J. Biochem. 130, 73–78 (2001)

Association of Phosphatidylinositol 3-Kinase Composed of p110β-Catalytic and p85-Regulatory Subunits with the Small GTPase Rab5[1]

Hiroshi Kurosu and Toshiaki Katada[2]

Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

Received March 27, 2001; accepted April 16, 2001

A family of phosphatidylinositol 3-kinases (PI 3-kinase), comprising three major classes (I–III) in terms of substrate specificity and regulation, play important roles in a variety of cell functions. We previously reported that the class-I heterodimeric PI 3-kinase consisting of p110β-catalytic and p85-regulatory subunits is synergistically activated by two different types of membrane receptors, one possessing tyrosine kinase activity and the other activating trimeric G proteins. Here we report an additional unique feature of the p110β/p85 PI 3-kinase. The small GTPase Rab5 was identified as a binding protein for the p110β-catalytic subunit in a yeast two-hybrid screening system. The interaction appears to require at least two separated amino-acid sequences present specifically in the β isoform of p110 and the GTP-bound form of Rab5. The expressions of constitutively active and dominant negative mutants of Rab5 in THP-1 cells induce the stimulation and inhibition, respectively, of protein kinase B activity, which is dependent on the PI 3-kinase product phosphatidylinositol 3,4,5-triphosphate. These results suggest that there is a specific interaction between GTP-bound Rab5 and the p110β/p85 PI 3-kinase, leading to efficient coupling of the lipid kinase product to its downstream target, protein kinase B.

Key words: G protein, phosphatidylinositol 3-kinase, phosphatidylinositol 3,4,5-triphosphate, protein kinase B, Rab5.

Phosphatidylinositol 3-kinase (PI 3-kinase) is a key signaling enzyme implicated in the regulation of a broad array of biological responses including membrane receptor–mediated mitogenesis, oxidative burst, glucose uptake, and cytoskeletal organization (*1*). Three different classes (I–III) of PI 3-kinase have been described, which differ in their substrate specificity for PI(4,5)P_2, PI(4)P, and PI, respectively, as well as in their regulation. The activation of PI 3-kinase results in an increase in intracellular levels of *D*-3 phosphorylated phosphoinositides, and these products have been proposed to act as second messengers and/or adaptor molecules. For example, PI(3,4)P_2 can activate a certain type of protein kinase C and PKB/Akt, and PI(3,4,5)P_3 is capable of binding to the PH domain of guanine nucleotide–exchange factor for the small GTPase ARF (*2*).

In addition, PI 3-kinase appears to regulate receptor trafficking in the endocytic pathway in mammalian cells (*3*) and vesicle transport from the Golgi to the vacuole in yeast (*4*). The docking and fusion of early endosomes, which is regulated by the small GTPase Rab5 (*5*), requires the class-III PI 3-kinase product, PI(3)P (*6*). A FYVE-finger motif was identified as the PI(3)P-binding domain in the endosomal protein EEA1 and Rabenosyn-5, which are an effector of Rab5 and a core component of the docking and fusion machinery, respectively (*7*, *8*). Moreover, two distinct PI 3-kinases, hVPS34 (class III) and p110β/p85α (class I), have recently been shown to associate with GTP-bound Rab5 (*9*). Thus, a class-III PI 3-kinase producing PI(3)P and the active form of Rab5 appear cooperatively to recruit EEA1 to endosomes.

We previously reported that the class-I heterodimeric PI 3-kinase consisting of p110β-catalytic and p85-regulatory subunits has unique properties in terms of the regulation of its lipid kinase activity (*10*). The p110β/p85α subtype can be synergistically activated by a tyrosine-phosphorylated peptide and the βγ subunits of G proteins (Gβγ). Such synergistic activation was, however, not observed for other class-I PI 3-kinases, p110α/p85α and p110γ/p101, whose activities are stimulated solely by the phosphorylated peptide and Gβγ, respectively (*11*). Thus, the p110β/p85α isoform appears to be responsible for the synergistic activation of PI 3-kinase by two different types of membrane receptors, one possessing tyrosine kinase activity and the other activating trimeric GTP-binding proteins (*12*).

