Phosphorylation of PDE3B by Phosphatidylinositol 3-Kinase Associated with the Insulin Receptor*

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Phosphatidylinositol 3-kinase mediates several actions of insulin including its antilipolytic effect. This effect is elicited by the insulin-stimulated serine phosphorylation and activation of cGMP-inhibited phosphodiesterase (PDE3B). In human adipocytes, we found that insulin differentially stimulated phosphatidylinositol 3-kinase activity; the lipid kinase activity was associated with IRS-1, whereas the serine kinase activity was associated with the insulin receptor and phosphorylated a number of proteins including p85, p110, and a 135-kDa protein identified as PDE3B. PDE3B phosphorylation was associated with enzyme activation, thus initiating the antilipolytic effect of insulin. These results show a novel pathway for intracellular signaling through the insulin receptor leading to the serine phosphorylation of key proteins involved in insulin action.

The antilipolytic effect of insulin is mediated through the activation of the cGMP-inhibitable phosphodiesterase PDE3B (1–5). This enzyme becomes activated by phosphorylation on serine 302 (5) through an insulin-regulated serine kinase which has yet to be identified. PDE3B, a protein of around 135 kDa (5), is associated with the plasma membrane (2) and is dependent on PI3-kinase for its activation. However, it is not known whether PDE3-kinase itself or other downstream kinases are involved in the phosphorylation and activation of PDE3B.

PI3-kinase is a heterodimer composed of two subunits: a p85 regulatory subunit, which contains two Src homology 2 (SH2) domains and one Src homology 3 (SH3) domain, as well as the p110 catalytic subunit (6–8). It is a dual specificity kinase (9, 10) that possesses both lipid and serine kinase activities, both of which can be abolished by the inhibitors wortmannin and LY 294002 (11, 12). Activation of PI3-kinase lipid kinase by insulin involves tyrosine phosphorylation of the large docking proteins IRS-1/2 by the insulin receptor tyrosine kinase and binding of the p85 subunit of PI3-kinase (13, 14). This binding activates the lipid kinase, which phosphorylates the phosphoinositol at the D-3 position of the inositol ring (15). In addition, the PI3-kinase serine kinase autophosphorylates both the p85 regulatory subunit and the p110 catalytic subunit (9, 10), which in turn appear to down-regulate the lipid kinase activity. However, it is not known whether insulin regulates this serine kinase activity.

In addition to the association with IRS-1/2, p85 has also been detected in anti-insulin receptor (IR) immunoprecipitates from insulin-stimulated cells transfected with human insulin receptor cDNA (15, 16). The insulin receptor β-subunit contains a C-terminal tyrosine residue present in the YTHM sequence (amino acid residues 1322–1325) that confers binding sites for the p85 subunit of PI3-kinase (16–18). Thus, the p85 subunit seems capable of binding directly to the phosphorylated insulin receptor although the potential role of this alternative mechanism is unknown. In the present study, using human fat cells, we investigated whether insulin activates PI3-kinase serine kinase. We present evidence that this kinase is activated by insulin through binding to insulin receptors and that PDE3B is a substrate.

EXPRESSIMENTAL PROCEDURES

Materials—Bovine serum albumin (fraction V), collagenase, leupeptin, phosphatidylinositol, phosphatidylserine,wortmannin, and LY 294002 were from Sigma. Radiochemicals were from Amersham Pharmacia Biotech or NEN Life Science Products. Anti-phosphotyrosine monoclonal antibodies, anti-PKB, anti-IRS-1 polyclonal antibodies, antiserum insulin receptor antibodies, and anti-PI3-kinase antibodies (p85 and p110) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) or Transduction Laboratories (Lexington, KY). Protein A/G-Sepharose was from Amersham Pharmacia Biotech and 4-(2-aminoethyl)benzenesulfonil fluoride was from Calbiochem.

Preparation of Cells, Lysates, and Incubation Procedures—Specimens of human subcutaneous adipose tissue were obtained from the abdominal region of nondiabetic subjects. The biopsies were placed in Medium 199 containing 25 mM Hepes, 4% bovine serum albumin, and 5.5 mM glucose at 37 °C. Adipose cells were prepared according to methods previously described (19). Briefly, adipocytes were isolated by digesting 0.6 g of tissue for 50 min at 37 °C in Medium 199 containing 25 mM Hepes, 4% bovine serum albumin, 5.5 mM glucose, and 0.8 mg/ml collagenase in a shaking water bath. The study was approved by the Ethical Committee of Goteborg University.

