We have investigated glycogen synthase (GS) activation in L6hIR cells expressing a peptide corresponding to the kinase regulatory loop binding domain of insulin receptor substrate-2 (IRS-2) (KRLB). In several clones of these cells (B2, F4), insulin-dependent binding of the KRLB to insulin receptors was accompanied by a block of IRS-2, but not IRS-1, phosphorylation, and insulin receptor binding. GS activation by insulin was also inhibited by >70% in these cells (p < 0.001). The impairment of GS activation was paralleled by a similarly sized inhibition of glycogen synthase kinase 3α (GSK3α) and GSK3β inactivation by insulin with no change in protein phosphatase 1 activity. PKD1 (a phosphatidylinositol triphosphate-dependent kinase) and Akt/protein kinase B (PKB) activation by insulin showed no difference in B2, F4, and control L6hIR cells. At variance, insulin did not activate PKCζ in B2 and F4 cells. In L6hIR, inhibition of PKCζ activity by either a PKCζ antisense or a dominant negative mutant also reduced by 75% insulin inactivation of GSK3α and β (p < 0.001) and insulin stimulation of GS (p < 0.002), similar to Akt/PKB inhibition. In L6hIR, insulin induced protein kinase kinase ζ (PKCζ) co-precipitation with GSK3α and β. PKCζ also phosphorylated GSK3α and β. Alone, these events did not significantly affect GSK3α and β activities. Inhibition of PKCζ activity, however, reduced Akt/PKB phosphorylation of the key serine sites on GSK3α and β by >80% (p < 0.001) and prevented full GSK3 inactivation by insulin. Thus, IRS-2, not IRS-1, signals insulin activation of GS in the L6hIR skeletal muscle cells. In these cells, insulin inhibition of GSK3α and β requires dual phosphorylation by both Akt/PKB and PKCζ.

Most insulin effects involve tyrosine phosphorylation of insulin receptor substrates (IRSs) by the receptor (1). IRSs include IRS-1 and IRS-2. These proteins feature a COOH terminus containing multiple tyrosine phosphorylation sites in various amino acid sequence motifs that bind to the Src homology 2 domain in enzymes and adapter molecules, conveying the insulin signal further downstream (2, 3). In addition to the phosphorylation sites, IRS proteins contain other domains to engage activated membrane receptors. At the NH2 terminus, the IRS proteins contain a pleckstrin homology (PH) domain (IH1PH). The IH1PH is essential for the physiological interaction of IRS-1 and IRS-2 with the insulin receptor (4). In addition to the PH domain, IRS-1 and IRS-2 contain a phosphotyrosine binding (PTB) domain (IH2PTB), which binds to the phosphorylated NPXY motif in the cytoplasmic region of the receptors for insulin, insulin-like growth factor-1, and interleukin (3, 5, 6). A third region encompassing residues 591–786 in IRS-2 engages the phosphorylated regulatory loop of the insulin receptor subunit (7–9). This region has been therefore termed kinase regulatory loop binding domain (KRLB) (7–9). Since IRS-1 does not contain a functional KRLB domain (9), we have previously proposed that the KRLB domain might contribute to a unique signaling potential of IRS-2 (7–9).

The signaling events by which insulin activates glycogen synthase have become much clearer in recent years. Insulin promotes the dephosphorylation of glycogen synthase (GS) and consequent stimulation of glycogen synthesis (10–12). Although glycogen synthase is a substrate for a large number of protein kinases, the 3a-3d cluster of phosphorylation sites are crucial to the activity of GS, and these sites are phosphorylated by glycogen synthase kinase 3 (GSK3) (13, 14). Insulin inactivates GSK3 by phosphorylation of Ser-21 (GSK3α) and/or Ser-9 (GSK3β) (13, 14) and also induces phosphorylation of the G-subunit of the glycogen-bound form of protein phosphatase 1 (PP1) (15). These two events cooperate to activate GS in the cells (16, 17), although their relative roles may vary during differentiation state of adipocytes (17). Insulin-dependent inactivation of GSK3 has been known to be dependent on Akt/PKB (also known as related to the A and C kinase kinase) (16, 17). Akt/PKB in turn was shown to be phosphorylated in response to insulin at Thr-308 and Ser-473 (20–22), and these phosphorylation events can be blocked by inhibitors of PI 3-ki-
nase (10, 13, 23, 24). PKD-1, a phosphatidylinositol 3,4,5-trisphosphate-dependent Akt/PKB kinase, was proved to phos-
phorylate Akt/PKB at Thr-308, which leads to a substantial but incomplete activation of Akt (21, 25). The identity of the Ser-
473 kinase is still unknown, but it is referred to as PKD-2, as it is expected that this kinase is also dependent upon phos-
phatidylinositol 3,4,5-trisphosphate. The central importance of
Akt/PKB induction for insulin inactivation of GSK3 is well
established (19–21). However, recent evidence indicates that
Akt/PKB activity is not sufficient for stimulation of GS in cells
(26). In addition, whether induction of Akt/PKB is sufficient for
insulin to inactivate GSK3 is unknown.

