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Biochemistry

Stimulation of glycogen synthesis by insulin in human erythroleukemia cells requires the synthesis of glycosyl-phosphatidylinositol

(protein phosphorylation/second messenger/src homology)

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ABSTRACT **Although the insulin-dependent hydrolysis of glycosyl-phosphatidylinositol (GPI) may play an important role in insulin action, an absolute requirement for this glycolipid has not been demonstrated. Human K562 cells were mutated to produce a cell line (IA) incapable of the earliest step in PI glycosylation, the formation of PI-GlcNAc. Another cell line (IVD) was deficient in the deacetylation of PI-GlcNAc to form PI-GlcN and subsequent mannosylated species. Each line was transfected with wild-type human insulin receptors. Similar insulin-stimulated receptor autophosphorylation was observed in all three lines, along with a nearly identical increase in the association of phosphorylated insulin receptor substrate 1 with endogenous PI 3-kinase. Both normal and GPI-defective lines also displayed a similar 2- to 3-fold increase in phosphorylation of the Shc protein and its association with growth factor receptor-bound protein 2 in response to insulin. In contrast to these results, striking differences were noted in insulin-stimulated glycogen synthesis. In normal cells, glycogen synthesis was significantly increased by insulin, whereas no insulin stimulation was observed in GPI-deficient IA cells, and only a trace of stimulation was detected in IVD cells. These results indicate that tyrosine phosphorylation produced by insulin is not dependent on GPI synthesis, and this effect is not sufficient to elicit at least some of the metabolic effects of the hormone. In contrast, GPI synthesis is required for the stimulation of glycogen synthesis by insulin in these cells. These findings support the existence of divergent pathways in the action of insulin.**

Although the precise intracellular events that mediate the actions of insulin remain elusive, the regulation of protein phosphorylation is thought to play an important role. Insulin stimulates the tyrosine phosphorylation of its receptor and subsequently induces changes in serine/threonine phosphorylation, paradoxically increasing activities of both phosphatases and kinases that target numerous intracellular proteins. Dephosphorylations are critical to many of the metabolic effects of insulin, including stimulation of glycogen and lipid synthesis, and inhibition of lipolysis. The consequences of insulin-stimulated serine/threonine phosphorylation are less well understood, although these effects are shared by other growth factors.

Many of the metabolic activities of insulin associated with protein dephosphorylation are correlated with glycosyl-phosphatidylinositol (GPI) turnover, due to the activity of a GPI-specific phospholipase C (1, 2). The resulting inositol phosphate glycan product fulfills many of the criteria for a second messenger (1–5). Its production is rapid and dependent on physiological levels of insulin. Moreover, the purified glycan has exhibited insulin-mimetic properties in a manner consistent with the promotion of dephosphorylation via serine/threonine phosphatase activities (reviewed in ref. 6). Although the precise structure of the molecule remains unknown, metabolic and chemical labeling studies (1–5) indicate a core structure of cyclic 1,2-inositol monophosphate-glucosamine-(mannose)$_3$, similar to the core structure of the glycan portion of the GPI protein anchor (7). This conclusion has been supported by the observed insulin-mimetic activities of an inositol glycan derived from the *Trypanosoma brucei* GPI anchor (8) and the blockade of some of the actions of insulin by antibodies raised against a *T. brucei* inositol phosphate glycan (9).

Although the insulin-mimetic activities of inositol glycans have been demonstrated in several systems, their absolute requirement in the various actions of insulin remains unknown. Human K562 erythroleukemia cells produce the common GPI core structure of phosphatidylinositol-glucosamine-(mannose)$_3$-phosphate-ethanolamine for anchoring of cell surface proteins, such as decay-accelerating factor (DAF) and CD59. Mutagenesis of these cells yielded one cell line (IA) incapable of the earliest step in PI glycosylation, the formation of PI-GlcNAc, and another (IVD) defective in the deacetylation of PI-GlcNAc to form PI-GlcN and subsequent mannosylated species (10, 11). Studies of insulin action in these cells have revealed an absolute requirement for GPI in the regulation of glycogen synthesis.

