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Stimulation of Protein Synthesis, Eukaryotic Translation Initiation Factor 4E Phosphorylation, and PHAS-I Phosphorylation by Insulin Requires Insulin Receptor Substrate 1 and Phosphatidylinositol 3-Kinase

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Insulin rapidly stimulates protein synthesis in a wide variety of tissues. This stimulation is associated with phosphorylation of several translational initiation and elongation factors, but little is known about the signaling pathways leading to these events. To study these pathways, we have used a myeloid progenitor cell line (32D) which is dependent on interleukin 3 but insensitive to insulin because of the very low levels of insulin receptor (IR) and the complete lack of insulin receptor substrate (IRS)-signaling proteins (IRS-1 and IRS-2). Expression of more IR permits partial stimulation of mitogen-activated protein kinase by insulin, and expression of IRS-1 alone mediates insulin stimulation of the 70-kDa S6 kinase ($pp70^{S6K}$) by the endogenous IR. However, expression of both IR and IRS-1 is required for stimulation of protein synthesis. Moreover, this effect requires activation of phosphatidylinositol 3-kinase (PI3K), as determined by wortmannin inhibition and the use of an IRS-1 variant lacking all Tyr residues except those which activate PI3K. Stimulation of general protein synthesis does not involve activation by IRS-1 of GRB-2–SOS–$p21^{ras}$ or SH-PTP2, since IRS-1 variants lacking the SH2-binding Tyr residues for these proteins are fully active. Nor does it involve $pp70^{S6K}$, since rapamycin, while strongly inhibiting the synthesis of a small subset of growth-regulated proteins, only slightly inhibits total protein synthesis. Recruitment of mRNAs to the ribosome is enhanced by phosphorylation of eIF4E, the cap-binding protein, and PHAS-I, a protein that specifically binds eIF4E. The behavior of cell lines containing IRS-1 variants and inhibition by wortmannin and rapamycin indicate that the phosphorylation of both proteins requires IRS-1-mediated stimulation of PI3K and $pp70^{S6K}$ but not mitogen-activated protein kinase or SH-PTP2.

Insulin influences a variety of cellular activities. It modulates glucose and amino acid transport; activates key enzymes of intermediary metabolism; increases the rates of protein, DNA, and RNA synthesis; enhances transcription and translation of specific genes; and generally promotes cellular growth and differentiation (53). These actions are mediated through the insulin receptor (IR), which phosphorylates itself as well as substrates such as the insulin receptor substrate (IRS)-signaling proteins (IRS-1 and IRS-2) and Shc (53, 60). The phosphorylated Tyr residues bind directly to proteins containing Src homology 2 domains (SH2 proteins) in a sequence-specific manner, leading to the activation of a wide variety of enzymatic activities. IRS-signaling proteins are required for insulin stimulation of phosphatidylinositol 3-kinase (PI3K), the Tyr phosphatase SH-PTP2, mitogen-activated protein kinase (MAPK), and the 70-kDa S6 kinase ($pp70^{S6K}$). Disruption of the IRS-1 gene in mice is not lethal but causes mild insulin resistance (56), a finding which led to the discovery of a second IRS protein, IRS-2 (55).

One of the principal end points of insulin action is the stimulation of protein synthesis. Insulin induces both a general increase in the rate of mRNA translation and preferential increases in the translation of specific mRNAs, e.g., mRNAs with polypyrimidine tracts in their 5′ untranslated regions (20, 37, 57) and mRNAs rich in secondary structure (32). Such mRNAs encode "growth-regulated" proteins which increase disproportionately during rapid growth and include those required for S phase events, components of the translational machinery, and transcription factors (2). Insulin appears to regulate both the initiation and elongation phases of translation (22), presumably by altering the phosphorylation of eukaryotic translation initiation factors (eIF2, eIF2B, eIF3, eIF4B, eIF4E, and eIF4G) and eukaryotic elongation factors (eEF1 and eEF2). (The names of initiation, elongation, and termination factors were revised on 8 April 1995 by an expert panel [Marianne Grunberg-Manago, convener] appointed by the IUBMB Nomenclature Committee. The new names are used in the present article. eIF4G was formerly referred to as either p220, eIF-4γ, or eIF-4Fγ.)

Despite the large number of protein synthesis factors phosphorylated in response to insulin, the specific effects of these phosphorylations on the overall rate of translation are not known in most cases. One of the best-characterized systems is the phosphorylation of eIF4E, the mRNA cap-binding protein. Phosphorylation of eIF4E correlates with an increase in the rate of protein synthesis under a variety of in vivo conditions, including stimulation by insulin (reviewed in reference 48). Phosphorylation of eIF4E increases its affinity for the cap structure of mRNA by three- to fourfold (35). The availability of eIF4E for translation is also regulated by insulin. In order for eIF4E to recruit mRNA to the ribosome, it must bind

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to the ribosome-associated factor eIF4G (26). The protein PHAS-I (also known as 4E-BP1) sequesters eIF4E in an inactive complex, but phosphorylation of PHAS-I in response to insulin causes release of the eIF4E (29, 47). The amino acid sequence in eIF4G which binds to eIF4E is homologous to the sequence in PHAS-I which binds to eIF4E (31), and PHAS-I competes with eIF4G for binding eIF4E, thereby preventing eIF4E (and mRNA) from binding to the 48S initiation complex (17). The identity of the physiological kinase which phosphorylates eIF4E is not clear. eIF4E is phosphorylated in vitro by protein kinase C, casein kinase I, cyclic GMP-dependent protein kinase, and an insulin-stimulated protamine kinase (reviewed in reference 22), but in vivo experiments are lacking. Similarly, in vitro studies showed that PHAS-I is phosphorylated by MAPK (29) and that the same site is phosphorylated in vivo in response to insulin (19). However, in vivo studies showed that rapamycin inhibited PHAS-I phosphorylation without affecting MAPK while PD098059 inhibited MAPK but not PHAS-I phosphorylation (30), arguing against the phosphorylation of PHAS-I by MAPK.