In the present study, we sought to identify the molecular
components of the signal transduction pathway involved in
insulin regulation of the glycogen synthetic machinery in skel-
etal muscle cells, a major target of insulin action. We demon-
strate that IRS-2, not IRS-1, signals insulin activation of gly-
cogen synthase in the L6hIR skeletal muscle cells. In these
cells we show that insulin inhibition of GSK3 requires dual
phosphorylation by both Akt/PKB and the atypical protein
 kinase C, PKCζ.

EXPERIMENTAL PROCEDURES

Materials—Media, sera, antibiotics for cell culture, the Lipo-
fectAMINE reagent, and rabbit polyclonal antibodies toward the
specific PKC isoforms were from Life Technologies, Inc. Polyclonal insulin
receptor antibodies were from Oncogene Science (Mahnasset, NY),
GSK3β antibodies were from Transduction Laboratories (Lexington, KY),
phosphotyrosine, IRS-1, IRS-2, Akt1/2, PDK1, and PPI antibodies
were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-
MAPK, phospho-Ser-Akt1/2, and phospho-Thr-Akt1/2 were purchased from
New England Biolabs (Beverly, MA). Phospho-GSK3β antibodies
were purchased from Quality Controlled Biochemicals (Hopkintown,
MA). SI33- and phospho-SI33α antibodies, the PDK and PKCζ
peptide, and the vector were synthesized by PRIMM (Milan, Italy). PKCζ
DN and wild-type DNAs were generous gifts of Dr. S. Gutkind (NCI, National Institutes of Health,
Bethesda, MD). Human IRS-2 cDNA has been described in Miranda
et al. (32). Individual G418-resistant clones were selected by
incubation for 30 min at 25 °C, reaction mixtures were spotted on
phosphocellulose discs. Disc-bound radioactivity was quantitated by liquid scintillation counting. Determinations of
PKCζ and PKCζ activities using either the acetyl-myelin basic protein
(4–14) peptide as substrate or the H-Arg-Phe-Ala-Val-Arg-Asp-
Met-Ang-Thr-Val-Ala-Val-Gly-Val-Ile-Lys-Ala-Val-Asp-Lys-
OH peptide (for PKCζ) or the PKCζ pseudosubstrate region (for
PKCζ) provided consistent results.

