A Critical Role for Phosphoinositide 3-Kinase Upstream of Gab1 and SHP2 in the Activation of Ras and Mitogen-activated Protein Kinases by Epidermal Growth Factor*

Received for publication, August 2, 2000, and in revised form, December 13, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.M006966200

Armelle Yart‡, Muriel Laffargue‡, Patrick Mayeux‡, Stany Chretien‡, Christine Peres‡, Nicholas Tonks§, Serge Roche, Bernard Payrastre‡, Hugues Chap‡, and Patrick Raynal‡**

From ‡INSERM U326, IFR 30, Hôpital Purpan, Toulouse 31059, §INSERM U363, Hôpital Cochin, 27 rue du Faubourg Saint-Jacques, Paris 75014, ICNRS UPR 1086, 1919 route de Mende, Montpellier 34293, France and the ¶Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724-2208

Although the mechanisms involved in the activation of mitogen-activated protein kinases (MAPK) by receptor tyrosine kinases do not display an obvious role for phosphoinositide 3-kinases (PI3Ks), we have observed in the nontransformed cell line Vero stimulated with epidermal growth factor (EGF) that wortmannin and LY294002 nearly abolished MAPK activation. The effect was observed under strong stimulation and was independent of EGF concentration. In addition, three mutants of class Ia PI3Ks were found to inhibit MAPK activation to an extent similar to their effect on Akt/protein kinase B activation. To determine the importance of PI3K lipid kinase activity in MAPK activation, we have used the phosphatase PTEN and the pleckstrin homology domain of Tec kinase. Overexpression of these proteins, but not control mutants, was found to inhibit MAPK activation, suggesting that the lipid products of class Ia PI3Ks are necessary for MAPK signaling. We next investigated the location of PI3K in the MAPK cascade. Pharmacological inhibitors and dominant negative forms of PI3K were found to block the activation of Ras induced by EGF. Upstream from Ras, although association of Grb2 with its conventional effectors was independent of PI3K, we have observed that the recruitment of the tyrosine phosphatase SHP2 required PI3K. Because SHP2 was also essential for Ras activation, this suggested the existence of a PI3K/SHP2 pathway leading to the activation of Ras. In addition, we have observed that the docking protein Gab1, which is involved in PI3K activation during EGF stimulation, is also implicated in this pathway downstream of PI3K. Indeed, the association of Gab1 with SHP2 was blocked by PI3K inhibitors, and expression of Gab1 mutant deficient for binding to SHP2 was found to inhibit Ras activation without interfering with PI3K activation. These results show that, in addition to Shc and Grb2, a PI3K-dependent pathway involving Gab1 and SHP2 is essential for Ras activation under EGF stimulation.

The mitogen-activated protein kinases (MAPK)1 extracellular signal-regulated kinases (ERK) 1 and 2 transduce proliferative signals to the nucleus (1). The mechanisms leading to their activation by ligands of receptor tyrosine kinases appear well understood and the GTPase Ras plays a central role (2). For example, epidermal growth factor (EGF) activates its receptor tyrosine kinase, which autophosphorylates, creating binding sites for SH2-domain containing proteins, including the adapter proteins Shc and Grb2. In addition to its SH2 domain, Grb2 binds through its SH3 domains to the guanine nucleotide exchange factor Sos. Thus, the binding of Grb2 to phosphorylated EGF receptor (EGFR) results in the recruitment of Sos to the plasma membrane and has been proposed as a model for activation of membrane-bound Ras (3). In addition, EGF-induced activation of Ras may be transduced via She, which binds to activated EGFR and becomes phosphorylated, creating an additional binding site for Grb2 (4). Once Ras has been activated by Sos, GTP-bound Ras stimulates the downstream kinase MAPK/ERK kinase (MEK), which in turn phosphorylates ERK. In addition, activation of ERK under EGF stimulation can be mediated by Ras-independent pathways, through protein kinase C (PKC) and calcium-mediated mechanisms (5).