MATERIALS AND METHODS

Materials. RPMI 1640 medium was from GIBCO, fetal bovine serum (FBS) was from HyClone, porcine insulin was from Eli Lilly, monoclonal antibodies (mAbs) to the insulin receptor α and β subunits were from Amersham and Oncogene Science, and anti-phosphotyrosine and anti-p85 subunit of PI 3-kinase antiserum and polyclonal antiserum to the insulin receptor α subunit were from Upstate Biotechnology (Lake Placid, NY). Anti-mouse immunoglobulin labeled with ^{125}I and horseradish peroxidase were from Amersham. Murine anti-DAF mAb was previously prepared (12). Murine anti-CD59 mAb was provided by H. Okada (13). Murine nonrelevant (IgG2a) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulins were purchased from Cappel. Growth factor receptor-bound protein 2 (Grb2) protein lacking the N-terminal 14 amino acids was expressed in a glutathione *S*-transferase (GST) fusion protein and was a generous gift of T. Takenawa (14). D-[U-^{14}C]Glucose and

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Abbreviations: GPI, glycosyl-phosphatidylinositol; PI, phosphatidylinositol; IRS-1, insulin receptor substrate 1; Grb2, growth factor receptor-bound protein 2; MAP, mitogen-activated protein; DAF, decay-accelerating factor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase.

UDP-[^{3}H]GlcNAc were from NEN. All other reagents were from Sigma and were of the highest quality.

Cell Culture. K562 wild-type cells and their GPI-deficient IVD (11) and IA (10) derivatives (0.2–1 × 10^6 cells per ml) were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. After transfection of cell lines for insulin receptor expression (see below), selective antibiotic (300–400 units of hygromycin B per ml) was maintained in the culture medium. Before insulin treatment, cells were washed two times with Ca^{2+}-free phosphate-buffered saline (PBS), resuspended in serum-free medium containing 0.1% bovine serum albumin (BSA), and incubated for 3 hr at 37°C. Cells were then pelleted (250 × g; 3 min) and resuspended in 5 ml of serum-free medium (0.1% BSA) at 5 × 10^6 cells per ml.

Transfection of Cell Lines with Human Insulin Receptor. Full-length human insulin receptor cDNA (15) was inserted into the pREP4 expression vector (Invitrogen) behind the Rous sarcoma virus promoter. Cell lines were transfected with this construct via Transfectam reagent (Promega), and clonal lines were selected by hygromycin B resistance.

Flow Cytometry. Clonal lines (1 × 10^6 cells per tube) were incubated with 2.5 μg of mouse anti-insulin receptor monoclonal IgG2b or the nonrelevant mouse IgG2b for 20 min on ice in 50 μl of PBS containing 10% BSA and 0.1% sodium azide (PBS/BSA/azide buffer). Cells were washed twice and then resuspended in 50 μl of the same buffer containing FITC-conjugated goat anti-mouse antibody (1:50 dilution). After an additional 20 min of incubation on ice, stained cells were washed as described above, resuspended in 1 ml, and analyzed on a FACScan flow cytometer (Becton Dickinson). For assessment of DAF and CD59 expression, cells were labeled with murine anti-DAF or anti-CD59 mAbs (10).

***In Vitro* Assay of GPI Synthesis.** *In vitro* studies of GPI synthesis were performed as described (10). In brief, after pretreatment with tunicamycin, cells were lysed and a microsomal particulate was prepared. The resulting suspensions were incubated with 10 μCi (1 Ci = 37 GBq) of UDP-[^{3}H]GlcNAc for 10 min at 30°C. Reactions were terminated with chloroform/methanol (1:1), and radiolabeled lipids were extracted by butanol and resolved on silica gel 60 TLC plates, which were then scanned with a TLC analyzer (Bioscan, Washington, DC).

