Phosphorylation *in vitro* of the 85 kDa subunit of phosphatidylinositol 3-kinase and its possible activation by insulin receptor tyrosine kinase

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Insulin causes a dramatic and rapid increase in phosphatidylinositol 3-kinase activity in the anti-phosphotyrosine immunoprecipitates of cells overexpressing the human insulin receptor. This enzyme may therefore be a mediator of insulin signal transduction [Endemann, Yonezawa & Roth (1990) *J. Biol. Chem.* **265**, 396–400; Ruderman, Kapeller, White & Cantley (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1411–1415]. At least two questions remain to be elucidated. Firstly, does the insulin receptor tyrosine kinase phosphorylate phosphatidylinositol 3-kinase directly, or does it phosphorylate a protein associated with the 3-kinase? Second, if the enzyme is a direct substrate for the insulin receptor tyrosine kinase, does tyrosine phosphorylation of phosphatidylinositol 3-kinase by the kinase alter the specific enzyme activity, or does the amount of the tyrosine-phosphorylated form of the phosphatidylinositol 3-kinase increase, with no change in the specific activity? We report here evidence that the 85 kDa subunit of highly purified phosphatidylinositol 3-kinase is phosphorylated on the tyrosine residue by the activated normal insulin receptor *in vitro*, but not by a mutant insulin receptor which lacks tyrosine kinase activity. We found that an increase in enzyme activity was detected in response to insulin not only in the anti-phosphotyrosine immunoprecipitates of the cytosol, but also in the cytosolic fraction before immunoprecipitation. In addition, we partially separated the tyrosine-phosphorylated form from the unphosphorylated form of the enzyme, by using a f.p.l.c. Mono Q column. The insulin-stimulated phosphatidylinositol 3-kinase activity was mainly detected in the fraction containing almost all of the tyrosine-phosphorylated form. This result suggests that tyrosine phosphorylation of phosphatidylinositol 3-kinase by the insulin receptor kinase may increase the specific activity of the former enzyme *in vivo*.

INTRODUCTION

Among efforts made to elucidate the molecular mechanisms of insulin action, determination of the structure of the human insulin receptor, which is responsible for signal transduction from insulin to the cell interior, has provided valuable information [1,2]. The insulin receptor is a transmembrane glycoprotein composed of two α and two β subunits. Upon insulin binding to the extracellular domain of the α subunit, the β subunit becomes autophosphorylated at tyrosine residues [3], and the kinase activity of the β subunit increases [4,5]. Experiments with mutant receptors lacking tyrosine kinase activity [6,7] and the use of monoclonal antibodies which inhibit the kinase [8,9] have suggested that the tyrosine kinase activity of the receptor is essential for insulin signal transduction. Cell lines which overexpress normal or kinase-deficient insulin receptors are useful for elucidating the molecular mechanisms of insulin signal transduction [6,7]. Although several proteins were found to be potential substrates for the receptor tyrosine kinase [4,5], the physiological targets of the insulin receptor have yet to be elucidated.

Phosphatidylinositol (PI) 3-kinase, which phosphorylates the D-3 position of the inositol ring of PI to produce PI 3-phosphate (PI-3P) [10–12], has been found to specifically associate with certain tyrosine kinases. These include the polyoma virus middle-size tumour antigen (middle T)–pp60^{c-src} complex [10–15], pp60^{v-src} [16,17], the platelet-derived growth factor receptor

[18–20], the colony-stimulating factor-1 receptor [21], the epidermal growth factor receptor [22,23] and *v-ros* [24,25]. Since the tyrosine kinase domain of the insulin receptor β subunit shares a partial sequence similarity with the protein encoded by *v-ros* [1,2], the activity of PI 3-kinase was examined after exposing the cells to insulin. Two different groups of investigators [26,27] reported that the activity of PI 3-kinase in the anti-phosphotyrosine immunoprecipitates was greatly increased after adding insulin to cells overexpressing the human insulin receptor. These observations suggested that PI 3-kinase itself, or a protein associated with this enzyme, acts as a substrate for the insulin receptor tyrosine kinase, and that tyrosine phosphorylation of the PI 3-kinase by the receptor kinase may activate the former enzyme.