The phosphoinositide 3-kinases (PI3Ks) also transduce proliferative signals. PI3Ks phosphorylate phosphoinositides at the 3′-position of the inositol ring, and their major lipid product is phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is produced during cell stimulation by various mitogens (6, 7). Three classes of PI3Ks have been defined, and class I enzymes are involved in mitogen signaling. The members of the subclass IA include the catalytic subunits p110α, p110β, and p110δ associated with a regulatory subunit p85 and activated through protein tyrosine kinases, and subclass IB is represented by p110γ, which is activated by heterotrimeric G proteins. On a functional point of view, subclass IA PI3Ks are required for growth factor-induced mitogenesis (8, 9), and it was recently reported that embryos of p110α knockout mice die at early age due to a profound proliferative defect (10). The mechanisms by which PI3Ks activate signaling pathways have been recently unraveled. PIP3 has binding affinity for a conserved peptidase; ERK, extracellular-regulated protein kinase; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; MBD, Met-binding domain; MEK, MAP kinase/ERK kinase; PDGF, platelet-deprived growth factor; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; RBD, Ras-binding domain of Raf; wt, wild type; HA, hemagglutinin; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

* This work was supported by grants from Ministère de la Recherche et de l'Enseignement Supérieur, Association pour la Recherche sur le Cancer, and Ligue Nationale Contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
** To whom correspondence should be addressed: Tel.: 33-561-779-412; Fax: 33-561-779-401; E-mail: raynal@purpan.inserm.fr.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; RBD, Ras-binding domain of Raf1; wt, wild type; HA, hemagglutinin; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
sequence called the pleckstrin homology (PH) domain, thereby inducing the localization of PH-domain-containing proteins to membrane-associated signaling complexes (6). Several targets for PI3K lipid products have been proposed, including the proto-oncogene product Akt/protein kinase B (PKB) and its upstream activators, the phosphoinositide-dependent kinases. These kinases activate various enzymes that are important for cell growth, including the p70-S6 kinase and the glycoprotein synthase kinase 3 (6, 7). In addition, the catalytic subunits of PI3Ks possess an intrinsic protein kinase activity, which is involved in the down-regulation of their lipid kinase activity (11, 12). Interestingly, at least in the case of p110γ, this protein kinase activity has been reported to participate to MAPK signaling, whereas its lipid kinase activity appeared not to be necessary (13).

Although the model for growth factor-induced MAPK activation described above does not show an obvious role for PI3K, many reports have documented inhibition of MAPK activation by pharmacological inhibitors of PI3K (14–16). However, recent data have suggested that the role of PI3K depends on signal strength and is in fact limited to weak activations. In Swiss 3T3 cells, PI3K was found to be required during stimulation induced by low, but not high, doses of platelet-derived growth factor (17). In insulin-treated Chinese hamster ovary cells, the requirement for PI3K was reported to depend on the number of insulin receptors expressed on the cell surface (17). Similarly, in COS cells, pharmacological inhibitors of PI3K were found to inhibit MAPK activation induced only by low doses of EGF (18). On a molecular point of view, two major mechanisms have been proposed to illustrate a possible involvement of PI3K in MAPK activation. One involves the ability of PIP_3 to activate members of the PKC family, directly or via the phosphoinositide-dependent kinases (19, 20). Activated PKC can then stimulate Raf (21). The second involves the ability of p21-activated kinase, a downstream target of PI3K, to stimulate Raf (22). The second mechanism involves the phosphoinositide-dependent kinases (19, 20). Activated PKC can then stimulate Raf (21). The second involves the ability of p21-activated kinase, a downstream target of PI3K, to stimulate Raf (22).

In the nontransformed cell line Vero, we have observed that various PI3K inhibitors block MAPK activation induced by EGF, independently of signal strength. This led us to show that a PI3K-dependent pathway involving Gab1 and SHP2 is required, in addition to Shc and Grb2, for the activation of Ras by EGF.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was from Calbiochem. Polyclonal antibodies against Grb2, SHP2, and EGFR and monoclonal anti-Myc were from Santa Cruz Biotechnology. Polyclonal antibodies against Gab1, Sos, Shc, p85, and mononclonal anti-phosphotyrosine (4G10) were from Upstate Biotechnology Inc. Anti-phospho-ERK antibody was from Promega. Monoclonal anti-pasbin Ras was from Oncogene Research. Monoclonal anti-His tag antibody was from Invitrogen, anti-HA tag was from Roche Molecular Biochemicals, and anti-T7 tag was from Novagen. Cell culture reagents were from Life Technologies, Inc.

