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# Requirement of Protein Kinase Cζ for Stimulation of Protein Synthesis by Insulin

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**The ability of insulin to stimulate protein synthesis and cellular growth is mediated through the insulin receptor (IR), which phosphorylates Tyr residues in the insulin receptor substrate-signaling proteins (IRS-1 and IRS-2), Gab-1, and Shc. These phosphorylated substrates directly bind and activate enzymes such as phosphatidylinositol 3′-kinase (PI3K) and the guanine nucleotide exchange factor for $p21^{Ras}$ (GRB-2/SOS), which are in turn required for insulin-stimulated protein synthesis, cell cycle progression, and prevention of apoptosis. We have now shown that one or more members of the atypical protein kinase C group, as exemplified by the ζ isoform (PKCζ), are downstream of IRS-1 and PI3K and mediate the effect of insulin on general protein synthesis. Ectopic expression of constitutively activated PKCζ eliminates the requirement of IRS-1 for general protein synthesis but not for insulin-stimulated activation of 70-kDa S6 kinase ($p70^{S6K}$), synthesis of growth-regulated proteins (e.g., c-Myc), or mitogenesis. The fact that PKCζ stimulates general protein synthesis but not activation of $p70^{S6K}$ indicates that PKCζ activation does not involve the proto-oncogene Akt, which is also activated by PI3K. Yet insulin is still required for the stimulation of general protein synthesis in the presence of constitutively active PKCζ and in the absence of IRS-1, suggesting a requirement for the convergence of the IRS-1/PI3K/PKCζ pathway with one or more additional pathways emanating from the IR, e.g., Shc/SOS/$p21^{Ras}$/mitogen-activated protein kinase. Thus, PI3K appears to represent a bifurcation in the insulin signaling pathway, one branch leading through PKCζ to general protein synthesis and one, through Akt and the target of rapamycin (mTOR), to growth-regulated protein synthesis and cell cycle progression.**

One of the principal endpoints 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 (37). Insulin appears to regulate both the initiation and elongation phases of translation by altering the phosphorylation of initiation factors (eIF2, eIF2B, eIF3, eIF4B, eIF4E, and eIF4G), elongation factors (eEF1 and eEF2), and modulatory proteins.

General protein synthesis is governed in part by eIF2. The activity of this factor can be regulated through the phosphorylation of its α subunit (19), which is diminished by insulin. eIF2 activity is also regulated by phosphorylation of the ε subunit of the guanine nucleotide exchange factor eIF2B (54) by glycogen synthase kinase-3, which is in turn inactivated by phosphorylation in response to insulin (37). Another insulin-regulated factor affecting general protein synthesis appears to be eEF2 (55).

Insulin also regulates the specific translation of a small subset of cellular mRNAs which have been termed growth regulated (5). This regulation is sensitive to the macrolide rapamycin, which was originally characterized as an inhibitor of cell cycle progression through $G_1$ (reviewed in reference 10). These growth-regulated mRNAs include mRNAs with polypyrimidine tracks at their extreme 5′ termini (e.g., those encoding ribosomal proteins, eEF1A, eEF2, and insulin-like growth factor II) and mRNAs rich in secondary structure (e.g., those for FGF-5, c-Myc, and ornithine decarboxylase). High levels of secondary structure make these mRNAs strongly dependent on the eIF4 factors, which collectively recognize the mRNA cap, unwind secondary structure, and recruit mRNA to the ribosome (44, 57, 64). Insulin regulates the cap-binding protein eIF4E in two ways. First, it increases phosphorylation of eIF4E itself, enhancing its affinity for the cap structure of mRNA (45). Second, it increases phosphorylation of PHAS-I (also called 4E-BP), a protein which, in the unphosphorylated state, binds to eIF4E (40, 53); this interferes with the binding of eIF4E and eIF4G and thereby blocks cap-dependent initiation of translation (33). The insulin-induced phosphorylations of both PHAS-I (6, 11, 22, 41, 43) and eIF4E (43) are sensitive to rapamycin.

Insulin receptor (IR) and IRS-1 are required for the insulin-mediated stimulation of both general and growth-regulated protein synthesis (43) as well as DNA synthesis (69) and $p70^{S6K}$ activation (47). Phosphatidylinositol 3′-kinase (PI3K) appears to be the only phosphotyrosine-activated effector downstream of IRS-1 that is required for stimulation of protein (43) and DNA (49) synthesis. The phosphorylation of glycogen synthase kinase-3 is mediated by the Ser/Thr protein kinase Akt (also called PKB) (16), which is itself activated by PI3K (29, 30). Similarly, the rapamycin-sensitive pathway leading to insulin-stimulated phosphorylation of PHAS-I proceeds through PI3K and Akt and also results in activation of $p70^{S6K}$ (14).