To further elucidate the relationship between the insulin receptor tyrosine kinase and PI 3-kinase activity, we examined phosphorylation of the purified PI 3-kinase by insulin receptors *in vitro*. We found that the 85 kDa subunit of the purified PI 3-kinase was phosphorylated on the tyrosine residue *in vitro* by the insulin receptor tyrosine kinase, and we suggest that this phosphorylation may increase the specific activity of PI 3-kinase, as deduced from experiments *in vivo*.

EXPERIMENTAL

Cells and materials

The cell lines used in this study were: (1) CHO-HIR, Chinese hamster ovary (CHO) cells overexpressing the normal human

Abbreviations used: PI, phosphatidylinositol; PIP, PI monophosphate; PI-3P, PI 3-phosphate; PI-4P, PI 4-phosphate; gPI-3P, glycerophosphatidylinositol 3-phosphate; gPI-4P, gPI 4-phosphate; CHO, Chinese hamster ovary; NP40, Nonidet P40; DTT, dithiothreitol; middle T, polyoma virus middle-sized tumour antigen.

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insulin receptor; and (2) CHO-HIR^{K→M}, CHO cells over-expressing a mutated human insulin receptor which lacks tyrosine kinase activity owing to substitution of methionine for lysine-1030 in the presumed ATP-binding region [6]. PI 3-kinase was purified to near-homogeneity as described [28]. Anti-(human insulin receptor) monoclonal antibodies 1G2 and 5D9 [29] and anti-phosphotyrosine monoclonal antibody PY20 [30] were kindly donated by Dr. R. A. Roth (Stanford University), and Dr. J. R. Glenney, Jr. (University of Kentucky) respectively.

Phosphorylation of PI 3-kinase *in vitro* by normal or mutated insulin receptor kinases

In vitro phosphorylation was performed in a 96-well plate according to the method of Morgan & Roth [31]. Confluent CHO-HIR^{K→M} or CHO-HIR cells in 100 mm dishes were incubated for 10 min at 37 °C in the presence or the absence of 0.1 μM-insulin, washed once with Hepes-buffered saline (pH 7.4) at 4 °C and lysed with 1 ml of lysis buffer [50 mM-Hepes, pH 7.5, 150 mM-NaCl, 1 % Triton X-100, 20 μM-(*p*-amidinophenyl)-methanesulphonyl fluoride hydrochloride, 1 mg of bacitracin/ml, 5 mM-EDTA, 5 mM-EGTA, 20 mM-sodium pyrophosphate, 1 mM-orthovanadate and 20 mM-NaF]. After centrifugation at 15000 *g* for 20 min at 4 °C, the supernatants (100 μl portions) were placed into each well of a 96-well plate on to which anti-(human insulin receptor) antibody 1G2 had previously been adsorbed. After 4 h of incubation at 4 °C, the wells were washed four times with buffer (50 mM-Hepes, pH 7.5, 150 mM-NaCl, 0.1 % Triton X-100, 0.1 % Tween 20 and 1 mg of BSA/ml). The amount of adsorbed insulin receptor in a 96-well plate was 4.4 fmol of insulin-binding capacity, calculated using an insulin-binding assay. The kinase reaction was initiated by adding 1 μM-[γ-³²P]ATP (specific radioactivity 100 μCi/nmol), with various amount of PI 3-kinase, in 20 μl of kinase buffer (50 mM-Hepes, pH 7.5, 150 mM-NaCl, 5 mM-MgCl₂ and 5 mM-MnCl₂). After the indicated periods at room temperature, the reaction was stopped by adding 20 μl of 2 × SDS sample buffer (125 mM-Tris/HCl, pH 6.8, 4 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol and 0.002 % Bromophenol Blue) and the preparation was then electrophoresed on 6 % and 15 % polyarylamide gels in the presence of SDS [32]. The 85 kDa and 110 kDa subunits of PI 3-kinase were identified by silver staining and autoradiography.

Insulin-binding assay

CHO-HIR cell lysates were prepared as described above and placed into each well of a 96-well plate, on to which anti-(human insulin receptor) antibody 1G2 had previously been adsorbed. After extensive washing, the wells were incubated with 0.04 μCi of ¹²⁵I-labelled insulin (NEN) in the presence of various concentrations of unlabelled insulin for 4 h at 4 °C [31]. Unbound insulin was removed by extensive washing, and then the bound insulin was solubilized with 100 μl of 1 % SDS and counted for radioactivity in a γ-radiation counter. The number of insulin receptors adsorbed to the well was estimated from a Scatchard plot.