Thus, although much has been learned about both the signal transduction pathways initially activated by insulin and the phosphorylation of initiation and elongation factors, the pathways which link insulin and protein synthesis are poorly understood. To characterize these pathways better, we have utilized a panel of cell lines which differ in the presence or absence of key components of the insulin signaling system. 32D is a myeloid progenitor cell line which is dependent on interleukin 3 (IL-3) but insensitive to insulin (59). 32D cells contain only small amounts of IR and completely lack IRS-1 and IRS-2. However, coexpression of IR and IRS-1 from cDNA constructs confers an insulin-induced mitogenic response (59). Thus, these cells enable one to investigate the signals mediating the stimulation of protein synthesis by insulin in the presence and absence of IR and IRS-1.

MATERIALS AND METHODS

Materials. [^{35}S]Met, [γ-^{32}P]ATP, and $^{32}P_i$ were purchased from DuPont-NEN. Antibodies against PI3K and MAPK were from UBI, Inc. (Lake Placid, N.Y.). Antibodies against pp70^{S6K} (38) and myc (9) have been described previously. Antibodies were raised against PHAS-I and affinity purified (33), using as the immunogen synthetic peptides corresponding to amino acid residues 69 to 82 and 101 to 114 of rat 4E-BPI (47) but with an additional C-terminal Cys residue and substitution of Val for Gly at position 109. The peptides (KTPPRDLPTIPG VTC and SPEDKRAGVEESQFC) were synthesized by Bio-Synthesis, Inc. (Lewisville, Tex.). Myelin basic protein, phosphatidylinositol, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma. m^7 GTP-Sepharose, protein A-Sepharose, and histidinol were purchased from Pharmacia, Pierce, and Bachem Bioscience, respectively. Insulin was from GIBCO and ELANCO (Indianapolis, Ind.).

Cell lines. WEHI-3 cells were purchased from the American Type Culture Collection and maintained at 37°C in RPMI 1640 containing 10% fetal calf serum. A variety of cell lines, all derived from the myeloid precursor 32D (59) but stably transfected with cDNAs for IR, IRS-1, or variants of IRS-1, were used in this study. IR/− cells express human IR but not IRS-1, −/IRS-1 cells express rat IRS-1 but not exogenous IR, etc. The cells used were 32D (−/− [59]), 1C (−/IRS-1 [59]), 5WC (IR/− [59]), 10W1 (IR/IRS-1 [59]), 329.9 (IR/IRS-1 [39]), 370.1W6 (IR/IRS-1^{Y895F} [40]), 665.2W2 (IR/IRS-1^{F18} [41]), and 665.3W5 (IR/IRS-1$^{Y608–658}$ [41]), as well as 418.2W1, 429.2W, and 429.2W2 (all IR/IRS-1$^{Y1172F/Y1222F}$ [39]). The cell lines were maintained at 37°C in RPMI 1640 containing 10% fetal bovine serum and 10% conditioned medium from WEHI-3 cells as a source of IL-3 (38, 40). The cell lines were tested for similar levels of IRS-1 expression, and whenever possible the experiments were repeated in several equivalent cell lines to check for clonal variability.

Incorporation of trichloroacetic acid-precipitable [^{35}S]Met. 32D cells and their derivatives were maintained at concentrations of 5×10^5/ml and starved for IL-3 in RPMI 1640 containing 10% fetal calf serum for 6 h. After this period, 1-ml aliquots were taken and the agent under study was added as indicated in the figure legends. Cells were pulse-labeled with 10 μCi of [^{35}S]Met for 60 min. After being washed once with phosphate-buffered saline (PBS) containing 10 mM Met, cells were lysed in 0.5 M NaOH for 30 min at 37°C. Protein was precipitated with 12% ice-cold trichloroacetic acid containing 10 mM Met, collected on glass fiber filters (GF/C; Whatman), and washed with 5% trichloroacetic acid and ethanol. The filters were air dried and subjected to liquid scintillation spectrometry in an aqueous fluor.

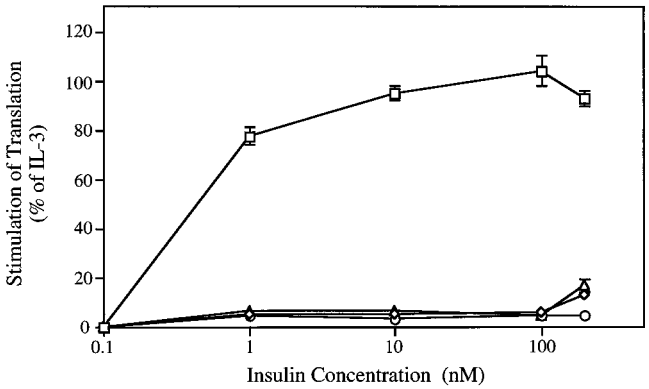

FIG. 1. Stimulation of translation by insulin in myeloid cells containing IR and IRS-1. 32D-derived cell lines (−/−, circles; IR/−, diamonds; −/IRS-1, triangles; and IR/IRS-1, squares) were starved for IL-3 and then grown in the indicated concentrations of insulin for 1 h. Protein synthesis was determined as described in Materials and Methods. Data are graphed as stimulation of treated cells (I) above control cells (no additions [C]) expressed as a percentage of the stimulation by IL-3 in WEHI-3 medium (W) versus control cells, i.e., (I − C)/(W − C) × 100. Protein synthesis under each condition for each cell line was determined in triplicate, and two to four independent experiments were conducted for each cell line. Error bars represent the standard errors of the means for all experiments.

MAPK, pp70^{S6K}, and PI3K activities. Cells in log-phase growth were washed and starved for IL-3 as described above, and 15-ml aliquots (8×10^6 cells) were treated with the various agents described in the figure legends. For MAPK, the quiescent cells were treated for 5 min at 37°C, diluted in ice-cold PBS, and collected by centrifugation. Cell lysis and assay of MAPK after immunoprecipitation were performed exactly as described previously (40). Measurement of pp70^{S6K} activity by immunoblotting was performed as previously described (6). For determination of PI3K activity, cells were treated for 10 min at 37°C, washed, immunoprecipitated with anti-PI3K antibodies, and assayed for PI3K activation as previously described (38).

