Central Role for Phosphatidylinositol 3-Kinase in the Repression of Glucose-6-phosphatase Gene Transcription by Insulin

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Transcription of the gene encoding the catalytic subunit of glucose-6-phosphatase (G6Pase) is stimulated by glucocorticoids and strongly repressed by insulin. We have explored the signaling pathways by which insulin mediates the repression of G6Pase transcription in H4IIE cells. Wortmannin, a phosphatidylinositol 3-kinase (PtdIns 3-kinase)-inhibitor blocked the repression of G6Pase mRNA expression by insulin. However, both rapamycin, which inhibits p70S6 kinase activation, and PD98059, an inhibitor of mitogen-activated protein kinase activation, were without effect. Insulin inhibited dexamethasone-induced luciferase expression from a transiently transfected plasmid that places the luciferase gene under the control of the G6Pase promoter. This effect of insulin was mimicked by the overexpression of a constitutively active PtdIns 3-kinase but not by a constitutively active protein kinase B. Taken together, these data demonstrate that PtdIns 3-kinase activation is both necessary and at least partly sufficient for the repression of G6Pase expression by insulin, but neither mitogen-activated protein kinase nor p70S6 kinase are involved. In addition, activation of protein kinase B alone is not sufficient for repression of the G6Pase gene. These results imply the existence of a novel signaling pathway downstream of PtdIns 3-kinase that is involved in the regulation of G6Pase expression by insulin.

The hydrolysis of glucose-6-phosphate to glucose by glucose-6-phosphatase (G6Pase) represents the final step of both gluconeogenesis and glycogen breakdown in the liver. G6Pase is therefore a key enzyme in the regulation of blood glucose. Expression of the gene encoding the catalytic subunit of G6Pase is increased in starved and diabetic animals and repressed by re-feeding or insulin treatment, respectively (1–3). In H4IIE rat hepatoma cells, insulin inhibits both basal and glucocorticoid-stimulated G6Pase gene transcription (4, 5).

A multi-component insulin response sequence (IRS) has recently been identified in the G6Pase promoter that mediates a strong repression of mouse G6Pase gene transcription by insulin (5). This IRS is composed of two promoter regions, one of which contains three copies of the sequence T(G/A)TTT(G/T) (5). The IRSs identified in the phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, and apolipoprotein CIII promoters all contain this same motif, whereas the IRS in the insulin-like growth factor-binding protein-1 promoter has two copies of this motif arranged as an inverted palindrome (6–11). Like G6Pase, insulin also inhibits the transcription of these other genes. This raises the possibility that the expression of these genes may be regulated in a coordinated fashion by insulin, both at the level of the transcription factors involved as well as in the signaling pathways utilized.

One of the central enzymes involved in insulin signaling is phosphatidylinositol 3-kinase (PtdIns 3-kinase (12)). This enzyme is activated when the SH2 domain of its p85 subunit binds to tyrosine-phosphorylated IRS-1 (13, 14). This leads to the production of 3-phosphorylated phosphatidylinositides, which serve to activate the downstream protein kinase, protein kinase B (PKB). Hence wortmannin, an inhibitor of PtdIns 3-kinase, is able to block activation of PKB by insulin (15–18). PtdIns 3-kinase and PKB appear to be upstream activators of p70S6 kinase; the stimulation of this enzyme being inhibited by wortmannin as well as rapamycin, the latter acting via FRAP/TOR (15, 19–22). PKB is also thought to be the enzyme responsible for the insulin-dependent phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3) (23). Insulin is also able to activate the small GTP-binding protein Ras, which then initiates the Raf1 → MAP kinase cascade (reviewed in Refs. 24 and 25).

Although the general definition of insulin signaling pathways has progressed dramatically, the elucidation of a complete signaling pathway from insulin receptor to transcription factor involved in the regulation of a specific gene remains to be established. In fact, the available data suggests that multiple divergent insulin signaling pathways regulate the expression of distinct genes (25–27). Thus, the activation of the Erk1 and Erk2 isoforms of MAP kinase appear to be important in the regulation of the AP-1 complex by insulin (28) but not of the phosphoenolpyruvate carboxykinase or hexokinase II genes (29, 30). By contrast, although the induction of hexokinase II and repression of phosphoenolpyruvate carboxykinase expression are blocked by wortmannin, only the former effect is blocked by rapamycin (29, 30).