MAPK was assayed as previously described (32). Briefly, cell lysates
(200 μg of protein/assay) were immunoprecipitated with MAPK anti-
odies and then incubated with protein A-Sepharose for 2 h. Immo-
obilized MAPK was washed three times with ice-cold TAT buffer (50 mM
HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na3P2O7, 2 mM
NaVO3, 10% glycerol, 1% Triton X-100), twice more with HNTGVa
buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM Na3P2O7, 10 mM
NaVO3, 1% Triton X-100) and then resuspended in HNTGVa supple-
mented with 60 mM magnesium acetate, 30 μM ATP, 6 mM dithio-
treitol, 1 μg/ml myelin basic protein, and 0.5 μCi of [γ-32P]ATP.
Upon phosphocellulose disc washing and three times with 1% (v/v) phosphoric acid and once more with ethanol. Disc-bound radioactivity was quantita-
ted by liquid scintillation counting. For quantitation of PI 3-kinase
activity, the cells were solubilized in 50 mM HEPES, pH 7.5, 150 mM
NaCl, 10% glycerol, 1% Triton X-100, and 4.5 nM okadaic acid.
Samples were incubated at 37 °C for 15 min, and the reaction
was initiated by the addition of 15 μCi of [γ-32P]-labeled phosphorylase
C, reaction mixtures were spotted on Immobilon-P
by guest on July 24, 2018http://www.jbc.org/Downloaded from
and immunoprecipitated with either GSK3α or GSK3β antibodies. Immuno-complexes were immobilized on protein G-Sepharose and washed twice with 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 5 mM Na2P2O7, and 10 mM sodium glycerophosphate and once more with reaction buffer (8 mM MOPS, 0.2 mM EDTA and 10 mM magnesium acetate). Pellets were resuspended in 20 μl of reaction buffer containing 250 mM phosphoglycerate synthase peptide-2, 500 mM ATP, 75 mM MgCl2, and 1 μCi/μl (3000 Ci/mmol) γ-32P]ATP and further incubated at 30 °C. Reaction mixtures were spotted on phosphocellulose discs and washed three times with 0.75% phosphoric acid and once more with acetone. Disc-bound radioactivity was quantitated by liquid scintillation counting. In some of the experiments, purified (0.1 mg) KRLB was used. In vitro phosphorylation of GSK was analyzed by incubating GSK3α or GSK3β immunoprecipitates with 0.1 mg of recombinant PKCζ in the presence of 0.5 mM ATP, 0.25 mM EGTA, 0.4 mM CaCl2, 0.1 mg/ml bovine serum albumin, 20 mM Tris, pH 7.5, 10 mM MgCl2, 0.32 mg/ml phosphatidylserine, and 10 μCi/ml (3000 Ci/mmol) γ-32P]ATP (final concentrations). The incubation was prolonged for 30 min at room temperature and then stopped by the addition of Laemmli sample buffer. Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and identified by autoradiography. In some of the experiments, purified (0.1 μg) rather than immunoprecipitated GSK3α and GSK3β were used in the phosphorylation assays.

**Thymidine Incorporation and Glycogen Synthase Assays**—The thymidine incorporation assay was accomplished as previously reported (34). Briefly, L6hIR myoblasts were seeded in six-well plates and, after 18 h, fed with Dulbecco’s modified Eagle’s medium supplemented with 0.25% bovine serum albumin. The cells were further incubated for 16 h in the absence or presence of 100 nM insulin followed by the addition of 500 nCi/ml [3H]thymidine. 4 h later the cells were washed with ice-cold 0.9% NaCl and then with ice-cold 20% trichloroacetic acid followed by solubilization with 1 N NaOH. Radioactivity was quantitated by liquid scintillation counting. Glycogen synthase activity was assayed as described in Formisano et al. (34).

**RESULTS**

**IRS-2 Block by Expression of the KRLB Peptide in L6hIR Cells**—The cDNA fragment corresponding to amino acids 591–786 of IRS-2 was transfected in L6hIR skeletal muscle cells. This fragment encodes the complete KRLB domain of IRS-2 protein (7–9). Several clones of L6hIR cells stably expressing the KRLB peptide were selected and characterized, and two of these clones expressing lower (B2) and higher levels of the peptide (F4) were studied in detail (Fig. 1A). In extracts from insulin-exposed L6hIR cells, the expressed KRLB peptide co-immunoprecipitated with insulin receptors (Fig. 1B). The co-precipitation was dependent on the concentration of insulin to which the cells were exposed (insulin EC50max 100 nM, EC50 5 nM) as well as on the KRLB peptide expression levels in the cells. In extracts from B2 and F4 cells, the KRLB peptide-insulin receptor interaction was accompanied by 75 and 95% decreased IRS-2 co-precipitation with the insulin receptor, respectively, as compared with those occurring in cells transfected with the plasmid alone (Fig. 1C). Expression of the KRLB peptide at increasing levels also caused a progressive decrease in insulin-dependent phosphorylation of IRS-2, with no change in the IRS-2 content of the cells (Fig. 2A). Decreased IRS-2 phosphorylation by the KRLB peptide corresponded to parallel increases in receptor co-precipitation of IRS-1 (Fig. 1C) as well as in insulin-dependent tyrosine phosphorylation of IRS-1 (Fig. 2B). As was the case for IRS-2, IRS-1 levels were unchanged in the B2 and F4 clones compared with L6hIR cells transfected with the empty vector.

