Phosphatidylinositol 3-Kinase Requirement in Activation of the ras/C-raf-1/MEK/ERK and p70^{s6k} Signaling Cascade by the Insulinomimetic Agent Vanadyl Sulfate†

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ABSTRACT: The mechanisms by which inorganic salts of the trace element vanadium mediate their insulinomimetic effects are not clearly understood and were investigated. We have shown previously that vanadium salts activate mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase activities (PI3-K) via a pathway that does not involve the insulin receptor (IR) tyrosine kinase function [Pandey, S. K., Anand-Srivastava, M. B., and Srivastava, A. K. (1998) *Biochemistry 37*, 7006−7014]. Herein, we have examined a possible role of PI3-K in the vanadyl sulfate (VS)-mediated increase in the level of ras-MAPK activation as well as the contribution of signaling components upstream to MAPK in this VS response. Treatment of IR-overexpressing cells with VS resulted in an increased level of tyrosine phosphorylation of p44^{mapk} (ERK-1) and p42^{mapk} (ERK-2) along with stimulation of MAPK, MAPK kinase (MEK), and C-raf-1 activities, and ras activation. Preincubation with wortmannin and LY294002, two structurally and mechanistically different inhibitors of PI3-K, blocked the VS-mediated increase in MAPK activity and phosphorylation of ERK-1 and ERK-2. Furthermore, wortmannin inhibited activation of ras, C-raf-1, and MEK in response to VS. The addition of a farnesyltransferase inhibitor, B581, to cells reduced the level of MAPK activation as well as ERK-1 and ERK-2 phosphorylation stimulated by VS. Finally, VS increased PI3-K activity in ras immunoprecipitates. A VS-mediated increase in p70^{s6k} activity was also found to be inhibited by wortmannin. Taken together, these results demonstrate that the insulinomimetic effects of VS may be mediated, in part, by PI3-K-dependent stimulation of the ras-MAPK and p70^{s6k} pathways.

The biological effects of insulin are initiated by binding to cell surface insulin receptor (IR)[1] α-subunits, which results in increased intrinsic tyrosine kinase activity of the receptor β-subunit (*1*, *2*). The activated receptor kinase phosphorylates its major substrate, insulin receptor substrate 1 (IRS-1) (*3*), which then serves as a binding site for Src homology 2 (SH2) domain-containing signaling proteins. These include the Grb2−SOS complex (*4*), Syp (SHP2), a protein tyrosine phosphatase (PTPase), and Nck (a linker protein whose function is not yet clear) (*5*) as well as the 85 kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) (*6*, *7*). The binding of the Grb2−SOS complex to tyrosine-phosphorylated IRS-1 leads to stimulation of ras by GTP loading with subsequent activation of raf-1, mitogen-activated protein kinase (MAPK), kinase (MEK), and two isozymic forms of MAPK, p44^{mapk} (extracellular signal-regulated kinase 1 or ERK-1) and p42^{mapk} (ERK-2) (*8*, *9*). The activated MAPK phosphorylates and activates downstream ribosomal protein kinase, p90^{rsk}, and transcription factors leading to increased gene transcription levels (*10*). Another pathway that radiates from the IRS-1-bound signaling intermediates upon insulin stimulation is that of PI3-K−PDK−PKB. PI3-K is composed of an 85 kDa (p85) regulatory subunit and a 110 kDa (p110) catalytic subunit which catalyzes the phosphorylation of PI on position D-3 of the inositol ring to produce PI3,4,5

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[1] Abbreviations: CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ERK, extracellular signal-regulated kinase; Grb, growth factor receptor binder; GSK-3, glycogen synthase kinase 3; GST, glutathione *S*-transferase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IDDM, insulin-dependent diabetes mellitus; IL, interleukin; IR, insulin receptor; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; mSOS, mammalian son of sevenless; NIDDM, non-insulin-dependent diabetes mellitus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDK, phosphatidylinositol-dependent kinase; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PMSF, phenylmethylsulfonyl fluoride; PTPases, protein tyrosine phosphatases; PVDF, polyvinylidine difluoride; ras, 21 kDa GTP binding protein; RBD, ras-binding domain of raf-1; rsk, ribosomal S-6 kinases; SDS, sodium dodecyl sulfate; SH2, src homology 2; VS, vanadyl sulfate.

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trisphosphate (PIP3) (*11*). PIP3, in turn, activates PI-dependent kinases (PDK) 1 and 2 (12). A downstream serine/threonine protein kinase named protein kinase B (PKB) has been shown to be one of the targets of PDK-1 and -2 (*12*, *13*). Insulin stimulates another ribosomal S6 protein kinase, $p70^{s6k}$, by a mechanism that is only partially characterized (*14*). However, a recent report suggests that PDK-1 directly phosphorylates and activates $p70^{s6k}$ (*13*).

Salts of the trace element vanadium mimic most of the actions of insulin, including stimulation of glucose transport, glycogen synthesis, glycogen synthase, and lipogenesis, while exerting inhibitory effects on lipolysis and gluconeogenesis (*15*, *16*; reviewed in ref *17*). Furthermore, vanadium salts, given orally, have been shown to normalize blood glucose levels in animal models of insulin-dependent diabetes mellitus (IDDM or type 1 diabetes) (*18*) and non-insulin-dependent diabetes mellitus (NIDDM or type 2 diabetes) (*19*). In addition, vanadium salts improve some abnormalities associated with diabetes mellitus in human subjects (*20*, *21*; reviewed in refs *22* and *23*).

Vanadium salts are believed to mimic insulin action by inhibiting PTPases (*17*, *20*, *22*, *23*) which results in enhanced cellular protein tyrosine phosphorylation concomitant with activation of various components of insulin signaling pathways. In earlier studies, the insulin-like action of vanadate was suggested to be due to the stimulation of IR kinase activity (*24*). Subsequently, it was observed that the site of vanadium action is at the postreceptor level (*25*−*29*) and does not involve IR tyrosine phosphorylation (*25*, *28*, *30*, *31*). Thus, the precise mechanism by which vanadium salts exert their effect remains poorly characterized.

