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Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation

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We have reported previously that Ras interacts with the catalytic subunit of phosphoinositide 3-kinase (PI 3-kinase) in a GTP-dependent manner. The affinity of the interaction of Ras·GTP with p85α/p110α is shown here to be ~150 nM. The site of interaction on the p110α and β isoforms of PI 3-kinase lies between amino acid residues 133 and 314. A point mutation in this region, K227E, blocks the GTP-dependent interaction of PI 3-kinase p110α with Ras *in vitro* and the ability of Ras to activate PI 3-kinase in intact cells. In addition, this mutation elevates the basal activity of PI 3-kinase in intact cells, suggesting a direct influence of the Ras binding site on the catalytic activity of PI 3-kinase. Using an *in vitro* reconstitution assay, it is shown that the interaction of Ras·GTP, but not Ras·GDP, with PI 3-kinase leads to an increase in its enzymatic activity. This stimulation is synergistic with the effect of tyrosine phosphopeptide binding to p85, particularly at sub-optimal peptide concentrations. These data show that PI 3-kinase is regulated by a number of mechanisms, and that Ras contributes to the activation of this lipid kinase synergistically with tyrosine kinases.

Keywords: phosphoinositide 3-kinase/Raf/Ras

Introduction

Phosphoinositide 3-kinase (PI 3-kinase) is a lipid kinase capable of phosphorylating phosphoinositides at the 3′ position of the inositol ring (Stephens *et al.*, 1993). The first characterized form of PI 3-kinase comprised a catalytic (p110) and a regulatory (p85) subunit (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Hiles *et al.*, 1992). Recently, it has become clear that there exists a family of PI 3-kinases with at least three p85 subunits and at least three p110 subunits (α, β and γ); in addition, a number of less closely related lipid and protein kinases exist including *Saccharomyces cerevisiae* Vps34p and TOR 1 and 2 and their mammalian homologues (Kunz *et al.*, 1993; Schu *et al.*, 1993; Brown *et al.*, 1994; Sabatini *et al.*, 1994), the product of the ataxia telangiectasia gene (Savitsky *et al.*, 1995) and a DNA-dependent protein kinase (Hartley *et al.*, 1995). p110α and β are found as heterodimers with p85 subunits, while p110γ does not bind p85. Phosphatidylinositol (3,4,5) trisphosphate (PIP_3) is thought to be the primary product of these kinases in intact cells: its level is elevated upon treatment of cells with any of a wide variety of agonists.

Several mechanisms for regulating the activity of PI 3-kinase in response to extracellular stimuli have been elucidated (Stephens *et al.*, 1993). The most thoroughly studied is the binding of tyrosine phosphorylated signalling proteins, such as the platelet-derived growth factor (PDGF) receptor, IRS-1 and CD28 to the SH2 domains of p85. This results in a few fold increase in the activity of the α form of PI 3-kinase *in vitro*, and in cells it may, in the case of PDGF receptor or CD28 for example, additionally stimulate by translocating the kinase to the plasma membrane where its substrate lipid is located. Other possible means of regulation through the p85 regulatory subunit include binding of the SH3 domain of Src family tyrosine kinases to proline-rich motifs (Liu *et al.*, 1993b; Prasad *et al.*, 1993; Pleiman *et al.*, 1994), binding of the Rho family proteins CDC42 and Rac to the Bcr-related domain (Zheng *et al.*, 1994; Tolias *et al.*, 1995), phosphorylation of p85 at Tyr580 (Hayashi *et al.*, 1993) and Tyr508 (Kavanaugh *et al.*, 1994) and autophosphorylation of p85 by the p110 kinase at Ser608 (Dhand *et al.*, 1994b). In addition, we have shown that Ras can bind the catalytic p110 subunit of PI 3-kinase in a GTP-dependent manner, and have found evidence that this interaction may be stimulatory within intact cells (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994). Other forms of PI 3-kinase which do not bind p85, including p110γ, are regulated by the βγ subunits of heterotrimeric G proteins (Stephens *et al.*, 1994; Stoyanov *et al.*, 1995).

Ras proteins are small monomeric GTP binding proteins which are key regulators of cell proliferation and are capable of causing cellular transformation when constitutively activated by point mutation (Lowy and Willumsen, 1993). They enter the active GTP-bound state upon treatment of cells with a wide variety of extracellular stimuli, including most of those that activate PI 3-kinase. Ras exerts its effect on cell behaviour through GTP-dependent interaction with a number of cellular targets, commonly known as effectors. The best characterized of these are the serine/threonine cytoplasmic protein kinases of the Raf family (Rapp, 1991). Ras interacts with the regulatory region of Raf (Moodie *et al.*, 1993; Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993) to cause its translocation to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), where further unknown events occur to activate its kinase activity (Dent *et al.*, 1995; Marais *et al.*, 1995). The activated Raf phosphorylates MEK and thereby switches on the MAP kinase cascade (Marshall, 1994). This pathway, which results in activation of a number of transcrip-

tion factors and other enzymes, is capable of stimulating proliferation of some cell types, at least when constitutively activated. There is good evidence, however, that other pathways are triggered by Ras that are important in transformation: Ras proteins containing certain mutations in the effector interaction site can synergize with other Ras mutants in this region, suggesting that multiple effector molecules are interacting with Ras at a similar site (White *et al.*, 1995). Transformation by Ras may involve both MAP kinase and Rac pathways (Qiu *et al.*, 1995). Genetic evidence for multiple effector pathways for Ras in the yeasts *S.cerevisiae* and *Schizosaccharomyces pombe* has been known for some time (Toda *et al.*, 1985; Wang *et al.*, 1991; Chang *et al.*, 1994).

A number of possible alternative effectors for mammalian Ras are known in the handful of proteins that are capable of interacting with the Ras effector site in a GTP-dependent manner. The GTPase-activating proteins $p120^{GAP}$, neurofibromin and Gap-1 are all negative regulators of Ras which stimulate its conversion from active GTP-bound form to inactive GDP-bound form (Lowy and Willumsen, 1993). In the case of $p120^{GAP}$, there is some evidence for downstream function, although its possible targets are not known. The other GAPs appear to be unlikely to have effector function. Ral·GDS and a related protein have been found to interact with the effector site of Ras in the yeast two-hybrid system (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). These proteins may be capable of inducing activation of the Ras family member Ral in response to activated Ras; at present the function of Ral is unknown, so the significance of the Ral·GDS pathway is unclear. A recent report has also indicated that protein kinase Cζ is able to interact with Ras·GTP *in vitro* in a manner that suggests it might be an effector (Diaz-Meco *et al.*, 1994). In addition, it has been reported that Ras can interact with MEKK, an activator of the Jun kinase pathway, in a GTP-dependent manner (Russell *et al.*, 1995).