Preparation of ^{32}P-labeled eIF4E and PHAS-I. Cells in log-phase growth (4×10^8 to 10×10^8) were washed and starved for IL-3 as described above. They were then incubated for 1 h in 10 to 15 ml of phosphate-free RPMI to which was added $^{32}P_i$ at 0.4 mCi/ml and for another 45 min with various agents as indicated in the figure legends. Cells were washed with ice-cold PBS and lysed with 4 ml of ice-cold 10 mM potassium phosphate containing 1 mM EDTA, 0.5% Nonidet P-40, 5 mM EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg each of aprotinin and leupeptin per ml. Insoluble material was removed by centrifugation, and the supernatant was diluted with 20 volumes of the same buffer without Nonidet P-40. The eIF4E was purified by m^7 GTP-Sepharose chromatography as previously described (21), and the PHAS-I was purified by immunoprecipitation (33) with both of the anti-PHAS-I antibodies described above. In both cases, the proteins were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

RESULTS

Both IR and IRS-1 are required for stimulation of translation by insulin. 32D cells starved for IL-3 were unresponsive to insulin with respect to protein synthesis (Fig. 1). Neither overexpression of IR alone nor overexpression of IRS-1 alone conferred responsiveness to insulin. Coexpression of IR and IRS-1, however, permitted a dose-dependent response to insulin, the final degree of stimulation being the same as with IL-3. The time course of mitogen action indicated that both IL-3 and insulin exerted maximal effects within the first hour of treatment and that the stimulation persisted for at least 6 h (data not shown). In IR/− cells, insulin mediates partial activation of MAPK through Shc, and in −/IRS-1 cells, insulin fully stimulates pp70^{S6K}, which needs only low levels of Tyr

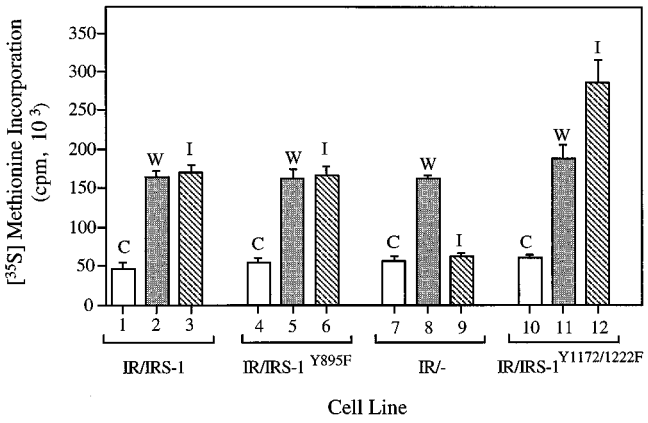

FIG. 2. Alteration of the GRB-2 or SH-PTP2 binding sites of IRS-1 does not diminish insulin-stimulated translation. The designated IL-3-starved cell lines were grown with no additions (C), in the presence of 10% WEHI-3 medium as a source of IL-3 (W), or in the presence of 100 nM insulin (I) for 1 h. Protein synthesis was determined as described in Materials and Methods. Data are the averages of at least three determinations and are graphed as total radioactivity. Error bars represent standard errors of the means.

phosphorylation in IRS-1 to become fully activated (38, 40). From the results shown in Fig. 1, it is clear that activation of either MAPK or pp70^{S6K} alone is not sufficient to stimulate protein synthesis.

Role of the IRS-1–GRB-2 and IRS-1–SH-PTP2 interactions in insulin-stimulated protein synthesis. Substitution of Phe for Tyr-895 prevents the binding of GRB-2 to IRS-1 both in vivo and in vitro (40), implicating this Tyr in the activation of p21^{ras} by insulin. The IRS-1^{Y895F} variant allowed us to study the potential role of the IRS-1–GRB-2 complex in insulin-stimulated translation. Insulin caused equal stimulation of cells containing IRS-1 (Fig. 2, column 3) and of cells with IRS-1^{Y895F} (column 6) but not of those lacking IRS-1 (column 9). This strengthens the above-mentioned conclusion that an interaction between GRB-2 and IRS-1, with the resulting activation of MAPK, is not required for insulin-stimulated protein synthesis. The hypothesis that insulin does not stimulate protein synthesis in IR/− cells because of the lack of sufficient MAPK activation can be ruled out; the degree of MAPK activation by insulin is the same in IR/− and IR/IRS-1^{Y895F} cells (40), but only in the latter is protein synthesis stimulated (Fig. 2).

The Tyr phosphatase SH-PTP2 binds to IRS-1 through Tyr-1172 and Tyr-1222 (39). An IRS-1 variant in which both Tyr-1172 and Tyr-1222 were replaced with Phe (IRS-1$^{Y1172F/Y1222F}$) enhanced rather than decreased the degree of insulin-stimulated protein synthesis compared with wild-type IRS-1 (Fig. 2, column 12). This suggests that IRS-1 is more effective in the absence of an SH-PTP2 interaction and that SH-PTP2 may down-regulate IRS-1 with regard to its effect on protein synthesis.

PI3K is required for insulin-stimulated protein synthesis. The preceding experiments indicated that the positive elements recruited into the insulin signaling pathway by IRS-1 for translation do not include GRB-2 or SH-PTP2. Another SH2 protein which associates with IRS-1 is PI3K (50). The fungal metabolite wortmannin has been shown to bind the 110-kDa catalytic subunit of PI3K, inhibiting its activity both in vivo and in vitro (46, 62). When tested in IR/IRS-1 cells, wortmannin prevented the stimulation of translation by both insulin and IL-3 but had no effect on protein synthesis in control (unstimulated) cells (Fig. 3A). The concentration of wortmannin necessary to reduce insulin-stimulated translation by 50% (10 nM) was the same as that needed for 50% inhibition of PI3K activity and insulin-induced membrane ruffling (23).