Phosphoamino acid analysis

Protein bands located by autoradiography were cut from the gel, extracted with 100 mM-NH₄HCO₃/0.2 % SDS and digested with 6 M-HCl for 1 h at 110 °C. Electrophoresis was carried out on cellulose plates (Merck) in pyridine acetate (pH 3.5) at 1000 V as described [33].

Assay of anti-phosphotyrosine-precipitated PI 3-kinase in cytosolic and particulate fractions

Confluent CHO-HIR^{K→M} or CHO-HIR cells in 100 mm dishes were incubated for 10 min at 37 °C in the presence or the absence

of 0.1 μM-insulin, and then washed once with Hepes-buffered saline (pH 7.4) at 4 °C. The cells from ten dishes were disrupted in 2 ml of buffer [20 mM-Tris/HCl, pH 7.4, 5 % glycerol, 0.5 mM-EDTA, 1 mM-orthovanadate and 20 μM-(*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride] using a Potter-Elvehjem homogenizer, and then sonicated (4 × 15 s bursts). The lysates were centrifuged at 200000 *g* for 20 min and the supernatants were used as the cytosolic fractions. The pellets were suspended in 2 ml of buffer [1 % Nonidet P40 (NP40), 20 mM-Tris/HCl, pH 7.4, 5 % glycerol, 0.5 mM-EDTA, 1 mM-orthovanadate and 20 μM-(*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride], and sonicated with 4 × 15 s bursts. After incubation for 30 min at 4 °C, the homogenates were centrifuged at 200000 *g* for 20 min and the supernatants were used as the particulate fractions. Portions of 200 μl of the cytosolic or particulate fractions were immunoprecipitated with 3 μg of anti-phosphotyrosine antibody PY20 and 40 μl of Protein G-Sepharose. Immunoprecipitates were washed twice with each of the following buffers: (i) 20 mM-Tris/HCl, pH 8.0, 150 mM-NaCl, 1 % NP40 and 1 mM-DTT (dithiothreitol), (ii) 0.1 M-Tris/HCl, pH 7.4, 0.5 M-LiCl and 1 mM-DTT, and (iii) 10 mM-Tris/HCl, pH 7.4, 100 mM-NaCl and 1 mM-DTT, as described [26]. The immunoprecipitates were subjected to the PI kinase assay in a 50 μl reaction mixture containing 20 mM-Tris/HCl, pH 7.4, 100 mM-NaCl, 10 mM-MgCl₂, 0.5 mM-EGTA, 100 μM-PI, 100 μM-phosphatidylserine, 120 μM-adenosine and [γ-³²P]-ATP (10 μM-ATP; 0.2 μCi/μl) [34]. After 10 min at 30 °C, the reaction was stopped by adding 200 μl of 1 M-HCl and 80 μl of chloroform/methanol (1:1, v/v). A 30 μl portion of the lower layer was spotted on to a Silica Gel 60 plate (Merck) and developed in chloroform/methanol/25 % NH₄OH/water (43:38:5:7, by vol.). The radioactive PIP spot was detected by autoradiography, scraped from the plate and extracted with 0.8 ml of diethyl ether/ethanol/0.04 M-KOH (10:7:5, by vol.) [35]. The extracted PIP was deacylated by methylamine and analysed by h.p.l.c. on a Partisphere SAX column (Whatman) with a linear gradient of 0–0.125 M-(NH₄)₂HPO₄ (pH 3.8) over 45 min [17]. The radioactivity in glycerophosphatidylinositol 3-phosphate (gPI-3P) was quantified by liquid scintillation counting.

Assay of total (tyrosine-phosphorylated and unphosphorylated) PI3-kinase in cytosolic and particulate fractions

Cytosolic and particulate fractions of CHO-HIR^{K→M} and CHO-HIR cells were prepared from ten 100 mm dishes. A 4 μl portion of the cytosolic or particulate fraction was used directly before immunoprecipitation for the PI kinase assay in a 50 μl reaction mixture, as described above. The final concentration of NP40 in the reaction mixtures for both the cytosolic and particulate fractions after Mono Q separation was 0.016 % (see below). This concentration of NP40 does not exert significant effects on PI 3-kinase activity [34,36]. The radioactivity gPI-3P was quantified by liquid scintillation counting.