**Insulin Action in Cells Expressing the KRLB Peptide**—To address the functional consequences of blocking IRS-2-mediated insulin signaling in L6hIR cells, we have compared proliferative and glycogen synthetic responses in KRLB expressors and untransfected cells. As shown in Fig. 3 (top panel), basal (non-insulin-dependent) incorporation of thymidine was increased by 25 and 40% in B2 and F4 cells, respectively, compared with control cells transfected with the vector alone or the untransfected cells (p < 0.001). Maximal insulin-stimulated thymidine incorporation was also increased by 18 and 40% in the B2 and F4 cell clones, respectively (p < 0.001). Increased thymidine incorporation into DNA in these cells was paralleled by 18 and 30% increased basal (p < 0.05) and a similarly sized (p < 0.05) increase in insulin-stimulated MAPK activity compared with the control cells (Fig. 3, bottom panel; p < 0.001). At variance with proliferative responses, basal glycogen synthase activity was unchanged in B2 and F4 cells (Fig. 4A). In the L6hIR control cells, insulin increased glycogen synthase activity in a dose and time-dependent fashion. Half-maximal and maximal insulin effects were achieved at 5 and 100 nM, respectively (Fig. 4A). Also, maximal stimulation was achieved within 30 min after insulin addition (Fig. 4B). Insulin-stimulated glycogen synthase activity was inhibited by 70 and 85% in B2 and F4 cells, respectively. Immunoprecipitated GSK3α and GSK3β activities toward the specific substrate phospho-glycogen synthase peptide 2 were also not different in basal F4 and in control cells (Fig. 5A). In the F4 cells, insulin, either at 1 nM or at 100 μM, did not induce any significant inhibition of either GSK3α or GSK3β (Fig. 5A). At these same concentrations,
insulin elicited 20 and 40% inhibition of GSK3α activity and 25 and 60% inhibition of GSK3β in the control cells (transfected with the empty vector). Phosphorylation of GSK3α and -β also exhibited no change after insulin stimulation of the F4 cells, whereas insulin stimulation increased in a dose-dependent manner in the L6hIR cells (Fig. 5A, inset). Similar results were obtained with the B2 cell clone (data not shown). Phosphatase activity in PP1 immunoprecipitates from L6hIR cells was well detectable but increased by only 25% (p < 0.05) upon insulin exposure of the cells (Fig. 5B). No significant difference in PP1 protein expression and activity were observed in the F4 compared with the control cells, whether in the absence or the presence of insulin.

PKCζ Activation in Cells Expressing the KRLB Peptide—To elucidate the insulin-signaling events responsible for the block of glycogen synthetic responses in the KRLB peptide-expressing cells, we first analyzed IRS-1 and IRS-2-associated PI 3-kinase activity. Basal IRS-1-coprecipitated PI 3-kinase in B2 and F4 cells featured 20 and 40% increased levels compared with that in control cells (Fig. 6, top panel; p < 0.05). Maximal insulin-stimulated PI 3-kinase activity associated to IRS-1 also showed 30 and 45% increase in cells expressing the KRLB peptide (p < 0.05), paralleling IRS-1 tyrosine phosphorylation. The PI 3-kinase activity measured in IRS-2 immunoprecipitates from basal B2 and F4 cells showed 40 and 60% lower levels as compared with control cells (Fig. 6, middle panel; p < 0.001). Insulin-stimulated activity of PI 3-kinase associated to IRS-2 was inhibited by 80 and 95% in B2 and F4 cells, respectively. Total PI 3-kinase activity associated with tyrosine-phosphorylated proteins featured no significant differences in the B2, the F4, and the control cells, however (Fig. 6, bottom panel).

It appeared, therefore, that IRS-1 and IRS-2 are redundant in transducing insulin activation of PI 3-kinase in L6hIR as in other models (32, 35).

Known proteins mediating insulin signals downstream PI 3-kinase include PDK1, Akt/PKB, and PKCζ (13, 14, 36). Insulin-dependent activation of PDK1 and Akt/PKB was unchanged in the F4 compared with control cells (Fig. 7A). Insulin-dependent phosphorylation of key phosphorylation sites on Akt/PKB (Ser473 and Thr308) were also unchanged in the F4 compared with control cells (Fig. 7B). Similarly, protein levels of both PDK1 and Akt/PKB were unaffected by expression of the KRLB peptide. In L6hIR control cells, insulin induced PKCα,-β,-δ, and -ζ in a dose-dependent fashion. In parallel with IRS-2 phosphorylation, however, maximal insulin-stimulated PKCζ activation (2-fold versus basal) was inhibited by 70 and >95% in the B2 and the F4 cells, respectively (Fig. 8C, bottom panel). PKCζ inhibition occurred with no detectable changes in the expression of PKCζ protein (Fig. 8A) or mRNA (data not shown) in the cells. The expression of PKCδ was also unchanged in the KRLB peptide-expressing cells, although maximal insulin-dependent activation was reduced by 20% (p < 0.05; Fig. 8C, top panel).
panel). At variance, PKCα and PKCβ expression and insulin-dependent activities were not significantly different in the KRLB and the control cells (Fig. 8, A and B).