We have shown previously that Ras can interact with the catalytic p110α subunit of PI 3-kinase in a GTP-dependent manner (Rodriguez-Viciana *et al.*, 1994). This interaction occurs through the effector region of Ras. In intact cells there is evidence that Ras can stimulate PI 3-kinase activity and is required for its optimal activation in response to growth factors (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994). It is possible that the Ras·GTP and the tyrosine phosphoprotein interactions with p85/p110 synergize to give full activation of PI 3-kinase. The ability of Ras to contribute to PI 3-kinase activation may be an important part of its downstream signalling, with major implications for cellular transformation. Here we provide further details of the nature of the interaction between Ras and PI 3-kinase. The Ras binding site of p110 is identified and a mutation in this region is shown to block Ras-induced activation of PI 3-kinase, and also to elevate basal lipid kinase activity. Data are presented from an *in vitro* reconstitution system showing that Ras·GTP can activate the lipid kinase activity of PI 3-kinase directly, and that this effect is synergistic with tyrosine phosphopeptide binding to p85. These data support the idea that multiple cellular signalling pathways are activated by Ras, including Raf and PI 3-kinase.

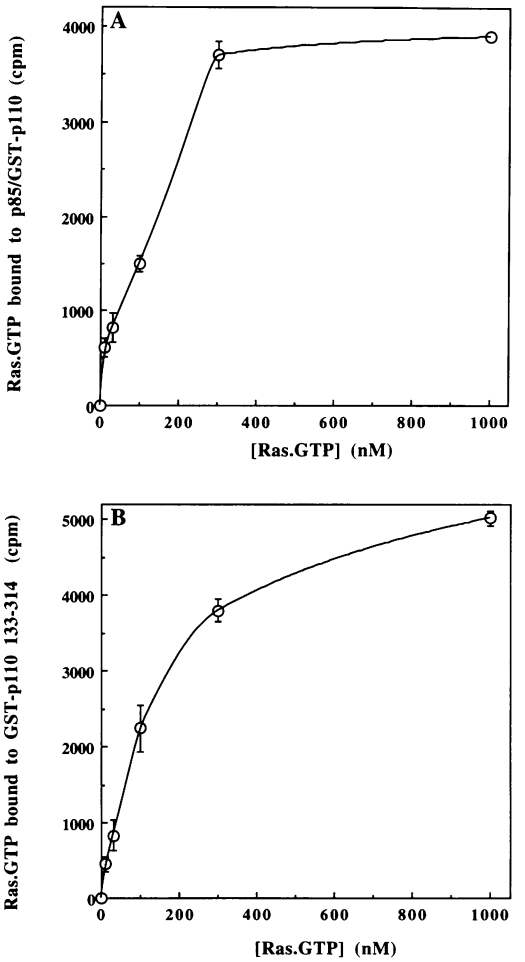

Fig. 1. Affinity of Ras binding to PI 3-kinase. (**A**) Purified baculovirus-expressed soluble V12 H-Ras was labelled with [γ-^{32}P]GTP and then varying amounts were mixed with soluble purified baculovirus-expressed p85α/GST–p110α. After 1 h, the PI 3-kinase was captured on glutathione–agarose, washed and the amount of labelled Ras bound was measured by Cerenkov counting. The background obtained when GST was used instead of p85α/GST–p110α was subtracted for each point. Data shown are the average of triplicates with standard error shown. (**B**) As above, but bacterially expressed p110 fragment, GST–p110 133–314, was used.

Results

Interaction of Ras·GTP with PI 3-kinase

In order to estimate the strength of the interaction between GTP-bound Ras and PI 3-kinase, purified baculovirus-expressed H-Ras protein was loaded with [α-^{32}P]GTP and then increasing amounts were allowed to bind to purified baculovirus-expressed p85/GST–p110 in solution in the presence of excess unlabelled guanine nucleotide. The PI 3-kinase fusion protein was recovered using glutathione–agarose, washed and the amount of [α-^{32}P]GTP-labelled Ras specifically bound determined by Cerenkov counting. As shown in Figure 1A, the amount of Ras binding to PI 3-kinase saturates at ~300 nM Ras. Half-maximal binding is achieved at ~150 nM Ras, giving a rough estimate of the binding affinity of Ras for PI 3-kinase. A slightly weaker binding was seen using the region of p110 that interacts with Ras (see below) expressed as a bacterial GST fusion protein. Figure 1B shows the binding of this PI 3-kinase fragment, immobil-