To characterize further the downstream effectors of IRS-1 for insulin-stimulated protein synthesis, we used a panel of cells which differ in the key components of the insulin signaling system they contain. 32D is a myeloid progenitor cell line which is dependent on interleukin 3 (IL-3) but insensitive to insulin (69). 32D cells contain little endogenous insulin receptor (IR) and completely lack IRS-1 and IRS-2, but ectopic expression of IR and IRS-1 permits cells to respond to insulin

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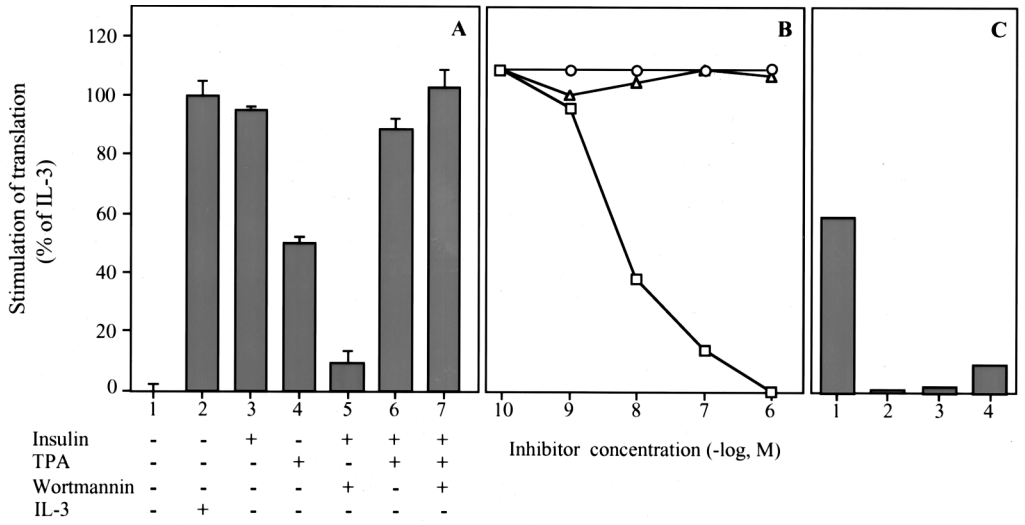


FIG. 1. Effects of PKC activators and inhibitors on insulin-stimulated translation. (A) IL-3-starved IR/IRS-1 cells were grown for 1 h with the additions indicated, and protein synthesis was measured as described in Materials and Methods. Translation was stimulated with insulin, 100 nM; TPA, 1 μM; wortmannin, 80 nM; and IL-3 (present in conditioned medium from WEHI-3 cells (69). Data are graphed as stimulation of treated cells (*I*) minus that of control cells with no additions (*C*) expressed as a percentage of the stimulation by IL-3 (*W*) minus that of control cells, i.e., $(I - C)/(W - C) \times 100$. (B) IL-3-starved IR/IRS-1 cells were analyzed for protein synthesis stimulation by insulin (100 nM) in the presence of the indicated concentrations of the PKC inhibitors Gö 6976 (circles), bisindolylmaleimide (triangles), or staurosporine (squares). (C) IL-3-starved IR/IRS-1 cells were analyzed for protein synthesis stimulation by TPA (1 μM) with no further additions (column 1) or in the presence of 0.1 μM staurosporine (column 2), Gö 6976 (column 3), or bisindolylmaleimide (column 4).

with increased cell division (69) and translation of both housekeeping and growth-regulated mRNAs (43). We have utilized this system to identify an atypical protein kinase C (PKC) as a component of the signaling pathway from PI3K to general, but not growth-regulated, protein synthesis.

## MATERIALS AND METHODS

**Materials.** [$^{35}$S]Met and [γ-$^{32}$P]ATP were purchased from DuPont NEN. Antibodies against PKC isoforms were from Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., and those against mitogen-activated protein kinase (MAPK) were from Upstate Biotechnology, Inc., Lake Placid, N.Y. Myelin basic protein, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and staurosporine were purchased from Sigma. Protein A-Sepharose, histidinol, and insulin were purchased from Pierce, Bachem Bioscience, and GIBCO, respectively. Glutathione (GSH)-Sepharose was purchased from Pharmacia. Bisindolylmaleimide and Gö 6976 were purchased from Calbiochem-Novabiochem Corporation.

**Cell lines.** WEHI-3 cells were purchased from the American Type Culture Collection. 32D cells were transfected with various cDNAs and maintained in selective media: cells expressing human IR were maintained with G418 (69), cells expressing rat IRS-1 or the IRS-1$^{F18}$ and IRS-1$^{Y608\text{-}658}$ variants (49) were maintained with histidinol (69), and cells expressing constitutively active PKCζ (see below) were maintained with puromycin. Lines are named according to the cDNAs expressed, e.g., IR/- cells express IR but not IRS-1, IR/-/ΔPKCζ cells express IR and ΔPKCζ but not IRS-1, and IR/IRS-1/ΔPKCζ cells express IR, IRS-1, and ΔPKCζ, etc. All cells that were not transfected with pBabe containing the ΔPKCζ insert were transfected with pBabe alone and maintained in puromycin. Cell lines were maintained at 37°C in RPMI 1640 containing 10% fetal bovine serum. 32D-derived cell cultures were supplemented with 10% conditioned medium from WEHI-3 cells as a source of IL-3.

**PKCζ subcloning.** pBabeΔPKCζ containing the catalytic domain of rat brain PKCζ fused with glutathione *S*-transferase (21) was constructed by PCR with the oligodeoxynucleotides 5′-GCTACGTATAGGAAACAGTATTCATG-3′ and 5′-GCGTCGACGTCACACGGACTCCTCA-3′ and subcloned into the *Sna*BI-*Sal*I sites of the pBabe-puro vector (46). The *Eco*RI fragment of the dominant-negative mutant of *Xenopus* PKCζ (7) was subcloned into the *Eco*RI site of pBabe-puro.

