Phosphatidylinositol Kinase or an Associated Protein Is a Substrate for the Insulin Receptor Tyrosine Kinase*

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The tyrosine kinase activity intrinsic to the insulin receptor is thought to be important in eliciting the intracellular responses to insulin; however, it has been difficult to determine the biochemical functions of the proteins which are substrates for this receptor. Treatment of Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor (CHO-T) with insulin results in a 38 ± 11 (mean ± S.E., n = 9)-fold increase in phosphatidylinositol (PtdIns) kinase activity in anti-phosphotyrosine immunoprecipitates of whole cell lysates. One minute of treatment of cells with insulin causes a dramatic increase in the PtdIns kinase activity in the anti-phosphotyrosine immunoprecipitates; the activity peaks within 5 min and remains elevated for at least 60 min after addition of insulin to the cells. This response is only slightly delayed compared with the time course we observe for activation of the insulin receptor tyrosine kinase. The insulin dose-response curves are also very similar for the activation of the insulin receptor tyrosine kinase activity and for the appearance of PtdIns kinase in the anti-phosphotyrosine immunoprecipitates. Stimulation of the endogenous insulin receptor of CHO cells also results in the association of PtdIns kinase activity with phosphotyrosine-containing proteins. However, CHO cells are less sensitive to insulin than CHO-T cells, and the maximal PtdIns kinase activity in anti-phosphotyrosine immunoprecipitates from CHO cells is one-sixth that of CHO-T cells. In contrast, immunoprecipitates from CHO-T cells made with anti-insulin receptor antibodies do not contain significant levels of PtdIns kinase activity. This demonstrates that the PtdIns kinase is either a substrate for the insulin receptor tyrosine kinase or is tightly associated with another tyrosine phosphoprotein, which is not the insulin receptor.

The cell surface receptor for insulin is responsible for transduction of the signal from this circulating hormone to the interior of the cell. The receptor is a transmembrane glycoprotein made up of two α subunits, which contain the extracellular insulin binding site, and two β subunits, which contain an intracellular protein tyrosine kinase activity (1, 2). Upon insulin binding, the β subunit becomes autophosphorylated at tyrosine residues, and the tyrosine kinase activity of the receptor increases toward other substrates(1). Evidence has accumulated from studies using monoclonal antibodies which inhibit the tyrosine kinase activity (3) and from studies of mutant receptors lacking kinase activity (1, 4) that the tyrosine kinase activity of the insulin receptor is essential in eliciting the complex cellular response to insulin. Although several proteins have been identified which are potential substrates for the receptor tyrosine kinase, none of these proteins has a known enzymatic activity (5–7).

Protein tyrosine kinase activity is limited to a class of molecules which have in common a role in regulation of cellular proliferation. These include the transmembrane receptors for epidermal growth factor, PDGF, and colony stimulating factor, and the proteins encoded for by several oncogenes including v-src, v-ros, and v-fms. In addition, association with pp60c-src and activation of its tyrosine kinase activity is thought to be essential for transformation of cells by the polyoma middle T antigen (8). The β subunit of the insulin receptor contains a domain which shares homology with other tyrosine kinases; the homology is highest for the protein encoded for by v-ros (9).

A feature common to several tyrosine kinases is an apparent association with a cellular PtdIns kinase activity. pp60c-src (10, 11), the polyoma middle T/pp60c-src complex (12, 13), and pp65–70 (14), have all been shown to co-immunoprecipitate with a PtdIns kinase activity. For a variety of mutants of middle T, the association with PtdIns kinase is coincident with the ability to transform cells (8, 12). Stimulation of cells with PDGF also leads to the appearance of a PtdIns kinase activity in immunoprecipitates made with antibodies to phosphotyrosine (15) and to the PDGF receptor (16). The PtdIns kinase which associates with activated tyrosine kinases has recently been shown to produce a novel phospholipid, PtdIns-3-P, of unknown function (13, 17). The major cellular PtdIns kinases produce PtdIns-4-P, which is further metabolized to PtdIns-4,5-bisphosphate and to inositol-1,4,5-trisphosphate, the second messenger responsible for rises in intracellular Ca2+ (18). To examine further the relationship between tyrosine kinases and PtdIns kinase activity, we have measured the association of this enzyme with the insulin receptor and with phosphotyrosine containing proteins. We find that insulin treatment of cells greatly increases the activity of PtdIns kinase in immunoprecipitates made with an antibody to phosphotyrosine.

