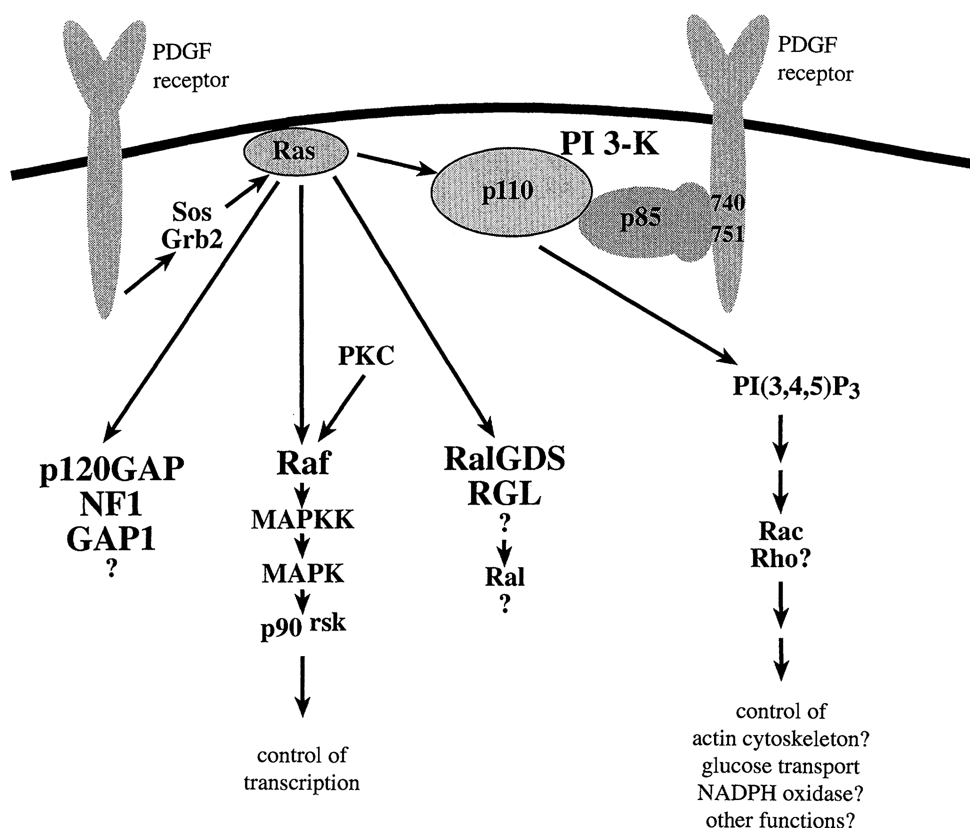

Figure 2. Ras is involved in the direct activation of multiple signalling pathways. (See text for details.)

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Activated Ras has been known for ten years to be able to induce changes in cellular morphology: when injected into fibroblasts it causes membrane ruffling within 30 minutes (Bar Sagi & Feramisco 1986). The relatively long time-period required is probably a result of the requirement for microinjected Ras to become post-translationally modified once it enters the cells. Similar effects are seen when activated mutants of the Ras-related protein Rac are microinjected into Swiss 3T3 cells (Ridley *et al.* 1992). Dominant negative mutants of Rac inhibit the effects of Ras, suggesting that Ras acts upstream of Rac in a pathway to control the organization of the cortical actin cytoskeleton. It is known that PDGF induces membrane ruffling in a manner that is sensitive to the phosphatidylinositol 3′ kinase inhibitor wortmannin; this effect is also blocked by mutations that remove the p85 binding phosphotyrosine residues from the PDGF receptor (Y740 and 751) or the overexpression of a mutant p85 that does not interact with p110 (Kotani *et al.* 1994; Wennström *et al.* 1994). Rac and phosphatidylinositol 3′ kinase thus both lie upstream of actin rearrangement. It is possible that Ras in part mediates the activation of phosphatidylinositol 3′ kinase in response to growth factors, which in turn induces activation of Rac which induces effects on the actin cytoskeleton. Because it is not possible to activate phosphatidylinositol 3′ kinase by transfecting activated Rac into COS cells (Rodriguez-Viciana *et al.* 1994), it is likely that Rac acts downstream of the lipid kinase products.

The effect of inhibiting Ras function on the ability of growth factors to induce membrane ruffling is not clear at present. The ability of dominant negative Ras mutants to inhibit short term effects of growth factors on a number of pathway is not straightforward: N17 Ras has no effect on the ability of EGF to activate MAP kinase, presumably because there are at least two alternative pathways to MAP kinase activation from the EGF receptor in addition to that involving Ras (Burgering *et al.* 1993). Similar redundancy may exist in the control of phosphatidylinositol 3′ kinase and the cytoskeleton. We do, however, find that the effects of Ras on membrane ruffling are sensitive to the phosphatidylinositol 3′ kinase inhibitor wortmannin (P. Rodriguez-Viciana and J.D., unpublished observations). It is very likely that there is a major component of the effects of growth factors on cell morphology that is mediated by Ras, and that a large part of the effects of Ras on cell morphology is mediated by phosphatidylinositol 3′ kinase.

Through the use of inhibitors such as wortmannin, phosphatidylinositol 3′ kinase has been implicated in the regulation of a number of other cellular activities, including glucose transport and NADP oxidase function in neutrophils. The possible importance of these specialized functions to the cellular effects of Ras is not clear. A more general effect of inhibiting phosphatidylinositol 3′ kinase function has been found on microinjecting antibodies against p110 into fibroblasts: DNA synthesis in response to a number of growth factors is inhibited (Roche *et al.* 1994). While it is not immediately obvious what pathway might be involved here, one possibility is that this could reflect regulation of certain protein kinase C isotypes by phosphatidylinositol (3,4,5) trisphosphate (Toker *et al.* 1994). It has been known for some time that the ability of activated Ras to induce DNA synthesis in fibroblasts is dependent on the presence of functional protein kinase C (Morris *et al.* 1989), whereas Ras induced morphological changes are independent of protein kinase C. It is conceivable that the protein kinase C dependent pathway leading from Ras to DNA synthesis may involve phosphatidylinositol 3′ kinase mediated protein kinase C activation, whereas the protein kinase C independent pathway from Ras to morphological transformation involves targets of phosphatidylinositol 3′ kinase other than protein kinase C.

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Discussion

M. Karin (*Department of Pharmacology, Univeristy of California, San Diego, U.S.A.*). What effect does over-expression of catalytically inactive p110 have on transformation and other Ras-driven functions?

J. Downward. Over-expressing p110 inhibits several Ras functions, including activation of MAP kinase, but this is

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probably because it ties up Ras in an inactive state rather than because of any specific effect on that downstream pathway. As a result, our analyses of transformants of this type have not been very productive.

C. J. Marshall (*Chester Beatty Laboratory, Institute of Cancer Research, London, U.K.*). What are the effects of wortmannin on the phenotypes produced by Ras?

J. Downward. Experiments of this type are difficult because the instability of wortmannin means it has to be re-applied at regular intervals throughout the experiment. In addition, it is now getting clear that the effects on the morphological effects of Ras, such as ruffling, vary between cells. As a result, it seems likely that there are multiple pathways between Ras and the effects on cytoskeletal organization. It should also be remembered that the specificity of wortmannin is sufficiently suspect for it to be unsafe to assume that all of its effects are due to inhibition of the phosphoinositide 3-kinase pathway.

M. Karin. Is there synergy in cells between p110 and the function of Raf?

J. Downward. Yes, we can see some synergy between normal p110 (combined with p85) and weakly activated mutants of Raf in transformation.

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