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A Heterotrimeric GTPase-Regulated Isoform of PI3K and the Regulation of its Potential Effectors [and Discussion]

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A heterotrimeric GTPase-regulated isoform of PI3K and the regulation of its potential effectors

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SUMMARY

We have purified two forms of phosphoinositide 3-kinase (PI3K) that are activated by heterotrimeric G-protein βγ-subunits. These novel isoforms of PI3K are structurally distinct to those forms of PI3K which have already been cloned. They are both heterodimers made up of a p120 and a p101 and a p117 and a p101 protein. The p101 species in both heterodimers are identical and show no substantial homology with any other proteins or DNA sequences. The p117 and p120 are highly related. The p101 and p120 species have been cloned from a pig neutrophil mRNA library. The p120 has similarities with other known P13K catalytic subunits. They may be responsible for conferring cells with the capacity to produce phosphatidylinositol(3,4,5)-trisphosphate in response to activation of G-protein-coupled receptors.

Activation of both the monomeric G-protein rac and PI3K(s) have been implicated in receptor-stimulated membrane-ruffling. We have observed that agonist-stimulated guanine nucleotide exchange on rac can be inhibited by a variety of PI3K inhibitors. This suggests PI3K may lie upstream of rac in receptor-driven pathways regulating cell movement.

1. INTRODUCTION

Phosphatidylinositol(3,4,5)-trisphosphate (PtdIns-(3,4,5)P_3) was identified initially in extracts from FMLP-stimulated neutrophils (Traynor-Kaplan *et al.* 1988; Stephens *et al.* 1991). Its levels were undetectable in unactivated cells but upon stimulation they rose rapidly to peak within 10–20 s before declining back toward basal. A large variety of extracellular transmitters, which utilise very different receptor transduction mechanisms, can activate PtdIns(3,4,5)P_3 accumulation. This knowledge, along with other factors, has lead to what has become a widely held belief; that PtdIns(3,4,5)P_3 is an intracellular messenger (Auger *et al.* 1989; Cantley *et al.* 1991; Hawkins *et al.* 1992; Stephens *et al.* 1993*a*; Kapeller & Cantley 1994). The recent use of inhibitors of PtdIns-(3,4,5)P_3 formation has supported this concept. Specifically blocking PtdIns(3,4,5)P_3 abolishes a number of complex cellular responses to hormone activation, including secretion, ruffling (see below), superoxide production and activation of glucose uptake (Stephens & Hawkins 1995). The molecular mechanisms by which the production of PtdIns-(3,4,5)P_3 is regulated by different agonists and receptors are becoming clearer. They are based, like other better understood signal transduction pathways, on the existence of a family of related enzymes that catalyse synthesis of PtdIns(3,4,5)P_3 (phosphoinositide 3-kinases; PI3Ks) that are individually adapted to interact with different upstream activators. The processes connecting the appearance of PtdIns(3,4,5)P_3 with the activation of other cellular responses are unclear, but analogy with the steps downstream of low-molecular mass messengers in other signalling cascades suggests certain common elements may be implicated in most or all of the diverse collection of cellular responses that lie downstream of PtdIns(3,4,5)P_3. Some recent work has suggested the small G-protein rac may serve such a role.

2. PI3Ks

PI3Ks capable of synthesising PtdIns(3,4,5)P_3 have been purified from several tissues (Carpenter *et al.* 1990; Morgan *et al.* 1990; Shibasaki *et al.* 1991; Thelen *et al.* 1994); they are heterodimers (with one possible exception; Shibasaki *et al.* 1991) made up 110 kDa and 85 kDa subunits. cDNAs encoding these proteins have been cloned and expressed (Escobedo *et al.* 1991; Otsu *et al.* 1991; Skolnik *et al.* 1991; Hiles *et al.* 1992). The 110 kDa proteins contain a catalytic domain which confers lipid kinase activity (Hiles *et al.* 1992) and appears to be the binding site for wortmannin, a highly potent inhibitor of PI3Ks (DeWald *et al.* 1988; Yano *et al.* 1993). The sequence of the catalytic domain has been used to isolate a substantial family of related cDNAs. Only the most closely related member of these proteins similar to p110 has also been shown to complex with the 85 kDa protein(s) found in purified PI3Ks (see below) and to synthesise PtdIns(3,4,5)P_3.