Cell Culture, Transfection, and Stimulations—Vero cells (a monkey kidney cell line, ATCC CCL 81) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal bovine serum and antibiotics. For transfection experiments, cells in 60-mm plates were incubated 3 h with 2 ml of Dulbecco’s modified Eagle’s medium containing 2 μg of total DNA, 6 μl of LipofectAMINE, and 6 μl of Plus reagent (Life Technologies, Inc.). Before stimulation, cells were blocked overnight by serum starvation. Unless otherwise indicated, cells were stimulated for 5 min with 10 ng/ml EGF. Before stimulation, cells were incubated for 15 min with 100 μM wortmannin (Sigma Chemical Co.) or 25 μM LY294002 (BioMol) where indicated.

Cell Lysis, Immunoprecipitation, and Immunoblotting—Cells were scraped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% glycerol, 1% Nonidet P-40, 10 μg/ml of each of autophosphorylated and leupeptin, and 1 μg/ml of each of autophosphorylated and leupeptin were incubated at 4 °C for 1 h, followed by addition of 10 μg/ml of GST-RBD fused to glutathione-Sepharose beads. To measure Ras activation, Vero cells were incubated with 1 μM EGF for 1 h, then washed three times with PBS and then boiled, and proteins were resolved by SDS-PAGE. Immunoblotting was performed with anti-ras antibodies. To study the activation of Ras in transfected cells, cells were transfected with 1 μg of each plasmid encoding HA-tagged wild type Ras (kindly provided by Dr. B. M. Burgersing, Utrecht, The Netherlands) and the indicated
**RESULTS**

**PI3K Is Required for EGF-induced MAPK Activation, Independently of Signal Strength**—By using anti-phospho-ERK immunoblotting, we have observed in Vero cells that the phosphorylation of ERK2 induced by 10 ng/ml EGF is abolished when cells are preincubated with wortmannin or LY294002 (Fig. 1A). Because the role of PI3K in ERK activation is thought to be limited to weak stimulations, we have measured the activation of transfected ERK1-His using an in vitro kinase assay. As shown in Fig. 1B, treatment with 10 ng/ml EGF induced a >10-fold increase in ERK1-His activity, in agreement with the strong phosphorylation of endogenous ERK2 observed using anti-phospho-ERK immunoblotting. Preincubation of the cells with PI3K inhibitors reduced by over 80% the activation of purified ERK1-His. In addition, the requirement for PI3K appeared independent of EGF concentration, because wortmannin treatment also inhibited the phosphorylation of endogenous ERK2 induced by 30 or 50 ng/ml EGF (Fig. 1C).