**MAPK depletion.** IR/IRS-1 cells ($5 \times 10^4$) were electroporated in 300 μl of electroporation buffer (69) with phosphorothioate oligonucleotides (59) corresponding to nucleotides 25 to 41 of mouse MAPK (5′-ATGGCGGCGGCGGC GGC-3′), where ATG is the initiation codon, or with the corresponding antisense probe (5′-GCCGCCGCCGCCGCCAT-3′). Cells were then diluted into 3 ml of IL-3-containing medium supplemented with a 1 μM concentration of the appropriate oligonucleotide. Oligonucleotide-supplemented media were changed every 24 h.

**Measurement of protein and DNA synthesis.** 32D cells and their derivatives were maintained at a concentration of $5 \times 10^5$ cells/ml and starved for IL-3 in RPMI 1640–10% fetal calf serum for 6 h. After this period, 1-ml aliquots were taken and various agents were 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. Filters were air dried and subjected to liquid scintillation spectrometry with aqueous fluor. Actin and c-Myc synthesis was determined as previously described (43). DNA synthesis was measured by incorporation of [$^3$H]thymidine (69).

**PKC and p70$^{S6K}$ activity.** Cells in log-phase growth were washed and starved for IL-3 as described above, and 15-ml aliquots ($8 \times 10^6$ cells) were treated with various agents as described in the figure legends. Cells were treated for 20 min at 37°C, diluted in ice-cold PBS, collected by centrifugation, and lysed with 0.5 ml of ice-cold lysis buffer consisting of 10 mM potassium phosphate, 1 mM EDTA, 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 each of aprotinin and leupeptin per ml. Insoluble material was removed by centrifugation, and the supernatant was collected. p70$^{S6K}$ phosphorylation was analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (15). To assay PKCζ, the cell extract was incubated with anti-PKCζ antibodies for 2 h at 4°C and the immune complex was adsorbed with protein A-Sepharose (pretreated with bovine serum albumin). The immobilized material was washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl, and twice with kinase buffer consisting of 25 mM Tris-HCl (pH 7.4), 10 mM MgCl, 1 mM dithiothreitol, and 0.5 mM EGTA. After the washes, the immunoprecipitated PKCζ activity was assayed in 50 μl of kinase buffer containing 500 μM [γ-$^{32}$P]ATP (0.02 Ci/mol) and 30 μg of myelin basic protein. Aliquots of 25 μl were adjusted to a concentration of 12.5% in trichloroacetic acid, and precipitated material was collected on GF/C filters and washed with 5% trichloroacetic acid and ethanol. Filters were air dried and subjected to liquid scintillation spectrometry with aqueous fluor.

**Northern blot analysis.** Total RNA was prepared from $2 \times 10^7$ cells (31), resolved by formaldehyde–1% agarose gel electrophoresis, and blotted to Nitro Plus 2000 membranes (MSI Separations, Inc.) (61). Antisense probes to *Xenopus* β-actin (23) and c-Myc (17) mRNA were transcribed with SP6 polymerase (Promega) and [α-$^{32}$P]UTP from linearized plasmids.

## RESULTS

**Role of PKCs in insulin-stimulated protein synthesis.** To test for a possible role of PKC in insulin-stimulated protein synthesis, we used TPA to activate the classical isoforms of

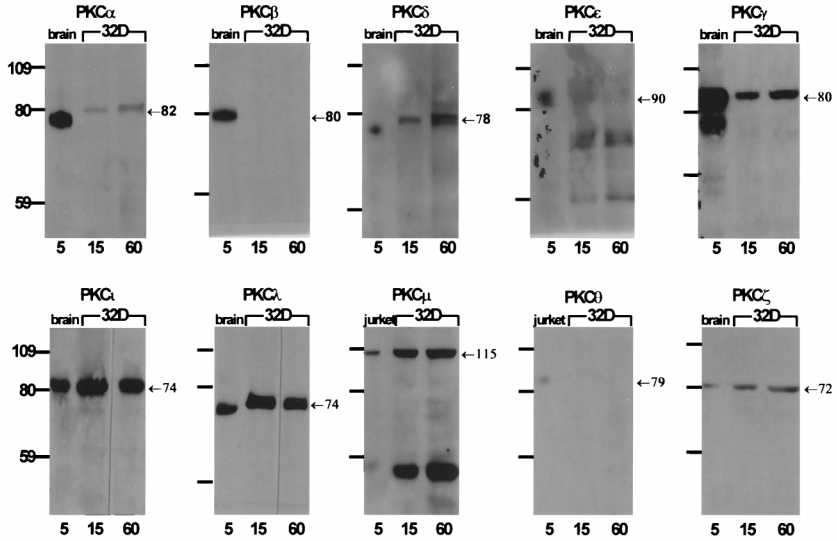


FIG. 2. Aliquots of 15 or 60 μg of 32D cell lysate were separated on a 12% polyacrylamide gel in parallel with 5-μg aliquots of either rat brain or Jurkat cell lysate as positive controls. Proteins were transferred to nitrocellulose and treated with mouse monoclonal antibodies against different PKC isoforms as indicated. Protein bands were visualized with secondary goat anti-mouse antibodies coupled to horseradish peroxidase by means of enhanced chemiluminescence. The molecular mass in kilodaltons of each PKC isoform is indicated to the right of each blot. The migration of prestained molecular mass markers of 109, 80, and 59 kDa is indicated to the left of the blots.