We have demonstrated previously that several vanadium salts activate MAPK and two ribosomal protein kinases, including $p90^{rsk}$ and $p70^{s6k}$, in an IR kinase-independent manner (*28*, *32*). Since vanadyl sulfate (VS) can stimulate PI3-K activity in IR-overexpressing Chinese hamster ovary cells (CHO-HIR) (*33*) and wortmannin, a known PI3-K inhibitor (*34*), blocks the insulin-induced ras/MAPK and $p70^{s6k}$ pathways in several cell types (*35*−*39*), the study presented here was undertaken in an effort to examine whether the increase in $p70^{s6k}$ and MAPK activities in response to VS can be inhibited by wortmannin. We also investigated the upstream signaling components of MAPK, including MEK, raf-1, and ras, and determined whether they are activated by VS via a wortmannin-sensitive cascade.

MATERIALS AND METHODS

Materials

Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) were a kind gift from M. F. White (Joslin Diabetes Center, Boston, MA). Insulin was from Eli Lilly Co. (Indianapolis, IN), and kinase-inactive MEK and polyclonal C-raf-1 antisera were generous donations from R. K. Jaiswal (Case Western Reserve University, Cleveland, OH). Polyclonal MEK antiserum and recombinant $p44^{mapk}$ were kindly provided by S. Meloche (University of Montreal). Wortmannin was purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 was from Biomol (Philadelphia, PA) and VS from Aldrich Chemical Co. (Milwaukee, WI), and monoclonal antiphosphotyrosine antibody (4G10), polyclonal MAPK antibody, and S6 peptide RRRLSSLRA were from Upstate Biotechnology (Lake Placid, NY). Goat anti-mouse and anti-rabbit IgG conjugated to alkaline phosphatase were obtained from Bio-Rad (Mississauga, ON). Phospho-specific p44/p42 MAPK (Thr202/Tyr204) antibody was from New England BioLabs (Beverly, MA). Protein A sepharose beads were from Pharmacia Biotech Inc. (Mississauga, ON), and $p70^{s6k}$ antiserum (raised against a peptide corresponding to amino acids 2−30 of rat $p70^{s6k}$) was generously provided by F. Hall (School of Medicine, University of Southern California, Los Angeles, CA). The farnesyltransferase inhibitor B581 was purchased from Bachem BioScience Inc. (King of Prussia, PA) and the enhanced chemiluminescense (ECL) detection system kit from Amersham Pharmacia Biotech (Baie d'Urfé, PQ); mouse monoclonal H-ras antibody and polyclonal antibodies against p85 regulatory subunit of PI3-K were from Santa Cruz Biotech (Santa Cruz, CA), and pan-ras antibody was from Calbiochem (San Diego, CA). Silica gel-60 plates were obtained from Merck & Co. Inc. (Rahway, NJ).

Methods

Cell Culture. 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 (*32*).

MAPK and $p70^{s6k}$ Assay. Both assays were carried out as described previously (*33*). Briefly, cells were lysed in buffer A [25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS)], and the lysates were clarified by centrifugation to remove insoluble material. The clarified lysates, normalized to contain equal amounts of protein (100 μg), were incubated for 4 h at 4 °C with either 5 μg of MAPK antibody or 3 μL of $p70^{s6k}$ antiserum preadsorbed to protein A−Sepharose beads. The immune complex was collected by centrifugation followed by washing three times with buffer A and once with buffer B [20 mM Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 mM β-glycerophosphate]. For the MAPK assay, 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 10 μL of 1 mg/mL myelin basic protein (MBP)] was added. The reaction was initiated by adding 5 μL of 1 mM adenosine 5′-triphosphate (ATP) containing 0.5 μCi of [γ-^{32}P]ATP (specific activity of 3000 Ci/mmol; Amersham). After 12 min at 30 °C, the reaction was stopped by spotting an aliquot of the supernatant on P-81 filter paper which was washed in 0.5% phosphoric acid and counted for radioactivity (*33*). For the $p70^{s6k}$ assay, the beads were resuspended in 20 μL of buffer B containing S6 peptide RRRLSSLRA. The phosphotransferase reaction was initiated by adding 5 μL of 100 μM ATP containing 2 μCi of [γ-^{32}P]ATP, and stopped 15 min later by spotting an aliquot of the supernatant on P-81 filter paper.

Immunoaffinity Purification of Phosphotyrosine-Containing Proteins and MAPK Immunoblotting. The clarified

lysates from control and stimulated cells were incubated with agarose-bound antiphosphotyrosine beads for 1 h at 4 °C. The beads were washed three times with buffer C [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 0.2 mM sodium vanadate, and 0.01% sodium azide], and phosphotyrosine-containing proteins were then eluted with 1 mM phosphotyrosine in buffer C. The eluates were boiled in 3× Laemmli's sample buffer, electrophoresed on 10% SDS−polyacrylamide gels (SDS−PAGE), transferred to polyvinylidine difluoride (PVDF) membranes, and blotted with anti-MAPK antibody (1:500), followed by signal detection using goat anti-rabbit IgG conjugated to alkaline phosphatase (1:3000).

Western Blotting of Cell Lysates. Cells incubated in the absence or presence of insulin or VS were lysed in 400 μL of buffer A on ice. The lysates were clarified by centrifugation for 12 min at 10000*g*. Equal amounts of protein were electrophoresed on 10% SDS−PAGE under reducing conditions, transferred to PVDF membranes, and incubated with monoclonal antiphosphotyrosine antibody (1:1000). The phosphotyrosylated proteins were detected by goat anti-mouse IgG conjugated to alkaline phosphatase (1:3000). Alternatively, a polyclonal phospho-specific p44/p42 MAPK antibody (1:2000) was used to probe the PVDF membranes in an ECL detection system.