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The abbreviations used are: PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; DTT, dithiothreitol; MfG, normal mouse immunoglobulins; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.
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PHOTYROSINE. However, the association of PtdIns kinase activity with the activated insulin receptor is not significant under these conditions.

EXPERIMENTAL PROCEDURES

Materials—Phosphoamino acids, DTT, PtdIns-4-P, poly(Glu, Tyr)4:1, Triton X-100, and Nonidet P-40 were purchased from Sigma. PtdIns (bovine liver) was purchased from Avanti Polar Lipids, Inc. Protein G-Sepharose from Genex Corp., pork insulin from Eliancgo, MlgG from Pel-Freez Biologicals, [γ-32P]ATP (3000 Ci/mmol) from Amersham Corp., and preadsorbed molecular weight markers for SDS-polyacrylamide gel electrophoresis from Bethesda Research Laboratories. The monoclonal antibody py20, which specifically recognizes photyrosine residues (10), was a gift from Dr. John R. Glenney, Jr., at the University of Kentucky, Lexington. Monoclonal antibodies 5D9 and 29B4 were developed to the human insulin receptor as described (20); 5D9 binds to an extracellular epitope of the α-subunit, and 29B4 recognizes an intracellular epitope on the β-subunit (20).

Preparation of Cells and Cell Extracts The CHO T cell line used in these studies is stably transfected with the human insulin receptor; ~10^6 receptors are expressed per cell compared with approximately 2000 receptors/CHO cell. Confluent 10-cm plates of CHO or CHO-T cells were washed with Ham's F-12 medium containing 20 mM Hepes and incubated in this medium for 30 min at 37 °C. Insulin or diluent was added to this medium, and cells were incubated at 37 °C for the indicated period of time. Plates were then washed with cold 0.1 M NaCl, scraped into cold lysis buffer (1 ml/plate; 157 mM NaCl, 20 mM Tris, pH 8, 1 mM MgCl2, 1 mM CaCl2, 1 mM EDTA, 1 mg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 0.4 mM sodium orthovanadate), and solubilized on ice for 10 min. The lysate was centrifuged at 15,000 rpm in an Eppendorf centrifuge for 15 min, and the supernatant (referred to as “lysate”) was removed.

Protein G-Sepharose (20-μl beads) was coated with 20 μg of MIgG, 29B4, or 5D9 or 5 μg of py20. The washed beads were incubated with the cell lysate for 2 h with gentle rotation on a rotating platform. Immunoprecipitates were washed twice with each of the following buffers: 1) phosphate-buffered saline, 1% Nonidet P-40, 0.1 mM DTT; 2) 0.1 M Tris, pH 7.4, 0.5 M LiCl, 1 mM DTT; 3) 10 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM DTT.

Assay of PtdIns Kinase Activity—PtdIns kinase activity was measured directly in immunoprecipitates in 50 μl of a reaction mix containing (final concentrations are final): 0.2 mg/ml PtdIns 20 mM Hepes pH 7.1, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM MgCl2, and [γ-32P]ATP (40 μM and 0.1 μCi/μl). PtdIns was dried with N2 and sonicated at 1 mg/ml in a bath sonicator (Heat Systems-Ultrasonics) for 3 × 5 min with cooling in between (12). PtdIns was incubated with the sample for 5 min at room temperature prior to addition of MgATP to start the reaction. After 5 min, the reaction was stopped by the addition of 15 μl of 4 N HCl, 1 μl of chloroform, and methanol (1:1). Tubes were mixed for 30 s, and 30 μl of the lower layer was spotted on a Silica Gel 60 plate (Merck, which had been coated with a solution of 1% potassium oxalate and activated at 100 °C for 1 h. Plates were developed as described (12), and the radioactive reaction product was quantitated using a Radioanalytic Imaging System (Amersham Corp.). This system does not separate the 3-P and 4-P isomers (17).

