Synergistic activation of PtdIns 3-kinase by tyrosine-phosphorylated peptide and $\beta\gamma$-subunits of GTP-binding proteins

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Stimulation of differentiated THP-1 cells by insulin led to rapid accumulation of PtdIns(3,4,5)P_3, a product of PtdIns 3-kinase. Stimulation of the GTP-binding-protein-linked receptor by *N*-formylmethionyl-leucyl-phenylalanine (fMLP) also induced the accumulation of PtdIns(3,4,5)P_3 in the cells. The effect of insulin was, while that of fMLP was not, accompanied by increased PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitate. The combination of insulin and fMLP induced more PtdIns(3,4,5)P_3 production than the sum of the individual effects. The insulin-induced recruitment of PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitate was unaffected by the combined treatment with fMLP. To investigate the mechanism underlying the synergistic accumulation of PtdIns(3,4,5)P_3, we separated the cytosolic proteins of THP-1 cells on a Mono Q column. PtdIns 3-kinase activities were eluted in two peaks, and one of the peaks markedly increased on the addition of $\beta\gamma$-subunits of GTP-binding proteins (G$\beta\gamma$). The other peak was affected only slightly by G$\beta\gamma$, but was synergistically increased by G$\beta\gamma$ and a tyrosine-phosphorylated peptide which was synthesized according to the amino acid sequence of insulin receptor substrate-1. The activity in the latter fraction was completely immunoprecipitated by an antibody against the regulatory subunit of PtdIns 3-kinase (p85). These results suggest that the conventional PtdIns 3-kinase (p85/p110), which has been implicated in insulin-induced cellular events, or a closely related isoenzyme is controlled by a combination of a tyrosine-phosphorylated protein and a GTP-binding protein in intact cells.

INTRODUCTION

PtdIns 3-kinase catalyses the phosphorylation of inositol phospholipids at the 3-position of the inositol ring [1]. This enzyme is activated following agonist occupation of a diverse range of cell-surface receptors, including growth-factor receptors, cytokine receptors and GTP-binding-protein-coupled receptors [2]. Though the biological activity of the products of this reaction, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, is currently unclear, these lipids are thought to act as novel second messengers, since they are poor substrates for phospholipase C [3]. It was observed that these lipids activate some protein kinase C isoforms *in vitro* [4,5]. In recent studies with the specific inhibitor of PtdIns 3-kinase, wortmannin, we and other investigators found that this kinase plays important roles in the regulation of various cell functions (for review see [6]): *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-induced respiratory burst in neutrophils [7,8], insulin-induced stimulation of glucose transport [9,10], Fcγ receptor-mediated phagocytosis of U937 cells [11], platelet-derived growth factor- and insulin-induced actin rearrangement [12,13], histamine secretion by basophilic leukaemia cells [14] and nerve growth factor-stimulated elongation of neurites [15].

PtdIns 3-kinase has been identified as a heterodimer of an 85 kDa adaptor subunit (p85) [16–18] and a 110 kDa catalytic subunit (p110) [19]. The p85 regulatory subunit possesses several regions exhibiting homology to those of other proteins involved in signal transduction cascades: one Src homology (SH) 3 domain, two SH2 domains and a region homologous to the GTPase-activating proteins for the family of small GTP-binding proteins (break-point cluster region domain) [2]. Ligand binding to growth-factor receptor tyrosine kinases induces the phosphorylation on a specific tyrosine residue located in the YXXM motif, which has been found in several proteins such as growth-factor receptors and insulin-receptor substrate-1 (IRS-1). Phosphorylation at these sites creates the specific binding sites for SH2 domains in p85. Direct activation of PtdIns 3-kinase through the binding of a tyrosine-phosphorylated peptide has also been demonstrated [20,21].

A chemotactic tripeptide, fMLP, is known to induce marked accumulation of PtdIns(3,4,5)P_3 in neutrophils through the activation of pertussis-toxin-sensitive GTP-binding proteins [7,22]. This response is thought to be independent of tyrosine phosphorylation, because the effect of fMLP is not accompanied by increased recovery of PtdIns 3-kinase activity in anti-phosphotyrosine antibody-directed immunoprecipitates, and is resistant to tyrosine kinase inhibitors [23]. Though the mechanism by which GTP-binding proteins activate PtdIns 3-kinase is not fully understood, the $\beta\gamma$-subunits of GTP-binding proteins (G$\beta\gamma$) were reported recently to activate the unidentified isoenzyme of PtdIns 3-kinase which is immunologically and chromatographically distinct from the well-characterized p85/p110 enzyme [24]. The occurrence of a novel catalytic subunit of PtdIns 3-kinase (p110γ) that is activated by G$\beta\gamma$ has also been reported [25,26]. These facts suggest that the fMLP-induced accumulation of PtdIns(3,4,5)P_3 is mediated by G$\beta\gamma$-sensitive PtdIns 3-kinases which are different from those stimulated by insulin. However, direct evidence that two distinct types of PtdIns 3-kinase function in intact cell systems has not been provided.