PKC and MAPK. TPA, in the absence of insulin, partially activated protein synthesis in IR/IRS-1 cells (Fig. 1A, column 4). The addition of insulin and TPA together produced the same response as insulin alone (column 6 versus column 3), suggesting that the pathways for insulin and TPA are redundant rather than synergistic or additive. TPA also overcame the inhibition of insulin-stimulated protein synthesis by the PI3K inhibitor wortmannin (column 7 versus column 5). These results suggest that the TPA-stimulated component is either downstream of PI3K or on a parallel but convergent pathway, i.e., activating the same translational factor(s).

To differentiate between isoforms of PKC in insulin-stimulated protein synthesis, we performed two kinds of experiments. First, we tested 32D cells for the presence of the known isoforms of PKC by Western analysis (Fig. 2). Second, we tested various PKC inhibitors: Gö 6976, which inhibits the "classical" (α and β) and "new" (δ and ε) PKCs but not the "atypical" isoforms (42); bisindolylmaleimide, which inhibits all isoforms of PKC except the atypical ones at concentrations less than $10^{-6}$ M (42); and staurosporine, a potent but unselective inhibitor of all PKCs (32, 65). Only staurosporine inhibited insulin-stimulated protein synthesis (Fig. 1B), whereas all three inhibited TPA-stimulated protein synthesis (Fig. 1C). Taking together the results of immunoanalysis and testing with isoform-specific inhibitors, we can conclude that PKCα, -β, -γ, -δ, -ε, -μ, and -θ are not responsible for insulin-stimulated translation since (i) the classical PKCs are at very low levels or are nonexistent in 32D cells compared with brain cells and are sensitive to the specific inhibitors; (ii) PKCδ is present in 32D cells but is sensitive to the specific inhibitors; (iii) PKCγ is at very low levels in 32D cells but is not activated by PI3K (68), whereas insulin-stimulated protein synthesis is (43); and (iv) PKCε and PKCθ are not present in 32D cells. On the other hand, PKCι, PKCλ, and PKCζ, which are all members of the atypical group of PKCs and which have very similar amino acid sequences (GenBank accession no. L18964 and X72973), are present in 32D cells and not inhibited by the isoform-specific inhibitors used. These results suggest that insulin stimulates protein synthesis by activating one or more atypical isoforms of PKC. The observed stimulation of protein synthesis and compensation of PI3K inhibition by TPA (Fig. 1A) may indicate that classical PKCs can substitute for insulin-stimulated atypical PKCs on a parallel pathway.

**Insulin activates PKCζ in an IRS-1- and PI3K-dependent manner.** A direct assay confirmed that an atypical PKC isoform, PKCζ, was indeed activated by both IL-3 and insulin (Fig. 3A, columns 2 and 3). The insulin response required the presence of IRS-1 (Fig. 3A, column 6 versus column 3) and was inhibited by the addition of wortmannin (Fig. 3B). Moreover, an IRS-1 variant in which all 18 potentially phosphorylated Tyr residues were replaced by Phe (IRS-$1^{F18}$) (49) failed to activate PKCζ (Fig. 3C, column 3), but a variant in which the three Tyr residues which bind and activate PI3K were restored to IRS-$1^{F18}$ (IRS-$1^{Y608-658}$) (49) activated PKCζ (Fig. 3C, column 9). This indicates that PKCζ is downstream of IRS-1 and PI3K but not of other pathways activated by the IR, e.g., the Shc-mediated activation of $p21^{Ras}$.

**Overexpression of constitutively activated PKCζ in the absence of IRS-1 supports protein synthesis but not cell cycle progression.** For more direct evidence of a cause-and-effect relationship between PKCζ and stimulation of protein synthesis, we transfected 32D cells with cDNA encoding a constitutively active form of PKCζ fused to glutathione *S*-transferase (ΔPKCζ) (21). ΔPKCζ contains the catalytic domain of PKCζ, which is 84% identical to that of the other atypical PKC isoforms, λ and ι. Evidence for the expression of ΔPKCζ is provided by the demonstration of a GSH-binding protein that phosphorylated myelin basic protein (Fig. 3D) and that cross-reacted with anti-PKCζ antibodies (Fig. 3D, inset). ΔPKCζ was active in the absence of either IL-3 or insulin (Fig. 3E, column 4).

