THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 46, Issue of November 18, pp. 28783–28789, 1994
Printed in U.S.A.

Insulin Receptor Substrate-1 Mediates Phosphatidylinositol 3′-Kinase and p70^{S6k} Signaling during Insulin, Insulin-like Growth Factor-1, and Interleukin-4 Stimulation*

(Received for publication, June 20, 1994, and in revised form, September 8, 1994)

Martin G. Myers, Jr.‡§, Timothy C. Grammer¶, Ling-Mei Wang‖, Xiao Jian Sun‡, Jacalyn H. Pierce‖, John Blenis¶‡‡, and Morris F. White‡§§**

From the ‡Research Division, Joslin Diabetes Center and Program in Cell and Developmental Biology, Harvard Medical School, Boston, Massachusetts 02215, the ¶Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, and the ‖Laboratory of Cell and Molecular Biology, National Institutes of Health, Bethesda, Maryland 20892

Insulin Receptor Substrate-1 (IRS-1) is an endogenous cellular protein that is tyrosine phosphorylated during stimulation of cells with insulin, IGF-1, and interleukin 4 (IL-4). Phosphorylated IRS-1 regulates multiple regulatory pathways by recruiting signaling molecules containing Src homology 2 domains (SH2 proteins). The 32D myeloid progenitor cell line contains few insulin receptors and no detectable IRS-1. Expression of the insulin receptor alone partially mediates insulin-stimulated microtubule-associated protein (MAP) kinase activation, and the addition of IRS-1 enhances this effect (Myers, M. G., Jr., Wang, L.-M., Sun, X. J., Zhang, Y., Yenush, L. P., Schlessinger, J., Pierce, J. H., and White, M. F. (1994) *Mol. Cell. Biol.* 14, 3577–3587). Alone, insulin receptors mediate phosphatidylinositol (PI) 3′-kinase and p70^{S6k} activation poorly if at all during insulin stimulation. Expression of IRS-1 alone in 32D cells mediates the stimulation of p70^{S6k} by insulin, IGF-1, or IL-4; addition of insulin receptor to these cells increases the sensitivity of the insulin response. In contrast, full insulin stimulation of PI 3′-kinase requires both the insulin receptor and IRS-1, suggesting that a high level of IRS-1 phosphorylation is required for insulin-stimulated PI 3′-kinase activation, whereas a low level of IRS-1 tyrosine phosphorylation transmits an essential signal to p70^{S6k}. Both insulin receptors and IRS-1 are required for mitogenic signaling in 32D cells suggesting that MAP kinase or p70^{S6k} alone are not sufficient, and that both or additional unknown IRS-1-mediated signals are necessary.

The initial steps on the molecular pathway between the insulin receptor and downstream effects of insulin have been clarified by the discovery of IRS-1[1] and its interaction with downstream signaling molecules which contain Src homology 2 (SH2) domains (1–3). IRS-1 interacts directly with the insulin receptor and undergoes tyrosine phosphorylation during insulin stimulation (1–3). Phosphorylated IRS-1 binds several SH2 domain-containing proteins (SH2 proteins), including isoforms of p85 (1, 4, 5), GRB-2 (6, 7), SH-PTP2 (*syp*, PTP1D) (8), and *nck* (9). Others are sure to be found, as IRS-1 contains many sites of tyrosine phosphorylation in distinctive amino acid sequence motifs (3, 10).

The presence of IRS-1 in most experimental systems exerts a dominant effect, interfering with the delineation of IRS-1-independent signaling mechanisms and the functional analysis of IRS-1 mutants (2). In contrast, the 32D myeloid progenitor cell line contains low levels of insulin receptor and no detectable IRS-1 or related molecules (11). Thus, the signals mediated by the insulin receptor can be investigated in the presence and absence of IRS-1 in 32D cells, and the signaling pathways controlled by IRS-1 can be clearly defined.

Previous studies using 32D cells demonstrated that expression of the insulin or the IL-4 receptor alone is insufficient for full insulin- or IL-4-stimulated mitogenesis in these cells (11). Similarly, in cells expressing only IRS-1, insulin poorly stimulates IRS-1 phosphorylation or mitogenesis. However, when expressed together in 32D cells, IRS-1 and the insulin receptor strongly mediate mitogenesis during insulin stimulation (11). Similarly, IRS-1 is essential for IGF-1 and IL-4-stimulated mitogenesis, and the response is increased by overexpression of the corresponding receptor. Thus, tyrosine-phosphorylated IRS-1 is an important mediator in insulin, IGF-1, and IL-4 mitogenic signaling.

Phosphatidylinositol (PI) 3′-kinase phosphorylates PI, PI 4-P, and PI 4,5-P_2 on the 3′-position of the inositol ring during stimulation of cells with a wide variety of growth factors or cytokines, including insulin, IGF-1, and IL-4 (12, 13). Site-directed mutagenesis and the use of inhibitors suggest that PI 3′-kinase is a necessary element in growth factor-stimulated mitogenesis, translocation of glucose transporters, receptor internalization, and chemotaxis (13–21). Most activated growth factor receptors associate directly with the SH2 domains in the 85-kDa regulatory subunit of PI 3′-kinase (p85) through autophosphorylation sites in YXXM motifs (13, 22). During insulin stimulation, however, p85 associates strongly with IRS-1, whereas only a small percentage of PI 3′-kinase associates with the insulin receptor (1). Although the insulin receptor directly binds and activates PI 3′-kinase *in vitro* (23), much of the receptor-associated PI 3′-kinase activity *in vivo* represents the association of the PI 3′-kinase/IRS-1 complex with the activated insulin receptor (24, 25).

* This work was supported in part by Grants DK 38712, DK 43808 (to M. F. W.), and CA 46595 (to J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Partially supported by the Albert J. Ryan Foundation at Harvard Medical School and a National Institutes of Health NRSA Training Grant DK 07260.

** Fellow of the Juvenile Diabetes Foundation.

‡‡ Established investigator of the American Heart Association and a recipient of Harvard Medical School Funds for Discovery.

§§ To whom correspondence and reprint requests should be addressed: Research Division, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. Tel.: 617-732-2578; Fax: 617-732-2593; E-mail: Whitemor@joslab.harvard.edu.

[1] The abbreviations used are: IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; IGF-1, insulin-like growth factor-1; IL-4, interleukin 4; αPY, antiphosphotyrosine; αIR, anti-insulin receptor; MAP, microtubule-associated protein; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein.