To confirm these data obtained with pharmacological inhibitors, we have studied the ability of three PI3K mutants to inhibit ERK1-His activation in cotransfection experiments. In parallel, we have determined their efficiency to inhibit PI3K signaling by measuring their ability to interfere with the activation of Akt/PKB. This was achieved in cotransfection experiments with HA-Akt/PKB and the same constructs as in A. Following cell stimulation, HA-Akt/PKB was immunoprecipitated and expression of PI3K mutants was verified in lysates from cells transfected with the indicated construct. B, cells were cotransfected with HA-tagged Akt/PKB and the same constructs as in A. Following cell stimulation, HA-Akt/PKB was immunoprecipitated and incubated with histones (H2B) and [γ-32P]ATP. Phosphorylation of histones was revealed using a PhosphorImager. The bottom graph represents the mean ± S.E. of three independent experiments.
PIP3 Is Essential for MAPK Activation—One important question regarding the involvement of PI3Ks in MAPK activation is the respective role of their lipid kinase and protein kinase activities, because PIP3 has been shown not to be necessary for p110α-mediated activation of MAPK (13). To define the importance of PI3K lipid products in EGF signaling, we have taken advantage of PTEN, a protein phosphatase that is also capable of dephosphorylating PI3K lipid products (25). As shown in Fig. 3, overexpression of PTEN inhibits ERK1-His activation induced by EGF. To determine whether the PTEN effect is due to its protein or lipid phosphatase activity, we have used as a control the “protein phosphatase only” mutant of PTEN (G129E), which does not interfere with PI3K signaling (25, 31). Transfection of PTEN-G129E did not significantly inhibit ERK1-His activation, whereas this mutant was somewhat more expressed than wild type PTEN (Fig. 3). To confirm the importance of PI3K lipid products, we have used the PH domain of Tec as a competitor for binding to PIP3. Tec belongs to a family of tyrosine kinases containing a PIP3-sensitive PH domain (32, 33). As shown in Fig. 3, expression of the Tec PH domain significantly inhibited ERK1-His activation induced by EGF. As a control, we have used the Tec PH domain containing the R29C mutation, which decreases the affinity of Tec kinases for PIP3 (32, 33). This mutant did not significantly inhibit ERK1-His activation. Altogether, these results suggested that PIP3 produced during EGF stimulation is necessary for MAPK activation.

PI3K Is Required Upstream of Ras and Sos—We next investigated the location of PI3K function in the MAPK pathway. Considering that PI3K-dependent mechanisms have been proposed at the level of Raf and MEK (19–23), and that MAPK activation by EGF can occur via Ras-independent pathways (5), we have first examined the role of Ras in Vero cells. Expression of dominant negative RasN17 was found to abolish ERK1-His activation induced by EGF (data not shown), indicating that MAPK activation is strictly dependent on Ras in Vero cells. Because MAPK activation also requires PI3K, we have determined whether PI3K was involved in the activation of Ras. This was achieved using a precipitation assay for activated Ras. Following cell stimulation and lysis, endogenous activated Ras was extracted using a GST fusion protein containing the Ras-binding domain of Raf (RBD), which interacts specifically with GTP-bound Ras (28). The amount of activated Ras in the GST-RBD pull-down assays was determined by anti-Ras immunoblotting. As shown in Fig. 4A, treatment of Vero cells with 10 ng/ml EGF induced the coprecipitation of Ras with GST-RBD, and preincubation of the cells with wortmannin or LY294002 strongly inhibited this association, suggesting that PI3K is involved in Ras activation. To confirm this hypothesis, the activation of HA-tagged Ras was studied in cells cotransfected with dominant negative PI3Ks. As shown in Fig. 4B, expression of Δp85 or p110β-K805R inhibited the amount of HA-Ras associated with the GST-RBD protein. Taken together, these results indicate that PI3K plays a critical role in the activation of Ras induced by EGF.

Upstream from Ras, membrane translocation of Sos is thought to be the limiting event for Ras activation. To determine whether PI3K was involved in Sos redistribution, we have prepared membrane fractions from EGF-treated cells preincubated or not with PI3K inhibitors. As shown in Fig. 4C, the membrane enrichment of Sos induced by EGF was nearly abolished by PI3K inhibitors. In addition, overexpression of Sos was found to partially overcome the need for PI3K in ERK activation without increasing the basal activation of ERK1-His (Fig. 4D). This suggested that PI3K was involved at the level or upstream of Sos. However, expression of constitutively activated PI3K (p110α-CAAX) was not sufficient to activate MAPK in unstimulated cells (data not shown). This indicated that PIP3 cannot directly induce the redistribution of Sos, suggesting the existence of a PIP3-dependent mechanism upstream from Sos.