Partial separation of the tyrosine-phosphorylated PI 3-kinase from the unphosphorylated form

The cytosolic and particulate fractions of CHO-HIR cells from ten 100 mm dishes were loaded on to a Pharmacia f.p.l.c. Mono Q (anion-exchange) column equilibrated with buffer [20 mM-Tris/HCl, pH 7.4, 0.2 % NP40, 5 % glycerol, 1 mM-orthovanadate and 20 μM-(*p*-amidinophenyl)methylsulphonyl fluoride hydrochloride], and eluted with a linear gradient of 0–0.5 M-NaCl. The fractions eluted at 0.1–0.2 M-, 0.2–0.3 M-, 0.3–0.4 M- and 0.4–0.5 M-NaCl were numbered I, II, III and IV respectively.

RESULTS AND DISCUSSION

In vitro phosphorylation of purified PI 3-kinase by insulin receptors

To investigate the direct phosphorylation of PI 3-kinase by the insulin receptor tyrosine kinase, we purified the former enzyme from the cytosolic fraction of bovine thymus to near-homogeneity [28]. We identified two types of PI 3-kinase: a 110 kDa monomer protein, and a heterodimer protein composed of 110 kDa and 85 kDa subunits. The PI 3-kinase activity is present in a 110 kDa protein [28]. However, Carpenter *et al.* [36] also reported that this enzyme from rat liver is a 110 kDa and 85 kDa heterodimer. Since the phosphorylation of the 85 kDa protein is closely associated with other tyrosine kinases [14,15,19], we used the highly purified PI 3-kinase heterodimer consisting of 110 kDa and 85 kDa subunits (the molar ratio of the 110 and 85 kDa subunits was 1:1) from bovine thymus for the *in vitro* phosphorylation experiments. The antibody-purified normal human insulin receptor protein was prepared from cells treated or not with insulin *in vivo*, as described in the Experimental section. We first examined the possibility that a contaminating or other associated tyrosine kinase in the antibody-purified human insulin receptor protein fraction was phosphorylated by the receptor tyrosine kinase and in turn phosphorylated the PI 3-kinase on a tyrosine residue. As shown in Fig. 1, only the β subunit of the human insulin receptor protein was phosphorylated in response to insulin. No other tyrosine kinase that was phosphorylated by the insulin-activated tyrosine kinase of the insulin receptor could be seen in the autoradiogram of either the 6% (Fig. 1a) or the 15% (Fig. 1b) polyacrylamide gels. Longer exposure of the gels had no effect (results not shown). Therefore the possibility of contamination by another tyrosine kinase seems remote. Insulin caused autophosphorylation of the β subunit of the normal receptor (Fig. 2a, lanes 5 and 6), but not of the kinase-deficient receptor (Fig. 2a, lanes 1 and 2). When highly purified PI 3-kinase was added to the reaction mixture containing the normal receptor, the 85 kDa subunit of the 3-kinase was phosphorylated in response to insulin (Fig. 2a, lanes 7 and 8). However, the mutant receptor lacked the potential to phosphorylate the 85 kDa

subunit (Fig. 2a, lanes 3 and 4). Phosphorylation of the 110 kDa subunit of PI 3-kinase did not change with insulin treatment of either the normal or the mutant receptors. We also examined the phosphorylation of the monomer form of the 110 kDa subunit, which has PI 3-kinase activity. This subunit was not phosphorylated on the tyrosine residue by the insulin-activated human insulin receptor *in vitro* (results not shown). To determine the phosphorylation site of the 85 kDa subunit, we cut out the regions of all the lanes corresponding to the area of this subunit and subjected them to phosphoamino acid analysis (Fig. 2b). The 85 kDa subunit was phosphorylated on the serine residue regardless of activation of the receptor tyrosine kinase by insulin (Fig. 2b, lanes 3, 4, 7 and 8). Activation of the tyrosine kinase of the insulin receptor by insulin led to phosphorylation of PI 3-kinase on the tyrosine residue; the amount of phosphoserine remained unchanged. The 110 kDa subunit of PI 3-kinase was phosphorylated only on the serine residue, and this phosphorylation was not influenced by the addition of insulin (results not shown). The serine phosphorylation of both subunits was apparently not associated with the insulin receptor tyrosine kinase, and further experiments are needed to elucidate the mechanism of serine phosphorylation of both subunits.