The signaling pathway(s) involved in the repression of G6Pase gene transcription by insulin have not been explored. In the current study, we have combined the use of membrane-permeant kinase inhibitors and the transient overexpression of...
PtdIns 3-Kinase and G6Pase Gene Transcription

**EXPERIMENTAL PROCEDURES**

**Materials**—General laboratory reagents were from BDH (Poole, UK). Dexamethason and bovine insulin were from Sigma. Wortmannin and rapamycin were from Calbiochem. The S6 peptide, KEAKERKQEQ-LAKKKRLSSLTSKSTSSQK (32-mer) and GSK3 phosphopeptide, RAAEEELSRAGS/P/QKL, were synthesized by Dr. Graham Bloomberg, Department of Biochemistry, University of Bristol. Rabbit polyclonal antipeptide antibody to human p70S6 kinase (residues 502–525, Ref. 17) and rabbit polyclonal anti-PKB antibody were kindly provided by Dr. Emily Foulstone, Department of Biochemistry, University of Bristol. Anti-active MAP kinase antibodies were a kind gift of Dr. E. Schafer (Promega Corp., Madison).

**Western Blotting**—H4IIE rat hepatoma cells were routinely cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum in an humidified atmosphere of 5% O2, 5% CO2 in 75 cm2 tissue culture flasks (Corning Inc., Corning, NY). Subconfluent cells were trypsinized and plated into 6-well plates (35-mm wells; Costar) for mRNA analysis, 12-well plates (22 mm wells; Costar) for transfection experiments and luciferase reporter assays, or 35-mm dishes (Fast-Ag) for p70S6 kinase assays.

**Constructions and Transfections**—A G6Pase firefly luciferase reporter plasmid designated pGL3-G6P was generated by subcloning a mouse G6Pase promoter fragment corresponding to bases −751 to +66 relative to the transcription start site into the polylinker of the plasmid PGL3-Basic (Promega). Transfection efficiencies were monitored by cotransfection with a pRL-SV40 plasmid (Promega) that expresses Renilla reniformis luciferase under control of a constitutively active SV40 promoter. Expression plasmids containing the p110 subunit of PtdIns 3-kinase have been described previously and were kind gifts from Dr. D. R. Alessi (18). HA epitope-tagged PKB was precipitated from H4IIE cell lysates using a rabbit anti-PKB polyclonal antibody (Boehringer Mannheim). Denatured RNA samples were separated on a 0.8% formaldehyde, agarose gel and transferred to nylon membrane. The membrane was blotted for G6Pase mRNA using a 32P-labeled 562-base pair probe generated by random priming of the RT-PCR product described above. The blots were visualized with a PhosphorImager (Molecular Dynamics).

**Assay Procedures**—Lysates were prepared from transfected H4IIE cells 18 h after ligand addition. Cells were washed in 2 ml of ice-cold phosphate-buffered saline, and cells were extracted by scraping into 80 μl of passive lysis buffer (Promega) or 200 μl of ice-cold immunoprecipitation buffer (IPB; 20 mM Hepes, pH 7.5, 137 mM NaCl, 25 mM β-glycerolphosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each peptatin, antipain, and leupeptin, 0.5 mM dithiothreitol, 1 mM Na3VO4). Lysates were centrifuged for 15 min at 14,000 rpm. Firefly and Renilla luciferases were assayed sequentially in 10 μl of lysis using the dual luciferase assay kit (Promega). The activity of the G6Pase promoter was expressed as the ratio of the firefly/Renilla luciferase activities and is thus corrected for differences in transfection efficiency and cell viability.

**Protein Kinase Assays**—p70S6 kinase was precipitated from 150 μl of cell extract by incubation with 5 μl of a rabbit polyclonal antibody raised to rat p70S6 kinase and 5 μg of pre-equilibrated protein A-Sepharose in a final volume of 500 μl of IPB. Precipitates were tumbled at 4 °C for 3 h and then washed with 3 × 1 ml IPB followed by 1 ml of kinase assay buffer (25 mM Hepes, pH 7.5, 25 mM β-glycerolphosphate, 25 mM MgCl2, 0.2 mM Na3VO4, 2 mM EGTA, 0.2 mM dithiothreitol). Immune complex kinase assays were performed in 50 μl of kinase assay buffer containing 50 μM [γ-32P]ATP (5 μCi/sample) and 500 μg/ml S6 peptide. Incubations were continued for 30 min at 30 °C and terminated by spotting 40-μl aliquots onto P81 phosphocellulose paper squares and washing extensively in 150 mM orthophosphoric acid. Papers were dried, and 32P incorporation into S6 peptide was determined by Cerenkov counting.