Insulin, like other growth factors, activates a network of serine/threonine kinases, including the MAP kinase/p90^{rsk} cascade, and p70^{S6k} (26–28). Each of these enzymes is implicated as an essential element in mitogenic signaling downstream of tyrosine kinases. The insulin receptor appears to engage the upstream elements of the MAP/p90^{rsk} cascade (including p21$^{H\text{-}ras}$) through the phosphorylation of IRS-1 or Shc which associate with Grb-2/SOS (7, 29, 30). In contrast, the regulation of p70^{S6k} by insulin is less well understood, although several drugs which inhibit cellular proliferation, including rapamycin and wortmannin, interfere with p70^{S6k} activation (21, 31, 32). Thus, p70^{S6k} may be an essential element in insulin-stimulated mitogenesis.

In this report, we demonstrate that IRS-1 is essential for insulin stimulation of both PI 3′-kinase and p70^{S6k} in 32D cells, which is consistent with recent reports that PI 3′-kinase is an upstream mediator of p70^{S6k} (21, 32). p70^{S6k} is much more sensitive than PI 3-kinase to IRS-1, however, suggesting either that very little PI 3′-kinase is required for full p70^{S6k} activation or that a separate pathway may branch from IRS-1 to regulate this kinase. Additional work with IRS-1 mutants will be necessary to fully evaluate this model.

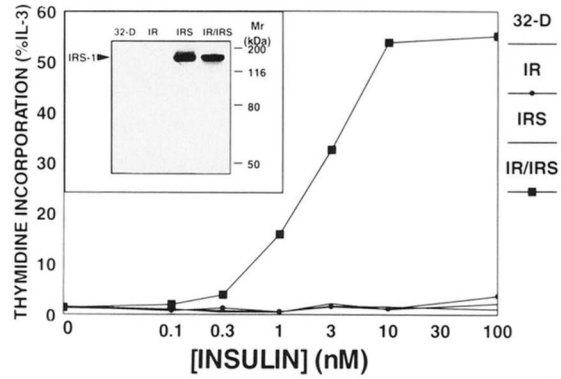

FIG. 1. **Thymidine incorporation into DNA in 32D cell lines.** 32D cell lines were grown in the absence or presence of various concentrations of insulin for 48 h. [^{3}H]Thymidine was then added to the media, and incubation was continued for 3 h before cells were harvested and DNA was collected on glass microfiber filters. Data are the average of triplicate determinations graphed as the percent of the positive control (IL-3-stimulated thymidine incorporation). *Inset*, 32D, 32D^{IR}, 32D/IRS-1, and 32D^{IR}/IRS-1 cells were lysed and analyzed by immunoblotting with αIRS-1. The data shown are representative of multiple experiments.

EXPERIMENTAL PROCEDURES

Cell Lines—32D cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum and 5% conditioned media from WEHI cells (a source of IL-3) (11). Cell lines expressing the insulin receptor, IRS-1, and both the insulin receptor and IRS-1 have been described (7, 11). Cell lines expressing IRS-1 were maintained in media containing 5–10 mM histidinol (Sigma).

Antibodies and Growth Factors—Affinity-purified rabbit polyclonal αPY antibodies (33) and polyclonal rabbit antibodies raised against baculovirus-produced IRS-1 protein (34) have been described previously. Mouse monoclonal αIR antibodies used to immunoprecipitate the insulin receptor were the gift of Dr. K. Siddle (Cambridge, UK). Rabbit antisera against MAP kinase and p70^{S6k} have been described previously (31). Affinity-purified rabbit antibodies to a synthetic peptide containing amino acids 146–161 of mouse αp85 were used for p85α immunoprecipitations. Insulin was from ELANCO (Indianapolis, IN), IGF-1 was from Calbiochem, and murine IL-4 was from UBI (Lake Placid, NY).

Incorporation of [^{3}H]Thymidine into DNA in 32D Cells—Insulin-stimulated thymidine incorporation was assayed as described previously (7, 11). Briefly, cells in log phase growth were washed and seeded into RPMI with 10% fetal bovine serum alone or containing various concentrations of insulin or IL-3-containing conditioned media (WEHI). Cells were grown for 48–72 h at 37 °C. [^{3}H]Thymidine (ICN) was added to a final concentration of 0.5 μCi/ml, and incubation was continued for 3 h. Cells were collected onto glass microfiber filters and lysed, and unincorporated nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

Phosphatidylinositol 3′-Kinase Activity—In vitro phosphorylation of phosphatidylinositol was carried out in immune complexes as described previously (12). 32D cell lines were starved for 4 h in unsupplemented Dulbecco's modified Eagle's medium at 37 °C. The quiescent cells were incubated with various concentrations of insulin, IGF-1, or IL-4 for 5 min, washed once with ice-cold phosphate-buffered saline, and collected by centrifugation. The cells were solubilized as described (12) and incubated with antibody for 1–2 h at 4 °C. Immune complexes were precipitated from the supernatant with Protein A-Sepharose (Pharmacia) and washed as described (12). Immune complexes were incubated with phosphatidylinositol (Avanti) and [γ-^{32}P]ATP (3000 Ci/mmol) for 10 min at 22 °C. Reactions were stopped with 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1) and centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck) which had been coated with 1% potassium oxalate (12). TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized and quantitated on a Molecular Dynamics PhosphorImager.

Immunoblotting—Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol by SDS-PAGE. Gels were transferred to nitrocellulose membranes (Schleicher & Schuell) in Towbin buffer containing 0.02% SDS and 20% methanol (35). Membranes were blocked, probed, and developed as described previously (7, 31). Blots were exposed to Kodak X-AR film or imaged on a Molecular Dynamics PhosphorImager.

In Vitro Assays for MAP and p70^{S6k} Kinase Activity—Cells were made quiescent, stimulated (5 min for MAP kinase assays or 30 min for p70^{S6k} assays, unless otherwise noted), and collected as described above. Cells were lysed in ice-cold 10 mM potassium phosphate, 1 mM EDTA (pH 7.05) containing 0.5% Nonidet P-40, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Anti-MAP kinase or anti-p70^{S6k} antibodies were added for 2 h and collected on protein A-Sepharose beads for 1 h at 4 °C. Immunoprecipitates were washed (31, 36) and incubated with [γ-^{32}P]ATP (50 μM final, 20 μCi/reaction) containing 2 μg of myelin basic protein/reaction (for MAP kinase assays) or 20 μg of 40 S ribosomes/reaction (for p70^{S6k} assays) (31, 36). Assays were incubated for 15 min at room temperature and were stopped by the addition of 2 × Laemmli sample buffer. Samples were denatured by boiling, and phosphorylated substrates were analyzed by SDS-PAGE.

