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Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling

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Insulin, IGF-1 or EGF induce membrane ruffling through their respective tyrosine kinase receptors. To elucidate the molecular link between receptor activation and membrane ruffling, we microinjected phosphorylated peptides containing YMXM motifs or a mutant 85 kDa subunit of phosphoinositide (PI) 3-kinase (Δp85) which lacks a binding site for the catalytic 110 kDa subunit of PI 3-kinase into the cytoplasm of human epidermoid carcinoma KB cells. Both inhibited the association of insulin receptor substrate-1 (IRS-1) with PI 3-kinase in a cell-free system and also inhibited insulin- or IGF-1-induced, but not EGF-induced, membrane ruffling in KB cells. Microinjection of nonphosphorylated analogues, phosphorylated peptides containing the EYYE motif or wild-type 85 kDa subunit (Wp85), all of which did not inhibit the association of IRS-1 with PI 3-kinase in a cell-free system, did not inhibit membrane ruffling in KB cells. In addition, wortmannin, an inhibitor of PI 3-kinase activity, inhibited insulin- or IGF-1-induced membrane ruffling. These results suggest that the association of IRS-1 with PI 3-kinase followed by the activation of PI 3-kinase are required for insulin- or IGF-1-induced, but not for EGF-induced, membrane ruffling.

Key words: IGF-1/insulin/IRS-1/membrane ruffling/PI 3-kinase

Introduction

Insulin, IGF-1 and EGF receptors belong to the family of structurally related transmembrane growth factor receptors with ligand-activated protein tyrosine kinase activity (Ullrich and Schlessinger, 1990). Several lines of evidence suggest that receptor kinase activity is essential for the ligand-activated signalling (Ullrich and Schlessinger, 1990). Insulin or IGF-1 treatment of various intact cells causes rapid tyrosine phosphorylation of a high molecular weight protein (M_r = 160 000–185 000) designated pp185 (White *et al.*, 1985; Izumi *et al.*, 1987; Kadowaki *et al.*, 1987). Tyrosine phosphorylation of pp185 was shown to be catalysed directly by the insulin receptor kinase and not by autophosphorylation (Tashiro-Hashimoto *et al.*, 1989). A cDNA encoding pp185 was isolated and the predicted protein was named insulin receptor substrate-1 (IRS-1) (Sun *et al.*, 1991). IRS-1 possesses 20 potential tyrosine phosphorylation sites, six of which were found in YMXM motifs and three in YXXM motifs (Myers and White, 1993).

The phosphoinositide (PI) 3-kinase phosphorylates the D-3 position of phosphatidylinositol (PtdIns) forming PtdIns 3-phosphate [PtdIns(3)P], PtdIns 3,4-bisphosphate [PtdIns $(3,4)P_2$] and PtdIns 3,4,5-trisphosphate [PtdIns$(3,4,5)P_3$] (Whitman *et al.*, 1988; Auger *et al.*, 1989; Hawkins *et al.*, 1992). D-3 phosphorylated inositides may be involved in the control of cellular growth and metabolism as a novel second messenger (Auger *et al.*, 1989). PI 3-kinase is a heterodimer consisting of 110 (p110) and 85 kDa (p85) subunits. cDNA cloning of these proteins revealed that p110 is a catalytic subunit (Hiles *et al.*, 1992) and that p85 is an adaptor subunit containing two Src homology region 2 (SH2) and one SH3 domain (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991). SH2 domains are reported to bind to tyrosine-phosphorylated proteins (Koch *et al.*, 1991). Insulin or IGF-1 treatment of cells increase PI 3-kinase activity in immunoprecipitates made using antibodies to phosphotyrosine (Endemann *et al.*, 1990; Ruderman *et al.*, 1990; Steel-Parkins and Roth, 1990; Yamamoto *et al.*, 1992). Recently, we and others have reported that insulin or IGF-1 drives a complex formation between PI 3-kinase and tyrosine-phosphorylated IRS-1 (Backer *et al.*, 1992; Lavan *et al.*, 1992; Yonezawa *et al.*, 1992b; Giorgetti *et al.*, 1993). Since phosphorylated peptides containing YMXM motifs inhibit the association of IRS-1 with PI 3-kinase in a cell-free system (Backer *et al.*, 1992; Yonezawa *et al.*, 1992b), these proteins associate via phosphorylated YMXM motifs of IRS-1 and SH2 domains of p85. In addition, tyrosine-phosphorylated IRS-1 or peptides containing phosphorylated YMXM motifs were reported to activate PI 3-kinase activity in a cell-free system (Backer *et al.*, 1992; Carpenter *et al.*, 1993). However, the role of this complex in insulin or IGF-1 signal transduction is not clearly established.

In many cell types, a number of growth factors including EGF, NGF, PDGF and IGF-1, phorbol esters and *ras* cause membrane ruffling (Brunk *et al.*, 1976; Chinkers *et al.*, 1979; Connolly *et al.*, 1979; Goshima *et al.*, 1984; Bar-Sagi and Feramisco, 1986; Kadowaki *et al.*, 1986; Mellström *et al.*, 1988). Ruffled membrane formation represents an alteration in cell morphology and cytoskeletal architecture that can be observed under a phase-contrast microscope a

few minutes after addition of these growth factors or the phorbol ester. The molecular events responsible for ruffle formation are not known but it is believed that polymerization of actin at the inner surface of the plasma membrane plays a crucial role (Mellström *et al.*, 1988). In human epidermoid carcinoma KB cells, we and others demonstrated that insulin, IGF-1 or EGF induces rapid membrane ruffling through their respective tyrosine kinase receptors (Goshima *et al.*, 1984; Kadowaki *et al.*, 1986). We also demonstrated that microinjection of the corresponding kinase-inhibitory antibody into the cytoplasm of KB cells blocked the ability of insulin, IGF-1 or EGF to induce membrane ruffling (Izumi *et al.*, 1988). Recently, the small GTP-binding protein *rac* was reported to be involved in the signal transduction of growth factor-induced membrane ruffling (Ridley *et al.*, 1992). However, other components of signal transduction pathways linking tyrosine kinase receptors to membrane ruffling are still missing. To dissect molecular and cellular events leading to membrane ruffling, we microinjected phosphorylated peptides containing YMXM motifs or a mutant p85α protein (Δp85), which lacks a binding site for p110 (Dhand *et al.*, 1994), both of which inhibited the association of IRS-1 with PI 3-kinase in a cell-free system, to observe directly the effects on membrane ruffling in KB cells. Finally, the effects of wortmannin, an inhibitor of PI 3-kinase activity (Yano *et al.*, 1993; Okada *et al.*, 1994), on insulin- or IGF-1-induced membrane ruffling in KB cells were examined.