Characteristics of the tyrosine phosphorylation of the 85 kDa subunit of PI 3-kinase

For quantitative determination of the amount of ^{32}P incorporated into PI 3-kinase by the insulin receptor, the bands corresponding to the 85 kDa subunit were cut from the dried gel and counted for radioactivity. First we analysed the ATP-concentration-dependence of PI 3-kinase phosphorylation by the insulin receptor under our assay conditions (see the Experimental section). The estimated K_m for ATP was 2.2 μM . Then we determined the kinetic parameters of PI 3-kinase phosphorylation in the presence of 1 μM -ATP. The time course of phosphorylation was linear for at least 30 min (Fig. 3a). Phosphorylation was also dependent on the concentration of PI 3-kinase (Fig. 3b). The estimated K_m and $V_{\max}$ values were 20 nM and 0.55 pmol/10 min per pmol of insulin-binding capacity respectively. Although this $V_{\max}$ was lower than those for some other substrates, such as *src*-related peptide, angiotensin II and histone (0.02–2.2 pmol/min per 10 fmol of insulin-binding capacity) [37,38], it was comparable with those for rather poor substrates such as β -lipoprotein and Arg-Tyr [37]. The K_m was somewhat lower than those for other substrates (30 nM–80 mM) [37–43]. The stoichiometry of phosphorylation was low (about 0.01 mol of P_i /mol PI 3-kinase), but was comparable with values for denatured *rasp21* [40], phosphofructokinase [41], calmodulin [42] and lipocortins [43] (0.004–0.1 mol of P_i /mol, of substrate). Therefore we concluded that the 85 kDa subunit of PI 3-kinase was directly phosphorylated at the tyrosine residue by the activated tyrosine kinase of the human insulin receptor *in vitro*, though the stoichiometry is low. However, the stoichiometry may be improved by cofactors such as insulin receptor substrate-1, as reported by Sun *et al.* [44].

Anti-phosphotyrosine-precipitated PI 3-kinase activity in CHO-HIR^{K→M} and CHO-HIR cells

We reported that the tyrosine kinase activity of the insulin receptor is essential for insulin signal transduction in CHO cells overexpressing normal or kinase-deficient receptors [6]. We examined the activity of PI 3-kinase in these cells to confirm that the stimulation of PI 3-kinase activity by insulin is a result of activation of the insulin receptor tyrosine kinase. The PI 3-kinase activities in the anti-phosphotyrosine immunoprecipitates of cytosolic fractions from cells overexpressing kinase-deficient (CHO-HIR^{K→M}) and normal (CHO-HIR) receptors were

Fig. 1. Autophosphorylation of antibody-purified human insulin receptor

Lysates of CHO cells overexpressing normal human insulin receptors were prepared with (lanes 2) or without (lanes 1) 0.1 μM -insulin and adsorbed to a 96-well plate, as described in the Experimental section. The kinase reaction was carried out by adding 2 μCi of [γ - ^{32}P]ATP in 20 μl of kinase buffer and incubating for 30 min. The reaction was halted by addition of 2 $\times$ SDS sample buffer, electrophoresed on a 6% (a) or a 15% (b) polyacrylamide gel, and autoradiographed. Arrowheads indicate the 95 kDa β subunit of human insulin receptor.

Fig. 2. Insulin-stimulated tyrosine phosphorylation of the 85 kDa subunit of purified PI 3-kinase by insulin receptors *in vitro*

(a) Lysates of CHO cells overexpressing normal (lanes 5–8) or mutated (lanes 1–4) human insulin receptors were prepared with (+) or without (–) 0.1 μ M-insulin (Ins) and adsorbed to a 96-well plate (4.4 fmol of insulin-binding capacity/well), as described in the Experimental section. The kinase reaction was carried out by adding 2 μ Ci of [γ - 32 P]ATP with (+) or without (–) purified PI 3-kinase (PI-3K; 50 ng) in 20 μ l of kinase buffer for 30 min. The reaction was halted by addition of 2 $\times$ SDS sample buffer, electrophoresed on a 6% polyacrylamide gel and autoradiographed. Arrowheads point to the 110 kDa and 85 kDa subunits of the purified PI 3-kinase and to the 95 kDa β subunit of the human insulin receptor (IR- β). (b) Regions (lanes 1–8 of a) which correspond to the area of the 85 kDa subunit of the gel were cut out and subjected to phosphoamino acid analysis, as described in the Experimental section. The locations of phosphoserine (P-Ser), phosphotyrosine (P-Tyr) and P_i are indicated.