In the present study, we found an additional feature of the p110β/p85α PI 3-kinase by searching for molecules that interact with the p110β-catalytic subunit. We suggest that

[1] This work was supported in part by research grants from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF 96L00505) and the Scientific Research Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

[2] To whom correspondence should be addressed. E-mail: katada@mol.f.u-tokyo.ac.jp

Abbreviations: EEA1, early endosome antigen 1; Gβγ, the βγ subunits of GTP-binding proteins; GSK, glycogen synthase kinase; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PKB, protein kinase B; p85, 85-kDa regulatory subunit of PI 3-kinase; p110, 110-kDa catalytic subunit of PI 3-kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH2, Src homology 2.

the GTP-bound form of Rab5 is capable of binding to the p110β isoform for efficient coupling of the lipid kinase product to its downstream target, protein kinase B.

MATERIALS AND METHODS

Materials—Polyclonal antibodies against human p110β, Rab5b, and GST were purchased from Santa Cruz Biotechnology. All other reagents were from commercial sources and of analytical grade.

DNA Constructs—The human Rab5b cDNA was obtained by reverse transcriptase polymerase chain reaction with THP-1 cell mRNAs and subcloned into pBlueScript(SK–) vector at the *Bam*HI site. The substitution mutations (S29V, S34N, and Q79L) of Rab5b were created by oligonucleotide-directed mutagenesis using the Künkel method. To construct epitope-tagged expression plasmids, we first inserted a DNA fragment encoding a start methionine followed by the Flag epitope (MDYKDDDDK) between the *Eco*RI and *Bam*HI sites of pCMV5 to produce the pCMV5-Flag vector. To express the Flag-tagged Rab5b and its mutants, fragments containing the Rab5b and its mutant coding regions were inserted at the *Bam*HI site of the pCMV5-Flag vector. pCMV6-Myc-PKB was a gift from Dr. T.F. Flanke, Columbia University.

Screening of Yeast Two-Hybrid Library—Yeast two-hybrid screening was performed according to the standard method of Fields and Song (*13*) utilizing the Matchmaker Two-hybrid System (Clontech). A rat liver cDNA library in the pGAD10 vector was screened using p110β linked to the internal region of the two SH2 domains of p85α (p85[iSH2]-p110β; see Fig. 1B), which was inserted into pGBT9, as bait. The library and bait were cotransformed into the yeast two-hybrid strain HF7c using a standard method with lithium acetate-polyethylene glycol. A total of 3.2×10^6 individual recombinant clones were screened. Positive clones grown on His^- medium were selected for activation of the HIS3 reporter gene, and the β-galactosidase assay was performed as described previously (*14*, *15*). Series of deletion mutants of p110β and p110α were constructed using a PCR-based strategy. PCR products encoding amino- and carboxyl-terminal truncations of p110β and p110α were subcloned into pGBT9 in-frame with the GAL4 DNA-binding domain. The two-hybrid vectors thus obtained were cotransformed into the yeast host-strain SFY526. Transformed colonies were replated on tryptophan- and leucine-deficient medium.

Production of Recombinant Proteins and In Vitro–Binding Assay—Recombinant baculoviruses for the expression of human Rab5b and p110β were constructed according to the standard methods described previously (*16*). The recombinant baculovirus for GST-p85α was a generous gift from Dr. Y. Fukui, University of Tokyo. Rab5b was purified from the membrane fraction of baculovirus-infected Sf9 cells as described previously (*17*). GST-fused p85α/p110β and GST alone were purified from the cytoplasmic fraction of baculovirus-infected Sf9 cells and pGEX2T-transformed *Escherichia coli* HB101 cells, respectively, using Glutathione Sepharose 4B (Amersham Pharmacia Biotech).

