Vanadyl Sulfate-Stimulated Glycogen Synthesis Is Associated with Activation of Phosphatidylinositol 3-Kinase and Is Independent of Insulin Receptor Tyrosine Phosphorylation†

Sanjay K. Pandey,‡,§ Madhu B. Anand-Srivastava,§ and Ashok K. Srivastava*,‡

Centre de Recherche, Centre Hospitalier Université de Montréal, Campus Hotel-Dieu, 3850, Saint Urbain Street, Montréal, Quebec, H2W 1T8, Canada, and Departments of Physiology and Medicine, University of Montréal, Montréal, Québec, H2W 1T8, Canada

Received October 29, 1997; Revised Manuscript Received March 18, 1998

ABSTRACT: Salts of the trace element vanadium, such as sodium orthovanadate and vanadyl sulfate (VS), exhibit a myriad of insulin-like effects, including stimulation of glycogen synthesis and improvement of glucose homeostasis in type I and type II animal models of diabetes mellitus. However, the cellular mechanism by which these effects are mediated remains poorly characterized. We have shown earlier that different vanadium salts stimulate the MAP kinase pathway and ribosomal-*S*-6-kinase (p^{70s6k}) in chinese hamster ovary cells overexpressing human insulin receptor (CHO−HIR cells) [Pandey, S. K., Chiasson, J.-L., and Srivastava, A. K. (1995) *Mol. Cell. Biochem. 153,* 69−78]. In the present studies, we have investigated if similar to insulin, VS also activates phosphatidylinositol 3-kinase (PI3-k) activity, and whether VS-induced activation of the PI3-k, MAP kinase, and p^{70s6k} pathways contributes to glycogen synthesis. Treatment of CHO−HIR cells with VS resulted in increased glycogen synthesis and PI3-k activity which were blocked by pretreatment of the cells with wortmannin and LY294002, two specific inhibitors of PI3-k. On the other hand, PD98059 and rapamycin, specific inhibitors of the MAP kinase pathway and p^{70s6k}, respectively, were unable to inhibit VS-stimulated glycogen synthesis. Moreover, VS-stimulated glycogen synthesis and PI3-k were observed without any change in the tyrosine phosphorylation of insulin receptor (IR) β-subunit but were associated with increased tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). In addition, PI3-k activation was detected in IRS-1 immunoprecipitates from VS-stimulated cells, indicating that tyrosine-phosphorylated IRS-1 was able to interact and thereby activate PI3-k in response to VS. Taken together, these results provide evidence that tyrosine phosphorylation of IRS-1 and activation of PI3-k play a key role in mediating the insulinomimetic effect of VS on glycogen synthesis independent of IR-tyrosine phosphorylation.

Inorganic salts of the trace element vanadium have been demonstrated to mimic most of the biological effects of insulin in several cell types, including stimulation of glucose transport (*1*−*3*), glycogen synthase (*4*), lipogenesis (*5*), and inhibition of lipolysis (*6*). They have also been shown to stimulate glycogen synthesis in the liver and diaphragm and inhibit gluconeogenesis in hepatocytes (*7*). In addition, oral administration of vanadium salts has been reported to improve glucose homeostasis in type I (insulin-dependent diabetes mellitus, IDDM)[1] (*8*, *9*) and type II (noninsulin-dependent diabetes mellitus, NIDDM) (*10*−*12*) animal models of diabetes mellitus as well as in limited human clinical trials (*13*, *14*, reviewed in refs *15*, and *16*).

Vanadium salts, potent inhibitors of protein tyrosine phosphatases (PTPases) (*17*), were initially thought to mimic insulin action by activating insulin receptor (IR) protein tyrosine kinase (PTK) activity by preventing dephosphorylation of the IR β-subunit (*18*). However, recent studies have suggested that the site of vanadium action may not involve IR−PTK (*19*−*23*). Thus, the molecular mechanism(s) of the insulinomimetic effect of vanadium salts remains poorly characterized and requires further elaboration.

Insulin elicits its biological effects by binding to the extracellular IR α-subunit which then stimulates the intrinsic

† Supported by a grant from the Medical Research Council of Canada.

* Corresponding author, Centre Hospitalier Université de Montréal. Tel: (514) 843-2917. Fax: (514) 843-2911. E-mail: srivasta@ere.umontreal.ca.

‡ Centre Hospitalier Université de Montréal and Department of Medicine.

§ Department of Physiology.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; CHO−HIR cells, chinese hamster ovary cells overexpressing human insulin receptor; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; ERK, extracellular signal regulated kinases; Grb, growth factor receptor binder; GSK-3, glycogen synthase kinase-3; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IDDM, insulin-dependent diabetes mellitus; IR, insulin receptor; IRS-1, insulin receptor substrate-1; JNK; c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; mSOS, mammalian son of sevenless; NIDDM, non-insulin-dependent diabetes mellitus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEPCK, phosphoenol pyruvate carboxykinase; PI3-k, phosphatidylinositol 3-kinase; PKB, protein kinase B.; PMSF, phenylmethanesulfonyl fluoride; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; rsk, ribosomal S-6 kinases; SDS, sodium dodecyl sulfate; SH2, src homology 2; VS, vanadyl sulfate.

S0006-2960(97)02678-0 CCC: $15.00
Published on Web 04/28/1998

PTK activity of the IR β-subunit (*24*−*27*). Activated IR−PTK phosphorylates insulin receptor substrate-1 (IRS-1), which appears to be the major substrate mediating insulin action (*28*). Tyrosine phosphorylated IRS-1 interacts with several src homology 2 (SH2) domains containing signaling proteins, such as adapter proteins growth factor receptor binder-2 (Grb-2) (*29*), Syp/SHP2 (*30*, *31*), Nck (*32*) as well as the 85 kDa subunit of phosphatidylinositol (PI) 3-kinase (PI3-k) (*33*, *34*) and initiates two main signaling pathways. In one pathway, Grb-2 binding to IRS-1 recruits a nucleotide exchange factor, mammalian son of sevenless (mSOS) which eventually activates p^{21ras}, a low molecular weight GTP-binding protein. p^{21ras} cycles between active GTP-bound conformation and inactive GDP-bound states. p^{21ras} activation leads to the sequential activation of several protein kinases such as Raf, a serine/threonine kinase, which phosphorylates and activates mitogen-activated protein (MAP) kinase kinase or MEK, which in turn phosphorylates and activates two isozymic forms of MAP kinases, p^{44mapk} (extracellular signal regulated kinase 1 or ERK 1) and p^{42mapk} (ERK 2) (*35*, *36*). Activated MAP kinase phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$ (*37*). Recently, using a selective inhibitor of MEK, PD98059, it was suggested that MAP kinase activation plays a role in DNA synthesis, cell growth, and transcriptional activation of c-fos (*38*, *39*). On the other hand, evidence has been presented to indicate that many other biological effects of insulin, such as glucose transport, glycogen synthesis, and lipid synthesis, as well as transcriptional regulation of several genes, such as phosphoenol pyruvate carboxykinase (PEPCK) and hexokinase II, may not require the insulin-induced ras/MAP kinase pathway (*39*−*41*). A 70 kDa ribosomal s6 kinase, p^{70s6k}, is also stimulated in response to insulin by a mechanism as yet uncharacterized (*42*).