In some cell lines, such as A431 and the rat skeletal muscle cell line L6, wortmannin inhibits not only PI3K but also MAPK (7, 51), whereas in other cell lines, such as 3T3-L1, CHO, and PC-12, only PI3K is inhibited (4, 18, 63). To determine whether the observed inhibition of insulin-stimulated translation by wortmannin was due to an inhibition of either MAPK or PI3K, we measured both enzymes in IR/IRS-1 cells. IL-3 and insulin induced MAPK activity to the same maximal levels obtained with the control treatment of TPA (Fig. 4A, columns 2 to 4). IL-3 and insulin activated PI3K (Fig. 4B, columns 2 and 3), but TPA had a much smaller effect (column 4). Wortmannin completely inhibited the insulin-induced activation of PI3K (Fig. 4B, column 5) but had no effect on MAPK activity (Fig. 4A, column 5). These results suggest that the inhibition of insulin-stimulated protein synthesis by wortmannin is due to an effect on PI3K but not MAPK, although other targets of wortmannin are known (see Discussion).

Wortmannin also inhibits phosphatidylinositol 4-kinase, although 10-fold higher concentrations are required than are needed to inhibit PI3K (43). The observed sensitivity of insulin-stimulated protein synthesis to wortmannin (Fig. 3A) matched that of PI3K. Nonetheless, we took an independent approach to test the involvement of PI3K. In cells containing the IRS-1^{F18} variant, in which all 18 Tyr residues from amino acid residues 147 to 1222 were changed to Phe (41), translation was stimulated by insulin only slightly more than in IR/− control cells (Fig. 5). Adding the Tyr residues which are involved in binding and activation of PI3K (Tyr-608, -628, and -658 [41]) back to IRS-1^{F18} restored the responsiveness of

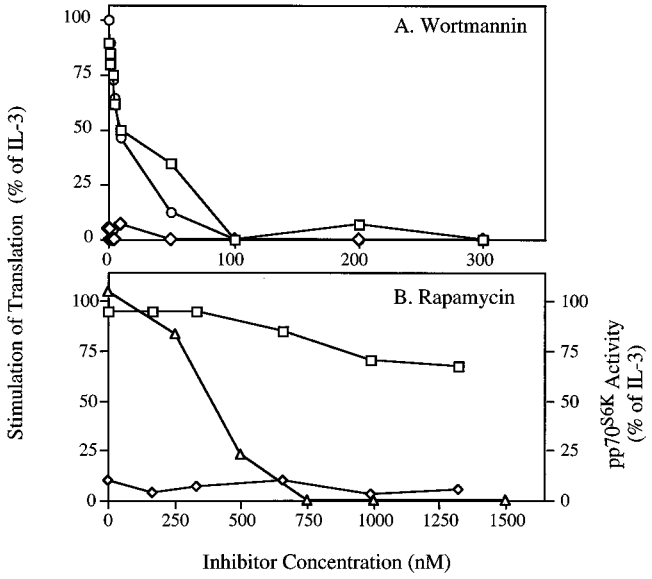

FIG. 3. Wortmannin, but not rapamycin, inhibits the stimulation of general translation by insulin. IL-3-starved IR/IRS-1 cells were grown for 1 h in the absence (diamonds) or presence (squares) of 100 nM insulin or 10% WEHI-3 medium (circles) with increasing concentrations of wortmannin (A) or rapamycin (B). Protein synthesis was determined as described in Materials and Methods. The data represent the averages of three independent experiments, graphed as described in the legend to Fig. 1, except that the positive control cells (W) contained no rapamycin or wortmannin. The activation of pp70^{S6K} (triangles) was determined as described in Materials and Methods and is expressed as a percentage of the stimulation by WEHI-3 medium above control cells containing no rapamycin.

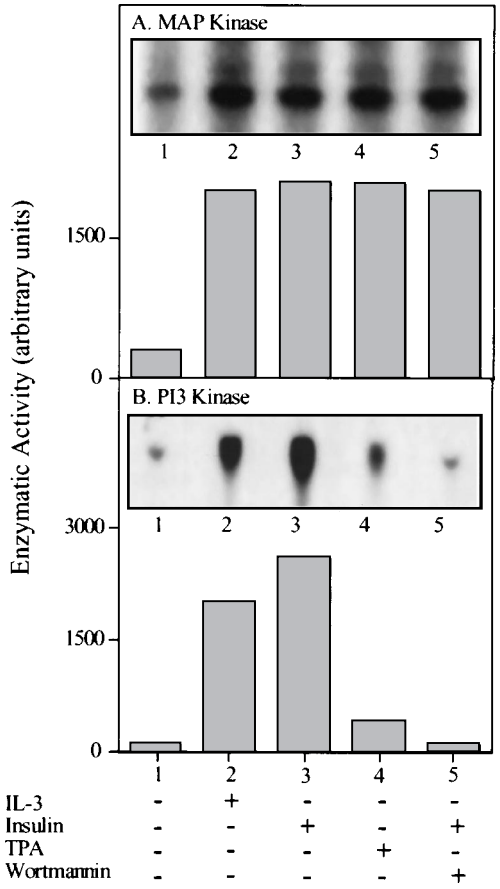

FIG. 4. Wortmannin inhibits PI3K but not MAPK. IL-3-starved IR/IRS-1 cells were treated with medium containing no additions (lanes 1), 10% WEHI-3 medium (lanes 2), 100 nM insulin (lanes 3), 1 μM TPA (as a positive control for MAPK; lanes 4), or 100 nM insulin plus 80 nM wortmannin (lanes 5). MAPK (A) and PI3K (B) were assayed as described in Materials and Methods. In both cases, the insets show autoradiograms prepared after SDS-PAGE (A) and thin-layer chromatography (B); the autoradiograms were scanned, and the results are presented as histograms.

protein synthesis to insulin. In IR/IRS-$1^{Y608–658}$ cells, the response was 80% of that of IL-3 at higher insulin concentrations and more than twice that of IL-3 at lower insulin concentrations. The stimulation by insulin was prevented by wortmannin (data not shown). These data indicate that PI3K is an obligatory component of the pathway leading from insulin to protein synthesis. Since IRS-$1^{Y608–658}$ does not contain Tyr-1172 and -1222, the more appropriate control cell line may be IR/IRS-$1^{Y1172F/Y1222F}$ (Fig. 2). The IR/IRS-$1^{Y608–658}$ cells were 50 to 60% as responsive to insulin as IR/IRS-$1^{Y1172F/Y1222F}$, indicating that PI3K alone is not sufficient to induce a full stimulation of protein synthesis and that other pathways mediated by IRS-1 may enhance the signal.