In Vitro Binding of Active ras to GST−RBD. Glutathione *S*-transferase fusion protein containing the minimal ras-binding domain of raf-1 (GST−RBD) was kindly provided by J. L. Bos (Utrecht University, Utrecht, The Netherlands) so the relative amount of active GTP-bound ras could be determined, as described previously (*40*). The GST−RBD fusion protein contains the minimal ras binding domain (amino acids 51−131) of raf-1. Briefly, GST−RBD was isolated and bound to glutathione−agarose beads for 45 min at room temperature. The bound GST−RBD was then incubated for 45 min at 4 °C with clarified cell extracts. The beads were pelleted by centrifugation, washed twice in lysis buffer, and solubilized in 50 μL of Laemmli's sample buffer. The samples were separated on 15% SDS−PAGE and immunoblotted with a mouse monoclonal pan-ras antibody.

PI3-K Assay. The clarified cell lysates were subjected to immunoprecipitation with 1 μg of anti-H-ras antibody for 2 h at 4 °C, followed by incubation with protein A−sepharose for an additional 2 h. The immunoprecipitates were washed and subjected to the in vitro PI3-K assay, as described previously (*33*). The phosphorylated lipid products were extracted and separated by ascending thin-layer chromatography (*33*). The radioactivity in the spots corresponding to PI3-phosphate were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

MEK Assay. MEK activity was assayed by measuring the ability to increase recombinant $p44^{mapk}$ kinase activity toward MBP as the exogenous substrate (*41*). The samples were prepared as described for the MAPK assay. Equal amounts of lysate proteins (100 μg) were incubated for 4 h with 1 μL of MEK antiserum preadsorbed to protein A−Sepharose beads. After incubation for 4 h, the immune complex was washed three times with lysis buffer and once with kinase assay buffer [20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT] and then resuspended in kinase buffer containing 50 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 300 ng of recombinant $p44^{mapk}$. After incubation for 30 min at 30 °C, MBP (0.25 mg/mL) was added and incubation was continued for an additional 10 min. The reaction was stopped by adding 40 μL of 3× Laemmli's sample buffer. Proteins were separated by 10% SDS−PAGE and electrotransferred onto PVDF, and the radioactivity associated with the labeled substrate was quantitated.

raf Assay. Samples were prepared as described for the MEK assay except that immunoprecipitation was performed with 1 μL of polyclonal C-raf-1 antisera. The reaction was carried out using "kinase-inactive MEK" as the substrate (*42*).

Protein Assay. The protein content was determined by the Bradford method (*43*).

RESULTS

Effect of Wortmannin on VS-Stimulated MAPK Activity and Protein Tyrosine Phosphorylation. These studies were aimed at investigating whether wortmannin, which has been shown to inhibit VS-stimulated PI3-K activity (*33*), could also attenuate VS-stimulated MAPK activity. As seen in Figure 1A, treatment of CHO-HIR cells with VS (100 μM) or insulin (100 nM) for 5 min resulted in a 4−5-fold increase in MAPK activity over the controls. This response was markedly suppressed by pretreatment for 10 min with 100 nM wortmannin. Wortmannin may exert its inhibitory effect by altering the levels of protein tyrosine phosphorylation in response to VS or insulin. Indeed, the ability of VS to increase the level of phosphorylation of two proteins having apparent molecular sizes of 44 (p44) and 42 kDa (p42) was markedly retarded by pretreatment of cells with wortmannin (Figure 1B). On the other hand, insulin treatment enhanced tyrosine phosphorylation of the 95 kDa IR β-subunit as well as of p44 and p42. Interestingly, wortmannin significantly attenuated the insulin-stimulated tyrosine phosphorylation of p44 and p42 but not that of the IR β-subunit. Notably, VS was unable to stimulate IR β-tyrosine phosphorylation (Figure 1B). Neither wortmannin alone nor its vehicle dimethyl sulfoxide (DMSO) had any effect on MAPK activity or protein tyrosine phosphorylation (Figure 1A,B).

To determine if the tyrosine-phosphorylated p44 and p42 proteins corresponded to $p44^{mapk}$ (ERK-1) and $p42^{mapk}$ (ERK-2), cell lysates were subjected to immunoaffinity purification using agarose-conjugated antiphosphotyrosine antibody. The eluted proteins were immunoblotted with anti-MAPK antibody which detects both ERK-1 and ERK-2 MAPK isoforms. As shown in Figure 2, pretreatment with wortmannin (100 nM) for 10 min markedly inhibited VS- and insulin-stimulated tyrosine phosphorylation of $p44^{mapk}$ (ERK-1) and $p42^{mapk}$ (ERK-2) proteins (Figure 2, lanes 3 and 6).

Effect of Wortmannin and LY294002 on VS-Induced MAPK Activation and ERK Phosphorylation. To further analyze the effect of wortmannin on VS-stimulated MAPK activity, we performed dose−response studies. As shown in Figure 3A, pretreatment of cells with increasing concentrations of wortmannin followed by stimulation with VS attenuated the activation of MAPK by VS in a dose-dependent manner. Wortmannin at 1 nM caused about 30% inhibition which increased to more than 95% at 100 nM. There was also a corresponding reduction in the level of VS-induced phosphorylation of both ERK-1 (p44) and ERK-2 (p42) in cells treated with wortmannintreated (Figure 3B).