In the present study, we examined the effects of insulin and fMLP on PtdIns(3,4,5)P_3 production by THP-1 cells. We found that these agonists were able to synergistically induce PtdIns(3,4,5)P_3 production by the cells. We also found that PtdIns 3-kinase activity partially purified from THP-1 cells was synergistically augmented by a tyrosine-phosphorylated peptide

Abbreviations used: fMLP, *N*-formylmethionyl-leucyl-phenylalanine; SH, Src homology; Gα, α-subunit of GTP-binding protein; G$\beta\gamma$, $\beta\gamma$-subunits of GTP-binding protein; p85, 85 kDa regulatory subunit of PtdIns 3-kinase; p110, 110 kDa catalytic subunit of PtdIns 3-kinase; IRS-1, insulin receptor substrate-1; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; DTT, dithiothreitol; PVDF, poly(vinylidene difluoride).

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and G$\beta\gamma$. We discuss the possibility that this activity is closely related to the well-characterized p85/p110 form of PtdIns 3-kinase.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP, [^{32}P]P_i and ^{125}I-labelled Protein A were purchased from DuPont/NEN. Protein A–Sepharose and anti-mouse IgG–agarose were from Pharmacia Biotech and American Qualex respectively. The tyrosine-phosphorylated peptide used in this study, NGDY(PO_4)MPMSPKS, was based on the sequence of IRS-1 [21] and was prepared by Dr. K. Nakajima of the Peptide Institute, Inc. (Osaka, Japan). A rabbit polyclonal anti-p85 antibody (KR1) was raised against the glutathione S-transferase–bovine p85 fusion protein. A monoclonal anti-p85 antibody (CB2) was kindly donated by Dr. Y. Fukui (Faculty of Agriculture, University of Tokyo, Tokyo, Japan). A monoclonal anti-phosphotyrosine antibody (PY20) was purchased from Leinco Technologies, Inc. All other reagents were from commercial sources and were of analytical grade.

Cell culture

THP-1 cells were maintained in RPMI 1640 medium at 37 °C under a 5% CO_2 atmosphere. The cells were cultured with the addition of 0.5 mM dibutyryl cAMP at 37 °C for 3 days before use.

Preparation of GTP-binding protein subunits

G$\beta\gamma$-subunits were purified from a particulate fraction of bovine brain, and stored at a concentration of 20 μM in 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/0.1 mM EDTA/0.5% CHAPS [27]. $G_i2\alpha$ was purified from *Escherichia coli* cells producing recombinant rat $G_i2\alpha$, and stored at a concentration of 20 μM in 50 mM Hepes (pH 7.4)/1 mM EDTA/1 mM dithiothreitol (DTT).

Radiolabelling of phospholipids in intact cells

PtdIns(3,4,5)P_3 production by cells was measured essentially according to the method described by Traynor-Kaplan et al. [22]. THP-1 cells were suspended at a density of 5×10^7 cells/ml in labelling medium consisting of 10 mM Hepes/NaOH (pH 7.4), 136 mM NaCl, 4.9 mM KCl and 5.5 mM glucose. After incubation at 37 °C for 30 min with the addition of [^{32}P]P_i (150 μCi/ml), the cells were washed twice and then resuspended at 10^7 cells/ml in the same medium supplemented with 1 mM $CaCl_2$. Aliquots (400 μl) of the cells were incubated at 37 °C for 5 min, and then for a further 30 s with the addition of 0.1 μM fMLP and/or 0.1 μM insulin. The cells were then quenched by the addition of 1.55 ml of chloroform/methanol/8% $HClO_4$ (50:100:5), followed by vigorous stirring. The mixture was mixed with 0.5 ml of chloroform and 0.5 ml of 8% $HClO_4$ to separate the organic phase, which was then washed with chloroform-saturated 1% $HClO_4$ before being dried. The lipids were dissolved in 20 μl of chloroform/methanol (95:5) before being spotted onto a TLC plate (Silica Gel 60, Merck), which had been impregnated with potassium oxalate by development with a solvent system comprising 1.2% potassium oxalate in methanol/water (2:3) and pre-activated by heating at 110 °C for 20 min before spotting. The plate, after being developed in chloroform/acetone/methanol/acetic acid/water (70:20:50:20:20), was dried, and radioactivity was visualized with a Fuji BAS2000 bioimaging analyser.

