Association of the insulin receptor and phosphatidylinositol 3-kinase requires a third component

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We have studied the interactions between the insulin receptor and PtdIns 3-kinase by a reconstitution system *in vitro* composed of highly purified PtdIns 3-kinase from rat liver and highly purified insulin receptors bound to insulin-agarose or to antibodies against insulin receptors. As a positive control, receptors for platelet-derived growth factor, which bind and phosphorylate PtdIns 3-kinase, were studied in parallel with insulin receptors. Our results indicate that the insulin receptor, regardless of its phosphorylation state, does not directly associate with purified PtdIns 3-kinase, whereas the autophosphorylated receptor does associate with PtdIns 3-kinase present in the crude CHO-cell lysate. Also, we could not detect phosphorylation of PtdIns 3-kinase by the insulin receptor, even through the receptor readily underwent autophosphorylation and phosphorylated an insulin-receptor substrate, poly(Glu-Tyr) (4:1). These findings argue that one or more cytosolic components link the receptor and the enzyme. Insulin-receptor substrate-1 (IRS-1) was evaluated as a

potential linking protein. In the absence of ATP, IRS-1 did not facilitate the coupling of the phosphorylated insulin receptor to PtdIns 3-kinase. Thus IRS-1 is unlikely to be the component in crude CHO-cell lysate that couples PtdIns 3-kinase to the phosphorylated insulin receptor. However, the addition of ATP, which allows phosphorylation of IRS-1 by the insulin receptor, also enhances the coupling of PtdIns 3-kinase to the insulin receptor. In support of this idea, immunoprecipitates of IRS-1 from insulin-treated CHO cells were found to contain both the insulin receptor and PtdIns 3-kinase. In conclusion, the insulin receptor does not appear to phosphorylate or bind directly to PtdIns 3-kinase, regardless of the receptor's state of phosphorylation. Association of PtdIns 3-kinase with the insulin receptor is mediated by one or more components, one of which may involve an unidentified factor in cell lysate and another that apparently involves phosphorylated IRS-1.

INTRODUCTION

PtdIns 3-kinase binds to several different growth-factor receptors that have undergone autophosphorylation [1–6], in addition to other proteins that contain phosphotyrosine in defined motifs [6–9]. Insulin stimulation of cells increases the activity of PtdIns 3-kinase, as measured by the formation of phospholipid products from this enzymic reaction [10]. Importantly, insulin stimulation increases the amount of enzyme activity in immunoprecipitates of phosphotyrosine or immunoprecipitates of the insulin receptor [10–14]. These results suggest the possibilities that the insulin receptor can phosphorylate PtdIns 3-kinase and that the enzyme can bind directly to the receptor, as demonstrated for other growth factor receptors. In fact, Tyr-1322, which undergoes autophosphorylation in the insulin receptor, was suggested to be a site that binds PtdIns 3-kinase [6].

To our knowledge, a direct test of the ability of the insulin receptor to interact with and possibly to phosphorylate PtdIns 3-kinase using highly defined components has not been reported. Other less direct experiments that have examined these questions have provided conflicting results. For example, one group has reported that activation of the insulin receptor and insulin treatment of cells resulted in the phosphorylation of the 85 kDa subunit of PtdIns 3-kinase [15–17]. In contrast, tyrosine phosphorylation of this enzyme was not detected by other groups [18–21] who examined insulin-stimulated cells. In those studies, PtdIns 3-kinase was found to be associated with insulin-receptor substrate 1 (IRS-1) [18,20–27]. Since the major effect of

insulin stimulation is to increase the amount of PtdIns 3-kinase activity in phosphotyrosine immunoprecipitates [10,19], of which a significant fraction is complexed to IRS-1 [18,20–27], the possibility exists that the insulin receptor does not act directly on the enzyme, but instead couples to the enzyme by a third component [27].

In the present study, we directly examined whether or not purified insulin receptor can bind and phosphorylate purified PtdIns 3-kinase. We also examined the possible involvement of IRS-1 in coupling the enzyme and the activated insulin receptor to form a tertiary complex. Overall, the findings show that the insulin receptor does not directly associate with or detectably phosphorylate PtdIns 3-kinase. The best explanation for the presence of PtdIns 3-kinase associated with the activated insulin receptor is that a third component links the two structures.