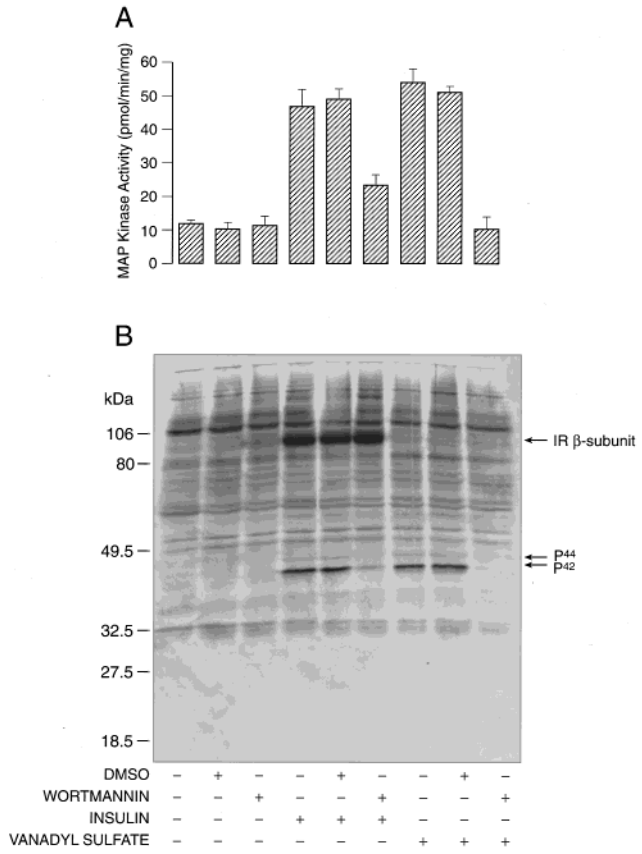

FIGURE 1: Effect of wortmannin on the stimulation of MAPK activity and protein tyrosine phosphorylation in response to VS or insulin. Confluent, serum-starved CHO-HIR cells were pretreated with wortmannin (100 nM) or vehicle (0.01% DMSO) for 10 min, followed by the addition of either vanadyl sulfate (100 μM) or insulin (100 nM) for 5 min. (A) Cell lysates were prepared and subjected to an immunoprecipitation-based MAPK assay using MBP as the exogenous substrate, as described in Materials and Methods. The values are from three separate experiments, each performed in triplicate. Each bar represents the mean ± the standard error. (B) The cells were stimulated as described for panel A, and cell lysates were subjected to antiphosphotyrosine immunoblotting. The arrows mark the positions of the insulin receptor (IR) β-subunit, ERK-1 (p44), and ERK-2 (p42). The immunoblot is representative of two independent experiments.

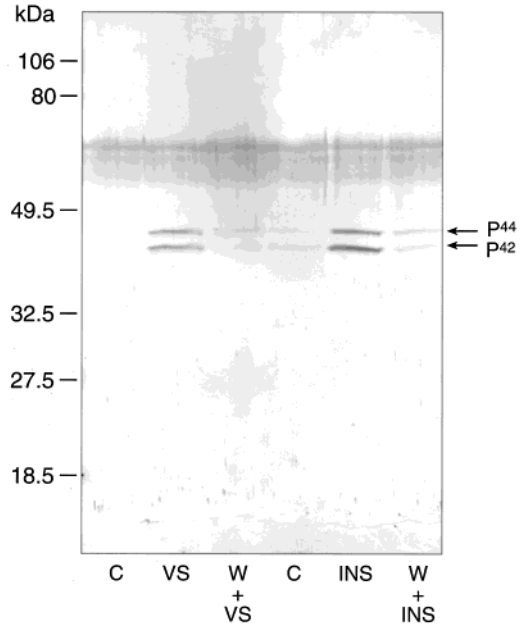

FIGURE 2: Identification of tyrosyl-phosphorylated p44 and p42 proteins. Confluent, serum-starved CHO-HIR cells were pretreated without (C) or with wortmannin (W, 100 nM) for 10 min, followed by no addition (C) or by the addition of either vanadyl sulfate (VS, 100 μM) or insulin (INS, 100 nM) for 5 min. The cell lysates were subjected to immunoaffinity purification with agarose-conjugated antiphosphotyrosine antibody, analyzed by SDS−PAGE, and immunoblotted with anti-ERK-1 or -ERK-2 antibody. The arrows mark the position of ERK-1 (p44) and ERK-2 (p42). The results are representative of two independent experiments.

The role of PI3-K in VS-stimulated MAPK activation was further confirmed by utilizing LY294002, another inhibitor of PI3-K, which is structurally and mechanistically different from wortmannin (*44*). As shown in Figure 3C, treatment of cells with LY294002 prior to stimulation with VS attenuated VS-stimulated phosphorylation of ERK-1 (p44) and ERK-2 (p42) in a dose-dependent manner.

The inhibitory effect of wortmannin was rapid since preincubation with 100 nM wortmannin resulted in a more than 95% reduction in the level of VS-stimulated MAPK activation and ERK-1 or ERK-2 phosphorylation within 10 min (Figure 4A,B). Therefore, in subsequent experiments, these conditions were used to test the effect of wortmannin in VS-mediated responses.

Effect of Wortmannin on VS-Induced Activation of MEK and C-raf-1. Since the increase in MAPK activity requires sequential activation of several upstream signaling molecules, such as MEK and C-raf-1, it was of interest to examine if VS activates these signaling components. VS treatment of CHO-HIR cells stimulated MEK activity approximately 3−4-fold when compared to unstimulated cells (Figure 5A,B). The fact that the VS-mediated increase in MEK activity was markedly inhibited by wortmannin pretreatment (Figure 5A,B) suggests that inhibition lies further upstream of MEK. Similarly, insulin-stimulated MEK activity was also downregulated by wortmannin (data not shown). These results are in agreement with those of Karnitz et al. (*45*), who reported that interleukin 2 (IL-2) stimulates MEK in a wortmannin-sensitive signaling cascade.

Increased raf-1 activity is the primary stimulus for MEK activation. Therefore, we examined the effect of VS on raf-1 activity and its modulation by wortmannin. CHO-HIR cells were pretreated with or without 100 nM wortmannin for 10 min, followed by stimulation with VS. raf kinase activity was determined in immunoprecipitates using kinase-inactive MEK as the substrate. As seen in Figure 5 (panels C and D), the 4-fold enhancement of C-raf-1 activity by VS over the controls was blocked by wortmannin pretreatment. Under these conditions, insulin-stimulated C-raf activity was also inhibited by wortmannin (data not shown). Taken together, these results suggest that a wortmannin-sensitive PI3-K pathway lies upstream of C-raf-1 and MEK.