SHP2 and Ras Are Activated Downstream of PI3K and Gab1—To identify a PI3K-dependent event upstream from Sos, we have analyzed by immunoblotting the proteins coimmunoprecipitated with Grb2. As expected, Fig. 5A shows that the EGFR and Shc readily precipitated with Grb2 upon EGF stimulation and this was not modified by PI3K inhibitors. In addition, PI3K inhibitors did not influence the constitutive association of Grb2 with Sos (data not shown). In contrast, the coimmunoprecipitation of the tyrosine phosphatase SHP2 with Grb2 was found to depend on PI3K (Fig. 5A). In agreement with this, the recruitment of SHP2 in anti-phosphotyrosine immunoprecipitates upon EGF stimulation was also reduced by PI3K inhibitors (Fig. 5B), suggesting that SHP2 is a downstream effector of PI3K in EGF signaling. To determine whether SHP2 was important for Ras activation in Vero cells, we have transfected a catalytically inactive mutant of SHP2 (SHP2-C/S) and analyzed the activation of HA-tagged Ras in cotransfection experiments. As shown in Fig. 5C, expression of SHP2-C/S completely inhibited the precipitation of HA-Ras with GST-RBD, whereas wild type SHP2 had no effect. This indicated that the catalytic activity of SHP2 is involved in the activation of Ras and suggested the existence of a PI3K/SHP2 pathway leading to Ras stimulation because SHP2 is recruited downstream of PI3K. We next attempted to identify a substrate of SHP2 involved in the activation of Ras using the SHP2-C/S mutant in a “substrate trapping” experiment. Cells transfected with SHP2-C/S, or wild type SHP2 as a control, were stimulated with EGF, subjected to SHP2 immunoprecipitation, then anti-phosphotyrosine immunoblotting. As shown in Fig. 5D, both wild type SHP2 and SHP2-C/S coimmunoprecipitated...
with three phosphoproteins of apparent molecular masses of around 180, 115, and 100 kDa, whereas only the SHP2-C/S mutant associated with a protein of approximately 135 kDa. Upon reblotting, the 180-kDa protein comigrated with the EGFR and the 115-kDa hyperphosphorylated protein comigrated with the docking protein Gab1 (data not shown). We attempted to identify the ~135-kDa protein using antibodies directed against Ras effectors, including Ras-GTPase-activating protein and Sos2, but we failed to label this protein (data not shown).

These results also suggested that the docking protein Gab1 could mediate the PI3K-dependent recruitment of SHP2 and the subsequent activation of Ras. Gab1 sequence displays three binding sites for p85 and is thought to mediate the activation of PI3K during EGF stimulation (34). In addition, Gab1 contains one binding site for SHP2 and a PIP3-specific PH domain (35, 36). However, the role of this PH domain in EGF signaling is not clear (37), and it is not known if the interaction of Gab1 with SHP2 requires PI3K. As shown in Fig. 6 (A and B), EGF induced the coimmunoprecipitation of Gab1 with SHP2, and this interaction was reduced by PI3K inhibitors. In addition, PI3K inhibitors nearly abolished the tyrosine phosphorylation of Gab1 induced by EGF (Fig. 6B). These data strongly suggested that Gab1 mediates the PI3K-dependent recruitment of SHP2. However, it is not known if Gab1 is important for Ras activation. To examine this question, we have produced Gab1 mutants deficient for binding to p85 or SHP2 and tested their effect on the activation of HA-Ras in cotransfection experiments. As shown in Fig. 6C, expression of Gab1-YF3 lacking the three p85 binding sites strongly inhibited the binding of HA-Ras to GST-RBD, and transfection of Gab1-Y627F deficient for SHP2 binding also blocked this association. This indicated that interaction of Gab1 with p85 and SHP2 is required for Ras activation. To determine whether Gab1 mutants blocked the pathway upstream or downstream of PI3K, we have studied their effect on the activation of HA-Akt/PKB. As shown in Fig. 6D, this activation was not impaired in cells transfected with Gab1-Y627F in comparison with cells expressing wild type Gab1. In contrast, activation of HA-Akt/PKB was strongly inhibited when Gab1-YF3 was expressed. In addition, Gab1-Y627F did not bind less p85 than wild type Gab1 in coimmunoprecipitation experiments (Fig. 6E). This demonstrates that Gab1-YF3 blocked the activation of Ras upstream of PI3K, whereas Gab1-Y627F interfered with Ras activation without preventing PI3K activation. Altogether, these results show that, in addition to Shc and Grb2, a PI3K-dependent pathway involving Gab1 and SHP2 participates to the activation of Ras under EGF stimulation.

