Specific activity of phosphatidylinositol 3-kinase is increased by insulin stimulation

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We investigated whether phosphatidylinositol 3-kinase (PI3K) is phosphorylated and whether its specific activity is increased by insulin stimulation *in vivo* using Fao cells and antibodies raised against the 85 kDa subunit of PI3K, insulin-receptor substrate-1 (IRS-1), and phosphotyrosine (pTyr). PI3K activity was detected in the immunoprecipitate produced with anti-PI3K at a basal state. The activity was increased 2–3-fold by insulin stimulation, although the protein concentration of kinase in the anti-PI3K immunoprecipitates was the same before and after insulin stimulation. Both anti-pTyr and anti-IRS-1 antibodies immunoprecipitated the kinase activity only after insulin stimulation. After the first immunoprecipitation with anti-pTyr, the supernatant was immunoprecipitated once more with anti-PI3K. PI3K activity in the second immunoprecipitate revealed little difference between the basal and insulin-stimulated states, suggesting that most of the insulin-activated portion of PI3K was precipitated by anti-pTyr. Both IRS-1 and the insulin-receptor β-subunit (95 kDa) were phosphorylated on tyrosine residues by insulin stimulation and immunoprecipitated with anti-pTyr. However, phosphorylation of neither subunit of PI3K (85 kDa or 110 kDa) was detectable in the immunoprecipitate produced with anti-pTyr. The 185 kDa pTyr-containing protein was immunoprecipitated with anti-PI3K after insulin stimulation, although there was little phosphorylation of the 85 kDa protein. pTyr in the 110 kDa protein immunoprecipitated with anti-PI3K was below detectable levels. These results indicate that the specific activity of PI3K is increased by insulin stimulation without detectable tyrosine phosphorylation of PI3K itself in Fao cells. The majority of the insulin-activated portion of PI3K is associated with pTyr-containing proteins including IRS-1, which suggests that this is important for activation of PI3K by insulin.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI3K; EC 2.7.1.137) phosphorylates the D-3 position of the inositol ring to produce phosphatidylinositol-3-phosphate (PIP) and its analogues [1–3]. PI3K consists of two major subunits of molecular masses 85 kDa and 110 kDa [4]. Cloning of the cDNA for 85 kDa subunit has revealed that the protein contains one SH3 and two SH2 regions (*src* homology regions) [5–7]. This enzyme associates with $pp60^{v\text{-}src}$ [8,9], polyoma middle-T-antigen (MTAg)–$pp60^{c\text{-}src}$ complexes [1,2,10], and receptors with tyrosine kinase [11–13], although the function of the polyphosphoinositide products in cell activation and proliferation remains unclear.

The current model of insulin signal transduction across the membrane is based on the tyrosine kinase nature of the insulin receptor [14]. According to this model, insulin binds to the extracellular α-subunit of the receptor, activating the tyrosine kinase of the transmembrane β-subunit. A number of proteins are tyrosine-phosphorylated in an insulin-dependent manner [15]. Recently, one of the major endogenous substrates of insulin receptor kinase, pp185, has been purified and its cDNA sequence reported as insulin-receptor substrate-1 (IRS-1) [16]. Furthermore, it has also been reported that PI3K rapidly associates with IRS-1 upon insulin stimulation. The significance of this association and the events subsequent to it, however, remain unknown. These issues are important to elucidate the role of IRS-1 in insulin signal transduction. The present study, using Fao cells, was therefore designed to examine whether PI3K is phosphorylated and whether its specific activity is increased by the association with IRS-1.

EXPERIMENTAL

Materials

$[^{32}P]P_i$ and $[\gamma\text{-}^{32}P]ATP$ were purchased from New England Nuclear; $Tran^{35}S$-label was from ICN; wheat germ agglutinin (WGA)–Sepharose was from Pharmacia; reagents for SDS/PAGE and the Bradford protein assay were from Bio-Rad Laboratories; human insulin was from NOVO (Bagsvaerd, Denmark); Nonidet P-40, phosphatidylinositol (bovine liver), phenylmethanesulphonyl fluoride (PMSF), aprotinin and other agents were from Sigma.

Antibodies

The anti-PI3K antibody was raised against the peptide (residues 599–616) of the 85 kDa subunit of murine PI3K [5], which is identical to the corresponding residues of the 85 kDa subunits of human [6] and bovine PI3K [7]. The anti-IRS-1 antibody was raised against the peptide (residues 489–507) of IRS-1, referred to as pep80 [16]. These antibodies were prepared by immunizing rabbits with the synthetic peptides coupled to keyhole-limpet haemocyanin. The antiphosphotyrosine (pTyr) antibody was prepared according to Pang et al. and Okamoto et al. [17,18].

Abbreviations used: PI3K, phosphatidylinositol 3-kinase; IRS-1, insulin-receptor substrate-1; pTyr, phosphotyrosine; WGA, wheat germ agglutinin; PMSF, phenylmethanesulphonyl fluoride; PIP, phosphatidylinositol-3-phosphate; TBS, Tris-buffered saline; T-TBS, TBS supplemented with 0.05% Tween-20; PDGF, platelet-derived growth factor; MTAg, polyoma middle-T-antigen; src, Rous sarcoma virus oncogene product.