Role of ras in VS-Mediated Stimulation of the MAPK Pathway. Activation of the raf/MEK/MAPK cascade can be mediated both by ras-dependent and -independent mechanisms (*46*−*48*). Insulin has been shown to activate ras in many cell types (*13*, *49*, *50*), thereby leading to MAPK stimulation (*51*). Farnesylation of ras is essential for its localization to the plasma membrane and, thus, its activation (*52*). Recent work has examined the role of ras in MAPK activation by using the farnesyltransferase inhibitor B581 (*53*). In this study, we examined the contribution of ras

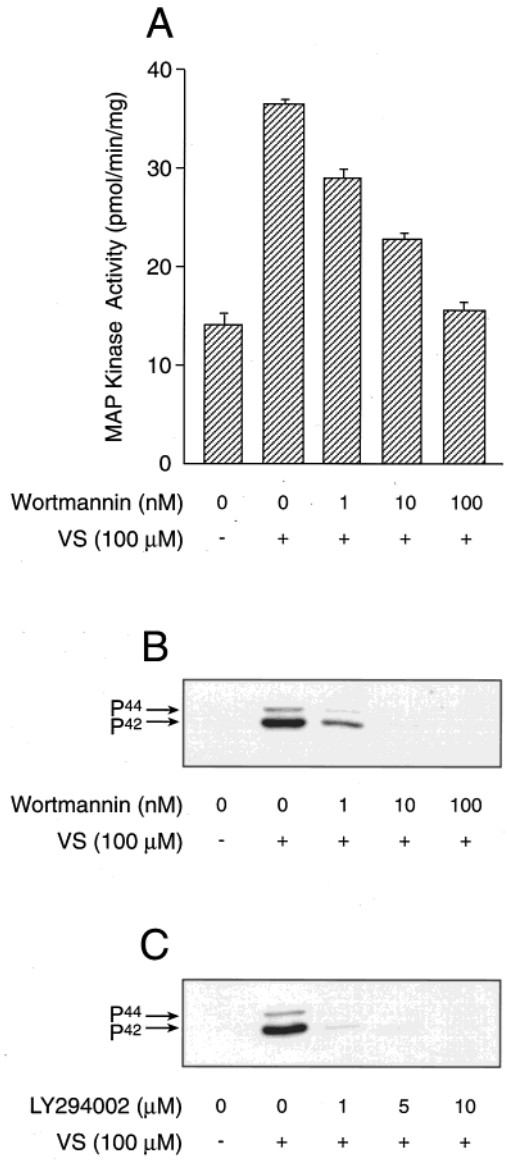

FIGURE 3: Dose−response effect of wortmannin and LY294002 on VS-stimulated MAPK activity and ERK phosphorylation. Confluent, serum-starved CHO-HIR cells were pretreated with the indicated concentrations of wortmannin for 10 min or LY294002 for 30 min prior to stimulation in the absence (−) or presence (+) of 100 μM vanadyl sulfate (VS) for 5 min. Cell lysates were prepared and subjected to (A) MAPK assay as described in Materials and Methods, and the results are means ± the standard error of three separate experiments. (B and C) Antiphospho-specific ERK-1 and ERK-2 immunoblotting. Arrows mark the positions of phosphorylated ERK-1 (p44) and ERK-2 (p42). The immunoblots are representative of two other experiments with similar results.

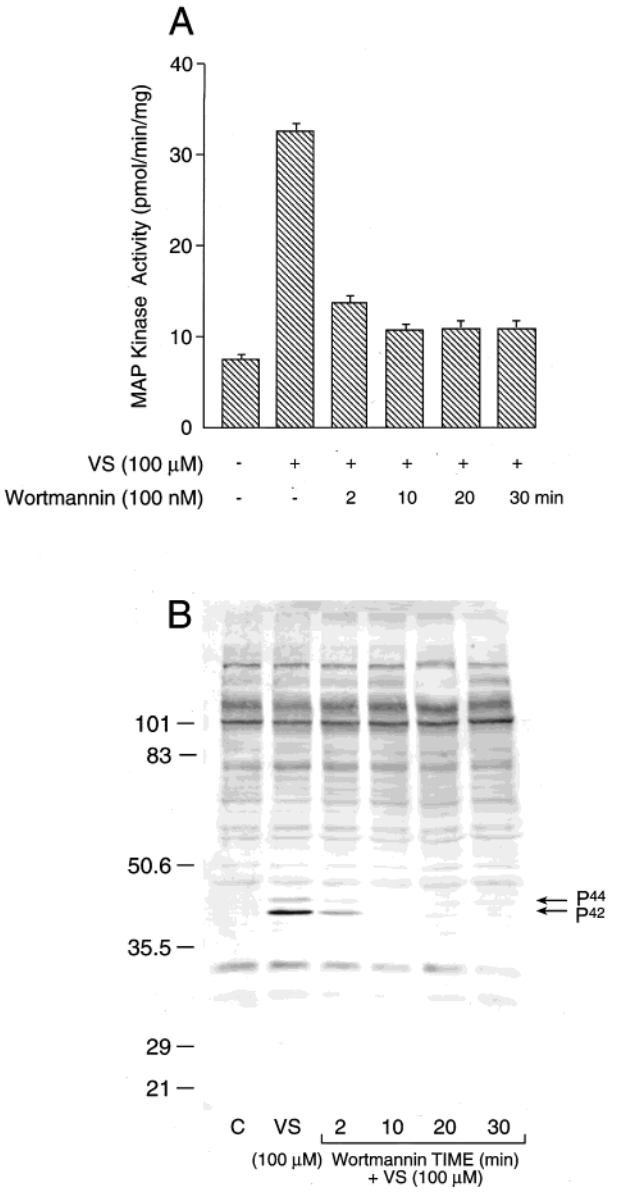

FIGURE 4: Time course effect of wortmannin on VS-stimulated MAPK activity and ERK phosphorylation. Confluent, serum-starved CHO-HIR cells were pretreated without (−) or with wortmannin (100 nM) for the indicated time periods prior to stimulation in the absence (−, C) or presence (+) of 100 μM vanadyl sulfate (VS) for 5 min. Cell lysates were prepared and subjected to (A) the MAPK assay as described in Materials and Methods (results are means ± the standard error of three separate experiments) or (B) antiphosphotyrosine immunobloting. Arrows mark the positions of phosphorylated ERK-1 (p44) and ERK-2 (p42). The immunoblot is a representative of two other experiments with similar results.

protein in the VS-stimulated increase of MAPK activity by using B581. Pretreatment of CHO-HIR cells with two different concentrations of B581 (50 and 100 μM) for 24 h inhibited MAPK activation (Figure 6A) and phosphorylation of both ERK-1 and ERK-2 (Figure 6B) in response to either VS or insulin. These results demonstrate that pharmacological inhibition of protein farnesylation blocks VS-induced phosphorylation and activation of MAPK and suggest a requirement of ras in these processes.