General protein synthesis was stimulated by insulin in IR/-/ΔPKCζ cells to the same extent as in IR/IRS-1 cells (Fig. 4A), indicating that the IRS-1 dependence is entirely replaced by PKCζ. The fact that protein synthesis in the IR/-/ΔPKCζ cells still required insulin suggests that an additional IR-driven pathway(s) is necessary, e.g., Shc activation of MAPK. Coexpression of IRS-1 and ΔPKCζ only slightly increased overall

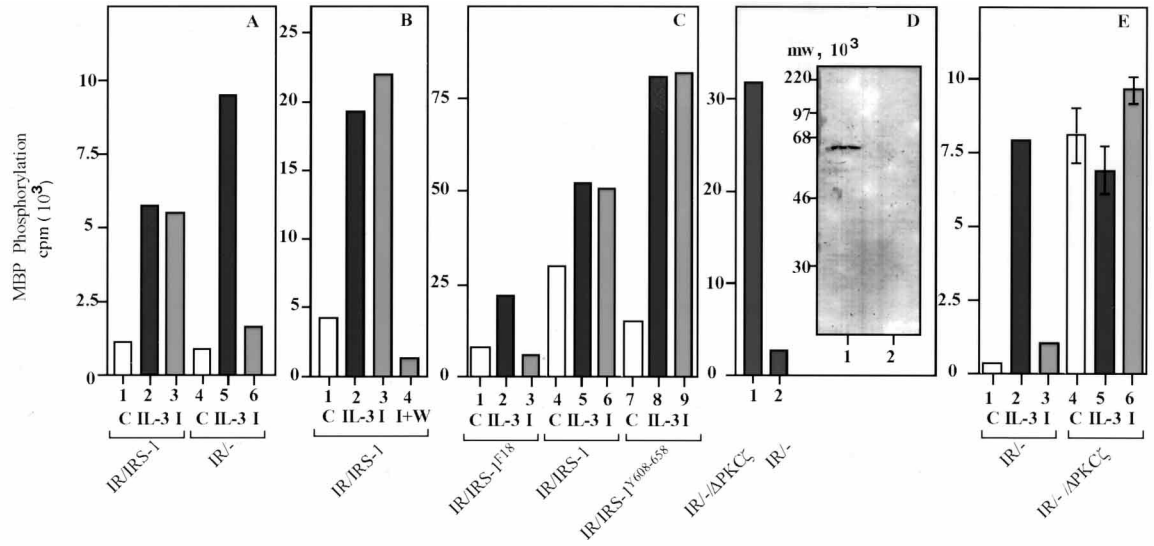


FIG. 3. Activation of endogenous PKCζ by insulin and expression of a constitutively activated form of PKCζ. (A) The indicated IL-3-starved cell lines were stimulated for 20 min at 37°C with IL-3 (columns 2 and 5), with 100 nM insulin (columns 3 and 6), or with no additions (columns 1 and 4), and PKCζ activity was measured as described in Materials and Methods. Bars indicate the amounts of $^{32}P$ radioactivity incorporated into myelin basic protein (MBP). (B) IL-3-starved IR/IRS-1 cells were assayed for PKCζ after treatment with IL-3 (lane 2), with 100 nM insulin (lane 3), or with 100 nM insulin plus 80 nM wortmannin (lane 4), or after no additions (lane 1). (C) The indicated IL-3-starved cell lines were stimulated and assayed for PKCζ as described for panel A. (D) The designated cell lines were treated as described for panel A, and the lysates were processed by affinity chromatography on GSH-Sepharose according to the manufacturer specifications. The material eluted with GSH was assayed for PKC as described in Materials and Methods (bar graph) and by Western blotting using anti-PKCζ antibodies (inset). (E) The indicated IL-3-starved cell lines were stimulated and assayed for PKCζ as described for panel A.

protein synthesis (Fig. 4A), as would be expected if PKCζ were in the IRS-1 pathway for protein synthesis stimulation. DNA synthesis, on the other hand, was not supported by the presence of ΔPKCζ, either alone or in conjunction with insulin-activated IR (Fig. 4B), suggesting that other IRS-1-mediated events are required. Since the IRS-$1^{Y608-658}$ variant supports IRS-1-mediated DNA synthesis (49), the missing activity for DNA synthesis is an effector downstream of PI3K other than PKCζ.

**Depletion of MAPK with antisense oligodeoxynucleotides prevents insulin-stimulated protein synthesis.** Previous studies showed that MAPK activation is not sufficient for insulin-stimulated protein synthesis (43). Also, MAPK activation by IRS-1 is not necessary for insulin-stimulated protein synthesis (43). We therefore tested whether MAPK activation (by any pathway) was necessary for insulin-stimulated protein synthesis. We prevented the expression of p44 and p42 MAP kinases by electroporating antisense phosphorothioate oligonucleotide analogs complementary to the 5′-untranslated region of MAPK mRNAs (59). IR/IRS-1 cells became unresponsive to insulin after depletion of MAPK (Fig. 5). This indicates that insulin-induced activation of MAPK, presumably via Shc (48, 52, 72), is necessary for the stimulation of protein synthesis.

**Overexpression of PKCζ does not affect $p70^{S6K}$ activity.** In IR/IRS-1 cells, insulin causes phosphorylation and consequent activation of $p70^{S6K}$, but this does not occur in IR/- cells (47). To test whether ΔPKCζ substitutes for this activity of IRS-1, we measured $p70^{S6K}$ activation in IR/IRS-1, IR/IRS-1/ΔPKCζ, IR/-, and IR/-/ΔPKCζ cells (Fig. 6). The results indicated that the expression of ΔPKCζ did not alter insulin-stimulated phosphorylation and activation of $p70^{S6K}$ in either the presence or absence of IRS-1. Thus, PKCζ is not sufficient to activate $p70^{S6K}$.