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Assay of PI3K activity

Assay of PI3K in the immunoprecipitates was performed using the method of Whitman et al. [10] as modified by Auger et al. and White and Backer [3,19]. In brief, after incubation with insulin for 10 min, Fao cells were frozen in liquid nitrogen, lysed in buffer containing 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 20 mM Tris/HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM sodium vanadate, 1 mM PMSF, 0.1 mg/ml aprotinin, and 1 μg/ml leupeptin, and then centrifuged (15000 *g*, 20 min). The supernatant was immunoprecipitated with the indicated antibody for 2 h, and then for 1 h with protein A–Sepharose at 4 °C. The immunoprecipitates were washed at 4 °C, twice with PBS containing 1% (v/v) Nonidet P-40 and 100 μM sodium vanadate, twice with 100 mM Tris/HCl (pH 7.5) containing 500 mM LiCl, and twice with 10 mM Tris/HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 μM sodium vanadate. The pellets were suspended in 50 μl of 10 mM Tris/HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA and 100 μM sodium vanadate, and PI3K activity was assayed directly on the beads at 15 °C. The reaction was initiated by the addition of 20 μM ATP (containing 10 μCi of [γ-^{32}P]ATP), 10 mM $MgCl_2$ and 10 μg of phosphatidylinositol/tube, and was stopped with 25 μl of 6 M HCl. After extraction with chloroform/methanol (1:1), phospholipids in the organic phase were separated by t.l.c. in methanol/chloroform/ammonia/water (100:70:15:25, by vol.), followed by autoradiography. The radioactivity in PIP was quantified by liquid scintillation counting.

Western-blot analysis

The proteins in the anti-PI3K immunoprecipitates described above were heated in boiling water for 3 min with Laemmli sample buffer, separated with SDS/PAGE, and transferred on to nitrocellulose papers (80 V for 16 h at 15 °C) using a Bio-Rad Trans Blot system. The papers were soaked in Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 7.4, 150 mM NaCl) for 30 min, then 10% (v/v) non-fat milk for 2 h at room temperature, washed three times with TBS (10 min each wash), and incubated with anti-PI3K in TBS for 10–16 h at 4 °C. After washing three times with T-TBS (TBS supplemented with 0.05% Tween-20), the sheets were incubated with ^{125}I-labelled protein A [5×10^5 c.p.m./ml, 10% (v/v) non-fat milk in T-TBS] for 3 h at room temperature, extensively washed with T-TBS, dried, autoradiographed, and densitometrically scanned as described previously [18].

Labelling and immunoprecipitations

Confluent cells were cultured in fetal-bovine-serum-free medium for 16 h, then incubated in phosphate-free or methionine-free RPMI-1640 medium. After labelling the cells with [^{32}P]P_i (final concentration of 400 μCi/ml, for 90 min at 37 °C) or with Tran^{35}S-label (final concentration of 100 μCi/ml for 12 h at 37 °C), insulin (10^{-7} M) was added for 10 min. The cells were washed, frozen in liquid nitrogen, and solubilized with 1% (v/v) Nonidet P-40 in 50 mM Hepes/100 mM NaF/10 mM $Na_4P_2O_7$/2 mM sodium vanadate/5 mM EDTA. The samples were then centrifuged at 200000 *g* for 60 min at 4 °C. The supernatant was immunoprecipitated with anti-PI3K, anti-IRS-1, or anti-pTyr antibodies, washed, then separated by SDS/PAGE, autoradiographed (with EN^{3}HANCE from NEN in the case of ^{35}S) and quantified as described previously [19,20].

Analysis of phosphorylated amino-acid residues

Tryptic phosphopeptides were obtained from the protein bands in polyacrylamide gel fragments as described previously [21]. Gel fragments containing phosphorylated proteins were excised, washed for 12 h at 37 °C in 20% (v/v) methanol, dried at 80 °C for 2 h, and digested with 2 ml of 50 mM NH_4HCO_3 containing 100 μg of trypsin (pH 8.0). After incubation for 6 h at 37 °C, another 100 μg of trypsin was added, and the enzymic digestion was continued for an additional 16 h. The supernatant solution was freeze-dried, and the phosphopeptides were dissolved in 100 μl of 6 M HCl and hydrolysed for 1.5 h at 110 °C. The hydrolysate was freeze-dried and dissolved in a phosphoamino-acid standard solution containing phosphoserine, phosphothreonine, and pTyr. The mixtures of the phosphoamino-acid residues were then separated by high-voltage electrophoresis on cellulose thin-layer plates (1000 V for 70 min at 15 °C) using a solution of water/acetic acid/pyridine (89:10:1, by vol.). Each phosphoamino-acid spot was identified by reaction with ninhydrin, and the radioactivity was located by autoradiography.

Two-dimensional phosphopeptide mapping

The phosphopeptides were eluted from the gel fragments as described above with 95% efficiency, and were dissolved in 0.1% trifluoroacetic acid. The phosphopeptides were then separated on cellulose thin-layer plates by electrophoresis (400 V for 70 min at 15 °C) in 30% (v/v) formic acid, and then by ascending chromatography in pyridine/butanol/acetic acid/water (40:60:12:48, by vol.). Phosphopeptide spots were located by autoradiography as described previously [22].

