Insulin-stimulated Phosphorylation of the Protein Phosphatase-1 Striated Muscle Glycogen-targeting Subunit and Activation of Glycogen Synthase*

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Protein phosphatase-1 (PP-1) in heart and skeletal muscle binds to a glycogen-targeting subunit (G_M) in the sarcoplasmic reticulum. Phosphorylation of G_M has been postulated to govern activity of PP-1 in response to adrenaline and insulin. In this study, we used biochemical assays and G_M expression in living cells to examine the effects of insulin on the phosphorylation of G_M, and the binding of PP-1 to G_M. We also assayed glycogen synthase activation in cells expressing wild type G_M and G_M mutated at the phosphorylation sites. In biochemical assays kinase(s) prepared from insulin-stimulated Chinese hamster ovary (CHO-IR) cells and C2C12 myotubes phosphorylated a glutathione S-transferase (GST) fusion protein, GST-G_M(1-240), at both site 1 (Ser48) and site 2 (Ser67). Phosphorylation of both sites was dependent on activation of the mitogen-activated protein kinase pathway, involving in particular ribosomal protein S6 kinase. Full-length G_M was expressed in CHO-IR cells and metabolic 32P labeling at sites 1 and 2 was increased by insulin treatment. The G_M expressed in CHO-IR cells or in C2C12 myotubes co-immunoprecipitated endogenous PP-1, and association was transiently lost following treatment of the cells with insulin. In contrast PP-1 binding to G_M(S67T), a version of G_M not phosphorylated at site 2, was unaffected by insulin treatment. Expression of G_M increased basal activity of endogenous glycogen synthase in CHO-IR cells. Insulin stimulated glycogen synthase activity the same extent in cells expressing wild type G_M or G_M mutated to eliminate phosphorylation site 1 and/or site 2. Phosphorylation of G_M is stimulated by insulin, but this phosphorylation is not involved in insulin control of glycogen metabolism. We speculate that other functions of G_M at the sarcoplasmic reticulum membrane might be affected by insulin.

Insulin stimulates glycogen synthesis in mammalian striated muscle by promoting the multisite dephosphorylation and activation of glycogen synthase, the rate-limiting enzyme in this process (1–3). Based on biochemical studies, glycogen synthase is inactivated primarily by cAMP-dependent protein kinase (PKA)1 and glycogen synthase kinase-3 (GSK-3) that together phosphorylate 7 separate sites in glycogen synthase (4–8), and activated by PP-1G, a glycogen-bound form of protein phosphatase-1 that shows specificity for dephosphorylation of these sites in glycogen synthase, relative to other substrates (9–11). The subcellular location, substrate specificity, and regulation of PP-1 activity are determined by its interaction with targeting subunits. In skeletal and cardiac muscle the major glycogen-associated form of PP-1 (PP-1G) is a heterodimer composed of the phosphatase catalytic subunit (PP-1) and a subunit called G_M or R_M-G_M directs PP-1 to glycogen-protein particles as well as membranes of the sarcoplasmic reticulum (11, 12), by directly binding glycogen and by virtue of a putative transmembrane segment near the C terminus. This localization presumably facilitates the dephosphorylation of glycogen-metabolizing enzymes and sarcoplasmic reticulum proteins, such as phospholamban (11, 13–15).

Since the discovery of G_M a family of glycogen-targeting subunits have been found, and these are closely related in sequence to the N-terminal domain of G_M. All members of this family bind glycogen and PP-1. Overexpression of one of these subunits, called PTG, promotes the activation of glycogen synthase (14). Curiously, although the structure and function seems to be conserved among this family of proteins, only G_M is phosphorylated. Purified preparations of rabbit skeletal muscle G_M were phosphorylated at Ser48 (site 1) and Ser67 (site 2) by PKA, which prevented binding of PP-1, as a possible way of reducing PP-1 activity toward glycogen-metabolizing enzymes (11, 16). As expected, phosphorylation of G_M at both sites 1 and 2 occurs in intact muscle in response to adrenaline (17). However, the phosphorylation of G_M in response to insulin, and the possible role of G_M phosphorylation in activation of glycogen synthase, has been a subject of controversy for 10 years.

Insulin reportedly increased phosphorylation of Ser48 (site 1) in G_M by activation of an insulin-stimulated protein kinase (18). Site 1 phosphorylation was said to produce a 2-fold increase in the PP-1G-catalyzed dephosphorylation of glycogen synthase (18). This offered a pathway for insulin activation of glycogen synthase, through site 1 phosphorylation of G_M. With