The specific role of IRS-2 in insulin activation of PKCζ and glycogen synthetic responses was further addressed by transient transfection of IRS-2 cDNA in L6hIR cells. As shown in Fig. 9 (left panels), overexpression of IRS-2 (10-fold versus control cells) was accompanied by a constitutive increase in PKCζ activity levels. Insulin further increased PKCζ activity by 40% in cells overexpressing IRS-2. The changes in PKCζ activity levels caused by IRS-2 overexpression were closely paralleled by very similar changes in glycogen synthase activity and GSK3α and -β phosphorylation (Fig. 9, right panels). Thus, the block of insulin activation of PKCζ and of glycogen synthetic responses in cells expressing the KRLB peptide seemed to be caused by a specific block of IRS-2 signaling.

Effects of PKCζ Block in L6hIR Muscle Cells—We tested the hypothesis that PKCζ is also involved in insulin activation of glycogen synthase in muscle cells. To address this issue, we transfected a PKCζ antisense oligonucleotide (PKCζ-AS) in wild-type L6hIR skeletal muscle cells. This antisense oligonucleotide reduced PKCζ protein levels by >70% in the cells without affecting those of PKCβ (Fig. 10A). Activation of PKCζ by insulin was also inhibited by about 70% by the PKCζ antisense (Fig. 10C). In parallel, PKCζ-AS transfection blocked insulin inhibition of GSK3α and -β by >75% (p < 0.001; Fig. 10B). Control oligonucleotides (PKCζ-S, Fig. 10B, S-ζ) neither decreased PKCζ (or PKCβ) levels nor GSK inhibition by insulin as compared with cells not treated with the antisense. In addition, we transfected the dominant negative Lys281→Trp
PKCζ mutant (PKCζ-DN) in L6hIR cells. Expression of this mutant at 50-fold higher levels than endogenous PKCζ significantly reduced insulin activation of PKCζ (p < 0.001; Fig. 10C). As in cells transfected with the PKCζ antisense, the lack of PKCζ insulin response in PKCζ-DN cells was accompanied by inhibition of insulin effect on GSK3α and -β (p < 0.001).
Similar to GSK3, insulin activation of glycogen synthase was largely prevented by PKCζ/AS (although not PKCζ-S) treatment and by transfection of the PKCζ-DN (Fig. 11). Different from PKCζ, however, inhibition of PKCζ activity with rottlerin (3 μM) had no effect on either glycogen synthase activation by insulin or GSK3ζ and -ζ inhibition (data not shown). This indicated that insulin effects on GSK3 and glycogen synthase in L6hIR skeletal muscle cells requires PKCζ, not PKCζ.

PKCζ Association and Phosphorylation of GSK3ζ and GSK3ζ—To further investigate the role of PKCζ in activating the glycogen synthetic machinery in L6hIR cells, we sought to identify potential PKCζ-GSK3 interactions. Insulin pretreatment of intact L6hIR cells was found to induce co-precipitation of PKCζ with GSK3ζ and GSK3ζ in the cell lysates (Fig. 12A). PKCζ co-precipitation with GSK3ζ and -ζ was detectable by either blotting PKC precipitates with GSK3 antibodies or vice versa (data not shown). The dominant negative PKCζ-DN mutant did not co-precipitate with either GSK3ζ or GSK3ζ either in the absence or the presence of insulin, indicating PKCζ activation is necessary for its association with GSK3. In vitro, activated recombinant PKCζ phosphorylated purified GSK3ζ and GSK3ζ (Fig. 12, B and C). Phosphorylation also occurred using GSK3ζ and GSK3ζ preparations from L6hIR cells (data not shown). PKCζ phosphorylation of GSK3ζ and GSK3ζ, however, did not reduce GSK3 activities toward the phospho-glycogen synthase peptide 2 substrate (Fig. 13A). This suggested that although necessary, PKCζ activation may not be sufficient to inhibit GSK3ζ or -ζ upon exposure of intact cells to insulin.