Isolated human adipocytes were distributed in plastic vials (12–15% cell suspension) in a final incubation volume of 400 μl. Cells were preincubated with or without 6.8 nM insulin (Novo-Nordisk) for 10 min in the presence of 0.1 μM -N(2-phenylisopropyl)-adenosine and 1 unit/ml teliposine deaminase; immediately separated by centrifugation through silicone oil; lysed in 0.4 ml of lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na3VO4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, and 0.1 mM 4-(2-aminoethyl)benzenesulfonil fluoride; and rocked for 40 min at
RESULTS AND DISCUSSION

Human adipocytes were incubated in the presence or absence of 6.9 nM insulin for 10 min; cells were lysed and immunoprecipitated with anti-IRS-1 or anti-IR antibodies. Immunoblots were probed with antibodies to the p85 and p110 subunits of PI3-kinase (Fig. 1). Insulin stimulated the association of both the p85 (apparently both the α- and β-isomers) and p110 subunits to both IRS-1 and IR, although the relative amount bound to IRS-1 was ~3-fold greater than that bound to the insulin receptors.

To investigate whether the association of PI3-kinase with the insulin receptor was due to a significant formation of ternary complexes where both p85 and the insulin receptor simultaneously bound to IRS-1, the same blots were probed with anti-phosphotyrosine, anti-IRS-1, and anti-IR antibodies. A small amount of IRS-1 was co-immunoprecipitated by anti-IR antibodies, and a similar small proportion of IR was co-immunoprecipitated with anti-IRS-1 antibodies (Fig. 1). Thus, in agreement with previous reports (16, 25), there is a small formation of these ternary complexes in response to insulin.

PI3-kinase lipid kinase activity was measured next in the same IR and IRS-1 immunoprecipitates from human adipocytes preincubated in the absence or presence of insulin. As shown in Fig. 2, insulin increased PI3-kinase lipid kinase activity associated with IRS-1 ~20-fold, but no increase was found in the anti-IR immunoprecipitates, although insulin clearly increased the association of both the p85 and p110 subunits with the IR (Fig. 1). In fact, insulin tended to further decrease the already low basal lipid kinase activity associated with the insulin receptors (Fig. 2).

Because PI3-kinase is a dual specificity kinase, we next examined whether the protein kinase activity was associated with IRS-1 or IR. Fig. 3A shows an autoradiogram of the cellular proteins in the IRS-1 and IR immunoprecipitates that became phosphorylated in response to insulin. When IRS-1 was immunoprecipitated from control and insulin-stimulated cells, no clearly phosphorylated proteins were visible, although p85

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4 °C. Detergent-insoluble material was sedimented by centrifugation at 12,000 × g for 10 min at 4 °C.

Immunoprecipitations and Immunoblotting—Cell lysates (100 μg of protein) were immunoprecipitated for 2 h with anti-IRS-1 C-terminal (4 μg/ml) or anti-IR (4 μg/ml) antibodies. Immunocomplexes were collected with Protein A/G-Sepharose, washed, solubilized in Laemmli sample buffer, and separated using 10 or 7.5% SDS-PAGE. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% fat-free milk. The blots were probed with the different primary antibodies (anti-IRS-1 C-terminal, anti-p85 (whole antiserum), anti-PY antibodies, anti-PKB/Akt C-terminal, anti-IR, and anti-p110 according to the recommendations of the manufacturer) or antiserum raised against PDE3B (peptide corresponding to amino acids 423–440 of rat PDE3B), and the proteins were detected by enhanced chemiluminescence using horseradish peroxidase-labeled secondary antibodies (Amersham Pharma Biotech). The intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics). Quantification was also verified using 125I-Protein A.

Phosphoamino Acids—Phosphoamino acid analysis was performed as described by Kamps and Sefton (21) by acid hydrolysis of phosphoproteins in 6 M HCl at 110 °C and separation on thin layer cellulose chromatography plates together with phosphoamino acid standards.

PI3-Kinase Lipid and Serine Kinases Activities—For PI3-kinase lipid kinase assays, immunoprecipitates were washed extensively, and the PI3-kinase reaction was performed using phosphatidylinositol as a substrate as described (20). For protein kinase activity, the immunoprecipitates were first preincubated with or without 100 nM wortmannin, 1 μM LY 294002, or other inhibitors for 30 min as indicated under "Results and Discussion." The immune complexes were then incubated in 20 μl of buffer containing 20 mM Hepes (pH 7.5), 3 mM MnCl2, 10 mM MgCl2, 50 μM ATP, and (γ-32P)ATP (10 μCi/assay) for 20 min at 30 °C. The complexes were washed twice with cold phosphate-buffered saline and then resuspended in Laemmli sample buffer. The samples were run on 10% SDS-polyacrylamide gels, and the products of the kinase reactions were visualized by autoradiography. The degree of phosphorylation was quantified by laser densitometry (Molecular Dynamics).