RESULTS AND DISCUSSION

PI3K activities in the immunoprecipitates

PI3K activity in the immunoprecipitates was analysed by t.l.c. (Figure 1a). The activity was barely detected in the immunoprecipitates prepared with control serum both before and after insulin stimulation. A significant amount of the kinase activity was detected in the immunoprecipitate produced with anti-PI3K before insulin stimulation. The activity was further increased 2–3-fold after insulin stimulation. It has been reported that insulin causes an increase in PI3K activity in the anti-pTyr immunoprecipitates [23–27]. Those observations might simply reflect increased recovery of PI3K in the immunoprecipitates by the antibodies, because increased amounts of the 85 kDa subunit of PI3K in the anti-pTyr immunoprecipitates have been demonstrated with anti-PI3K after platelet-derived-growth-factor (PDGF) stimulation, and PI3K assays of anti-p85 immunoprecipitates did not demonstrate a major increase in activity after PDGF treatment [28]. Translocation of PI3K might be important for insulin signal transduction with PI3K [29]. However, it remains unknown whether specific activity (activity/amount of protein) of PI3K is increased by insulin stimulation, since the protein concentration of PI3K in the anti-pTyr and anti-IRS-1 immunoprecipitates was not determined by Western blotting with anti-PI3K in previous reports [23–27]. To measure the alteration in specific activity of PI3K, the protein concentration of the 85 kDa subunit of PI3K in the anti-PI3K immunoprecipitates was estimated by Western blotting (Figure 1b). It was not changed after insulin stimulation, suggesting that the specific activity of PI3K is increased with insulin stimulation. If the 110 kDa (catalytic) and 85 kDa (binding) subunits can exist separately and if the 110 kDa subunit can bind to tyrosine-phosphorylated proteins without the 85 kDa subunit, the amount of the 85 kDa subunit estimated by the Western blotting may not reflect the amount of the 110 kDa subunit. However, our result was consistent with that of Hayashi et al., who reported that an

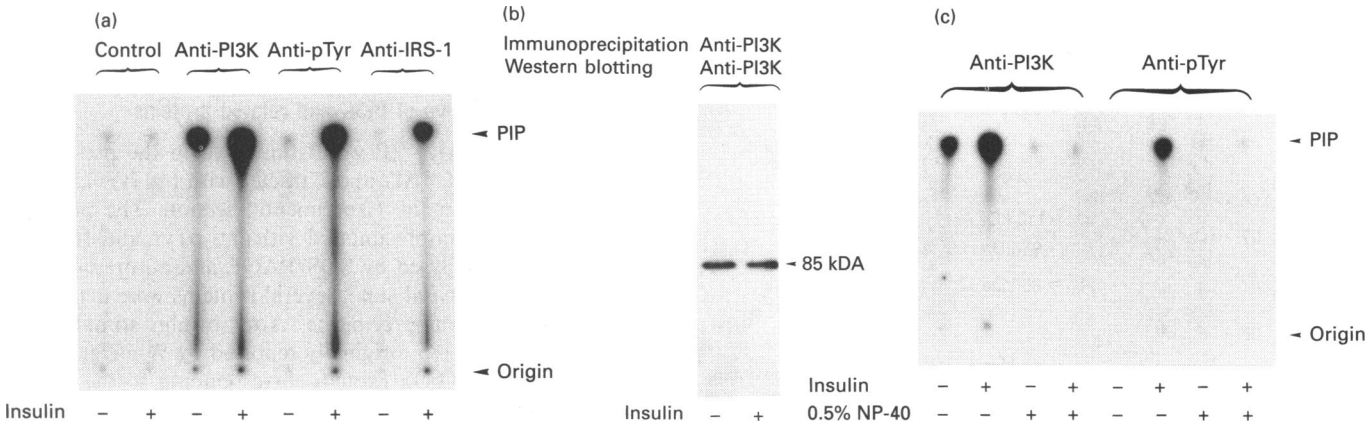

Figure 1 T.l.c. (a) and Western blotting (b) of immunoprecipitates showing PI3K activity, and the effect of detergent on this activity (c)

(**a**) After stimulation with insulin (10^{-7} M) for 10 min, Fao cells were frozen in liquid nitrogen, and extracted with 1% (v/v) Nonidet P-40 as described in the Experimental section. Extracted supernatants were immunoprecipitated with the indicated antibodies, and the resulting precipitates were incubated with [γ-^{32}P]ATP, and 10 μg of phosphatidylinositol/tube. The resulting phosphoinositides were extracted with chloroform/methanol and analysed by t.l.c. The first two lanes are control serum before and after insulin stimulation. (**b**) The protein concentration of PI3K in the anti-PI3K immunoprecipitates before and after insulin stimulation was determined by Western blotting using anti-PI3K antibody. Anti-PI3K immunoprecipitates were prepared as described above. The proteins in the immunoprecipitates were separated by SDS/PAGE, followed by Western blotting with anti-PI3K as described in the Experimental section. An autoradiogram of the nitrocellulose paper is shown. (**c**) PI3K activity was immunoprecipitated as described in (**a**). Then a PI3K assay was performed in the absence or presence of Nonidet P-40 (NP-40) (final concentration, 0.5%). The resulting phosphopeptides were analysed by t.l.c. as described in (**a**).

increase in the enzyme activity was detected in response to insulin not only in the anti-pTyr immunoprecipitates, but also in the cytosolic fraction before immunoprecipitation [30].

The kinase activities in the immunoprecipitates produced with anti-pTyr and anti-IRS-1 before insulin stimulation were as low as that in the immunoprecipitate produced with control serum (Figure 1a). A marked increase in PI3K activity, however, was observed in the immunoprecipitates with these antibodies after insulin stimulation. The ratios of basal/insulin-stimulated kinase activity in the immunoprecipitates produced with anti-pTyr and anti-IRS-1 were more than 30-fold and 20-fold respectively. These results confirmed previous studies which suggested that PI3K is tightly associated with tyrosine phosphoproteins, including IRS-1, after insulin stimulation [16].