Results

PI 3-kinase activation by insulin, IGF-1 or EGF in KB cells

Human epidermoid carcinoma KB cells, treated for 1 min with or without 10^{-7} M insulin, IGF-1 or EGF, were lysed and the PI 3-kinase activity in immunoprecipitates with anti-phosphotyrosine or anti-IRS-1 antibodies was measured. Insulin stimulated PI 3-kinase activity 15- to 20-fold in immunoprecipitates with anti-phosphotyrosine antibody and 3- to 4-fold in immunoprecipitates with anti-IRS-1 antibody respectively (Figure 1A). IGF-1 stimulated PI 3-kinase activity in a similar manner (Figure 1A). In the case of EGF stimulation, PI 3-kinase activity increased only slightly in anti-phosphotyrosine immunoprecipitates but not in anti-IRS-1 immunoprecipitates, suggesting that PI 3-kinase itself or an associated protein, other than IRS-1, is slightly tyrosine-phosphorylated in response to EGF. To confirm these results, after ligand treatment KB cells were immunoprecipitated with mAb F12. As we described previously (Yonezawa *et al.*, 1992b), F12 immunoprecipitates p85 complexed with p110 of PI 3-kinase from several species including human. The immunoprecipitates were then immunoblotted with anti-phosphotyrosine antibody. mAb F12 was able to coimmunoprecipitate IRS-1 from cells treated with insulin or IGF-1, but not with EGF (Figure 1B). Immunoblotting of the same immunoprecipitates with anti-EGF receptor antibody did not detect EGF receptors (data not shown), suggesting that the p85 subunit of PI 3-kinase was not associated with EGF receptors in KB cells.

Effects of phosphopeptides on the association of PI 3-kinase activity with IRS-1 in a cell-free system

IRS-1 has multiple putative tyrosine phosphorylation sites (Sun *et al.*, 1991). Five tyrosine-phosphorylated synthetic peptides and nonphosphorylated analogues corresponding to putative tyrosine phosphorylation sites of IRS-1 were prepared as described previously and designated as IRP-1, IRP-2, IRP-3, IRP-4 and IRP-5 (Yonezawa *et al.*, 1992b) (Figure 2). The IRS-1 from KB cells was immobilized with anti-IRS-1 antibody on the protein G−agarose and then tyrosine-phosphorylated by partially purified insulin receptors. This tyrosine-phosphorylated IRS-1 was exposed to lysates from KB cells in the presence of various peptides (final concentration 0.1 mM) and the PI 3-kinase activity associated with IRS-1 was measured (Figure 2). PI 3-kinase activity from KB cells was associated with tyrosine-phosphorylated IRS-1, but not with nonphosphorylated IRS-1. The phosphorylated peptides containing the YMXM motif blocked the association of PI 3-kinase activity with tyrosine-phosphorylated IRS-1. In contrast, the phosphoryl-

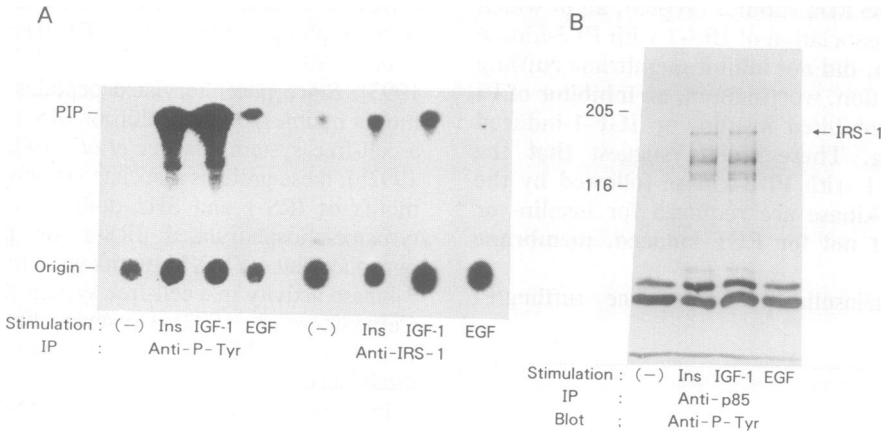

Fig. 1. The association of PI 3-kinase and IRS-1 in insulin-, IGF-1- or EGF-stimulated KB cells. KB cells were stimulated with 10^{-7} M of insulin, IGF-1 or EGF for 1 min at 37°C. **(A)** Insulin-, IGF-1- or EGF-stimulated PI 3-kinase activity in KB cells. PI 3-kinase activities were determined in anti-phosphotyrosine (PY20) or anti-IRS-1 (6G5) immunoprecipitates made from lysates of KB cells. ^{32}P-labelled lipids were separated by thin layer chromatography (TLC). Origin and PIP indicate the position of the TLC origin and migration of a phosphatidylinositol 4-phosphate standard, respectively. **(B)** The association of IRS-1 and the p85 subunit of PI 3-kinase. After ligand stimulation, KB cells were lysed and immunoprecipitated with monoclonal anti-p85 antibody (F12). The immunoprecipitates were electrophoresed on SDS−polyacrylamide gels, transferred and then blotted with anti-phosphotyrosine antibodies. The strong doublet found in all lanes is derived from IgG reaction with the detection antibody.

ated peptide IRP-5 containing the EYYE motif as well as nonphosphorylated analogues failed to block this association in KB cells.

Effects of the microinjection of peptides on growth factor-induced membrane ruffling

The addition of growth factors to serum-starved KB cells rapidly induced alterations in their morphology and cytoskeletal structure as described previously (Kadowaki *et al.*, 1986). Insulin (10^{-7} M) or IGF-1 (10^{-8} M) rapidly induced membrane ruffling, which looked like dark and irregular rims along a part or around all of the margin of cells. Without growth factors, the percentage of cells showing membrane ruffling was very low. EGF (10^{-8} M) also induced membrane ruffles, but the ruffles were visible as curtain-like folds or rod-like spikes.