For *in vitro*–binding assay, GDP- or GTPγS-bound Rab5b (50 pmol) was incubated at 25°C for 60 min with the purified GST-fused p85α/p110β or GST alone (10 pmol) in 0.25 ml of 50 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, 5 mM EDTA, 0.5 mM dithiothreitol, and 100 mM NaCl, and mixed with Glutathione Sepharose resin (20 μl). The resin was washed four times with 500 μl of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM $MgCl_2$, 2 mM EDTA, and 0.2% (w/v) Nonidet P-40 (buffer A). Proteins were eluted from the resin with 30 μl of buffer A containing 10 mM glutathione. After centrifugation, 24 μl of the supernatant was mixed with 8 μl of 4 × SDS–polyacrylamide sample buffer and boiled for 5 min, followed by SDS-PAGE (12% of acrylamide). Immunoblots were performed with rabbit polyclonal antibodies raised against the human p110β, Rab5b, and GST.

DNA Transfection into THP-1 Cells and Assay of PKB Activity—For the production of a GST-GSK3β peptide corresponding to the sequence of GSK3β (GRPRTSSFAEG) containing a phosphorylation site (Ser9) by PKB, a PCR product encoding the corresponding sequence was subcloned into pGEX4T1 in-frame with the GST. The expressed protein was purified according to the manufacturer's instructions and resuspended in 25 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, and 150 mM NaCl.

THP-1 cells expressing insulin receptors were maintained in RPMI1640 containing 10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 0.6 mg/ml of glutamine at 37°C in 95% air and 5% CO_2. For electroporation, exponentially growing THP-1 cells (1×10^7 cells) were washed twice and resuspended in 0.2 ml of Opti-MEM. The cell suspension was mixed with 2 μg of pCMV6 containing a cDNA encoding Myc-tagged PKB and 8 μg of pCMV5 containing the cDNA encoding Flag-tagged Rab5b (or its mutants) and transferred to a cuvette (0.4-cm gap; BioRad). The cells, after being electroporated (230 V, 960 μF), were diluted into 20 ml of RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 0.6 mg/ml of glutamine, and cultured at 37°C for 24 h.

The transfected THP-1 cells were suspended at a density of 1×10^7 cells/ml in 10 mM Hepes-NaOH (pH 7.4), 136 mM NaCl, 4.9 mM KCl, 5.5 mM glucose, 1 mM $CaCl_2$, and 0.2% (w/v) bovine serum albumin. Aliquots (150 μl) of cell suspensions that had been incubated at 37°C for 10 min in the presence or absence of 0.1 μM wortmannin were further incubated with 0.1 μM insulin for 1.5 min, and the reaction was terminated according to the method described previously (*18*). Cell extracts precleared with Sepharose 4B resin were immunoprecipitated with anti-Myc antibody 9E10 (1 μg) and anti-mouse IgG-agarose resin. After incubation, the resin was washed three times with 500 μl of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% (w/v) Nonidet P-40, and three more times with 20 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ (buffer B). The resin was resuspended in 15 μl of buffer B containing 0.05 μCi/μl of [γ-^{32}P]ATP, 0.2 mM ATP, and 0.2 μg/ml of GST-GSK3β peptide, and further incubated at 30°C for 10 min. The reaction was terminated by the addition of 5 μl of 4× SDS–polyacrylamide sample buffer and boiled for 5 min, followed by SDS-PAGE (13% of acrylamide). The incorporation of $^{32}P_i$ into the GST-GSK3β peptide was visualized with a bioimaging analyzer. The amounts of PKB expressed in the transfected cells were monitored by means of SDS-PAGE and immunoblot with anti-Myc antibody.