**Active PKCζ supports actin but not c-Myc synthesis.** The finding that constitutive activation of PKCζ in the absence of IRS-1 is not able to support DNA synthesis (Fig. 4B) or $p70^{S6K}$

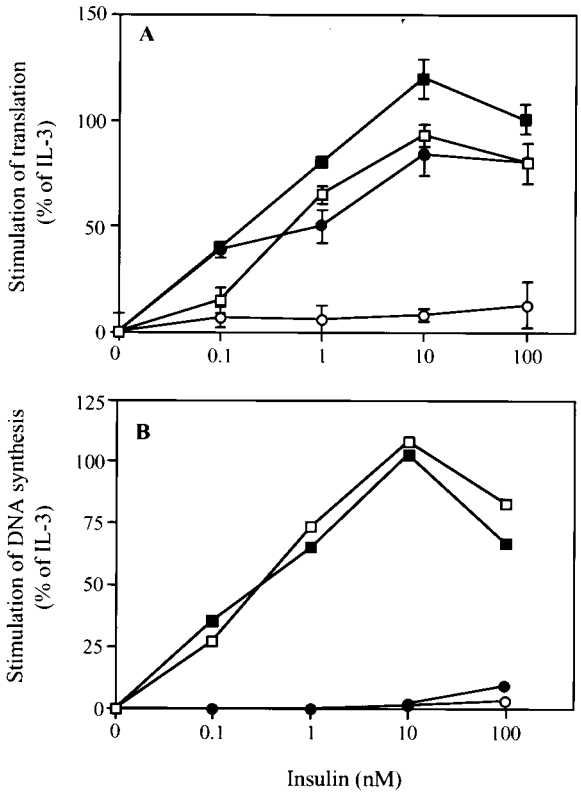


FIG. 4. PKCζ replaces IRS-1 for protein synthesis but not for DNA synthesis. 32D-derived cell lines (IR/- [open circles], IR/IRS-1 [open squares], IR/-/ΔPKCζ [solid circles], and IR/IRS-1/ΔPKCζ [solid squares]) were starved for IL-3 and grown in the presence of the indicated concentrations of insulin. Protein synthesis (A) and DNA synthesis (B) were determined as described in Materials and Methods. Data are graphed as described in the legend for Fig. 1.

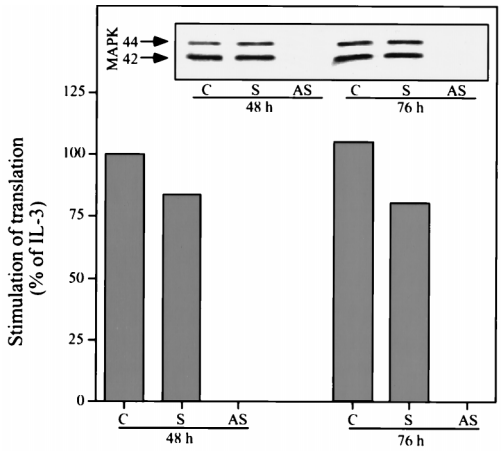


FIG. 5. Insulin-stimulated translation requires MAPK. IR/IRS-1 cells were electroporated with phosphorothioate oligonucleotides (59) corresponding to nucleotides 25 to 41 of mouse MAPK (S) or with the corresponding antisense probe (AS) or were mock treated (C). Cells were grown in the presence of the oligonucleotides (1 μM) for the indicated times, after which MAPK expression was determined by Western blotting (inset) and stimulation of protein synthesis by insulin was determined as described in the legend for Fig. 1 (bars).

activation (Fig. 6) suggests that insulin and PKCζ are mediating stimulation of general but not growth-regulated (rapamycin-sensitive) protein synthesis. To test this hypothesis, we measured the synthesis of a rapamycin-insensitive protein, actin (43), and a rapamycin-sensitive, growth-regulated protein, c-Myc (17, 43). Constitutively active PKCζ, in conjunction with insulin, stimulated actin synthesis, regardless of whether IRS-1 was present (Fig. 7A). Insulin-stimulated c-Myc synthesis, on the other hand, required IRS-1 (Fig. 7B, lane 9 versus lane 3), and this requirement was not replaced by ΔPKCζ (lane 6). These changes in c-Myc and actin protein levels were due to changes at the translational level, since mRNAs for c-Myc and actin were nearly the same regardless of treatment (Fig. 7C; quantitation given in the figure legend). This is consistent with earlier results showing that rapamycin treatment of IR/IRS-1

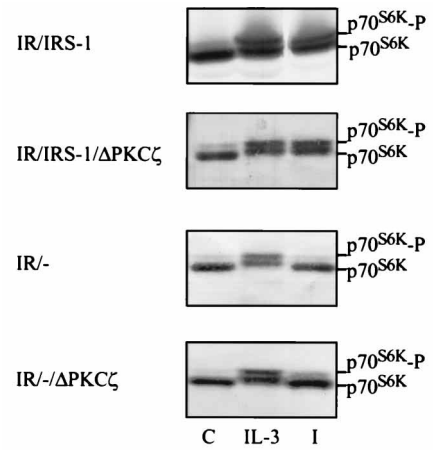


FIG. 6. Expression of ΔPKCζ does not affect $p70^{S6K}$ activation. The indicated 32D cell lines in log phase were washed and starved for IL-3 and were treated with 100 nM insulin (I) or IL-3 or were left untreated (C) for 25 min at 37°C. Then they were diluted in ice-cold PBS and collected by centrifugation. Measurements of $p70^{S6K}$ activity by Western blotting were performed as described in Materials and Methods.