These data also suggested that the major function of PI3K in the activation of Ras is to promote the recruitment of Gab1 in EGF signaling, leading to the recruitment of SHP2. However, Gab1 can associate to the activated EGFR through Grb2 (34) and directly through the Met-binding domain (MBD), which constitutes a novel phosphotyrosine-binding motif (36, 38). We have thus further examined the role of PI3K in Gab1 recruitment by preparing a mutant deleted of the PH domain (Gab1-PH). As expected, this mutant has lost the ability to associate with membrane fractions in a PI3K-dependent manner (Fig. 7A). We next studied its involvement in EGF signaling. As shown in Fig. 7B, the phosphorylation of Gab1-ΔPH and its coimmunoprecipitation with SHP2 were reduced in comparison to Gab1-wt, and these events were insensitive to wortmannin. This indicates that the PH domain is important for the recruitment of Gab1 in EGF signaling. However, it is not clear if the interaction between the PH domain and PI3K simply stabilizes the association of Gab1 with the EGFR, or whether PI3K promotes the recruitment of additional Gab1 molecules in the vicinity of the receptor.

As a first approach to answer this question, we have studied the ability of Gab1-ΔPH to interfere with EGF signaling. This was achieved by measuring MAPK activation in cotransfection experiments. Fig. 7C shows that Gab1-ΔPH did not significantly modify Erk1-His activation. As a control, transfection of
the Gab1-Y627F mutant that inhibited the activation of Ras (Fig. 6C) was found to reduce by more than 70% MAPK activation (Fig. 7C). Because Gab1-ΔPH is less phosphorylated than Gab1-wt (Fig. 7B), the fact that Gab1-ΔPH did not inhibit EGF signaling suggested that this mutant, albeit overexpressed, could not prevent the recruitment of endogenous wild type (wt) Gab1. To test this hypothesis, we have determined whether overexpression of Gab1-ΔPH interferes or not with the phosphorylation of Gab1-wt induced by EGF. This was achieved by studying the phosphorylation of HA-Gab1-wt cotransfected with Gab1-ΔPH in a 1:20 ratio. As shown in Fig. 7D, overexpression of Gab1-ΔPH did not modify the phosphorylation of HA-Gab1-wt. As a control, overexpression of other Gab1 constructs (Gab1-Myc-wt or Gab1-Myc-Y627F) strongly inhibited the phosphorylation of HA-Gab1-wt. These results indicate that Gab1-ΔPH cannot prevent the phosphorylation of Gab1-wt by the EGFR, suggesting that PIP3 alone is sufficient to recruit Gab1 in the vicinity of the receptor.

**DISCUSSION**

Although the mechanisms involved in growth factor-induced activation of MAPK do not display an obvious role for PI3K, pharmacological inhibitors of PI3K were found to strongly interfere with MAPK activation in Vero cells stimulated with EGF. In agreement with this, expression of mutants of class Ia PI3Ks were found to strongly inhibit the activation of MAPK to an extent similar to their effect on the activation of Akt/PIKB, a major effector of PI3K. Moreover, the requirement for PI3K was observed under strong activation and independently of EGF concentration, which indicated that this enzyme can play an important function in the mechanisms leading to MAPK activation.

We have first studied the role of PI3K lipid products in this pathway, because it has been reported that only the protein kinase activity of p110γ is necessary for MAPK activation (13). We have shown that overexpression of two proteins interfering with PIP3 impaired MAPK activation, suggesting that, in contrast to p110γ, the lipid kinase activity of class Ia PI3Ks is essential for MAPK signaling. PTEN has already been shown to interfere with growth factor-induced MAPK activation, but it has been proposed that this effect was due to its ability to dephosphorylate proteins potentially involved in MAPK signaling, including the Fak kinase and Shc (39, 40). We have thus used as a control the G129E mutant of PTEN, which retains the catalytic activity but has lost its ability to interact with phosphoinositides and, consequently, does not interfere with PI3K signaling (25, 31). In our model, this mutant was not active on MAPK signaling, suggesting that the lipid phosphatase activity of PTEN is primarily responsible for MAPK inhibition. In agreement with this, overexpression of the PH domain of Tec also inhibited MAPK, whereas mutation of an amino acid residue involved in PIP3 binding produced an inactive protein.