Insulin stimulates ras activity through the exchange of GDP to GTP by SOS (*4*). To examine the GTP-binding status (i.e., activation) of ras, we utilized the RBD of raf-1 to precipitate GTP-bound ras, as described recently by de Rooij and Bos (*40*). In the presence of VS or insulin, GST−RBD precipitated significantly higher amounts of ras from whole cell extracts compared to the controls (Figure 7, left panel). There was a marked reduction in the amount of ras precipitated by GST−RBD following preincubation for 10 min with wortmannin (Figure 7, left panel, lanes 3 and 5), suggesting a possible interaction between ras and PI3-K. It should be noted that under these conditions, no change in the amount of ras in cell lysates was detected (Figure 7, right panel). ras and PI3-K interaction was investigated by directly measuring PI3-K activity present in ras immunoprecipitates. As shown in Figure 8, VS increased PI3-K activity in a dose-dependent manner with about 3-fold stimulation by 100 μM

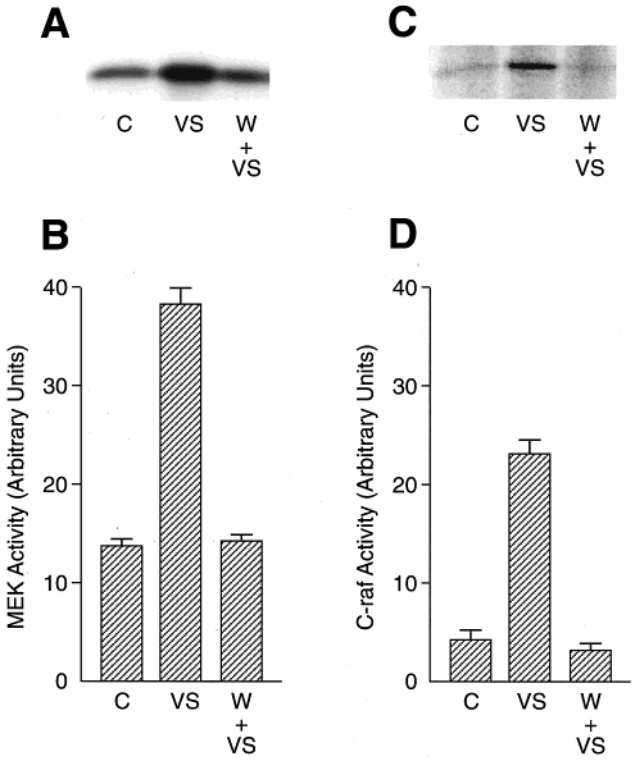

FIGURE 5: Effect of wortmannin on VS-stimulated MEK and C-raf-1 activities. Serum-starved, confluent CHO-HIR cells were pretreated without (C) or with wortmannin (W, 100 nM) for 10 min, followed by stimulation in the absence or presence of VS (100 μM) for 5 min. MEK activity was determined in cell lysates by following the ability of recombinant ERK to phosphorylate MBP (A and B), as described in Materials and Methods. The kinase reaction products were separated on SDS−PAGE, transferred to PVDF membrane, and visualized by autoradiography. (A) An autoradiograph from a representative experiment is shown. (B) The phosphorylated substrate was quantified by PhosphorImager, and the results are the means ± the standard error of three separate experiments. C-raf-1 activity was assessed by using "kinase-dead" MEK as the substrate (C and D). The kinase reaction products were processed as described above, and an autoradiograph from a representative experiment is shown in panel C. (D) The phosphorylated substrate was quantified, and the results are expressed as the means ± the standard error of three separate experiments.

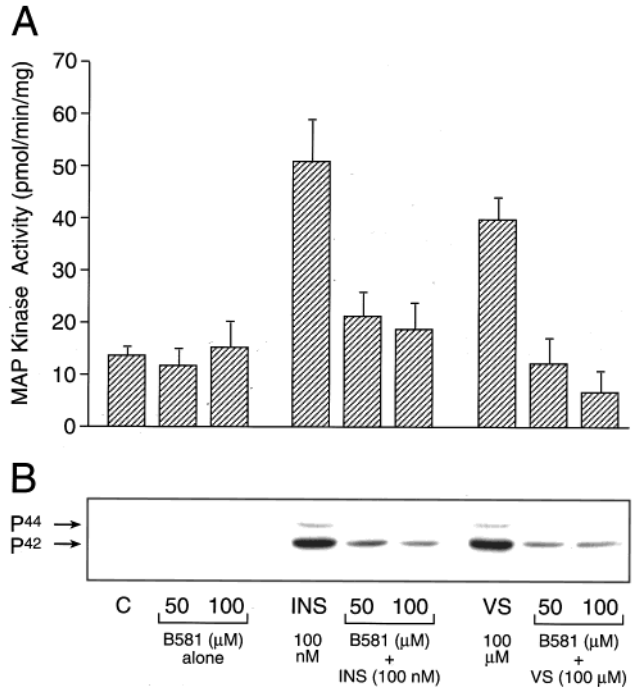

FIGURE 6: Effect of the farnesyltransferase inhibitor B581 on VS-stimulated MAPK activity and ERK-1 and ERK-2 phosphorylation. Confluent, serum-starved CHO-HIR cells were pretreated with B581 at the indicated concentrations for 24 h and then stimulated with VS (100 μM) or insulin (INS, 100 nM) for 5 min. (A) Cell lysates were prepared and subjected to the MAPK assay, as described in Materials and Methods. The results are means ± the standard error of three separate experiments, each performed in triplicate. (B) The cells were stimulated as described for panel A, and lysates were subjected to antiphospho-specific ERK-1 and ERK-2 immunoblotting. A representative immunoblot from two separate experiments is shown, and arrows mark the positions of phosphorylated ERK-1 (p44) and ERK-2 (p42).

