Regulation of Phosphatidylinositol 3,4,5-trisphosphate 5'-phosphatase Activity by Insulin*

(Received for publication, August 22, 1996, and in revised form, October 1, 1996)

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Polyposphoinositides are thought to be mediators of cellular signaling pathways as well as regulators of cytoskeletal elements and membrane trafficking events. It has recently been demonstrated that a class of phosphatidylinositol (PI) 3,4,5-P₃ 5'-phosphatases contains SH2 domains and proline-rich regions, which are present in many signaling proteins. We report here that insulin stimulation of Chinese hamster ovary cells (CHO-T) expressing human insulin receptors causes an 8–10-fold increase in PI 3,4,5-P₃ 5'-phosphatase activity in anti-phosphotyrosine immunoprecipitates of the cell lysates. This insulin-sensitive polyposphoinositide 5'-phosphatase did not catalyze dephosphorylation of PI 4,5-P₃. No change in 5'-phosphatase activity was detected in insulin receptor or IRS-1 immune complexes in response to insulin. However, insulin treatment of CHO-T cells markedly increased the PI 3,4,5-P₃ 5'-phosphatase activity associated with Shc and Grb2. The insulin-regulated polyposphoinositide 5'-phosphatase was not immunoreactive with antibody raised against the recently cloned SHIP 5'-phosphatase reported to associate with Shc and Grb2 in B lymphocytes. These data demonstrate that insulin causes formation of complexes containing a PI 3,4,5-P₃ 5'-phosphatase, and Shc or Grb2, or both, suggesting an important role of this enzyme in insulin signaling.

The insulin receptor belongs to a family of structurally related transmembrane growth factor receptors that exhibit ligand-activated protein-tyrosine kinase activity (1–3). The insulin receptor kinase activity is thought to be essential for cellular responses to insulin (4–6). Activation of insulin receptor kinase promotes the rapid autophosphorylation of insulin receptor β-subunits as well as tyrosine phosphorylation of several cytoplasmic proteins such as IRS-1,¹ Shc, and pp60 that appear to be involved in the insulin signaling pathway (3, 7–9). Evidence indicates that a primary function of the insulin receptor kinase is to place tyrosine phosphate docking sites on these proteins for the recruitment of signaling proteins containing Src homology (SH) 2 domains (1, 10, 11). Thus, insulin-induced phosphorylation of IRS-1, Shc, and pp60 promotes their association with specific SH2-containing proteins, which in turn can stimulate the catalytic activity of these SH2 proteins (12–15). One such SH2-containing protein is the p85 regulatory subunit of the p110 phosphatidylinositol (PI) 3-kinase which catalyzes phosphorylation of the 3-position on PI (12, 13, 16).

Strong evidence supports a pivotal role for signaling complexes containing IRS-1 and the p85 regulatory subunit of PI 3-kinases in mediating insulin action on GLUT4 glucose transporter redistribution to the plasma membrane leading to increased glucose uptake as well as glycogen synthesis. Inhibition of PI 3-kinase activity by wortmannin (17–19) or LY294002 (20), microinjection of a fusion protein consisting of an SH2 domain of the p85 regulatory subunit of PI 3-kinase (21), and disruption of PI 3-kinase recruitment to IRS-1 by dominant inhibitory constructs of p85 (22) ablate the stimulation of glucose transport and glycogen synthesis by insulin. Expression of IRS-1 antisense RNA in isolated fat cells also inhibits insulin-mediated translocation of epitope-tagged GLUT4 glucose transporters to the cell surface (23). Further, insulin causes the localization of IRS-1:PI 3-kinase complexes to intracellular membrane vesicles containing GLUT4 (24), while other growth factors that stimulate PI 3-kinase activity but fail to activate glucose transport do not.² These data are consistent with the hypothesis that one or more 3'-phosphoinositide species generated in intracellular membranes in response to insulin regulate cellular components involved in membrane trafficking of GLUT4.

