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Association of p21^{ras} with phosphatidylinositol 3-kinase

(growth factors/inositolphospholipids)

ANITA SJÖLANDER*, KYOHEI YAMAMOTO, BRIAN E. HUBER, AND EDUARDO G. LAPETINA†

Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC 27709

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ABSTRACT **In mammalian cells, *ras* genes code for 21-kDa GTP-binding proteins. Increased expression and mutations in specific amino acids have been closely linked to alterations of normal cell morphology, growth, and differentiation and, in particular, to neoplastic transformation. The signal transduction induced by these p21^{ras} proteins is largely unknown; however, the signaling pathways of several growth factors have been reported to involve phosphatidylinositol (PtdIns) 3-kinase. In the present study of a Ha-*ras*-transformed epithelial cell line, we demonstrated increased PtdIns 3-kinase activity in anti-phosphotyrosine and anti-receptor (insulin and hybrid insulin-like growth factor I) immunoprecipitates of cells that had been stimulated with insulin or insulin-like growth factor I. The PtdIns 3-kinase activity was also immunoprecipitated in these experiments by the anti-Ras monoclonal antibody Y13-259. The specificity of this association with p21^{ras} was ascertained by the neutralizing effect of the antigen peptide and the absence of PtdIns 3-kinase activity in Y13-259 immunoprecipitates from cells in which the *ras* gene was turned off. These data indicate that PtdIns 3-kinase activity is an important step in the cascade of reactions in p21^{ras} signal transduction, suggesting that the alterations of the cytoskeleton and growth in *ras*-transformed cells could be mediated by PtdIns 3-kinase activity.**

In mammalian cells, *ras* genes code for a family of closely related 21-kDa proteins (p21^{ras}) (1, 2). The function of these proteins seems to be regulated by GTP and GDP in a manner similar to that of the heterotrimeric GTP-binding proteins involved in transmembrane signaling (2–4). Activated *ras* oncogenes have been identified in various forms of human cancer, including carcinomas of the lung, colon, and pancreas (5). These cancer cells, as well as cells that have been experimentally transformed with *ras*, exhibit gross morphological changes (6, 7). A direct link between morphological changes and the Ras proteins has been demonstrated by microinjection of p21^{ras} (7). Since Ras proteins act as signal-transduction elements (3), they may affect the cytoskeleton and the behavior of a cell via alterations of its signaling system. The biochemical mechanism of Ras proteins might therefore be related to the metabolism of inositolphospholipids. Phosphatidylinositol (PtdIns) 3-kinase is likely to be involved in the dynamic modulation of the cytoskeleton (8) and the effects of certain growth factors (9, 10).

Stimulation of growth factor receptors leads to receptor autophosphorylation, tyrosine phosphorylation of intracellular substrates, and an increase in PtdIns 3-kinase activity (9–12). This latter kinase phosphorylates PtdIns at the D-3 position of the inositol ring to generate potential signals for cell growth (3, 13). PtdIns 3-kinase activity has been found in anti-phosphotyrosine immunoprecipitates from intact cells stimulated with various growth factors (3, 9–11, 13). In addition, work with polyoma-transformed cells has demonstrated that PtdIns 3-kinase activity is associated with the oncogenic complex formed by the polyoma middle-sized tumor antigen (middle t) and the product of the c-*src* gene (pp60$^{c\text{-}src}$) (14–16). PtdIns 3-kinase has been purified and was reported to be a heterodimer of an 85-kDa protein and a 110-kDa protein (17). The recently cloned 85-kDa protein contains one SH3 and two SH2 Src-homology domains, explaining its tight association with tyrosine phosphorylated receptors. The 85-kDa protein lacks PtdIns 3-kinase activity, which strongly suggests that the activity is located on the 110-kDa protein (18, 19).