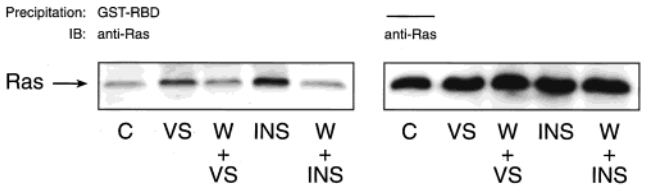

FIGURE 7: Effect of wortmannin on VS-stimulated ras activation. Serum-starved CHO-HIR cells were preincubated without or with wortmannin (W, 100 nM) for 10 min, followed by stimulation in the absence (C) or presence of VS (100 μM) or insulin (INS, 100 nM) for 5 min. Cell lysates were prepared and incubated in the presence of GST−RBD fusion protein, as described in Materials and Methods. The samples were then immunoblotted (IB) with a pan-ras antibody. A representative immunoblot from two separate experiments is shown in the left panel. Similar levels of ras protein were present under each experimental condition that was tested, as judged by immunoblotting the crude lysates with pan-ras antibody. A representative immunoblot from two separate experiments is shown in the right panel.

VS compared to the controls. Under similar conditions, insulin stimulated PI3-K activity about 2.0-fold.

Effect of Wortmannin on VS-Stimulated p70^{s6k} Activity. The 70 kDa ribosomal protein kinase (p70^{s6k}) is a downstream effector of PI3-K that is activated by both VS and insulin (*32*, *54*). Because wortmannin can inhibit p70^{s6k} in response to insulin (*54*), we were interested in determining whether VS-mediated activation of this enzyme was also sensitive to wortmannin. The p70^{s6k} activity present in crude cell lysates was determined by performing an immune complex kinase assay. As shown in Figure 9, treatment of CHO-HIR cells with 100 μM VS or 100 nM insulin for 5 min augmented p70^{s6k} activity by about 4−5-fold over control values. Preincubation with wortmannin markedly inhibited the ability of insulin or VS to stimulate p70^{s6k} activity, suggesting a possible role of wortmannin-sensitive PI3-K in VS-induced p70^{s6k} activation.

DISCUSSION

In the studies presented here, we have examined the effect of VS, an insulinomimetic agent, on activation of the ras/MAPK pathway in CHO-HIR cells. Our results show that wortmannin and LY294002, two structurally and mechanistically different inhibitors of PI3-K, blocked both VS- and insulin-stimulated MAPK activity and protein phosphorylation of p44^{mapk} (ERK-1) and p42^{mapk} (ERK-2) in these cells. On the basis of our earlier findings that demonstrated a marked reduction in the VS-mediated increase in PI3-K activity by wortmannin (*33*), this work provides the first evidence to support a possible involvement of PI3-K and

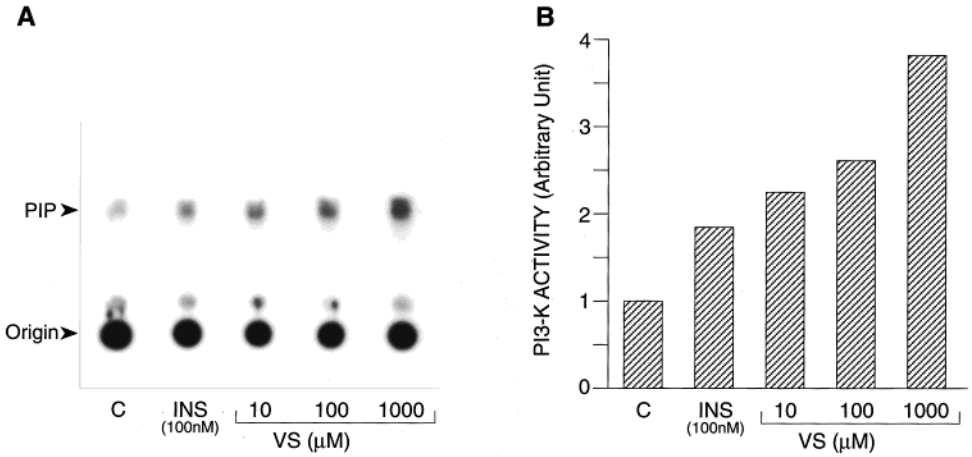

FIGURE 8: Effect of VS on ras-associated PI3-K activity. Serum-starved CHO-HIR cells were stimulated without any addition (C) or with either insulin (INS, 100 nM) or different concentrations of VS for 5 min. The cell lysates were subjected to immunoprecipitation with pan-ras antibody followed by the determination of PI3-K activity as described in Materials and Methods. (A) A representative autoradiogram from two separate experiments is shown. (B) The level of radioactivity incorporated into phosphatidylinositol 3-phosphate (PIP) was quantified and arbitrarily set at 1.0 for the control (C) samples.

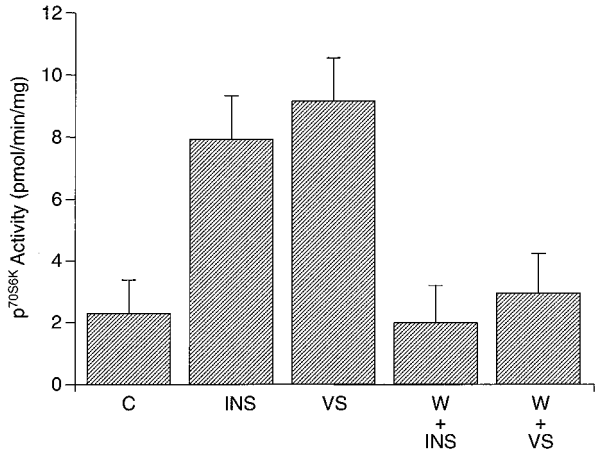

FIGURE 9: Effect of wortmannin on VS-stimulated $P70^{s6k}$ activity. Confluent, serum-starved CHO-HIR cells were pretreated without or with wortmannin (W, 100 nM) for 10 min, followed by stimulation in the absence (C) or presence of VS (100 μM) or insulin (INS, 100 nM) for 5 min. Cell lysates were prepared, and $P70^{s6k}$ activity was determined in an immunoprecipitation-based kinase assay using the S6 peptide RRRLSSLRA as the substrate. The activities are expressed as picomoles of phosphate incorporated into S6 peptide per minute per milligram of lysate protein. The results are means ± the standard error of four separate experiments, each performed in triplicate.

ras activation in the insulinomimetic responses to vanadium salts.