Assay of Insulin Receptor Kinase Activity—The insulin receptor was immunoprecipitated from cell lysates as described above using the monoclonal antibody to the insulin receptor, 5D9. The washed beads were incubated for 10 min at room temperature in a 50-μl reaction mixture containing (final concentrations): 0.5 mg/ml poly(Glu,Tyr)4:1, 50 mM Hepes, pH 7.6, 1 mM DTT; 10 mM MgCl2, 3 mM NaCl, 0.1% Triton X-100, and [γ-32P]ATP at 1 μCi/tube. Following addition of Laemmli SDS sample buffer (21), samples were heated at 100 °C for 3 min and electrophoresed on a 7.5% SDS-polyacrylamide gel. Gels were stained and autoradiographed, and the bands were quantitated by Cerenkov counting.

Preparation of Polyclonal Anti-insulin Receptor Antibody—Human placental insulin receptor was highly purified by sequential gel filtration, affinity chromatography, and electrophoresis as described (22). A rabbit was injected in the popliteal lymph nodes with ~50 μg of purified insulin receptor emulsified in complete Freund's adjuvant. After 3 weeks, the rabbit was injected intramuscularly with the same amount of receptor in incomplete Freund's adjuvant. A second boost was performed 4 weeks later, and the rabbit was bled 10 days following this injection.

Comparison of Anti-insulin Receptor and Anti-photyrosine Antibodies—Immunoprecipitates were made from control or insulin-treated CHO-T cells with 20 μg of either MIgG, 29B4, or 5D9 or 5 μg of py20, as described above. Replicate samples were used to both assay PtdIns kinase activity and to compare the amount of immunoprecipitated insulin receptor. For the latter purpose, samples were separated on a 4–15% gradient gel, electrophoretically transferred to nitrocellulose, and blotted with the polyclonal anti-insulin receptor antibody. The bound rabbit immunoglobulin was detected with an anti-rabbit immunoglobulin alkaline phosphatase conjugate (Promega Biotech, as detailed in their instructions).

RESULTS

PtdIns Kinase Activity Is Present in Anti-photyrosine Immunoprecipitates from Insulin-treated Cells—CHO cells transfected with the human insulin receptor (CHO-T) were treated with insulin, a detergent lysate was prepared, and photyrosine-containing proteins were immunoprecipitated with the monoclonal antibody, py20. When immunoprecipitates were washed and assayed in 0.1% Triton X-100, insulin treatment was found to result in a 2-fold increase in PtdIns kinase activity. However, when the pellets were washed with 1.0% Triton X-100 to more thoroughly remove nonspecifically associated proteins, the effect was no longer observed. These assays were carried out in the presence of nonionic detergent, which has been reported to stimulate certain PtdIns kinases (23) but to completely inhibit the PtdIns kinase which associates with the polyoma middle T-ecd-c complex and the activated PDGF receptor (10). When we assayed the py20 immunoprecipitates in the absence of detergent, we found that insulin treatment of cells caused a large increase in PtdIns kinase activity. In nine separate experiments using 10^-6 to 10^-7 M insulin for 5–10 min, the average increase due to insulin was 38 ± 11 (S.E.)-fold. Fig. 1 shows that the PtdIns kinase in the anti-photyrosine immunoprecipitates is completely inhibited by 0.2% Nonidet P-40, while the remainder of the PtdIns kinase activity in the cell lysate is stimulated by this detergent.