analysed on t.l.c. plates. Each of the PIP products was analysed by h.p.l.c. to separate PI-3P from PI-4P, as described in the Experimental section. When insulin was added there was a marked increase in PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitates of the CHO cells overexpressing the normal receptor (CHO-HIR) (inset of Fig. 4b), but not the kinase-deficient receptor (CHO-HIR^{K→M}) (inset of Fig. 4a). Thus the increase in activity seems to be due to activation of the insulin receptor tyrosine kinase in response to insulin.

It has been reported that PI 3-kinase physically associates with several tyrosine kinases [11,12]. However, under our experimental conditions the amount of PI 3-kinase activity precipitated with two different anti-(human insulin receptor) antibodies was less than 1% of the amount of PI 3-kinase activity precipitated with anti-phosphotyrosine antibody from an equivalent portion of the same extract (results not shown). Almost the same result was observed by Endemann *et al.* [26]. Thus PI 3-kinase does not seem to be physically associated with the insulin receptor.

Total (tyrosine-phosphorylated and unphosphorylated) PI 3-kinase activities in cytosolic fractions of CHO-HIR^{K→M} and CHO-HIR cells

We next examined whether the increase in PI 3-kinase activity in response to insulin is detectable in the cytosolic fraction, which includes both unphosphorylated and tyrosine-phosphorylated forms. The cytosolic fractions of the cells overexpressing normal or kinase-deficient insulin receptors, treated with or without insulin, were assayed for PI 3-kinase activity as described in the

Fig. 3. Time course (a) and concentration-dependence (b) of PI 3-kinase phosphorylation by the insulin receptor

Lysates of CHO cells overexpressing normal human insulin receptors were prepared with 0.1 μ M-insulin and adsorbed to a 96-well plate (4.4 fmol of insulin-binding capacity/well) as described in the Experimental section. (a) The kinase reaction was carried out by adding 1 μ M-[γ - 32 P]ATP to purified PI 3-kinase (60 ng) in 20 μ l of kinase buffer for the indicated periods of time at room temperature. After electrophoresis on a 6% polyacrylamide gel, 32 P incorporated into the 85 kDa band was quantified. (b) The kinase reaction was carried out by adding 1 μ M-[γ - 32 P]ATP to various concentrations of PI 3-kinase in 20 μ l of kinase buffer for 10 min. 32 P incorporated into the 85 kDa band was quantified. Values represent means of two separate experimental determinations.

Experimental section. PI 3-kinase activity was increased in response to insulin in cells expressing the normal receptor (CHO-HIR) (inset of Fig. 5b) but not the kinase-deficient receptor (CHO-HIR^{K→M}) (inset of Fig. 5a). Using the total cytosolic fraction, containing both the tyrosine-phosphorylated and unphosphorylated forms of the enzyme, we noted an increase in PI 3-kinase activity in response to insulin. The results shown in Figs. 4 and 5 suggest that the increase in PI 3-kinase is due to tyrosine phosphorylation of the enzyme.

Partial separation of the tyrosine-phosphorylated form of PI 3-kinase from the unphosphorylated form on a f.p.l.c. Mono Q column

To examine whether or not the tyrosine phosphorylation of PI 3-kinase causes an increase in the specific activity of the enzyme, we attempted to separate the tyrosine-phosphorylated form from the unphosphorylated form of the enzyme present in the cytosolic fraction. After treatment with or without insulin, the cytosol of the CHO cells overexpressing the normal insulin receptor (CHO-HIR) was applied to a f.p.l.c. Mono Q column and developed

Fig. 4. Anti-phosphotyrosine-precipitated PI 3-kinase activity in cytosolic fractions of CHO-HIR^{K→M} (a) and CHO-HIR (b) cells

Cytosolic fractions were prepared from CHO-HIR^{K→M} or CHO-HIR cells in the absence (○) or the presence (●) of 0.1 μM-insulin, and immunoprecipitated with anti-phosphotyrosine antibody and protein G-Sepharose. The immunoprecipitates were subjected to the PI 3-kinase assay, and the products were separated by t.l.c. The ³²P-labelled PIPs on t.l.c. were extracted, deacylated and analysed by h.p.l.c., as described in the Experimental section. The elution times of gPI-3P and gPI-4P are indicated. Insets show integrated radioactivities of gPI-3P as PI 3-kinase activity in the absence (□) or the presence (■) of insulin. Results shown are means ± S.E.M. of triplicate determinations.