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; PP-1, protein phosphatase 1; G_M, skeletal muscle glycogen-targeting subunit; PP-1G, glycogen associated form of protein phosphatase 1; PKI, protein kinase inhibitor; GST, glutathione S-transferase; HA, epitope tag of hemagglutinin A with sequence YPYDVPDYA; (HA)_3, triple HA tagged; GK-3, glycogen synthase kinase-3; RSK, ribosomal protein S6 kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase kinase; EGF, epidermal growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; DMEM, Dulbeco’s modified Eagle’s medium; G6P, glucose 6-phosphate; PAGE, polyacrylamide gel electrophoresis; UDP-glucose, uridine 5’-diphosphoglucose; wt, wild type; CHO-IR, Chinese hamster ovary-insulin receptor; PBS, phosphate-buffered saline.
the discovery that insulin-stimulated protein kinase was RSK-2 (19), a downstream substrate for MAP kinase (20), site 1 phosphorylation of G_M was linked to insulin activation of the MAP kinase pathway. However, multiple groups provided convincing evidence that MAP kinase was not involved in glycogen synthase activation. Experiments used the selective MEK inhibitor, PD98059, or the potent MAP kinase activator, EGF, and showed that activation of MAP kinase and RSK was neither necessary nor sufficient for the activation of glycogen synthase (21–24). This was shown in both skeletal muscle cells and adipocytes. The role for MAP kinase in activation of glycogen synthase through activation of PPIG was discounted, but there remained the possibility that other insulin-stimulated kinases phosphorylated site 1 in G_M. Experiments reported after original submission of this paper contradicted earlier results from the same group, because phospho-specific antibodies against sites in G_M did not detect significant increase in phosphorylation of G_M from freeze-clamped rat hindlimb, in response to insulin injection (17). Therefore, two key questions remain: does insulin stimulate phosphorylation of G_M, and is phosphorylation of G_M required for insulin activation of glycogen synthase?

In the present study, we examined the phosphorylation of G_M in response to insulin using: 1) extracts of insulin-stimulated cells plus recombinant GST-G_M and 2) expression of full-length tagged G_M in CHO-IR and C2C12 muscle cells. Our data shows that insulin-stimulated phosphorylation of G_M at both site 1 and site 2, and this is dependent on the activation of MAP kinase pathway. Insulin-stimulated phosphorylation of site 2 in G_M decreased the association of PP-1 in intact cells. Furthermore, we found the stimulation of glycogen synthase activity by insulin remained unchanged in cells expressing G_M. Expression of wild type G_M or G_M mutated in sites 1 and 2 produced the same activation of glycogen synthase in response to insulin, suggesting that phosphorylation of G_M is not required for glycogen synthase activation, parallel to the function of PTG (14, 25).

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO cells overexpressing insulin receptors (CHO-IR cells) were from Dr. J. E. Pessin (University of Iowa), and C2C12 myoblasts were from Dr. L. Mei (University of Virginia). Tissue culture reagents and the kit for reverse transcription (Preamplification System for First Strand cDNA Synthesis) were purchased from Life Technologies (Grand Island, NY). PGEX vectors and glutathione-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [U-14C]Uridine diphosphoglucone (U-14C)UDP was purchased from NEN Life Science Products Inc. (Boston, MA). Bovine insulin, PD98059, and Microcystin-LR were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). EGF and PKI were purchased from Sigma. Mouse anti-PP-1 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY), and mouse anti-phospho-MAPK antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-RSK-2 and mouse anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). G_M antisera were generated by immunizing rabbits with recombinant GST-G_M(1–240) fusion protein. Restriction enzymes were from Promega Life Science (Madison, WI). All the oligonucleotides were synthesized by ITD (Coralville, IA).

**Mutagenesis of G_M(1–240)—** Rabbit cDNA encoding G_M(1–240) was cloned and inserted into pGEX-4T2 vector to produce recombinant GST-G_M protein. Mutated forms of G_M(1–240) used in the following experiments were all confirmed by DNA sequencing.

**Cloning and Construction of Expression Vectors with G_M cDNA—** The entire coding sequence of rabbit G_M was cloned into pGEX-4T2 vector by reverse transcriptase-polymerase chain reaction and subcloning methods as described previously (27). (HA),-tagged G_M was constructed by insertion of G_M cDNA into pKHHI mammalian expression vector. Point mutations in full-length G_M were made by using Quick-Change Mutagenesis kit according to the manufacturer’s protocol. Mutations were all confirmed by DNA sequencing.

**Tissue Culture**—CHO-IR cells were maintained in minimal Eagle’s medium containing nucleotides and 10% fetal bovine serum. C2C12 myoblasts were maintained in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum until confluent, at which point differentiation was initiated by conversion to Dulbecco’s modified Eagle’s medium with 4% horse serum. Cell fusion was apparent after 48 h. CHO-IR cells were routinely deprived of serum for 3 h prior to analysis using Ham’s F-12 medium. C2C12 myotubes were serum starved for 4 h in Kres-Ringer buffer with 30 mM Hepes (pH 7.4) containing 0.5% bovine serum albumin and 2.5 mM glucose.

**Transient Transfection of CHO-IR and C2C12 Muscle Cells—** CHO-IR cells and C2C12 myoblasts were grown to 50–60% confluency and transfected by FuGene reagent according to the manufacturer’s instructions (Roche Molecular Biochemicals). Plasmid DNA (6 µg/60-mm dish) was usually transiently transfected for 36 h in the complete medium. While CHO-IR cells were then used for experiments, the confluent C2C12 myoblasts were induced to form myotubes by switching to Dulbecco’s modified Eagle’s medium with 4% horse serum. The cells were serum starved as described above before the treatment with insulin ± PD98059 or EGF. To obtain a higher degree of transfection efficiency for glycogen synthase activity assay, CHO-IR cells were electroporated with 40 µg of plasmid DNA as described previously (28). Under these conditions, approximately 25% of the cells remained viable, and over 80% of the surviving cells were transfected as determined by parallel transfection with a green fluorescent protein construct.