Based on biochemical approaches, multiple reports have suggested an involvement of PI3K in MAPK activation downstream of Ras, considering that the activation of Raf or MEK...
can be mediated by targets of PI3K signaling (19–23). In contrast, we have observed using inhibitors and dominant negative mutants that PI3K has a function upstream of Ras during EGF stimulation. PI3K was also found to be necessary for the redistribution of Sos, but expression of constitutively activated PI3K was not sufficient to activate MAPK. This suggested that PIP3 cannot directly induce the membrane translocation of Sos, although Sos contains a PH domain that has some affinity for this lipid (33). Nevertheless, we cannot exclude that PIP3 participates in Sos redistribution by stabilizing its interaction with the plasma membrane. In addition, although PI3K does not seem to contribute to the formation of the complex between Grb2 and the EGFR, Shc, or Sos, we have observed that the recruitment of Sos in response to EGF was also strongly dependent on Sos. Because the activation of Ras in response to EGF was also strongly dependent on Sos, this suggested the existence of a PI3K/Sos pathway that is important, in addition to Shc and Grb2, for the activation of Ras.

The docking protein Gab1 was found to participate in the process at two different levels (Fig. 8). First, we have verified that Gab1 is effectively involved in PI3K activation during EGF stimulation, because expression of Gab1 deficient for p85 binding sites abolished the activation of Akt/PKB. Second, Gab1 mediates the recruitment of Sos to the surface by SHP2 downstream of PI3K and the subsequent activation of Ras. Indeed, the phosphorylation of Gab1 and its association with SHP2 induced by EGF were blocked by PI3K inhibitors, and disruption of its SHP2 binding site suppressed the stimulation of Ras without interfering with PI3K activation. In addition, we have observed that the PH domain is important for efficient phosphorylation of Gab1 and binding to SHP2. Therefore, these data support the notion that PIP3 is essential for the recruitment of Gab1 in EGF signaling, as suggested by the fact that Gab1 contains a PH domain that binds specifically PIP3 (35, 36). Yet, one may wonder what precise role PIP3 plays, because Gab1 can associate to the phosphorylated EGFR through the MBD and indirectly through Grb2 (34, 36). PIP3 could simply stabilize the association of Gab1 with the receptor through Grb2- or MBD-mediated interactions. Nevertheless, overexpression of Gab1-ΔPH suggest that PIP3 alone could promote the recruitment of Gab1 molecules in the vicinity of the EGFR. Indeed, the observations that Gab1-ΔPH is phosphorylated upon EGF stimulation, albeit less strongly than Gab1-wt, and that this phosphorylation is independent of PI3K suggest that Gab1-ΔPH can compete with Gab1-wt for binding to the receptor through Grb2- or MBD-mediated interactions. Nevertheless, overexpression of Gab1-ΔPH does not have a dominant negative effect on EGF signaling, i.e. phosphorylation of Gab1-wt and MAPK activation. This indicates that Gab1-wt is effectively recruited to the receptor in cells overexpressing Gab1-ΔPH, through a mechanism for which Gab1-ΔPH cannot compete. This is most likely the interaction between the PH domain and PIP3. In any case, this question must be further investigated, by studying, for example, the recruitment of Gab1 mutants deleted of the MBD and the Grb2-binding regions.