EXPERIMENTAL

Materials

Chinese-hamster ovary (CHO) cells were obtained from the American Type Culture Collection (A.T.C.C.) and cultured in 10 cm diam. dishes (Corning Glass Works, Corning, NY, U.S.A.) in Ham's F12 medium (Hyclone Laboratories, Logan, UT, U.S.A.) with 10% (v/v) fetal-bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and NaHCO₃ (1.176 g/l). The COS A₂ (COS) cell line was a gift from Dr. Tom Broker (University of Rochester, Rochester, NY, U.S.A.), and the C-127 cell line was obtained from A.T.C.C. The cells were main-

Abbreviations used: a-IR-1, antibody against human insulin receptor; WGA, wheat-germ agglutinin; SH2, src homology-2; PDGF, platelet-derived growth factor; CHO, Chinese-hamster ovary; IRS-1, insulin-receptor substrate 1; PtdInsP, phosphatidylinositol monophosphate.

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tained in 10 cm dishes in Dulbecco's modified Eagle's medium (DMEM; from Sigma Chemical Co., St. Louis, MO, U.S.A.) that contained 10 % fetal-bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. PtdIns 3-kinase was purified approx. 5000-fold from rat liver as described previously [28]. A monoclonal antibody specific against human insulin receptor α -subunit (α -IR-1) [29] was produced by a myeloma cell line from A.T.C.C. Monoclonal antibody against phosphotyrosine, polyclonal antibody against type B human platelet-derived growth factor (PDGF) receptor (amino acids 1099–1111) and recombinant baculovirus-expressed IRS-1 were purchased from UBI, Lake Placid, NY, U.S.A. PtdIns, PtdIns-(4,5) P_2 , PtdIns(3,4,5) P_3 and bovine insulin were purchased from Sigma. [γ - 32 P]ATP (3000 Ci/mmol) and 125 I[A-14]insulin (2000 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Pre-coated silica-gel 60 t.l.c. plates were from EM Science Corp., Gibbstown, NJ, U.S.A. Protein G-Sepharose beads were purchased from Pharmacia, Uppsala, Sweden. Wheat-germ agglutinin (WGA) gel was obtained from EY Laboratories Inc., San Mateo, CA, U.S.A.

Stable cell lines

CHO cells were transfected by the calcium phosphate method with a neomycin-resistance gene alone (PNull) or with either the wild-type human insulin-receptor gene (HIRC) or the gene encoding a double mutant insulin receptor (F2). The wild-type HIRC cDNA present in an SV40 expression vector was a gift from Dr. C. Ronald Kahn, Joslin Diabetes Center [30], and the mutations in the insulin-receptor cDNA were produced by mutagenesis *in vitro* (Promega Corp.) as directed by the manufacturer. This receptor has a Phe-for-Tyr substitution at position 1316 and at position 1322. Both cell lines express similar numbers of receptor and were grown in Ham's F12 medium as described above.

Assay for PtdIns 3-kinase activity *in vivo*

CHO cells that express human insulin receptors were cultured in six-well plates and starved overnight after they had reached confluence. The cells were stimulated with the indicated concentrations of insulin for 5 min, and lysed with 1 ml of lysis buffer [10]. After centrifugation at 15000 *g* for 10 min, the supernatant was incubated with 2 μ g of α -IR-1 absorbed to 50 μ l of Protein G-Sepharose beads and rotated at 4 °C for 3 h. The samples were washed and PtdIns 3-kinase activity was measured as described [10].

Association between purified PtdIns 3-kinase and PDGF receptors

Confluent C-127 cells were serum-starved overnight and lysed with 1 ml of lysis buffer [10]. After centrifugation at 15000 *g* for 10 min, the supernatant (0.5 ml) was incubated with 1 μ l of anti-PDGF-receptor antibody (whole serum) absorbed to 50 μ l of packed Protein G-Sepharose beads. The immunoprecipitates were washed twice with buffer A (50 mM Tris/HCl, pH 7.4, and 0.1 % Triton X-100) and treated for 30 min with 10 mM $MnCl_2$ in the absence or presence of 100 μ M ATP. After washing each sample three times with buffer A, 175 ng of purified PtdIns 3-kinase in a volume of 0.5 ml was added and rotated at 4 °C for 3 h. The samples were washed and PtdIns 3-kinase activity in the immunoprecipitates was measured as described [10].

Cell lysate prepared from baculovirus-infected Sf9-cells that expressed the PDGF receptor was a gift from Dr. David Kaplan, Frederick, MD, U.S.A. [5]. Lysate (0.5 ml) from 2×10^6 cells was added to 12 μ l of anti-PDGF-receptor antibody bound to 360 μ l of packed Protein A-Sepharose beads. After 3 h at 4 °C, the immunoprecipitates were treated with 10 mM Mn^{2+} in the absence or presence of 100 μ M ATP for 30 min at 24 °C. The samples were washed with buffer A and the PDGF-receptor immunoprecipitate was incubated in a total volume of 0.5 ml with 175 ng of purified PtdIns 3-kinase for 3 h at 4 °C. PtdIns 3-kinase activity associated with the receptor in the immunoprecipitates was determined as described above.