In the present study we used a *ras*-transformed rat liver epithelial cell line in which expression of the *ras* oncogene is under the transcriptional control of the mouse mammary tumor virus long terminal repeat. This *ras* gene can therefore be turned off by the absence of glucocorticoid in the extracellular medium (20–22). Expression of the *ras* oncogene causes distinct morphological/cytoskeletal alterations of the cell and loss of contact inhibition of growth (20). Furthermore, Western blot analysis confirmed that these cells have an active p21^{ras} with a mutation in position 12 (20). These cells express receptors for insulin and insulin-like growth factor I (IGF-I) as well as a hybrid receptor for IGF-I (C. Moxham, personal communication). On the *ras*-transformed cells, the first two types of receptors exhibit binding characteristics similar to those of receptors found on normal liver cells (23). The hybrid receptor for IGF-I exhibits characteristics similar to those of hybrid IGF-I receptors found on *ras*-transformed NIH 3T3 mouse fibroblasts and HepG2 human hepatoma cells (C. Moxham, personal communication; ref. 24). Insulin receptors as well as IGF-I receptors are transmembrane glycoproteins that possess cytosolic tyrosine kinase activity (25, 26). Agonist binding triggers autophosphorylation of tyrosine resulting in increased receptor tyrosine kinase activity toward other intracellular substrates (9). The signaling mechanism by which the receptors of these and other growth factors couple to functional responses is still unclear. Based on these previous findings, we chose to examine the possible association between Ras proteins and PtdIns 3-kinase activity in insulin- and IGF-I-stimulated *ras*-transformed liver epithelial cells.

MATERIALS AND METHODS

Materials. IGF-I was from Imcera (Terre Haute, IN) and anti-insulin receptor antibody that binds both insulin receptors and hybrid IGF-I receptors was a gift of S. Jacobs (24). The anti-Ras antibody Y13-259 was obtained from Oncogene Science (Manhasset, NY), and the anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY). The anti-Ras antibody M90 was a gift of J. C. Lacal and S. A. Aaronson (27).

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Abbreviations: PtdIns, phosphatidylinositol; IGF-I, insulin-like growth factor I; middle t, middle-sized tumor antigen.

*On leave from the Department of Cell Biology, University of Linköping, Linköping, Sweden.
†To whom reprint requests should be addressed.

Cells. The *ras*-transformed Fischer rat liver epithelial cell line was maintained in culture in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum, penicillin (55 units/ml), and streptomycin sulfate (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Under serum-free conditions, the DMEM medium was supplemented with epidermal growth factor (10 ng/ml), transferrin (500 ng/ml), insulin (500 ng/ml), fetuin (500 μg/ml), oleic acid (500 ng/ml), selenious acid (5 ng/ml), and the cell culture flasks were precoated with vitrogen (GIBCO) (25 mg/ml) and fibronectin (25 mg/ml) for 30 min at 37°C (20).

Immunoprecipitation. Confluent cultures of rat liver epithelial cells overexpressing *ras* were washed twice with phosphate-buffered saline and then incubated in a physiologically balanced Ca^{2+}-containing medium (28) for 2 min at 37°C in the absence or presence of insulin or IGF-I. The stimulation with these growth factors was terminated by addition of an ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 0.1 mM phenylmethanesulfonyl fluoride, 50 μg of leupeptin per ml, and 1% Nonidet P-40. The cells were kept in this buffer for 1 hr at 4°C. The lysates were then cleared by centrifugation and incubated for 1 hr at 4°C with anti-phosphotyrosine or anti-insulin receptor (24) antibodies at a 1:1000 dilution. After an additional 1-hr incubation at 4°C with protein A-Sepharose, the immunoprecipitates were recovered by centrifugation. When the anti-Ras monoclonal antibody Y13-259 bound to agarose was used, the incubation time was 2 hr at 4°C before the immunoprecipitates were recovered. The collected immunoprecipitates were washed twice with the lysis buffer, twice with 0.5 M LiCl/0.1 M Tris, pH 7.4, and finally twice with 10 mM Tris, pH 7.2/100 mM NaCl/1 mM EDTA (TNE buffer; ref. 29).

Kinase Assay. The washed immunoprecipitates were resuspended in 50 μl of TNE buffer, and sonicated PtdIns micelles (0.2 mg/ml) were added together with 20 μCi (740 kBq) of [γ-^{32}P]ATP and 20 mM $MgCl_2$ (29). The kinase reaction was allowed to continue for 20 min at room temperature. The phospholipids were extracted and were separated by thin-layer chromatography (TLC), and the radioactive spots were detected by autoradiography (29, 30).

Gel Electrophoresis and Immunoblotting. Immunoprecipitates obtained with monoclonal antibody Y13-259 were solubilized in 0.125 M Tris, pH 6.8/0.1% SDS/10% glycerol/100 mM dithiothreitol, heated at 100°C for 5 min, and electrophoresed in an SDS/10–20% polyacrylamide gel. This was followed by electophoretic transfer of the proteins to nitrocellulose. The Y13-259 immunoprecipitates were assayed by Western blotting with monoclonal antibody M90 (1:500 dilution), which detects epitope 107–130 of $p21^{ras}$ (27).