To investigate the effect of peptides on membrane ruffling, 1 mM of the phosphorylated form of peptide IRP-1 (IRP-1P) (Yonezawa *et al.*, 1992b) was microinjected into the cytoplasm of the two KB cells on the left of panel 1 (Figure 3A). The two KB cells on the right in panel 1 were microinjected with the same concentration of the nonphosphorylated form of IRP-1 (IRP-1NP). After 30 min, all these cells were treated with 10^{-7} M insulin. As shown in panel 1 of Figure 3A, the two KB cells on the right showed, with phase-contrast microscopy, membrane ruffles visible as dark and irregular rims along the cell margin. However, the two KB cells on the left showed no membrane ruffles. These KB cells were stained with tetramethyl-rhodamine isothiocyanate (TRITC)-labelled phalloidin, a fluorescent phallotoxin used to identify filamentous/polymerized actin. TRITC-phalloidin stained clearly along the cell margin of two KB cells on the right localized to membrane ruffles (Figure 3A, panel 4), suggesting that filamentous/polymerized actin accumulates at the plasma membrane in membrane ruffles. Next, the phosphorylated form of peptide IRP-5 (IRP-5P) or the nonphosphorylated form of peptide IRP-5 (IRP-5NP) was microinjected into KB cells. As shown in panels 2 and 5 of Figure 3A, the six KB cells on the left which were microinjected with IRP-5P, and the four KB cells on the right which were microinjected with IRP-5NP, showed membrane ruffles after insulin treatment. In panels 3 and 6 of Figure 3A, 1 mM of IRP-1P was microinjected into the five KB cells on the left. The four KB cells on the right were microinjected with the same concentration of IRP-1NP. These KB cells were treated with 10^{-8} M EGF. All of the KB cells showed membrane ruffling as curtain-like folds or rod-like spikes, suggesting that these peptides had no effect on EGF-induced membrane ruffling.

Membrane ruffle formation was quantitatively assessed using the scoring system (ruffling index) defined in Materials and methods. This scoring system provides reproducible values for both untreated and growth factor-treated cells. Several concentrations of peptides were microinjected into KB cells and insulin (10^{-7} M)-induced membrane ruffling was assessed quantitatively. As shown in Figure 3B, IRP-1P inhibited insulin-induced membrane ruffling in a dose-dependent manner, while IRP-5P did not.

To investigate the specificity of phosphorylated peptides, other tyrosine-phosphorylated synthetic peptides and nonphosphorylated analogues were microinjected into KB cells and their effects on growth factor-induced membrane ruffling were quantitatively measured. As shown in Figure 3C, the phosphorylated form of IRP-1, IRP-2, IRP-3 and IRP-4 inhibited insulin- or IGF-1-induced membrane ruffles with similar potencies when 1 mM of each peptide was microinjected. However, the phosphorylated form of IRP-5 and all of the nonphosphorylated analogues had no effect on insulin- or IGF-1-induced membrane ruffling. Neither phosphorylated nor nonphosphorylated peptides had clear effects on EGF-induced membrane ruffling in KB cells.

Recently, the SH2 domains of GRB2 and SH PTP2 were reported to bind most strongly to tyrosine-phosphorylated

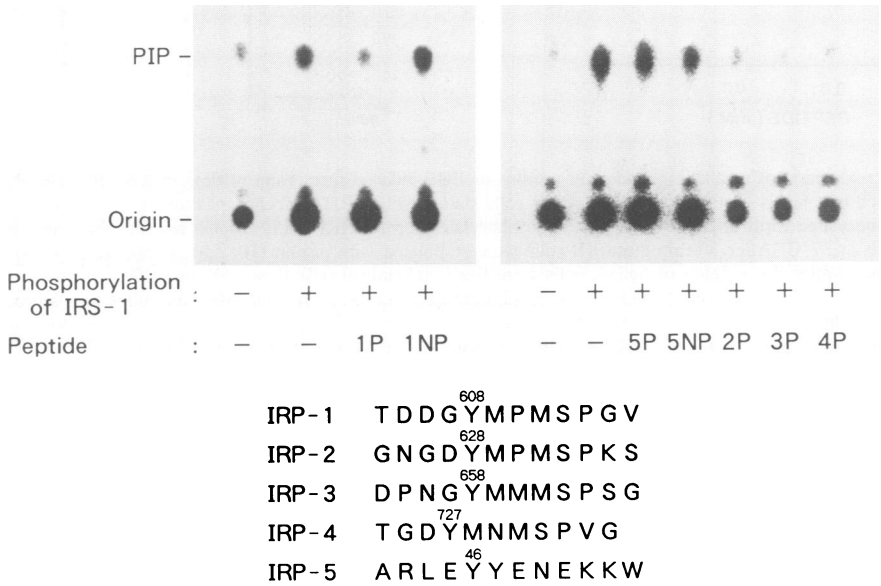

Fig. 2. Effects of synthetic peptides on the association of PI 3-kinase activity with IRS-1. The IRS-1 from KB cells was immobilized with anti-IRS-1 antibody (6G5) on protein G–agarose, tyrosine-phosphorylated by partially purified insulin receptors and exposed to lysates from KB cells in the presence of various peptides (final concentration 0.1 mM). The PI 3-kinase activity associated with IRS-1 was measured. 1P–5P indicate the phosphorylated forms of IRP-1–IRP-5, respectively. 1NP and 5NP indicate the nonphosphorylated forms of IRP-1 and IRP-5, respectively.