The second pathway stimulated by insulin involves the activation of PI3-k, which is a heterodimer consisting of an 85 kDa (p85) regulatory subunit with two SH2 domains and a 110 kDa (p110) catalytic subunit (*27*). The p110 catalytic subunit has lipid kinase activity which phosphorylates PI on the D-3 position of the inositol ring (*43*, *44*). Recently, a serine/threonine kinase named protein kinase B (PKB) has been shown to be the target of PI3-k (*45*). By using two specific inhibitors of PI3-k, wortmannin and LY294002, it has been demonstrated that insulin-induced activation of PI3-k plays a key role in mediating several biological effects of insulin, such as glucose transport (*46*), glycogen synthesis (*47*), and transcriptional regulation of several genes (*40*, *41*).

Vanadium salts have been reported to mimic the cardinal response of insulin on glycogen synthesis in various in vitro systems (*7*, *18*) and to improve the depressed levels of glycogen in animal models of diabetes mellitus (*48*). However, the possible contribution of insulin-induced signaling pathways in vanadium salt-stimulated glycogen synthesis has not been investigated. We have shown earlier that various vanadium salts activate MAP kinase and p^{70s6k} signaling pathways in an IR−PTK-independent manner (*22*, *49*). In the present studies, we have investigated the contribution of the MAP kinase, p^{70s6k}, and PI3-k pathway in vanadyl-sulfate (VS)-stimulated glycogen synthesis in chinese hamster ovary cells overexpressing human insulin receptor (CHO−HIR cells). The data presented here demonstrate, for the first time, that VS-stimulated glycogen synthesis and PI3-k activity in CHO−HIR cells, and the inhibitors of PI3-k, wortmannin, and LY294002, blocked VS-stimulated glycogen synthesis. On the other hand, inhibition of either the MAP kinase or p^{70s6k} pathway failed to inhibit the glycogen synthesis induced by VS. We demonstrate further that VS-induced PI3-k activation occurred in the absence of any detectable tyrosine phosphorylation of IR β-subunit, but was accompanied by enhanced tyrosine phosphorylation of IRS-1.

MATERIALS AND METHODS

Materials. CHO−HIR cells and IRS-1 antibody were kind gifts from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was obtained from Eli Lilly Co. (Indianapolis, IN). Antibody against p85 regulatory subunit of PI3-k was a generous gift from Dr. Louise Larose (McGill University, Montreal) and was also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Radiolabeled D-[U-^{14}C]glucose was purchased from Dupont, NEN (Boston, MA). Insulin receptor antibody was a kind gift from Dr. Barry I. Posner (McGill University, Montreal) or purchased from Upstate Biotechnology (Lake Placid, NY). Wortmannin and staurosporine were obtained from Sigma Chemical Co. (St. Louis, MO). MEK inhibitor PD98059 was kindly provided by Dr. Alan Saltiel of Parke-Davis Pharmaceutical Research, (Ann Arbor, MI). Rapamycin was from Calbiochem (La Jolla, CA), LY294002 was from Biomol (Philadelphia, PA), and VS was from Aldrich Chemical Co. (Milwaukee, WI). Anti-phosphotyrosine and anti-MAP kinase antibodies as well as S6 peptide RRRLSSLRA were sourced from Upstate Biotechnology. Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase was from Bio-Rad (Mississauga, ON). Silica gel-60 plates were purchased from Merck & Co., Inc. (Rahway, NJ). Protein A sepharose beads were bought from Pharmacia Biotech Inc. (Mississauga, ON). p^{70s6k} antiserum (raised against a peptide corresponding to amino acids 2−30 of rat p^{70s6k}) was generously donated by Dr. Frederic Hall of the USC School of Medicine at Los Angeles.

Methods. Cell Culture. CHO−HIR cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 20 h prior to the experiment, as described earlier (*49*).

Glycogen Synthesis. Glycogen synthesis was measured by following the incorporation of D-[U-^{14}C]glucose into glycogen, essentially according to the method described by Lazar et al. (*39*). Briefly, near-confluent, serum-deprived CHO−HIR cells in 100 mm dishes were stimulated with VS or insulin with or without pretreatment with inhibitors as described in figure legends. The assay was initiated by adding D-[U-^{14}C]glucose (1 μCi/dish) and 5 mM glucose (final concentration). It was terminated after 2.5 h by washing three times with ice-cold phosphate-buffered saline (PBS) and then solubilized in 30% KOH. The solubilized cells were transferred to glass tubes and heated at 100 °C for 20 min in a dry bath, then 2.0 mg of carrier glycogen was added, and the mixture was boiled for another 30 min. Glycogen was precipitated by the addition of 2 vol of 95% ethanol, and the precipitate was dissolved in water. The radioactivity incorporated into glycogen was determined by counting the precipitate in a scintillation counter.

PI3-k Assay. The assay was performed essentially as described by Fukui and Hanafusa (*50*) with minor modifications. Serum-deprived, confluent CHO−HIR cells were stimulated with VS or insulin with or without pretreatment with inhibitors, as described in the figure legends. The cells were washed twice with cold PBS and lysed in buffer A [25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM sodium *o*-vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis(oxyethylenenitrolo)tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS)]. The cells were scraped and centrifuged at 12000*g* for 12 min. Equal amounts of protein lysates were incubated with 5 μL of p85 antibody for 2 h at 4 °C. Protein A sepharose beads were then added, and the resulting mixture was incubated for an additional 2 h at 4 °C. The beads were centrifuged in a microtube for 15 s, and the pellet was washed 3 times with HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% *G*lycerol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM PMSF), once each with PBS, 0.5 M LiCl, and 0.1 M Tris, pH 7.5, H_2O and, finally, with 0.1 M NaCl/1 mM EDTA/20 mM Tris-HCl, pH 7.5. The beads were then resuspended in 50 μL of kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 0.5 mM EGTA), followed by the addition of 1.0 μL of 20 mg/mL PI dissolved in dimethyl sulfoxide (DMSO). The mixture was vortexed, preincubated at room temperature for 10 min, and the kinase reaction initiated by adding [γ-^{32}P]adenosine 5′-triphosphate (ATP), (10 μCi/assay) and $MgCl_2$ (10 mM, final concentration) at room temperature (30 °C). The reaction was stopped after 3 min by the addition of 150 μL of $CHCl_3$:CH_3OH:HCl (100:200:2), and 100 μL of $CHCl_3$ was added to separate the phases. The organic (inferior) phase was taken and washed with 1 vol of CH_3OH:HCl (1:1). Lipid samples were concentrated in vacuo and spotted onto silica gel-60 plates which were later developed in $CHCl_3$:CH_3OH:NH_4OH:H_2O (43:38:5:7). The phosphorylated lipids were then visualized by autoradiography, and areas corresponding to each spot were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Insulin Receptor Tyrosine Phosphorylation. Serum-deprived, confluent CHO−HIR cells were stimulated with insulin (100 nM) or VS (100 μM) for 5 min, washed with ice-cold PBS and lysed on ice in 400 μL of buffer A. The lysates were clarified by centrifugation at 10,000g for 12 min, precleared with protein A sepharose for 30 min, and incubated with insulin receptor antibody for 4 h at 4 °C. Immunoprecipitates were collected with protein A sepharose, washed 3 times with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (Hepes) buffer, pH 7.5, containing 0.1% Triton X-100 and 0.1% SDS, and 2 times with the above buffer without SDS. The immunoprecipitates were solubilized by boiling in 2× sample buffer for 10 min, electrophoresed on 10% SDS−polyacrylamide gels (SDS−PAGE) and immunoblotted with anti-phosphotyrosine antibody (1:1000). The blots were developed by using goat anti-mouse IgG conjugated to alkaline phosphatase (1:3000).