RESULTS AND DISCUSSION

Identification of the Small GTPase Rab5b as a Binding Protein for p110β/p85-Heterodimeric PI 3-Kinase—In a previous study (*10*), we reported that a class-I heterodimeric PI 3-kinase consisting of p110β-catalytic and p85-regulatory subunits has unique properties in terms of lipid kinase regulation. The PI 3-kinase composed of the p110β isoform could be synergistically activated by a tyrosine-phosphorylated peptide and Gβγ. To investigate further the properties of this PI 3-kinase isoform, we searched for its binding proteins in a yeast two-hybrid screening system. A rat liver cDNA library was screened with p110β linked to the internal region of the two SH2 domains of p85α (p85-[iSH2]-p110β; Fig. 1). The screening of 3.2×10^6 independent clones resulted in the identification of 6 positive clones, all of which encoded sequences of the small GTPase Rab5b.

Mapping of Rab5-Binding Sequences on the p110β-Catalytic Subunit of PI 3-Kinase—In order to identify a sequence(s) required for Rab5b binding, the varying lengths of p110β were further analyzed in the two-hybrid system. As shown in Fig. 1B, we observed strong β-galactosidase activity with the full-length sequence (#1–1070) of p110β and Rab5b. Experiments with the carboxyl-terminal deletion mutants of p110β revealed that the sequence #658–759 is required for Rab5b binding. Interestingly, this Rab5b-binding sequence, which is located near a conserved lipid-kinase domain (see Fig. 1A), has the lowest homology to the corresponding region of p110α. Amino-terminal deletion experiments showed that another sequence (#136–270) of p110β is also required for Rab5b binding. The second Rab5b-binding sequence corresponds to a putative H-Ras–binding domain previously reported in p110α (*19*). In contrast, the full-length sequence (#1–1068) of another p110 isoform, p110α, or its partial sequence (#127–760) containing the H-Ras–binding domain and a region corresponding to the p110β-specific Rab5b-binding sequence, failed to associate with the small GTPase. Thus, at least two separated sequences on p110β, which are different from the conserved p85-binding or lipid kinase domains, appear to be responsible for Rab5b binding. Recently, the X-ray crystallographic structure of the p110γ PI 3-kinase has been reported (*20, 21*). On the assumption that its tertiary structure is similar to that of the p110β isoform, the two separated sequences interacting with Rab5 are located closely and oriented toward the same face. Moreover, this predicted Rab5-interacting region on p110β appears to be related to the putative Ras-binding domain on p110γ (*21*).

Interaction of p110β with the Active Form of Rab5b—The specificity of the interaction between p110β and Rab5b was further investigated in a yeast two-hybrid system (Fig. 1C). In addition to the wild type Rab5b (Rab5b/WT), constitu-