Determination of PtdIns 3-kinase activity

The assays were conducted in a final volume of 50 μl of an assay mixture comprising 20 mM Tris/HCl (pH 7.4), 0.5 mM EGTA, 5 mM $MgCl_2$, 1 mM phosphatidylethanolamine (PtdEtn), 0.1 mM PtdInsP_2 and 0.1 mM (1 μCi) [γ-^{32}P]ATP. In the experiments shown in Figures 2 and 6, 0.2 mM phosphatidylserine (PtdSer) and 0.2 mM PtdIns were used instead of 1 mM PtdEtn and 0.1 mM PtdInsP_2. In the experiments performed to examine the effects of the subunits of GTP-binding proteins, G$\beta\gamma$ and/or $G_i\alpha$ were added to the lipid mixtures, followed by incubation on ice for 5 min before the addition of the enzyme fractions. When the effect of $G_i\alpha$ was examined, 10 μM GDP was also included in the assay mixture. The kinase reaction was allowed to proceed at 30 °C for 6 min and was stopped by the addition of 20 μl of 8% $HClO_4$ and 0.45 ml of chloroform/methanol (1:2). Phospholipids were then extracted and separated on TLC plates as described above.

Determination of PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitate

Aliquots (400 μl) of cells were incubated at 37 °C for 5 min and then for a further 30 s with the addition of 100 μl of 0.1 μM fMLP and/or 0.1 μM insulin. The cells were then quenched by the addition of 500 μl of a solubilization buffer (40 mM Hepes/NaOH, pH 7.4/1% Nonidet P-40/100 mM NaF/10 mM $Na_4P_2O_7$/1 mM Na_3VO_4/5 mM EDTA/1 mM PMSF/10 μg/ml leupeptin/2 μg/ml aprotinin) and then centrifuged at 10000 ***g*** for 10 min. The supernatant was incubated first with an anti-phosphotyrosine antibody (PY20) for 1 h and then with anti-mouse IgG–agarose beads for 1 h. The precipitate was washed vigorously and assayed for PtdIns 3-kinase activity.

Separation of PtdIns 3-kinase from THP-1 cells

THP-1 cells (5×10^7 cells) were disrupted with a Branson Sonifier in 10 ml of a buffer consisting of 20 mM Hepes (pH 7.6), 2 mM EGTA, 0.2 mM EDTA, 3 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 10 μg/ml leupeptin and 2 μg/ml aprotinin. The homogenate was centrifuged at 125000 ***g*** for 1 h. The supernatant was loaded onto a Mono Q HR5/5 column (Pharmacia LKB) at 0.5 ml/min. The column was washed with 10 ml of buffer A containing 20 mM Tris/HCl (pH 7.4), 1 mM EGTA, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin and 2 μg/ml aprotinin, and then eluted (0.5 ml/min) with an NaCl gradient: 0 min, 0% B; 0.5 min, 5% B; 24 min, 25% B; 45 min, 100% B (buffer B = buffer A with 1 M NaCl).

Immunoblotting

A sample of the column eluate was mixed with an equal volume of 3 × SDS/PAGE sample buffer, boiled, and then subjected to standard SDS/PAGE (10% gel). Proteins were electrotransferred to poly(vinylidene difluoride) (PVDF) filters (Bio-Rad). After blocking in TBS (20 mM Tris/HCl, pH 8.0/100 mM NaCl) containing 5% fatty acid-free BSA, the blot was incubated with an anti-p85 antibody, washed, and then incubated with 0.05 μCi/ml ^{125}I-labelled Protein A. Following repeated washings, the radioactivity associated with anti-p85 antibody was located using a Fuji BAS2000 bioimaging analyser.

Immunoprecipitation of PtdIns 3-kinase

An aliquot (50 μl) of the fraction eluted from the Mono Q column was incubated at 4 °C for 1 h with Protein A–Sepharose,

which had been coupled to a polyclonal anti-p85 antibody (KR1). Another aliquot was incubated with anti-mouse IgG–agarose coupled to a monoclonal anti-p85 antibody (CB2). After the incubation, PtdIns 3-kinase activity in the supernatant was determined as described above. In some cases, peptides in the supernatant were separated by SDS/PAGE and probed with the anti-p85 antibody.

RESULTS

Synergistic effect of fMLP and insulin on PtdIns(3,4,5)P_3 production by THP-1 cells

To investigate the roles of PtdIns 3-kinase isoenzymes in intact cells, we used differentiated THP-1 cells, in which both insulin and fMLP elicit rapid accumulation of PtdIns(3,4,5)P_3. Figure 1(A) shows an autoradiogram of a TLC plate on which ^{32}P-labelled phospholipids from THP-1 cells were separated. When the cells were stimulated with either 0.1 μM insulin or 0.1 μM fMLP for 30 s, increased incorporation of ^{32}P into the PtdIns(3,4,5)P_3 fraction was observed. As we showed previously [7,9], the effects of insulin and fMLP on [^{32}P]PtdIns(3,4,5)P_3 accumulation were abolished by treatment with wortmannin, a potent and specific inhibitor of PtdIns 3-kinase (results not shown). Pertussis toxin blocked the ability of fMLP to stimulate PtdIns(3,4,5)P_3 accumulation, but had no effect on the response elicited by insulin. These results clearly suggest that the fMLP receptor is coupled to PtdIns(3,4,5)P_3 accumulation via a pertussis toxin-sensitive G-protein, while the insulin receptor is not. Interestingly, simultaneous treatment of THP-1 cells with insulin and fMLP resulted in PtdIns(3,4,5)P_3 production that was strongly enhanced as compared with the sum of the individual responses (Figure 1B).