**In Vitro Phosphorylation Assay**—CHO-IR cells and C2C12 myotubes were serum-starved and incubated with or without PD98059, wortmannin, and rapamycin for 30 min prior to the addition of 100 nM insulin. After 10 min of insulin treatment, cells (150-mm dishes) were washed twice with ice-cold PBS, then lysed in 0.5 ml of lysis/kinase buffer (50 mM Tris, pH 7.4, 50 mM sodium fluoride, 100 mM NaCl, 1% Nonidet P-40, 5 mM MgCl₂, 2 mM EGTA, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 µM Microcystin-LR, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin). Lysates were clarified at 10,000 x g for 10 min. GST-G_M fusion proteins (~7.5 µg) were mixed with 22 µl of cell lysate in a final volume of 45 µl containing 50 mM Tris (pH 7.4), 50 mM sodium fluoride, 100 mM NaCl, 0.5% Nonidet P-40, 5 mM MgCl₂, 2 mM EGTA, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 µM Microcystin-LR, 10 µg/ml PKI, and 0.5 mM [γ-32P]ATP (2000 dpm/ml). The mixture was incubated for 30 min at 30 °C before the reaction was stopped by addition of 500 µl of ice-cold PBS containing 1 mM dithiothreitol and 1 µM Microcystin-LR, 50 µl of glutathione-Sepharose beads was added, mixed and the mixture was kept constantly for 30 min at room temperature. The beads were then pelleted by centrifugation at 20,000 x g for 20–30 s and washed 3 times with ice-cold PBS. Samples were analyzed by SDS-PAGE and autoradiography.

**Phosphopeptide Mapping by Alkaline PAGE—** Phosphopeptide mapping experiments were performed as described previously (29, 30). Transfected CHO-IR cells were lysed in 50 mM sodium phosphate, 10% SDS-PAGE, Coomassie Blue-stained GST-G_M proteins were excised from the gel and exhaustive trypsin digestion was performed. After repeated lyophilization, phosphopeptides were resolved on a 40% alkaline polyacrylamide gel (pH 9.0) and then analyzed by autoradiography.

**In Vitro PP-1 Binding and Activity Assays—** This assay used 7.5 µg of GST-G_M(1–240) protein coupled to glutathione-Sepharose beads to pull-down PP-1 from NIH3T3 cell lysates as described previously (26). The binding of PP-1 was analyzed by anti-PP-1 immunoblotting and PP-1 activity assay. To assay activity following in vitro PP-1 pull-down assay, the GST-G_M(1–240) on glutathione beads were pelleted by centrifugation at 20,000 x g for 20–30 s. After washing twice with ice-cold PBS and once with PP-1 assay buffer, the beads were resuspended in 20 µl of PP-1 assay buffer. PP-1 activity was determined against 60 µg of [32P]-labeled phosphorylase a in 20 µl of PP-1 assay buffer for 10 min at 30 °C as described previously (31).

**Immunoprecipitation and Immunoblotting—** Cells lysates were prepared by detergent solubilization in a lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM sodium fluoride, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM NaVO₄, 1 µM Microcystin-LR, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin) for 30 min at 4 °C. The resultant cell lysates were clarified at 10,000 x g for 10 min. Immunoprecipitations were performed by incubating cell lysate with anti-HA antibodies for 1 h at 4 °C. The samples were then incubated with protein G-Sepharose for 1 h at 4 °C. The resulting immunoprecipitates were washed three times with the same lysis buffer and then subjected to 10% SDS-PAGE. For immunoblotting, proteins on the
gel were electrophoretically transferred to nitrocellulose membranes, which were then blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat milk. Membranes were incubated with the appropriate antibodies and washed as described previously (26). Antibody binding was detected by enhanced chemiluminescence with horseradish peroxidase-conjugated secondary antibodies. 

Metabolic $^{32}$P Labeling—36 h post-transfection, confluent CHO-IR cells in 100-mm dishes were serum-starved for 3 h in phosphate-free Dulbecco's modified Eagle's medium supplemented with pyruvate. Cells were then incubated for 1 h in the same medium containing 1 mM/ml $[^{32}$P]orthophosphate. After stimulation with insulin, cells were washed three times with ice-cold PBS. Lysate preparation and anti-HA immunoprecipitation were performed as described above. Precipitates were resuspended in SDS sample buffer and resolved by SDS-PAGE. After Coomassie Blue stain, tryptic phosphopeptide mapping was performed essentially as described above.

Glycogen Synthase Activity Assay—Glycogen synthase activity was measured as described previously (24, 32) with some modifications. After two washes with ice-cold PBS, confluent CHO-IR cells (100-mm dishes) were scraped into 500 µl of lysis/glycogen synthase assay buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 100 mM NaCl, 50 mM sodium fluoride, 1 µM Microcystin-LR, 1% Nonidet P-40, and protease inhibitors as above). Lysates were clarified at 10,000 × g for 10 min. Then 50 µl of the cell lysate was added to an equal volume of glycogen synthase assay buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 50 mM sodium fluoride, and 1 µM Microcystin-LR) containing 10 µM UDP-$[^{14}$C]glucose (0.15 µCi/µmol) and 15 mg/ml glycogen, in the presence or absence of 10 mM glucose 6-phosphate. After 15 min of incubation at 37 °C, assay tubes were chilled on ice for 15 min. The entire contents of the tubes were spotted on a Whatman filter paper (GF/A; 2.4 cm) which was immediately immersed in 25 ml of ice-cold 70% ethanol. Filters were air-dried, and radioactivity was counted with 5 ml of scintillation mixture.