HPLC Identification of the Inositolphospholipids from the Kinase Assay. Phospholipids from the TLC analysis were deacylated with the methylamine reagents described by Clarke and Dawson (31). The glycerophosphoinositol phosphates so obtained were then separated using an NH_4HCO_3 (pH 3.7) gradient, as described by Nolan and Lapetina (32).

RESULTS

We assayed for PtdIns 3-kinase activity in anti-phosphotyrosine immunoprecipitates of both nonstimulated and agonist-stimulated cells (Fig. 1*A*). Increased PtdIns 3-kinase activity was immunoprecipitated following stimulation with insulin (100 μg/ml; lane 1) or IGF-I (100 ng/ml; lane 2). We found only minor basal PtdIns 3-kinase activity in anti-phosphotyrosine immunoprecipitates from unstimulated cells (lane 3), generally 10 times less than that of agonist-stimulated cells. No PtdIns 3-kinase activity above background levels was detected in immunoprecipitates obtained with preimmune serum (data not shown). Endemann *et al.* (9) demonstrated that in insulin-stimulated Chinese hamster ovary cells, the majority of the PtdIns 3-kinase activity that was precipitated with anti-phosphotyrosine was not associated with the insulin receptor itself (9). Similar observations have been obtained in studies of the IGF-I receptor in the mouse fibroblast cell line LISN C4 (K.Y., E.G.L., and C. P. Moxham, unpublished data).

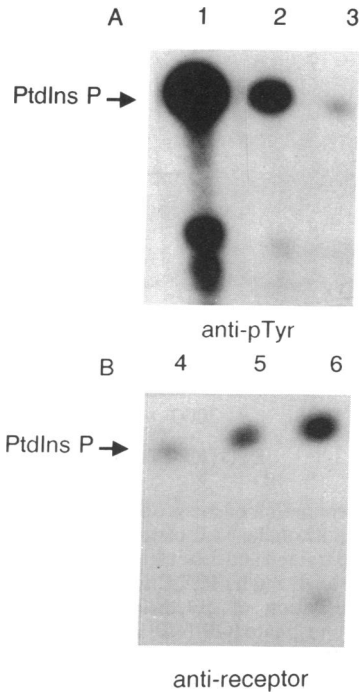

FIG. 1. (*A*) PtdIns 3-kinase activities in anti-phosphotyrosine (anti-pTyr) immunoprecipitates. Shown are kinase reaction products of immunoprecipitates from cells stimulated with insulin at 100 μg/ml (lane 1), from cells stimulated with IGF-I at 100 ng/ml (lane 2), and from unstimulated cells (lane 3). (*B*) Receptor kinase reaction products in immunoprecipitates obtained with anti-insulin receptor antibody, which immunoprecipitates both insulin receptors and the receptor hybrid for IGF-I. Immunoprecipitates were from unstimulated cells (lane 4), from cells stimulated with insulin at 100 μg/ml (lane 5), and from cells stimulated with IGF-I at 100 ng/ml (lane 6). Position of PtdIns monophosphate is indicated.

In our studies of enzyme–receptor association, we found PtdIns 3-kinase activity in immunoprecipitates obtained with an anti-insulin receptor antibody that immunoprecipitates both insulin and hybrid IGF-I receptors (Fig. 1*B*; ref. 24). Immunoprecipitates from insulin-stimulated (lane 5) and IGF-I-stimulated (lane 6) cells contained at least 5 times more PtdIns 3-kinase activity than those obtained from unstimulated cells (lane 4). The identity of the lipids produced in the PtdIns kinase assay was determined by HPLC analysis of the deacylated products. As an example of this, Fig. 2 shows the identification of the deacylated products from the PtdIns kinase assays of anti-phosphotyrosine immunoprecipitates. The HPLC elution profile is identical to that of authentic deacylated PtdIns 3-monophosphate. These data were identical to those obtained with both anti-insulin receptor and anti-Ras immunoprecipitates (data not shown). The PtdIns kinase activity immunoprecipitated by anti-phosphotyrosine, anti-insulin receptor, and anti-Ras antibodies is, therefore, PtdIns 3-kinase activity. It is obvious from Fig. 1 that anti-phosphotyrosine immunoprecipitates from stimulated cells had more PtdIns 3-kinase activity than immunoprecipitates obtained with anti-insulin receptor from similarly stimulated cells. These data suggest that the PtdIns 3-kinase