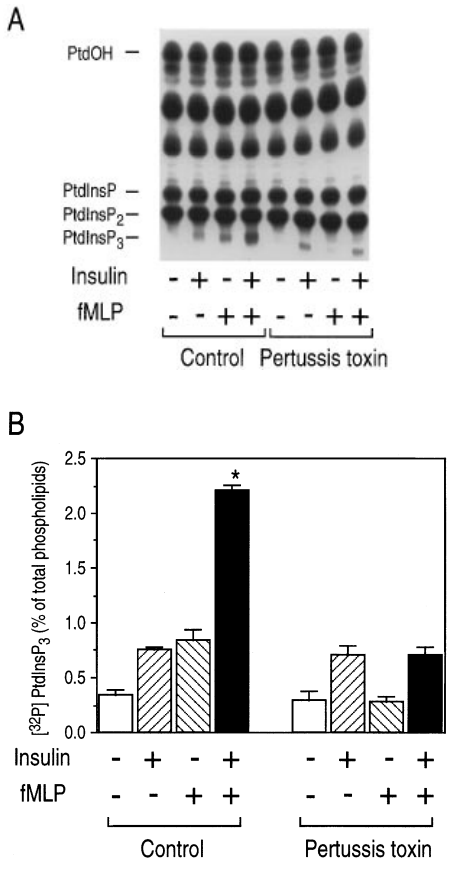

Figure 1 Synergistic effect of insulin and fMLP on the incorporation of ^{32}P into PtdIns(3,4,5)P_3 in differentiated THP-1 cells

THP-1 cells were incubated with or without 100 ng/ml pertussis toxin for 4 h, labelled with [^{32}P]P_i and then incubated in the absence or presence of 0.1 μM insulin or 0.1 μM fMLP at 37 °C for 30 s. Phospholipids were extracted and separated on an oxalate-impregnated TLC plate, as described in the Materials and methods section. (**A**) Autoradiogram of the TLC plate. PtdOH, phosphatidic acid. (**B**) Radioactivity in the PtdIns(3,4,5)P_3 fraction in (**A**) was determined. The data are presented as means ± S.E.M. of triplicate determinations. * The synergism between fMLP and insulin is significant ($P < 0.005$).

Table 1 Failure of fMLP to augment the insulin-induced increase in the PtdIns 3-kinase activity of anti-phosphotyrosine immunoprecipitates

THP-1 cells were incubated in the absence or presence of 0.1 μM insulin or 0.1 μM fMLP at 37 °C for 30 s. After solubilization of the cells, tyrosine-phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine antibody. PtdIns 3-kinase activity associated with the immunoprecipitate was determined as the ability to phosphorylate PtdInsP_2. The results are presented as means ± S.E.M. of triplicate determinations.

Treatment	PtdIns 3-kinase activity (pmol/10 min)
Control	0.193 ± 0.006
Insulin	0.696 ± 0.026
fMLP	0.119 ± 0.015
Insulin + fMLP	0.404 ± 0.035

In insulin-treated cells, the p85 subunit of PtdIns 3-kinase was shown to associate with tyrosine-phosphorylated IRS-1 and thus to be immunoprecipitated by an anti-phosphotyrosine antibody [28]. Insulin actually augmented the PtdIns 3-kinase activity associated with phosphotyrosyl proteins in THP-1 cells (Table 1). In contrast, fMLP did not increase the activity, indicating that fMLP activated PtdIns 3-kinase through a mechanism distinct from that of insulin. The addition of fMLP with insulin did not enhance the insulin-induced increase in PtdIns 3-kinase activity (Table 1), suggesting that the synergistic production of PtdIns(3,4,5)P_3 was not due to enhancement of tyrosine phosphorylation.

Synergistic activation of PtdIns 3-kinase in a cell-free system

We next examined whether or not this synergistic activation of PtdIns 3-kinase is also observed in a cell-free system. Cytosolic proteins of THP-1 cells were separated by chromatography on a Mono Q column. PtdIns 3-kinase activities were eluted as at least two peaks: peaks I and II eluting at 0.18 M and 0.35 M NaCl respectively (Figure 2). These activities were completely inhibited by 0.1 μM wortmannin (results not shown). Western blot analysis using an anti-p85 antibody showed that peak I contained p85, a regulatory subunit of PtdIns 3-kinase, and thus heterodimeric PtdIns 3-kinase was eluted in this fraction (Figure 2, inset).