RESULTS

IRS-1 Mediates Insulin-stimulated Mitogenesis in 32D Cells—The 32D cells, like the related myeloid progenitor cells FDC-P1 and FDC-P2, proliferate rapidly during interleukin-3 (IL-3) stimulation. However, unlike typical myeloid cell lines, mitogenesis in 32D cells is completely unresponsive to insulin, IGF-1, and IL-4 (11). Even overexpression of the cognate receptors for these growth factors does not restore responsiveness in 32D cells (Fig. 1). Co-expression of the insulin receptor with IRS-1 enables 32D cells to proliferate vigorously during insulin stimulation (Fig. 1). Expressed alone, low levels of IRS-1 have no effect on insulin-stimulated mitogenesis because the receptor level is so low (Fig. 1), although expression of much higher levels of IRS-1 mediates a weak but detectable mitogenic response to insulin (11). IGF-1 and IL-4 stimulate mitogenesis with lower levels of IRS-1 expression, since the level of their endogenous receptors is higher, and overexpression of the receptors increases the response (11). The requirement for IRS-1 in insulin-stimulated mitogenesis in 32D cells suggests that IRS-1 contributes important elements to the insulin signal which the insulin receptor alone is unable to provide.

PI 3′-Kinase Signaling in 32D Cells—We directly examined the role of IRS-1 in the activation of PI 3′-kinase by insulin in the 32D cells by assaying the activity found in αp85 immunoprecipitates (Fig. 2). PI 3′-kinase was not activated significantly during insulin stimulation of 32D or 32D cells expressing the insulin receptor (32D^{IR}) (0.7 ± 0.3-fold in 32D cells and

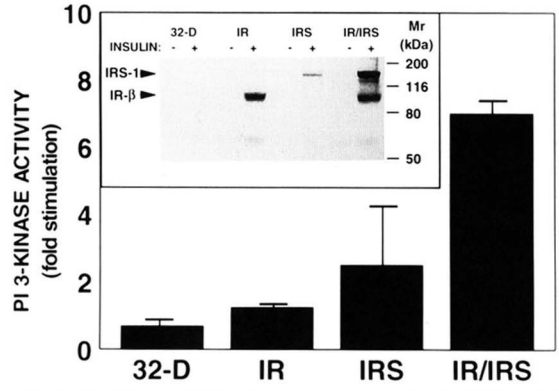

FIG. 2. **Activation of PI 3′-kinase by insulin in 32D cell lines.** 32D cell lines were stimulated with 100 nM insulin for 5 min, lysed, and immunoprecipitated with αp85 antibodies. Immunoprecipitates were washed, and PI 3′-kinase activity was quantitated in an *in vitro* kinase assay. Data are plotted as -fold stimulation for each cell line during insulin stimulation ± S.E. for triplicate determinations. *Inset*, 32D, 32D^{IR}, 32D/IRS-1, and 32D^{IR}/IRS-1 cells were incubated in the absence (−) or presence (+) of insulin (+), lysed, and analyzed by immunoblotting with αPY. The data shown are representative of at least three independent experiments.

1.25 ± 0.35-fold in 32D^{IR} cells), although the β-subunit of the insulin receptor was strongly tyrosine-phosphorylated in the latter (Fig. 2). PI 3′-kinase was stimulated slightly in 32D cells expressing only IRS-1 (32D/IRS-1), consistent with the low level of IRS-1 tyrosine phosphorylation mediated by the endogenous insulin receptor and cross-reacting IGF-1 receptor (Fig. 2, *inset*). However, significant stimulation occurred only in cells expressing both insulin receptor and IRS-1 (32D^{IR}/IRS-1), in which IRS-1 is strongly tyrosine-phosphorylated during insulin stimulation (Fig. 2). Thus, the activation of PI 3′-kinase by insulin in 32D cells requires tyrosyl phosphorylation of IRS-1, as activation of the insulin receptor alone has no effect.

Immune complex PI 3′-kinase assays with antibodies to specific components of the insulin signaling system confirmed our conclusions. PI 3′-kinase strongly associated with αIRS-1 immunoprecipitates from 32D^{IR}/IRS-1 cells, weakly with immunoprecipitates from 32D/IRS-1 cells, and not at all from 32D and 32D^{IR} cells (Fig. 3*A*). This relative ranking reflects the level of IRS-1 tyrosine phosphorylation that occurs in the cells. Parallel immunoprecipitates with antibodies against the insulin receptor (αIR) showed clearly that the PI 3′-kinase does not associate directly with the insulin receptor *in vivo* (Fig. 3*C*). However, αIR strongly immunoprecipitated PI 3′-kinase in 32D^{IR}/IRS-1 cells confirming that a tertiary complex (IR/IRS-1/PI 3′-kinase) forms during insulin stimulation, as described previously (25). Interestingly, PI 3′-kinase activity was weakly detected in αPY immunoprecipitates (relative to IRS-1 immunoprecipitates) from 32D^{IR}/IRS-1 cells (Fig. 3*B*). Although the arbitrary values obtained from the PhosphorImager for the experiments shown are not directly comparable, the activity in αIRS-1 immunoprecipitates was generally 5–10-fold greater than in αPY or αIR immunoprecipitates. These results suggest that hyperphosphorylation of IRS-1 or the formation of the ternary (IR/IRS-1/PI 3′-kinase) complex is necessary for successful immunoprecipitation by αPY.