There is a small family of 85 kDa proteins (Otsu *et al.* 1991; Gout *et al.* 1992), none of which possess lipid kinase activity, but which do contain a number of protein:protein interaction domains known to be shared with other key signalling enzymes (Kapellar *et al.* 1994). The best understood of these are two

phosphotyrosine-binding SH2 domains (src region 2 homology domains). The SH2 domains on a particular protein, although closely related to other SH2 domains, appear to display a unique specificity for phosphotyrosyl residues within a precise local sequence (Cantley *et al.* 1991; Songyang *et al.* 1993). They are the structure which enables PI3K to bind to certain activated (and hence tyrosine phosphorylated) receptor tyrosine kinases such as the PDGF receptor (Valius & Kazlauskas 1993; Wennström *et al.* 1994). Interaction of the activated tyrosine kinase with PI3K leads to activation of the lipid kinase activity of the latter and accumulation of PtdIns(3,4,5)P_3 (Backer *et al.* 1992; Carpenter *et al.* 1993).

However, the majority of G-protein-linked receptors on myeloid-derived cells can also activate PtdIns(3,4,5)P_3 accumulation via a mechanism that appears to involve heterotrimeric G-proteins and to be independent of protein tyrosine kinases (Stephens *et al.* 1993b, c) . By fractionating cytosolic proteins from these cells a novel form of PI3K that was activated by G-protein βγ-subunits was identified (Stephens *et al.* 1994). This enzyme was inhibited by wortmannin, but was insensitive to tyrosine-phosphorylated peptides that can activate the p85/p110 forms of PI3K. This was not recognised by a panel of antibodies directed against either the p85 or p110 proteins. This βγ-regulated PI3K has been purified from pig neutrophils. Two very similar isoforms were resolved which behaved similarly during *in vitro* assays of their properties. Both were heterodimers made up a 101 kDa protein and a 117 or 120 kDa protein, each of which bound [^{3}H]-wortmannin specifically. The two subunits of each isoform were subjected to proteolytic cleavage and the resulting peptides were purified and some were sequenced. The pattern of peptides derived from the p101s from the two PI3K isoforms were indistinguishable and sequencing suggested they were identical. The p120 and p117 species were almost identical; they differed on the basis of a single peptide derived from p117 which was NH_2-terminally blocked (and was hence assumed to be derived from the amino terminus), suggesting p117 and p120 are likely to be translated from different messages (and do not result from differential post-translational modification or proteolysis).

A full length cDNA encoding, in a single open reading frame, all of the 11 peptides derived from the p120 subunit was isolated from a polyA-selected pig neutrophil cDNA library. It has a predicted relative molecular mass of 125 kDa (1150 residues), and possesses significant homology with the proven PI3Ks (mammalian p110α-and β and the yeast PI3K (VPS34; vacuolar protein sorting) and its mammalian homologue). This homology is highest in two distinct blocks which represent the lipid kinase domain of these enzymes. p120 lacks any similarity to the domain towards the NH_2-terminus of p110α and β that interacts with p85 proteins, suggesting that the region that mediates interaction between p101 and p120 is quite different to that limiting p110/p85 heterodimers.

A full length cDNA encoding, in a single open reading frame all of the sequenced p101-derived peptides (16 peptides) has also been isolated. The predicted translation product would have a relative molecular mass of 95 kDa. At present, no substantial regions of similarity between p101 and any other sequences in the current databases (either nucleotide or protein level) have been detected. This tallies with the observation that this form of PI3K was insensitive to p85-SH2 domain-directed tyrosine phosphorylated peptides that activate p85/p110 PI3Ks.

These wortmannin-sensitive, and βγ-activated PI3Ks appear to be candidates for the wortmannin target in the FMLP-driven pathways that stimulate superoxide production, degranulation and MAPK (mitogen-activated protein kinase) activation in neutrophils.