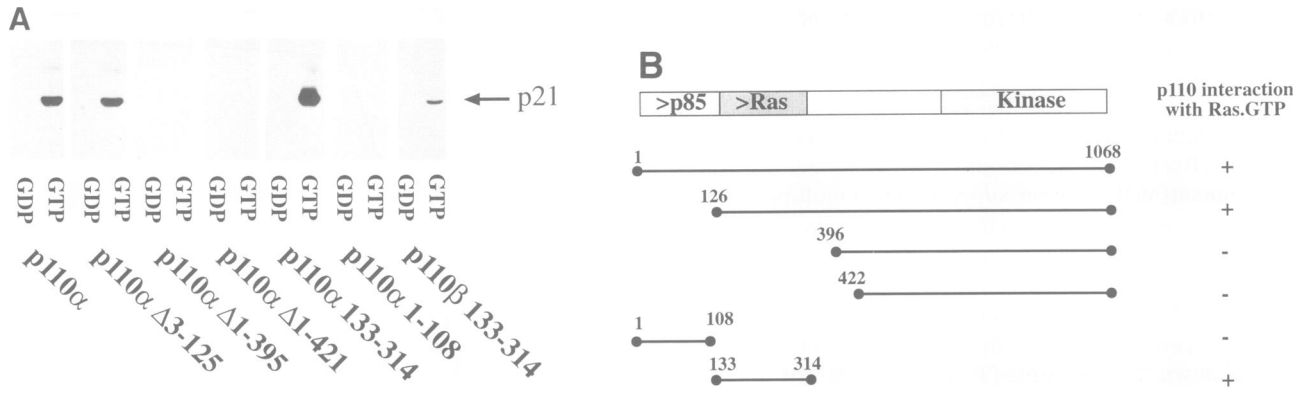

Fig. 2. Determination of the binding site for Ras on the catalytic domain of p110. (**A**) Purified deletions mutants of p110α, expressed as GST fusion proteins in baculovirus, or bacterially expressed GST fusion proteins of short fragments of p110α or β, were mixed with soluble Ras bound to either GTP or GDP. The GST fusion proteins were recovered on glutathione–agarose, washed and probed for the presence of Ras by Western blotting using anti-Ras antibodies. (**B**) Summary of the Ras binding behaviour of the p110 mutants shown in (A).

ized on glutathione–agarose, to soluble Ras protein loaded with [α-^{32}P]GTP. The binding assays shown were carried out at close to physiological ionic strength. At low ionic strength (with omission of the added 100 mM NaCl), the binding of Ras to p85/GST–p110 was ~3-fold stronger (data not shown).

Determination of the site on p110 that binds Ras

PI 3-kinase interacts with Ras through its catalytic p110 subunit: p110 will bind to Ras·GTP in the absence of the regulatory p85 subunit (Rodriguez-Viciana *et al.*, 1994). Addition of increasing amounts of monomeric p85 does not inhibit the binding of monomeric p110 to Ras·GTP *in vitro*, even though the stoichiometric formation of p85/p110 heterodimers can be demonstrated (data not shown). In order to determine where on the p110α catalytic subunit Ras·GTP interacts with PI 3-kinase, a number of deletion mutants were expressed in baculovirus. One mutant, Δ3–125, lacked the minimal p85 binding site (Dhand *et al.*, 1994a), while others lacked larger stretches from the amino-terminus. The interaction of these purified mutant proteins with immobilized Ras was examined: as shown in Figure 2, only Δ3–125 interacted with Ras, while other mutants, including Δ1–395, failed to bind. This suggested that the interaction site may lie between amino acid residues 125 and 395 of p110. A GST fusion protein containing most of this area (amino acids 133–314) was therefore expressed in bacteria and was found to be able to interact with Ras in a GTP-dependent manner, thus defining amino acid residues 133–314 of p110α as a sufficient binding site for Ras·GTP. A similar region of p110β was also expressed as a GST fusion protein in bacteria and found to interact with Ras in a GTP-dependent manner; p110β is therefore also likely to be a target for Ras.

Effect of a point mutation in the Ras binding site of p110 on regulation of enzymatic activity

The sequence of the three cloned forms of p110 in this region is compared in Figure 3A. Conserved residues are boxed and a consensus sequence is written below. The homology over this region is 25% identity between p110α and β, 18% between p110α and γ and 20% between p110β and γ. This compares with overall homologies between the entire catalytic subunits of 42% identity between p110α and β, 36% between p110α and γ and 34% between p110β and γ (Hiles *et al.*, 1992; Hu *et al.*, 1993; Stoyanov *et al.*, 1995). In order to investigate the significance of the Ras–p110 interaction in the activation of PI 3-kinase in cells, a number of point mutations were made in p110. One of these, the substitution of the conserved lysine residue 227 of p110α by glutamic acid, resulted in generation of a protein that was unable to interact with Ras. When expressed in an *in vitro* transcription–translation system, p110α K227E displayed phosphoinositide 3-OH kinase activity, suggesting that the overall folding of the protein was not disrupted (data not shown). However, this mutation completely blocked the ability of a GST fusion protein containing p110α amino acids 133–314 to bind to Ras·GTP (Figure 3B). The effect of this mutation on the ability of Ras to activate PI 3-kinase in intact cells was also investigated. As shown in Figure 3C, transient transfection of either V12 Ras or p85/p110 into COS cells causes modest elevation of the cellular levels of PIP_3. Transfection of both activated Ras and p85/p110 together gives a much greater than additive elevation of PIP_3 levels, suggesting that Ras is activating PI 3-kinase directly. Use of the K227E mutant p110 in these transfections abolishes the synergistic activation of PI 3-kinase by Ras, confirming that this effect relies on the interaction of Ras with the catalytic p110 subunit of PI 3-kinase. However, the basal activity of K227E mutant p110 is elevated by >4-fold over that of wild-type p110. This suggests the possibility that this mutation in the Ras binding site is able to give rise to a protein that is unable to interact with Ras but has undergone a conformational change that mimics Ras binding.

Effect of interaction of p110 with p85 and with Ras on PI 3-kinase activity in cells

The COS cell transient co-transfection assay was used further to explore aspects of the regulation of PI 3-kinase activity. As shown in Table I, the p85 regulatory subunit had minimal effect on the phosphatidylinositol (3,4) bisphosphate [$PI(3,4)P_2$] and PIP_3 levels in cells transfected with the p110 catalytic subunit or p110 plus activated Ras. Immunoprecipitation of p110 through a carboxy-terminal myc epitope tag followed by immunoblotting for p85 revealed that, in the absence of co-