Immunoblots. After insulin treatment, cells were washed with PBS, pelleted, and lysed with 1 ml of HNTG buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 200 μM Na_3VO_4, 100 mM NaF, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride (16). Cell lysates were centrifuged (10,000 × g; 10 min) to preclear insoluble material. Supernatants were then incubated for 1 hr with the indicated antiserum followed by a 30-min incubation with Protein G-Plus/Protein A Agarose beads (Oncogene Science). Alternatively, precleared cell lysates were incubated for 90 min with bacterially expressed GST–Grb2 fusion protein previously immobilized on glutathione agarose beads (14). Pelleted beads were washed two times with HNTG lysis buffer and resuspended in 50 μl of Laemmli sample buffer. For immunoblotting of whole cell lysates, precleared lysates were diluted directly into Laemmli sample buffer. Samples were resolved by SDS/8% PAGE, transferred to nitrocellulose paper, and blocked with 1% BSA/1% ovalbumin. Immunoblotting was performed with the indicated antisera followed by autoradiography or enhanced chemiluminescence detection.

Assay of Glycogen Synthesis. This was assayed by an adaptation of the method of Lawrence *et al.* (17). After serum deprivation for 3 hr, cells were washed twice with Krebs–Ringer buffer (pH 7.4) containing 0.1% BSA and then resuspended in the same buffer at 5 × 10^5 cells per ml. Multiple aliquots (0.5 ml each) of this cell suspension were then placed into borosilicate tubes (12 × 75 mm) and incubated with increasing concentrations of insulin (15 min; 37°C). Cells were then incubated with 2 mM D-[U-^{14}C]glucose (2 μCi per tube) for 90 min in a 37°C shaking water bath. The assay was terminated by immersion of tubes into an ice bath (15 min) followed by two washes with ice-cold PBS. Cells were then solubilized in 30% KOH, and glucose incorporation into glycogen was determined (18).

RESULTS

Transfection of K562 Cell Lines with Human Insulin Receptor. To evaluate the role of GPI in insulin action, untransfected human K562 cells and their GPI-deficient derivatives were evaluated for insulin responsiveness. However, because insulin receptor was barely detectable, cells were transfected with wild-type human insulin receptor. Clonal lines were screened for receptor expression by immunoblotting with a polyclonal antibody to the α subunit (Fig. 1*A*). Clonal lines identified with nearly identical levels of receptor expression were examined by flow cytometry, revealing cell populations homogeneous with respect to surface expression (Fig. 1*B*). ^{125}I-labeled insulin binding to whole cells revealed ≈3 × 10^5 binding sites per cell with high-affinity K_d values ranging from 3 to 6 nM (data not shown). These data indicate that the mutations disrupting GPI biosynthesis in these cell lines do not globally interfere with protein glycosylation that is required for insulin receptor processing and cell surface expression (19).

To confirm that the receptor-transfected cell lines retained their deficiencies in GPI synthesis, each cell line was eval-

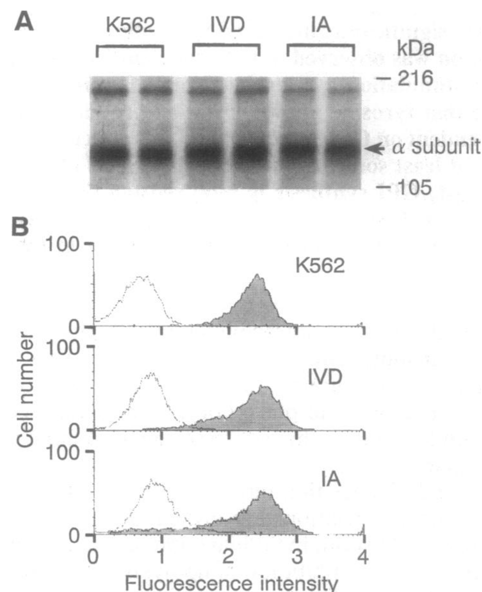

FIG. 1. Equivalent expression of human insulin receptor in K562, IVD, and IA cell lines. After transfection with wild-type insulin receptor, resistant clonal lines were evaluated for levels of receptor expression. (*A*) Whole cell lysates (5 × 10^5 cell equivalents) were subjected to SDS/PAGE and immunoblotted with antiserum to the α subunit of the insulin receptor. Determinations were made in duplicate for the clonal lines presented. The 125-kDa α subunit is designated by the arrow. (*B*) Insulin receptor expression on the cell surface for colonies examined in *A* was assessed by flow cytometry as described. Prior to staining with FITC-conjugated anti-IgG, cells were incubated with either mAb to insulin receptor α subunit (shaded regions) or the nonrelevant IgG (open regions). Histograms of cell fluorescence intensity are representative of three or more separate determinations.