(*a*) *Downstream of PI3Ks*

Characterization of a number of independent and specific methods for inhibiting PI3Ks (see below) provides the opportunity to define cellular responses downstream of PI3K. The number of responses now implicated as targets for PI3K is substantial, but we have concentrated on the possibility that PI3K is involved in regulation of the 'ruffling' response that is produced by many growth factors in the hope that the resulting information may shed light on how PI3K regulates other responses (see above). Ruffling responses are detected by staining fixed, and permeabilized cells with phalloidin, a toxin that binds specifically to filamentous actin. Ruffling responses are most readily characterized by the appearance of membraneous protrusions or lamellipodia at points around the periphery of the cell surface in contact with the substratum.

Four independent ways of inhibiting PI3K have been used to address the question of whether PI3K is implicated in growth factor-stimulated ruffling. The mechanisms by which these inhibitions are inflicted are significant because they can reveal which of the potential signal relaying ports of PI3K (e.g. its lipid product(s), the targets of its potential protein kinase activity, or its SH3 domain, BCR domain etc) are employed. The method first applied was to use point-mutated receptors in which the potentially PI3K-binding tyrosine residues and other phosphocylatable Tyr residues were substituted with phenylalanines (Wennström *et al.* 1994). These data suggest that of the range of effector proteins that are recruited by the activated PDGF receptor, it was PI3K that was implicated in development of a ruffling response. Hormone-stimulated ruffling has also been inhibited by intracellular injection of tyrosine phosphorylated peptides known to bind and activate PI3K (Kotani *et al.* 1994). It si assumed that these peptides are effective because they can bind to cytosolic PI3K in unstimulated cells and hence prevent the active protein from translocating to the plasma membrane. These results give no information on which of the intramolecular domains in PI3K was responsible for delivering the stimulus to the downstream effector(s).

A dominant negative p85 deletion construct, which did not bind the catalytic subunit but still translocated

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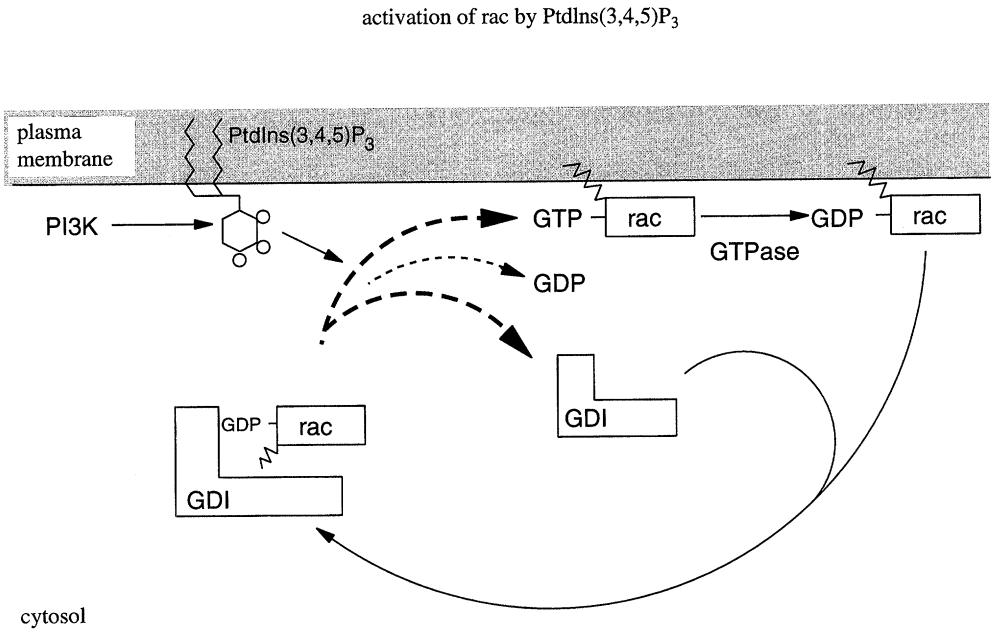

Figure 1. Rac is thought to be bound to a cytosolic GDI protein (guanine nucleotide dissociation inhibitor) when in its GDP bound form. By analogy with what is understood of regulation of the rab family of small GTPases it is suggested activation of rac (i.e. conversion to its GTP bound form) requires dissociation of the GDI protein. How this event is related to the exchange of GDP for GTP is unclear; they may occur at the same or independent steps; furthermore the step(s) at which PtdIns(3,4,5)P_3 acts to accelerate this process is also unknown.