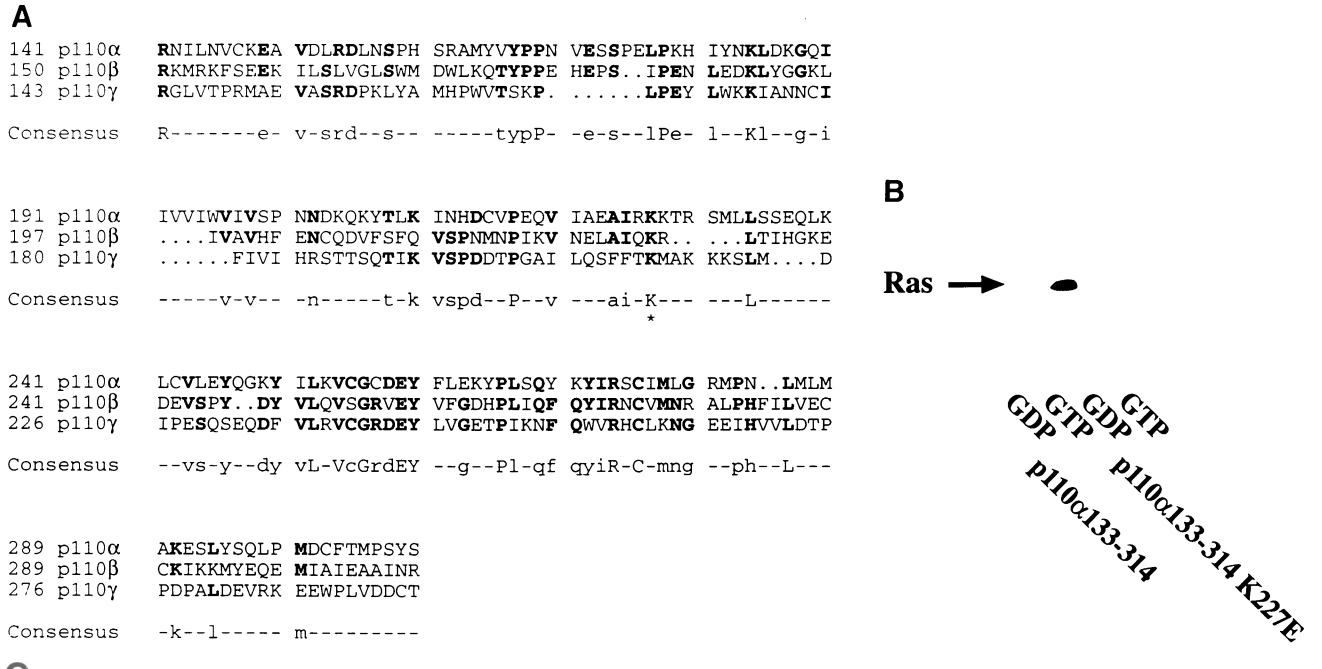

Fig. 3. Mutation in p110α that blocks binding to Ras. (**A**) Alignment of sequences from the Ras binding site of the catalytic p110 subunit of PI 3-kinase isoforms. The sequences of p110α, p110β and p110γ are shown. Conserved residues are in bold. A consensus sequence is written below: residues conserved in all three sequences are in upper case, those conserved in two out of three are in lower case. Lysine residue 227 in p110α is marked with an asterisk. (**B**) Binding to Ras of GST fusion proteins containing residues 133–314 of p110α: effect of the mutation K227E. Performed as in Figure 2. (**C**) Effect of the K227E mutation on Ras elevation of PIP_3 levels in intact COS cells. V12 Ras was transfected into COS cells along with p85α and p110α, either mutant or wild-type. After 24 h, cells were metabolically labelled with [^{32}P]orthophosphate in the absence of serum for 16 h prior to measurement of the levels of cellular PIP_3.

transfected p85, p110 was >95% monomeric, or at least not bound to p85α or β (data not shown). This suggests that the stimulation of PI 3-kinase activity of p110 by Ras seen in this system does not rely on signalling through p85, such as phosphotyrosine–SH2 domain interactions. Co-transfection of the inter-SH2 domain of p85 (the binding site for p110) with p110 leads to a relatively weak elevation of $PI(3,4)P_2$ and PIP_3 levels, as suggested by previous reports (Hu *et al.*, 1995). However, inclusion of the p85 binding site of p110 (amino acids 1–125) as a separate fragment, which might be expected to sequester endogenous p85, did not alter the ability of p110 to elevate $PI(3,4)P_2$ and PIP_3 levels either in the presence or absence of Ras. Immunoprecipitation of this fragment using an epitope tag showed that it was capable of binding to endogenous or co-transfected p85 (data not shown). Furthermore, co-transfection of a S608A mutant of p85, which is resistant to phosphorylation by p110 (Dhand *et al.*, 1994b), did not greatly affect the activity of PI 3-kinase in this system.

COS cell transient transfection was also used to study whether membrane localization of Ras was required in order for it to activate PI 3-kinase. Farnesylation of Ras at C186 is absolutely required for it to influence the activity of PI 3-kinase: very similar results have been reported for Ras activation of Raf and MAP kinase (Leevers and Marshall, 1992). By contrast, removal of the second membrane localization signal, either palmitoylation at C181 and C184 for H- and N-Ras (Hancock *et al.*, 1989) or a polybasic region near the carboxy-terminus for K-Ras (Hancock *et al.*, 1990), only slightly reduces Ras activation of PI 3-kinase. Although these mutants are

not associated predominantly with the plasma membrane (Hancock *et al.*, 1989, 1990), they do retain a significant amount of transforming activity and are still able to activate Raf and MAP kinase quite efficiently (Cadwallader *et al.*, 1994). It is possible that there is a large excess of Ras expressed in these assays, such that only a minor fraction at the membrane is required for activation of PI 3-kinase: however, the absolute requirement for farnesylation is clear.

Activation of PI 3-kinase by Ras in a purified liposome reconstitution system

Post-translationally modified wild-type Ras protein was purified from the membrane fraction of Ras baculovirus-infected Sf9 cells. The membranes were solubilized and purified in the non-ionic detergent *n*-octylglucoside (40 mM). Phospholipid liposomes containing phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylinositol (4,5) bisphosphate [PI(4,5)P_2] were made based on the procedure of Stephens *et al.* (1994). Reconstitution of modified Ras into the liposomes was achieved by adding a concentrated stock solution of Ras to the liposomes in the absence of additional detergent, such that the final detergent concentration in the mixture was only 1 mM *n*-octylglucoside, ~5% of the critical micellar concentration. The total lipid:detergent ratio was 1.6:1. Following mixing and incubation on ice for 10 min, the liposomes were collected by ultracentrifugation. As shown in Table II, ~35% of the modified Ras could be recovered stably associated with the liposomes, as judged from the inclusion of a pre-labelled Ras·[α-^{32}P]GTP tracer. Unmodified Ras was unable to associate with liposomes. When the liposome pellet was resuspended using sonication, ~80% of the associated Ras was accessible to the medium, as judged by the ability of EDTA to exchange labelled for unlabelled GTP.