Autophosphorylation of IR β-Subunit. CHO−HIR cells were stimulated as described above with either insulin or VS for 5 min, and lysates were prepared. Antibodies against phosphotyrosine or IR β-subunit were incubated with protein A sepharose for 2 h at 4 °C. After this, equal amounts of protein from cell lysates were added to the complexes of protein A sepharose and antibody, with incubation continuing for another 4 h at 4 °C. The beads were centrifuged in a microtube for 15 s, and the pellet was washed 3 times with HNTG and once with PBS. They were then resuspended in 50 μL of kinase buffer. The mixture was vortexed and preincubated at room temperature with $MgCl_2$ (10 mM) and $MnCl_2$ (5 mM) for 10 min. The reaction was started by adding [γ-^{32}P]ATP (5 μCi per assay), and incubation was continued for another 10 min at 30 °C. It was stopped by the addition of 50 μL of 2× sample buffer. The contents were boiled, centrifuged, and the solubilized fractions electrophoresed on 10% SDS−PAGE, followed by autoradiography.

IRS-1 Tyrosine Phosphorylation. CHO−HIR cells were stimulated with different concentrations of VS for 5 min. Equal amounts of proteins from cell lysates were added to the anti-IRS-1 antibody−protein A sepharose complex, and incubation was continued overnight at 4 °C. The immunoprecipitates were collected by centrifugation in a microtube for 15 s, and the pellet was washed 3 times with ice-cold PBS. The beads were boiled in 2× Laemmli's sample buffer and processed further as described above for IR tyrosine phosphorylation.

Assay of MAP Kinase and p^{70s6k} Activity. Clarified cell lysates prepared in buffer A were normalized to contain equal amounts of protein (100 μg) and incubated for 4 h at 4 °C with either 3 μL of MAP kinase antibody or p^{70s6k} antibody preadsorbed to protein A sepharose beads. The immunocomplexes were collected by centrifugation, followed by washing 3 times with buffer A and once with buffer B [20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 mM β-glycerophosphate]. For MAP kinase assay, the immunecomplexes were suspended in 40 μL of kinase buffer [25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 μM staurosporine, 0.5 mM EGTA, and 15 μg of myelin basic protein (MBP)], and the reaction was initiated by adding 5 μL of 40 μM ATP containing 0.5 μCi [γ-^{32}P]ATP. For p^{70s6k} assay, the immunecomplexes were suspended in 20 μL of buffer B containing the S6 peptide RRRLSSLRA (3 μg), and the reaction was initiated by adding 5 μL of 100 μM ATP containing 2 μCi [γ-^{32}P]ATP. After 15 min at 30 °C, the reaction was stopped by spotting the aliquots on P-81 filter paper, followed by washing in 0.5% phosphoric acid and counting for radioactivity (*49*).

Statistical Analysis. For some experiments, comparison of the data was performed by Student's paired *t*-test using SigmaStat 2.0 program (Jandel Scientific, San Rafael, CA). The *P*-values of <0.05 were considered as significantly different.

RESULTS

Effect of Wortmannin on VS-Stimulated Glycogen Synthesis. Incubation of CHO−HIR cells with increasing concentrations of the insulinomimetic agent VS stimulated the rate of glycogen synthesis in a concentration-dependent manner, as determined by [^{14}C]glucose incorporation into glycogen (Figure 1A). Stimulation of glycogen synthesis could be detected with VS doses as low as 1 μM (1.7-fold

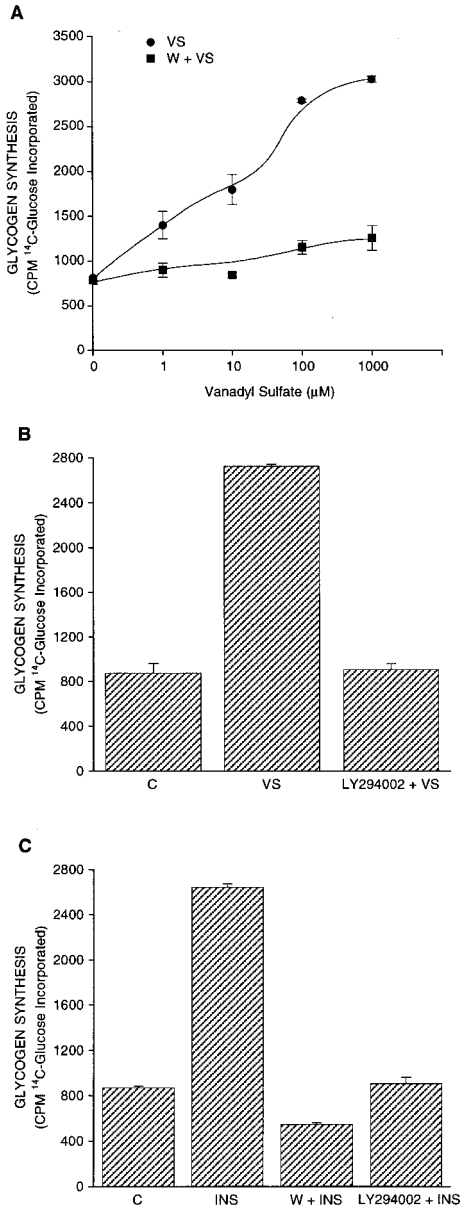

FIGURE 1: Effect of wortmannin and LY294002 on VS-stimulated glycogen synthesis. (A) Confluent, serum starved CHO−HIR cells were treated with wortmannin (100 nM, filled squares) or vehicle (0.01% DMSO used to dissolve wortmannin, filled circles) for 10 min prior to stimulation with different concentrations of VS for 5 min. After this period, 5 mM glucose and D-[U-^{14}C]glucose (1 μCi/sample) was added to the dishes and incubation continued for an additional 2.5 h. The reaction was terminated by washing with ice-cold PBS, and the cells were solubilized in 30% KOH. Glucose incorporation into glycogen was determined by following the incorporation of [^{14}C]glucose into glycogen as described in the Materials and Methods. Values from three separate experiments, each performed in triplicate, are shown as mean ± SE. (B) Confluent serum-starved CHO−HIR cells were incubated without (control, C) or with VS (100 μM for 5 min) or pretreated with LY294002 (10 μM, 30 min) followed by stimulation with VS (LY294002 + VS). Glycogen synthesis was determined as described in Figure 1A. Values are from three experiments, each performed in triplicate, are shown as means ± SE. (C) Confluent serum-starved CHO−HIR cells were incubated without (control, C) or with insulin (INS, 100 nM for 5 min) or pretreated with wortmannin (100 nM, 10 min) or LY294002 (10 μM, 30 min), followed by stimulation with INS (W + INS) or (LY294002 + INS), respectively. Glycogen synthesis was determined as described under Figure 1A. Values are from three separate experiments, each performed in triplicate, are shown as means ± SE.

stimulation) and was maximal at 100 μM, where about 3-fold stimulation was observed (Figure 1A).