to activated receptor tyrosine kinases inhibited PDGF-stimulated ruffling in endothelial cells (Wennström *et al.* 1994). This protocol translocates an apparently full set of the protein:protein interaction domains found in wild type p85 (i.e. SH2, SH3, proline-rich SH3 target sequences, and BCR domains), suggesting the essential component is the catalytic subunit. This interpretation is supported and further refined by the observations that wortmannin and the structurally unrelated PI3K inhibitor LY294002 both inhibit receptor-stimulated ruffling (Kotani *et al.* 1994; Wennström *et al.* 1994). In the presence of these reagents endogenous PI3K is still translocated to activated growth factor receptors but its lipid and protein kinase catalytic activities are inhibited. This suggests that the product of the lipid kinase (PtdIns(3,4,5)P_3), that is responsible for driving the ruffling reaction (at present the only known target for the protein kinase activity of p110 is serine 608 in p85) (Dhand *et al.* 1994).

Before this work implicated PI3K and its product PtdIns(3,4,5)P_3 in ruffling, the small monomeric GTPase rac had been shown to be involved in the same ruffling response. Intracellular injection of a potentially dominant negative form of rac (N17 rac) blocked PDGF-stimulated ruffling, whereas similar application of a constitutively active form (V12 rac) produced a ruffling phenotype (Ridley *et al.* 1992). These results failed to establish a connection between rac and receptor activation, because in contrast to the readily detectable interaction between PI3K and activated receptors, no link to receptors had been observed for rac proteins. Hence it was natural to suggest that PI3K might lie upstream of rac in this signalling pathway (Wennström *et al.* 1994), and this hypothesis was tested by investigating the effects of PI3K inhibitors on the ability of V12 rac to stimulate ruffling. None of the inhibitors prevented V12 rac-induced ruffling, so the question arose whether PI3K migth lie upstream of the activation of rac.

To establish this point it was necessary to measure the activation rac. This was achieved by generating clonal cell lines (porcine aortic endothelial cells; PAE cells) expressing epitope-tagged rac-1 and quantitating the guanine nucleotides bound to the tagged rac when rapidly purified (by immunoprecipitation with anti-epitope tag monoclonal antibodies) from control or PDGF-stimulated cells. These experiments showed that PDGF stimulated an increase in the ratio of GTP to GDP bound to rac i.e. they indicated the activation of rac, and this response was inhibited by prior treatment with wortmannin (Hawkins *et al.* 1995). These results strongly indicate that PDGF-stimulated activation of PI3K and production of PtdInsP_3 can lead to the activation of rac, but the number of steps involved in these pathways and the mechanism(s) leading to activation of rac are still unclear.

To attempt to gain a clearer understanding of the PI3K-dependent regulatory events controlling rac activation, we again utilized PAE cells expressing epitope-tagged rac. We attempted to estimate the rate of exchange of GDP for GTP on rac and the rate of guanine nucleotide hydrolysis by rac in the presence and absence of PDGF. This was accomplished by measuring the rate of incorporation of [α^{32}P]-GTP into tagged-rac in streptolysin-O permeabilized cells. These experiments showed that PDGF-stimulated a PI3K-dependent increase in guanine nucleotide exchange on rac (Hawkins *et al.* 1995). Various mechanisms by which this might occur are shown in figure 1.

At present, the only other transduction pathway that appears to implicate both PI3K(s) and rac is that by which FMLP stimulates superoxide production in

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neutrophils. In this situation, rac is known to be an essential component of the minimal complex that can reconstitute superoxide production *in vitro* (indeed, the complex can be activated by addition of GTPγ-S bound rac). Wortmannin can inhibit FMLP-stimulated superoxide formation. In the light of the information discussed above, it seems possible that activation of PI3K and production of PtdIns(3,4,5)P_3 are responsible for stimulation of rac and hence for the release of superoxide. It is not clear whether rac will also play a part in regulating any of the other cellular responses thought to be downstream of PI3K.

REFERENCES

Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. & Cantley, L. C. 1989 PDGF-dependent trrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167–175.