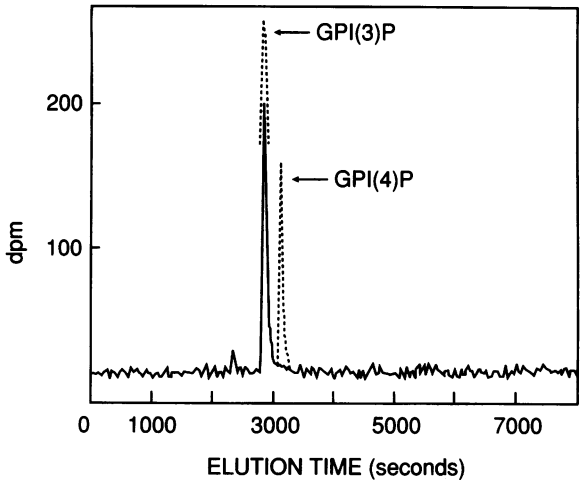

FIG. 2. Determination of the product of PtdIns 3-kinase activity by HPLC. The spots on the TLC plates (see Fig. 1) were scraped off, and lipids were extracted and deacylated to glycerophosphoinositols, which were then analyzed by HPLC together with known standards. The relative migration of glycerophosphoinositol 3-phosphate [GPI(3)P] and 4-phosphate [GPI(4)P] is represented by broken lines. These data are representative of at least five replicate experiments.

activity can associate with a protein(s) different from that of the receptors for insulin and IGF-I.

Immunoprecipitation of cell lysates with the anti-Ras monoclonal antibody Y13-259 showed an association between PtdIns 3-kinase and Ras proteins in unstimulated cells (Fig. 3, lane 1). However, stimulation of the cells with either insulin or IGF-I significantly increased the PtdIns 3-kinase activity associated with Ras proteins (lanes 2–4). In identical parallel experiments carried out in the absence of serum, under conditions where the transformed *ras* gene is turned off, no PtdIns 3-kinase activity was detected (data not shown). Furthermore, the specificity of the Y13-259 immunoprecipitation was also tested by addition of the antigen peptide to which the anti-Ras monoclonal antibody Y13-259 was originally raised. This antigen peptide will consequently compete with the 63–73 epitope of the $p21^{ras}$. Addition of this peptide (Y13-259:peptide, 1:10) blocked the immunoprecipitation of PtdIns 3-kinase in lysates of both unstimulated and stimulated cells (Fig. 4*A*). The specificity was documented by the finding that an irrelevant peptide [the carboxyl terminus of the GTPase-activating protein (GAP)] at the same concentration had no blocking effect (data not shown). In Y13-259 immunoprecipitates of unstimulated and stimulated cells, the presence of $p21^{ras}$ could be readily detected by the monoclonal antibody M90, which recognizes the 107–130 epitope of the Ha-Ras protein (Fig. 4*B*). When the immunoprecipitations were performed in the presence of the peptide that was used to raise the antibody Y13-259, $p21^{ras}$ could not be detected on the Western blots (Fig. 4*B*).

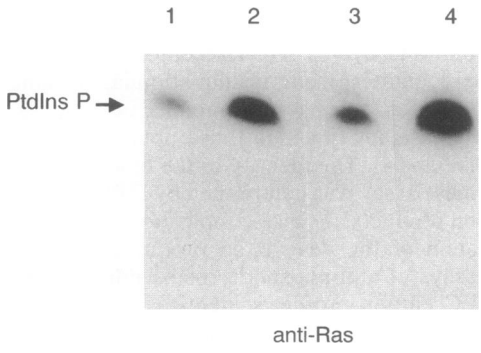

FIG. 3. Association of PtdIns 3-kinase activity with Ras proteins. The experimental conditions were as described in the legend to Fig. 1 except that the immunoprecipitates were obtained with the anti-Ras monoclonal antibody Y13-259 coupled to protein G-agarose (25 μl). Shown are PtdIns 3-kinase reaction products of immunoprecipitates from unstimulated cells (lane 1), from cells stimulated with insulin at 100 μg/ml (lane 2) or 100 ng/ml (lane 3), and from cells stimulated with IGF-I at 100 ng/ml (lane 4). These data are representative of three replicate experiments.