RESULTS

Lysates from Insulin-stimulated Cells Phosphorlyate GST-GM at Site 1 and Site 2—The recombinant fusion protein GST-GM(1–240) was used as a substrate for kinases from CHO-IR cells that we treated with insulin. Lysates were prepared and incubated with the fusion protein plus [$\gamma$-$^{32}$P]ATP and PKI, a protein that specifically inhibits PKA activity. As seen in Fig. 1, GST-GM(1–240) was phosphorylated in this assay and insulin stimulation of the CHO-IR cells produced more than a 5-fold increase in the kinase activity in the lysate. Pretreatment of cells with the MEK inhibitor PD98059 nearly eliminated insulin stimulation of GST-GM(1–240) phosphorylation. However, pretreatment of cells with either wortmannin to block PI-3 kinase or rapamycin to inhibit the mammalian target of rapamycin did not change insulin stimulation of kinase activity (Fig. 1, upper panel). Coomassie staining showed that a comparable amount of the substrate GST-GM(1–240) was present in each reaction (Fig. 1, lower panel) and the same amount of lysate protein was added. These results showed that insulin stimulated the specific activity of a kinase(s) on the MAP kinase pathway that phosphorylated GST-GM(1–240).

Phosphopeptide mapping was performed following $^{32}$P labeling of GST-GM(1–240) to determine the site(s) of phosphorylation. Wild type and mutated GST fusion proteins were incubated with cell lysates in the presence of [$\gamma$-$^{32}$P]ATP and an excess of PKI to block PKA activity. The GST fusion proteins were resolved by SDS-PAGE, digested exhaustively by trypsin, and phosphopeptides were resolved by alkaline PAGE and detected by autoradiography. The identity of site 1 and site 2 phosphopeptides were deduced by comparing migration of phosphopeptides using the following collection of proteins: GST-GM, GST-GM(1–240), GST-GM(1–240)/S48A, and GST-GM(1–240)/S51A (Fig. 2A). Next, GST-GM(1–240) was phosphorylated by lysates from (i) control cells, (ii) cells treated with insulin, and (iii) cells treated with PD98059 plus insulin. Insulin treatment increased phosphorylation of site 1 and 2 in parallel, and pretreatment of cells with PD98059 significantly inhibited insulin-stimulated phosphorylation of both sites (Fig. 2B). Site 2 is known as a substrate for PKA, but the kinases here were active in the presence of PKI to block PKA. Similar results were obtained using a skeletal muscle model, C2C12 myotubes differentiated in culture (Fig. 2C). Lysates of control, insulin-treated, or EGF-treated C2C12 myotubes were used to phosphorylate GST-GM(1–240).
Insulin treatment of C2C12 myotubes activated a kinase(s) that equally phosphorylated site 1 and site 2. EGF is a more effective activator of the MAP kinase pathway in muscle cells compared with insulin, and indeed EGF enhanced phosphorylation of site 1 and site 2 in GST-GM(1–240) even more than insulin (Fig. 2C). These data showed that insulin or EGF-stimulated kinases that phosphorylated site 1 as well as site 2, and activation of MAP kinase was necessary for the stimulation.

Immunodepletion and Immunoprecipitation Show RSK-2 Is an Insulin-stimulated Kinase That Phosphorylates GST-GM—We found that phosphorylation of GM was catalyzed by RSK-2, a kinase activated by MAP kinase (Fig. 3). A specific RSK-2 antibody was used to immunodeplete endogenous RSK-2 from lysates prepared from insulin-stimulated CHO-IR cells (Fig. 3A). Immunoblotting of the control and RSK-2-depleted lysates with the same antibody showed almost complete removal of RSK-2 from the lysate. RSK-2 depletion caused about 50% decrease in the phosphorylation of GST-GM(1–240).

Moreover, we immunoprecipitated RSK-2 from lysates of control and insulin-treated CHO-IR cells. The specific activity toward GST-GM(1–240) in this assay was 8-fold higher in immunoprecipitates from insulin-treated cells (Fig. 3B). Phosphopeptide mapping showed that the RSK-2 recovered from either control cells or insulin-treated cells phosphorylated both sites in GM, but had preferential activity toward site 1, compared with site 2 (Fig. 3C). Therefore, using GST-GM(1–240) as substrate, RSK-2 was a prominent insulin-stimulated kinase in cell lysates. But, for insulin and EGF to stimulate phosphorylation of site 1 and site 2 to the same extent, there must have been another kinase(s) activated in concert with RSK-2.

Phosphorylation of PP-1 Glycogen-targeting Subunit

FIG. 3. Immunodepletion and immunoprecipitation of RSK-2 followed by in vitro phosphorylation of GST-GM(1–240). A, lysates from insulin-treated CHO-IR cells were incubated with or without anti-RSK-2 antibodies and Protein G-Sepharose. After centrifugation, GST-GM(1–240) was phosphorylated by incubation with the supernatants. Upper panel, 32P-labeled fusion proteins shown by autoradiography; middle panel, fusion proteins shown by Coomassie Blue stain; lower panel, anti-RSK-2 Western blotting of the supernatants. B, RSK-2 was immunoprecipitated from lysates of insulin-treated or untreated CHO-IR cells. Immuno complex kinase assay was performed by using GST-GM(1–240) as substrate. Upper panel, 32P-labeled fusion proteins shown by autoradiography; middle panel, fusion proteins shown by Coomassie Blue stain; lower panel, anti-RSK-2 Western blotting of the immunoprecipitates. C, phosphopeptide mapping of 32P-labeled GST-GM fusion proteins from panel B.