Since wortmannin, a highly selective inhibitor of PI3-k, has been shown to block insulin-stimulated glycogen synthesis (*47*, *51*), we were interested to determine if PI3-k was also implicated in VS-stimulated glycogen synthesis. Figure 1A illustrates that treatment of CHO−HIR cells with 100 nM wortmannin for 10 min prior to stimulation with different concentrations of VS markedly blocked the stimulatory effect of VS on glycogen synthesis. Under similar conditions, insulin treatment (100 nM for 5 min), as a positive control, also stimulated the glycogen synthesis by about 3-fold over control values which was almost completely blocked by wortmannin pretreatment (Figure 1C).

Effect of LY294002 on VS-Stimulated Glycogen Synthesis. Since wortmannin has also been shown to inhibit other enzymes, such as phospholipase A_2 (*52*), we utilized another inhibitor of PI3-k, LY294002, to further confirm a role of PI3-k in VS-induced glycogen synthesis. This inhibitor is structurally different from wortmannin and acts on the ATP-binding site of the enzyme (*53*). As shown in Figure 1, panels B and C, pretreatment of CHO−HIR cells with LY294002 (10 μM) for 30 min markedly inhibited VS- and insulin-induced activation of glycogen synthesis, further supporting a role of PI3-k in VS- as well as insulin-stimulated glycogen synthesis.

Effect of VS on PI3-k Activity. To investigate if VS-induced activation of glycogen synthesis was correlated with enhancement of PI3-k activity in CHO−HIR cells, PI3-k activity in lysates of cells treated with different concentrations of VS was assayed in immunecomplexes obtained by using an antibody to the p85 subunit of PI3-k. As shown in Figure 2, panels A and B, treatment of CHO−HIR cells with VS for 5 min stimulated PI3-k activity in a concentration-dependent manner. At 100 μM, VS stimulated PI3-k activity by about 3-fold as compared to the controls, and this was markedly inhibited by treatment of cells with wortmannin (100 nM for 10 min) prior to stimulation with VS (Figure 2, panels C and D). Similar results were obtained while using insulin as a positive control (Figure 2, panels C and D).

Effect of PD98059 on VS-Stimulated MAP Kinase Activity and Glycogen Synthesis. Recently, PD98059, which blocks insulin-induced activation of the MAP kinases ERK 1 and ERK 2, has been used to study the role of the MAP kinase pathway in mediating various biological effects of insulin (*39*). We, therefore, investigated whether PD98059 modulates VS-induced MAP kinase activation and glycogen synthesis in CHO−HIR cells. As shown in Table 1, both VS- and insulin-stimulated MAP kinase activity by 4−5-fold, which was markedly inhibited by pretreatment of cells with PD98059 (10 μM for 30 min). However, PD98059 treatment failed to exert an inhibitory effect on insulin- or VS-stimulated glycogen synthesis (Table 1). These data demonstrate that, similarly to insulin, VS-induced stimulation of glycogen synthesis is independent of MAP kinase activation.

Effect of Rapamycin on VS-Induced Activation of P^{70s6k} and Glycogen Synthesis Rapamycin, an immunosuppressant drug which potently inhibits p^{70s6k} activation induced by insulin and other growth factors has been used as a tool to investigate the role of p^{70s6k} in mediating various physiological effects of insulin (*54*−*56*). Studies in which rapamycin