To confirm that the enzymic activity shown in Figure 1(a) is due to the PI3K activity, the effect of a non-ionic detergent on activity was examined, since the PI3K activity is inhibited completely by the presence of more than 0.2% Nonidet P-40 [13,23]. The addition of Nonidet P-40 to the reaction mixture caused a complete inhibition of the activity to the basal level in the anti-pTyr immunoprecipitates. It is noteworthy that the activities in the anti-PI3K immunoprecipitates, both before and after insulin stimulation, were inhibited to the same level as those in anti-pTyr immunoprecipitates by Nonidet P-40. These observations indicate that not only the activity in the anti-pTyr immunoprecipitates after insulin stimulation, but also the activity in anti-PI3K immunoprecipitates, both before and after insulin stimulation, are due to PI3K activity.

Sequential immunoprecipitation with anti-pTyr and other antibodies

To study whether all of the insulin-activated forms of PI3K are associated with tyrosine phosphoproteins, a Nonidet P-40 extract of the insulin-stimulated cells was sequentially immunoprecipitated. After the first immunoprecipitation with anti-pTyr, the supernatant was again immunoprecipitated with anti-pTyr, anti-IRS-1, or anti-PI3K. The PI3K activity in the precipitates was

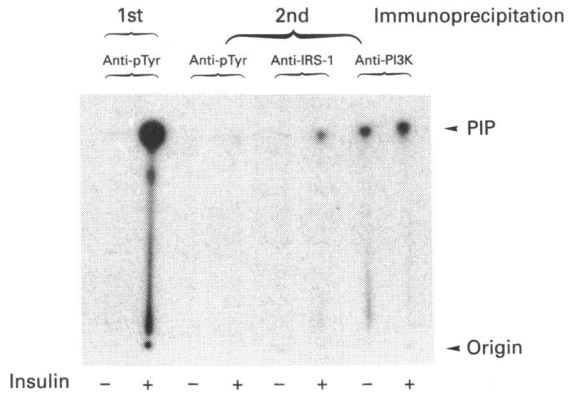

Figure 2 PI3K activities in the immunoprecipitates after sequential immunoprecipitation

Procedures were identical to those described in the legend of Figure 1, except that Nonidet P-40 extracts were sequentially immunoprecipitated with the indicated antibodies.

determined as described above (Figure 2). The kinase activity in the first immunoprecipitate was increased more than 30-fold after insulin stimulation, which is in agreement with the results shown in Figure 1(a). PI3K activity in the second immunoprecipitate with anti-pTyr revealed that more than 95% of the anti-pTyr-immunoprecipitable kinase activity from the insulin-stimulated cells was immunoprecipitated by the first immunoprecipitation. A small amount of kinase activity was detected in the second immunoprecipitation with anti-IRS-1 after insulin stimulation. However, the activity was markedly decreased in comparison with that in Figure 1(a), suggesting that a large portion of the anti-IRS-1-precipitable PI3K activity was removed by the first immunoprecipitation with anti-pTyr. The insulin-induced increase in the kinase activity in the immunoprecipitates produced with anti-PI3K (see Figure 1a) was lost in the second immunoprecipitate with anti-PI3K. These results indicate that the

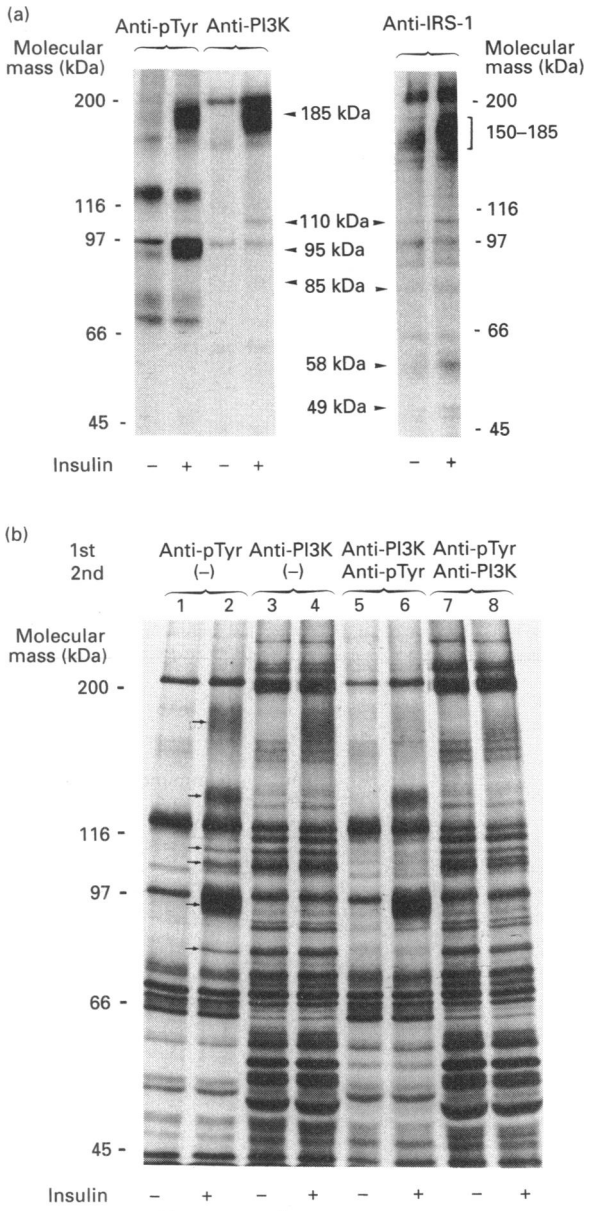

Figure 3 Autoradiograms after SDS/PAGE showing (a) phosphorylated proteins in the immunoprecipitates and (b) ^{35}S-labelled proteins in the immunoprecipitates produced with anti-pTyr and anti-PI3-kinase