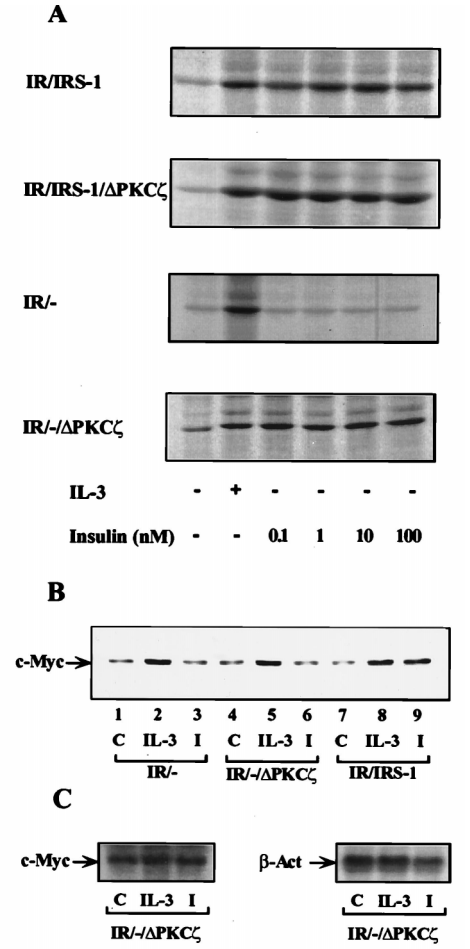


FIG. 7. Expression of constitutively active PKCζ supports the synthesis of actin but not c-Myc. (A) The indicated IL-3-starved 32D cell lines were labeled with [$^{35}$S]Met in medium containing no additions, IL-3, or the indicated concentrations of insulin. Actin synthesis was analyzed as described in Materials and Methods. Scanning the autoradiogram yielded actin values for IR/IRS-1 cells treated with 0, 0.1, 1, 10, and 100 nM insulin of 41.6, 87.2, 100.3, 103.6, and 86.3, respectively, relative to IL-3 treatment, which was normalized to 100. For IR/-/ΔPKCζ cells, the corresponding values were 60.5, 113.5, 101.1, 113.2, and 113.6, respectively. (B) The indicated IL-3-starved 32D cell lines were incubated in medium containing no additions (lanes 1, 4, and 7), IL-3 (lanes 2, 5, and 8), or 100 nM insulin (lanes 3, 6, and 9). After 3 h cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, briefly sonicated, and analyzed by Western blotting with anti-Myc antibody. Scanning the immunoblot yielded the following values for lanes 1 to 9 (relative to lane 8, which was normalized to 100): 38.4, 113.9, 33.8, 39.0, 102.5, 29.9, 21.3, 100.0, and 103.2, respectively. (C) mRNA levels for c-Myc and β-actin (β-Act) were analyzed by Northern blotting as described in Materials and Methods. Scanning the autoradiograms yielded values, normalized to 100 for IL-3, of 87.7, 100.0, and 91.5 for c-Myc and 100.2, 100.0, and 77.7 for β-actin.

cells prevents the insulin stimulation of c-Myc but not actin synthesis (43).

## DISCUSSION

To understand the effects of insulin on protein synthesis and the signaling pathways involved, it is necessary to differentiate between the translation of housekeeping and growth-regulated mRNAs (5, 10, 35, 56). Overexpression of eIF4E causes rapid growth and cellular transformation (18, 39) and specifically increases the translation of growth-regulated mRNAs whose products are required for cell cycle progression, e.g., ornithine

decarboxylase (63), ornithine aminotransferase (24), cyclin D1 (58), P23 (9), NF-AT (4), c-Myc (17), and FGF (36). This preferential stimulation of translation is thought to be related to the presence of extensive secondary structure at the 5′-untranslated regions of growth-regulated mRNAs and the role of initiation factors of the eIF4 group in unwinding mRNA secondary structure (44, 57, 64). Translational regulation of housekeeping mRNAs, on the other hand, is likely to involve components which are equally required for translation of all mRNAs, e.g., active 43S initiation complexes. Studies to date indicate that insulin stimulates translation of both growth-regulated and housekeeping mRNAs (37).

The signaling pathways by which insulin stimulates growth-regulated protein synthesis are partially understood. Both types of protein synthesis have a common requirement for IR and IRS-1 (43) (Fig. 7). The only phosphotyrosine-mediated signal from IRS-1 necessary for protein synthesis is that which activates PI3K, since the IRS-$1^{Y608-658}$ variant stimulates both general and growth-regulated protein synthesis (43). MAPK activation does not play a role in the IRS-1-mediated pathway to protein synthesis, since an IRS-1 variant unable to activate MAPK (48) nonetheless activates protein synthesis (43). Moreover, MAPK activation in the absence of PI3K activation does not support stimulation of protein synthesis, since insulin activates MAPK (48) but not protein synthesis (43) in IR/- cells.