Fig. 5. Total (tyrosine-phosphorylated plus unphosphorylated) PI 3-kinase activity in cytosolic fractions of CHO-HIR^{K→M} (a) and CHO-HIR (b) cells

Products of the PI 3-kinase assay in cytosolic fractions from unstimulated (○) and 0.1 μM-insulin-stimulated (●) CHO-HIR^{K→M} (a) or CHO-HIR (b) cells were separated by t.l.c. After autoradiography, the ³²P-labelled PIP products were extracted, deacylated and analysed by h.p.l.c., as described in the Experimental section. The elution times of gPI-3P and gPI-4P are indicated. Insets show integrated radioactivities of gPI-3P as PI 3-kinase activity in the absence (□) or the presence (■) of insulin. Results shown are means ± S.E.M. of triplicate determinations.

with a linear gradient of salt. The eluted fractions were collected and divided into four groups, as described in the Experimental section. Aliquots from each group were used for the PI 3-kinase assay before or after immunoprecipitation with the anti-phosphotyrosine antibody (Table 1). In the absence of insulin, the unphosphorylated form of PI 3-kinase was mainly eluted in fraction group II, as the tyrosine-phosphorylated form was not detected in this fraction. With insulin treatment, tyrosine-phosphorylated PI 3-kinase activity appeared mainly in fraction group III. The apparent increase in PI 3-kinase activity in the cytosol was observed in the same fraction group III in response to insulin. An increase in the 3-kinase activity in cytosol fraction group III was apparently related to the presence of the tyrosine-phosphorylated form of the enzyme. This indicates that tyrosine phosphorylation of the enzyme by the insulin receptor kinase causes an increase in enzyme specific activity *in vivo*. However, the overall (sum of fractions I–IV) increase in total (tyrosine-phosphorylated plus unphosphorylated) PI 3-kinase activity following insulin treatment after Mono Q fractionation (1.6-fold increase) (Table 1) was less than that in unfractionated cytosol

(3.1-fold increase) (Fig. 5b). The overall (sum of fractions I–IV) increase in anti-phosphotyrosine precipitates following insulin treatment after Mono Q fractionation (7.0-fold increase) (Table 1) was also less than that in unfractionated immunoprecipitates (22.1-fold increase) (Fig. 4b). These results suggest that the tyrosine-phosphorylated PI 3-kinase activated by insulin treatment may be partially deactivated owing to dephosphorylation by phosphatases in the cell lysates during preincubation before Mono Q loading and separation.

There was no reciprocal decrease in the total PI 3-kinase activity in cytosol fraction II after insulin treatment (Table 1). This can be explained by postulating that fraction II contained a small but consistent amount of the tyrosine-phosphorylated form of the enzyme, and that phosphorylation stoichiometry of the enzyme may not be very high even *in vivo*. Ruderman *et al.* [27] also concluded that the specific activity of PI 3-kinase in the anti-phosphotyrosine immunoprecipitates was increased, because the products of the PI 3-kinase activity increased in quantity in response to insulin *in vivo*.

On the other hand, Cohen *et al.* [45] reported that their middle

Table 1. Partial separation of the tyrosine-phosphorylated form of PI 3-kinase from the unphosphorylated form on a f.p.l.c. Mono Q column

Cytosolic fractions prepared from CHO-HIR cells in the absence or the presence of 0.1 μ M-insulin were loaded on to a Mono Q column, and separated into fractions I–IV as described in the Experimental section. For anti-phosphotyrosine (anti-*P*-Tyr)-immunoprecipitated PI 3-kinase activity, 200 μ l of each fraction was immunoprecipitated with anti-phosphotyrosine antibody and the resulting PIP products were analysed sequentially by t.l.c. and h.p.l.c. Radioactivities of gPI-3P in the absence (Control) or the presence of 0.1 μ M-insulin are shown as the PI 3-kinase activity. Total (tyrosine-phosphorylated plus unphosphorylated) PI 3-kinase activity was measured using 4 μ l of each fraction. Results shown are means $\pm$ S.E.M. of triplicate determinations.