(**a**) Fao cells labelled with [^{32}P]P_i for 90 min were stimulated with or without insulin (10^{-7} M), and extracted with 1% (v/v) Nonidet P-40 as described in the Experimental section. Phosphoproteins were immunoprecipitated with the indicated antibodies, and then analysed by SDS/PAGE and autoradiography. (**b**) After labelling the cells with Tran^{35}S-label for 12 h, insulin (10^{-7} M) was added for 10 min, and extracted with 1% (v/v) Nonidet P-40. The ^{35}S-labelled proteins were immunoprecipitated with anti-pTyr or anti-PI3K, and then analysed by SDS/PAGE and autoradiography (lanes 1–4). Furthermore, the supernatant from the first immunoprecipitation with anti-PI3K was subjected to the second immunoprecipitation with anti-pTyr, and the supernatant from the first immunoprecipitation with anti-pTyr was subjected to the second immunoprecipitation with anti-PI3K (lanes 5–8).

majority of the insulin-activated portion of PI3K was immunoprecipitated with anti-pTyr, suggesting that insulin-activated PI3K associates with IRS-1 or tyrosine phosphoproteins. It seems that this association is important for increasing the specific activity of the PI3K by insulin stimulation. However, at this moment, it is still unclear whether PI3K itself is phosphorylated on tyrosine residues, or simply associates with tyrosine phosphoproteins. To clarify this point phosphorylated proteins were analysed *in vivo*.

Phosphorylation *in vivo* of PI3K and related proteins

Fao cells labelled with ^{32}P were stimulated in the presence or absence of insulin (10^{-7} M), and extracted with 1% (v/v) Nonidet P-40 as described in the Experimental section. The phosphoproteins were immunoprecipitated with anti-pTyr, anti-IRS-1, or anti-PI3K, then analysed by SDS/PAGE and autoradiography (Figure 3a). In the basal state, several proteins were detected in the anti-pTyr immunoprecipitate. After insulin stimulation a 185 kDa protein pp185, originally reported by White et al. [31], appeared, and a 95 kDa protein corresponding to the insulin-receptor β-subunit increased, whereas the phosphorylation of other proteins was not affected. Phosphorylation of both the 85 kDa and the 110 kDa subunits of PI3K was barely detectable (less than 5% of the 185 kDa protein). In the anti-PI3K immunoprecipitates, a 185 kDa phosphoprotein was observed after insulin stimulation. A 110 kDa protein was also immunoprecipitated with anti-PI3K. However, the level of phosphorylation was quite low compared with that of the 185 kDa protein in the same precipitate (less than 5%), or compared with that of the 185 kDa and 95 kDa proteins immunoprecipitated with anti-pTyr. It is not clear, at this moment, whether this protein is the 110 kDa subunit of PI3K. Phosphorylation of the 85 kDa subunit of PI3K was slight (less than 20% of the 110 kDa protein), and of the 95 kDa β-subunit of the insulin receptor in the immunoprecipitates produced with anti-PI3K was below the detectable level.

PI3K was previously reported to be tyrosine-phosphorylated upon MTAg transformation [32], PDGF stimulation [3,32], or colony-stimulating factor 1 stimulation of cells [12]. Recently, Hayashi et al. reported that the insulin-stimulated tyrosine kinase of the receptor phosphorylates the 85 kDa subunit of purified PI3K, and they suggested that tyrosine phosphorylation of PI3K by the insulin receptor kinase increases the specific activity of the enzyme [30]. However, in our studies *in vivo*, tyrosine phosphorylation of the 85 kDa and 110 kDa subunits of PI3K was barely detectable even after insulin stimulation. Recent studies have demonstrated that activation of PI3K by PDGF occurs without tyrosine phosphorylation of the 85 kDa subunit [28]. Therefore, the activating mechanism suggested by Hayashi et al. may play a minor role *in vivo*. However, the 85 kDa subunit is phosphorylated on tyrosine residues in cells that transiently overexpress the 85 kDa subunit of the kinase and the PDGF receptor [28]. Since PI3K is not an abundant protein, it is possible that labelling was not sufficient to make it detectable even if it occurred.

PI3K associates physically with molecules with tyrosine kinase as described in the introductory statement. However, according to the present results, insulin receptors immunoprecipitated with anti-PI3K were undetectable. In agreement with our results, previous studies have shown that a significant amount of PI3K activity was not immunoprecipitated with anti-insulin receptor antibodies [23,24,33]. These results suggest that only a small portion of PI3K associates with the insulin receptor, or that affinity of PI3K to the insulin receptor is relatively low *in vivo*.