FIG. 4. Pull-down of PP-1 from NIH3T3 cell lysates by GST-GM(1–240) phosphorylated in vitro. GST-GM(1–240) was phosphorylated by incubation with lysates from basal or insulin-treated CHO-IR cells. After coupled to GSH beads, 3.5 μg of fusion proteins was used to pull-down PP-1 from NIH3T3 cell lysates. A, PP-1 coprecipitated with GST-GM(1–240) were detected by anti-PP-1 Western blotting (upper panel). The fusion protein used were shown by Coomassie Blue stain (lower panel). B, PP-1 activity coprecipitated with GST-GM(1–240) was measured by using 32P-labeled phosphorylase a as substrate. Shown are the mean ± S.E. of three separate experiments.
PP-1 binding, as seen by immunoblotting, there was nearly a 10-fold decrease in PP-1 activity associated with GST-GM(1–240), measured with [32P]orthophosphate and treated with insulin stimulation produced little in CHO-IR cells. Cells were lysed and immunoprecipitated by using anti-HA antibodies. Immunoprecipitates were separated by SDS-PAGE. As Coomassie Blue stain of (HA)3-GM. A, autoradiography of (HA)3-GM, C, phosphopeptide mapping of [32P]-labeled (HA)3-GM.

**Insulin Stimulation of the 32P-Phosphorylation of Full-length GM at Site 1 and Site 2 in Living Cells**—Our biochemical experiments suggested that insulin-activated kinases that phosphorylate sites 1 and 2 in the N-terminal domain of GM. To test this we expressed the full-length GM protein in CHO-IR cells. The cells were metabolically labeled with [32P]. and stimulated with insulin, then the GM protein was recovered by immunoprecipitation. The same amount of GM protein was recovered (Fig. 5) and insulin increased its [32P] labeling in two independent experiments by 67 and 89% (average 78%) (Fig. 5). Phosphopeptide mapping of the GM from unstimulated, control and from insulin-stimulated showed sites 1 and 2 were phosphorylated in control cells and insulin increased the phosphorylation of both sites (Fig. 5).

**Insulin Disrupts Association of PP-1 with Wild-type GM but Not GM(S67T)**—Phosphorylation of site 2 in GM in living cells should cause a loss of PP-1 association. To test this prediction, epitope-tagged full-length GM was transiently expressed in CHO-IR cells. The experiments were designed to evaluate PP-1 binding in living cells and to determine whether or not the insulin and immunoprecipitates prepared with anti-HA antibodies (Fig. 6A, upper panel). Immunoblotting showed increased insulin treatment of the cells decreased the amount of PP-1 as co-immunoprecipitated with GM by 57% (Fig. 6A, lower panel). Insulin stimulation also decreased PP-1 association with the mutated full-length GM(S48A), indicating that site 1 phosphorylation was not involved in this response. Consistent with the change being due to site 2 phosphorylation, insulin treatment of CHO-IR cells did not reduce PP-1 binding to full-length GM(S67T) (Fig. 6A, lower panel). We previously showed that S67T version of GM binds PP-1 the same as wild type GM and PP-1 binding is unaffected by reaction with PKA (27). In addition, we expressed the epitope-tagged N-terminal domain (1–240) of GM in CHO-IR cells and co-immunoprecipitation of PP-1 was reduced by insulin treatment of the cells (not shown). Our results show that insulin stimulated phosphorylation of Ser67 in GM, thereby causing reduced association with PP-1 in CHO-IR cells. In C2C12 cells transiently transfected and then differentiated into myotubes insulin stimulation produced little change in association of PP-1 with full-length GM (Fig. 6B). If these cells were treated with propanol, a β-antagonist, there was an 55% increase in the amount of PP-1 associated with GM.

**Insulin Stimulation of Cells Pre-treated with Propanol showed a detectable decrease in PP-1 association with GM (Fig. 6B).**

**Transient MAP Kinase Activation Is Coincident with Diminished GM**—Treatment of CHO-IR cells with insulin robustly activated MAP kinase, assayed by immunoblotting of the cell lysates with a phospho-specific antibody (Fig. 7A, lower panel). Insulin stimulation of the cells severely reduced co-immunoprecipitation of PP-1 with the full-length GM (Fig. 7A, middle panel; also see Fig. 6). Pretreatment of the cells with the MEK inhibitor PD98059 prior to insulin stimulation reduced MAP kinase activation and partially blocked the loss of PP-1 binding (Fig. 7B, lower panel). The co-immunoprecipitation of PP-1 with full-length GM from these same cells mirrored the time course of MAP kinase activation, with low recovery at 5 or 10 min, and nearly full recovery by 20 min (Fig. 7B, middle panel). Recoveries of GM at various times was identical (Fig. 7B, upper panel). Taken together, these data showed that in living cells insulin stimulated transient phosphorylation of site 2 in GM and reduced binding of PP-1, involving activation of MAP kinase.