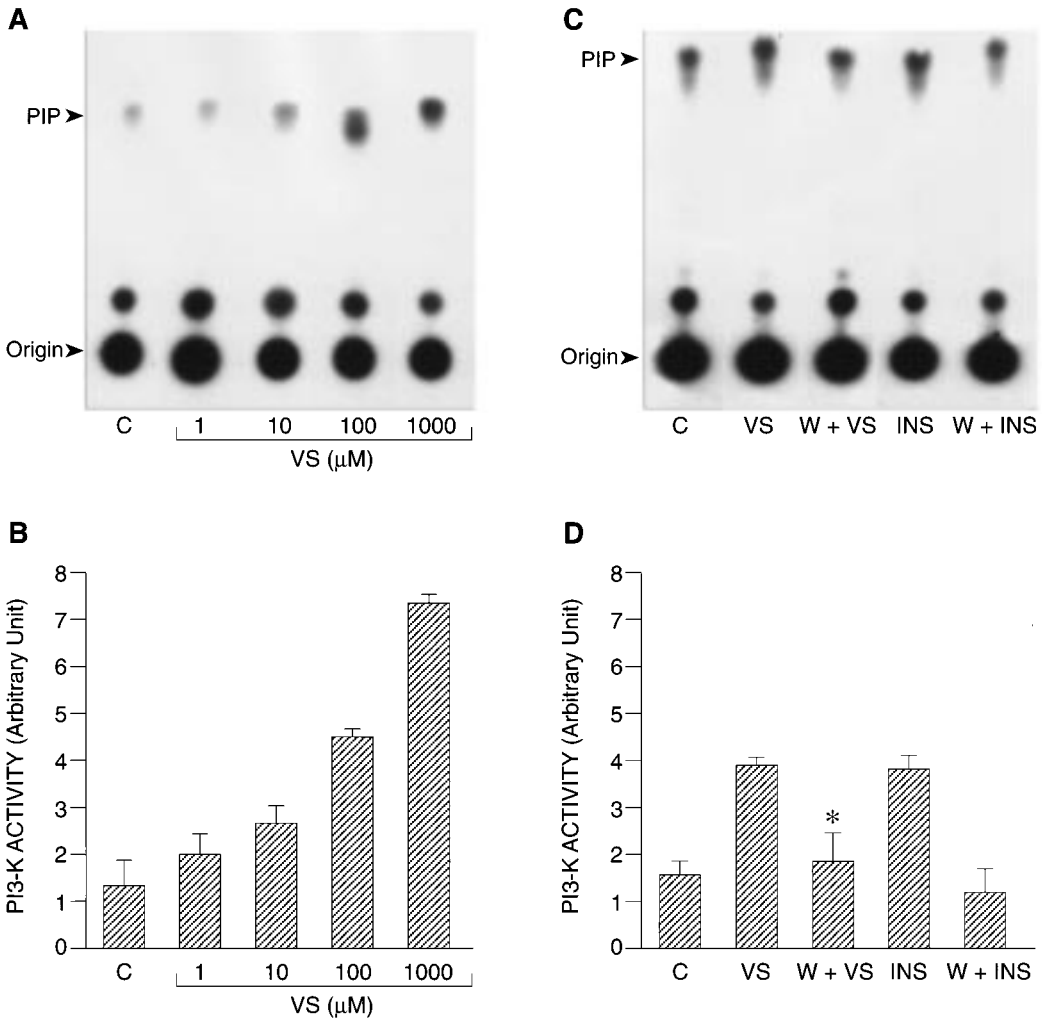

FIGURE 2: Effect of wortmannin on VS-stimulated PI3-k activity. (A) Confluent, serum-starved CHO−HIR cells were incubated without (control, C) or with increasing concentrations of VS for 5 min. Cell lysates were prepared and proteins immunoprecipitated with anti-p85 antibody. Immunecomplexes were assayed for PI3-k activity using PI as substrate. Phospholipids were resolved by thin-layer chromatography and then exposed to X-ray film. An autoradiogram of a representative experiment is shown. Arrowheads indicate the origin and position of phosphorylated substrate PIP. (B) PIP, the phosphorylated product, formed was quantified by PhosphorImager and values from two experiments are shown as means ± SE. (C) Confluent, serum-starved CHO−HIR cells were pretreated with wortmannin (100 nM) or vehicle for 10 min followed by stimulation in the absence, C, or presence of VS (100 μM) or insulin (INS, 100 nM) for 5 min. Lysates were prepared, and PI3-k assay was performed as described above. (D) The phosphorylated product (PIP) was quantified by PhosphorImager. Values from four experiments are shown as means ± SE. *P = 0.01 versus VS-stimulated activity.

was employed to examine its effect on insulin-stimulated glycogen synthesis or glycogen synthase activation have produced conflicting results. It has been shown that rapamycin, despite blocking insulin-induced activation of p^{70s6k}, fails to block glycogen synthesis in rat adipocytes (*57*, *58*), mouse skeletal muscle (*59*), CHO (*60*), and PC12 cells (*51*). On the other hand, rapamycin treatment has been reported to inhibit insulin-stimulated activation of glycogen synthase and glycogen synthesis in 3T3-L1 adipocytes (*47*) and rat diaphgram muscle (*61*). Therefore, it was of interest to investigate if rapamycin could also block VS-stimulated glycogen synthesis in CHO−HIR cells. The results in Table 1 indicate that pretreatment of cells with 50 ng/mL rapamycin for 30 min blocked VS- and insulin-stimulated p^{70s6k} activity without affecting glycogen synthesis. These data suggest that, like insulin (*60*), p^{70s6k} activation is not required for VS-induced glycogen synthesis in CHO−HIR cells.

Effect of VS on IR Tyrosine Phosphorylation and Autophosphorylation. Insulin-induced activation of the PTK activity of IR β-subunit is essential for mediating several biological responses of insulin including glycogen synthesis (*62*). We therefore, investigated if conditions which stimulate glycogen synthesis in response to VS also stimulate tyrosine phosphorylation of the IR β-subunit. CHO−HIR cells were incubated with insulin (100 nM) as well as VS (100 μM) for 5 min. Cell lysates were prepared and subjected to immunoprecipitation by an antibody directed against IR β-subunit followed by immunoblotting with anti-phosphotyrosine antibody. As shown in Figure 3A, VS treatment did not cause any detectable increase in tyrosine phosphorylation of the IR β-subunit, whereas, as expected, insulin markedly stimulated tyrosine phosphorylation of the IR β-subunit as compared to unstimulated cells. Since IR β-subunit tyrosine phosphorylation is associated with stimulation of its autophosphorylation, this observation was further confirmed by performing in vitro autophosphorylation reaction in anti-phosphotyrosine and anti-IR immunoprecipitates from control, VS-, and insulin-stimulated cells. As shown in Figure 3, panels B and C, VS did not cause any increase in autophosphorylation, whereas, as expected, heightened

Table 1: Effect of PD98059 and Rapamycin on VS- and Insulin-Stimulated MAP Kinase and p^{70S6k} Activities as well as Glycogen Synthesis[a]

treatment	kinase activity (pmol/min/mg) MAP kinase	p^{70s6k}	glycogen synthesis (cpm ± SE)
control	9.6 ± 3.2	2.2 ± 0.5	871 ± 25
vanadyl sulfate	52 ± 7.4	8.87 ± 0.4	2738 ± 54
insulin	50 ± 5.8	8.93 ± 1.0	2646 ± 29
VS + PD98059	19.3 ± 4		2793 ± 73
Ins + PD98059	10.6 ± 5		2522 ± 51
VS + rapamycin		3.2 ± 0.3	2897 ± 88
Ins + rapamycin		2.71 ± 0.4	2650 ± 72

[a] CHO−HIR cells were incubated with or without PD98059 (10 μM) or rapamycin (50 ng/mL) for 30 min, followed by stimulation in the absence (control) or presence of vanadyl sulfate (VS, 100 μM) or insulin (Ins, 100 nM) for 5 min. MAP kinase or P^{70s6k} activity in lysates was determined as described in the Materials and Methods section by using MBP or a synthetic peptide RRRLSSLRA as exogenous substrate, respectively. Glycogen synthesis was determined by following the incorporation of [^{14}C]glucose into glycogen as described in Figure 1. Values represent the means ± SE from three experiments performed in triplicate.

autophosphorylation of the 95 kDa IR β-subunit was observed in insulin-treated cells. This suggests that, in contrast to insulin, VS-stimulated glycogen synthesis is not associated with activation of IR−PTK.

Effect of VS on Tyrosine Phosphorylation of IRS-1 and PI3-k Activity Associated with IRS-1. PI3-k activation by insulin requires increased tyrosine phosphorylation of IRS-1. We, therefore, examined whether PI3-k activation by VS was correlated with enhanced tyrosine phosphorylation of IRS-1. CHO−HIR cells were incubated with various concentrations of VS, and cell lysates were subjected to immunoprecipitation with an antibody against IRS-1. Immunoprecipitates were then analyzed by immunoblotting with anti-phosphotyrosine antibody. As shown in Figure 4A, VS treatment of cells stimulated the tyrosine phosphorylation of IRS-1 in a concentration-dependent manner.

To determine if VS-stimulated tyrosine phosphorylation of IRS-1 results in its association with and activation of PI3-k, we studied the PI3-k activity in IRS-1 immunoprecipitates prepared from CHO−HIR cells stimulated with VS. As shown in Figure 4, panels B and C, about a 6-fold rise in PI3-k activity was detected in IRS-1 immunoprecipitates after VS stimulation (100 μM, 5 min) of cells compared to unstimulated control cells. This increase correlated well with an enhanced tyrosine phosphorylation of IRS-1 in response to VS (Figure 4A).

DISCUSSION

In the present study, we have demonstrated that VS, which exerts a variety of insulin-like effects, including the ability to serve as an orally active antidiabetic agent, stimulates PI3-k activity as well as glycogen synthesis in CHO−HIR cells. Our results also show that two structurally and mechanistically distinct inhibitors of PI3-k, wortmannin, and LY294002 attenuate VS-induced PI3-k activation as well as glycogen synthesis. Similar observations have been made in relation to insulin-induced activation of glycogen synthesis or glycogen synthase in several systems, such as 3T3-L1 adipocytes (*47*), PC-12 cells (*51*), and rat adipocytes (*63*).