PKCζ Inhibition of GSK3ζ and GSK3ζ—In most cell types, phosphorylation by Akt/PKB is believed to be necessary for...
FIG. 10. Effect of blocking of PKCζ activity on GSK3α and -β function. A, L6hIR cells were transiently transfected with the dominant negative PKCζ cDNA (PKCζ-DN), PKCζ antisense (AS-ζ), or control antisense (S-ζ). The cell were then stimulated with 100 nM insulin as indicated, solubilized, and immunoprecipitated with GSK3α or GSK3β antibodies. GSK3 activity was then assayed in the immunoprecipitates, as described under “Experimental Procedures.” B, for the control, aliquots of the cell lysates were blotted with PKCζ or PKCδ antibodies. Filters were revealed by ECL and autoradiographed. C, alternatively, aliquots of the cell lysates were precipitated with PKCζ antibodies, and precipitates were assayed for PKC activity, as outlined in the legend to Fig. 8. The bars represent the mean ± S.D. of duplicate determinations in four (B) and three (C) independent experiments.

From either basal or insulin-treated cells or with an active recombinant Akt/PKB. As shown in Fig. 13A, incubation of PKCζ-phosphorylated GSK3α with the activated Akt/PKB preparations reduced GSK3α activity by >70%. At variance, Akt/PKB phosphorylation alone elicited only a 35–40% inhibition of GSK3α in the absence of PKCζ pretreatment. No significant inhibition of GSK3α activity occurred upon incubation with inactive Akt/PKB both in the absence or in the presence of previous PKCζ phosphorylation. The same results were obtained with GSK3β (data not shown). At variance with Akt/PKB, PKCζ did not phosphorylate Ser21 on GSK3α and Ser21 on GSK3β in vitro (Fig. 13B). However, a 2.5-fold increase in Akt/PKB-induced phosphorylation of Ser21 and Ser21 was detectable when GSK3α and -β were incubated with both PKCζ and Akt/PKB.

To address the relevance of PKCζ phosphorylation to GSK3 control by Akt/PKB in vivo, we analyzed the key Akt/PKB phosphorylation sites on GSK3α and -β in cells transfected with either PKCζ antisense or the dominant negative PKCζ-DN mutant. In both PKCζ antisense-transfected cells and in cells overexpressing the PKCζ dominant negative mutant, Ser21 phospho-GSK3α and Ser21 phospho-GSK3β were almost absent compared with the untransfected cells either in the absence or in the presence of insulin (Fig. 13C). This paralleled the lack of insulin effect on GSK3 activity occurring in cells when PKCζ expression or function is blocked. Thus, PKCζ phosphorylation appears to be necessary and permissive for further phosphorylation of GSK3α and -β by Akt/PKB as well as for insulin constraint of GSK3α and -β activities.

**DISCUSSION**

In the present report, we describe a novel approach to investigating IRS-2-mediated events in insulin activation of glycogen synthase in cultured cells. We have expressed a peptide corresponding to the KRLB domain of IRS-2 (amino acids 591–
786 (7–9)) in L6hIR skeletal muscle cells. Consistent with our previous in vitro data (7–9), expression of this peptide specifically blocked IRS-2 association and phosphorylation by the active insulin receptor kinase. It appears, therefore, that the KRLB domain is necessary for enabling IRS-2 to interact with the insulin receptor in intact cells as well as in vitro.
Block of IRS-2 binding to the receptor by the KRLB peptide was accompanied by increased receptor binding and phosphorylation of IRS-1, with no change in IRS-1 protein levels. The KRLB domain is unique to IRS-2. In addition to the KRLB, however, IRS-2 possesses PI and phosphotyrosine binding domains homologous to those enabling IRS-1 to interact with the insulin receptor (3). Prevention of IRS-2 binding to the receptor through the expression of the KRLB peptide may remove IRS-2 competition for PI and phosphotyrosine binding sites on the receptor, fostering binding of IRS-1. Consistent with this possibility, muscles from IRS-1 knockout mice and L6hIR cells transfected with IRS-1 ribozyme have also been reported to feature increased phosphorylation of IRS-2 (32, 35).