PDGF receptors were dephosphorylated by potato phosphatase by adding 20 mg/ml phosphatase to the immunoprecipitated receptor as described [5]. The reaction was conducted for 30 min at 30 °C. The immunoprecipitates were washed three times with buffer A and then underwent the standard autophosphorylation reaction. The samples were incubated with purified PtdIns 3-kinase and PtdIns 3-kinase activity associated with the immunoprecipitates was measured as described above.

Association between purified PtdIns 3-kinase and the insulin receptor

A portion (500 μ l; 50 μ g of protein) of WGA extract from COS cells that transiently express wild-type insulin receptor was incubated for 3 h with 2 μ g of α -IR-1 antibody absorbed to 50 μ l of packed Protein G-Sepharose beads. The beads were washed twice with buffer A, and incubated for 30 min at 24 °C with 10 mM $MnCl_2$ in the absence or presence of 100 nM insulin and 100 μ M ATP. After washing with buffer A, 175 ng of purified PtdIns 3-kinase in a 0.5 ml volume was added to each sample and incubated at 4 °C for 3 h. The immunoprecipitate was washed and the PtdIns 3-kinase activity was determined as described [10]. The existence of insulin receptors in the immunoprecipitates was shown by conducting autophosphorylation studies.

Insulin receptors immobilized to insulin-agarose beads were also used in an attempt to demonstrate receptor binding of purified PtdIns 3-kinase. To prepare the insulin-agarose beads, bovine insulin (15 mg) was suspended in 300 μ l of 0.1 M HCl, and this suspension was added to 5 ml of 0.1 M phosphate buffer, pH 7.4, containing 4 M urea and tracer 125 I-insulin [31]. Then 3 ml of packed Affigel-10 agarose beads, rapidly washed twice in ice-cold 0.1 M phosphate buffer, was added to the insulin solution. After rotating at 21 °C for 3 h, the beads were collected by centrifugation and incubated for 15 min with 10 ml of 0.1 M phosphate buffer, pH 7.4, containing 150 μ l of ethanolamine. The beads were extensively washed with 4 M urea and stored in 0.1 M NaCl in buffer A at 4 °C. The efficiency of insulin immobilization was monitored by 125 I-insulin; approx. 0.22 mg of insulin was coupled to 50 μ l of packed beads, the amount used in each assay. WGA-purified insulin receptors from 6 g of rat liver were incubated in the absence or presence of 1 μ g/ml native insulin. Each receptor preparation was cycled over a 300 μ l (packed) insulin-agarose-beads column. The numbers of receptors immobilized on insulin-agarose beads by this procedure were measured by determining the insulin-binding activity in the WGA extract before and after the cycling step. Based on Scatchard analysis of insulin-binding data, approx. 0.74 pmol of insulin-binding activity was present in 50 μ l of packed agarose beads that was used in each assay. The insulin-agarose beads were washed with buffer A that contained 1 M NaCl to remove any associated proteins, followed by a wash in buffer A alone. The highly purified receptors immobilized on 50 μ l of beads were

phosphorylated by a 30 min incubation with 10 mM MnCl_2 in the absence or presence of 100 μM ATP. The beads were washed three times with buffer A to remove ATP, and 175 ng of purified PtdIns 3-kinase was added to each assay in a total volume of 0.5 ml. After incubation for 3 h at 4 °C, the samples were processed as described for the PtdIns 3-kinase assay, except that dithiothreitol was removed and the concentration of Nonidet P-40 was lowered to 0.1 % to preserve the binding of the receptor to insulin-agarose. The presence of insulin receptors on insulin-agarose beads was demonstrated by autophosphorylation studies.

Cell lysates and PtdIns 3-kinase association with the insulin receptor

COS cells transiently expressing the wild-type insulin receptor were serum-starved for 5 h, lysed as described previously [32], and the insulin receptor was immunoprecipitated with 2 μg of $\alpha\text{-IR-1}$ antibody. The immunoprecipitates were incubated for 30 min at 24 °C with 10 mM MnCl_2 , 20 $\mu\text{g}/\text{ml}$ pepstatin, 50 mM Hepes, pH 7.5, and 0.1 % Triton X-100 in the absence or presence of 1 μM insulin and 1 mM ATP. After the precipitates were washed twice with 50 mM Hepes (pH 7.5)/0.1 % Triton X-100, 1 ml of CHO lysate prepared as described previously [32] was added to the immunoprecipitates and rotated at 4 °C for 3 h. The immunoprecipitates were washed and the associated PtdIns 3-kinase activity was determined.