Mono Q fraction	Activity (c.p.m.)			
	Anti- <i>P</i> -Tyr precipitates of cytosol		Cytosol before immunoprecipitation	
	Control	Insulin	Control	Insulin
I	16 $\pm$ 16	12 $\pm$ 12	36 $\pm$ 3	38 $\pm$ 5
II	16 $\pm$ 10	87 $\pm$ 25	253 $\pm$ 5	321 $\pm$ 18
III	28 $\pm$ 7	355 $\pm$ 18	117 $\pm$ 6	313 $\pm$ 18
IV	12 $\pm$ 12	52 $\pm$ 20	52 $\pm$ 3	52 $\pm$ 20

Table 2. Subcellular localization of PI 3-kinase activity in CHO-HIR cells

Cytosolic and particulate fractions prepared from CHO-HIR cells in the absence (control) or the presence of 0.1 μ M-insulin were loaded on to a Mono Q column and separated into fractions I–IV as described in the Experimental section. PI 3-kinase activity in fraction III, which contains the phosphorylated form of PI 3-kinase, was measured before and after immunoprecipitation by anti-phosphotyrosine antibody as in Table 1. Results shown are means $\pm$ S.E.M. of triplicate determinations.

Subcellular fraction	Activity (c.p.m.)			
	Anti-phosphotyrosine precipitates		Before immunoprecipitation	
	Control	Insulin	Control	Insulin
Cytosol	28 $\pm$ 7	355 $\pm$ 12	117 $\pm$ 16	313 $\pm$ 19
Particulate	30 $\pm$ 10	267 $\pm$ 13	102 $\pm$ 8	214 $\pm$ 23

T-associated 85 kDa protein (the 85 kDa subunit of PI 3-kinase) was recruited from the cytosol to the membrane by tyrosine phosphorylation. We examined whether or not the increase in PI 3-kinase activity in the cytosol in response to insulin was due to the translocation of the enzyme from the particulate to the cytosolic fraction on tyrosine phosphorylation. After subcellular fractionation and Mono Q separation, we assayed total (tyrosine-phosphorylated and unphosphorylated) and anti-phosphotyrosine-antibody-precipitated PI 3-kinase activities in both the cytosolic and the particulate fractions. With insulin treatment, the total and anti-phosphotyrosine-precipitated PI 3-kinase activities were increased in both the cytosolic and the particulate fractions (Table 2). Thus it is likely that tyrosine phosphorylation of PI 3-kinase by the insulin receptor exerted no significant effects on the subcellular localization of the kinase.

The specific activity of the 110 kDa monomer is five times greater than that of the heterodimer form of PI 3-kinase [28]. When we applied the purified 110/85 kDa heterodimer and the 110 kDa monomer to a Mono Q column, the heterodimer was eluted broadly in fractions II–IV and the monomer was mainly in fraction III (F. Shibasaki, Y. Homma & T. Takenawa, unpublished work). The broad elution of the purified dimer may be due to modifications such as phosphorylation or heterogeneity, as reported by Otsu *et al.* [46]. Thus another possible explanation for the increase in PI 3-kinase activity in response to insulin is that the tyrosine phosphorylation of the 85 kDa subunit may decrease its affinity for the 110 kDa subunit, and the dissociated monomer form of 110 kDa may then be eluted in fraction III with the tyrosine-phosphorylated heterodimer from the Mono Q column (Table 1). In this case, the tyrosine phosphorylation of the 85 kDa subunit may increase the 110 kDa monomer form, and the total amount of PI 3-kinase composed of both heterodimer and monomer forms, may show enhanced specific activity.

In summary, we have obtained evidence that the insulin receptor tyrosine kinase directly phosphorylates the 85 kDa subunit of purified PI 3-kinase *in vitro*. We also suggest that this may increase the specific activity of the enzyme *in vivo*. Acquisition of an antibody against the PI 3-kinase should clearly reveal the effects of phosphorylation. Although the biochemical fate of products of this enzyme is unknown [47,48], it has been suggested that they mediate not only cell growth [11,12], but also secretion [49,50] and differentiation [22].

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