**Expression of Wild Type and Phosphorylation Site Mutants of GM in CHO-IR Cells Increases Glycogen Synthase Activity, Without a Change in Stimulation by Insulin**—We tested the effects of GM on insulin-stimulated glycogen synthase activity in CHO-IR cells. Cells expressing full-length GM were treated with or without PD98059 (50 μM) prior to the addition of insulin. Cells were lysed and glycogen synthase activity (−/+ G6P) was measured in the total cell lysates, as described under “Experimental Procedures.” Control CHO-IR cells have a low basal activity of glycogen synthase (−/+ G6P) activity ratio of 0.015), and this increased by 2-fold upon insulin treatment (Fig. 8A). Transient expression of GM in CHO-IR cells resulted in a 2.2-fold increase in the basal (− insulin) glycogen synthase activity ratio (Fig. 8A, blank bars). Treatment of these cells with insulin produced a further 2-fold increase in glycogen synthase activity ratio (Fig. 8A, shaded bars). This 2-fold activation of glycogen synthase by insulin in either control cells or cells expressing full-length GM was not significantly affected by pretreatment with PD98059 (Fig. 8A, solid bars). These results indicate that insulin stimulation of glycogen synthase was not coupled to the activation of MAP kinase, and the MAP kinase-dependent phosphorylation of GM. Furthermore, pretreatment of control CHO-IR cells with wortmannin (100 nM) or wortmannin + PD98059 inhibited insulin activation of glycogen synthase by 48 and 45%, respectively. Likewise in CHO-IR cells expressing GM, wortmannin or wortmannin + PD98059 inhibited insulin activation of glycogen synthase by 59 and 50%, respectively (average of two independent experiments, data not shown). Therefore, wortmannin inhibition of the PI3K-PKB-GSK3 pathway that regulates glycogen synthase was relatively insensitive to expression of GM.

Mutations in site 1 (Ser68) and site 2 (Ser67) of GM to preclude phosphorylation did not alter insulin stimulation of glycogen synthase in CHO-IR cells. Cells were transfected with empty vector or vectors encoding: (a) HA-tagged wild type GM, (b) S48A mutant, (c) S67T mutant, or (d) S48A/S67T double mutant. The amount of vectors was adjusted so that the full-length GM proteins were expressed at comparable levels, as judged by Western blotting of the cell lysates (not shown). Transfected cells were treated with insulin for different times and the endogenous glycogen synthase activity was measured in cell lysates. As shown in Fig. 8B, overexpression of wild type GM or any of the three mutants of GM increased the glycogen synthase activity.
activity 2-fold in the absence of insulin (Fig. 8B, blank bars). This showed that the phosphorylation sites in G_{M} were not necessary for increasing basal glycogen synthase activity. At either 10 (Fig. 8B, shaded bars) or 40 min (Fig. 8B, solid bars) following insulin treatment there was a further 2-fold increase in glycogen synthase activity, regardless of which mutated version of full-length G_{M} was expressed, showing that phosphorylation of site 1 or site 2 was not necessary for the effect of insulin. There was not a relationship between the amount of PP-1G and insulin activation of glycogen synthase because association of PP-1 with full-length G_{M} was diminished at 10 min following insulin addition and was restored at 40 min (see Fig. 8B), although the glycogen synthase activity was elevated at both times (Fig. 8B). Furthermore, 10 min after insulin addition less PP-1 was associated with wild type G_{M} compared with G_{M}(S67T), although levels of glycogen synthase activity were the same in both cells. Taken together, the results show insulin stimulation of glycogen synthase activity was enhanced by expression of full-length G_{M}, but this did not require phosphorylation of either G_{M} site 1 or site 2.

**DISCUSSION**

Insulin activates glycogen synthase by inducing dephosphorylation. This occurs via PKB inactivation of the kinase GSK3 (33, 34) and has been thought to also involve activation of glycogen-bound PP-1 (PP-1G). The proposed mechanism for activation of PP-1G involved selective phosphorylation of Ser48 in the G_{M} subunit, a model that has been influential for 10 years (11, 18). We used lysates of insulin-stimulated CHO-IR cells and C2C12 myotubes as a source of kinases and examined phosphorylation of the PP-1G glycogen-targeting subunit G_{M} with a panel of mutated GST-G_{M}(1–240) proteins as substrates in biochemical assays. We demonstrated by phosphopeptide mapping the surprising result that insulin activated kinases in both cell types that phosphorylated GST-G_{M}(1–240) at both site 1 (Ser^{48}) and site 2 (Ser^{67}). Phosphorylation of both sites involved the MAP kinase pathway, evidenced by (i) treatment of CHO-IR cells with PD98059, a selective MEK inhibitor which significantly decreased the kinase activity and (ii) treatment of C2C12 myotubes with EGF, a potent activator of the MAP kinase pathway that stimulated phosphorylation of both sites to a greater extent than insulin. Immunodepletion of RSK-2 from lysates supported the idea that RSK-2 was an insulin-activated kinase for GST-G_{M}(1–240). Immunocomplex kinase assays showed that RSK-2 preferentially phosphorylated site 1. These results agree with Cohen and co-workers (18, 19) who showed that RSK-2 (then called insulin-stimulated protein kinase), one of the substrates for MAP kinase (20), could phosphorylate site 1 of G_{M} in vitro. However, we observed that GST-G_{M}(1–240) also was phosphorylated at site 2 by lysates from insulin-stimulated cells and this was not due to PKA because PKI was added to the assays. Therefore another kinase besides RSK-2 was activated by MAP kinase to phosphorylate site 2. Ser^{67} (site 2) lies within a sequence motif (RRVSFA) that could be a substrate for other members of the pp90 family (35, 36), i.e. RSK-1 and/or RSK-3. Alternatively, other MAP kinase-activated kinases, such as MAPK-interacting kinase 1 or 2 (37, 38), or mitogen- and stress-activated protein kinase 1 or 2 (39, 40), could phosphorylate site 2. Site 2 phosphorylation is well known to prevent binding of PP-1, and indeed we found decreased PP-1 binding to GST-G_{M}(1–240) following reaction with insulin-activated kinases from CHO-IR cells. Wortmannin did not reduce insulin-stimulated phosphorylation of GST-G_{M}(1–240), arguing that the kinases involved did not require activation of PI3K. The identity of the site 2 kinase in these lysates remains undetermined. Nonetheless, results with biochemical assays using cell lysates implicated phosphorylation of both site 1 and site 2 in G_{M} in response to insulin and prompted us to study this with full-length G_{M} in intact cells.