In some experiments 30-mm dishes of confluent H4IIE cells were transfected with 2 μg of pcDNA3-GSK3β (or pcDNA3 control vector) and 2 μg of pcCMV5-PKB expression plasmid using 10 μl of Trk-50 in 1.5 ml of serum-free DMEM. The cells were lysed in 0.5 ml of IPB, and the kinase assay was performed as described above except that immunoprecipitations were performed with 3 μg of anti-HA antibody (Boehringer Mannheim) and 5 μg of GSK3 peptide used as substrate in the kinase assay (32). In control experiments (not shown), we observed no detectable phosphorylation of this peptide by the transfected HA-tagged PKB, which was also present in the immunoprecipitates.

**RESULTS AND DISCUSSION**

**Repression of G6Pase Transcription by Insulin Is Blocked by Wortmannin**—PtdIns 3-kinase is potently activated by insulin in many cell types and has been shown to be important in the actions of insulin on a number of cellular processes (25). To determine whether PtdIns 3-kinase activation is also required for insulin to suppress G6Pase gene expression, H4IIE cells were incubated in the absence or presence of various combinations of 100 nM wortmannin and 200 nM insulin. Total mRNA was then isolated, and the levels of G6Pase and β-actin mRNA were determined by quantitative RT-PCR. As shown in Fig. 1, there was a significant repression of G6Pase mRNA expression by insulin at both 2 and 6 h. Wortmannin completely blocked the repression by insulin at both time points and caused an additional elevation of G6Pase mRNA levels in both the absence and presence of insulin as compared with nontreated cells. Neither insulin nor wortmannin had any effect on β-actin mRNA expression. These data indicate that PtdIns 3-kinase activity is necessary for the suppression of G6Pase gene expression by insulin. In addition, the data also suggest that there is
a tonic level of PtdIns 3-kinase activity in the basal state which partially represses G6Pase gene expression but that is also inhibited by wortmannin.

**Constitutively Active PtdIns 3-Kinase Mimics the Effect of Insulin on G6Pase Gene Transcription**—A transient transfection strategy was used to determine whether PtdIns 3-kinase activation was sufficient to repress G6Pase gene expression. H4IIE cells were co-transfected with a reporter plasmid (pGL3-G6P) containing the mouse G6Pase promoter (nucleotides −751 to +166) upstream of the firefly luciferase reporter gene and either of two different plasmids that express constitutively active forms of PtdIns 3-kinase. One plasmid encodes a PtdIns 3-kinase catalytic subunit (p110-CAX) made constitutively active by virtue of a carboxyl-terminal CAAX motif that directs isoprenylation and membrane targeting. The other encodes a p110 subunit with a single point mutation (K227E) in the Ras interaction domain, which appears to increase its basal activity (31).

Since the basal activity of the transfected G6Pase promoter is weak in H4IIE cells, the synthetic glucocorticoid dexamethasone was used to elevate reporter gene expression. As shown in Fig. 2, dexamethasone treatment induces luciferase expression 7-fold, but in the presence of insulin this effect was completely blocked, and luciferase expression was reduced to below basal levels. Overexpression of p110-CAX caused a striking reduction in the stimulation of G6Pase gene transcription by dexamethasone, thus mimicking the effect of insulin (Fig. 2A). In contrast, a kinase-dead p110-CAX-R916P mutant was ineffective (Fig. 2A). Co-transfection of the G6Pase-luciferase reporter construct with a plasmid encoding the other form of the constitutively active PtdIns 3-kinase catalytic subunit, p110-K227E (Fig. 2B). These data demonstrate that activation of PtdIns 3-kinase is sufficient at least for a large part of the effect of insulin on the G6Pase promoter and that this effect is dependent on the catalytic activity of the enzyme and independent of the regulatory subunit (p85). That the constitutively active versions of PtdIns 3-kinase do not repress the G6Pase promoter to the
same extent as insulin might suggest that another pathway is additionally involved in the full effect; if it exists, then this pathway must also be inhibitable by wortmannin (Fig. 1). Alternatively, the signal(s) generated by the expressed constructs, but not insulin, may be subjected to feedback inhibition perhaps because they generate much higher levels of intracellular PtdIns(3,4,5)P3. Due to the low transfection efficiency of those transfected constructs, but not insulin, may be subjected to feedback inhibition perhaps because they generate much higher levels of intracellular PtdIns(3,4,5)P3. Due to the low transfection efficiency of H4IIE cells, we cannot compare the levels of PtdIns(3,4,5)P3 generated in cells treated with insulin versus those transfected with the constitutively active PtdIns 3-kinase constructs. Thus we cannot discount either of these possibilities.