uated for cell surface expression of the GPI-linked proteins DAF and CD59 (Fig. 2A). Flow cytometry analysis with anti-DAF and anti-CD59 antibodies indicated no expression of GPI-linked protein in the IA clonal line and only modest expression of these proteins in the IVD line. *In vitro* assessment of GPI biosynthetic activity in microsomes isolated from these same clonal lines (Fig. 2B) verified the previously characterized deficiencies (10, 11). While parental K562 cells synthesized both PI-GlcNAc and PI-GlcN, IVD cells produced significant levels of only PI-GlcNAc, and IA cells were deficient in both GPI precursors.

GPI Deficiency Does Not Block Insulin Receptor Function. The autophosphorylation and subsequent activation of the insulin receptor tyrosine kinase upon hormone binding is essential for the full expression of insulin action. To ensure that mutations in GPI synthesis do not nonspecifically alter receptor function, the autophosphorylation of the receptor and the insulin-stimulated tyrosine phosphorylation of known substrates were evaluated. Cells were treated with insulin, receptors were immunoprecipitated, and tyrosine phosphorylation was evaluated by SDS/PAGE followed by immunoblotting with anti-phosphotyrosine antibody (Fig. 3A). In all three cell lines, insulin stimulated the autophosphorylation of its receptor, although the maximal phosphorylation obtained in IA cells was slightly diminished compared to wild-type cells.

To further characterize insulin receptor function in these different cell lines, we compared the effect of insulin on the tyrosine phosphorylation of the major substrates of the receptor. One of these substrates, insulin receptor substrate 1 (IRS-1), associates with the 85-kDa subunit (p85) of PI 3-kinase, stimulating activity of this enzyme by occupancy of SH2 domains on p85 (21, 22). After treatment, cells were lysed and immunoprecipitated with anti-p85 antibodies, followed by SDS/PAGE and immunoblotting with anti-phosphotyrosine antibody (Fig. 3B). As previously reported, insulin did not stimulate the tyrosine phosphorylation of p85 but induced association of a 185-kDa tyrosine-phosphorylated protein with p85 in all three cell lines. This was paralleled by a similar 2- to 3-fold stimulation of PI 3-kinase activity in all cell types (data not shown). This 185-kDa tyrosine phosphorylated protein is likely to be IRS-1 or its functional homologue 4PS (23).

Like other growth factors, insulin stimulates the activity of mitogen-activated protein (MAP) kinase in cultured cells, thought to play a central role in the regulation of cell growth.

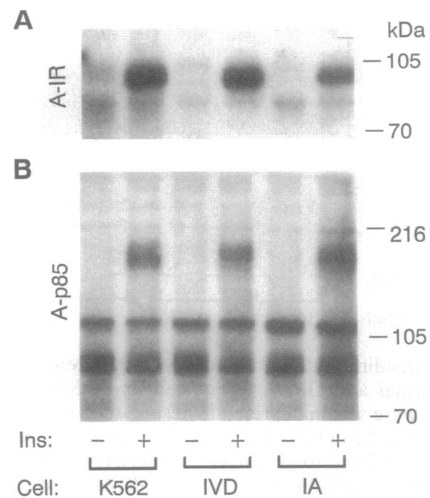

FIG. 3. Insulin-stimulated tyrosine phosphorylation in normal and GPI-deficient cell lines. K562, IVD, and IA clonal lines were serum deprived for 3 hr and then treated with 100 nM insulin (Ins) for 2.5 min. Whole cell lysates were immunoprecipitated with either mAb to the insulin receptor β subunit (A-IR) (*A*) or antiserum to the p85 regulatory subunit of PI 3-kinase (A-p85) (*B*). Samples were resolved by SDS/PAGE and immunoblotted with anti-phosphotyrosine mAb.