To characterize the two PtdIns 3-kinase activities obtained on the Mono Q column, we used a tyrosine-phosphorylated peptide that had been shown to activate PtdIns 3-kinase by binding via the SH2 domains of the p85 subunit [21]. We also utilized the G$\beta\gamma$ that had been reported to activate a novel PtdIns 3-kinase in U937 cells [24]. The results revealed that the two activities showed different sensitivities to these activators (Figure 3). The tyrosine-phosphorylated peptide augmented the PtdIns 3-kinase activity in the peak I fraction (Figures 3A and 4A), suggesting again that this fraction contained p85/p110 PtdIns 3-kinase. G$\beta\gamma$ by themselves also activated the enzyme activity at higher

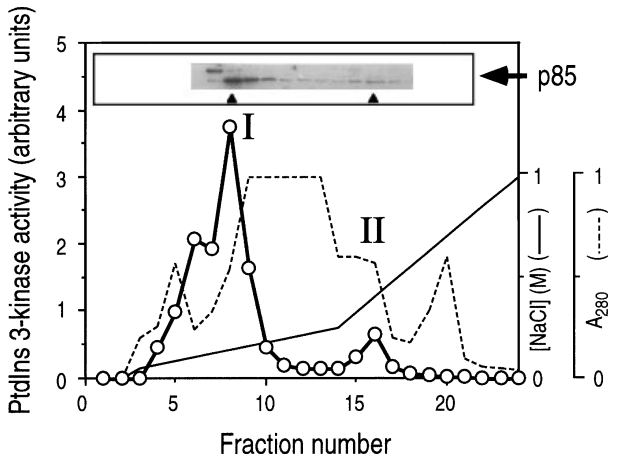

Figure 2 Separation of PtdIns 3-kinase activity in the THP-1 cell cytosol by Mono Q column chromatography

The cytosolic fraction of THP-1 cells was loaded onto a Mono Q column. The column was washed and eluted with a gradient of NaCl. Each fraction was assayed for the ability to phosphorylate PtdIns (○). Inset, peptides in each fraction were separated by SDS/PAGE, transferred to a PVDF membrane and then probed with an anti-p85 antibody. The peak I and II fractions corresponding to fractions 8 and 16 respectively are indicated by arrowheads. Similar results were obtained in a repeated experiment.

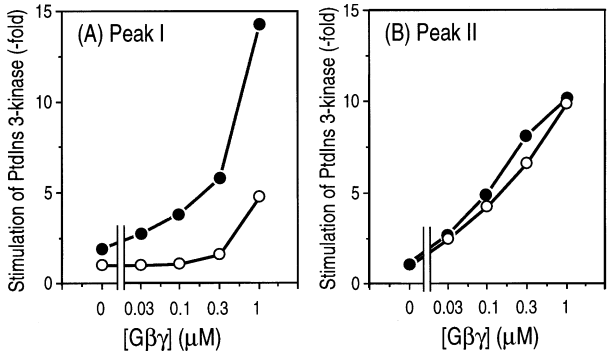

Figure 3 Dose-dependent effects of G$\beta\gamma$ on the Mono Q-purified PtdIns 3-kinase activities

The peak I (**A**) and peak II (**B**) fractions in Figure 2 were assayed for ability to phosphorylate PtdInsP_2. Various concentrations of bovine brain G$\beta\gamma$ were included in the assay mixture with (●) or without (○) 250 μg/ml of the tyrosine-phosphorylated peptide. The control activities of the peak I and II fractions, which were determined in the absence of the activators, were 0.89 pmol/10 min and 0.08 pmol/10 min respectively. Similar results were obtained in a repeated experiment.

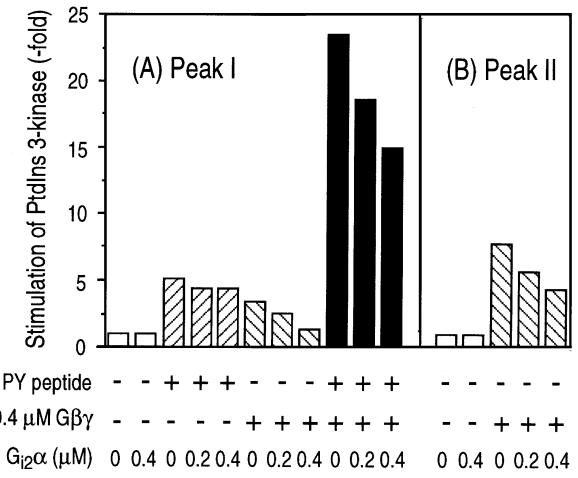

Figure 4 Inhibition of G$\beta\gamma$ responsiveness of PtdIns 3-kinase by GDP-liganded $G_i\alpha$

The peak I (**A**) and peak II (**B**) fractions in Figure 2 were assayed for ability to phosphorylate PtdInsP_2 in the presence or absence of 0.4 μM bovine brain G$\beta\gamma$. Where indicated, G$\beta\gamma$, which had been pre-incubated with various concentrations of $G_i\alpha$ loaded with GDP, was used. The tyrosine-phosphorylated peptide (PY peptide) was included in the assay mixture at a concentration of 250 μg/ml. Similar results were obtained in a repeated experiment.

concentrations (Figure 3A). When the tyrosine-phosphorylated peptide was added together with G$\beta\gamma$, the activity in the peak I fraction was augmented synergistically (Figure 3A). The results indicate that some isoenzyme of PtdIns 3-kinase that was sensitive to the tyrosine-phosphorylated peptide was also sensitive to G$\beta\gamma$.