Repression of G6Pase Transcription by Insulin Is Not Blocked by the MAP Kinase Kinase Inhibitor, PD98059—Since the data suggest that PtdIns 3-kinase is both necessary and substantially sufficient for the ability of insulin to repress the expression of G6Pase, the nature of the downstream target for the action of this lipid kinase was investigated. It has been reported in some cell types that wortmannin can block the activation of MAP kinase by insulin (33, 34). Thus, to examine a role for MAP kinase in the repression of G6Pase gene transcription by insulin, we used the MAP kinase kinase inhibitor PD98059 (35). We found by Western blotting using anti-active MAP kinase antibodies that MAP kinase was stimulated by insulin in H4IIE cells and that this effect was completely reversed by PD98059 (Fig. 3A). Despite this, PD98059 had no effect on the ability of insulin to repress G6Pase mRNA levels but did cause an approximate 2-fold increase in the effect of dexamethasone (Fig. 3B; as observed with wortmannin in Fig. 1). This excludes a role for MAP kinase in the repression of G6Pase expression by insulin, but we speculate that the activity of the glucocorticoid receptor may be regulated by phosphorylation by MAP kinase. Such a phenomenon has been previously reported for both the oestrogen receptor (36) and peroxisome proliferator-activated receptor γ (37). However, this type of regulation does not form the basis for the effect of insulin on G6Pase promoter activity.

Rapamycin Does Not Block the Suppression of G6Pase Gene Transcription by Insulin—It is well established that PtdIns 3-kinase is responsible for the activation of the p70S6 kinase (22). To determine whether p70S6 kinase activation is required for the repression of G6Pase gene transcription by insulin, H4IIE cells were transiently transfected with the G6P-luciferase fusion gene and treated with various combinations of dexamethasone (DEX), 200 nM insulin (INS), or 20 nM rapamycin (RAPA) as indicated. Cells were incubated at 37 °C and extracted at 2 or 18 h as indicated by scraping into immunoprecipitation buffer. Each lysate was assayed for p70S6 kinase activity (panel A) and firefly and Renilla luciferase (panel B). The data represent the mean ± S.E. of three separate experiments. The effect of rapamycin on G6Pase mRNA at 2 h in H4IIE cells treated with 500 nM dexamethasone with or without 200 nM insulin was determined by Northern blotting (panel B, inset).

At both time points rapamycin completely blocked the activation of p70S6 kinase by insulin but had no effect on the basal kinase activity (Fig. 4A). Interestingly, treatment of cells for 18 h with dexamethasone caused a small but significant stimulation of p70S6 kinase activity, but this induction was also suppressed by rapamycin.
Little detectable luciferase activity accumulated during the first 2-h period post-transfection (Fig. 4B), so Northern blotting was used to allow determination of the acute effect of rapamycin on G6Pase gene expression. Fig. 4B (inset) demonstrates the repression of G6Pase mRNA by insulin at 2 h. This effect was not altered by the presence of rapamycin. By 18 h a potent induction of luciferase expression by dexamethasone was detected. In the presence of insulin this induction was completely repressed, an effect not altered by the presence of rapamycin (Fig. 4B).

The fact that rapamycin completely abolishes the stimulation of p70S6 kinase activity by insulin in H4IIE cells without affecting the ability of insulin ability to repress G6Pase gene transcription clearly demonstrates that p70S6 kinase activation is not required for this action of insulin.

**Constitutively Active Protein Kinase B Does Not Mimic the Action of Insulin on G6Pase Gene Transcription**—Another, perhaps more direct, target for PtdIns 3-kinase is PKB (15), which is activated by insulin in H4IIE cells. To assess the potential role of PKB in mediating the action of insulin on G6Pase gene transcription, the effect of co-transfecting a plasmid encoding a constitutively active derivative of this enzyme on G6Pase-luciferase fusion gene expression was investigated. In this PKB derivative, the phosphorylation sites responsible for activation of the enzyme have been substituted with acidic residues (T308D/S473D; Ref. 16). Overexpression of PKB-T308D/S473D in 3T3 L1 adipocytes has a potent insulin-like effect on the translocation of GLUT4 to the plasma membrane, which is similar to the effect of overexpression of a constitutively active membrane-targeted GagPKB (39, 40).