Activation of this enzyme occurs downstream of p21^{ras}, which is itself activated by the nucleotide exchange factor, SOS, due to a complex formation between SOS and its upstream activators Grb2 and Shc (24, 25). The formation of this activating complex is initiated by the tyrosine phosphorylation of Shc, inducing binding to the SH2 domains of Grb2, and the subsequent association of Grb2 with SOS. To examine the possible effects of GPI deficiency on this aspect of insulin signaling, K562, IVD, and IA cells were treated with 100 nM insulin for 2.5 min, and cell lysates were incubated with an immobilized GST fusion protein containing the SH2 and SH3 domains of Grb2. These precipitates were subjected to SDS/PAGE followed by anti-Shc immunoblotting (Fig. 4A). Shc levels, which were monitored by anti-Shc immunoprecipitation followed by immunoblotting, were similar in all three cell lines (Fig. 4B). Insulin treatment produced a 2- to 3-fold increase in the association of the 52-kDa Shc protein with the Grb2 beads. This insulin-dependent association,

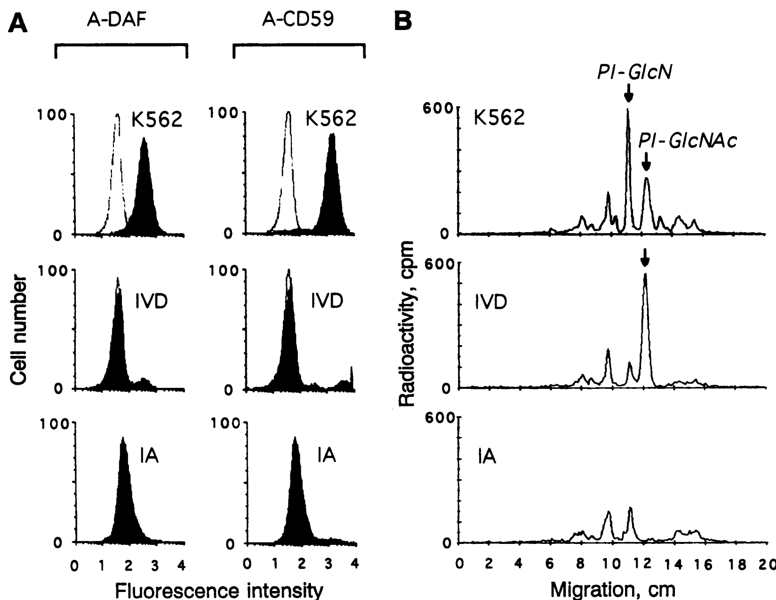

FIG. 2. Verification of GPI-deficiency in clonal cell lines. Cells previously characterized for insulin responsiveness were reevaluated for their ability to express GPI-linked proteins (DAF and CD59) and *in vitro* GPI biosynthetic activity. (*A*) Prior to flow cytometry analysis, cells were incubated with either anti-DAF or anti-CD59 antibodies (shaded regions) or the corresponding nonrelevant IgG (open regions) followed by staining with FITC-conjugated anti-IgG. (*B*) Cell lysates were incubated with UDP-[^{3}H]GlcNAc, and butanol-extractable radiolabeled products were analyzed by TLC in chloroform/methanol/1 M ammonia (10:10:3). Positions of PI-GlcNAc and PI-GlcN standards (20) are shown.

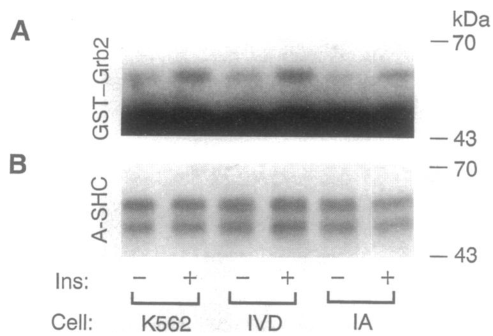

FIG. 4. Insulin treatment increases Shc protein association with Grb2 in normal and GPI-deficient cell lines. K562, IVD, and IA clonal lines were serum deprived (3 hr) and then treated with 100 nM insulin (Ins) for 2.5 min. Cell lysates were incubated with GST–Grb2 fusion protein affixed to glutathione beads (*A*) or anti-Shc antiserum for immunoprecipitation (*B*) as described. After SDS/PAGE resolution of bound protein, immunoblotting was performed with anti-Shc antiserum.

which is paralleled by the tyrosine phosphorylation of Shc (data not shown), was similar in all three cell lines.