The ability of purified baculovirus-expressed PI 3-kinase (p85α/GST–p110α) to phosphorylate the PI(4,5)P_2 in these liposomes was tested. As shown in Figure 4, PI 3-kinase efficiently phosphorylated PI(4,5)P_2 to give PIP_3. In the absence of Ras, the activity of the lipid kinase could be stimulated ~3-fold by the addition of a tyrosine phosphopeptide based on the autophosphorylation site Y751 of the human PDGF receptor β. Similar stimulations were seen using a tyrosine phosphopeptide based on the autophosphorylation site Y740 of the PDGF receptor, and a slightly stronger stimulation using a doubly phosphorylated peptide spanning both sites. The principal effect of using a doubly, as opposed to a singly, phosphorylated peptide was to reduce the concentration of peptide required to give maximal stimulation of lipid kinase activity by >10-fold: the optimal stimulation achieved was not greatly different with the various phosphopeptides. In contrast, a tyrosine phosphopeptide based on the sequence around Tyr1068 of the epidermal growth factor (EGF) receptor, which binds to the SH2 domain of Grb2 but not p85, has no influence on the activity of PI 3-kinase in this system at concentrations up to 30 μM (data not shown).

In order to study the effect of Ras on PI 3-kinase activity, Ras reconstituted into liposomes was loaded with either GTP or GDP in the absence of magnesium. PI 3-kinase phosphorylation of PI(4,5)P_2 to give PIP_3 was then measured. As shown in Figure 5A, GDP-bound Ras did not significantly affect the activity of PI 3-kinase in this system. GDP or GTP alone were also without effect in the absence of Ras. However, GTP-bound Ras caused

Table I. The levels of PI(3,4)P_2 and PIP_3 in transiently transfected ^{32}P metabolically labelled COS cells

Plasmids transfected	Percentage of level with V12 Ras + p85 + p110	
	PI(3,4)P_2	PIP_3
None	0	0
p110α	12.7	5.1
p110 + V12 H-Ras	106	122
p110 + p85α	10.6	6.1
p110 + p85 + V12 Ras	100	100
p85 inter-SH2 + p110	23.3	11.4
p85 inter-SH2 + p110 + V12 Ras	106	123
p110 (1–125) + p110	14.8	5.5
p110 (1–125) + p110 + V12 Ras	108	124
p110 + p85 S608A	18.6	9.9
p110 + p85 S608A + V12 Ras	112	57.2
p110 + V12 Ras S181/184	77.0	54.9
p110 + V12 Ras S186	7.7	2.9
p110 + V12 K-Ras	102	98.2
p110 + V12 K-Ras K6Q	108	84.3

Phosphoinositides were analysed by HPLC [200 000 c.p.m. of PI(4,5)P_2]. Phospholipid levels are expressed as a percentage of the level seen in cells transfected with V12 H-Ras plus p85 and p110 plasmids. Background corresponding to that seen in the same fractions from untransfected cells was subtracted. The results shown are representative of at least three different experiments. The background subtracted was no more than 3% [800 c.p.m. for PIP_3 and 600 c.p.m. for PI(3,4)P_2].

Table II. Reconstitution of Ras into liposomes

	Percentage of total input		
	Modified Ras	Unmodified Ras	No Ras
Total Ras·[α-^{32}P]GTP added	100	100	
Ras·[α-^{32}P]GTP associated with liposomes	34.8 ± 0.6	0.4 ± 0.1	
[α-^{32}P]GTP dissociating from liposomes in the presence of EDTA	28.3 ± 0.3	0.2 ± 0.04	
Total [α-^{32}P]GTP added without Ras			100
[α-^{32}P]GTP associated with liposomes			0.09
[α-^{32}P]GTP dissociating from Ras in liposomes in the presence of EDTA			0.02

Post-translationally modified Ras, or unmodified Ras, was labelled with [α-^{32}P]GTP and reconstituted into liposomes as described in Materials and methods. Ras associating with liposomes was estimated by counting anti-Ras monoclonal antibody Y13-259 immunoprecipitates of solubilized washed liposomes. Total Ras input was also measured by immunoprecipitation. Label released with EDTA and the 'No Ras' controls were measured by direct counting of liposome or supernatant fractions.

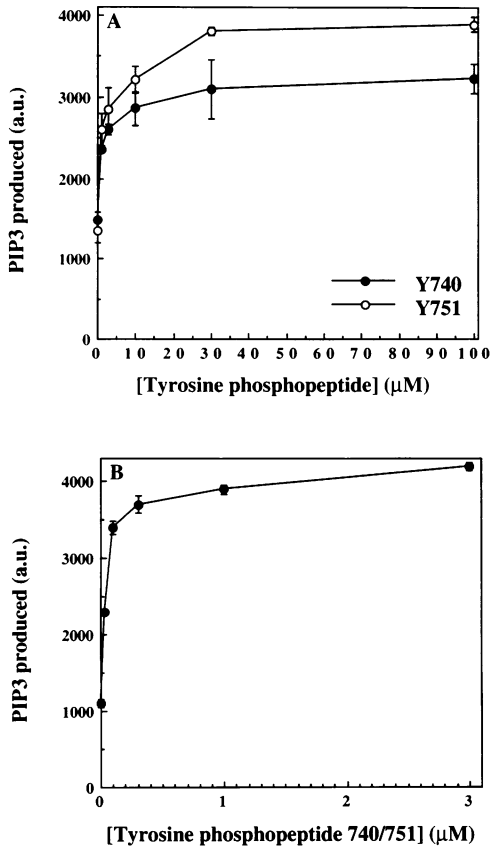

Fig. 4. Tyrosine phosphopeptide stimulation of kinase activity of p85α/GST–p110α towards $PI(4,5)P_2$-containing liposomes. (**A**) Purified p85α/GST–p110α was added to $PI(4,5)P_2$-containing liposomes in the presence of increasing concentrations of singly phosphorylated tyrosine phosphopeptides, based on the human PDGF receptor β autophosphorylation sites Y740 and Y751, and [γ-^{32}P]ATP. Levels of PIP_3 produced were determined by TLC. (**B**) As (A), but the doubly phosphorylated tyrosine phosphopeptide Y740/Y751 was used. Average of duplicates with standard errors shown. Representative of four experiments.