In the basal state, a broad (150–175 kDa) band was detected together with several other phosphorylated proteins in the anti-IRS-1 immunoprecipitate. After insulin stimulation, the band was increased, with a slightly slower mobility on SDS/PAGE (150–185 kDa) (Figure 3a). Anti-IRS-1 was raised against pep80 (a portion of IRS-1), which is a component of pp185. This antibody clearly immunoprecipitated the PI3K activity (see Figure 1a), and pTyr was detected in the 150–185 kDa protein

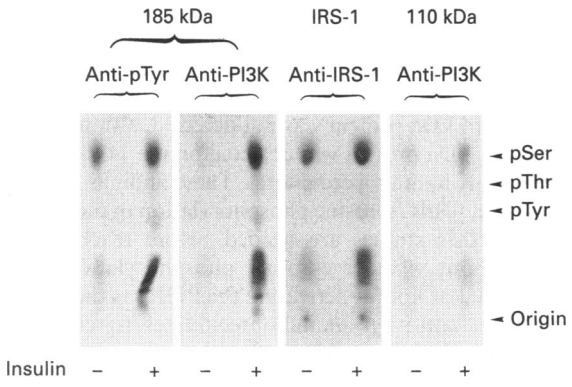

Figure 4 Phosphoamino-acid analysis

The bands corresponding to the 185 kDa proteins in the immunoprecipitate with anti-pTyr and anti-PI3K, the IRS-1, and the 110 kDa protein were excised from SDS/polyacrylamide gel. The fragments were then trypsinized to elute labelled peptides and partially hydrolysed with 6 M HCl for 90 min. Phosphoamino acids were separated by high-voltage electrophoresis on a cellulose thin-layer plate as described in the Experimental section. Abbreviations: pSer, phosphoserine; pThr, phosphothreonine.

after insulin stimulation (results shown in Figure 4) although the band of phosphoproteins immunoprecipitated with anti-IRS-1 was broad and migrated slightly more rapidly on SDS/polyacrylamide gels than pp185 in the anti-pTyr immunoprecipitate. There are at least two explanations for these results. One is that pp185 consists of at least two proteins, both of which can bind to PI3K after insulin stimulation. The other is that our anti-IRS-1 antibody recognizes only part of IRS-1 (a non- or partially tyrosine-phosphorylated form). IRS-1 consists of a lot of 'turn' sequences as determined by Chou and Fasman analysis [34], and pep80 is not as hydrophilic, as determined by Kite–Doolittle plot. These characteristics could induce a conformational change in the pep80 portion of IRS-1 due to insulin stimulation, so that highly tyrosine-phosphorylated IRS-1 would not be recognized by our anti-IRS-1 antibody.

Phosphorylation of several other proteins was also increased by insulin stimulation (110 kDa, 85 kDa, 58 kDa, and 49 kDa) in anti-IRS-1 immunoprecipitates. Phosphorylation of the 110 kDa protein was less than 5% of IRS-1. Phosphorylation of the 85 kDa protein was 60% of the 110 kDa protein in the anti-IRS-1 immunoprecipitate, although it was less than 20% of the 110 kDa protein in the anti-PI3K immunoprecipitate. Further characteristics of the 58 kDa and 49 kDa proteins are unknown. The β-subunit of the insulin receptor in this immunoprecipitate was below the detectable level.

^{35}S-labelled proteins in the immunoprecipitates produced with anti-pTyr and anti-PI3K

The amount of the 110 kDa (catalytic) subunit in the immunoprecipitates was not determined in previous reports. It is still unclear whether the 110 kDa (catalytic) and 85 kDa (binding) subunit can exist separately, or whether the 110 kDa subunit can bind to tyrosine-phosphorylated proteins without the 85 kDa subunit. To solve these problems and to confirm the findings described above, the amounts of PI3K, IRS-1, insulin receptor, and other proteins in the immunoprecipitates were evaluated by ^{35}S-labelling (Figure 3b).

In the anti-pTyr immunoprecipitates, several proteins were detected before insulin stimulation. After insulin stimulation, several other proteins appeared (185 kDa, 135 kDa, 114 kDa, 110 kDa, 95 kDa, 85 kDa which are indicated by the arrows in Figure 3b). Among these proteins, the 135 kDa, 114 kDa, 110 kDa, and 85 kDa proteins were barely phosphorylated by insulin stimulation (cf. Figure 3a). The 185 kDa protein is supposed to be pp185, and the 135 kDa and the 95 kDa proteins are the α- and β-subunits of the insulin receptor respectively.

The 185 kDa protein was detected in the anti-PI3K immunoprecipitates only after insulin stimulation, which directly confirmed the association of the 185 kDa protein with PI3K after insulin stimulation. When the supernatant of the first immunoprecipitation produced with anti-PI3K was subjected to the second immunoprecipitation with anti-pTyr, the amount of the 185 kDa protein in the immunoprecipitates was considerably decreased (lane 6 in Figure 3b) in comparison with that in the first immunoprecipitates with anti-pTyr (lane 2). When the supernatant of the first immunoprecipitation with anti-pTyr was subjected to the second immunoprecipitation with anti-PI3K, the amount of 185 kDa protein in the immunoprecipitates was again dramatically decreased (lane 8) in comparison with that in the first immunoprecipitate with anti-PI3K (lane 4). These findings suggest that the 185 kDa proteins in the anti-pTyr immunoprecipitates and anti-PI3K immunoprecipitates are identical or closely related. A small amount of the 185 kDa protein was still observed in the second immunoprecipitate produced with anti-pTyr (lane 6, Figure 3b), which suggested that a small portion of the tyrosine-phosphorylated 185 kDa protein is not associated with PI3K. A small amount of the 185 kDa protein was observed in the second immunoprecipitate produced with anti-PI3K (lane 8, Figure 3b), which suggested that a small portion of the PI3K-associated 185 kDa protein was not immunoprecipitated with our anti-pTyr antibody.