PDE3B Activity—Immunoprecipitates and supernatants were assayed for PDE3B activity as described (22) in the presence or absence of the specific PDE3B inhibitor OPC3911 (3 μM). Samples were incubated for 30 min at 30 °C in a total volume of 0.3 ml containing 50 mM HEPES, pH 7.5, 0.1 mM EDTA, 8.3 mM MgCl2, and 0.5 μM [3H]cAMP (200–500 cpm/pmol).

FIG. 1. PI3-kinase co-immunoprecipitates with IRS-1 and the insulin receptor. Human adipocytes were stimulated with 6.9 nM insulin (Ins) for 10 min where indicated. Cell lysates were immunoprecipitated using anti-IRS-1 or anti-IR antibodies, separated on 10% SDS-PAGE, and immunoblotted with anti-IR and anti-IRS-1 antibodies or with anti-p85 and anti-p110 antibodies. The intensity of the bands was quantitated with a laser densitometer. IP, immunoprecipitate.
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Fig. 3. PI3-kinase serine kinase is associated with the insulin receptor. Human adipocytes were incubated as described with (+) or without (−) insulin (INS). IRS-1 or insulin receptors were isolated by immunoprecipitation with anti-IRS-1 and anti-IR antibodies, and the immunoprecipitates (IP) were assayed for protein kinase activity as described under "Experimental Procedures." A, the proteins phosphorylated in the immune complexes were separated by 10% SDS-PAGE, transferred, and visualized by autoradiography. B, the same membrane was probed with anti-p85 antibodies. C, IR immunoprecipitates were incubated with or without 100 nM wortmannin (Wort) for 30 min at room temperature and then assayed for protein kinase activity as described.

association was markedly increased (Fig. 3B). In contrast, in the IR immunoprecipitates from cells preincubated with insulin, phosphorylation was increased in proteins with molecular masses of 85, 97, 110, and 135 kDa, respectively (Fig. 3A). The 85-kDa phosphoprotein co-migrates with the p85 regulatory subunit of PI3-kinase and was identified by immunoblotting with anti-p85 antibodies in the same membrane. In addition, the p110 subunit was also identified as the catalytic subunit of the PI3-kinase, and phosphoamino acid analysis showed that these proteins were phosphorylated on serine residues (not shown). Both p85 and p110 are known substrates of PI3-kinase serine kinase (9, 10), so these results are consistent with the presence of this serine kinase in the IR immunoprecipitates.

We next studied the effect of wortmannin, which inhibits both the PI3-kinase serine and lipid kinase activities (11, 12), to determine whether the protein kinase associated with the IR was indeed PI3-kinase itself. The IR immunoprecipitates were preincubated with or without 100 nM wortmannin for 30 min and then assayed for in vitro protein kinase activity (Fig. 3C). Wortmannin inhibited the insulin-stimulated phosphorylation of the 85-, 97-, 110-, and 135-kDa proteins in the IR immunoprecipitates, suggesting that the protein kinase associated with the insulin receptor was indeed the PI3-kinase with serine kinase activity. Similar results were obtained with 1 μM LY 294002 (data not shown). The identity of the phosphorylated p97 band is currently unclear. Although the insulin receptor is a likely candidate, this cannot be the only phosphorylated protein because control experiments showed that insulin receptor phosphorylation was not inhibited by either wortmannin or LY 294002 (data not shown). Several other previous studies have also reported that wortmannin at the concentration used has no effect on insulin receptor phosphorylation or tyrosine kinase activity (11, 12, 24).

Wortmannin inhibits the antilipolytic effect of insulin as well as the insulin-stimulated serine phosphorylation and activation of PDE3B (2, 4, 5), the key step in eliciting the antilipolytic action, supporting the involvement of PI3-kinase in this action. To examine whether the 135-kDa protein, which became phosphorylated by insulin and was recovered in the IR immunoprecipitates, could be PDE3B, we probed the membranes with anti-PDE3B antibodies. PDE3B co-migrated with the 135-kDa phosphoprotein, suggesting that PDE3B was indeed a possible substrate for the PI3-kinase serine kinase. Phosphoamino acid analysis also showed that a protein of this molecular weight was phosphorylated on serine residues (Fig. 4A). In addition, sequential immunoprecipitations of cell lysates using anti-IR antibodies first and then anti-PDE3B antibodies were performed, and the proteins were immunoblotted with anti-PDE3B antibodies (Fig. 4B). PDE3B was mainly detected in the IR immunoprecipitate (∼70%), but ∼30% was found in the supernatant. This association did not appear to be regulated by insulin because PDE3B was recovered with the insulin receptor in both basal and insulin-stimulated cells. Consequently, IR and PI3-kinase (p85) were also co-immunoprecipitated with the PDE3B immunocomplexes (Fig. 4C).