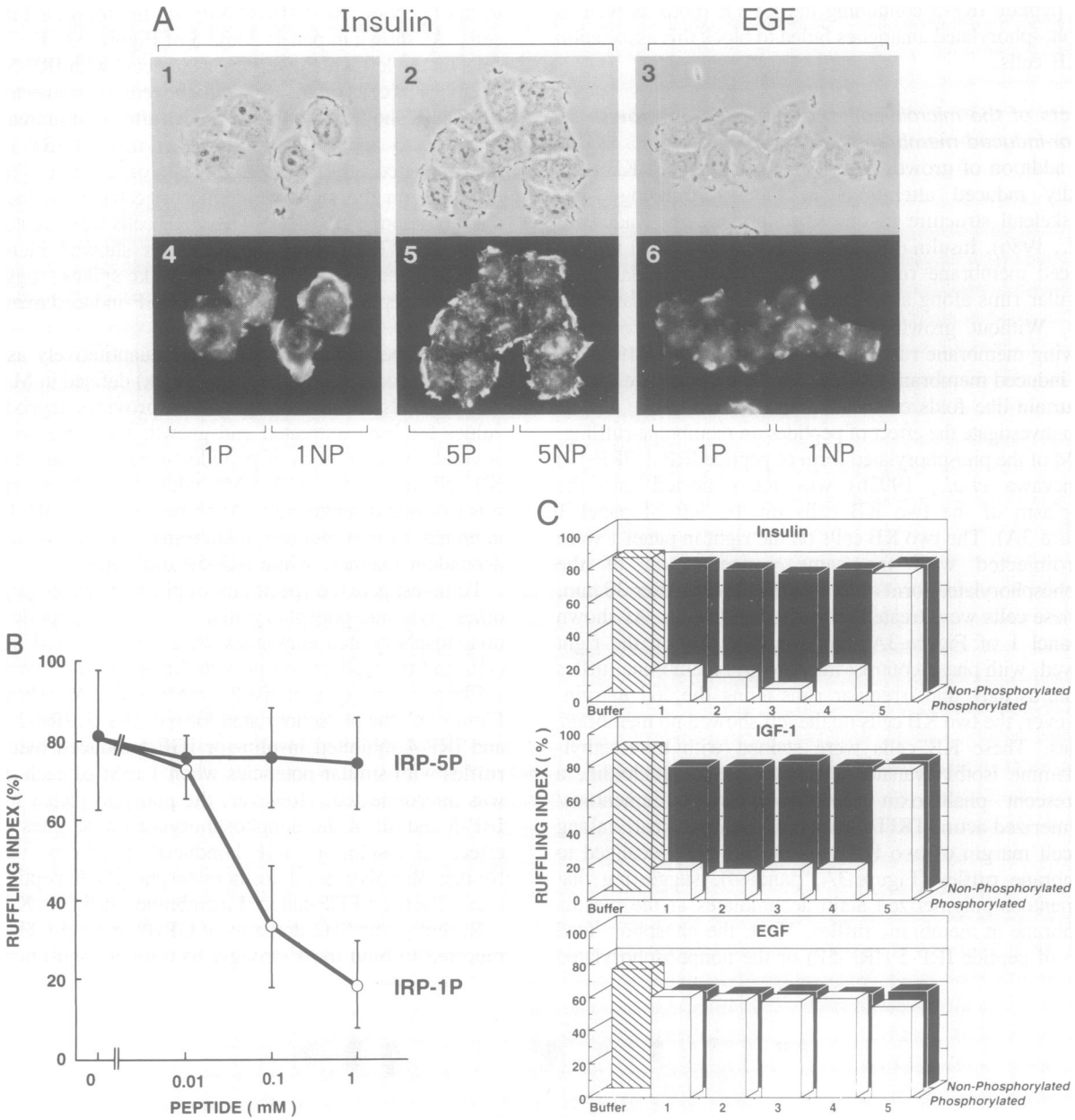

Fig. 3. Effects of the microinjection of synthetic peptides on insulin- or EGF-induced membrane ruffling in KB cells. (**A**) Phase-contrast (top; panels 1−3) and fluorescence (bottom; panels 4−6) micrographs of KB cells stained with TRITC-labelled phalloidin. 1 mM of the phosphorylated form of IRP-1 (1P), the nonphosphorylated form of IRP-1 (1NP), the phosphorylated form of IRP-5 (5P) or the nonphosphorylated form of IRP-5 (5NP) was microinjected into KB cells at 37°C. After 30 min these KB cells were stimulated with insulin (10^{-7} M; panels 1, 2, 4 and 5) or EGF (10^{-8} M; panels 3 and 6). Five minutes after the addition of ligand, cells were fixed and stained. (**B**) Dose-response effects of the microinjected phosphorylated forms of IRP-1 (IRP-1P) or IRP-5 (IRP-5P) on insulin-induced membrane ruffling. Ruffling index was scored as defined in Materials and methods. Data are presented as the mean ± SE from five separate experiments. (**C**) The specificity of the phosphorylated form or the nonphosphorylated form of 1 (IRP-1), 2 (IRP-2), 3 (IRP-3), 4 (IRP-4) and 5 (IRP-5) on insulin- (top), IGF-1- (middle) or EGF (bottom)-induced membrane ruffling. 1 mM of the indicated peptide was microinjected into KB cells.

Y_{895}VNI and Y_{1172}IDL sequences of IRS-1 *in vitro*, respectively (Skolnik *et al.*, 1993; Sun *et al.*, 1993). To investigate the specificity of phosphorylated peptides more directly, two tyrosine-phosphorylated synthetic peptides and nonphosphorylated analogues (SPGE Y_{895}VNIEFGS and NGLNY_{1172}IDLDLVK) were prepared and microinjected into KB cells. Neither phosphorylated nor nonphosphorylated forms of these peptides had effects on insulin- or IGF-1-induced membrane ruffling.

Effects of the microinjection of p85 of PI 3-kinase

To disrupt the association of tyrosine-phosphorylated IRS-1 and PI 3-kinase, we tried to saturate the p85 binding site of IRS-1 by the microinjection of a mutant p85α (Δp85) (Hara *et al.*, submitted) which lacks a binding site for p110 (Dhand *et al.*, 1994) into the cytoplasm of KB cells. For this purpose, we expressed full-length bovine wild-type p85α (Wp85) and Δp85 as glutathione *S*-transferase (GST) fusion proteins. The ability of fusion proteins to bind IRS-1 or the

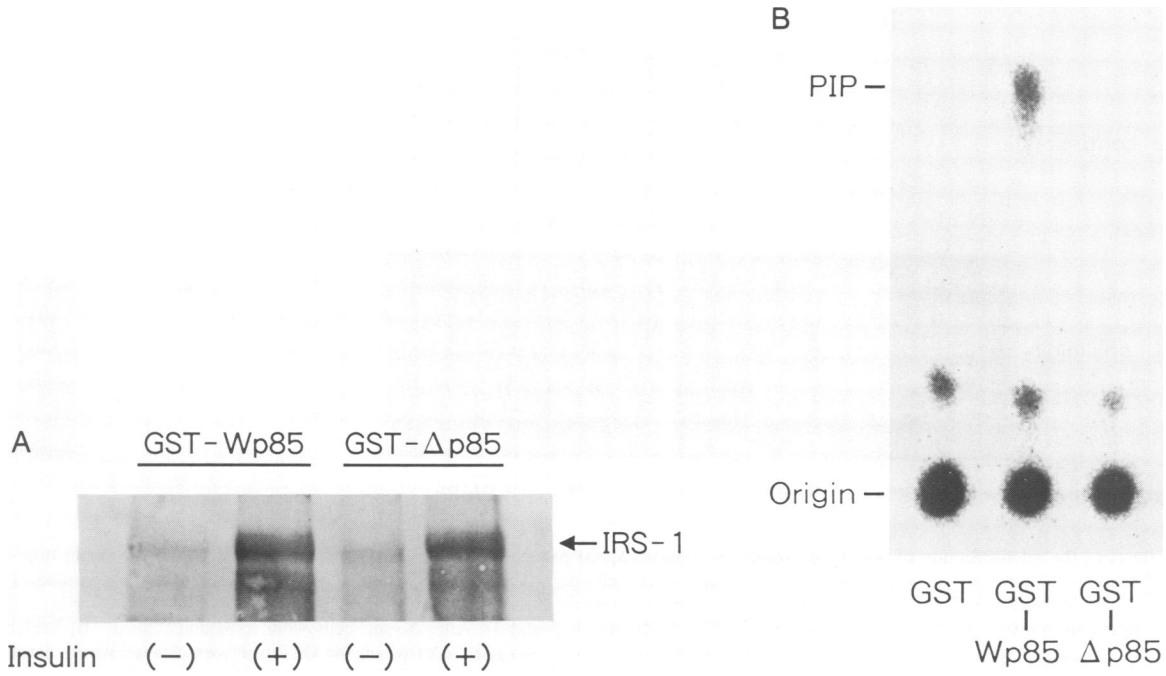

Fig. 4. The association of GST−Wp85 or GST−Δp85 with IRS-1 and PI 3-kinase activity. **(A)** The association of GST−Wp85 or GST−Δp85 with IRS-1. Full-length bovine wild-type p85α (Wp85) or a mutant p85α (Dp85) lacking the binding site for the catalytic p110 subunit of PI 3-kinase was expressed as GST fusion proteins. GST−Wp85 or GST−Δp85 fusion proteins immobilized to glutathione−Sepharose beads were incubated with lysates of KB cells treated with or without 10^{-7} M insulin for 1 min at 37°C and then immunoblotted with anti-phosphotyrosine antibodies. **(B)** The association of GST−Wp85 or GST−Δp85 with PI 3-kinase activity. GST, GST−Wp85 or GST−Δp85 fusion proteins immobilized to glutathione−Sepharose beads were incubated with the lysates of KB cells and the associated PI 3-kinase activity was measured.