The Insulin Dose-Response Relationship Is Similar for Activation of the Insulin Receptor Tyrosine Kinase and the Photyrosine-associated PtdIns Kinase Activity—In order to determine whether the effect of insulin on PtdIns kinase activity is likely to be of physiological importance, PtdIns kinase activity was measured following treatment of cells with various doses of insulin (Fig. 2A). In immunoprecipitates from

Fig. 1. Effect of Nonidet P-40 on PtdIns kinase activities. PI kinase activity was assayed in anti-photyrosine immunoprecipitates from insulin-stimulated cells (●) and in the supernatant from the immunoprecipitation (○). The supernatant was diluted to 0.02% Nonidet P-40, and Nonidet P-40 was added back to the indicated level.
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**FIG. 2.** PtdIns kinase and insulin receptor kinase dependence on insulin concentration. Activities were measured in immunoprecipitates made from lysates of CHO (open circles) or CHO-T (closed circles) cells treated with the indicated concentrations of insulin for 5 min. A, PtdIns kinase activity was measured in anti-phosphotyrosine immunoprecipitates. B, tyrosine kinase activity of the insulin receptor was measured in anti-insulin receptor immunoprecipitates using the synthetic substrate poly(Glu,Tyr)4:1. C, autophosphorylation of the insulin receptor was measured in anti insulin receptor immunoprecipitates. Data points are the mean values from two to three separate experiments.

CHO-T cells made with py20, PtdIns kinase activity increases significantly with 10^-10 M insulin and is half-maximal at ~3 x 10^-9 M. Activation of the endogenous insulin receptor of CHO cells also leads to increased association of PtdIns kinase with phosphotyrosine-containing proteins. Consistent with CHO cells possessing ~3000 insulin receptors compared with 10^6 on CHO-T cells, approximately 1000-fold more insulin is required to obtain equal amounts of phosphatidylinositol kinase activity from CHO cells compared with CHO-T cells. In parallel studies, activation of receptor tyrosine kinase was measured in CHO-T cells by immunoprecipitation of the insulin receptor with the monoclonal antibody 5D9 and by assaying either phosphorylation of poly(Glu,Tyr)4:1 (Fig. 2B) or autophosphorylation of the β-subunit of the receptor (Fig. 2C). Half-maximal activation of both reactions occurs at ~3 x 10^-9 M insulin.

**The Insulin Receptor Tyrosine Kinase and the Phosphotyrosine-associated PtdIns Kinase Are Stimulated Similarly Over Time by Insulin**—Within 1 min of addition of insulin to cells, a large increase is measurable in PtdIns kinase activity in py20 immunoprecipitates (Fig. 3A), the phosphorylation of poly(Glu,Tyr)4:1 by the insulin receptor (Fig. 3B), and the autophosphorylation of the β-subunit of the insulin receptor (Fig. 3C). PtdIns kinase activity reaches a maximum within 5 min of insulin treatment, while tyrosine kinase activities reach plateau values within 2 min. All three activities remain elevated for at least 60 min of insulin treatment.

**Insulin Increases PtdIns Kinase Activity Associated with Phosphotyrosine-containing Proteins but Not with the Insulin Receptor**—The presence of a PtdIns kinase activity in immunoprecipitates made from lysates of CHO-T cells treated with 10^-7 M insulin for the indicated times. The assays used in A, B, and C are described in the legend to Fig. 2. Data points are the mean values from three separate experiments.