IRS-1 Is Required for Insulin-stimulated Activation of p70^{S6k}—Certain insulin signaling pathways are independent of IRS-1 because other molecules mediate the response. In particular, insulin partially stimulated MAP kinase activity in the immune complex assay from 32D^{IR} cells, whereas insulin had no effect on MAP kinase from parental 32D cells or 32D/IRS-1 cells (Fig. 4*A*). Previous work suggests that IRS-1-independent stimulation of Shc tyrosine phosphorylation and its subsequent binding to Grb-2/SOS mediates the activation of MAP kinase by

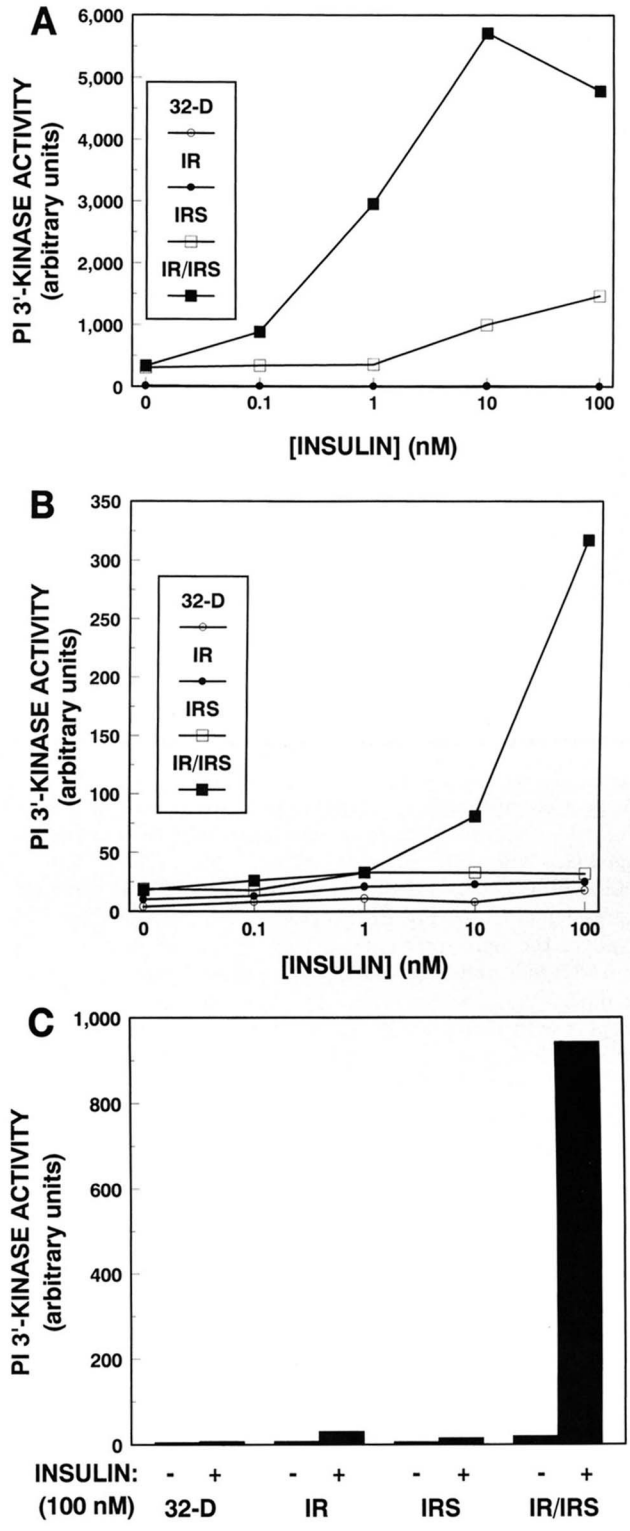

FIG. 3. **Association of PI 3′-kinase with insulin-stimulated phosphoproteins in 32D cell lines.** Equivalent numbers of 32D cells or 32D cells expressing IR, IRS-1, or IR and IRS-1 were stimulated with the indicated concentrations of insulin for 5 min, lysed, and immunoprecipitated with αIRS-1 (*A*), αPY (*B*), and αIR antibodies (*C*). Immunoprecipitates were washed and assayed for associated PI 3′-kinase activity. Counts incorporated into PI 3′-phosphate were quantified on a PhosphorImager; the absolute amount of activity is not directly comparable between different immunoprecipitates, as assays were run and quantified independently. The data shown are representative of at least two independent experiments.

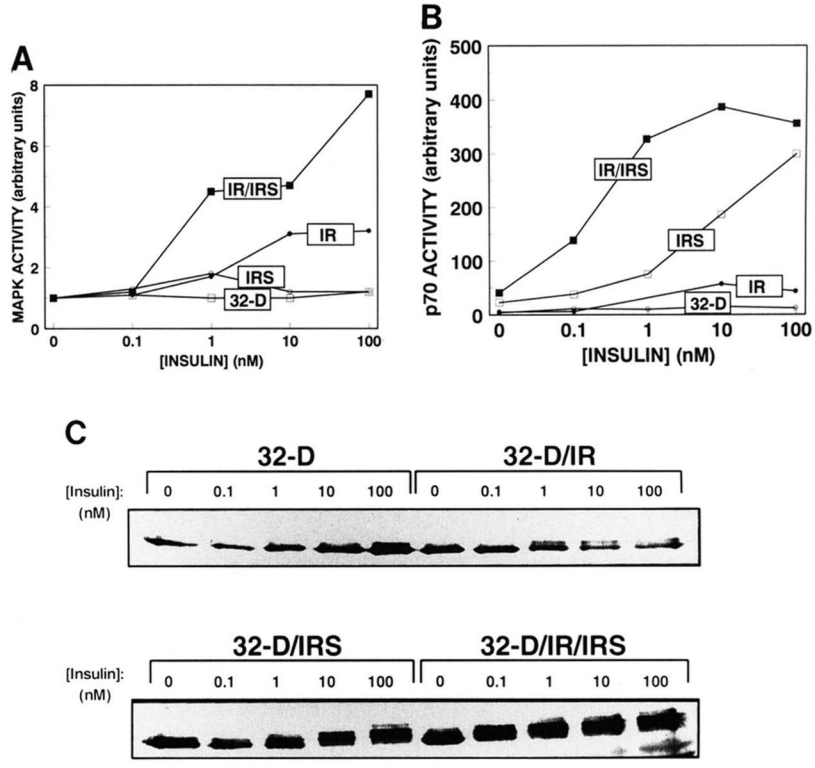

FIG. 4. **Activation of MAP and p70^{S6k} kinases in 32D cell lines.** Cell lines were stimulated with the indicated concentrations of insulin and lysed. *A*, MAP kinase (*MAPK*) was precipitated and assayed for its ability to phosphorylate MBP *in vitro*. Phosphate incorporation was quantified on a PhosphorImager. *B*, p70^{S6k} was immunoprecipitated from the lysates and assayed for its ability to phosphorylate ribosomal S6 protein *in vitro*. Phosphate incorporation was quantified on a PhosphorImager. *C*, lysates were resolved by SDS-PAGE and immunoblotted with αp70^{S6k} antibodies. Migration of p70^{S6k} is indicated. The data shown are representative of multiple independent experiments.

insulin in 32D^{IR} cells (7). Moreover, the effect of insulin on MAP kinase was enhanced by expression of IRS-1 in 32D^{IR}/IRS-1 (Fig. 4A), since the hyperphosphorylation of IRS-1 further engaged Grb-2/Sos (7).