It is established that the insulin-sensitive p85/p110 PI 3-kinase can catalyze formation of PI 3-P, PI 3,4-P₂, and PI 3,4,5-P₃ from PI, PI 4-P, and PI 4,5-P₂, respectively (24–26). However, no information is available about which of these 3'-phosphoinositide species actually participates in the mechanisms of insulin action. Interestingly, interconversion of these species appears to occur through the action of 3'-polyposphoinositide 4'- and 5'-phosphatases (27–34). Recent reports have described PI 3,4,5-P₃ 5'-phosphatases that contain SH2 and proline-rich domains characteristic of signaling proteins (30, 31, 33). Recent reports demonstrate the association of PI 3,4,5-P₃ 5'-phosphatase with Shc (30, 31, 33). Also, a PI 3,4,5-P₃ 5'-phosphatase which forms a complex with p85/p110 PI 3-kinase in platelets has been described (32). The combination of actions of this stimulated PI 3-kinase activity and 5'-phosphatase activity is expected to produce cellular PI 3,4-P₂ which may be uniquely important in triggering downstream cellular effects. These considerations prompted us to investi-

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ilenames used are: IRS-1, insulin receptor substrate-1; IR, insulin receptor; PI, phosphatidylinositol; SH, Src homology; SHIP, SH2-containing inositol 5'-phosphatase; CHO, Chinese hamster ovary cells; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

¹ R. A. Heller-Harrison, M. Morin, A. Guilherme, E. Skolnik, and M. P. Czech, submitted for publication.
gate whether insulin action might also modulate polyphosphoinositide 5-phosphatase activity. We report here a marked insulin-mediated recruitment of 5-phosphatase activity specific for PI 3,4,5-P₃ to complexes containing Shc and Grb2. This novel action of insulin is likely to play an important role in one or more downstream cascades important to this hormone’s function.

EXPERIMENTAL PROCEDURES

Material—4G10 anti-phosphotyrosine (anti-Tyr(P)) mouse monoclonal and anti-p85 polyclonal antibodies were purchased from UBI and used according to manufacturer’s specifications. Rabbit polyclonal anti-IRS-1 immunoglobulin used for immunoprecipitation was prepared by injecting a peptide of the COOH-terminal 15 amino acids derived from the sequence of rat IRS-1 conjugated to keyhole limpet hemocyanin into New Zealand White rabbits. An IgG fraction from the resultant serum was prepared by Protein A-Sepharose chromatography. Insulin receptor-specific monoclonal antibody CT-1 was from mouse ascites (a kind gift of Dr. Ken Siddle). Anti-Shc monoclonal and polyclonal antibodies were purchased from Transduction Laboratories. Anti-Grb2 polyclonal antibody was from Santa Cruz. Phosphatidylinositol and phosphatidylserine were from Avanti Polar Lipids. PI4-P and PI4,5-P2 and Protein A-Sepharose were from Sigma. [γ-32P]ATP, [3H]PI 4-P, and [3H]PI 4,5-P2 were purchased from DuPont NEN.

Cell Culture—CHO-T cells were maintained in Ham’s F-12 media, 10% fetal bovine serum, 50 μg/ml streptomycin/penicillin, and grown to confluence before use.

Cell Lysis and Immunoprecipitation—CHO-T cells were serum-starved for 18–24 h, stimulated with 100 nM insulin for 15 min, and then washed twice on ice in phosphate-buffered saline (PBS). Cells were then lysed in ice-cold lysis buffer composed of 0.5% Nonidet P-40, 50 mM Heps pH 7.4, 100 mM NaF, 10 mM NaPO₄, 2 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, 40 μg/ml aprotinin, 40 μg/ml leupeptin. After 20 min of incubation on ice, insoluble material was removed by centrifugation (14,000 × g for 15 min). The supernatant was removed and assayed for total protein content, using a BCA protein determination kit with bovine serum albumin as standard. Equal amounts of protein (typically 1 mg of protein) from each cleared lysate were then incubated overnight at 4°C on a end-over-end mixer with 4 μg of anti-Tyr(P), 60 μg of anti-IRS-1 IgG, 25 μg of anti IR, 4 μg of anti-Shc, 1 μg of anti-Grb2, or 2 μl of anti-SHIP antibodies. Mouse or rabbit antibodies were then adsorbed to anti-mouse IgG-Sepharose (40 μl) or Protein A-Sepharose (40 μl), respectively, for 2 h at 4°C by end-over-end mixing. The Sepharose-bound immune complexes were then collected by centrifugation (10,000 × g, 30 s) and washed five times in ice-cold PBS containing 0.1% Nonidet P-40. The samples were then used in immunoblots or in PI 3,4,5-P₃ phosphatase assays.