In order to determine whether the PtdIns kinase is physically associated with the insulin receptor, immunoprecipitates were made from insulin-treated cells using two monoclonal antibodies to the insulin receptor. A small and variable amount of PtdIns kinase activity is immunoprecipitated by 29B4, and immunoprecipitates made with 5D9 contained no PtdIns kinase activity (Fig. 4A). Data on PtdIns kinase activity were pooled from experiments using either 10^-8 M insulin for 10 min or 10^-7 M insulin for 5 min, as the effects were similar and there was no systematic difference between the two treatments. Equal amounts of the activated insulin receptor are immunoprecipitated by 29B4, 5D9, and py20 (Fig. 4B). These data from cells stimulated with 10^-8 M insulin for 10 min are very similar to that seen...
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Fig. 4. Immunoprecipitation of PtdIns kinase and the insulin receptor by anti-phosphotyrosine and anti-insulin receptor antibodies. Immunoprecipitates were made from control or insulin-treated cells using control (MlgG), anti-phosphotyrosine (py20), or anti-insulin receptor (5D9, 29B4) antibodies. A, PtdIns kinase activity in the immunoprecipitates; data (mean ± S.E.M.) are pooled from four separate experiments as described under “Results.” B, the amount of insulin receptor in the immunoprecipitates from insulin-treated cells was compared by Western blot analysis using a polyclonal anti-insulin receptor antibody which recognizes the β-subunit and the insulin receptor precursor. IR-β, insulin receptor β-subunit; pre-IR, insulin receptor precursor.

with 10⁻⁷ M insulin for 5 min. Thus PtdIns kinase is either a substrate for the insulin receptor tyrosine kinase or it is closely associated with another tyrosine kinase substrate.

Discussion

The identification of physiological substrates for the insulin receptor tyrosine kinase will be necessary for understanding how the cellular responses to insulin are elicited. In studies designed to identify such substrates, we have measured PtdIns kinase activity in phosphotyrosine-containing proteins from insulin-treated cells. In CHO-T cells, which stably express the human insulin receptor at high levels, treatment with insulin results in an approximately 38-fold increase in the PtdIns kinase activity in immunoprecipitates made with an anti-phosphotyrosine antibody, py20. Activation of the endogenous insulin receptor of CHO cells similarly results in an increase in PtdIns kinase activity associated with phosphotyrosine-containing proteins; however, higher concentrations of insulin are required to elicit the response in CHO cells than in CHO-T cells, as previously observed with other biological responses (4). We have also observed that insulin treatment of rat adipocytes causes a 20-fold increase in PtdIns kinase activity associated with phosphotyrosine-containing proteins. The PtdIns kinase in anti-phosphotyrosine immunoprecipitates from CHO-T cells is distinct from the bulk of CHO cell PtdIns kinase and from previously studied PtdIns kinase activities associated with the insulin receptor (24, 25) in that it is completely inhibited by the nonionic detergent Nonidet P-40 at concentrations close to or above the critical micelle concentrations. Whitman et al. (10) observed identical inhibition of the polyoma middle T- c-src-associated PtdIns kinase by Nonidet P-40. This contrasts with other PtdIns kinases from fibroblasts (11) and brain (23), which are greatly stimulated by nonionic detergents.

The presence of PtdIns kinase activity in anti-phosphotyrosine immunoprecipitates does not appear to be due to a physical association with the autophosphorylated insulin receptor, as immunoprecipitates made with two different monoclonal antibodies to the insulin receptor do not contain significant amounts of PtdIns kinase activity. These antibodies, 5D9 and 29B4, recognize an extracellular domain on the α-subunit and an intracellular domain on the β-subunit, respectively (20), making it unlikely that the lack of PtdIns kinase activity is due to inhibition by the antibodies. PtdIns kinase activity has also been reported to be present in anti-phosphotyrosine immunoprecipitates from cells stimulated with PDGF (10, 15, 16) and from cells transformed by polyoma middle-T antigen, v-fms, or v-sti (16). In these cases, the data do not distinguish between PtdIns kinase associated with the tyrosine-phosphorylated receptor or oncogene product and the possibility that PtdIns kinase is a substrate for the activated tyrosine kinases. Unlike our observations with the insulin receptor, PtdIns kinase activity is apparently associated with purified pp60+src (26) and immunoprecipitates of pp60+src (10, 11), p68+src (14), the polyoma middle-T-pp60+src complex (8, 12, 13), and the activated PDGF receptor (16). The insulin receptor may be unique among tyrosine kinases in that the PtdIns kinase may be a substrate without necessarily becoming part of a relatively stable complex with the receptor.