$pp70^{S6K}$ is required for insulin-stimulated synthesis of specific proteins but not for general stimulation of translation. To trace the signaling pathway further, we investigated potential components downstream of PI3K. Activation of PI3K leads to the stimulation of $pp70^{S6K}$ (5). The macrolide rapamycin is a specific inhibitor of $pp70^{S6K}$ (5, 6) but does not affect the activity of $pp90^{S6K}$ or MAPK (6). We therefore tested the effect of rapamycin on insulin-stimulated protein synthesis. Treatment of IR/IRS-1 cells with concentrations of rapamycin sufficient to completely inhibit $pp70^{S6K}$ caused only a minor inhibition of translation (10% at 750 nM) (Fig. 3B), suggesting that $pp70^{S6K}$ activity is not necessary for insulin stimulation of general protein synthesis.

Previous studies have shown that rapamycin preferentially inhibits translation of mRNAs of growth-regulated proteins (see the introduction), especially those mRNAs containing polypyrimidine tracts (20, 57). To determine whether the modest drop in protein synthesis caused by rapamycin (Fig. 3B) was due to inhibition of a selective group of mRNAs in 32D cells, we examined a typical growth-regulated protein (myc) and a typical constitutive protein (actin). Between 100 and 1000 nM rapamycin reduced myc levels to the value of unstimulated cells (Fig. 6A), but actin synthesis was virtually unchanged at the same concentrations (Fig. 6B). Synthesis of essentially all other ^{35}S-labeled proteins detectable after SDS-PAGE was similar to that of actin (data not shown). These results suggest that the increase above basal protein synthesis caused by insulin can be divided into two parts: approximately 90% is a general stimulation of translation of all mRNAs, while approximately 10% is a preferential stimulation of the growth-regulated mRNAs, i.e., those which respond specifically to activation of $pp70^{S6K}$.

eIF4E and PHAS-I phosphorylations are mediated by IRS-1, PI3K, and $pp70^{S6K}$. As reviewed in the introduction, changes in the phosphorylation of numerous initiation and elongation factors correlate with stimulation of translation by insulin, and in the case of eIF4E and PHAS-I, phosphorylation promotes mRNA recruitment to the ribosome. Insulin rapidly stimulates phosphorylation of eIF4E (32, 36) and PHAS-I (29, 47), but the signal transduction pathways and kinases involved are poorly understood. Hence, it was of interest to determine the involvement of IRS-1 and downstream pathways leading to these two phosphorylation events.

In IR/IRS-1 cells, insulin caused maximal phosphorylation of both eIF4E and PHAS-I compared with IL-3, but in IR/− cells, insulin was completely ineffective (Fig. 7). Interestingly, there was a differential response in −/IRS-1 cells: eIF4E phosphorylation was partially stimulated by insulin, while PHAS-I phosphorylation was fully stimulated. Considering that insulin does not enhance protein synthesis in −/IRS-1 cells (Fig. 1), it is apparent that eIF4E and PHAS-I phosphorylations are not sufficient for insulin-stimulated protein synthesis, although they may be necessary.

The response of eIF4E and PHAS-I phosphorylations to

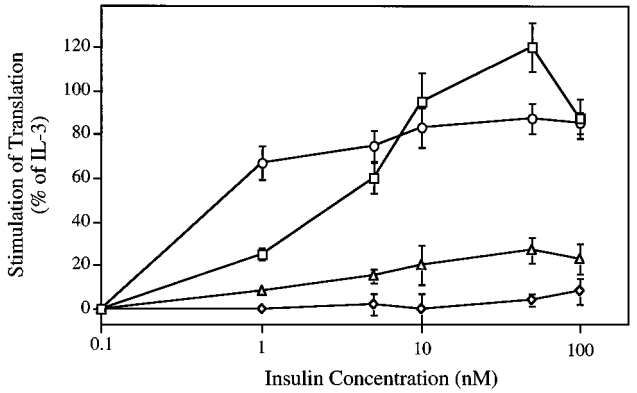

FIG. 5. Stimulation of translation by insulin in myeloid cells containing IR, IRS-1, IRS-1^{F18}, and IRS-$1^{Y608–658}$. Starved 32D-derived cell lines (IR/−, diamonds; IR/IRS-1, squares; IR/IRS-1^{F18}, triangles; and IR/IRS-$1^{Y608–658}$, circles) were grown in the presence of the indicated concentrations of insulin for 1 h, and protein synthesis was measured as described in the legend to Fig. 1.