In the experiments involving protein immunoblots, after washing as described above, immune complexes were resolved on SDS-PAGE (7.0%) in the presence of vanadate, nearly quantitative conversion of PI 3,4,5-P₃ to PI 3,4-P₂ was observed, measured either as the reaction product was analyzed by thin layer chromatography. NA indicates parallel tubes containing PI 3,4,5-P₃, but with no addition of immune complexes. B, the graph shows the effect of insulin on PI 3,4,5-P₃ 5-phosphatase activity in immunoprecipitates. Spots corresponding to PI 3,4-P₂ formed upon thin layer plate were cut out and counted using a β-counter. The data presented are the average values from 3 independent experiments ± S.E. C, cell lysates were immunoprecipitated with anti-phosphotyrosine, anti-IR, or anti-IRS-1, and immune complexes were resolved by SDS-PAGE on a 7.0% gel and electrophoretically transferred to nitrocellulose for 12 h at 125 mA. The filter was blocked and incubated with anti-Tyr(P) antibody and then horseradish peroxidase-anti-mouse followed by detection with chemiluminescence.

RESULTS AND DISCUSSION

Initial experiments were conducted to establish assay conditions for quantifying polyphosphoinositide 5-phosphatase activity in lysates of CHO-T cells expressing human insulin receptors. Dephosphorylation of [32P]PI 3,4,5-P₃ to di- and monophosphoinositides in cell lysates was readily observed in the absence of vanadate upon thin layer chromatography analysis of the reaction products (not shown). However, inclusion of vanadate in the assay buffer almost completely blocked PI 3,4-P₂ phosphatase activity with little effect on the PI 3,4,5-P₃ 5-phosphatase activity. This is consistent with a previous report that 3-phosphatase is inhibited by vanadate (27). Thus, in the presence of vanadate, nearly quantitative conversion of PI 3,4,5-P₃ to PI 3,4-P₂ was observed, measured either as the disappearance of the former or appearance of the latter (not shown). All subsequent assays were therefore performed under these conditions.

To determine whether insulin regulates PI 3,4,5-P₃ 5'-phosphatase, lysates of CHO-T cells incubated with or without insulin were immunoprecipitated with anti-Tyr(P) antibody, and the precipitates were assayed as described above. The results revealed a marked increase in PI 3,4,5-P₃ 5'-phosphatase activity in the anti-Tyr(P) precipitates due to insulin action (Fig. 1, A and B). Tyrosine-phosphorylated insulin receptor and IRS-1 were immunoprecipitated when cells were treated with insulin under these conditions, as evidenced by Western blot analysis (Fig. 1C). However, when either insulin receptors or IRS-1 were specifically immunoprecipitated with anti-insulin receptor or anti-IRS-1 antibody (Fig. 1C), no insulin-stimu-
Insulin-regulated PI 3,4,5-P₃ 5'-phosphatase activity with Shc and Grb2. Lysates were prepared from CHO-T cells treated with (+) or without (−) 100 nM insulin for 15 min. A, cell lysates were immunoprecipitated with anti-Tyr(P), anti-Shc, or anti-Grb2, and immunoprecipitates were assayed for PI 3,4,5-P₃, 5'-phosphatase by incubation with [³²P]PI 3,4,5-P₃. NA indicates assay, without immune complex. B, graph shows the effect of insulin on PI 3,4,5-P₃ 5'-phosphatase activity in immune complexes. Spots corresponding to PI 3,4,5-P₃ in the thin layer chromatography plate were cut out and quantitated using a β-counter. The data presented are average values from 3 independent experiments ± S.E., normalized by equating each value in the absence of insulin to 1. The actual counts/min values in basal conditions were: 1014, anti-Tyr(P); 313, anti-Shc; and 890, anti-Grb2.