Coupling of PtdIns 3-kinase to the insulin receptor by IRS-1

WGA-purified insulin receptor (5 μg of protein) was autophosphorylated by using 100 μM ATP and 10 mM Mn^{2+} in buffer A during a 25 min incubation in the absence or presence of 100 nM insulin. IRS-1 (3 ng) was added as indicated and the incubation was continued for another 5 min. Then 300 ng of purified PtdIns 3-kinase in 0.5 ml volume was added, followed by 2 μg of $\alpha\text{-IR-1}$ antibody absorbed to 50 μl of packed Protein G-Sepharose beads. After incubation for 3 h at 4 °C, the beads were washed and the PtdIns 3-kinase activity measured.

Phosphorylation of PtdIns 3-kinase

PtdIns 3-kinase and the PDGF receptor were prepared exactly as described previously. However, rather than measuring PtdIns 3-kinase activity, the immunoprecipitates were incubated for 10 min at 24 °C with 50 μl of 10 mM MnCl_2 /20 mM Tris/HCl, pH 7.4, and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After washing twice with buffer A, the samples were subjected to SDS/PAGE and autoradiography.

Insulin receptors bound to insulin-agarose beads were prepared exactly as described above. After treatment of the beads for 30 min at 24 °C with 100 μM ATP and 10 mM MnCl_2 in the absence and presence of 1 μM insulin, the beads were washed twice with buffer A. For some samples, 175 ng of PtdIns 3-kinase was added in a total volume of 0.5 ml and rotated for 3 h at 4 °C. The agarose beads were washed as described above for measuring PtdIns 3-kinase activity associated with insulin-agarose. The samples were then incubated for 10 min at 24 °C with 50 μl of 10 mM MnCl_2 , 20 mM Tris/HCl, pH 7.4, and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The incubation was terminated by pelleting the agarose beads and addition of sample buffer for SDS/PAGE. The supernatant from the beads was treated with poly(ethylene

glycol) [33] and the precipitate collected by centrifugation. Both the precipitate and the material from the agarose beads were subjected to SDS/PAGE and autoradiography.

RESULTS AND DISCUSSION

Prior work has shown that treatment of a number of different cell types with insulin causes an increase in the activity of PtdIns 3-kinase in immunoprecipitates that contain the insulin receptor [10,12,19,27]. These findings raise the possibility that SH2 (*src* homology-2) domains of PtdIns 3-kinase bind to the C-terminus region of the receptor at the autophosphorylation site of Tyr-1322 [6], since this region has some homology to the autophosphorylation site of PDGF receptor known to bind PtdIns 3-kinase. Therefore, in order to study this signalling pathway during insulin stimulation, we began by examining a mutant insulin receptor in which Tyr-1316 and Tyr-1322 were replaced by Phe.

Figure 1 shows that removal of the two autophosphorylation sites does not prevent the cells from responding to insulin in the same manner as cells that express the wild-type receptor. No differences were evident in the amount of PtdIns 3-kinase activity in the insulin-receptor immunoprecipitates taken from cells expressing either the wild-type or the mutant receptor. During maximum insulin stimulation, the amount of PtdIns 3-kinase activity in insulin-receptor immunoprecipitates was about 10 times the basal activity (no insulin stimulation) for both cell types. These findings indicate that PtdIns 3-kinase is not binding to the autophosphorylation sites at Thr-1322 or Tyr-1316.

It is possible that another domain in the insulin receptor is responsible for binding the enzyme. Recently the sequence IADGMAYLNAKK in the insulin receptor was pointed out as being a potential site for binding the SH2 domain of Sem-5 protein [34]. However, this is not a known phosphorylation site [35,36] and, in the absence of phosphorylation, the SH2 domains of PtdIns 3-kinase cannot form a complex with another protein.

Another possibility is that the enzyme and the insulin receptor are linked together by a third protein, e.g. IRS-1, which is phosphorylated by the insulin receptor and which will bind to

Figure 1 Comparison of PtdIns 3-kinase activity in insulin-receptor immunoprecipitates from CHO-HIRC and CHO-F2 cell lysates

Starved cells in six-well plates were treated with the indicated concentrations of insulin, lysed and immunoprecipitated with 2 μg of $\alpha\text{-IR-1}$ antibody absorbed to Protein G beads. PtdIns 3-kinase activity was measured as described in the Experimental section. In most Figures: Ori., origin.