As the 85 kDa subunit of PI3K has two SH2 regions and the 185 kDa protein in the anti-PI3K immunoprecipitate is supposed to be associated with PI3K by these two regions, it is reasonable to speculate that the amount of the 185 kDa protein in the anti-PI3K immunoprecipitates is less than two times the amount of PI3K in the same immunoprecipitates. In other words, the amount of PI3K is more than half of the 185 kDa protein. Therefore, the 85 kDa and 110 kDa subunits are expected to show explicit bands, as does the 185 kDa protein in the anti-PI3K immunoprecipitates. The three proteins detected in the anti-pTyr immunoprecipitates after insulin stimulation (114 kDa, 110 kDa, and 85 kDa) were clearly detected in the anti-PI3K immunoprecipitates before and after insulin stimulation. Furthermore, when the supernatant of the first immunoprecipitation by anti-PI3K was subjected to the second immunoprecipitation by anti-pTyr, these proteins were hardly detectable (compare lanes 1 and 2 with lanes 5 and 6 in Figure 3b), which suggests that these proteins were removed by the first immunoprecipitation with anti-PI3K. These findings strongly suggest that the 85 kDa protein is a subunit of PI3K. However, it is still unclear if both the 114 kDa and 110 kDa proteins are forms of PI3K (e.g. the 114 kDa protein is a non-phosphorylated form and the 110 kDa protein is a phosphorylated form), or if one of them is a subunit of PI3K and the other is another protein tightly associated with PI3K. Carpenter et al. identified two forms of the 110 kDa subunit in their purified PI3K preparation [4], and Auger et al. reported that both the 110 kDa doublet and the 85 kDa proteins are associated with the MTAg–pp60$^{c\text{-}src}$ complex [35]. Furthermore, according to Hiles et al., the Southern-blotting data of p110 genes suggest there may be two genes for PI3K in rats and humans [36]. So two forms of the 110 kDa subunit might be associated with the 185 kDa protein.

As Figure 3(b) shows, the amount of the 85 kDa subunit of PI3K in the anti-PI3K immunoprecipitates are the same before

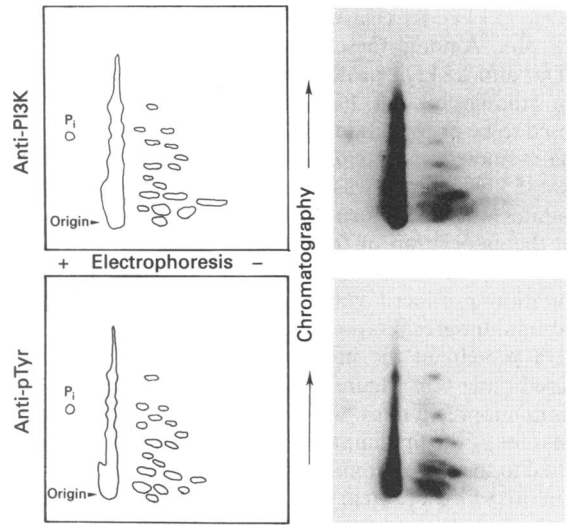

Figure 5 Two-dimensional tryptic peptide mapping of the 185 kDa proteins

The bands corresponding to the 185 kDa protein in the immunoprecipitate with anti-PI3K and with anti-pTyr were excised from SDS/polyacrylamide gel. The phosphopeptides were eluted from the gel fragments as described in the Experimental section. The phosphoamino acids were then separated on cellulose thin-layer plates by electrophoresis and then by ascending chromatography. Phosphopeptide spots were located by autoradiography. Results from the anti-PI3K immunoprecipitates and from the anti-pTyr immunoprecipitates were shown in the upper and lower panel respectively. In each panel, the autoradiogram is shown on the right and the corresponding schematic diagram of phosphorylated spots is shown on the left.

and after insulin stimulation. The amounts of both the 114 kDa and 110 kDa proteins in the anti-PI3K immunoprecipitates were also the same before and after insulin stimulation, which indicates that the same amount of catalytic subunit(s) of PI3K was present in the anti-PI3K immunoprecipitates before and after insulin stimulation. These findings further support the view that the increase in the kinase activity in the anti-PI3K immunoprecipitates shown in Figure 1(a) was due to an increase in the specific activity of the kinase, and also that the phosphorylation of the 110 kDa protein detected in the anti-PI3K immunoprecipitates after insulin stimulation (cf. Figure 3a) was not due to an increase in the recovery of the protein but due to *de novo* phosphorylation.

The ratio of the amount of ^{35}S in the 85 kDa, 110 kDa, and 114 kDa proteins in the anti-PI3K immunoprecipitates (60:100:40) was the same before and after insulin stimulation, which suggested that the association state (ratio) of the 85 kDa, 110 kDa, and 114 kDa proteins was not affected by insulin stimulation. This ratio was similar in the anti-pTyr immunoprecipitates, which represented the portion of PI3K associated with pTyr-containing proteins after insulin stimulation. These findings indicate that these proteins are tightly associated and the association state (ratio) of the subunits is not affected by insulin stimulation or by the association of the complex to pTyr-containing protein(s), although the actual amount of these proteins is still unknown.