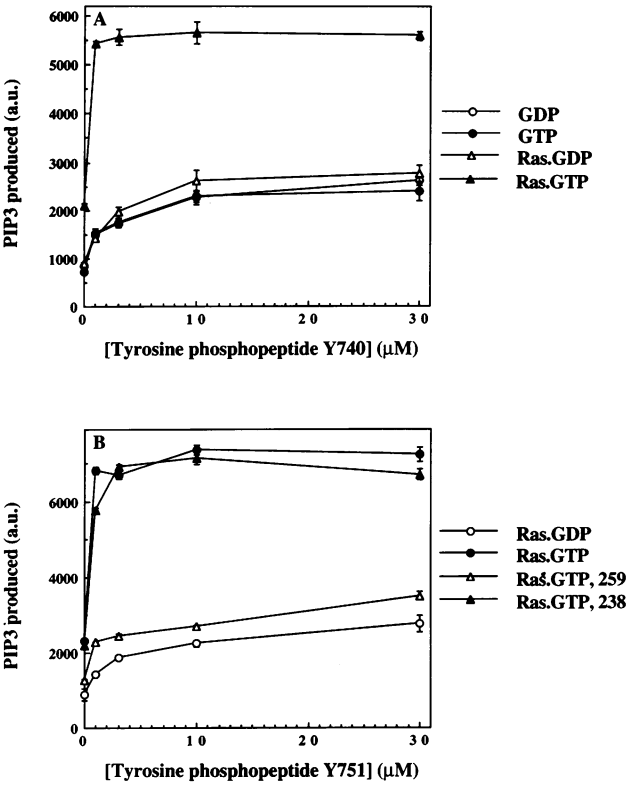

Fig. 5. Effect of Ras·GTP on PI 3-kinase activity in the liposome system. (**A**) The kinase activity of p85α/GST–p110α towards $PI(4,5)P_2$ was measured in liposomes into which had been reconstituted post-translationally modified Ras that was bound to GTP or GDP. The influence of increasing concentrations of PDGF receptor β Y740 phosphopeptide was determined. The effect of GTP or GDP alone in the absence of Ras was also measured. Average of triplicates with standard error shown. Representative of five experiments. (**B**) The influence of anti-Ras monoclonal antibodies Y13-259 and Y13-238 on the stimulation of PI 3-kinase activity by Ras·GTP was measured as in (A), except that tyrosine phosphopeptide Y751 was used. Average of duplicates with standard error shown. Representative of three experiments.

a significant increase in the activity of PI 3-kinase. The Ras·GTP-induced stimulation observed in the absence of phosphopeptide Y740 was ~2.5-fold and was similar in the presence of saturating concentrations of peptide. However, the activation of PI 3-kinase activity by Ras·GTP was most marked at low concentrations of tyrosine phosphopeptide. For example, at 1 μM Y740 phosphopeptide there was only a small stimulation of PI 3-kinase activity in the absence of Ras·GTP, but maximal stimulation in the presence of Ras·GTP, a stimulation by Ras·GTP of >4-fold. Similar results are seen for the Y751 phosphopeptide (Figure 5B): in the absence of peptide the Ras-induced stimulation was 3-fold, while in the presence of saturating peptide Ras·GTP stimulated 3.5-fold. At 1 μM Y751, Ras·GTP induced a 5-fold activation of PI 3-kinase. Comparable results were found for the doubly phosphorylated peptide, including the leftward shift of the dose–response curve induced by Ras·GTP (data not shown). To be certain that this effect was due to Ras and not contaminants in the Ras preparation, the neutralizing Ras monoclonal antibody Y13-259 was added to the Ras preparation prior to reconstitution. Excess antibody was removed during the preparation of the Ras liposomes. As shown in Figure 5B, treatment of Ras in this way with Y13-259, but not the non-neutralizing Y13-238 antibody, blocked the ability of Ras·GTP to stimulate PI 3-kinase activity. These data indicate that GTP-bound Ras can activate the enzymatic activity of PI 3-kinase in a defined system, and that the interaction is synergistic with tyrosine phosphopeptides that bind to the SH2 domains of p85.

Discussion

The data presented here provide new evidence for the importance of the interaction between Ras and PI 3-kinase in the control of cell growth. We have established that the interaction with full-length p85/p110 occurs with a relatively high binding affinity of ~150 nM for V12 H-Ras. This compares with ~50 nM for the interaction of this transforming Ras mutant with the amino-terminal part of c-Raf1 (Herrmann *et al.*, 1995), which is thought to be stronger than the interaction with full-length Raf. Given the established physiological importance of the Ras–Raf interaction, the strength of the interaction between Ras and PI 3-kinase suggests that it is at least a good candidate for a biologically significant effector pathway. It should be noted, however, that biologically significant interactions

need not be of high affinity: Ras·GTP binds to $p120^{GAP}$ with an affinity of ~2 μM (Vogel *et al.*, 1988).

The site of the interaction of Ras with PI 3-kinase is shown here to be between residues 133 and 314 on the catalytic p110 subunit, with lysine residue 227 being essential for the interaction. This is a region immediately carboxy-terminal to the site of interaction between p110 and the regulatory subunit p85. This suggests a model in which the lipid kinase receives regulatory signals through two domains in its amino-terminal region. One signal comes from tyrosine phosphoproteins, and possibly from SH3 domains of Src family kinases and from Rho family proteins, through p85 and its interaction with the first 150 amino acids of p110. The other signal comes from the direct interaction of Ras·GTP with the neighbouring region of p110, roughly spanning the next 150 amino acid residues. It is likely that optimal activation of PI 3-kinase requires input from both signalling pathways. Since growth factors such as PDGF stimulate both tyrosine phosphorylation of p85 binding sites and activation of Ras, both these pathways will be activated in response to the growth factor, allowing potentiation of the activation signals being transmitted to PI 3-kinase. This is analogous to the stimulation of MAP kinase by growth factors: multiple signalling pathways feeding into MAP kinase activation are triggered by a single growth factor, including Ras activation, protein kinase C activation and intracellular calcium elevation (Burgering *et al.*, 1993). Ras contributes to Raf activation by translocating it to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), while protein kinase C can phosphorylate and activate Raf directly (Sözeri *et al.*, 1992; Kolch *et al.*, 1993). It is possible that this growth factor-induced activation of multiple signalling pathways, that then synergize together to activate Ras effectors such as Raf and PI 3-kinase, allows the generation of strong downstream signals from activation of small numbers of receptors. It may also allow for the greatest flexibility in the range of responses generated by different growth factors that activate seemingly similar sets of signalling pathways.