PtdIns 3-kinase activity in U937 cells, corresponding to the peak II fraction of THP-1 cells, was reported previously to be enhanced by G$\beta\gamma$ but not to be affected by phosphorylated peptides at all [24]. In agreement with those results, the PtdIns 3-kinase activity in the peak II fraction increased 10-fold on the addition of 1 μM G$\beta\gamma$ (Figure 3B). The addition of the tyrosine-phosphorylated peptide had no effect on the activity (Figure 3B). These results suggest that an isoform of G$\beta\gamma$-sensitive PtdIns 3-kinase lacking the SH2 domain might be eluted in this fraction. Alternatively, it can be considered that p85/p110 was eluted in the peak II fraction as a complex with an intrinsic tyrosine-phosphorylated peptide, and thus the addition of the phosphopeptide could not induce further activation. This possibility seems, however, unlikely because no phosphoprotein was detected by the anti-phosphotyrosine antibody in the peak II fraction (results not shown). Furthermore, treatment of THP-1 cells with insulin before the preparation of a cell extract did not increase the activity in the peak II fraction (results not shown).

The effect of G$\beta\gamma$ could be inhibited by pre-incubation of the subunits with the GDP-liganded recombinant $G_i\alpha$-subunit. As shown in Figure 4(A), a stoichiometric amount of GDP–$G_i\alpha$ inhibited the G$\beta\gamma$-stimulated PtdIns 3-kinase activity in the peak I fraction without affecting the basal activity or the tyrosine-phosphorylated-peptide-stimulated activity in this fraction. The synergistically enhanced activity in the peak I fraction and the G$\beta\gamma$-stimulated activity in the peak II fraction were also reduced on the addition of GDP–$G_i\alpha$ (Figure 4B). In these cases, however, a stoichiometric amount of $G_i\alpha$ only partially inhibited the effect of G$\beta\gamma$. This discrepancy in the effective concentration of GDP–$G_i\alpha$ is probably due to the different sensitivity of the activities to G$\beta\gamma$ (see Figures 3A and 3B); a low concentration of G$\beta\gamma$ freed from $G_i\alpha$, which exists even after the addition of $G_i\alpha$ at the highest concentration used, can still stimulate the activities in these cases. It has been reported that the non-myristoylated recombinant $G_i\alpha$ used in this study has a reduced affinity for G$\beta\gamma$ [29]. Thus, for complete inhibition, the myristoylated form of $G_i\alpha$ would be necessary.

Effect of immunoprecipitation with antibodies to p85

The results in Figure 3 indicate that some isoenzyme of PtdIns 3-kinase eluted in the peak I fraction was sensitive to both tyrosine-phosphorylated peptides and G$\beta\gamma$. To determine whether or not this PtdIns 3-kinase is identical with the well-known isoenzyme of the p85/p110 type, we used anti-p85 polyclonal (KR1) and monoclonal (CB2) antibodies, which had been immobilized to

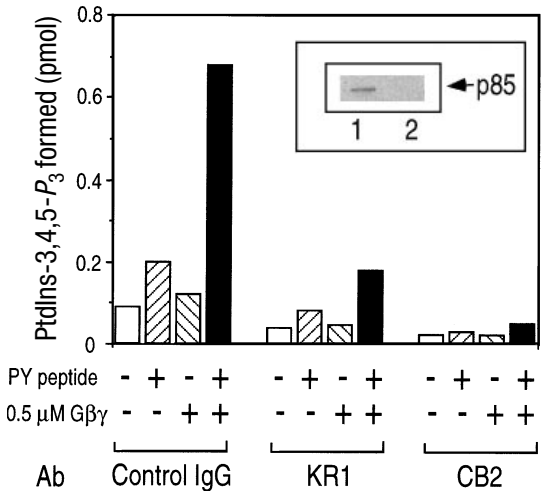

Figure 5 Immunoprecipitation of the G$\beta\gamma$-sensitive PtdIns 3-kinase activity with monoclonal and polyclonal antibodies against p85 from the peak I fraction

The peak I fraction in Figure 2 was incubated for 1 h with polyclonal (KR1) or monoclonal (CB2) anti-p85 antibodies, or control IgG, which had been immobilized to Sepharose beads, followed by sedimentation. The supernatant was assayed for the ability to phosphorylate PtdInsP_2 with or without the addition of 0.5 μM G$\beta\gamma$ and/or 250 μg/ml of the tyrosine-phosphorylated peptide (PY peptide). Inset: the peak I fraction was immunoprecipitated with control IgG (lane 1) or CB2 (lane 2) as above. Peptides in the post-immunoprecipitation supernatant were separated by SDS/PAGE, transferred to a PVDF membrane and then probed with a polyclonal anti-p85 antibody (KR1). Similar results were obtained in a repeated experiment.