catalytic subunit of PI 3-kinase p110 in a cell-free system was examined. Either GST−Wp85 or GST−Δp85 fusion proteins were incubated with lysates of KB cells treated with or without 10^{-7} M insulin and then immunoblotted with anti-phosphotyrosine antibodies. Both GST−Wp85 and GST−Δp85 were capable of binding to the tyrosine-phosphorylated high molecular weight protein (M_r = 185 000) only after insulin stimulation (Figure 4A). Next, either GST alone, GST−Wp85 or GST−Δp85 fusion proteins were incubated with lysates of KB cells. As shown in Figure 4B, PI 3-kinase activity bound to GST−Wp85, but not to GST−Δp85 or to GST alone. These results suggest that GST−Δp85 can bind to IRS-1, but not to the catalytic subunit of PI 3-kinase, p110, from KB cells in a cell-free system.

To see the effect of GST fusion proteins on membrane ruffling, GST−Δp85 proteins at a concentration of 1.0 mg/ml (~10 μM) were microinjected into four KB cells on the left of panel 1 (Figure 5A). The same concentration of GST−Wp85 proteins was microinjected into four KB cells on the right of panel 1 (Figure 5A). After 30 min, these cells were treated with 10^{-7} M insulin. GST−Δp85 inhibited insulin-induced membrane ruffling compared with GST−Wp85 (Figure 5A, panels 1 and 3). IGF-1-induced membrane ruffling was also inhibited by microinjection of GST−Δp85 into KB cells (data not shown). However, both proteins had no effect on the EGF-induced membrane ruffling (Figure 5A, panels 2 and 4).

Next, membrane ruffling was assessed quantitatively. The same concentration (1.0 mg/ml) of GST alone, GST−Wp85 or GST−Δp85 was microinjected into >100 serum-starved KB cells and the ruffling index was scored. GST−Δp85 inhibited insulin-induced membrane ruffling while GST alone or GST−Wp85 did not (Figure 5B). To confirm this result, bacterially expressed Wp85 or Δp85 protein (0.5 mg/ml; ~6 μM) from which the GST had been cleaved was microinjected. Again, only Δp85 inhibited insulin-induced membrane ruffling (Figure 5B).

Effect of wortmannin on insulin- or IGF-1-induced membrane ruffling

Recently, wortmannin was found to bind to the 110 kDa protein, but not to the PI 3-kinase 85 kDa regulatory subunit, and to inhibit PI 3-kinase activity both *in vivo* and *in vitro* (Yano *et al.*, 1993; Okada *et al.*, 1994). To confirm our data, KB cells were treated with several concentrations of wortmannin at 37°C for 10 min and stimulated with 10^{-7} M insulin or IGF-1. After 5 min, PI 3-kinase activity in immunoprecipitates with anti-phosphotyrosine antibodies and membrane ruffling were evaluated. Wortmannin inhibited insulin- or IGF-1-stimulated PI 3-kinase activity in a dose-dependent manner in KB cells (Figure 6A). Wortmannin also inhibited insulin- or IGF-1-induced membrane ruffling with a similar dose-dependent manner (Figure 6B). However, wortmannin had no effects on the tyrosine-phosphorylation of insulin or IGF-1 receptors and IRS-1 (data not shown).

Discussion

Microinjection of phosphopeptides containing the YMXM motif into the cytoplasm of serum-starved KB cells inhibited

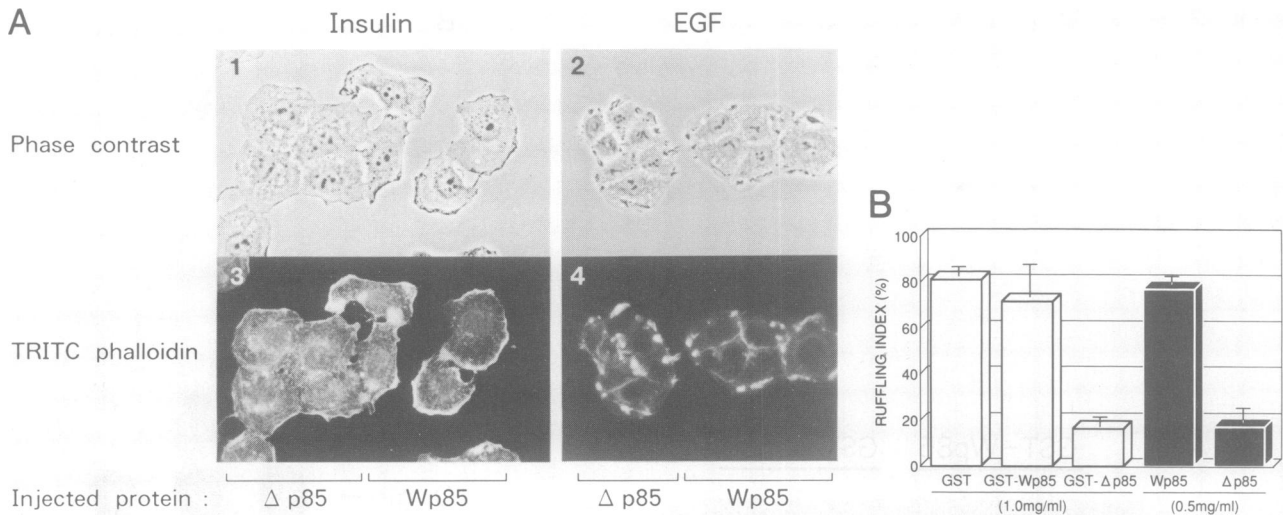

Fig. 5. Effects of the microinjection of wild-type (Wp85) or mutant (Δp85) p85 on membrane ruffling in KB cells. (**A**) Phase-contrast (top; panels 1 and 2) and fluorescence (bottom; panels 3 and 4) micrographs of KB cells stained with TRITC-labelled phalloidin. The same concentration (1.0 mg/ml; ~10 μM) of GST−Wp85 or GST−Δp85 fusion proteins was microinjected into serum-starved KB cells at 37°C. After 30 min, cells were stimulated with insulin (10^{-7} M) (panels 1 and 3) or EGF (10^{-8} M) (panels 2 and 4). After 5 min, cells were fixed and stained. (**B**) Bacterially expressed GST fusion proteins (1.0 mg/ml; ~10 μM) or proteins (0.5 mg/ml; ~6 μM) from which the GST had been cleaved were microinjected into KB cells at 37°C. After 30 min, these cells were stimulated with 10^{-7} M insulin. Five minutes after the addition of insulin, cells were fixed, stained and membrane ruffling was assessed by the ruffling index. Data are presented as the mean ± SE from three separate experiments.