It is now clear that a number of different forms of growth factor-regulated PI 3-kinases exist, including p110α, β and γ. We have shown here that both p110α and β interact with Ras in a GTP-dependent manner. The sequence similarity of p110γ to p110α and β in the Ras binding site indicates that it too is likely to interact with Ras. In this case, stimuli that activate heterotrimeric G proteins could activate p110γ synergistically both through direct interaction and through stimulation of Ras (Koch *et al.*, 1994), in a situation analogous to that described above for tyrosine kinase pathways.

The behaviour of the K227E mutant of p110 establishes not only that this residue is essential for interaction with Ras, although not for *in vitro* basal PI 3-kinase activity of the catalytic subunit by itself, but also that the direct interaction of Ras with PI 3-kinase is necessary for the elevation of PIP_3 levels in COS cells transfected with activated Ras and p85/p110. This indicates that Ras-induced production of autocrine growth factors is not sufficient by itself to cause the increase in PIP_3 seen, but that direct interaction with Ras is also required, supporting the model in which multiple signals are required for PI 3-kinase activation, including direct interaction of Ras with p110 and interaction of p85 with tyrosine phosphoproteins. The fact that the basal activity of K227E p110 is high in Cos cells suggests that this reversal of charged residues at an essential site for the Ras–p110 interaction may cause a conformational change that in some ways mimics that occurring when Ras binds. We currently are exploring whether less radical amino acid changes result in a PI 3-kinase that does not bind Ras but also does not have elevated basal activity.

Use of the *in vitro* reconstitution system described here clearly shows that Ras interaction with PI 3-kinase can lead to activation of its lipid kinase activity. This appears to occur synergistically with the previously described activation of PI 3-kinase by tyrosine phosphopeptide interaction with the SH2 domains of the p85 subunit, with the strongest effects being seen at suboptimal phosphopeptide concentrations, although Ras stimulation of PI 3-kinase activity is seen both in the absence of tyrosine phosphopeptide and in the presence of saturating amounts of peptide. The exact mechanism by which this stimulation is achieved is not clear: one possibility is a simple translocation of PI 3-kinase to the liposome where both Ras and its substrate $PI(4,5)P_2$ are located. The stimulation is dependent on the post-translational modification of Ras: this is not needed for Ras binding to p110 but is essential for Ras to associate with the liposomes. Bacterially expressed Ras will bind to PI 3-kinase but will not stimulate its activity in the liposome system. However, there is precedent for Ras farnesylation being required for a productive interaction with another protein, Sos (Porfiri *et al.*, 1994), although it is not absolutely required for binding (Liu *et al.*, 1993a). In addition, the activation of the kinase activity of p110 by the mutation K227E suggests that a conformational change might also be involved, as does the fact that the presence of Ras·GTP alters the concentration dependence of the stimulation of the kinase by tyrosine phosphopeptides. The Ras-induced change in the concentration dependence for tyrosine phosphopeptide could reflect an increase in the affinity of the SH2 domains of p85 for the tyrosine phosphopeptide induced by Ras·GTP. Since Ras binds to p110 at the site next to that where p110 binds to p85, this is perhaps not unlikely. Whatever the mechanism, the stimulation of PI 3-kinase activity by Ras reported here is the first time that the enzymatic activity of a mammalian effector of Ras has been shown to be controlled by Ras in a completely defined system. No regulation of the protein kinase activity of purified Raf by interaction with Ras·GTP has yet been reported.

It has been suggested that the Rho family protein Rac lies on a signalling pathway downstream of PI 3-kinase which controls, amongst other things, the polymerization of actin beneath the plasma membrane to create membrane ruffles (Ridley *et al.*, 1992; Kotani *et al.*, 1994; Wennström *et al.*, 1994a,b). Activated Ras can induce activation of Rac, at least as judged by downstream morphological events (Ridley *et al.*, 1992). PIP_3 may be able to control the activation state of Rac through decreasing Rac–GAP activity or increasing Rac exchange factor activity (Hawkins *et al.*, 1995). A recent report has shown that an activated mutant of Rac has some transforming activity, which can synergize strongly with activated Raf, but not Ras (Qiu *et al.*, 1995), suggesting that both Rac and the

MAP kinase pathway are separate effector mechanisms used by Ras. Taken together, these reports plus the data presented here suggest that Ras activates the MAP kinase pathway through interaction with Raf and the Rac pathway through interaction with PI 3-kinase, and that the function of both of these pathways is required for efficient transformation of fibroblasts by Ras. For the activation of both Raf and PI 3-kinase pathways in Ras-transformed cells, it is likely that direct Ras–effector interaction is potentiated by the autocrine production of growth factors leading to receptor tyrosine kinase activation. In support of the possibility that PI 3-kinase is required for cell proliferation and could be playing an essential role downstream of Ras, it has also been reported that microinjection of neutralizing antibodies against PI 3-kinase blocks the ability of a number of growth factors to induce DNA synthesis in fibroblasts (Roche *et al.*, 1994).

Altogether, the data presented here provide strong evidence for a physiological role for PI 3-kinase in addition to Raf in signalling downstream of Ras. At least five different effector systems have now been identified that might operate downstream of Ras, raising the possibility that a very complex set of signals emerges from Ras which could co-operate in a various ways to cause cellular transformation. At present, it is not clear how important other putative effectors of Ras are in its control of cell growth, but it is clearly possible that GAPs, RalGDSs, MEKKs or PKCζ could also be contributing to the overall phenotype of Ras transformation. When strongly activated mutants of Ras are highly expressed as in a transformation assay, it is possible that just one potently activated effector pathway is required for measurable loss of growth control. This may be very different from the situation in normal cells stimulated with physiological levels of growth factors where the expression level of Ras is low and its activation is moderate and transient: under these circumstances, function of several signalling pathways downstream of Ras may be required in order for cell proliferation to result.