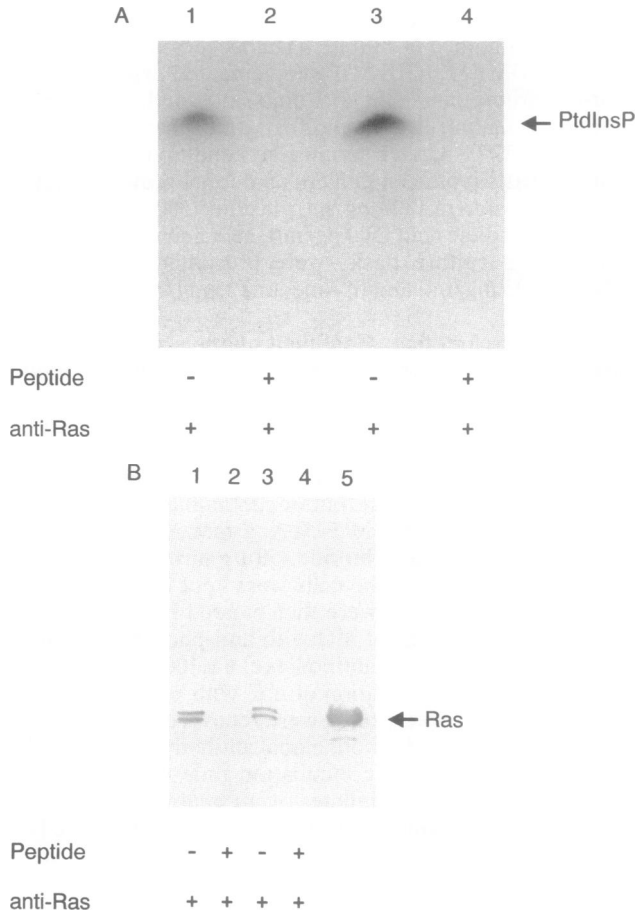

FIG. 4. Specificity of the PtdIns 3-kinase association with Ras proteins. (*A*) The peptide to which the anti-Ras monoclonal antibody Y13-259 was raised was used to block immunoprecipitation with the Y13-259 antibody (lanes 2 and 4). Shown are PtdIns 3-kinase reaction products of precipitates from unstimulated cells (lanes 1 and 2) and from cells stimulated with IGF-I at 100 ng/ml (lanes 3 and 4). (*B*) Western blot analysis of Ras protein in the same immunoprecipitates described in *A*. In addition, a purified recombinant Ha-Ras protein was used as a marker in lane 5. The blotted proteins were visualized with another anti-Ras monoclonal antibody (M90). These data are representative of three replicate experiments.

DISCUSSION

Earlier studies suggested a close biochemical link between the activity of $p21^{ras}$ and the actions of growth factor receptors and oncogenes possessing tyrosine kinase activity (33). Studies in fibroblasts and other cell types showed that ligand-induced activation of several kinds of tyrosine kinase receptors coincided with activation of $p21^{ras}$, indicating that the GTP-bound form of $p21^{ras}$ is part of the positive signal in cell proliferation (34). These findings also suggest that growth factors can directly or indirectly cause the release of GDP from $p21^{ras}$, thus enabling its conversion to an active, GTP-bound state. These results imply that within the mitogenic signaling pathway, $p21^{ras}$ resides downstream of several growth factor receptors. Beyond this central role of $p21^{ras}$ in

the mitogenic signaling pathway, little is known about the signaling properties of $p21^{ras}$.