Figure 2 PDGF receptors immunoprecipitated from C-127 cells associate with purified PtdIns 3-kinase

PDGF receptors (PDGF-R) were immunoprecipitated from C-127 cell lysate by anti-PDGF antibody and incubated with 10 mM Mn^{2+} in the presence (+) or absence (-) of 1 mM ATP. After washing away Mn^{2+} and ATP, the samples were incubated with purified PtdIns 3-kinase and the receptor immunoprecipitates were collected by centrifugation, washed, and PtdIns 3-kinase activity was measured. Densitometer measurements shown an approx. 4-fold increase in PtdInsP for the phosphorylated PDGF receptor over the non-phosphorylated receptor.

Figure 3 PDGF receptors immunoprecipitated from baculovirus lysate associate with purified PtdIns 3-kinase

(a) PDGF receptors were immunoprecipitated from baculovirus lysate by PDGF antibody and incubated with 10 mM Mn^{2+} in the presence (+) or absence (-) of 1 mM ATP. The samples were then washed and incubated with purified PtdIns 3-kinase. The receptor immunoprecipitates were collected by centrifugation, washed, and PtdIns 3-kinase activity was determined. (b) The immunoprecipitated PDGF receptor was treated with potato phosphatase as described in the Experimental section before undergoing autophosphorylation and incubation with purified PtdIns 3-kinase.

PtdIns 3-kinase [18,20-27]. In order to examine directly this and other possibilities, the following studies have used a reconstitution system with highly purified PtdIns 3-kinase and the insulin receptor.

Figures 2 and 3 show the results of control experiments that examine the catalytic activity of purified PtdIns 3-kinase and its

Figure 4 Purified PtdIns 3-kinase does not associate with the insulin receptor in insulin-receptor immunoprecipitates

(a) Results from incubation of purified PtdIns 3-kinase with immunoprecipitates of the wild-type insulin receptor (IR) prepared from WGA extracts of HIRC-transfected COS cells. Insulin (100 nM) was incubated with the insulin receptor in the absence or presence of 1 mM ATP before addition of PtdIns 3-kinase. (b) Autoradiograph of an SDS/PAGE gel for the immunoprecipitated insulin receptor after addition of 100 μ M ATP, 1 μ Ci of [γ - 32 P]ATP, 10 mM Mn^{2+} and in the absence or presence of 100 nM insulin.

ability to bind to the autophosphorylated PDGF receptor. In Figure 2, the PDGF receptor is immunoprecipitated from C-127 cells and incubated without or with ATP to induce full receptor autophosphorylation. As evident in the results, the fully phosphorylated PDGF receptor bound approx. 4-fold more PtdIns 3-kinase activity than the receptor incubated in the absence of ATP.

The findings in Figure 3 show that the purified PtdIns 3-kinase from rat liver retains its ability to bind phosphorylated PDGF receptor, which in this case is generated from a baculovirus expression system. Unlike the experiments with the receptor from C-127 cells, ATP treatment of the receptor did not result in an increase in PtdIns 3-kinase association with the receptor (Figure 3a). The failure of ATP treatment to cause an increase in association may be caused by prior phosphorylation of the receptor in the baculovirus expression system. This idea is supported by the ability of potato phosphatase treatment to decrease markedly the amount of enzyme associated with the receptor. The addition of ATP, which allows the rephosphorylation of the receptor, also allows PtdIns 3-kinase to bind to the receptor (Figure 3b). Overall, the studies with the PDGF receptor indicate that purified PtdIns 3-kinase retains its ability to bind normally to a phosphorylated receptor and that the conditions of the reconstitution system are appropriate to detect this association.

Experiments with the insulin receptor are shown in Figure 4. The partially purified receptor was immunoprecipitated and treated with ATP by using the same protocol as used with the PDGF receptor (Figure 4a). However, unlike the findings with the PDGF receptor, purified PtdIns 3-kinase did not associate with the insulin receptor regardless of whether or not it has undergone phosphorylation. The SDS/PAGE gel (Figure 4b) demonstrates the presence of the insulin receptor in the immunoprecipitate and its ability to undergo insulin-mediated autophosphorylation on the β -subunit under these conditions.