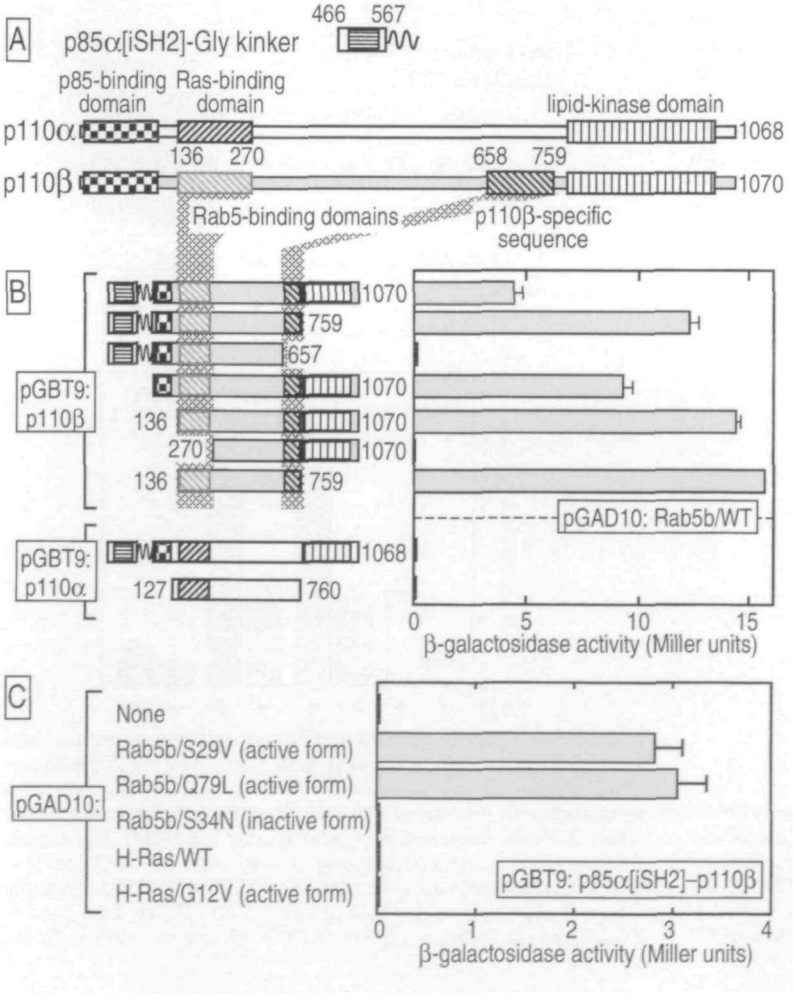

Fig. 1. **Schematic representation of the primary structure of PI 3-kinase/p110-catalytic subunits and the association of p110β with Rab5b.** A, the p110α- and p110β-catalytic subunits contain amino-terminal p85-binding and carboxyl-terminal lipid-kinase domains. A Ras-binding domain (amino acid sequence #136–276) is also present in the p110α-isoform (Ref. *19*). B, series of amino- and carboxyl-terminal deletion mutants of p110β/p110α and Rab5b/WT were inserted into pGBT9 and pGAD10 vectors, respectively, and transformed into SFY526 cells. β-Galactosidase activity was assayed as described in "MATERIALS AND METHODS." C, various forms of Rab5b (and H-Ras) and p110β linked to the internal region (#466–567) of the two SH2 domains of p85α (p85-[iSH2]-p110β) were inserted into pGAD10 and pGBT9 vectors, respectively, and the β-galactosidase activity of the cell lysates was measured. Values shown are the means ± SD of triplicate determinations.

tively active forms of Rab5b, Rab5b/S29V and Rab5b/Q79L, associated with p85[iSH2]-p110β. However, a dominant negative form of the small GTPase, Rab5b/S34N, failed to interact with p110β. Although H-Ras has been reported to interact with the p110α-isoform of PI 3-kinase in a yeast two-hybrid system (*22*), neither the wild type (H-Ras/WT) nor active form (H-Ras/G12V) associates with p110β. Thus, Rab5b appears to interact selectively with the p110β isoform of PI 3-kinase.

GTPγS-Bound Form of Rab5b Required for In Vitro Binding to p110β/p85 PI 3-Kinase—The direct interaction between p110β and Rab5b was further confirmed by an *in vitro*–binding assay with recombinant proteins purified from baculovirus-infected Sf9 cells. GST-fused p85α/p110β or GST alone was incubated with the GDP- or GTPγS-bound form of Rab5b, and glutathione-Sepharose was added to the reaction mixture. Proteins that associated with the Sepharose resin were separated by SDS-PAGE and immunoblotted with anti-Rab5b, p110β, and GST antibodies (Fig. 2). In accordance with the results shown in Fig. 1C, GTPγS-bound Rab5b, but not the GDP-bound form, was capable of binding to p110β/p85. This interaction, however, was not observed upon incubation of Rab5b with another isoform, p110α/p85 (data not shown).