We have demonstrated an association between $p21^{ras}$ and PtdIns 3-kinase activity. At least two groups of PtdIns kinases are distinguishable: (*i*) enzymes in the classical phosphatidylinositol pathway and (*ii*) enzymes in a distinct pathway (13, 17, 29). The classical enzymes are part of a signaling pathway that, upon agonist-induced activation of phospholipase C, results in hydrolysis of phosphatidylinositol 4,5-bisphosphate and the formation of two key second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (for review see ref. 35). PtdIns 3-kinase, which is associated with several growth factor receptors (platelet-derived growth factor, epidermal growth factor, colony-stimulating factor 1, insulin, and IGF-I receptors; refs. 3 and 12) and a nonreceptor family of tyrosine kinases ($pp60^{v\text{-}src}$, middle t-$pp60^{c\text{-}src}$, middle t-$pp62^{c\text{-}yes}$, $pp160^{gag\text{-}abl}$, $pp130^{gag\text{-}fps}$, and $pp59^{c\text{-}fyn}$; refs. 3, 14, and 15), is an important element of a fundamentally distinct signaling pathway. The phospholipids formed by PtdIns 3-kinase do not appear to function as substrates for phospholipase C, in contrast to the products formed by the classical PtdIns kinases (3, 35). These findings suggest that at least two parallel pathways of phosphatidylinositol metabolism occur in cellular signal transduction. Our present observations and the finding that overexpression of $p21^{ras}$ affect ligand-induced activation of phospholipase C (36) suggest the existence of cross-talk between these two parallel signaling pathways. Very little is known about how the products formed by PtdIns 3-kinase (PtdIns 3-phosphate and its relatives PtdIns 3,4-bisphosphate and PtdIns 3,4,5-trisphosphate) function in cell regulation. It seems reasonable to suggest that they are important for cell growth and the associated alterations of the cytoskeleton.

Cellular proliferation is closely associated with profound changes of the actin network of the cytoskeleton. The cellular state of actin is regulated by rapid and dynamic polymerization and depolymerization. Mainly from studies in cell-free systems, it has been proposed that PtdIns 4,5-bisphosphate, a metabolite of the classical phosphatidylinositol pathway, could serve as a key metabolite triggering dynamic alterations of the actin network (37, 38). However, studies on such a role for PtdIns 4,5-bisphosphate in various types of cells do not support this concept (39, 40). Recently, a close correlation between actin polymerization and the formation of PtdIns trisphosphate has been noted (8). In fact, PtdIns trisphosphate is the only inositolphospholipid that is not detectable before stimulation of actin polymerization that then increases upon stimulation (8). *ras*-transformed cells, and normal cells into which $p21^{ras}$ has been microinjected, exhibit gross morphological changes (6, 20, 41). These changes can be blocked by $p21^{ras}$ antibodies (7). Our finding of a close association between $p21^{ras}$ and PtdIns 3-kinase activity supports the idea that the 3-phosphate-containing phosphatidylinositol pathway is, perhaps via the formation of PtdIns 3,4,5-trisphosphate, involved in the modulation of the cytoskeleton.

The direct physical association of both nonreceptor and receptor families of tyrosine kinases with PtdIns 3-kinase is explained by the presence of SH2 Src-homology domains on the PtdIns 3-kinase. These SH2 domains have the capacity to recognize and bind autophosphorylated tyrosine residues of the tyrosine kinases. This type of direct physical association is not possible with $p21^{ras}$ because $p21^{ras}$ has neither SH2 domains nor phosphotyrosine residues. Although the mechanism is unknown, a direct association that is unrelated to SH2 domains and phosphotyrosine residues might exist. Alternatively, and perhaps more likely, our data could be explained by $p21^{ras}$ immunoprecipitating together with another protein that could both directly bind $p21^{ras}$ and be phosphorylated on its tyrosine residues. This could also explain our observation that ligand-induced activation of receptor tyrosine kinases resulted in increased PtdIns 3-kinase activity in the $p21^{ras}$ immunoprecipitates. Indeed, the Ras-specific GAP both binds to the effector site of $p21^{ras}$ and can be phosphorylated on its tyrosine residues (42, 43).

The *ras*-transformed liver epithelial cell line used in this study was transformed with an active form of *ras*. This mutated *ras* encodes a form of $p21^{ras}$ that does bind to GAP, although this complex formation does not result in an increased GTPase activity and inactivation of $p21^{ras}$. It has been suggested that GAP acts not only as a regulator of $p21^{ras}$ activity but also as a direct downstream target for $p21^{ras}$ in its mitogenic signaling pathway. The possibility that GAP could be a part of the $p21^{ras}$–PtdIns 3-kinase complex is compatible with such a role for GAP.

This close association between $p21^{ras}$ and PtdIns 3-kinase provides a biochemical basis for how Ras proteins affect the cytoskeleton, cell growth, and transformation. In addition, it can be speculated from these experiments that the Ras proteins and PtdIns 3-kinase activity are only two constituents of a larger complex, which may include the agonist receptor and other tyrosine-phosphorylated protein(s).

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