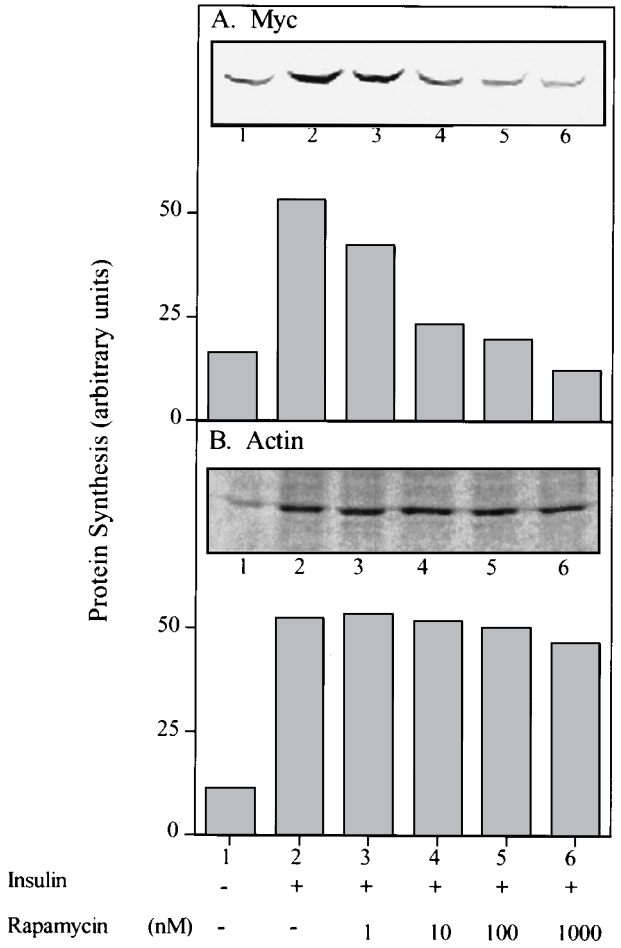

FIG. 6. Rapamycin inhibits the synthesis of myc but not actin. IL-3-starved IR/IRS-1 cells were labeled with [^{35}S]Met in medium containing no additions (lanes 1), 100 nM insulin (lanes 2), or 100 nM insulin plus 1 to 1000 nM rapamycin (lanes 3 to 6) as described in Materials and Methods, except that the labeling period was 4 h. The cells were lysed in SDS-PAGE sample buffer, briefly sonicated, and analyzed by immunoblotting with anti-myc antibody (A, inset) and SDS-PAGE autoradiography (B, inset). Actin was identified by its abundance and mobility. In both cases the insets were scanned, and the results are shown in the histograms.

insulin in the various cell lines correlated with the activation of PI3K and pp70^{S6K} (38, 40). To determine whether these phosphorylations in fact occurred downstream of PI3K and pp70^{S6K}, the inhibitors wortmannin and rapamycin were employed (Fig. 8). Insulin-stimulated phosphorylation of both proteins was inhibited by both wortmannin and rapamycin, further strengthening the hypothesis that the pathways leading from insulin to PHAS-I and eIF4E phosphorylations involve PI3K and pp70^{S6K}.

The variant forms of IRS-1 which indicated a PI3K requirement for total protein synthesis (Fig. 5) were also employed to investigate phosphorylation of eIF4E and PHAS-I (Fig. 9). The IRS-1^{F18} variant was unable to transmit the insulin signal, but restoration of Tyr-608, -628, and -658 allowed even greater insulin-stimulated phosphorylation than that of the positive control (IL-3). This indicates that the only Tyr residues in IRS-1 needed for phosphorylation of eIF4E and PHAS-I are those which activate PI3K (41). These results confirm the conclusion drawn from wortmannin inhibition (Fig. 8) that PI3K is a component of the pathway leading from insulin to eIF4E and PHAS-I phosphorylation.

DISCUSSION

A major conclusion from these studies is that insulin-stimulated protein synthesis in 32D cells requires both IR and IRS-1. The 32D cell line provides a favorable system for studying the pathways leading from insulin to protein synthesis since these cells lack IRS-1 and express very little endogenous IR. Increased expression of IR activates MAPK partially, whereas expression of IRS-1 alone is sufficient to mediate a partial activation of PI3K and a full activation of pp70^{S6K} by the endogenous IR (38, 40). Yet expression of both IR and IRS-1 is required for insulin-stimulated mitogenesis (59). Our studies indicate that both IR and IRS-1 are required for insulin-stimulated protein synthesis and, hence, that activation of MAPK or pp70^{S6K} alone cannot stimulate it.

Although numerous signaling proteins bind to IRS-1 (see

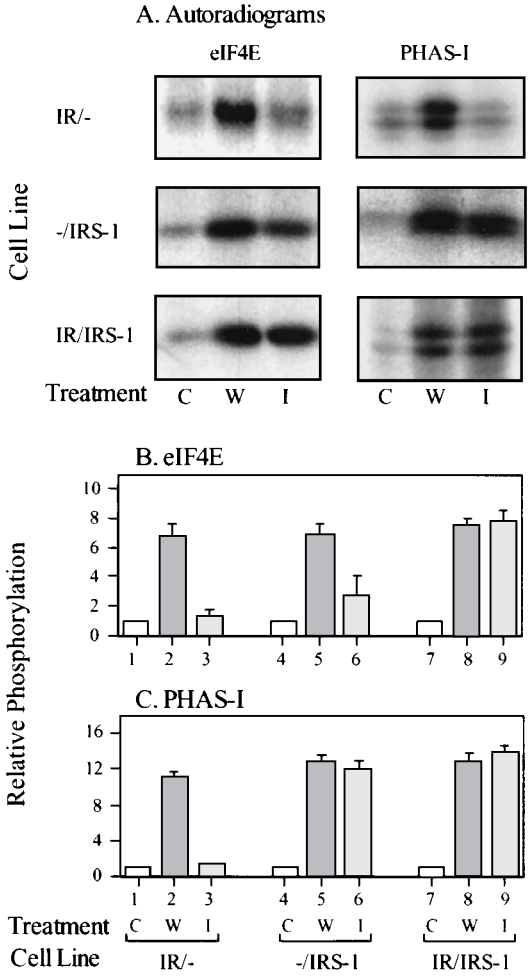

FIG. 7. Phosphorylation of eIF4E and PHAS-I in response to insulin requires IR and IRS-1. IL-3-starved IR/−, −/IRS-1, and IR/IRS-1 cells were preincubated with $^{32}P_i$ and then incubated with either no addition (C), 10% WEHI-3 medium (W), or 100 nM insulin (I) as described in Materials and Methods. eIF4E was purified by affinity chromatography, and PHAS-I was purified by immunoprecipitation. The proteins were subjected to SDS-PAGE and detected by autoradiography (A). The experiment was performed five times with IR/IRS-1 cells, twice with −/IRS-1 cells, and three times with IR/− cells. The autoradiograms were scanned and normalized to the corresponding bands from unstimulated cells (C), and the results were averaged and expressed as fold stimulation above the control (eIF4E [B] and PHAS-I [C]). Error bars represent the standard errors of the means of the different experiments, but there are no error bars for control cells since they were used in each case for normalization.