An important question is the physiological significance of...
The next step in the understanding of this PI3K-dependent pathway will be brought by the identification of the SHP2 substrates involved in the activation of Ras. It has been proposed that SHP2 can function as an adaptor in PDGF signal-

ing, because it can bind to both the receptor and the SH2 domains of Grb2 and therefore contributes to the recruitment of Grb2-Sos (43). However, we have found that expression of catalytically inactive SHP2 abolished Ras activation in EGF-treated Vero cells, which is consistent with the fact that catalytically inactive SHP-2 was shown to inhibit MAPK activation induced by EGF in other cell types (37, 44). This suggests that SHP2 activates by dephosphorylation a protein promoting the translocation of Sos or down-regulates an inhibitor of Sos redistribution. A candidate could be the 135-kDa protein that is associated with catalytically inactive SHP-2 in our substrate trapping experiment. Another candidate could be an unidentified 90-kDa protein associated to Gab1 and for which phosphorylation is up-regulated in SHP2 knockout mice (41). Further efforts are required to identify these proteins.

Acknowledgments—We are grateful to the following researchers for constructs used in this study: Drs. P. Hu, M. Wymann, F. R. McKenzie, El. vanObberghen, C. Susini, N. Rivard, J. Downward, W. Ogawa, P. Chardin, P. van Bergen en Henegouwen, B. M. Burgering, and A. Ullrich.

REFERENCES
1. Schaeffer, H. J., and Weber, M. J. (1999) Mol. Cell. Biol. 19, 2435–2444
2. McCormick, F. (1993) Nature 363, 15–16
3. Buday, L., and Downward, J. (1993) Cell 73, 611–620
4. Pelici, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelici, P. G. (1992) Cell 70, 93–104
5. Burgering, B. M., de Vries-Smits, A. M., Medema, R. H., van Weeren, P. C., Tertoolen, L. G., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 7248–7256
6. Rameh, L. E., and Cantley, L. C. (1999) J. Biol. Chem. 274, 8347–8350
7. Levers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) Curr. Opin. Cell Biol. 11, 219–225
8. Roche, S., Koegl, M., and Courtneidge, S. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9185–9189
9. Roche, S., Downward, J., Raynal, P., and Courtneidge, S. (1998) Mol. Cell. Biol. 18, 7119–7129
10. Bi, L., Okabe, I., Bernard, D. J., Wynshaw-Boris, A., and Nussbaum, R. L. (1999) J. Biol. Chem. 274, 10963–10968
11. Hunter, T. (1995) Cell 83, 1–4
12. Vanhaesebroeck, B., Higashi, K., Raven, C., Welham, M., Anderson, S., Brennan, P., Ward, S. G., and Waterfield, M. D. (1999) EMBO J. 18, 1292–1302
13. Bondeva, T., Pirla, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., and Wymann, M. P. (1998) Science 282, 293–296
14. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O.
Role of Phosphoinositide 3-Kinase Upstream of Ras

15. Marra, F., Pinzani, M., DeFranco, R., Laffi, G., and Gentilini, P. (1995) FEBS Lett. 376, 141–145
16. Sajan, M. P., Standaert, M. L., Bandopadhyay, G., Quon, M. J., Burke, T. R., and Farese, R. V. (1995) J. Biol. Chem. 270, 30495–30500
17. Wennstrom, S., and Downward, J. (1999) Mol. Cell. Biol. 19, 4279–4288
18. Wennstrom, S., and Downward, J. (1999) Mol. Cell. Biol. 19, 4279–4288
19. Duckworth, B. C., and Cantley, L. C. (1997) J. Biol. Chem. 272, 27665–27670
20. Legood, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2042–2045
21. Schonwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, P. J. (1998) Mol. Cell. Biol. 18, 790–798
22. Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E., and Cobb, M. H. (1997) EMBO J. 16, 6426–6438
23. Schonwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, P. J. (1998) Mol. Cell. Biol. 18, 790–798
24. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13513–13518
25. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344
26. August, A., Sadra, A., Dupont, B., and Hanafusa, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11227–11232
27. de Rooij, J., and Bos, J. L. (1997) Oncogene 14, 623–625
28. Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M., and Hirano, T. (2000) Mol. Cell. Biol. 20, 3695–3704
29. Bennett, A. M., Hausdorff, S. F., Oreilly, A. M., Freeman, R. M., and Neel, B. G. (1996) Mol. Cell. Biol. 16, 1189–1202