To further verify that the 135-kDa 32P-polypeptide was PDE3B, we dissociated the IR immunoprecipitates with 100 mM glycine after in vitro phosphorylation, and the supernatants were re-immunoprecipitated with anti-PDE3B antibodies. The 135-kDa 32P-peptide that was phosphorylated by insulin in the IR immunoprecipitates was re-immunoprecipitated by anti-PDE3B antibodies, thus verifying that the 135-kDa protein was indeed PDE3B (Fig. 4D).

To further explore the role of PI3-kinase serine kinase for PDE3B phosphorylation, human adipocytes were preincubated with or without insulin for 15 min. IR immunoprecipitates as well as the supernatants were assayed for protein kinase activity in the presence or absence of 100 nM wortmannin using purified recombinant PDE3B as a substrate. Fig. 4E shows that insulin stimulated the phosphorylation of recombinant PDE3B by a wortmannin-sensitive kinase associated with the IR, presumably PI3-kinase serine kinase. There was no remaining phosphorylating activity in the supernatants of the IR immunoprecipitates in the presence of wortmannin (data not shown).
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Fig. 4. PI3-kinase serine kinase associated with the insulin receptor phosphorylates and activates PDE3B. Cells were stimulated with (+) or without (−) insulin (INS) and lysed. A, phosphoamino acids were analyzed after partial hydrolysis of the p135 band by SDS-PAGE following the kinase assay with the insulin receptor immunoprecipitate (IP). B, cell lysates were immunoprecipitated using first anti-IR and then anti-PDE3B antibodies or vice versa. Immune complexes were separated by 10% SDS-PAGE mini-gels, transferred, and probed with anti-PDE3B antibodies. Phosphorylated PDE3B was then visualized by autoradiography-

To further corroborate that the kinase involved was indeed PI3-kinase, IR and p110 (PI3-kinase) immunoprecipitates obtained from the same cells, preincubated with or without insulin, were assayed for protein kinase activity in the presence of different protein kinase inhibitors using recPDE3B as a substrate. Fig. 5A shows that insulin stimulated the phosphorylation of recPDE3B −2-fold by a wortmannin-sensitive kinase associated with the IR and with p110. This kinase was insensitive to other inhibitors like staurosporine, H-7, and olo-

FIG. 4.

To examine whether phosphorylation of PDE3B by the insulin-stimulated PI3-kinase serine kinase in the IR immunoprecipitates was associated with an increase of the PDE3B phosphodiesterase activity, IR immunoprecipitates and their supernatants from cells preincubated with or without insulin were assayed for phosphodiesterase activity in the presence or absence of the specific PDE3B inhibitor OPC3911. Fig. 5B shows that phosphodiesterase activity was recovered in the IR immunoprecipitates as well as in the supernatants. However, preincubation with insulin mainly increased the activity in the IR immunoprecipitate. In the absence of insulin, approximately one-third of the total PDE3B activity was associated with the insulin receptor, and two-thirds remained in the supernatant. However, insulin exerted a major effect in activating the PDE3B associated with the insulin receptor (−100% increase) whereas only a small increase (−30%) was seen in the supernatant. These findings suggest that phosphorylation and activation of PDE3B associated with the insulin receptor may lead to the dissociation and translocation of the enzyme to different subcellular sites. Although PDE3B is mainly associated with the plasma membrane (2) the precise subcellular localization is still unclear. It also remains to be identified if there are other kinases and pathways for PDE3B phosphorylation and activation. For instance, the presently identified mechanism seems unlikely to account for the ability of cAMP protein kinase A to activate PDE3B.

In conclusion, PDE3B is associated with the insulin receptor, but this association does not appear to be regulated by insulin. In contrast, insulin increases binding and activity of the PI3-
kinase serine kinase associated with the insulin receptor, leading to an increased phosphorylation and activation of PDE3B and, thus, initiation of the antilipolytic effect of insulin. This mechanism is similar to that of a recent report showing that interferon increased the association of PI3-kinase serine kinase with the interferon receptor and that this elicited the phosphorylation of STAT3 (28). Recently, the role of PI3-kinase lipid kinase and serine kinase activities on intracellular signaling pathways was examined in cells expressing catalytically inactive lipid kinase. Bondeva et al. (29) found PKB activation to be dependent on PI3-kinase lipid kinase, whereas MAPK activation was dependent on the protein kinase activity.

Another salient finding in the present study is that PI3-kinase activity in human adipocytes is differentially expressed in two key docking proteins involved in insulin’s signal transduction cascade: lipid kinase activity associated with IRS-1 and serine kinase activity associated with the insulin receptor. The inverse relationship between these activities is in agreement with previous findings with purified PI3-kinase (9, 10) and probably reflects the binding conformation of the protein. Whether this inverse relationship is peculiar to human fat cells or is a general phenomenon remains to be established. Further studies are also in progress to elucidate the binding sites for PI3-kinase and PDE3B as well as to characterize other proteins that are phosphorylated on serine sites by this novel pathway of insulin action.

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