PI 3-Kinase–Dependent Activation of PKB Is Affected by the Active and Inactive Forms of Rab5b—To investigate the physiological significance of the association between GTP-bound Rab5b and p110β, we further studied the effect of the small GTPase on PI 3-kinase–dependent cell responses (Fig. 3). For this analysis, the THP-1 cell line was used because it contains p110β/p85 PI 3-kinase and insulin receptors (*12*). Various forms of the small GTPase and Myc-tagged PKB, which is a downstream target enzyme of the PI 3-kinase product PI(3,4,5)P_3, were co-expressed in THP-1 cells, and the cells were incubated with or without insulin in the presence or absence of a PI 3-kinase inhibitor, wortmannin. Myc-tagged PKB was immunoprecipitated from the cell lysate (Fig. 3A), and the protein kinase activity was assayed with [γ-^{32}P]ATP and GSK3β peptide as substrates. As shown in Fig. 3, B and C, non-stimulated and insulin-stimulated PKB activities were markedly enhanced by the constitutively active Rab5b/Q79L and Rab5b/S29V. In contrast, the dominant negative Rab5b/S34N inhibited the insulin-stimulated PKB activity. As expected, wortmannin completely inhibited PKB activity in all transfected cells (Fig. 3B), indicating that PI 3-kinase is certainly involved in the stimulation of PKB under the present conditions. Since the insulin-stimulated PKB activity observed in THP-1 cells is markedly potentiated by stimulation of G protein-coupled chemotactic receptors (T. Okada, *et al.*, unpublished observation), it is very likely that the p110β/p85-subtype PI 3-kinase is responsible for the kinase activation in this cell line. Thus, the activated Rab5b appears to exert its influence on p110β/p85 PI 3-kinase–dependent PKB stimulation in intact-cell levels. It will be very interesting to investigate that how activated Rab5b exerts its influence on other wortmannin-sensitive signal transduction pathways, including the activation of extracellular signal-regulated kinase 1/2 and the synergistic stimulation of PKB activity by Tyr kinase-linked and G protein–coupled receptors. These important issues are currently under investigation in our laboratory.

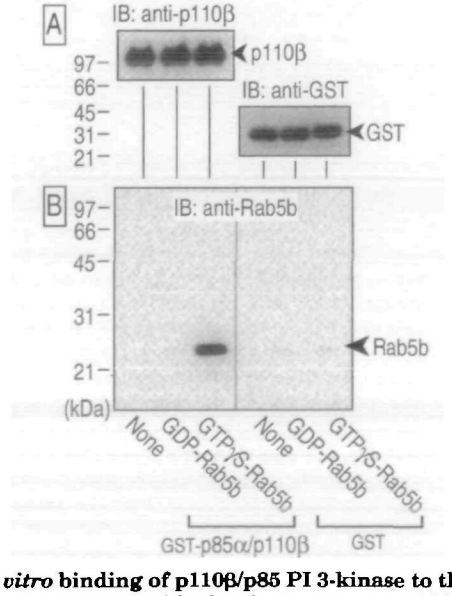

Fig. 2. ***In vitro* binding of p110β/p85 PI 3-kinase to the GTPγS-bound form of Rab5b.** GST-fused p85α/p110β or GST alone was incubated with the GDP- or GTPγS-bound form of Rab5b. Proteins bound to glutathione-Sepharose resin were separated by SDS-PAGE and immunoblotted with anti-p110β, anti-GST (Panel A), and anti-Rab5b (Panel B) antibodies as described in "MATERIALS AND METHODS."