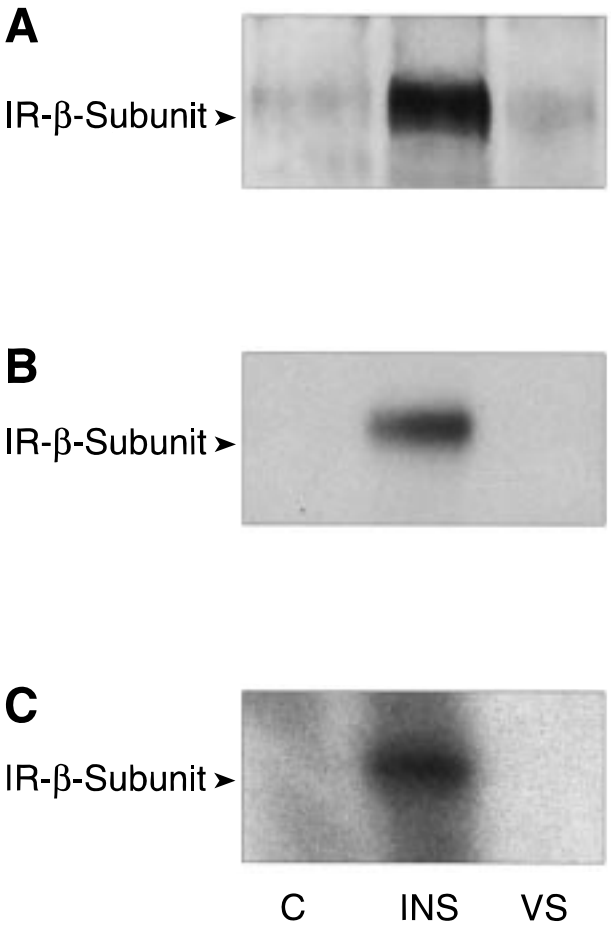

FIGURE 3: Effect of VS on insulin receptor β-subunit tyrosine phosphorylation and autophosphorylation. (A) Confluent, serum-starved CHO−HIR cells were either unstimulated, C, or stimulated with 100 nM insulin (INS) or 100 μM VS for 5 min. Cells were lysed and lysates subjected to immunoprecipitation with anti-IR β-subunit antibody, followed by immunoblotting with anti-phosphotyrosine antibody. This immunoblot is the representative of two other experiments with similar results. (B, and C). Confluent serum-starved, CHO−HIR cells were either unstimulated, C, or stimulated with 100 nM insulin (INS) or 100 μM VS for 5 min. Cell lysates were prepared and then immunoprecipitated with either anti-phosphotyrosine antibody (B) or anti-IR β-subunit antibody (C). The respective immunoprecipitates were subjected to autophosphorylation reaction followed by autoradiography as described in Methods. The autoradiograms shown are representative of two other experiments with similar results.

However, the data presented here are the first to show that VS-stimulated activation of glycogen synthesis as well as PI3-k activation are blocked in concert by wortmannin and LY294002 (Figures 1 and 2) and suggest that VS and insulin employ wortmannin/LY294002-inhibitable signaling intermediates to stimulate glycogen synthesis.

A serine/threonine protein kinase, cAKt/PKB, is activated in response to several growth factors including insulin (*45*, *64*−*65*) and vanadate (*66*). The enzyme is stimulated in vitro by PI3-k-generated phospholipid products (*64*) and has been implicated in the regulation of glycogen synthesis (*65*). This contention is based on the fact that glycogen synthase kinase-3 (GSK-3), a potent protein kinase capable of phosphorylating and decreasing the activity of glycogen synthase, is a substrate of cAKt/PKB (*65*). Interestingly, cAKt/PKB-catalyzed phosphorylation and inactivation of GSK-3 was also inhibited by wortmannin and LY294002

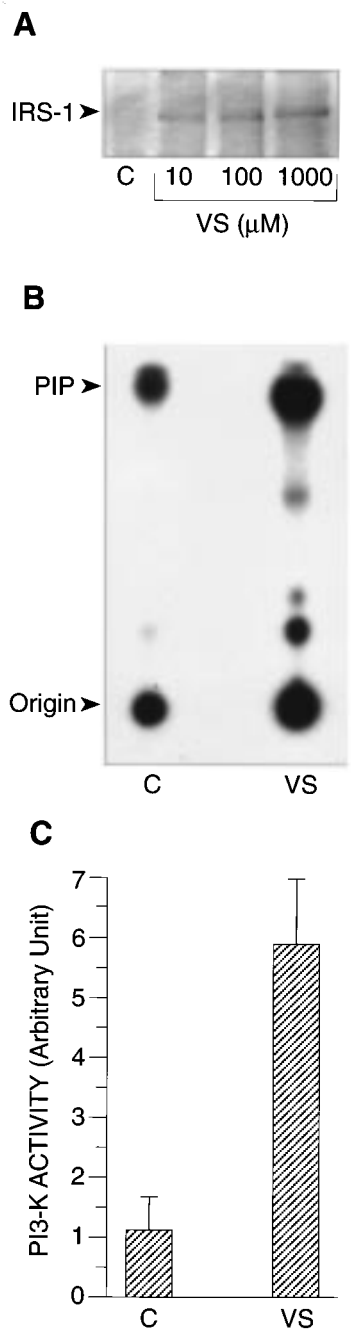

FIGURE 4: Effect of VS on insulin receptor substrate (IRS-1) tyrosine phosphorylation and IRS-1 associated PI3-k activity. (A) Confluent, serum-starved CHO−HIR cells were stimulated with the indicated concentrations of VS. Lysates were prepared and subjected to immunoprecipitation with antibody to IRS-1. Immunoprecipitates were separated on 10% SDS−PAGE followed by immunoblotting with anti-phosphotyrosine antibody as described in the Materials and Methods. The data are representative of three independent experiments with similar results. (B) Confluent, serum starved CHO−HIR cells were either unstimulated, C, or stimulated with VS (100 μM) for 5 min. They were lysed and immunoprecipitated with anti-IRS-1 antibody. PI3-k activity was measured in IRS-1 immunecomplexes as described in Figure 2. This autoradiogram is representative of two experiments with similar results. (C) The phosphorylated product (PIP) was quantified by PhosphorImager. Values from two separate experiments are represented as means ± SE.

(*65*). Recent studies have shown that overexpression of constitutively active cAKt/PKB did not reproduce the ability of insulin to stimulate glycogen synthesis in 3T3-L1 adipocytes (*67*). It is not known, however, if cAKt/PKB overexpression was associated with decreased GSK-3 activity in the above studies. Moreover, insulin-induced glycogen synthesis was significantly attenuated in the cells overexpressing the constitutively active form of cAKt/PKB (*67*).

Studies using cells overexpressing a dominant negative mutant of the 85 kDa subunit of PI3-k have shown that, despite markedly attenuated insulin-dependent activation of PI3-k, glycogen synthase activation was normal (*60*). This, together with the observation that PI3-k activation in response to other growth factors was not associated with glycogen synthase activation, suggests that PI3-k may not be involved in the process (*68*). Therefore, it is possible that wortmannin and LY294002 exert their inhibitory effect on glycogen synthase activation by suppressing some other form of PI3-k and/or an uncharacterized enzyme involved in glycogen synthase activation. Thus, the contribution of PI3-k or cAKt/PKB in regulating glycogen synthesis appears to be complex and needs further investigation.