Insulin Stimulation of Glycogen Synthesis Is Dependent on GPI Expression. While the growth-promoting effects of insulin are generally associated with increased serine/threonine phosphorylation, due to mitogen-dependent enzymes such as MAP kinase, many of the metabolic effects of the hormone, such as glycogen synthesis, depend predominantly on serine/threonine dephosphorylation. To explore the role of GPI in the regulation of glycogen synthesis by insulin, K562, IVD, and IA clonal lines expressing similar levels of wild-type insulin receptor were treated with increasing concentrations of insulin, and radiolabeled glucose incorporation into glycogen was assessed (Fig. 5). Insulin stimulated glycogen synthesis in wild-type K562 cells expressing mature GPI in a dose-dependent manner, with an EC_{50} of ≈5 nM. In contrast, little or no significant increases in glycogen accumulation were obtained in IVD and IA cell lines, respectively, after insulin treatment. The only activity in IVD cells that was statistically significant ($P < 0.05$) was observed at 10 nM insulin. No significant stimulation was observed at any insulin concentration in the IA cells. A similar pattern of results was obtained with both mixed

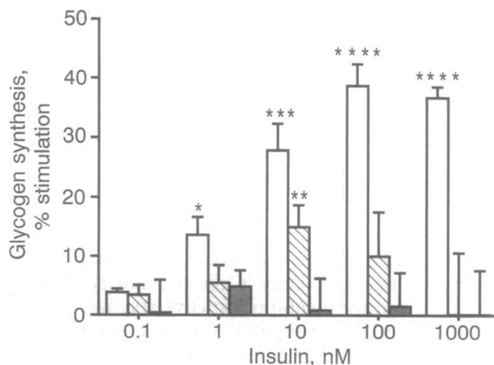

FIG. 5. Stimulation of glycogen synthesis by insulin correlates with the expression of mature GPI. After a 3-hr serum deprivation of K562 (open bars), IVD (hatched bars), and IA (solid bars) clonal cell lines, insulin stimulation of D-[U-^{14}C]glucose incorporation into glycogen was determined as described. Results presented are means + SE of at least four experiments, each performed in triplicate. Average basal activities for K562, IVD, and IA cells were 3605, 1968, and 3797 cpm, respectively. Statistically significant differences between activity in the presence of insulin vs. the corresponding basal activity were determined by administration of the Student *t* test (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$; ****, $P < 0.005$).

populations of each cell type and with other clonal lines (data not shown). Furthermore, stimulation of glycogen synthase activity by the potent allosteric activator glucose 6-phosphate was nearly identical in broken cell preparations from all cell lines (data not shown), demonstrating that GPI deficiency does not affect the level of glycogen synthase or responsiveness of the enzyme to modulation by glucose 6-phosphate. In addition, insulin did not stimulate glucose uptake in either normal or GPI-deficient cells (data not shown), presumably due to the absence of Glut4 transporters. Therefore, potential effects of GPI deficiency on insulin-stimulated glucose uptake do not complicate the interpretation of glycogen synthesis data obtained in this study.

DISCUSSION

Although the precise molecular mechanisms involved in insulin signal transduction have not been delineated, it is likely that regulation of protein phosphorylation plays an essential role. Activation of the β-subunit tyrosine kinase and receptor autophosphorylation following hormone binding appear to be the initiating events in most of the cellular responses to insulin, including generation of the inositol phosphate glycan (26). However, many of the downstream signaling events involve changes in serine/threonine phosphorylation, with the simultaneous increase in phosphorylation of some proteins and the dephosphorylation of others. Differences in the patterns of insulin-stimulated activities have been observed in cells overexpressing truncated (27) or mutant receptors (28–30), indicating that divergence in insulin signaling occurs at or near the receptor level. Furthermore, the mitogenic activities of insulin have been dissociated from its metabolic actions in numerous studies (27, 28, 31–33).