**Phosphorylation of Full-length G_{M} Site 1 and Site 2 in Living Cells in Response to Insulin**—We tested for phosphorylation of full-length G_{M} using transient expression in CHO-IR cells and C2C12 myotubes. Myotubes are syncytial cells that serve as a model for skeletal muscle and CHO-IR cells have overexpressed insulin receptors. Metabolic ^{32}P labeling of CHO-IR cells showed the full-length G_{M} protein was phosphorylated in

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**Fig. 6.** Co-immunoprecipitation of PP-1 with (HA)\textsubscript{3}-G\textsubscript{M} transiently expressed in CHO-IR and C2C12 cells. A, CHO-IR cells transfected with (HA), full-length versions of G\textsubscript{M} wild type, S48A, or S67T, were treated with or without 100 nM insulin for 10 min. Cell lysates were lysed and immunoprecipitation was performed by using anti-HA antibodies. (HA)\textsubscript{3}-G\textsubscript{M} in the precipitates was detected by anti-G\textsubscript{M} Western blotting (upper panel), and coprecipitated PP-1 was detected by anti-PP-1 Western blotting (lower panel). B, C2C12 myotubes transfected with (HA), full-length G\textsubscript{M} were treated with 10 μM propanolol + 100 nM insulin for 10 min. Cell lysates were lysed and anti-insulin immunoprecipitation was performed. (HA)\textsubscript{3}-G\textsubscript{M} in the precipitates was detected by anti-G\textsubscript{M} Western blotting (upper panel), and coprecipitated PP-1 was shown by anti-PP-1 Western blotting (lower panel).

**Fig. 7.** MAP kinase activation and association of PP-1 with (HA)\textsubscript{3}-G\textsubscript{M}. A, (HA)\textsubscript{3}-G\textsubscript{M} transfected CHO-IR cells were treated with and without 25 μM PD98059 for 30 min prior to the addition of 100 nM insulin for 10 min. Cell lysates were lysed and immunoprecipitation was performed by using anti-HA antibodies. (HA)\textsubscript{3}-G\textsubscript{M} in the precipitates was detected by anti-G\textsubscript{M} Western blotting (upper panel), and coprecipitated PP-1 was detected by anti-PP-1 Western blotting (middle panel). Phospho-MAPK was detected in the cell lysate by anti-phospho-MAPK Western blotting (lower panel). B, (HA)\textsubscript{3}-G\textsubscript{M} transfected CHO-IR cells were treated with 100 nM insulin for 0, 5, 10, 20, or 40 min. Cell lysates were lysed and immunoprecipitation was performed by using anti-HA antibodies. (HA)\textsubscript{3}-G\textsubscript{M} in the precipitates was detected by anti-G\textsubscript{M} Western blotting (upper panel), and coprecipitated PP-1 was detected by anti-PP-1 Western blotting (middle panel). Phospho-MAPK was detected in the cell lysate by anti-phospho-MAPK Western blotting (lower panel).
Phosphorylation of PP-1 Glycogen-targeting Subunit

unstimulated cells, and the phosphorylation of both sites was stimulated by insulin. In these cells insulin caused loss of PP-1 binding to G$_M$, consistent with phosphorylation of site 2. Pretreatment with PD98059 prior to insulin addition partially increased co-immunoprecipitation of PP-1 with G$_M$, suggesting that MAP kinase activation led to site 2 phosphorylation. Insulin caused a transient loss of PP-1 binding to G$_M$ coincident with a peak of activation of MAP kinase, providing a correlation between MAP kinase activation and the loss of PP-1 binding. The S67T mutant of full-length G$_M$ bound PP-1 constitutively in living cells and treatment of cells with insulin did not reduce its association with PP-1. Full-length G$_M$ mutated at site 1 (S48A) gave responses the same as wild type, showing that this site did not affect PP-1 binding. Altogether, the results showed that site 2 phosphorylation of G$_M$ occurred in response to insulin and this reduced PP-1 binding.

Our results seem at odds with the view that insulin activates PP-1G by causing phosphorylation of both site 1 and site 2 in G$_M$ (45). We propose that a transient phosphorylation of site 2 and loss of PP-1 binding were not detected when insulin was injected into animals and muscle was extracted and homogenized. The rates of site 1 versus site 2 dephosphorylation following insulin stimulation could be much different. In this regard it is interesting to note that PP2A was reported to selectively dephosphorylate site 2 compared with site 1 (18, 46). With insulin the site 2 phosphorylation could be rapidly reversed, with restoration of PP-1 binding, whereas with adrenaline site 2 would be phosphorylated more persistently and PP-1 binding eliminated. Thus, we envision that G$_M$ is phosphorylated at both sites in response to either insulin or adrenaline, but by different kinases, and for different lengths of time. While this article was under review a report appeared claiming that whereas adrenaline increased phosphorylation of G$_M$ (sites 1 and 2) in rat muscle cytosol, insulin did not (17). This new study failed to confirm previous results of insulin stimulation of G$_M$ phosphorylation, and suggested that the earlier report with rabbit muscle was “likely to be artifactual” due to kinases activated during contraction of the muscle during excision (17). This new report differs from the results reported here.