Like PI 3′-kinase, insulin stimulation of p70^{S6k} required the expression of IRS-1 (Fig. 4*B*). Insulin activated p70^{S6k} poorly if at all in the immune complex assay prepared with extracts from 32D cells and 32D^{IR} cells, whereas insulin strongly stimulated p70^{S6k} in 32D/IRS-1 (Fig. 4*B*). The presence of the insulin receptor with IRS-1 (32D^{IR}/IRS-1) increased the sensitivity of activation compared to 32D/IRS-1 cells, but did not increase the maximal response. Therefore, a low level of IRS-1 phosphorylation is sufficient for maximal activation of p70^{S6k}, whereas the hyperphosphorylation of IRS-1 is required for increased IRS-1-mediated MAP kinase signaling or full PI 3′-kinase activation.

The p70^{S6k} is activated by multisite serine phosphorylation, which is readily detected by a retarded migration of the enzyme during SDS-PAGE (31). Only the most highly phosphorylated forms of p70^{S6k} (the slowest migrating) display increased kinase activity (31). Insulin had no effect on p70^{S6k} phosphorylation in 32D cells, whereas it caused a partial, but incomplete, phosphorylation in 32D^{IR} cells (Fig. 4*C*). This was consistent with the absence of increased p70^{S6k} activity in the immune complex assay. The p70^{S6k} was completely phosphorylated in 32D/IRS-1 cells during stimulation with high insulin concentrations, consistent with the full activation of the enzyme in the immune complex assay (Fig. 4*C*). The insulin sensitivity of the IRS-1-mediated phosphorylation and activation of p70^{S6k} was significantly increased by co-expression of the insulin receptor with IRS-1 (Fig. 4*C*).

Insulin (100 nM) stimulated p70^{S6k} with similar time courses in 32D/IRS and 32D^{IR}/IRS cells suggesting that partial IRS-1 phosphorylation was sufficient for the response (Fig. 5*A*). Moreover, during stimulation with 100 nM insulin, the activation of p70^{S6k} did not vary greatly with various levels of IRS-1 expression in 32D/IRS-1 cells (Fig. 5*B*). Thus, IRS-1 is a sensitive mediator of p70^{S6k} activation, although under identical conditions IRS-1 is unable to support mitogenesis, enhanced MAP kinase signaling, or full PI 3′-kinase activation during insulin signaling.

IRS-1 binds PI 3′-kinase and mediates p70^{S6k} activation in response to IGF-1 and IL-4—In addition to insulin, IRS-1 mediates IGF-1- and IL-4-stimulated responses in 32D/IRS-1 cells (11, 37). Since these cells contain a sufficient level of endogenous IGF-1 and IL-4 receptors, mitogenesis is stimulated by these growth factors without expression of additional receptors (11). Moreover, both IGF-1 (10 nM) and IL-4 (50 ng/ml) strongly stimulated the association of PI 3′-kinase with IRS-1 (Fig. 6*A*). In contrast, 10 nM insulin had a small or no effect on the association of PI 3′-kinase with IRS-1 in 32D/IRS-1 cells (Figs. 3*A* and 6*A*). Paradoxically, co-expression of the insulin receptor with IRS-1 in 32D^{IR}/IRS-1 cells decreased the IGF-1- and IL-4-stimulated association of PI 3′-kinase with IRS-1, whereas it strongly enhanced the insulin-stimulated effect (Fig. 6*A*). Perhaps the insulin receptor competes for IRS-1 binding, thus decreasing the availability of IRS-1 for the IGF-1 and IL-4 receptors.

Immune complex and gel shift assays demonstrated that insulin, IGF-1, and IL-4 poorly activated p70^{S6k} in 32D or 32D^{IR} cells, whereas all of these factors activated p70^{S6k} in 32D/IRS-1 and 32D^{IR}/IRS-1 cells (Fig. 6, *B* and *C*). Thus, IRS-1 mediates the activation of p70^{S6k} by insulin, IGF-1, and IL-4, but this response does not exactly parallel the activation of the PI 3′-kinase.

DISCUSSION

The 32D cell provides an ideal system for studying the role for IRS-1 in insulin signaling, since these cells contain no functional IRS-1. Not only is IRS-1 immunologically undetectable in these cells with a variety of αIRS-1 antibodies (11),[2] it is undetectable by immunoblotting with αPY antibodies, which amplifies the signal on phosphorylated IRS-1 and detects the related 4PS (11, 38). Furthermore, expression of IRS-1 protein

[2] M. F. White, J. H. Pierce, M. G. Myers, Jr., and L. M. Wang, unpublished data.

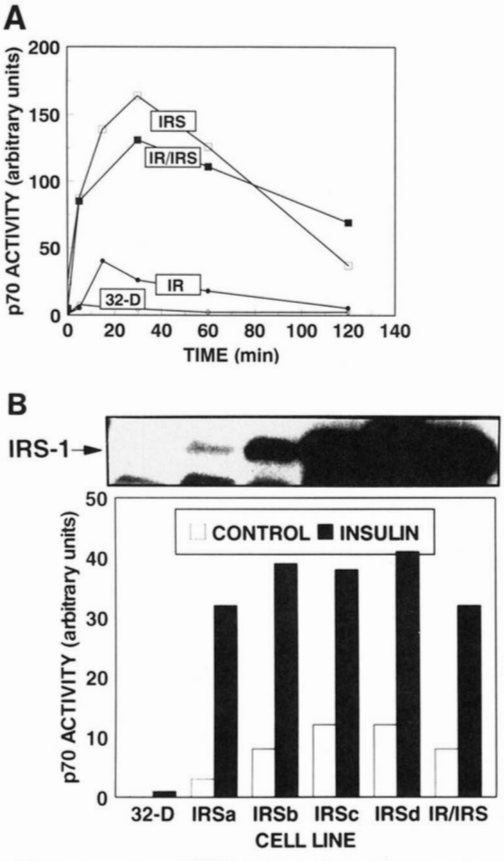

FIG. 5. **Time course and IRS-1 dose dependence of p70^{S6k} activation in 32D cell lines.** *A*, 32D cell lines were stimulated with 100 nM insulin for the indicated times, lysed, and assayed for p70^{S6k} activity. Phosphate incorporation was quantified on a PhosphorImager. *B*, 32D cells, 32D cell lines expressing various levels of IRS-1, and 32D^{IR}/IRS-1 cells were stimulated for 30 min with 100 nM insulin, lysed, and assayed for p70^{S6k} activity. Phosphate incorporation was quantified on a PhosphorImager. *Inset*, lysates from the cell lines in *B* were resolved by SDS-PAGE and immunoblotted with αIRS-1 antibodies. Migration of IRS-1 is indicated. The data shown are representative of several independent experiments.