The patterns of serine/threonine phosphorylation stimulated by insulin are generally reproduced by other growth factors. However, the dephosphorylation reactions are unique to insulin. Thus, it has been suggested that many of the "growth-promoting" activities of the hormone may result, in part, from the well-known growth factor cascade of serine/threonine kinases. One of the best characterized of these, MAP kinase, is regulated after activation of the *ras* protooncogene (34–36), due to a series of protein–protein interactions initiated by receptor tyrosine kinases (37, 38). However, much less is known about the processes governing the metabolic responses to insulin. Studies utilizing the specific phosphatase inhibitor okadaic acid have demonstrated the importance of serine/threonine phosphatase activity in many of the metabolic activities of the hormone, including stimulation of glycogen synthesis (18, 39).

Cohen and coworkers (40) have suggested that the insulin-dependent activation of ribosomal S6 kinase II by MAP kinase may mediate a cascade resulting in stimulation of glycogen synthesis and inhibition of glycogenolysis via phosphorylation of site 1 on glycogen-associated protein phosphatase 1. However, in 3T3-L1 adipocytes, where insulin and epidermal growth factor produce nearly identical activation of MAP kinase, only insulin stimulates glycogen synthesis (41). Furthermore, insulin stimulates glycogen synthesis in PC12 cells in the apparent absence of any stimulation of MAP kinase activity (42). Similarly, overexpression of the Ser^{1200} insulin receptor mutant in CHO cells produced a dominant negative phenotype with respect to activation of MAP kinase by insulin, yet insulin stimulated glycogen synthesis to an extent identical to that observed in cells expressing the wild-type receptor (28, 31). These observations indicate that MAP kinase activation is not universally required for insulin stimulation of glycogen synthesis, suggesting the importance of alternative pathways.

In contrast to the lack of correlation between glycogen synthesis and MAP kinase, there has been a consistent correlation with insulin-dependent GPI hydrolysis. The inositol glycan product of this reaction exhibits insulin-mimetic properties on the activity and phosphorylation state of glycogen phosphorylase in intact cells (43–45). The glycan also promotes serine/threonine dephosphorylation in adipocyte extracts via a mechanism requiring PP1, the phosphatase that regulates the activity of both glycogen synthetase and phosphorylase (46). Moreover, reduced cellular GPI content and insulin-stimulated turnover was accompanied by a marked decrease in stimulation of glycogen synthase activity and net glycogen accumulation in hepatocytes isolated from insulin-resistant, genetically obese rats (47). While these observations indicated a correlation between GPI metabolism and glycogen synthesis, an absolute dependence has not been demonstrated. Results reported here on mutant K562 cells indicate that the stimulation of glycogen synthesis by insulin depends critically on the presence of cellular GPI. Moreover, GPI deficiency did not influence receptor autophosphorylation and early postreceptor phosphorylation events, including phosphorylation of Shc and activation of PI 3-kinase, via IRS-1 phosphorylation. These data suggest that these phosphorylation events may lie upstream, or on a different pathway from GPI hydrolysis, and that they are not sufficient to elicit stimulation of glycogen synthesis. Unfortunately, it was not possible to evaluate the role of GPI in mitogenic signaling. The wild-type cell line used in this study does not respond mitogenically to insulin. Furthermore, both raf and MAP kinase appear to be constitutively active, presumably due to the transformed phenotype caused by the activity of BCR-Abl (48). However, since GPI deficiency does not affect the formation of IRS-1/p85 and Shc/Grb2 complexes, it seems unlikely that GPI molecules play an important role in these two pathways, which are frequently associated with mitogenesis.

This study demonstrates an absolute requirement for GPI in insulin action. While the exact mechanism by which the inositol glycan product of GPI hydrolysis increases glycogen synthesis remains to be elucidated, an essential role for inositol phosphate glycan as a second messenger for some of the metabolic activities of insulin is supported by these findings. Identification of the genes involved in GPI synthesis (49, 50) should allow a more detailed analysis of the role of GPI and its turnover in insulin metabolic responses in classical insulin-responsive cell lines and tissues.

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