Insulin Activation of Glycogen Synthase Does Not Require G$_M$ Phosphorylation—The function of PP-1 in glycogen metabolism is based on years of investigations that implicated a type-1 phosphatase in regulation of phosphorylase, phosphorylase kinase, and glycogen synthase. Even after substantial effort, it remains uncertain whether and how the glycogen-bound form of PP-1, called PP-1G, is activated in response to insulin. A pathway proposed for MAP kinase phosphorylation and activation of PP1G in the insulin activation of glycogen synthase has been controversial (47–50). Results obtained with EGF and PD98059 in both adipocytes and muscles indicated that MAP kinase and RSK-2 activation were neither sufficient nor necessary for glycogen synthase activation (21–24). Therefore, the relationship between G$_M$ phosphorylation and glycogen synthase activation also was called into question. We address this issue in the present study and provide evidence that although there may be a role for G$_M$ in the activation of glycogen synthase, the phosphorylation of G$_M$ is not necessary. Expression of wild type G$_M$ resulted in an increase in the basal and insulin-stimulated glycogen synthase activity ($+/+G6P$), indicating that indeed G$_M$ is capable of promoting the dephosphorylation and activation of glycogen synthase in living cells. The increase in basal glycogen synthase activity probably involves binding of glycogen synthase in addition to binding of PP1 to G$_M$. The related PP1-binding protein, called PTG, directly binds glycogen synthase (25) and we have found direct binding of glycogen synthase to G$_M$ as well.$^2$

In cells expressing G$_M$, addition of insulin further activated glycogen synthase. However, this effect of insulin did not require G$_M$ phosphorylation. Addition of PD98059 did not reduce glycogen synthase activation by insulin. Also, wortmannin inhibited insulin activation of glycogen synthase with or without G$_M$ expression. None of the three mutations in G$_M$, S48A, S67T, or S48A/S67T, appeared to have an effect on insulin-stimulated synthase activity. Together, the results clearly separate G$_M$ phosphorylation at sites 1 and 2 from glycogen synthase activation by insulin. Thus, G$_M$ may simply function in the same way as other members of the PP-1 glycogen-binding subunit family. None of the other family members are reportedly phosphoproteins, but at least PTG is capable of enhancing the activity of glycogen synthase (14). This would be consistent

$^2$ J. Liu and D. L. Brautigan, unpublished results.
Phosphorylation of PP-1 Glycogen-targeting Subunit

with a preliminary report that insulin activation of glycogen synthase is unaffected in mice with a knock out of the G_M gene (51).

Our results do not necessarily rule out the possibility that PP-1 is required for full activation of glycogen synthase by insulin. Although insulin has been reported to inhibit GSK-3 by phosphorylation via protein kinase B (33, 34), inhibition of GSK-3 cannot account for the insulin stimulated dephosphorylation of all critical sites in glycogen synthase (49). Insulin was found to promote dephosphorylation of 5 sites in rabbit skeletal muscle glycogen synthase (52–54). Studies have shown that insulin stimulates PP-1 activity in both cultured muscle and fat cells, and skeletal muscle isolated from mice (24, 55), using phosphorylase α as the substrate in the assays. The molecular basis for the changes in PP-1 activity, indeed even these ideas require further investigation.

These proteins via transient phosphorylation of PP-1G, and

tine Palazzolo for assistance in preparing the manuscript.

providing CHO-IR cells and C2C12 myoblasts, respectively. We thank

Insulin might regulate the binding and/or function of GM. Insulin might regulate the binding and/or function of specific protein phospholamban (13, 15, 58). It is possible that putative transmembrane region for association with the sarcoplasmic membrane, may interact with the C-terminal region of GM. Insulin might regulate the binding and/or function of these proteins via transient phosphorylation of PP-1G, and these ideas require further investigation.

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REFERENCES

1. Villar-Palasi, C., and Larner, J. (1960) Biochem. Biophys. Acts 39, 171–173
2. Lawrence, J. C. (1992) Annu. Rev. Physiol. 54, 177–193
3. Cohen, P. (1986) in The Enzymes (Boyer, P., and Krebs, E. G., eds) pp. 461–497, Academic Press, Orlando, FL
4. Roach, P. J., Takeda, Y., and Larner, J. (1976) J. Biol. Chem. 251, 1913–1919
5. Roach, P. J., and Larner, J. (1976) Trends Biochem. Sci. 1, 110–112
6. Roach, P. J. (1990) FEBS Lett. 4, 2961–2968
7. Parker, P. J., Caudwell, F. B., and Cohen, P. (1983) Eur. J. Biochem. 130, 227–234
8. Rylatt, D. E., Atkin, A., Bilham, T., Condon, G. D., and Cohen, P. (1980) Eur. J. Biochem. 107, 529–537
9. Strålfors, P., Hiraga, A., and Cohen, P. (1985) Eur. J. Biochem. 149, 295–303
10. Hubbard, M. J., and Cohen, P. (1989) Eur. J. Biochem. 166, 711–716
11. Hubbard, M. J., and Cohen, P. (1995) Trends Biochem. Sci. 18, 173–177
12. Tang, P. M., Bondor, J. A., Swindler, K. M., and DePaoli-Roach, A. A. (1991) J. Biol. Chem. 266, 15782–15789
13. Hubbard, M. J., Dent, P., Smythe, C., and Cohen, P. (1990) Eur. J. Biochem.