from exogenous promoters is required for insulin-mediated mitogenesis, suggesting that if any small amount of IRS-1 exists in these cells, it is insufficient to function by this measure of insulin signaling. Moreover, association of tyrosine-phosphorylated IRS-1 with PI 3′-kinase is undetectable in cells expressing only the insulin receptor. Using these 32D cells and derivative lines expressing insulin receptor and/or IRS-1, we have previously defined a role for IRS-1 as a component of MAP kinase activation by insulin (7), although the insulin receptor is able to stimulate pathways which partially activate MAP kinase in the absence of IRS-1. Here, we demonstrate an absolute requirement for IRS-1 in signaling to p70^{S6k} and PI 3′-kinase during insulin stimulation (Fig. 7). Furthermore, IRS-1 plays a similar role in p70^{S6k} and PI 3′-kinase during signaling by IGF-1 and IL-4.

We observed that IRS-1 is required for the association of PI 3′-kinase with insulin-stimulated tyrosyl phosphoproteins in αIRS-1, αPY, and αIR immunoprecipitates. Thus, the majority of PI 3′-kinase associated with the insulin receptor in most cell types (which express some IRS-1) is likely associated indirectly with the insulin receptor by binding receptor-associated IRS-1 (25). We previously reported that IRS-1 forms stable complexes with the insulin receptor (2) and that overexpression of IRS-1 increases the amount of PI 3′-kinase associated with the insulin receptor during insulin stimulation (25). This suggested that some αIR-precipitable PI 3′-kinase activity represents PI 3′-kinase bound to insulin receptor-associated IRS-1 and not bound directly to the receptor. Our data here suggest that virtually no PI 3′-kinase associates directly with the insulin receptor *in vivo*. Although the insulin receptor directly associates with and activates PI 3′-kinase *in vitro* (23), this pathway does not operate in the absence of IRS-1 in the intact cell, as expression of receptor alone in 32D^{IR} cells is insufficient for detectable activation of PI 3′-kinase in αp85 immunoprecipitates. Moreover, mutation of the C-terminal phosphorylation sites on the insulin receptor (those shown to activate PI 3′-kinase *in vitro*) does not detectably alter PI 3′-kinase activation or PI 3′-kinase/insulin receptor association *in vivo* (24). In contrast, mutation of the insulin receptor juxtamembrane region, which is required for IRS-1-mediated events, abrogates insulin-stimulated PI 3′-kinase and growth (24). Thus, by multiple criteria, IRS-1 is an essential component in insulin signaling to PI 3′-kinase in many cells and tissues.

Homologous knockout of IRS-1 from mice impairs organismal growth and insulin metabolic signaling. Furthermore, it reduces, but does not eliminate, insulin-stimulated PI 3′-kinase from several tissues.[3] Thus, alternative routes must exist for the activation of PI 3′-kinase by insulin. Our data demonstrate that the insulin receptor alone is insufficient to mediate PI 3′-kinase signaling, hence another mediator must exist. One promising candidate for this mediator is 4PS, a 185-kDa IRS-1-related protein which is tyrosine-phosphorylated during insulin, IGF-1, and IL-4 stimulation of several myeloid cell lines, but which is absent in 32D cells (11). Although 4PS co-migrates with IRS-1 during SDS-PAGE, it reacts poorly if at all with αIRS-1 antibodies; interestingly, a similar protein is found in αp85 immunoprecipitates from tissues of IRS-1 knockout mice.[3]

In parental 32D and 32D^{IR} cells, insulin, IGF-1, and IL-4 are unable to activate p70^{S6k}. These growth factors activate p70^{S6k} in 32D/IRS or 32D^{IR}/IRS-1 cells, however, demonstrating that IRS-1 lies on the pathway between the receptors for these factors and p70^{S6k}. The hormone sensitivity is increased by co-expression of the insulin receptor suggesting a role for tyrosine phosphorylation of IRS-1; although, above some low threshold, increases in the amount of tyrosine-phosphorylated IRS-1 fail to further activate p70^{S6k}. Thus, relative to IRS-1 mediation of mitogenesis, MAP kinase activation, and PI 3′-kinase activation, activation of p70^{S6k} is very sensitive to IRS-1.

9 of 21 potential (and 5 of the 8 known) tyrosine phosphorylation sites on IRS-1 lie in canonical PI 3′-kinase recognition motifs (1, 3), and low levels of incompletely phosphorylated IRS-1 bind PI 3′-kinase, although they do not effectively activate it. The relative importance of PI 3′-kinase/phosphoprotein associations and PI 3′-kinase activation in signal transduction is unknown. However, during the analysis of *in vivo* phospholipids in 32D cell lines, we have noted high levels of 3′-phosphorylated lipids even in unstimulated 32D cell lines[4]; these lipids may be a product of the novel G protein-regulated PI 3-kinase recently characterized in myeloid cells (39). Thus, the association of PI 3′-kinase with the signaling complex may be the important factor in signal transmission, rather than the absolute level of 3′-phosphorylated phosphoinositides. The homology between PI 3′-kinase p110 and the yeast protein trafficking protein Vps34 (40) suggests that such an association could be required for proper trafficking of a signaling complex.

While the signaling element(s) engaged by IRS-1 to mediate p70^{S6k} activation is unknown, the sensitivity of the p70^{S6k} response to low levels of incompletely phosphorylated IRS-1 suggests that PI 3′-kinase binding may be involved. Indeed, recent

[3] C. R. Kahn, personal communication.
[4] M. G. Myers, Jr. and M. F. White, unpublished data.