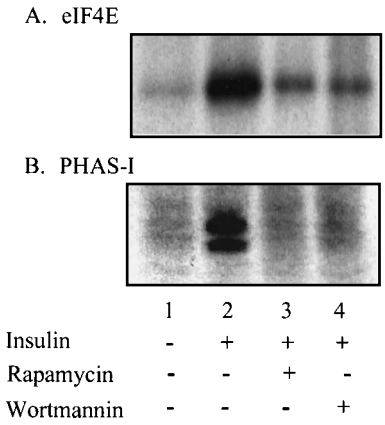

FIG. 8. eIF4E and PHAS-I phosphorylations are inhibited by wortmannin and rapamycin. IL-3-starved IR/IRS-1 cells were preincubated with $^{32}P_i$ and then incubated with either no additions (lanes 1), 100 nM insulin (lanes 2), 100 nM insulin plus 750 nM rapamycin (lanes 3), or 100 nM insulin plus 100 nM wortmannin (lanes 4). eIF4E (A) and PHAS-I (B) phosphorylations were detected as described in the legend to Fig. 7.

the introduction), the present work indicates that binding of GRB-2–SOS to IRS-1 is not required for the stimulation of protein synthesis. This conclusion is drawn from the fact that wild-type IRS-1 enhances MAPK activation by insulin but IRS-1^{Y895F} does not, underscoring the critical role for Tyr-895 in GRB-2–SOS binding, yet IRS-1 and IRS-1^{Y895F} are equally capable of mediating insulin-stimulated protein synthesis. Thus, Tyr-895 does not participate in this process. We conclude that although the p21^{ras} pathway may be necessary for insulin-stimulated protein synthesis (through Shc phosphorylation), it is not sufficient.

The second ligand of IRS-1 that can be ruled out for a positive role in protein synthesis is the Tyr phosphatase SH-PTP2. This enzyme has been implicated in the activation of p21^{ras} (45), in the dephosphorylation of IRS-1 (24), and in the insulin-induced activation of MAPK and mitogenesis (34, 61). Our finding that removing the SH2 binding site for SH-PTP2 (IRS-$1^{Y1172F/Y1222F}$) stimulates translation may indicate that SH-PTP2 acts on phosphotyrosine residues in IRS-1 that are critical for the stimulation of protein synthesis, thereby down-regulating IRS-1. Such a role has been shown for LAR, a Tyr phosphatase which can act on IR; when depleted, LAR increases the insulin response, in particular increasing PI3K by 350% (25).

Another known ligand of IRS-1 is PI3K (38). Unlike GRB-2–SOS and SH-PTP2, this enzyme is implicated in the pathway on the basis of two criteria: first, both PI3K activity and insulin-stimulated protein synthesis are inhibited by wortmannin; second, an IRS-1 molecule containing only those Tyr residues which are involved in PI3K activation, Tyr-608, -628, and -658, is also capable of mediating insulin-stimulated protein synthesis, while the IRS-1^{F18} variant lacking these Tyr residues is inactive in both properties. Yet both of these results must be interpreted with caution. First, PI3K may not be the only enzyme affected by wortmannin; it has been shown that wortmannin also inhibits bombesin-stimulated phospholipase A_2 activity in CHO (52) and Swiss 3T3 (8) cells. Also, the fact that IRS-$1^{Y608–658}$ confers insulin-stimulated protein synthesis but IRS-1^{F18} does not (Fig. 5) could be due to a specific conformation assumed by IRS-$1^{Y608–658}$ rather than to binding to Tyr residues. For example, the Y825F point mutation of the platelet-derived growth factor (PDGF) receptor blocks both PDGF-stimulated DNA synthesis and the ligand-induced change in receptor conformation (12). Finally, other downstream signaling components in addition to PI3K may dock to Tyr-608, -628, and -658 of IRS-1. For instance, both PI3K and Nck bind to phosphorylated Tyr-751 in the platelet-derived growth factor receptor (44). Nck is, in fact, known to associate with IRS-1, but its binding site was shown by phosphopeptide competition studies to be different from the binding sites of PI3K and GRB2 (28). Thus, the two experimental approaches used in the present study are consistent with, but do not unequivocally prove, involvement of PI3K in insulin-stimulated protein synthesis.

PI3K is also required for glucose transport but does not affect the p21^{ras} pathway in CHO-IR cells (18). Similarly, PI3K is essential for insulin-stimulated DNA synthesis, glucose uptake, and GLUT 4 translocation to the plasma membrane in 3T3-L1 cells, activating pp70^{S6K} but not MAPK or pp90^{S6K} (4). Finally, in PC-12 cells, prevention of apoptosis requires PI3K but not p21^{ras} (63). Thus, the PI3K branch of insulin signaling seems to be independent of the MAPK branch. Our findings indicate that the IRS-1-mediated stimulation of protein synthesis proceeds through the PI3K branch but not the MAPK branch.