![Graph](image-url)

**Fig. 2.** Insulin-regulated PI 3,4,5-P₃ phosphatase dephosphorylates PI 3,4,5-P₃ at the 5'-position of the inositol ring. Lysates from CHO-T cells treated with or without insulin (control) were immunoprecipitated with anti-Tyr(P), and PI 3,4,5-P₃ 5'-phosphatase activity was assayed as described in Fig. 1. Shown are the HPLC profiles of ³²P-labeled deacylated polyphosphoinositides (gPI₅s) after the phosphatase assays. Top panel, assay containing PI 3,4,5-P₃ with no addition of immune complexes (NA). Bottom panels, assays with immune complexes from control cells (CON) and insulin-treated (INS) cells, as indicated. Dashed arrows indicate retention time of the deacylated [⁹²]PI 4,5-P₂ standard.
nase and PI 3,4,5-P_3 phosphatase in combination promote the conversion of PI 4,5-P_2 to PI 3,4-P_2. The fact that insulin and other growth factor receptor tyrosine kinases regulate both of these enzymes indicates that at least part of the cellular PI 3,4-P_2 generated in response to these signaling pathways derives from newly formed PI 3,4,5-P_3. Further, these considerations strongly suggest an important role for PI 3,4-P_2 in signaling by these receptors. PI 3,4-P_2 may be an effector that is unique and specific for one or more downstream signaling pathways such as the cAKt/Rac protein kinase (40). Thus, the PI 3,4,5-P_3 phosphatase reaction may serve as a branch point for polyphosphoinositide signaling in which PI 3,4,5-P_3 activates a set of events distinct from those activated by PI 3,4-P_2. It will be important to search for cellular proteins that specifically bind each of these phosphoinositides.

The association of 5-phosphatase with Shc and Grb2 in response to insulin reported here (Fig. 3) suggests a potential role of this phosphatase in regulating the p21ras pathway. Both Shc and Grb2 are components of multiprotein complexes containing the guanine nucleotide exchange factor son of sevenless that catalyzes GTP loading of p21ras (8, 11, 41, 42). Interactions between p21ras and the p110α subunit of PI 3-kinase have been reported (43), and in some cell types a downstream effect of p21ras, mitogen-activated protein kinase activation, in response to insulin or growth factors is blocked by wortmannin, a potent inhibitor of PI 3-kinases (44). Further studies on this issue are clearly warranted.

In addition to a potential positive signaling function of the insulin-sensitive PI 3,4,5-P_3 5-phosphatase described above, a negative role in signal transmission is also possible. If PI 3,4,5-P_3 generated by PI 3-kinases is a positive effector of downstream signaling events, as appears likely (16), its concentration is expected to be reduced by the action of the 5-phosphatase. Thus, the signaling potential of PI 3,4,5-P_3 may be reduced or desensitized by insulin-regulated PI 3,4,5-P_3 phosphatase. It is perhaps desirable to control the cellular localization of PI 3,4,5-P_3 and to restrict it from regions containing ShcGrb2 complexes. This hypothesis requires rigorous testing subsequent to identification of additional cellular targets of PI 3,4,5-P_3. In any case, the data reported here indicate an important function of PI 3,4,5-P_3 5-phosphatase activity in one or more signaling pathways emanating from the insulin receptor.

Acknowledgment—We thank Jane Erickson for her expert assistance in the preparation of this manuscript.