Materials and methods

Expression and purification of proteins

Unmodified wild-type and V12 c-Ha-Ras was purified from a detergent-free homogenate of recombinant baculovirus-infected Sf9 cells as described in Page *et al.* (1989). Post-translationally modified wild-type Ras was purified from the particulate pellet from the above cells. The pellet was solubilized in the same homogenization buffer to which 40 mM *n*-octylglucoside had been added. After clarification by centrifugation at 350 000 *g* for 10 min, the solubilized Ras was purified as in Page *et al.* (1989) but with all buffers including 40 mM *n*-octylglucoside. To prepare PI 3-kinase, Sf9 cells were co-infected with GST–p110α and p85α baculoviruses and the GST–p110/p85 complex purified according to the manufacturer's protocol.

Amino-terminal deletion mutants of p110α were made by ExoIII deletion of bovine p110, inserted in the pAcG3 vector (Davies *et al.*, 1993).

p110 constructs. p110 in pBluescript was digested with Fsp1, a *Bam*HI linker ligated and, after digestion with *Bam*HI, the resulting fragment ligated into the *Bam*HI site of pcDNA3 (Invitrogen).The 9E10 epitope was inserted at the amino-terminus of p110 by PCR. Point mutations in p110 and p85 were done using the U.S.E. *in vitro* mutagenesis kit (Pharmacia) according to the manufacturer's protocol. For COS transfections, all the p110 variants were subcloned into the *Bam*HI site of pSG5 (Stratagene).

The p85 binding domain of p110 (amino acids 1–108) and the inter-SH2 domain of p85 (amino acids 358–617) were amplified and a 9E10 epitope inserted by PCR, and cloned into the *Bam*HI site of pcDNA3. For transfections they were subcloned as *Kpn*I–*Xho*I fragments into pMT2.

Interaction of Ras with PI 3-kinase in vitro

Either baculovirus-expressed p85α/GST–p110α or bacterially expressed GST–p110 133–314 was used in solution at 50 nM. The binding buffer used was 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1 mg/ml bovine serum albumin (BSA), 5 mM $MgCl_2$. To determine the affinity of the Ras–PI 3-kinase interaction, unmodified Ras was loaded with [γ-^{32}P]GTP (36 μCi per set of 30 assays, diluted with 3 μmol of cold GTP). Varying concentrations of Ras were incubated with PI 3-kinase in 50 μl assays for 1 h at 4°C. Five μl of glutathione–agarose was then added per assay for 30 min at 4°C with shaking, and then the beads were washed with 4× 1 ml of cold 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1 mg/ml BSA, 5 mM $MgCl_2$/0.1% Triton X-100, and the pellets counted. Backgrounds without PI 3-kinase were subtracted.

For detection of Ras binding to PI 3-kinase by immunoblotting, assays were performed as above, using 1 μM Ras which was bound to either unlabelled GTP or GDP. Ras stably associated with PI 3-kinase was detected by immunoblotting using pan-Ras antibody (Oncogene Science).

Measurement of PIP_3 in COS-7 cells

Cells were harvested, washed and resuspended at 5×10^6 in HeBS and transfected by electroporation (250 V, 125 μF) with the indicated plasmids and 50 μg of sonicated herring sperm DNA. The amount of plasmid in different transfections was made equal with empty vector. Cells were allowed to stand at room temperature for 10 min and seeded in a 60 mm dish. At 40–48 h after transfection, cells were washed with phosphate-free DMEM and labelled overnight with 200 μCi [^{32}P]orthophosphate per dish. Reactions were terminated and samples analysed as described in Chung *et al.* (1992). One-tenth of the combined $CHCl_3$ phase was run on an oxalated silica gel 60 TLC plate, using a $CHCl_3$:methanol:acetone:acetic acid:water (40:13:15:12:7) system. The rest of the $CHCl_3$ phase was dried in a Speedvac and deacylated by incubating for 60 min at 50°C with methylamine. The dried pellet was resuspended in 500 μl of butanol and then re-extracted twice with 500 μl of water. Samples were run on a Spherisorb HPLC column (Anachem) and fractions counted by Cerenkov emission. Expression of each protein was checked by Western blotting of whole cell lysates with the appropriate antibodies.

Reconstitution of Ras into liposomes

Liposomes were made by sonication of dried lipids in kinase buffer (40 mM HEPES pH 7.5, 1 mg/ml fatty acid-free BSA, 2 mM EGTA, 100 mM NaCl, 5 mM $MgCl_2$, 50 μM ATP). The final lipid concentration was: 640 μM phosphatidylethanolamine, 600 μM phosphatidylserine, 280 μM phosphatidylcholine, 60 μM sphingomyelin, 60 μM PI(4,5)P_2. Sonication was on ice for 4× 20 min with a 4 mm probe at 15 μm amplitude in an MSE Soniprep 150. Modified Ras was added to the liposomes at a final concentration of 1 μM and incubated on ice for 20 min. Liposomes were collected by centrifugation at 350 000 *g* for 20 min. The supernatant was removed and liposome pellet resonicated in the same volume of kinase buffer. To load Ras with nucleotide, the mixture was made 10 mM in EDTA, and then 20 μM GTP or GDP added. After incubation at 30°C for 10 min, 15 mM $MgCl_2$ was added. When antibodies were used to block Ras effects, 300 μg/ml of purified monoclonal antibody was added to the initial incubation of Ras with liposomes.

PI 3-kinase assays in liposomes

Liposome mixture was made as described above, either with or without Ras. Tyrosine phosphopeptides were added as indicated, along with 10 ng of p85α/GST–p110α and 1 μCi [γ-^{32}P]ATP per point. The total assay volume was 25 μl, which represented a 50% dilution of the liposome preparation. Assays were mixed continuously for 20 min at 20°C, then stopped by adding 400 μl of chloroform:methanol (1:2) and 100 μl of 2.4 M HCl. After vortexing and centrifugation (15 000 *g*, 3 min), the lower chloroform layer was put into fresh tubes and dried down in a Speedvac. The residue was dissolved in 25 μl of chloroform and run on a silica TLC plate in chloroform:methanol:acetone:acetic acid:water (40:13:15:12:7). The PIP_3 spot was quantitated using a Molecular Dynamics PhosphorImager.

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