Backer, J. M., Myers, M. G. Jr, Shelson, S. E., Chin, D. J., Sun, X. -J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, Y., Schlessinger, J. & White, M. F. 1992 Phosphatidylinositol 3-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* **11**, 3469–3479.

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. & Soltoff, S. 1991 Oncogenes and signal transduction. *Cell* **64**, 281–302.

Carpenter, C. C., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. & Cantley, L. C. 1990 Purification and characterisation of phosphoinositide 3-kinase from rat liver. *J. biol. Chem.* **265**, 19704–19711.

Carpenter, C. L., Auger, K. R., Chaudhuri, M., Yoakim, M., Schaffhausen, B., Schoelson, S. & Cantley, L. C. 1993 Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85 kDa subunit. *J. biol. Chem.* **268**, 9478–9483.

Dewald, B., Thelen, M. & Baggiolini, M. 1988 Two transduction sequences are necessary for neutrophil activation by receptor agonists. *J. biol. Chem.* **263**, 16179–16184.

Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Kasuga, M. & Waterfield, M. D. 1994 P13 kinase: structural and functional analysis of intersubunit interactions. *EMBO J.* **13**, 511–521.

Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milftay, D., Fried, V. A. & Williams, L. T. 1991 CDNA cloning of a novel 85 kDa protein that has SH2 domains and regulates PI3-kinase binding to the PDGFβ-receptor. *Cell* **65**, 75–82.

Gout, I., Dhand, R., Panayotou, G., Fry, M. J., Hiles, I., Otsu, M. & Waterfield, M. D. 1992 Expression and characterisation of the p85 subunit of the phosphatidylinositol 3-kinase complex and a related p85β-protein by using the baculovirus expression system. *Biochem. J.* **288**, 395–405.

Hawkins, P. T., Jackson, T. R. & Stephens, L. R. 1992 Platelet-derived growth factor stimulated synthesis of PtdIns(3,4,5)P_3 by activating a PtdIns(4,5)P_2 3-OH kinase. *Nature, Lond.* **358**, 157–159.

Hawkins, P. T., Eguinoa, A., Qiu, R. -G., Stokoe, D., Cooke, F. T., Walters, R., Jackson, T., Wennström, S., Claesson-Welsh, L., Evans, T., Symons, M. & Stephens, L. 1995 PDGF stimulates an increase in GTP-rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5,** 393–403.

Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J. & Waterfield, M. D. 1992 Phosphatidylinositol 3-kinase: structure and expression of the 110 kDa catalytic subunit. *Cell* **70**, 419–429.

Kapeller, R. & Cantley, L. C. 1994 Phosphoinositide 3-kinase. *Bioessays* **16**, 565–576.

Kotani, K., Yonezowa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Pondo, A., Chavanieu, A., Calas, B., Grigorescu, F., Nishiyama, M., Waterfield, M. D. & Kasuga, M. 1994 Involvement of phosphoinositide 3-kinase in insulin or IGF-1 induced membrane ruffling. *EMBO J.* **13,** 2313–2321.

Morgan, S. J., Smith, A. D. & Parker, P. J. 1990 Purification and characterisation of bovine brain type phosphatidylinositol kinase. *Eur. J. Biochem.* **191**, 761–767.

Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. & Waterfield, M. D. 1991 Characterisation of two 85 kDa proteins that associate with receptor tyrosine kinases, middle T/pp60$^{c\text{-}src}$ complexes and P13K- kinase. *Cell* **65**, 91–104.

Ridley, A., Paterson, H. F., Johnston, C. L., Diekmann, D. & Hall, A. 1992 The small GTP-binding protein rac regulates growth factor induced membrane ruffling. *Cell* **70**, 401–410.

Shibasaki, F., Homma, Y. & Takenawa, T. 1991 Two types of phosphatidylinositol 3-kinase from bovine thymus. *J. biol. Chem.* **266**, 8108–8114.

Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. & Schlessinger, J. 1991 Cloning of PI-3K associated p85 utilising a novel method for expressing/cloning of targets for receptor tyrosine kinases. *Cell* **65**, 83–90.

Songyang, Z., Shoelson, S. E., Chauduri, M., Gish, G., Pawson, T., Haswer, W. G. *et al.* 1993 SH2 doamins recognise specific phosphopeptide sequences. *Cell* **72**, 767–778.