Figure 5 Purified PtdIns 3-kinase does not associate with the insulin receptor bound to insulin-agarose beads

(a) Experiment similar to that of Figure 4, except that insulin receptor (IR) from rat liver was immobilized on insulin-agarose and used in place of the immunoprecipitated receptor. The amount of immobilized insulin receptor in each assay corresponded to 0.74 pmol of insulin-binding activity (see the Experimental section). PtdIns 3-kinase activity associated with immobilized insulin receptor was measured as in receptor immunoprecipitates, except that milder washing conditions were used with the insulin-agarose beads, i.e. dithiothreitol was removed from the wash buffer and Nonidet P-40 was decreased to 0.1% concentration. (b) SDS/PAGE results for the insulin receptor after autophosphorylation of the immobilized receptor. Note that the insulin receptors were incubated with 1 μ g/ml insulin (right lane) before they were added to insulin-agarose beads, a procedure that specifically blocks the receptors from binding to immobilized insulin.

Figure 5 extends the studies with the insulin receptor to include a different means of receptor purification and immobilization. Here the receptor was partially purified from rat liver by WGA chromatography and the WGA extract was incubated with insulin-agarose beads. After extensive washing of the beads, a highly purified and active insulin receptor was obtained bound to the affinity matrix. This approach has an advantage over the immunoprecipitation protocol because the receptor is tethered through its normal interaction with insulin, thus decreasing the chance that a binding site for PtdIns 3-kinase is blocked. However, as shown by the data, no association between PtdIns 3-kinase and the phosphorylated receptor (or non-phosphorylated receptor) could be detected (Figure 5a). In Figure 5(b), the presence of the insulin receptor on the agarose beads was shown by its ability to undergo autophosphorylation on the β -subunit. Note that insulin was used as a control to prevent the receptor from binding to insulin-agarose, and thus the excess free insulin provides a measure of non-specific interaction. This explains why little autophosphorylated insulin receptor was found in the presence of insulin. The findings of these studies argue strongly that the insulin receptor does not bind PtdIns 3-kinase, or at least that the receptor does not bind the enzyme with the same high affinity shown by other phosphorylated receptors [1–6].

Not only do other receptors bind the enzyme, they also phosphorylate PtdIns 3-kinase complexed to the receptor. We therefore examined this possibility with our reconstituted system (Figure 6). Studies with the PDGF receptor (Figure 6) show that the addition of PtdIns 3-kinase results in phosphorylation of an 85 kDa protein, a finding consistent with the phosphorylation of

Figure 6 Phosphorylation of PtdIns 3-kinase by PDGF and insulin receptors

(a) shows the ability of the PDGF receptor (PDGF-R) from baculovirus preparation to phosphorylate an 85 kDa band after addition of purified PtdIns 3-kinase. Autophosphorylation of the PDGF receptor (+ ATP) resulted in greater phosphorylation of the 85 kDa band. (b) shows an over-exposed autoradiograph of proteins phosphorylated by the insulin receptor (IR) immobilized on insulin-agarose beads. Note that the presence of free insulin significantly decreased the amount of receptor bound to insulin-agarose. No 85 kDa band was detected after different periods of film exposure.

the regulatory subunit of the enzyme, which is known to be phosphorylated by the activated PDGF receptors [1,28,37].

Figure 6(b) illustrates the results when the insulin receptor is used in place of the PDGF receptor. In these studies the insulin receptor tethered to insulin-agarose was incubated with the purified enzyme. After the incubation with enzyme and radiolabelled ATP, the proteins associated with the beads were subjected to SDS/PAGE and autoradiography. As shown, the only phosphorylated protein is the 95 kDa subunit of the insulin receptor, and shorter film exposures did not uncover an 85 kDa protein. The supernatant from the incubation was also examined for phosphorylated 85 or 110 kDa proteins, by using poly(ethylene glycol) to precipitate the enzyme. Again, no phosphorylated 85 or 110 kDa bands were detected (results not shown). We considered that the PtdIns 3-kinase preparation might contain an inhibitor of the tyrosine kinase activity of the insulin receptor that allowed receptor autophosphorylation but blocked protein substrate phosphorylation. Using the artificial substrate poly(Glu-Tyr) (4:1), we found that the kinase activity of the insulin receptor was active under the experimental conditions. Approx. 0.8 pmol of phosphate was incorporated into poly(Glu-Tyr) during the assay. In comparison, 'non-specific' activity was 0.11 pmol of phosphate incorporated into poly(Glu-Tyr) by 'non-specific' beads, i.e. insulin-agarose beads previously incubated with insulin receptors in the presence of 1 μ g/ml free insulin to decrease the amount of receptor attached to insulin-agarose. When PtdIns 3-kinase was added, the incorporation of phosphate into poly(Glu-Tyr) was 1.1 pmol, an increase of about 45% over the amount incorporated in the absence of the enzyme. As in the previous studies, the increase in phosphate incorporation was not due to phosphorylation of PtdIns 3-kinase, since SDS/PAGE showed only the radiolabelled poly(Glu-Tyr) (results not shown). Finally, another method was used in which the insulin receptor present in WGA extracts was incubated with purified PtdIns 3-kinase in the absence and presence of insulin. Phosphorylation studies were carried out under conditions known to facilitate the phosphorylation of substrates for the insulin receptor [38]. After the incubation, antibodies against PtdIns 3-kinase or against phosphotyrosine were used to try to identify phosphorylated protein. However, no evidence was