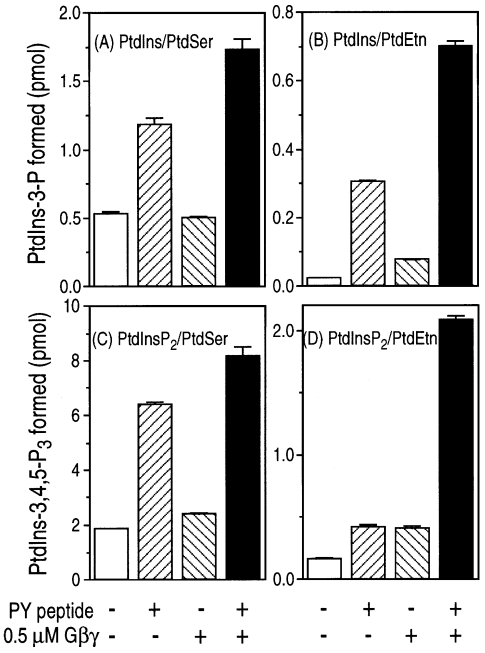

Figure 6 Dependence of the actions of tyrosine-phosphorylated peptide and G$\beta\gamma$ on the composition of substrate vesicles

The PtdIns 3-kinase activity in the peak I fraction in Figure 2 was determined in the presence or absence of bovine brain G$\beta\gamma$, with or without 250 μg/ml of the tyrosine-phosphorylated peptide (PY peptide). PtdIns (0.2 mM)/0.2 mM PtdSer (**A**), 0.2 mM PtdIns/0.2 mM PtdEtn (**B**), 0.1 mM PtdInsP_2/1 mM PtdSer (**C**) and 0.1 mM PtdInsP_2/1 mM PtdEtn (**D**) were included in the assay mixture as substrate vesicles. The results shown are the means $\pm$ range for duplicate determinations.

Sepharose beads. As shown in Figure 5, both antibodies could decrease the G$\beta\gamma$-stimulated PtdIns 3-kinase activity in the post-immunoprecipitation supernatant. When CB2 was used, almost no p85 was observed in the supernatant of the peak I fraction (Figure 5, inset). In this case, no stimulation of PtdIns 3-kinase activity was found in the supernatant, even when the tyrosine-phosphopeptide and G$\beta\gamma$ were added as activators (Figure 5). The addition of CB2 to the assay mixture did not, by itself, impair the PtdIns 3-kinase activities in peaks I and II (results not shown). Western blotting analysis with CB2 or KR1 of the CB2 immunoprecipitate revealed no proteins other than that which migrated with the 85 kDa peptides (results not shown). These results suggest that a conventional p85/p110 or a closely related isoenzyme is sensitive to the synergistic stimulation.

Dependence of G$\beta\gamma$ action on the carrier phospholipid

We have shown that the ability of the peak I fraction to phosphorylate PtdInsP_2 is stimulated at higher concentrations of G$\beta\gamma$, even in the absence of the tyrosine-phosphorylated peptide. In U937 cells, however, the PtdIns 3-kinase activity corresponding to the peak I fraction is reported to be unaffected by G$\beta\gamma$ [24]. Because the carrier phospholipid we used to prepare the substrate vesicles (PtdEtn) was different from that in the previous study, we next investigated the effect of the phospholipid composition on the actions of G$\beta\gamma$ and the tyrosine-phosphorylated peptide. Figure 6(A) shows the results when PtdSer and PtdIns were used to prepare the substrate vesicles. Under those conditions, G$\beta\gamma$ had little effect on the basal activity of PtdIns 3-kinase. The activity in the presence of the tyrosine-phosphorylated peptide was, however, potentiated slightly but significantly by G$\beta\gamma$. When PtdEtn was used instead of PtdSer, G$\beta\gamma$ effectively potentiated both the basal and the phosphopeptide-stimulated PtdIns 3-kinase activities (Figure 6B). Similar results were obtained when PtdInsP_2 was used as substrate (Figures 6C and 6D). The PtdIns 3-kinase activity of the peak II fraction was stimulated by G$\beta\gamma$ and was unaffected by the tyrosine-phosphorylated peptide, regardless of the vesicle composition (results not shown).