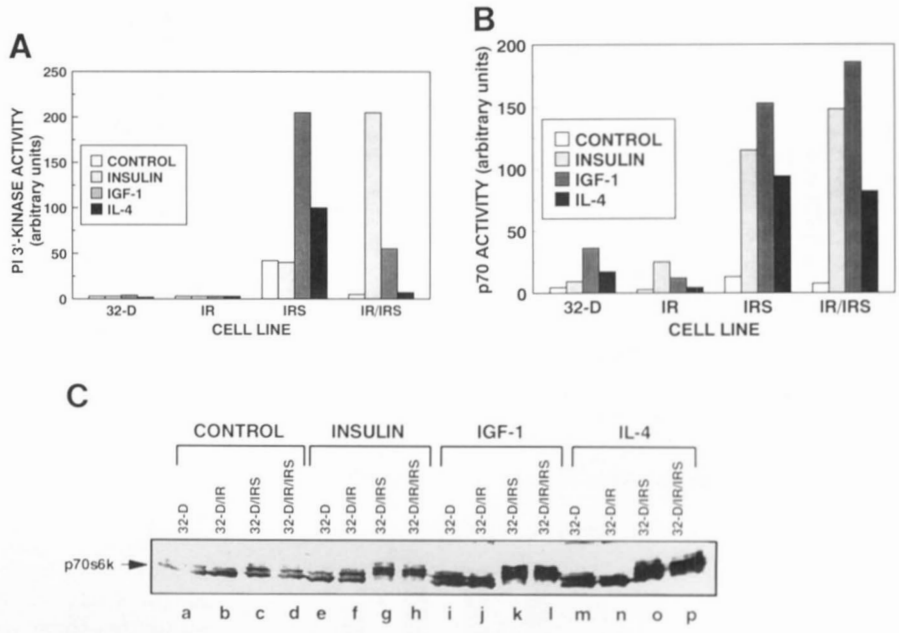

FIG. 6. **Formation of PI 3′-kinase/IRS-1 complexes and p70^{S6k} activation during stimulation with IGF-1 or IL-4 in 32D cell lines.** 32D cell lines were stimulated with 10 nM insulin, 10 nM IGF-1, or 50 ng/ml IL-4 and lysed. *A*, IRS-1 was immunoprecipitated, and associated PI 3′-kinase activity was assayed. Activity was quantified on a PhosphorImager. *B*, p70^{S6k} activity was determined by *in vitro* immune complex assay. *C*, 32D cell lysates were resolved by SDS-PAGE, and the migration of p70^{S6k} was determined by immunoblotting. Migration of p70^{S6k} is indicated. The data shown are representative of two independent experiments.

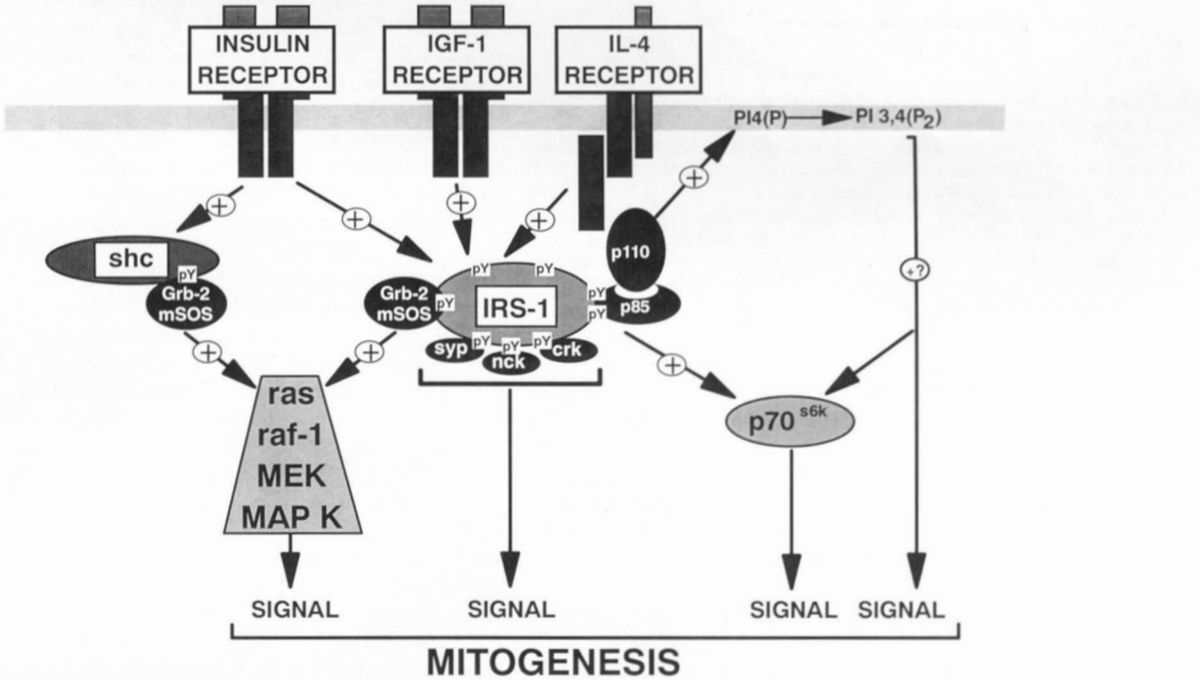

FIG. 7. **The role of IRS-1 in signal transmission.** Engagement of tyrosine kinases during stimulation with insulin, IGF-1, or IL-4 results in the tyrosine phosphorylation of IRS-1 and the recruitment of SH2 domain-containing signaling molecules to these sites of phosphorylation. IRS-1 binds PI 3′-kinase and is an essential link in the pathway(s) leading to activation of p70^{S6k} and mitogenesis. There is evidence linking PI 3′-kinase to mitogenic signaling and p70^{S6k} activation. During insulin signaling, IRS-1 plays a requisite role in PI 3′-kinase signaling. The insulin receptor engages the Shc and MAP kinase pathways independently of IRS-1, although IRS-1 contributes to MAP kinase signaling by binding to GRB-2/mSos. The relative roles of IRS-1 and Shc in MAP kinase signaling are not known in IGF-1 and IL-4 signaling.

data suggest a central role for PI 3′-kinase in p70^{S6k} activation (21, 32). The PI 3′-kinase association sites on the platelet-derived growth factor receptor are required for activation of p70^{S6k} in response to platelet-derived growth factor stimulation (32). Furthermore, LY294002 and wortmannin, two unrelated inhibitors of PI 3′-kinase, abrogate p70^{S6k} activation in response to insulin, platelet-derived growth factor, and IL-2 (21, 32).