Expression of the KRLB peptide was accompanied by increased insulin mitogenic activity through the MAPK system but block of insulin activation of the glycogen synthetic apparatus. In addition, IRS-2 overexpression constitutively activated GSK3 phosphorylation and glycogen synthase activity in L6hIR cells. Thus, IRS-1 appears to transduce insulin mitogenic effects, whereas IRS-2 is the main molecule involved in glycogen synthetic responses in L6hIR muscle cells. In these same cells, ribozyme block of IRS-1 expression prevents insulin mitogenic but not glycogen synthetic responses (32). Also, the expression of an insulin receptor mutant (IR1152) featuring constitutively increased phosphorylation of IRS-2 induces constitutive activation of the glycogen synthetic apparatus in the L6 cells (7). This effect can be prevented by expressing the KRLB peptide in the cells (data not shown). In addition to the muscle cells, IRS-2 signaling appears to be prominent in transducing insulin activation of the glycogen synthetic apparatus in isolated liver cells (37). Thus, the prominent role of IRS-2 in insulin activation of glycogen synthesis may be common to the two major tissues accomplishing this function in the organism.

Akt/PKB activation is known to represent a major mechanism leading insulin to stimulate glycogen synthase activity in cells (13, 21). Accordingly, treatment of L6hIR cells with the Akt/PKB inhibitor ML-9 (38) prevents insulin stimulation of glycogen synthesis. In the present paper, however, we report that Akt/PKB phosphorylation and activation occurs at normal levels in cells expressing the KRLB peptide despite the block of insulin-stimulated glycogen synthase activity. This indicates that Akt/PKB activation is necessary but not sufficient for enabling insulin control of glycogen synthase in L6hIR cells. PDK1 and PDK2 phosphorylation of Akt/PKB in response to insulin also occurred at the same levels in cells expressing the KRLB peptide and in untransfected cells. Thus, in cells expressing the KRLB peptide, increased IRS-1 phosphorylation is paralleled by a similar increase in MAPK activity and insulin mitogenic signaling but unchanged PDK1 and Akt/PKB activities. This may occur because of the following possibilities. (i) Insulin activation of PDK1 and Akt/PKB (at variance with that of MAPK) is already saturated by IRS-1 signaling in untransfected cells; (ii) IRS-1 and IRS-2 may be redundant in signaling activation of PDK1 and Akt/PKB but not of MAPK activation; so that increased IRS-1 phosphorylation in KRLB-expressing cells exactly compensates for the lack of IRS-2 phosphorylation in activating PDK1 and Akt/PKB but not MAPK; (iii) insulin activation of PDK1 and Akt/PKB, different from that of MAPK, may not require IRS-1- or IRS-2-associated PI 3-kinase, as recently proposed by Whitehead et al. (39). These possibilities are currently under investigation in our laboratory.

At variance with PDK1 and Akt/PKB, insulin induction of PKCζ activity was blocked in cells expressing the KRLB peptide, indicating a major role of IRS-2 in signaling PKCζ activation. It is possible that expression of the KRLB peptide reduces the amount of phosphatidylinositol 3-phosphate produced after insulin stimulation of the cells and that PDK1, Akt/PKB, and PKCζ feature a differential need for phosphatidylinositol 3-phosphate for activation. This hypothesis is unlikely to explain the differential activation of PDK1, Akt/PKB, and PKCζ in KRLB and control cells, since the total amount of PI 3-kinase activity associated with tyrosine phosphoproteins was not different in the two cell types. Alternatively, activation of PKCζ by IRS-2-associated PI 3-kinase may occur in an intracellular compartment different from that where PDK1 and Akt/PKB are activated by IRS-1-associated PI 3 kinase. Consistent with this possibility, tyrosine-phosphorylated IRS-1 and IRS-2 are differentially localized inside the cell (40, 41). Other PI 3-kinase docking substrates such as IRS-4 and Grb2-associated binder (GAB) may also be differentially phosphorylated by insulin in cells expressing the KRLB peptide and contribute to the differential activation of PDK1, Akt/PKB, and PKCζ. This latter possibility is presently under investigation in our laboratory.