MAPK (ERK-1 and ERK-2) activation requires phosphorylation on both tyrosine and threonine residues (*55*) catalyzed by a single dual-specificity kinase, MEK (*56*). Further examination of the protein kinase cascade revealed that VS and insulin caused an increase in MEK activity which was also sensitive to wortmannin. In this regard, Cross et al. have reported an inhibitory effect of wortmannin on insulin/IGF-1-induced activation of MEK in rat L6 skeletal muscle cells (*35*). Thus, the results presented in these studies suggest that VS-induced activation of MEK may require PI3-K activation in a manner similar to that of insulin. Our next search for possible candidates that may relay the signal to MAPK in response to VS led us to demonstrate that C-raf-1 is also activated in response to VS. Interestingly, wortmannin treatment also inhibited VS-stimulated raf-1 kinase. A similar observation was made by Suga and coworkers (*57*), who reported that insulin-stimulated raf-1 kinase was blocked by wortmannin in CHO-HIR cells whereas EGF-stimulated raf-1 kinase activity was not altered in these cells.

The attenuating effect of PI3-K inhibitors is not limited to insulin-responsive cells, since several other studies have shown that wortmannin blocks MAPK activation by various growth factors and hormones (*58*−*60*). For example, Scheid and Duronio have demonstrated that PI3-K inhibitors suppressed IL-3-induced activation of MAPK in MC-9 hematopoietic cells (*59*). Similarly, Von Willebrand et al. reported that wortmannin attenuated anti-CD3-induced MAPK in Jurkat T cells (*60*). Moreover, studies by Karnitz and coworkers showed a wortmannin-sensitive activation of MAPK and MEK by IL-2 in the T cell line CTLL-2 (*45*). However, these same authors also reported that raf-1 kinase stimulated by IL-2 was not inhibited by wortmannin (*45*). Such conflicting observations appear to arise from either the nature of the agonists, the cell lines that were used, or the experimental conditions that were employed. Our results strongly suggest that VS stimulates the raf/MEK/MAPK cascade via activation of a wortmannin-sensitive PI3-K pathway in CHO-HIR cells.

Ras activation is critical for stimulating the MAPK signaling pathway in response to external stimuli. The participation of ras in the VS-stimulated raf/MAPK cascade was evaluated by utilizing the specific farnesyltransferase inhibitor B581. Pretreatment of CHO-HIR cells with B581 prevented MAPK activation as well as phosphorylation of ERK-1 and ERK-2 in response to VS. This effect was presumably due to ras inhibition since ras farnesylation is essential for its localization to the plasma membrane and for its activation (*52*). These data suggest that ras activation may be involved in VS-stimulated activation of raf, MEK, and MAPK. This notion was further supported by the observation that VS increased the amount of GTP-bound ras to GST−RBD and that pretreatment of cells with wortmannin markedly reduced this interaction. Hence, we have provided strong evidence indicating that ras is indeed activated by VS and lies downstream to PI3-K. Furthermore, our data on

PI3-K activity measured in ras immunoprecipitates provide additional support for the view that ras and PI3-K interact with each other to propagate a signal.

The precise mechanism by which PI3-K activates ras remains unclear. However, it is possible that downstream effectors of PI3-K such as PDK-1 and PKB or other phospholipid-dependent kinase(s) play a role in mediating this response. In this regard, we have recently shown that VS stimulated the PDK-1-catalyzed phosphorylation of PKB in Thr^{308} which was blocked by wortmannin and LY294002 (J.-F. Théberge, S. K. Pandey, and A. K. Srivastava, unpublished data), but any role of PKB in ras activation has not been established.

Stimulation of the ras/MAPK pathway by insulin requires PI3-K activation which depends on the IR β-subunit and enhanced tyrosine phosphorylation of IRS-1 (*11*). In contrast, the activation of MAPK cascade in response to VS appears to be independent of IR autophosphorylation but is associated with the tyrosine phosphorylation of IRS-1 (*28*, *33*). The mechanism by which VS enhances IRS-1 phosphorylation remains unclear at present. However, it is possible that VS-induced inhibition of IRS-1-specific PTPase and/or activation of a nonreceptor, src-related protein tyrosine kinase mediates this effect. The demonstration of Janus kinase-dependent tyrosine phosphorylation of IRS-1 in response to IL-4, oncostatin, interferon (*61*), and growth hormone (*62*) is noteworthy in this regard. PI3-K can transmit the signal to the ras/MAPK pathway in both a receptor-independent and -dependent manner. In this context, Hu and co-workers have demonstrated that a constitutively active form of PI3-K can transmit signals in a ras-dependent manner without requiring receptor stimulation (*63*). In another study, Yamauchi and co-workers reported that PI3-K can act upstream of ras and raf in mediating insulin stimulation of c-fos transcription (*64*).

Ribosomal protein kinases are important in translational control by insulin and other mitogens (*65*). The observation that $p70^{s6k}$ activation by VS was sensitive to wortmannin suggests the participation of PI3-K in this response. It has been shown previously that $p70^{s6k}$ stimulation in response to PDGF, insulin, or serum is also attenuated by wortmannin (*54*, *66*). Indeed, coexpression of PI3-K with $p70^{s6k}$ enhances $p70^{s6k}$ phosphorylation at Thr^{252} which can be blocked by wortmannin (*67*). Our results indicate that VS-induced activation of $p70^{s6k}$ may also be regulated by PI3-K. Whether other components of the $p70^{s6k}$ cascade such as mTOR are also activated by VS is not yet known.

In summary, we have elucidated the upstream signaling components involved in vanadium salt-induced ERK activation. We demonstrate that stimulation of the ras-ERK cascade by VS is dependent on PI3-K activation and requires a protein farnesylation step. It is suggested that stimulation of the PI3-K/ras/ERK pathway plays a key role in mediating the insulinomimetic effects of inorganic vanadium salts.

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