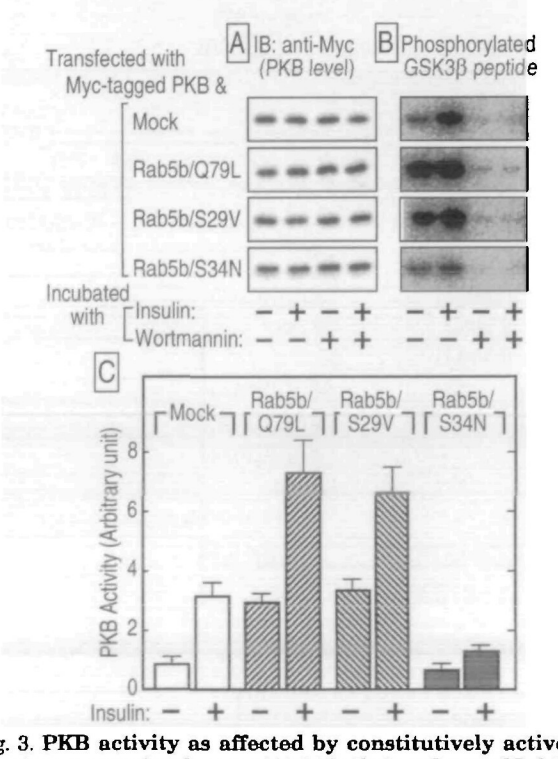

Fig. 3. **PKB activity as affected by constitutively active and dominant negative forms of Rab5b.** Various forms of Rab5b and Myc-tagged PKB were expressed in THP-1 cells, and the cells were incubated with or without insulin (0.1 μM) in the presence or absence of PI 3-kinase inhibitor, wortmannin (100 nM). Myc-tagged PKB was immunoprecipitated from the cell lysate (Panel A), and the kinase activity was assayed with [γ-^{32}P]ATP and GSK3β peptide (Panel B) as described in "MATERIALS AND METHODS." Data in Panel C are the means ± SD of triplicate determinations as shown in Panel B.

A Possible Role for the Interaction between Activated Rab5b and p110β/p85 in PI 3-Kinase–Dependent Cellular Signaling—We investigated the direct effect of GTP-bound Rab5b on the PI 3-kinase activity of the purified p110β/p85 enzyme. There was, however, no marked stimulation of lipid kinase activity upon the addition of Rab5b under our *in vitro*–assay conditions (data not shown). This is in sharp contrast to the findings observed in intact THP-1 cells (Fig. 3). These results suggest that the interaction between GTP-bound Rab5b and p110β/p85 PI 3-kinase may not necessarily be required for the stimulation of lipid kinase activity *per se*. A more likely role of Rab5b may be the translocation of the PI 3-kinase to confine the production of $PI(3,4,5)P_3$ to a restricted membrane region, where the lipid product can associate efficiently with its target molecules, such as PKB. This mechanism may be comparable to the interaction between hVPS34 (class III PI 3-kinase) and Rab5 for the recruitment of EEA1 to endosomes (*6*, *9*).

In relation to this, recent studies have revealed that class-I heterodimeric PI3-kinases play an important role in receptor-mediated endocytosis. Phosphoinositides possessing an inositol phosphate at the D-3 position, which increases the affinity of the adaptor protein-2 complex for peptides containing a tyrosine-based endocytic motif, are required for the formation of clathrin-coated pits (*23*, *24*). Moreover, PDGF-receptor mutants that lack a binding site for heterodimeric PI 3-kinase fail to sort in juxtanuclear vesicular structures after activation with PDGF (*25*, *26*). The inhibition of PI 3-kinase activity by wortmannin has similar effects (*27*), and p110α/p85 PI 3-kinase appear not to be involved in the down-regulation of PDGF receptors (*28*). Taken together with the present results, it is tempting to speculate that the p110β/p85-isoform of PI 3-kinase may be responsible for PDGF-receptor endocytosis.

In addition to the involvement of Rab5 in the docking and fusion processes of endocytic vesicles, it has been reported that the small GTPase that associates with its guanine-nucleotide dissociation inhibitor (GDI) is required for the earliest stages of the endocytic pathway, the formation of clathrin-coated pits (*29*). Moreover, there is a report showing that fluid-phase endocytosis measured by the uptake of horseradish peroxidase is stimulated in a cooperative manner by Rab5 and PKB, the downstream target of heterodimeric PI 3-kinase (*30*, *31*). It thus appears that several steps in the endocytic pathway are generally regulated in a cooperative manner by the products of PI 3-kinase and the small GTPase Rab5.

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