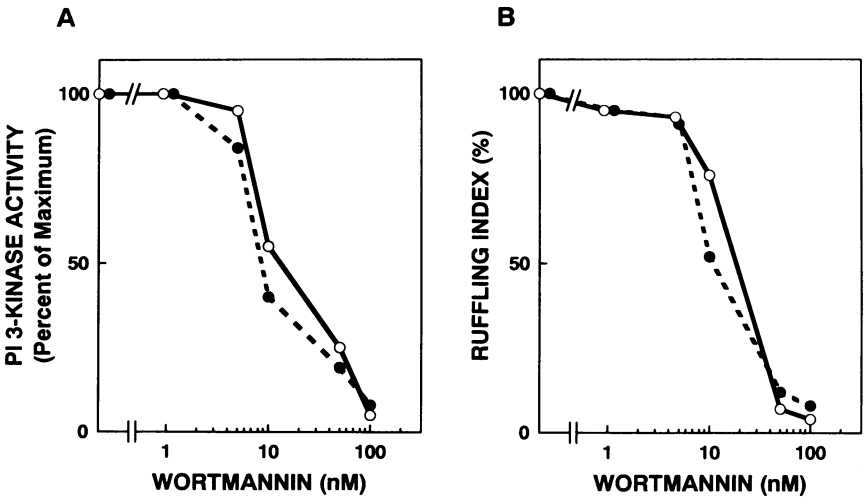

Fig. 6. Effects of wortmannin on insulin- or IGF-1-stimulated PI 3-kinase activity and membrane ruffling in KB cells. KB cells were treated with the indicated concentration of wortmannin at 37°C for 10 min and stimulated with 10^{-7} M insulin (○) or IGF-1 (●). After 5 min, PI 3-kinase activity in immunoprecipitates with anti-phosphotyrosine antibodies was measured (**A**). Membrane ruffling was also assessed by the ruffling index (**B**). The data shown are representative of two independent experiments.

insulin- or IGF-1-induced membrane ruffling in a dose-dependent manner, whereas non-phosphorylated analogues and a phosphopeptide containing the EYYE motif had no effect. Microinjection of these phosphopeptides did not block EGF-induced membrane ruffling. Insulin or IGF-1 drove a complex formation between tyrosine-phosphorylated IRS-1 and the p85 subunit of PI 3-kinase, and stimulated PI 3-kinase activity in immunoprecipitates made from KB cells using antibody to phosphotyrosine or IRS-1. However, EGF only slightly stimulated PI 3-kinase activity in these immunoprecipitates from KB cells. Phosphopeptides containing the YMXM motif inhibited a complex formation between tyrosine-phosphorylated IRS-1 and PI 3-kinase in an *in vitro* system of KB cells. In contrast, analogous nonphosphorylated peptides and a phosphopeptide containing the EYYE motif were ineffective. The specificity of the peptide is very similar to that observed in the inhibition seen during insulin- or IGF-1-induced membrane ruffling in KB cells. Microinjection of phosphopeptides containing the YVNI or the YIDL motif of IRS-1, which have a higher affinity for the SH2 domain of GRB2 or SH PTP-2 respectively (Skolnik *et al.*, 1993; Songyang *et al.*, 1993; Sun *et al.*, 1993), into KB cells had no effect on insulin- or IGF-1-induced membrane ruffling. Taking into consideration that the phosphorylated YMXM motif has a higher affinity only for the SH2 domain of the p85 subunit of PI 3-kinase (Songyang *et al.*, 1993), our data suggest that the association of IRS-1 with PI 3-kinase resulted in the

activation of PI 3-kinase which may be required for insulin- or IGF-1-induced, but not for EGF-induced, membrane ruffling.

It was reported recently that the binding of tyrosine-phosphorylated IRS-1 or peptides containing phosphorylated YXXM motifs to the SH2 domain of p85 activates PI 3-kinase *in vitro* (Backer *et al.*, 1992; Carpenter *et al.*, 1993). Microinjection of phosphopeptides containing the YMXM motif did not induce membrane ruffling (data not shown), although these phosphopeptides must specifically bind to and activate PI 3-kinase. These data may suggest that not only the activation, but also the correct localization of PI 3-kinase directed by tyrosine-phosphorylated IRS-1, are necessary for the induction of membrane ruffling. It is possible that these phosphopeptides are dephosphorylated and/or degraded in the cytoplasm of cells during incubation at 37°C. Therefore, we examined the effects of incubation time of these phosphopeptides on insulin-induced membrane ruffling. Thirty minutes after microinjection of these phosphopeptides, only 16% of microinjected cells showed insulin-induced membrane ruffling. However, 42% (90 min), 79% (180 min) and 93% (300 min) of microinjected cells showed insulin-induced membrane ruffling. These data suggest that these microinjected phosphopeptides in the cytoplasm may be inactivated by longer incubation periods.

Next, we microinjected a mutant Δp85 protein which lacks a binding site for p110 into the cytoplasm of serum-starved KB cells. GST−Δp85 fusion proteins bound to IRS-1 but not to PI 3-kinase activity in an *in vitro* system of KB cells, while GST−Wp85 fusion proteins bound to both. In contrast to GST alone or GST−Wp85, microinjection of the GST−Δp85 fusion protein inhibited insulin- or IGF-1-induced membrane ruffling. Microinjection of GST−Δp85 may disrupt the association of tyrosine-phosphorylated IRS-1 and PI 3-kinase activity by saturating the p85 binding site of IRS-1 with GST−Δp85. Finally, wortmannin, an inhibitor of PI 3-kinase activity (Yano *et al.*, 1993; Okada *et al.*, 1994), inhibited insulin- or IGF-1-induced membrane ruffling in KB cells. At the same time, wortmannin inhibited insulin- or IGF-1-stimulated PI 3-kinase activity in a similar dose-dependent manner. However, pretreatment of Chinese hamster ovary cells overexpressing insulin receptors (CHO-IR cells) (Yonezawa *et al.*, 1992b) with 50 nM wortmannin did not inhibit insulin stimulation of *ras* activation (unpublished observation).