Figure 7 Association of insulin receptor with PtdIns 3-kinase activity from CHO-cell lysate

COS cells expressing wild-type insulin receptors were lysed and the insulin receptors were immunoprecipitated by a-IR-1. After treatment (lanes 2 and 4) or no treatment (lanes 1 and 3) of the insulin receptors with ATP/Mn²⁺ and insulin, the immunoprecipitated insulin receptors were incubated with purified PtdIns 3-kinase (lanes 1 and 2) or CHO-cell lysate (lanes 3 and 4). The precipitates were washed extensively and PtdIns 3-kinase activity in the immunoprecipitates was measured as described in the Experimental section. The amount of PtdIns 3-kinase activity added to the incubations for the purified enzyme (lanes 1 and 2) was 4 times the PtdIns 3-kinase activity present in the CHO-cell lysate (lanes 3 and 4). The amount of PtdInsP in lane 4 was approx. 3 times the amount present in lane 3.

Figure 8 IRS-1 effects on the putative association between PtdIns 3-kinase and insulin receptor

WGA-purified insulin receptor (WGA IR) was autophosphorylated with ATP in the absence or presence of Mn²⁺ and 100 nM insulin by using various combinations in the incubations as shown. The samples were incubated for 5 min with 3 ng of IRS-1 as indicated. Purified PtdIns 3-kinase (300 ng) was then added to all of the incubations with 2 µg of a-IR-1 absorbed to Protein G beads. After 3 h, PtdIns 3-kinase activity was measured in the insulin-receptor immunoprecipitates.

found of a 110 or 85 kDa protein that showed insulin-dependent phosphorylation.

Overall, the evidence is strong that the insulin receptor does not directly bind PtdIns 3-kinase. Nor were we able to detect the receptor-mediated phosphorylation of the enzyme. These results therefore cannot explain the findings from cells that have undergone insulin stimulation in which PtdIns 3-kinase activity is co-precipitated with the insulin receptor (see Figure 1). The most likely explanation for this discrepancy is the involvement of a third component that helps to link the enzyme to the receptor.

This possibility was examined by the use of CHO-cell lysates

Table 1 Effects of insulin stimulation on the ability of IRS-1 immunoprecipitates to associate with the insulin receptor and PtdIns 3-kinase

Confluent CHO-HIRC cells in 10 cm dishes were starved overnight and incubated with or without 100 nM insulin for 5 min. The cells were lysed and immunoprecipitated with 5 µl of anti-IRS-1 antibody. The immunoprecipitates were washed and the amount of PtdIns 3-kinase activity was measured (c.p.m. in PtdInsP). Specific insulin-binding activity was determined in the immunoprecipitates by standard insulin-receptor analysis as reported previously [38]. The data shown are representative of three separate experiments.

Insulin concn. (nM)...	Activity (c.p.m.)			
	PtdIns 3-kinase		Insulin binding	
	0	100	0	100
	115.1	670.2	113.2	650.3

that contain PtdIns 3-kinase activity (Figure 7). In this study, experiments similar to those described in Figures 4 and 5 were conducted, except that cell lysates were used as well as purified PtdIns 3-kinase. As shown previously, purified PtdIns 3-kinase will not bind to the phosphorylated insulin receptor. However, in agreement with previous findings [32], PtdIns 3-kinase in cell lysate associates with the receptor in the immunoprecipitate, and the amount of association was increased by using an auto-phosphorylated receptor. In the present work the increase was approx. 3-fold, a small increase, but one that was reproduced in each of three separate experiments.

One candidate for linking the insulin receptor to PtdIns 3-kinase is the protein IRS-1 [27]. This putative docking protein is phosphorylated by the insulin receptor, thereby providing phosphorylation attachment sites for PtdIns 3-kinase SH2 domains [18,20–27]. Attempts to show that IRS-1 produced by a baculovirus system could facilitate the coupling of purified PtdIns 3-kinase to the phosphorylated and immunoprecipitated insulin receptor were not successful (results not shown). However, it is possible to devise a system in which IRS-1 facilitates receptor–enzyme coupling by adding ATP along with purified PtdIns 3-kinase and the other components. Figure 8 shows the results typical of two separate experiments in which the absence or presence of Mn²⁺ was used to regulate the tyrosine kinase activity of the insulin receptor.