The observation that an increase in PtdIns kinase, but not tyrosine kinase, activity is detectable in immunoprecipitates from CHO-T cells treated with 10⁻⁹ M insulin is indicative that an amplification of the insulin signal has occurred. The similar dose-response curves for the three kinase activities from 10⁻⁸ to 10⁻⁷ M insulin suggest that the presence of PtdIns kinase in anti-phosphotyrosine immunoprecipitates is a direct response to receptor kinase stimulation. The effects of insulin on PtdIns kinase and tyrosine kinase activities which we observe occur rapidly; receptor tyrosine kinase activity peaks within 1–2 min of insulin addition, and the activity of PtdIns kinase in anti-phosphotyrosine immunoprecipitates increases dramatically within 1 min and peaks within 5 min of insulin addition. This time course suggests that if PtdIns kinase is not itself a substrate for the receptor tyrosine kinase, under resting conditions it is already associated with such a substrate. Kaplan et al. (15) observed a similarly rapid association of PtdIns kinase activity with the activated PDGF receptor. This observation raises the possibility that activation of PtdIns kinase is involved in one or more of the cellular responses to insulin which take place on the second to minute time scale including changes in gene transcription, stimulation of hexose and ion transport, receptor internalization, and alterations in intracellular enzyme activities (1). PtdIns kinase activity remains elevated in anti-phosphotyrosine immunoprecipitates for at least 60 min; thus it is possibly

involved in eliciting longer term responses to insulin including synthesis of protein, lipid, and nucleic acid, maximal down-regulation of the receptor, and stimulation of cell growth (1). It has recently been reported that the PtdIns kinase activity associated with polyoma middle T-pp60c-src (13, 17) and the activated PDGF receptor (27) phosphorylates the 3-position on the inositol ring, while the detergent-stimulated activity from fibroblasts phosphorylates the 4-position (17). The two isomers are not distinguished by the commonly used assay. The formation of PtdIns-4-P contributes to "classical" polyphosphoinositide turnover and generation of the second messenger, inositol-1,4,5-trisphosphate (18), it is not known what the function(s) or biochemical fate(s) of PtdIns-3-P are. The PtdIns kinase, which appears in anti-phosphotyrosine immunoprecipitates in response to insulin, and the PtdIns 3-kinase share the unique characteristic of inhibition by detergent. Further, we have found that over 90% of the PtdIns P formed by anti-phosphotyrosine immunoprecipitates from insulin-treated cells is PtdIns-3-P.3 The presence of a Ptdlns kinase in uitro, may be preferred substrates for PtdIns-4,5-P2, which have been shown to be substrates for polymerase turnover and generation of the second messenger, inositol-1,4,5-trisphosphate (18), it is not known what the function(s) or biochemical fate(s) of PtdIns-3-P are. The PtdIns kinase activity which appears in anti-phosphotyrosine immunoprecipitates in response to insulin, and the PtdIns 3-kinase share the unique characteristic of inhibition by detergent. Further, we have found that over 90% of the PtdIns P formed by anti-phosphotyrosine immunoprecipitates from insulin-treated cells is PtdIns-3-P.3 The presence of a PtdIns 3-kinase activity in uitro, may be preferred substrates for PtdIns-4,5-P2, which have been shown to be substrates for the PtdIns-3-P and PtdIns-4-P isomers. We are grateful to Dr. John R. Glenney, Jr. for his gift of the monoclonal antibody, py20, and to T. Hansen, J. Stagsted, S. Ziehe, and L. Olsson for providing us with adipocytes. We thank Neil Ruderman, Rosanna Kapeller, Morris White, and Lewis Cantley for sharing their findings with us prior to publication.

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