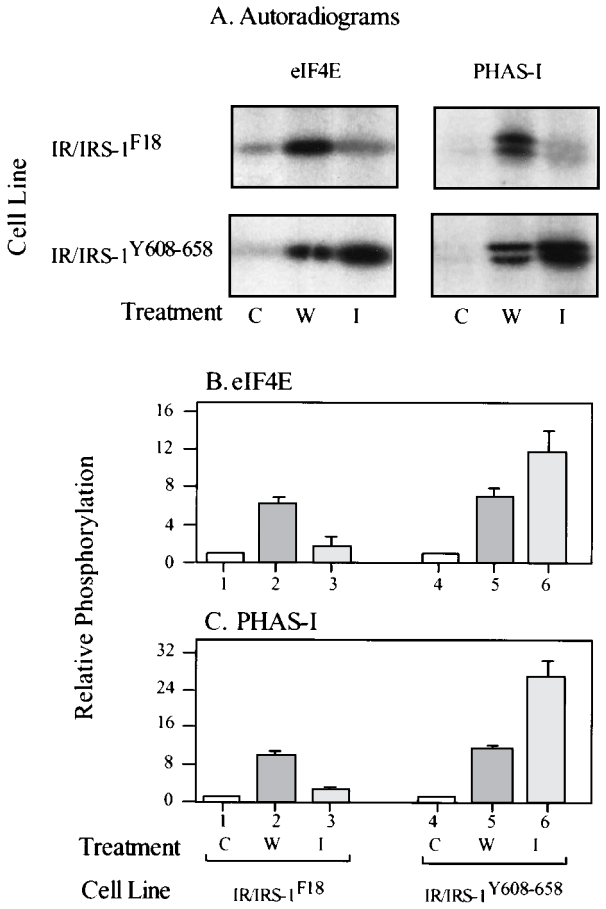

FIG. 9. IRS-1 mediates phosphorylation of eIF4E and PHAS-I through PI3K. IL-3-starved IR/IRS-1^{F18} and IR/IRS-$1^{Y608–658}$ cells were preincubated with $^{32}P_i$ and incubated with either no additions (C), 10% WEHI-3 medium (W), or 100 nM insulin (I) as described in Materials and Methods. eIF4E and PHAS-I phosphorylations (A) were detected by autoradiography as described in the legend to Fig. 7. The experiment was performed twice with each cell line, and the average results are presented as histograms (B and C).

Several enzymes are activated downstream of PI3K, among them certain isoforms of protein kinase C (13, 42, 58), the Ser/Thr kinase Akt (15), and $pp70^{S6K}$ (5). Two lines of evidence support the idea that $pp70^{S6K}$ is not a component of the pathway to the general increase in protein synthesis rate mediated by insulin in IR/IRS-1 cells. First, insulin activates $pp70^{S6K}$ (38) but not protein synthesis (Fig. 1) in −/IRS-1 cells. Second, rapamycin only slightly inhibits insulin-stimulated protein synthesis at concentrations which completely inhibit $pp70^{S6K}$ (Fig. 3B). In this sense, PI3K-stimulated general protein synthesis is like PI3K-stimulated glucose transport in 3T3-L1 cells, which is not inhibited by rapamycin (14). Yet $pp70^{S6K}$ is a component of the pathway to insulin-stimulated translation of specific mRNAs, e.g., myc (Fig. 6), a representative of the growth-regulated class of mRNAs. The fact that rapamycin inhibits only 10% of insulin-stimulated protein synthesis but completely inhibits DNA synthesis (5) could reflect the importance of these proteins in cell cycle progression.

How the insulin-induced signal ultimately causes up-regulation of the translational machinery is not known, but phosphorylation of eIF4E and its binding protein, PHAS-I, has been the subject of intense investigation recently. Both phosphorylation events promote cap recognition and recruitment of mRNA to the ribosome. However, it is not known whether one phosphorylation event enhances or facilitates the other.

In this work, we show that both eIF4E and PHAS-I phosphorylations require IRS-1 and are inhibited by wortmannin. Moreover, $IRS\text{-}1^{Y608–658}$, which contains only the Tyr residues that activate PI3K (41), induces eIF4E and PHAS-I phosphorylations to maximal levels, indicating that both eIF4E and PHAS-I kinases are downstream components of the PI3K pathway. In the −/IRS-1 cell line, in which insulin activation of $pp70^{S6K}$ is maximal but that of PI3K is only partial (38, 40), PHAS-I phosphorylation is maximal (Fig. 7), suggesting that $pp70^{S6K}$ is a component of the pathway to PHAS-I phosphorylation. This is confirmed by the finding that rapamycin inhibits the phosphorylation of PHAS-I (Fig. 8). Initial studies showed that PHAS-I is phosphorylated by MAPK in vitro (29) and that the same site is phosphorylated in vivo in response to insulin (19). However, more recent in vivo studies showed that rapamycin, but not the MAPK inhibitor PD098059, inhibits PHAS-I phosphorylation, indicating that the kinase involved is $pp70^{S6K}$ or one which is downstream of it (30). Our results therefore support the latter interpretation.

The pathway to eIF4E phosphorylation seems to be similar but not identical to the pathway to phosphorylation of PHAS-I. In −/IRS-1 cells, where (i) MAPK is not activated, (ii) PI3K is partially activated, and (iii) $pp70^{S6K}$ is fully activated (38, 40), eIF4E phosphorylation is only half maximal (Fig. 7B), suggesting that eIF4E phosphorylation occurs downstream of PI3K but not of $pp70^{S6K}$. Yet rapamycin inhibits eIF4E phosphorylation (Fig. 8). This could mean that the (as yet unidentified) eIF4E kinase is not activated by $pp70^{S6K}$ directly but that prior dissociation of eIF4E from PHAS-I, which is stimulated by $pp70^{S6K}$ (16), enhances eIF4E phosphorylation.

The fact that rapamycin inhibits PHAS-I and eIF4E phosphorylations but not general insulin-stimulated protein synthesis suggests that making more eIF4E available for translation (PHAS-I phosphorylation) or increasing its affinity for mRNA (eIF4E phosphorylation) is primarily important for enhancing the translation of a small subset of mRNAs. Indeed, overexpression of eIF4E in cell lines specifically increases the translation of the highly growth-regulated mRNAs for ornithine decarboxylase (54), ornithine aminotransferase (11), cyclin D1 (49), P23 (3), NF-AT (1), and myc (9). The complete inhibition of insulin-stimulated phosphorylation of eIF4E and PHAS-I by rapamycin (Fig. 8) (30) could provide a mechanism for the decrease in growth-regulated proteins like myc (Fig. 6) and inhibition of cell cycle progression (5). Conversely, overexpression of eIF4E, which is known to accelerate cell growth (10) and to lead to malignant transformation (27), causes excessive production of the same subset of proteins involved in cell cycle progression.

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