It is clear that formation of the receptor–enzyme complex requires all of the ingredients needed to support a phosphorylation reaction. The lack of insulin-stimulated tyrosine kinase activity, the lack of IRS-1 or the lack of added PtdIns 3-kinase (results not shown) prevented PtdIns 3-kinase from being included in the immunoprecipitate of the insulin receptor. Another more physiological approach for showing the coupling activity of IRS-1 is presented in Table 1, which is representative of three separate experiments. CHO cells that over-express the wild-type insulin receptor were incubated in the absence or presence of insulin. Immunoprecipitates formed by anti-IRS-1 antibody were examined for the insulin receptor by a standard insulin-binding assay and assayed for PtdIns 3-kinase activity. Clearly, insulin stimulation increased both the amount of insulin receptor in the IRS-1 precipitates and the level of PtdIns 3-kinase activity. These latter findings support, but do not prove, that IRS-1 can link the insulin receptor and PtdIns 3-kinase, since the immunoprecipitates may contain only insulin receptor–IRS-1 and PtdIns 3-kinase–IRS-1 complexes.

We presume that insulin–receptor phosphorylation of IRS-1 is

an important feature of the process that couples the receptor, IRS-1 and PtdIns 3-kinase. A phosphorylated 185 kDa band can be demonstrated in immunoprecipitates formed by antibodies against phosphotyrosine and PtdIns 3-kinase prepared from these cells after insulin treatment (results not shown). These findings, however, do not conclusively establish the importance of IRS-1 phosphorylation for forming the tertiary complex.

In summary, the findings from the reconstitution system argue strongly that the insulin receptor and PtdIns 3-kinase cannot directly interact. This lack of interaction also includes a lack of detectable phosphorylation of the enzyme by the receptor. Moreover, the ability of the F-2 insulin-receptor mutation to generate a normal PtdIns 3-kinase response to insulin in the receptor immunoprecipitates (see Figure 1) clearly shows that the enzyme is not binding to Tyr-1322, as previously suggested [6]. Although these are negative findings, the results with a positive control, i.e. the PDGF receptor, indicate that the insulin receptor does not interact with PtdIns 3-kinase in the same manner as do other growth-factor receptors. Since PtdIns 3-kinase can be detected in the immunoprecipitates of the insulin receptor prepared from insulin-stimulated cells, some mechanism must exist to link the receptor with the enzyme. This idea is supported by the ability of PtdIns 3-kinase in cell lysates to bind to the phosphorylated form of the insulin receptor, a finding previously reported [32]. Furthermore, the studies with cell lysates raise an interesting question regarding the mechanism for the association of PtdIns 3-kinase with the insulin receptor. It is important to realize that the insulin receptor was previously phosphorylated and the ATP was removed before lysate was added to the system. Thus the mechanism does not require a significant phosphorylation reaction to operate, which argues against the involvement of IRS-1. This explains the failure of IRS-1 to couple the phosphorylated receptor and purified PtdIns 3-kinase. It also explains why adding IRS-1 to cell lysate (in the absence of Mn^{2+} and ATP) failed to enhance the amount of enzyme bound to the phosphorylated receptor (results not shown).

It is possible that a small amount of phosphorylated IRS-1 exists in lysates from unstimulated cells. Although we have not detected such phosphorylated IRS-1, some of the coupling between the receptors and PtdIns 3-kinase could be mediated by this phosphorylated protein.

Although IRS-1 does not appear to participate in the 'kinase minus' mechanism described above, this protein will link the receptor and PtdIns 3-kinase if ATP/ Mn^{2+} are available, i.e. a 'kinase-dependent' mechanism. Under these conditions, IRS-1 is phosphorylated and can bind PtdIns 3-kinase, an interaction that is well established. The ability of IRS-1 antibodies to immunoprecipitate the insulin receptor after insulin stimulation of cells establishes the receptor-IRS-1 association. Thus, under physiological conditions, it is likely that a major portion of the PtdIns 3-kinase associates with the insulin receptor through IRS-1, a conclusion reached in a recent study which used another approach to study this question [39]. However, it is intriguing that other components may exist that facilitate the interaction of the insulin receptor with this or other signal-transduction proteins [40]. More work is needed to evaluate the types of interactions that the insulin receptor may undergo and the role of its tyrosine kinase activity. One role may be to phosphorylate proteins such as IRS-1 to initiate signal transduction, and a second role may be the autophosphorylation of the receptor itself to provide sites for the binding of other proteins.

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