We employed three different approaches to clarify the molecular link between PI 3-kinase activity and membrane ruffling. Each approach had some limitations. For example, it is difficult to exclude completely the possibility that microinjected phosphopeptides containing YMXM motif may interact with SH2 domains of proteins other than p85. Microinjected Δp85 may interact with proteins other than IRS-1. Wortmannin may interact with proteins other than PI 3-kinase. However, the fact that three different approaches reached the same conclusion strongly suggests that PI 3-kinase activity is involved in insulin- or IGF-1-induced membrane ruffling. Furthermore, we have established a stable CHO-IR cell line overexpressing a mutant Δp85 protein (CHO-IR/Δp85 cells). In this cell line, a complex formation between IRS-1 and the catalytic subunit of PI 3-kinase during insulin stimulation was disrupted and insulin-stimulated accumulation of PtdIns(3,4,5)P_3 was decreased, while activation of insulin receptor kinase and the tyrosine-phosphorylation of IRS-1 remained unaffected (Hara *et al.*, submitted). Consistent with our results, insulin- or IGF-1-induced membrane ruffling was also markedly impaired in this cell line (unpublished observation).

In many cell types, a number of growth factors cause membrane ruffling. However, it is possible that the molecular event(s) responsible for growth factor-induced ruffled membrane formation is different. For example, in KB cells insulin or IGF-1 induce membrane ruffling, appearing as dark and irregular rims along a part or the whole of the margin of the cells. In contrast, EGF induced membrane ruffling which is visible as curtain-like folds or rod-like spikes. PDGF was reported to induce unique membrane ruffles which form circular arrangements on the dorsal side of human fibroblasts (Mellström *et al.*, 1988; Hammacher *et al.*, 1989). Accordingly, signal transduction mechanisms of growth factor-induced membrane ruffling must be also different. Our data suggest that PI 3-kinase may be involved in insulin- or IGF-1-induced, but not EGF-induced, membrane ruffling in KB cells. Consistent with this result, it was reported that protein kinase C is required for the membrane ruffling induced by EGF but not for that induced by insulin or IGF-1 in KB cells (Miyata *et al.*, 1989). Using a mutant form of the PDGF receptor lacking the binding site for PI 3-kinase, it was suggested that PI 3-kinase activity may be involved in PDGF-induced actin reorganization (Severinsson *et al.*, 1990; Wennström *et al.*, 1994). It will be interesting to examine this possibility using a similar approach. The small GTP binding protein *rac* was reported to be involved in the signal transduction of growth factor-induced membrane ruffling (Ridley *et al.*, 1992). Microinjection of *rac* protein in KB cells caused membrane ruffling more similar to that induced by insulin or IGF-1 than to that induced by EGF (data not shown). These data may suggest that both PI 3-kinase and *rac* are involved in insulin- or IGF-1-induced membrane ruffling. A study of the interaction between PI 3-kinase and *rac* proteins would be very interesting. Furthermore, in the light of the recent report that the SH3 domains of GRB2 target this protein to membrane ruffles (Bar-Sagi *et al.*, 1993), it will be interesting to examine the role of the SH3 domain of p85 in the localization of this molecules during insulin- or IGF-1-induced membrane ruffling.

The recent cloning of the p110 subunit of PI 3-kinase has provided some interesting insights into the possible function of this enzyme (Hiles *et al.*, 1992). The catalytic subunit, p110, has a region of significant homology with the yeast VPS34 gene product. This protein has been shown to be involved in the targeting of proteins to the yeast vacuole, as well as vacuole morphogenesis during budding (Herman and Emr, 1990). The VPS34 protein was recently shown to possess intrinsic PI 3-kinase activity (Schu *et al.*, 1993).These data suggest that PI 3-kinase may be involved in vesicle trafficking in mammalian cells.

In this study we present data suggesting that PI 3-kinase activity is involved in membrane ruffling. PtdIns(4,5)P_2 was found to promote actin polymerization *in vitro* by stimulating dissociation of gelsolin−actin complexes (Lassing and Lindberg, 1985) and the dissociation of profilactin (Janmey and Stossel, 1987). The accumulation of PtdIns(3,4,5)P_3 was found to be in parallel with filamentous actin polymerization following chemotactic peptide stimulation of neutrophils (Eberle *et al.*, 1990).

These data may suggest that PI 3-kinase activity and actin polymerization are closely linked processes. At the same time, a close association between membrane ruffling and fluid-phase pinocytosis has been noted (Brunk *et al.*, 1976; Chinkers *et al.*, 1979; Bar-Sagi and Feramisco, 1986). Microinjection of *rac* also induced pinocytosis leading to vesicle accumulation in subconfluent cells (Ridley *et al.*, 1992). We also found that insulin-induced fluid-phase pinocytosis was impaired in CHO-IR/Δp85 cells (unpublished observation). The underlying common mechanism for vesicle trafficking, membrane ruffling and pinocytosis mediated by PI 3-kinase will be the focus of further investigations.

Materials and methods

Cell culture and microinjections

KB cells (derived from a human epidermoid carcinoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Insulin, IGF-1 and EGF were obtained from sources as described previously (Izumi *et al.*, 1988). Microinjection and induction of membrane ruffling were performed as described previously (Izumi *et al.*, 1988) with a slight modification. Briefly, KB cells were seeded into 35 mm grid tissue culture dishes (Nunc) at a density of 2×10^4 cells per dish in 2.5 ml DMEM containing 10% FCS. After 2–3 days, the cells were serum-starved for 36 h in DMEM without FCS. Microinjections were performed with glass capillaries drawn to a tip diameter of <1 μm as described (Izumi *et al.*, 1988). Several concentrations of tyrosine-phosphorylated synthetic peptides and nonphosphorylated analogues (Yonezawa *et al.*, 1992b) or proteins, diluted with the buffer containing 20 mM Tris–HCl pH 7.4, 20 mM $MgCl_2$, 0.1 mM EDTA and 1 mM 2-mercaptoethanol, were microinjected into the cytoplasm of living KB cells at 37°C. After 30 min incubation at 37°C, cells were stimulated with each growth factor and 5 min later at 37°C fixed with 3.7% formaldehyde/PBS for 20 min at 25°C. About 100 cells in an area surrounded by four grids were confirmed to be microinjected by lucifer yellow. Trypan blue exclusion showed that >95% of the cells survived the microinjection procedure. About 5×10^{-14} l of sample was microinjected by one injection (Bar-Sagi and Feramisco, 1986), which may be 1/20–1/10 of the total volume of cells.