DISCUSSION

We have shown that insulin and fMLP exert a synergistic effect on PtdIns(3,4,5)P_3 formation in intact THP-1 cells (Figure 1). This finding suggests that there is cross-talk between the tyrosine phosphorylation-dependent signal-transduction pathway and the GTP-binding protein-dependent pathway to activate PtdIns 3-kinase. Significantly, this synergism was also seen in a cell-free system: the tyrosine-phosphorylated peptide and the G$\beta\gamma$ synergistically augmented the PtdIns 3-kinase activity in a fraction separated from the cytosol of THP-1 cells by Mono Q column chromatography (Figure 3). This activity could be precipitated with monoclonal and polyclonal anti-p85 antibodies (Figure 5), suggesting that the enzyme responsible for the activity contains p85 or a closely related protein.

Recently, Stephens et al. [24] reported the occurrence in U937 cells of a G$\beta\gamma$-sensitive PtdIns 3-kinase which was chromatographically distinct from the conventional p85/p110 enzyme. Consistent with their study, the activity in the peak II fraction in our study was stimulated by G$\beta\gamma$ but was not affected by the tyrosine-phosphorylated peptide (Figure 3). Our results indicate that the conventional p85/p110 (peak I fraction) was also activated by G$\beta\gamma$, although the sensitivity to G$\beta\gamma$ in the absence of the tyrosine-phosphorylated peptide was less than that of the

peak II fraction. The combined use of the tyrosine-phosphorylated peptide greatly improved the sensitivity to G$\beta\gamma$ and markedly augmented the activity. This synergistic effect indicates that the two activators act on the same enzyme. Our findings differ from those of Stephens et al., who reported that G$\beta\gamma$ did not affect the activity in the p85/p110 fraction, though they did not examine the effect of the combination of G$\beta\gamma$ and the phosphotyrosyl peptides [24]. It is important to note that our assay system is different from theirs. Actually, the basal activity of PtdIns 3-kinase and the effect of G$\beta\gamma$ were greatly dependent upon the lipids used for the assay (see Figure 6). Under our conditions (1 mM PtdEtn and 0.1 mM PtdInsP_2), a low basal activity and high sensitivity to G$\beta\gamma$ were observed. When we used the other lipids, such as 0.2 mM PtdSer and 0.2 mM PtdIns, the effect of G$\beta\gamma$ as an activator was weaker, though the synergistic effect of the phosphotyrosyl peptide and G$\beta\gamma$ could still be observed. Recently, a novel G$\beta\gamma$-sensitive catalytic subunit of PtdIns 3-kinase (p110γ) was identified [25,26]. The isoenzyme is unlikely to explain the PtdIns 3-kinase activity in the peak I fraction, because p110γ does not contain a binding site for the p85 subunit [25,26] and thus is expected to be insensitive to the phosphotyrosyl peptide. Furthermore, p110γ was found to be activated by both Gα and G$\beta\gamma$ [26], while the activity in the peak I fraction was not activated by Gα at all (Figure 4A). Interestingly, our results are consistent with those of Thomason et al., who reported that a G$\beta\gamma$-sensitive PtdIns 3-kinase was co-precipitated with p85 [30].

Our findings do not indicate that phosphopeptides and G$\beta\gamma$ directly interact with the immunoprecipitable PtdIns 3-kinase. The possibility that another component is involved in mediation of the action of G$\beta\gamma$ cannot be excluded at present. This possibility is consistent with the fact that PtdIns 3-kinase activity in CB2 immunoprecipitates is not stimulated by G$\beta\gamma$, in spite of the finding that CB2 itself did not interfere with the effects of G$\beta\gamma$ and the phosphopeptide (results not shown). We have also found that a crude preparation of p85/p110 from rat liver is, but a highly purified preparation is not, sensitive to the synergistic activation by G$\beta\gamma$. Further study is necessary to determine whether or not the synergistic effect requires a component other than p85/p110. In any event, it is very attractive to consider that activation of a single PtdIns 3-kinase molecule by the phosphopeptide and G$\beta\gamma$ could be the basis of the cross-talk between the tyrosine kinase-coupled receptors and GTP-binding-protein-coupled receptors in the regulation of cellular functions.

In summary, we have found that tyrosine kinase-dependent and GTP-binding-protein-dependent signals caused synergistic activation of PtdIns 3-kinase in both intact cells and in a cell-free system. A previously identified PtdIns 3-kinase (p85/p110), or a closely related isoenzyme, is responsible for this synergism. Though the physiological consequence of the synergism has not been determined as yet, it has been shown in the literature that pertussis toxin, which abolishes the functions of specific subtypes of GTP-binding proteins, attenuates the metabolic action of insulin in intact cell systems [31–33]; in spite of that, direct evidence showing the insulin-induced stimulation of pertussis toxin-sensitive GTP-binding proteins has not been obtained. Because activation of PtdIns 3-kinase is now recognized to be the essential mechanism underlying many insulin actions [9,10,34–36], it would be intriguing to examine the possible involvement in such cases of synergistic stimulation of PtdIns 3-kinase by G$\beta\gamma$.

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