Our observation that VS-stimulated glycogen synthesis is not blocked by PD98059, a specific inhibitor of MEK1 and MEK2, the immediate upstream kinase in the ERK1 and ERK 2 signaling cascade (Table 1), demonstrated that ERK activation does not contribute to VS-stimulated glycogen synthesis. Similar conclusions were drawn by Lazar et al. after studying the mechanism of insulin-induced glycogen synthesis in 3T3-L1 adipocytes and L6 myotubes (*39*). In addition, experiments using dominant negative ras mutants have revealed that activation of the ras/ERK signaling cascade is not required for insulin-activated glycogen synthesis in PC-12 cells (*51*) and 3T3-L1 adipocytes (*69*).

The lack of an effect of rapamycin on VS-stimulated glycogen synthesis (Table 1), which suggests that p^{70s6k} may not have a role in this process is consistent with findings in PC12 cells (*51*), skeletal muscle (*59*), rat adipocytes (*57*, *58*), and CHO−HIR cells (*60*) in response to insulin. In some studies, however, rapamycin treatment was shown to inhibit insulin-induced glycogen synthesis (*47*, *61*). Thus, the existence of cell-specific pathways for activation of glycogen synthesis may be postulated. Evidence has been presented recently showing that c-jun NH_2-terminal kinase (JNK) and ribosomal kinase III (rsk III) may contribute to insulin-stimulated activation of glycogen synthesis in skeletal muscle in vivo (*59*).

Whereas insulin-induced glycogen synthesis requires functionally active IR−PTK (*62*), VS-induced glycogen synthesis occurs in the absence of a detectable IR tyrosine phosphorylation. In fact, using three different experimental approaches, we have failed to find any significant increase in tyrosine phosphorylation and PTK activation of IR in VS-stimulated CHO−HIR cells. Similar observations have been made in the rat diaphragm where vanadate-stimulated glycogen synthesis was not associated with enhanced IR tyrosine phosphorylation (*19*). No effect of vanadate on IR tyrosine phosphorylation in rat adipocytes has also been demonstrated (*70*). Interestingly, despite having no effect on tyrosine phosphorylation of the IR β-subunit, VS stimulated the tyrosine phosphorylation of IRS-1. Moreover, the fact that PI3-k activation was observed in IRS-1 immunoprecipitates from VS-treated cells suggests that recruitment of PI3-k by binding to tyrosine phosphorylated IRS-1 may be one of the mechanisms by which VS activates PI3-k. The

precise mechanism by which VS stimulates the tyrosine phosphorylation of IRS-1 is not clearly understood at present. Since vanadium salts are potent inhibitors of PTPases (*17*), it is possible that by suppressing a constitutively active IRS-1 specific-PTPase, VS causes an increase in the phosphotyrosine content of IRS-1. Alternatively, VS may stimulate a nonreceptor PTK which, in turn, may phosphorylate IRS-1. It is noteworthy that some cytokines and other peptide hormones have been shown to stimulate the tyrosine phosphorylation of IRS-1 by activating cytosolic PTKs (*71*, *72*). Clearly, additional studies are required to test these possibilities and to identify putative PTKs or PTPases responsible for IRS-1 phosphorylation in response to VS.

In summary, our results demonstrate, for the first time, that VS induces the tyrosine phosphorylation of IRS-1 and activates PI3-k in an IR−PTK-independent manner. Thus, it may be suggested that activation of PI3-k and wortmannin/LY294002-inhibitable signaling pathway constitute one of the mechanisms by which inorganic vanadium salts exert insulin-like effects on glycogen synthesis.

ACKNOWLEDGMENT

We are grateful to FCAR (Fonds pour la formation des chercheurs et l'aide à la recherche) Québec, Canada, for awarding a studentship to S.K.P. We thank Dr. M. F. White of the Joslin Diabetes Center, Boston, for providing CHO cells and antibody to IRS-1, Drs. Barry I. Posner and Louise Larose of McGill University, Montreal, for the gift of insulin receptor and p85 antibodies respectively, Dr. Frederic Hall, USC School of Medicine at Los Angeles, for the antibody to p^{70s6k}, and Dr. Alan Saltiel, Parke-Davis, Warner Lambert Pharmaceutical Research Division, Ann Arbor, for the MEK inhibitor PD98059. Finally we also thank Mr. Ovid M. DaSilva for editing the manuscript and Mrs. Susanne Bordeleau-Chénier for her excellent secretarial help.