That is not to say, however, that signaling elements in addition to PI 3′-kinase may not be required for the activation of p70^{S6k}. Furthermore, the role of PI 3′-kinase in signal transduction is not well defined: PI 3′-kinase activation appears to be required for movement of the facilitated glucose transporter GLUT4 (18, 21), which is on a divergent pathway from p70^{S6k} (36), as well as for mitogenesis (14–17), chemotaxis (19), and receptor endocytosis (20). The involvement of PI 3′-kinase in

multiple disparate responses suggests that it may not activate specific signaling pathways, but facilitate the activation of multiple pathways, perhaps by controlling vesicle movement.

The ability of high doses of insulin to activate p70^{S6k} but not mitogenesis in 32D/IRS-1 cells suggests that activation of p70^{S6k} is not sufficient for mitogenesis. Similarly, in 32D^{IR} cells, insulin activates p21$^{H\text{-}ras}$ and MAP kinase in response to insulin, although these cells fail to respond mitogenically to insulin (7). Therefore, neither p21$^{H\text{-}ras}$, MAP kinase, nor p70^{S6k} is sufficient for mitogenic signaling, but the combination of these three and possibly others may be required. This delineation of IRS-1-independent (insulin receptor-dependent) and IRS-1-dependent signaling pathways, neither of which is sufficient for the mediation of mitogenic signaling, underscores the importance of coordinating multiple signaling pathways in the generation of complex biological phenomena.

Acknowledgments—We thank Bentley Cheatham, Jon Backer, Mary Elizabeth Patti, and Lynne Yenush for helpful discussions, Ron Kahn for communication of unpublished data, and Charles Knickley for excellent technical assistance. Thanks to Montse Miralpeix and Tomo Asano for help in the production of αp85 antibodies.

REFERENCES

1. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77
2. Sun, X. J., Miralpeix, M., Myers, M. G., Jr., Glasheen, E. M., Backer, J. M., Kahn, C. R., and White, M. F. (1992) *J. Biol. Chem.* **267,** 22662–22672
3. Myers, M. G., Jr., and White, M. F. (1993) *Diabetes* **42,** 643–650
4. Backer, J. M., Myers, 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.* **11,** 3469–3479
5. Myers, M. G., Jr., Backer, J. M., Sun, X. J., Shoelson, S. E., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354
6. Skolnik, E. Y., Lee, C. H., Batzer, A. G., Vicentini, L. M., Zhou, M., Daly, R. J., Myers, M. G., Jr., Backer, J. M., Ullrich, A., White, M. F., and Schlessinger, J. (1993) *EMBO* **12,** 1929–1936
7. Myers, M. G., Jr., Wang, L.-M., Sun, X. J., Zhang, Y., Yenush, L. P., Schlessinger, J., Pierce, J. H., and White, M. F. (1994) *Mol. Cell. Biol.* **14,** 3577–3587
8. Lechleider, R. J., Freeman, R. M., Jr., and Neel, B. G. (1993) *J. Biol. Chem.* **268,** 13434–13438
9. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A., Myers, M. G., Jr., White, M. F., Schlessinger, J., and Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11713–11717
10. Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993) *Mol. Cell. Biol.* **13,** 7418–7428
11. Wang, L. M., Myers, M. G., Jr., Sun, X. J., Aaronson, S. A., White, M. F., and Pierce, J. H. (1993) *Science* **261,** 1591–1594
12. Ruderman, N., Kapeller, R., White, M. F., and Cantley, L. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1411–1415
13. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302
14. Coughlin, S. R., Escobedo, J. A., and Williams, L. T. (1989) *Science* **243,** 1191–1193
15. Escobedo, J. A., and Williams, L. T. (1988) *Nature* **335,** 85–87
16. Escobedo, J. A., Kaplan, D. R., Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1991) *Mol. Cell. Biol.* **11,** 1125–1132
17. Valius, M., and Kazlauskas, A. (1993) *Cell* **73,** 321–334
18. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3568–3573
19. Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T., and Zetter, B. R. (1994) *Nature* **367,** 474–476
20. Joly, M., Kazlauskas, A., Fay, F. S., and Corvera, S. (1994) *Science* **263,** 684–687
21. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14,** 4902–4911
22. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, J. C. (1993) *Cell* **72,** 767–778
23. Van Horn, D. J., Myers, M. G., Jr., and Backer, J. M. (1994) *J. Biol. Chem.* **269,** 29–32
24. Backer, J. M., Schroeder, G., Kahn, C. R., Myers, M. G., Jr., Wilden, P. A., Cahill, D. A., and White, M. F. (1992) *J. Biol. Chem.* **267,** 1367–1374
25. Backer, J. M., Myers, M. G., Jr., Sun, X. J., Chin, D. J., Shoelson, S. E., Miralpeix, M., and White, M. F. (1993) *J. Biol. Chem.* **268,** 8204–8212
26. Blenis, J., Chung, J., Erikson, E., Alcorta, D. A., and Erikson, R. L. (1991) *Cell Growth & Differ.* **2,** 279–285
27. Erikson, R. L. (1991) *J. Biol. Chem.* **266,** 6007–6010
28. Pelech, S. L., and Sanghera, J. S. (1993) *Trends Biochem. Sci.* **17,** 233–238
29. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) *Cell* **68,** 1041–1050
30. McCormick, F. (1993) *Nature* **363,** 15–16
31. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) *Cell* **69,** 1227–1236
32. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) *Nature* **370,** 71–75
33. White, M. F., and Backer, J. M. (1991) *Methods Enzymol.* **201,** 65–79
34. Miralpeix, M., Sun, X. J., Backer, J. M., Myers, M. G., Jr., Araki, E., and White, M. F. (1992) *Biochemistry* **31,** 9031–9039
35. Towbin, H., Staehelin, T., and Gordon, G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
36. Fingar, D. C., Hausdorff, S. F., Blenis, J., and Birnbaum, M. J. (1993) *J. Biol. Chem.* **268,** 3005–3008
37. Myers, M. G., Jr., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., and White, M. F. (1993) *Endocrinology* **132,** 1421–1430
38. Wang, L.-M., Keegan, A. D., Li, W., Lienhard, G. E., Pacini, S., Gutkind, J. S., Myers, M. G., Jr., Sun, X. J., White, M. F., Aaronson, S. A., Paul, W. E., and Pierce, J. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4032–4036
39. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1994) *Cell* **77,** 83–93
40. Hiles, I. D., Otsu, M., Volinna, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1992) *Cell* **70,** 419–429