The amount of these proteins in the anti-pTyr immunoprecipitates after insulin stimulation was about 25% of those in the anti-PI3K immunoprecipitates, which indicates that only a part of the PI3K is associated with IRS-1 after insulin stimulation. This might be why phosphorylation of the 110 kDa protein was barely detectable in the anti-pTyr immunoprecipitates. However, the phosphorylation of the 85 kDa protein in the anti-PI3K immunoprecipitates could hardly be detected (see Figure 3a), although about 60% of ^{35}S on the 110 kDa protein was detected on the 85 kDa protein in the anti-PI3K immunoprecipitates (Figure 3b). The tyrosine phosphorylation of the 85 kDa protein was below the detectable level (results not shown). Phosphorylation of the 114 kDa protein was not detected, although 40% of ^{35}S on the 110 kDa protein was detected on the 114 kDa protein in the anti-PI3K immunoprecipitates. These findings suggest that there was little, if any, tyrosine phosphorylation of these proteins. However, further studies are needed before reaching a final conclusion about whether tyrosine phosphorylation of PI3K itself is essential or not for activating the PI3K, as the amount of the protein in anti-pTyr immunoprecipitates is relatively low (lane 2, Figure 3b).

The 135 kDa and 95 kDa proteins were not detected in the anti-PI3K immunoprecipitates (Figure 3b). When the supernatant of the anti-PI3K immunoprecipitate was subjected to the second immunoprecipitation with anti-pTyr, 135 kDa and 95 kDa insulin-receptor subunits were detected after insulin stimulation, and the amounts of these were comparable with those in the first immunoprecipitates with anti-pTyr (compare lanes 1 and 2 with lanes 5 and 6, Figure 3b). This supports the assumption that PI3K is hardly associated with the insulin receptor, or that the affinity of PI3K for the insulin receptor is relatively low *in vivo*.

Phosphoamino-acid analysis

PI3K itself was barely phosphorylated at tyrosine residues (Figures 3a and 3b), and it associates with tyrosine phosphoprotein(s) [Figures 1(a) and 2]. To test whether the 110 kDa protein is a non-tyrosine phosphoprotein, and whether the 185 kDa protein immunoprecipitated by anti-PI3K is a tyrosine phosphoprotein, phosphoamino-acid analysis was performed. The 185 kDa protein immunoprecipitated by anti-pTyr, pp185, and IRS-1 were also studied to confirm that both of them are phosphorylated on tyrosine residues by insulin stimulation (Figure 4). Phosphoamino-acid analysis of the 185 kDa protein in the immunoprecipitate produced with anti-pTyr revealed that the major phosphoamino-acid residue in the basal state was phosphoserine. pTyr was a significant constituent of the protein after insulin stimulation. In addition, the phosphoserine level was increased and a small amount of phosphothreonine appeared. It remains unknown whether these increases in phosphoserine and phosphothreonine arise from *de novo* phosphorylation or simply reflect improved recovery by the pTyr antibody following tyrosine phosphorylation. Phosphoamino-acid analysis of the 185 kDa protein in the immunoprecipitate with anti-PI3K gave results almost identical to those from the 185 kDa protein in the immunoprecipitates produced with anti-pTyr, except that phosphoserine levels at the basal state were somewhat lower than that in the latter. Anti-pTyr might immunoprecipitate some proteins with a molecular mass of about 185 kDa before insulin stimulation, which were not immunoprecipitated by anti-PI3K. pTyr and phosphoserine levels in the 150–185 kDa protein immunoprecipitated with anti-IRS-1 were increased by insulin stimulation. However, the amount of pTyr and the increase in phosphoserine after insulin stimulation were somewhat less than those of the 185 kDa proteins in the anti-pTyr and anti-PI3K immunoprecipitates, suggesting that anti-IRS-1 recognizes only a portion of the 185 kDa proteins immunoprecipitable with anti-pTyr or anti-PI3K. Phosphothreonine was detected in the basal state, which was not stimulated by insulin. Insulin stimulated phosphorylation of the 110 kDa protein in the immunoprecipitates produced with anti-PI3K mainly on serine residues, and pTyr was below detectable levels. The results again confirmed

that PI3K itself is phosphorylated only a little, if at all, on tyrosine residues by insulin stimulation.

Two-dimensional tryptic-peptide mapping

Both of the 185 kDa proteins immunoprecipitated with anti-pTyr and with anti-PI3K were phosphorylated on tyrosine residues after insulin stimulation (see Figure 4). To test whether these 185 kDa proteins are the same or not, two-dimensional phosphopeptide mapping of these proteins was performed (Figure 5). Before insulin stimulation, phosphopeptides were barely detectable (results not shown). After insulin stimulation, several phosphopeptides were detected. The pattern of the maps of the 185 kDa protein in the anti-pTyr immunoprecipitates and the 185 kDa protein in the anti-PI3K immunoprecipitates, were virtually identical, indicating that the predominant component(s) of these two 185 kDa proteins were identical.

In conclusion, this study indicates that the specific activity of PI3K is increased by insulin stimulation without significant tyrosine phosphorylation of PI3K itself. The majority of the insulin-activated portion of PI3K is associated with pTyr-containing protein including IRS-1, suggesting that association of PI3K with pTyr-containing protein is important in activation of PI3K by insulin.

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