To confirm that the transfected PKB-T308D/S473D was catalytically active when expressed in H4IIE cells, its ability to inhibit GSK3 as reported (23) was measured. As the transfection efficiency of H4IIE cells is very low (<2%), PKB-T308D/S473D was co-expressed with HA-tagged GSK3β. In cells overexpressing wild-type PKB, insulin caused an approximate 60% inhibition of HA-GSK3β activity (Fig. 5A). Overexpression of PKB-T308D/S473D caused a quantitatively similar repression of HA-tagged GSK3β to that observed in the presence of insulin, although insulin still appeared to promote a small additional inhibition (Fig. 5A). Western blotting also demonstrated the expression of both wild-type and constitutively active HA-tagged PKB in H4IIE cells but not in mock-transfected (control) cells (Fig. 5B). Taken together, these results confirm that PKB is expressed in transfected H4IIE cells and that the PKB-T308D/S473D construct is constitutively active in H4IIE cells as determined by its ability to repress GSK3 activity.

Overexpression of the constitutively active PKB-T308D/S473D had little apparent effect on G6Pase-luciferase fusion gene expression apart from an enhancement of the effect of dexamethasone, whereas insulin had a substantial suppressive effect (Fig. 5C). This is in contrast to expression of a constitutively active PtdIns 3-kinase, which suppresses the effect of dexamethasone (Fig. 2).

The fact that constitutively active PKB can suppress the activity of GSK3β but does not mimic the effect of insulin on G6Pase gene transcription suggests very strongly that neither PKB activation nor GSK3 inactivation are sufficient alone for the ability of insulin to suppress G6Pase gene transcription.

**General Conclusions**—The data presented in this study support a model for the inhibitory action of insulin on G6Pase gene transcription in which PtdIns 3-kinase activation, but not MAP kinase, PKB, or p70S6 kinase activation, plays a central role.

**FIG. 5. Constitutively active PKB does not suppress the glucose 6-phosphate promoter.** In panel A, H4IIE cells were co-transfected with vectors expressing wild-type GSK3β with either wild-type (WT) PKB or constitutively active PKB-T308D/S473D. Cells were serum-starved for 18 h and incubated in 2 ml of serum-free DMEM with or without 200 nM insulin (INS) as indicated for 10 min at 37 °C. Cells were lysed, and GSK3 was precipitated with anti-HA antibody (upper section) or assayed for firefly and Renilla luciferases (lower section). Reporter activity is expressed as the normalized ratio of the firefly and Renilla luciferases. The data represent the mean ± S.E. of four (panel A) or three (panel B) separate experiments, all performed in triplicate.

Future work must be directed at identifying the target for PtdIns 3-kinase that regulates the signaling pathway leading to the G6Pase promoter and how activation of this pathway affects the transcriptional machinery. As yet such a target has not been identified, but the likely protein may possess a pleckstrin-homology domain, which appears to be a recognition motif for 3-phosphorylated lipids (41). Calcium-insensitive isoforms

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2 M. Dickens and J. M. Tavare´, unpublished data.

3 P. Outey and J. M. Tavare´, unpublished data.
of protein kinase C are activated by 3-phosphorylated inositol lipids (42, 43), and the possibility that these isoforms of protein kinase C may mediate the effect of insulin on the G6Pase and other promoters warrants further investigation.

In view of the homology between the G6Pase IRS and those in several other hepatic genes (see the Introduction), it will be of considerable interest to determine whether the transcription of these other genes can be similarly repressed by constitutively active PtdIns 3-kinase and whether PKB plays a role. Thus, although it was previously reported that PtdIns 3-kinase but not p70S6 kinase was required for the effect of insulin on phosphoenolpyruvate carboxykinase gene expression (29), it remains to be shown whether the activation of PtdIns 3-kinase is sufficient. However, it appears likely that the expression of these two crucial gluconeogenic enzymes is regulated by insulin and glucocorticoids in a co-ordinated fashion by very similar signaling pathways and transcriptional apparatus.

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