We have also shown that antisense inhibition of PKCζ expression or block of PKCζ activity with a dominant negative PKCζ mutant prevents an insulin effect on GSK3 (both α and β) and on glycogen synthase, as in the case of Akt/PKB block. It appears therefore that both PKCζ and Akt/PKB are necessary for insulin control of GSK3 and glycogen synthase in intact L6hIR cells. In these same cells, antisense block of PKCζ expression or inhibition of PKCζ activity with a dominant negative PKCζ mutant abolished insulin phosphorylation of the key
Akt/PKB phosphorylation sites in GSK3α and -β (Ser21 and Ser3, respectively). These sites might undergo promiscuous phosphorylation by PKCζ in the L6hIR cells. This is an unlikely possibility, however, since, in vitro PKCζ does not phosphorylate either GSK3α on Ser21 or GSK3β on Ser3. Alternatively, PKCζ phosphorylation of GSK3α and -β may be permissive for phosphorylation and inactivation by Akt/PKB. In vitro recombinant PKCζ phosphorylates GSK3α and -β but is unable to inhibit its activity. Recombinant Akt/PKB alone exhibits only modest effects on GSK3 activity. We have found, however, that PKCζ phosphorylation of GSK3α or -β enables Akt/PKB to further inhibit the GSK3s in vitro. This sequential phosphorylation of GSK3 by PKCζ and Akt/PKB was accompanied by almost complete block of GSK3 activity. Thus, in vitro full inhibition of GSK3α or -β activities requires phosphorylation by both PKCζ and Akt/PKB. We propose, therefore, that dual phosphorylation of GSK3α and -β by PKCζ and Akt/PKB may also be necessary for full inactivation of GSK3 by insulin, at least in intact L6hIR muscle cells. It appears that PKCζ is involved in transducing insulin action to GSK3 and glycogen synthase in addition to regulating insulin-mediated glucose uptake and general protein synthesis (42–44).

Phosphorylation of GSK3 by Akt/PKB is believed to represent a major mechanism responsible for insulin control of GSK3 and glycogen synthase activities in cells. This is also the case for the L6hIR myotubes, since insulin elicited only a slight effect on PP1 in these cells. To our knowledge, however, the present report provides the first evidence that PKCζ phosphorylation of GSK3 is permissive for insulin-dependent Akt/PKB regulation of GSK3. Mapping the relevant PKCζ phosphorylation sites on GSK3 is presently in progress in our laboratory.

A recent report by Tsujo et al. (45) shows that activation of PKCζ rather than -ζ is involved in insulin signaling to GSK3 in neuroblastoma cells, suggesting that different PKC isoforms may accomplish this function in different cell types. In addition, in liver PKCζ is not involved in insulin signaling to glycogen synthase (10). Also, the expression of the KRLP peptide in mouse liver cells inhibits insulin activation of Akt/PKB despite the unchanged induction occurring in the L6hIR muscle cells. Thus, the molecular mechanisms responsible for insulin inactivation of GSK3 may feature tissue specificity.

Acknowledgments—We are grateful to Dr. E. Consiglio for continuous support and advice during the course of this work. We also thank Dr. L. Beguinot (DIBIT, H. S. Raffaele, Milan) for advice and critical reading of the manuscript and Dr. D. Liguoro for technical help.

REFERENCES

1. Virkamaki, A., Ueki, K., and Kahn, C. R. (1999) J. Clin. Invest. 103, 931–943
2. Sudol, M. (1998) Oncogene 17, 1469–1474
3. White, M. F. (1997) Diabetologia 40, 2–17
4. Yamasaki, H., Machida, K. J., Smith-Hall, J., Ishibashi, O., Myers, M. G., Jr., and White, M. F. (1996) J. Biol. Chem. 271, 24300–24306
5. Eck, M. J., Dhe-Paganon, S., Trub, T., Nolan, R. T., and Shoelson, S. E. (1996) Cell 85, 695–705
6. Wolf, G., Trub, T., Oettinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 27407–27410
Insulin Receptor Substrate-2 Phosphorylation Is Necessary for Protein Kinase Cζ Activation by Insulin in L6hIR Cells

Francesco Oriente, Pietro Formisano, Claudia Miele, Francesca Fiory, Maria Alessandra Maitan, Giovanni Vigliotta, Alessandra Trencia, Stefania Santopietro, Matilde Caruso, Emmanuel Van Obberghen and Francesco Beguinot

J. Biol. Chem. 2001, 276:37109-37119.
doi: 10.1074/jbc.M104405200 originally published online July 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104405200

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 26 of which can be accessed free at http://www.jbc.org/content/276/40/37109.full.html#ref-list-1