REFERENCES

1. Shechter, Y., and Karlish, S. J. D. (1980) *Nature 284*, 556−558.
2. Okumura, N., and Shimazu, T. (1992) *Biochem. J. 112*, 107−111.
3. Meyerovitch, J., Shechter, Y., and Amir, S. (1989) *Physiol. Behav. 45*, 1113−1116.
4. Tamura, S., Brown, T. A., Dubler, R. E., and Larner, J.(1983) *Biochem. Biophys. Res. Commun. 113*, 80−86.
5. Shechter, Y., and Ron, A. (1986) *J. Biol. Chem. 261*, 14951−14954.
6. Degani, H., Gochin, M., Karlish, S. J. D., and Shechter, Y. (1981) *Biochemistry 20*, 5795−5799.
7. Tolman, E. L., Barris, E., Burns Pansini, A., and Partridge, R. (1979) *Life Sci. 25*, 1159−1164.
8. Heyliger, C. E., Tahiliani, A. G., and McNeill, J. H. (1985) *Science 277*, 1474−1477.
9. Meyerovitch, J., Farfel, Z., Sack, J., and Shechter, Y. (1987) *J. Biol. Chem. 262*, 6658−6662.
10. Brichard, S. M., Pottier, A. M., and Henquin, J. C. (1989) *Endocrinology 125*, 2510−2516.
11. Pugazhenthi, S., Angel, J. F., and Khandelwal, R. L. (1991) *Metabolism 40*, 941−946.
12. Meyerovitch, J., Rothenberg, P., Bonner-Weir, S., and Kahn, C. R. (1991) *J. Biol. Chem. 87*, 1286−1294.
13. Goldfine, A. B., Simonson, D. C., Folli, F., Patti, M.-E., and Kahn, C. R. (1995) *Mol. Cell. Biochem. 153*, 217−231.
14. Cohen, N., Halberstam, M., Shlimovich, P., Chang, C. J., Shamoon, H., and Rossetti, L. (1995) *J. Clin. Invest. 95*, 2501−2509.
15. Srivastava, A. K. (1995) *Exp. Opin. Invest. Drugs 4*, 525−536.
16. Sekar, N., Li, J., and Shechter, Y. (1996) *Crit. Rev. Biochem. Mol. Biol. 31*, 339−359.
17. Swarup, G., Cohen, S., and Garbers, D. L. (1982) *Biochem. Biophys. Res. Commun. 107*, 1104−1109.
18. Tamura, S., Brown, T. A., Whippel, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K., and Larner, J. (1984) *J. Biol. Chem. 259*, 6650−6658.
19. Strout, H. V., Vicario, P. P., Saperstein, R., and Slater, E. E. (1989) *Endocrinology 124*, 1918−1924.
20. Shisheva, A., and Shechter, Y. (1992) *Biochemistry 31*, 8059−8063.
21. Mooney, R. A., Bordwell, K. L., Luhowsky, S., and Casnelli, J. E. (1989) *Endocrinology 124*, 422−426.
22. D'Onofrio, F., Le, M. Q. U., Chiasson J.-L., and Srivastava, A. K. (1994) *FEBS Lett. 340*, 269−275.
23. Blondel, A., Simon, J., Chevalier, B., and Portha, B. (1990) Am. J. Physiol. *258*, E459−E467.
24. Rosen, O. (1987) *Science 237*, 1452−1458.
25. Olefsky, J. M. (1990) *Diabetes 39*, 1009−1116.
26. Myers, M. G., and White, M. F. (1993) *Diabetes. 42*, 643−650.
27. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem. 269*, 1−4.
28. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahil, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature 352*, 73−77.
29. Skolnik, E. Y., Lee, C.-H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Mayers. M. G, Jr., Backer, J. M., Ullrich, A., White, M. F., and Schlessinger, J (1993) *EMBO J. 12*, 1929−1936.
30. Kuhne, M. R., Pawson, T., Lienhard, G. E., Feng, S. E. (1993) *J. Biol. Chem. 268*, 11479−11481.
31. Adachi, M., Fischer, E. H., Ihle, J., Imai, K., Jirik, F., Neel, B., Pawson, T., Shen, S.-H., Thomas, M., Ullrich, A., and Zhao, Z. (1996) *Cell 85*, 15.
32. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G. Jr., White, M. F., Schlessinger, J., Skolinik, E. Y. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 11713−11717.
33. Lavan, B. E., Kuhne, M. R., Garner, C. W., Anderson, D., Reedijik, M., Pawson, T., and Lienhard, G. E. (1992) *J. Biol. Chem. 267*, 11631−11636.
34. Backer, J. M., Mayers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix,. M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) *EMBO J. 9*, 3469−3479.
35. Crew, C. M., and Erikson, R. L. (1993) *Cell 74*, 215−217.
36. Eagan, S. E., and Weinberg, R. A (1993) *Nature 365*, 781−782.
37. Ahn, N. G., and Krebs, E. (1990) *J. Biol. Chem. 265*, 11495−11501.
38. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7686−7689.
39. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P., and Saltiel, A. R. (1995) *J. Biol. Chem. 270*, 20801−20807.
40. Gabbay, R. A., Sutherland, C., Gnudi, L., Kahn, B. B., O'Brien, R. M., Granner, D. K. and Flier, J. S. (1996) *J. Biol. Chem. 271*, 1890−1897.
41. Osawa, H., Sutherland, C., Robey, B. R., Printz, R. L., and Granner, D. K. (1996) *J. Biol. Chem. 271*, 16690−16694.
42. Hara, K., Yonezawa, K., Sakaue, H., Kotani, K., Kozima, A., Waterfield, M. D., and Kasuga, M. (1995) *Biochem. Biophys. Res. Commun. 208*, 735−741.
43. Carpenter, C. L., Duckworth, B. C., Auger, K, R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. (1990) *J. Biol. Chem. 265*, 19704−91711.
44. Fry, M. J. (1994) *Biochim. Biophys. Acta 1226*, 237−268.

45. Burgering, B. M.T and Coffer, P. J. (1995) *Nature 376*, 599−602.
46. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J, and Kahn, C. R. (1994) *Mol. Cell. Biol. 14*, 4902−4911.
47. Shepherd, P. R., Nave, B. T., and Siddle, K. (1995) *Biochem. J. 305*, 25−28.
48. Pugazhenthi, S., and Khandelwal, R. L. (1990) *Diabetes 39*, 821−827.
49. Pandey, S. K., Chiasson, J.-L., and Srivastava, A. K. (1995) *Mol. Cell. Biochem. 153*, 69−78.
50. Fukui, Y., and Hanafusa, H. (1989) *Mol. Cell. Biol. 9*, 1651−1658.
51. Yamamoto-Honda, R., Kazuyuki, T., Kaburagi, Y., Ueki, K., Asai, S., Yachi, M., Shirouza, M., Yodoi, J., Akanuma, Y., Yokohama, S., Yazaki, Y., and Kadowaki, T. (1995) *J. Biol. Chem. 270*, 2729−2734.
52. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995) *J. Biol. Chem. 270*, 25352−25355.
53. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) *J. Biol. Chem. 269*, 5241−5248.
54. Chung, J., Crabtree, G. R., and Blenis, J. (1992) *Cell 69*, 1227−1236.
55. Price, D. J., Grove, J. R., Calvo, V., Avruch, J., and Bierer, B. E. (1992) *Science 257*, 973−977.
56. Calvo, V., Crews, C. M., Vik, T. A., and Bierer, B. E. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7571−7575.
57. Lin, T.-A.; Lawerence, J. C., Jr. (1994) *J. Biol. Chem. 269*, 21255−21261.
58. Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G., and Denton, R. M. (1995) *Biochem. J. 311*, 595−601.
59. Moxham, C. M., Tabrichi, A., Davis, R. J., and Malbon, C. C. (1996) *J. Biol. Chem. 271*, 30765−30773.
60. Sakaue, H., Hara, K., Noguchi, T., Matozaki, T., Kotani, K., Ogawa, W., Yonezawa, K., Waterfield, M. D., and Kasuga, M. (1995) *J. Biol. Chem. 270*, 11304−11309.
61. Azpiazu, I., Saltiel, A,. Depaoli-Roach, A. A., and Lawrence, J. C., Jr. (1996) *J. Biol. Chem. 271*, 5033−5039.
62. McClain, D. A., Malgawa, H., Lee, J., Dull, T. J., Ullrich, A., and Olefsky, J. M. (1987) *J. Biol. Chem. 262*, 14663−14675.
63. Standaert, M. L., Bandyopadhyay, G., and Farese, R. V. (1995) *Biochem. Biophys. Res. Commun. 209*, 1082−1088.
64. Franke, T. F., Yang, S. I., Chan, T. O., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell 81*, 727−736.
65. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovitch, M., and Hemmings, B. A. (1995) *Nature 378*, 785−789.
66. Wijkander, J., Holst, L. S., Rahn, T., Resjo, S., Castan, I., Manganiello, V., Belfarge, P., and Degerman, E. (1997) *J. Biol. Chem.* 272, 21520−21526.
67. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem. 271*, 1372−31378.
68. Weise, R. J., Mastick, C. C., Lazar, D. F., and Saltiel A. (1995) *J. Biol. Chem. 270*, 3442−3446.
69. Dorrestijn, J., Ouwens, D. M., Berghe, N. V., Bos, J. L., and Massen, J. A. (1996) *Diabetologia 39*, 558−563.
70. Fantus, I. G., Kadota, S., Deragon, G., Foster, B., and Posner, B. I. (1989) *Biochemistry 28*, 8864−8871.
71. Ridderstrale, M., Degerman, E., and Tornqvist, H. (1995) *J. Biol. Chem. 270*, 3471−3474.
72. Chauvel-Kowalski, A., Pradayrol, L., Vaysse, N., and Seva, C. (1996) *J. Biol. Chem. 271*, 26356−26361.

BI9726786