Stephens, L. & Hawkins, P. T. 1995 *Experimental medicine* (Japanese) vol.13, no 7, pp. 43–50.

Stephens, L. R., Hughes, K. T. & Irvine, R. F. 1991 Pathway of phosphotidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature, Lond.* **351**, 33–39.

Stephens, L., Jackson, T. J. & Hawkins, P. T. 1993*a* Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim. Biophys. Acta* **1179**, 25–75.

Stephens, L., Eguinoa, A., Corey, S., Jackson, T. J. & Hawkins, P. T. 1993*b* Receptor stimulated accumulation of phosphatidylinositol(3,4,5)-trisphosphate by G protein mediated pathways in human myeloid derived cells. *EMBO J.* **12**, 2265–2273.

Stephens, L., Jackson, T. & Hawkins, P. T. 1993*c* Synthesis of phosphatidylinositol (3,4,5)-trisphosphate in permeabilised neutrophils. *J. biol. Chem.* **268**, 17162–17172.

Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. J., Sternweis, P. & Hawkins, P. T. 1994 A novel phosphoinositide 3-kinase activity in myeloid derived cells is activated by G protein βγ subunits. *Cell* **77**, 83–93.

Thelen, M., Wymann, M. P. & Langen, H. 1994 Wortmannin binds specifically to L-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein coupled signalling in neutrophil leukocytes. *Proc. natn Acad. Sci. U.S.A.* **91**, 4960–4964.

Traynor-Kaplan, A. E., Harris, A., Thompson, B., Taylor, P. & Sklar, L. A. 1988 An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature, Lond.* **334**, 353–356.

Valius, M. & Kazlauskas, A. 1993 Phospholipase C and phosphatidylinositol 3 kinase are downstream mediators of the PDGF receptors mitogenic signal. *Cell* **73**, 321–334.

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Wennström, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. & Stephens, L. 1994 *Curr. Biol.* **4**, 385–393.

Yano, H., Nakanashi, S., Kimura, K., Hani, N., Saitoh, Y., Fumkui, Y., Nonomura, Y. & Matsuda, Y. 1993 Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. biol. Chem.* **268**, 25846–25856.

Discussion

J. Saklatvala (*Cytokine Laboratory, The Babraham Institute, Cambridge, U.K.*). Does Dr Stephens' p120 interact with p85?

L. Stephens. Professor Waterfield's lab has defined a motif in p110 that interacts with p85, and this is absent from the p120 sequence.

E. Rozengurt (*Imperial Cancer Research Fund, London, U.K.*). Does Dr Stephens know whether the activation of his kinase is influenced by the composition of the preparation that he uses?

L. Stephens. No. All of our experiments were done with bovine brain preparations of βγ, which will be predominantly derived from G_o and G_i. However, I would predict that we would find very similar activation with most βγ preparations, maybe with $\beta_1\gamma_1$ from transducin rather less active.

M. Karin (*Department of Pharmacology, Univeristy of California, San Diego, U.S.A.*). Dr Stephens has suggested that phosphoinositide 3-kinase may be upstream to Rac. Can he comment on that?

L. Stephens. We have evidence that the activation of Rac in cells, as measured by an increase in the proportion of Rac-GTP, is prevented by phosphoinositide 3-kinase inhibitors of the wortmannin type (including Ly294002) or by peptides that interact with the SH3 domains of phosphoinositide 3-kinase.

Question. What other G protein-coupled receptors activate phosphoinositide 3-kinase?

L. Stephens. We have not demonstrated directly that other receptors can activate this enzyme, but we do know that there are a number of receptors that can activate phosphatidylinositol 3,4,5-trisphosphate accumulation in a pertussis toxin-sensitive manner. These include receptors for fMLP, ATP, PAF and some of the leukotrienes. This is probably also true of a modest proportion of the response of platelets to thrombin.

Question. Does Dr Stephens see recruitment of his enzyme to membranes during cell activation?

L. Stephens. We have not yet tested that. We have looked at its distribution in unstimulated cells, where it is largely cytosolic but with 10–20% associated with membranes.

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