Analysis of cell morphology and scoring of membrane ruffling

Cell morphology was analysed by phase-contrast microscopy (model IMT-2, Olympus, Tokyo, Japan). For the analysis of filamentous/polymerized actin, cells fixed with 3.7% paraformaldehyde/PBS were permeabilized with 0.2% Triton X-100 for 1 min at 25°C. About 500 μl of 10 μg/ml TRITC-labelled phalloidin was applied to each dish. The dishes were incubated in a humidified chamber for 60 min at 25°C, washed with PBS and mounted in 90% glycerol/PBS containing 1 μg/ml *p*-phenylenediamine. The samples were examined by a fluorescence microscope (model Axiophot, Zeiss, Germany). Membrane ruffling was scored by TRITC-labelled phalloidin staining as described (Izumi *et al.*, 1988). In brief, cells whose margins were strongly stained were scored as 1 point. Cells that showed atypical or equivocal ruffling were scored as 0.5 points. Cells that showed no ruffling were scored as 0 points. More than 100 cells were examined for one assay. Ruffling indices were calculated by dividing total points by the total number of cells examined, followed by multiplying by 100.

Immunoprecipitation and PI 3-kinase assay

Immunoprecipitation and PI 3-kinase assay were performed as described (Yonezawa *et al.*, 1992b). Briefly, serum-starved KB cells were treated with or without 10^{-7} M insulin, IGF-1 or EGF for 1 min at 37°C and lysed in the buffer containing 137 mM NaCl, 20 mM Tris (pH 7.6), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40 and 1 mM sodium vanadate. The insoluble debris was removed by centrifugation at 15 000 *g* for 20 min at 4°C, and the supernatant was immunoprecipitated with monoclonal anti-phosphotyrosine antibody (PY20; ICN), anti-human IRS-1 antibody (6G5) or anti-p85 antibody (F12) bound to protein G–agarose beads. PI 3-kinase activity in the immunoprecipitates was assayed as described previously (Yonezawa *et al.*, 1992a). For immunoblotting, the beads were incubated in Laemmli's sample buffer and boiled for 2 min. Proteins were separated on 7.5% SDS–polyacrylamide gel, transferred to Immobilon-P membranes and blotted with polyclonal anti-phosphotyrosine antibody (UBI). Proteins were detected with horseradish peroxidase-conjugated anti-rabbit IgG followed by ECL detection (Amersham).

In vitro association of IRS-1 with PI 3-kinase and peptide competition studies

IRS-1 from KB cells was immobilized with monoclonal anti-human IRS-1 antibody (6G5) bound to protein G–agarose beads and was tyrosine-phosphorylated by partially purified activated human insulin receptors for 2 h at 4°C in the presence of 100 μM ATP, 10 mM $MgCl_2$ and 3 mM $MnCl_2$. Lysates from quiescent KB cells were preincubated for 2 h at 4°C in the absence or presence of the indicated synthetic peptides (final concentration 0.1 mM). Synthesis and purification of phosphorylated and nonphosphorylated peptides were performed as described previously (Chavanieu *et al.*, 1991; Yonezawa *et al.*, 1992b). The phosphopeptide containing the YVNI motif and the YIDL motif were synthesized by Fujiya Co. Ltd (Hatano, Japan). The lysates were added to immobilized tyrosine-phosphorylated IRS-1 on protein G–agarose beads for 2 h at 4°C and the beads were washed and IRS-1-associated PI 3-kinase activity was determined as described above.

Expression, purification and binding of recombinant proteins

Plasmids for the expression of full-length wild-type p85α (Wp85) or p85α lacking the p110 binding site (Δp85) in bacterial cells were constructed as follows. For Wp85, a PCR product (A1) encompassing nucleotides 1–1026 was introduced with a *Bam*HI site at the 5′ end and an *Eco*RI site at the 3′ end. A unique *Stu*I site in the p85α cDNA (43 bp downstream of the stop codon) was modified by the addition of *Eco*RI linkers and then digested with *Xho*I and *Eco*RI. This *Xho*I–*Eco*RI fragment of p85α and a *Bam*HI–*Xho*I fragment of A1 were ligated into the *Bam*HI–*Eco*RI site of the pGEX-2T plasmid (pGEX-Wp85α). For Δp85, a *Bam*HI–*Xho*I fragment of A1 and an *Xho*I–*Eco*RI fragment of the SRα-Δp85 plasmid, which was constructed as described previously (Hara *et al.*, submitted), were ligated into the *Bam*HI–*Eco*RI site of the pGEX-2T plasmid (pGEX-Δp85). Expression of Δp85 results in the deletion of 35 amino acids from 479 to 513 of bovine p85α and the insertion of two other amino acids (Ser-Arg) in this deleted region. To express and isolate GST–p85 fusion proteins, *Escherichia coli* transformed with pGEX-Wp85, pGEX-Δp85 or pGEX alone was incubated with 0.1 mM isopropyl 1-thio-β, D-galactopyranoside for 4 h at 37°C, and expressed GST–p85 fusion proteins were isolated from the bacterial lysates by incubation with glutathione–Sepharose beads. These GST fusion proteins were eluted with 5 mM reduced glutathione at 4°C for 2 min and incubated with bovine thrombin (final concentration 1.5 U/ml; Sigma) at 4°C for 12 h in the buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 1 mM dithiothreitol to cleave GST. The supernatant, containing the majority of the p85 proteins, was treated with *p*-aminobenzamidine agarose beads (Sigma) to remove thrombin. Protein concentrations were determined with bovine serum albumin as a standard by densitometric tracing of protein bands stained with Coomassie Brilliant Blue on an SDS–polyacrylamide gel as described previously (Weber *et al.*, 1972).

To examine binding of IRS-1 or PI 3-kinase activity, purified GST alone, GST–Wp85 or GST–Δp85 fusion proteins were incubated with 10 μl glutathione–Sepharose beads for 4 h at 4°C. Proteins bound to the beads were ~1 μg/μl. Lysates (1 ml) from quiescent KB cells (1×10^7 cells), with or without insulin (10^{-7} M) treatment, were incubated with the beads (10 μl) for 8 h at 4°C. The beads were washed and Western blotted with anti-phosphotyrosine antibodies, or PI 3-kinase activity on the beads was measured as described above.

Wortmannin experiments

Wortmannin was kindly provided by Dr Y.Matsuda (Kyowa Hakko Kogyo Co. Ltd, Machida, Japan). Stock solution of wortmannin (10 mM) was prepared in Me_2SO, stored at −20°C in the dark and diluted with the incubation medium just before use. KB cells were treated with the indicated concentration of wortmannin at 37°C for 10 min, then cells were treated with 10^{-7} M insulin or IGF-1. After 5 min, membrane ruffling and PI 3-kinase activity in immunoprecipitates with anti-phosphotyrosine antibodies were evaluated.

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