At some point after PI3K, the pathways to growth-regulated and general protein synthesis diverge, as demonstrated by the use of the fungal macrolide rapamycin. Activation of a rapamycin-sensitive pathway, most commonly measured by the activation of $p70^{S6K}$ (15), is required for growth-regulated but not general protein synthesis. Rapamycin specifically prevents increased synthesis of highly cap-dependent, growth-regulated mRNAs by both insulin (43) and other growth factors (6, 35, 51, 67). Rapamycin blocks the phosphorylation of PHAS-I (6, 11, 22, 41, 43) and eIF4E (43), although blocking the phosphorylation of the latter could be an indirect effect since PHAS-I binding prevents eIF4E phosphorylation by PKC in vitro (71). Yet the findings that eIF4E phosphorylation in CHO cells overexpressing IR is sensitive to MEK inhibitors (28) and that PHAS-I and eIF4E phosphorylations appear to be regulated independently in 293 cells (26) and 32D cells (43) suggest distinct pathways to eIF4E and PHAS-I. In any case, preventing both PHAS-I and eIF4E phosphorylation renders eIF4E less active or less available for participation in the translation of highly cap-dependent mRNAs. Rapamycin, however, does not prevent insulin-stimulated general protein synthesis or the synthesis of a typical housekeeping protein, actin (43). Thus, rapamycin can serve as a discriminator between general and growth-regulated protein synthesis.

Activation of the rapamycin-sensitive pathway by insulin is downstream of PI3K (13, 14). The Ser/Thr protein kinase Akt is on the pathway from PI3K to $p70^{S6K}$ activation by both insulin (12) and platelet-derived growth factor (30), and, in fact, Akt can be activated by direct interaction with phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, the products of PI3K (29). The actual rapamycin-sensitive component is downstream from Akt. Rapamycin binds to the 12-kDa protein FKBP, which in turn binds to a novel protein kinase called TOR (3) or mTOR in mammalian systems (reference 11 and references therein). mTOR is on the pathway to PHAS-I phosphorylation (2, 6, 11, 41). Rapamycin also inhibits DNA synthesis and cell cycle progression through $G_1$ (15, 38, 66). These two effects of rapamycin, inhibition of highly cap-dependent, growth-regulated mRNA translation and inhibition of cell cycle progression, can be accommodated

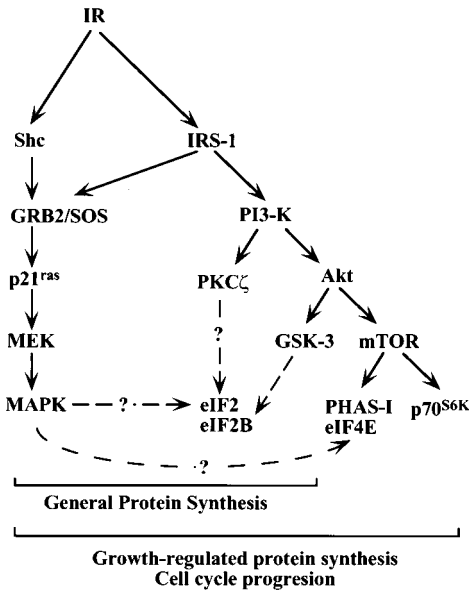


FIG. 8. Model for the signaling pathways leading to stimulation of general and growth-regulated protein synthesis by insulin.

in a model whereby increasing the activity and availability of eIF4E yields products (cyclins and transcription factors, etc.) that promote cell division. An elegant demonstration of the link between the translational and mitogenic roles of TOR has been published (3).

In contrast to the wealth of information about the rapamycin-sensitive branch of insulin signaling, relatively little is known about the pathway from PI3K that controls general protein synthesis. Notably, insulin still stimulates protein synthesis when the rapamycin-sensitive pathway is blocked (43). The signal, which accounts for approximately 90% of insulin-stimulated protein synthesis, emanates from PI3K but is independent of TOR (Fig. 8). The results presented in this study indicate that this pathway involves PKCζ. Insulin is capable of activating PKCζ through IRS-1 and PI3K (Fig. 3). Furthermore, constitutively active PKCζ is able to functionally replace IRS-1 in the stimulation of general protein synthesis (Fig. 4A and 7A). More specifically, PKCζ functionally replaces the ability of IRS-1 to activate PI3K, since IRS-$1^{Y608-658}$, which contains only the Tyr residues which activate PI3K (49), was able to fully activate PKCζ (Fig. 3C). However, PKCζ replaces some but not all functions of PI3K: general protein synthesis and actin synthesis are supported (Fig. 4A and 7A) but not growth-regulated protein synthesis, as exemplified by c-Myc (Fig. 7B), activation of $p70^{S6K}$ (Fig. 6), or cell cycle progression (Fig. 4B). This result agrees with the finding that expression of constitutively active or dominant-negative forms of PKCζ does not affect the activation of $p70^{S6K}$ by serum (8). Thus, it seems likely that PI3K is independently activating two different pathways involved in protein synthesis: the atypical PKC pathway for general protein synthesis and the Akt pathway for growth-regulated protein synthesis (Fig. 8).

Atypical PKC isoforms are thus named because they are neither activated nor down-regulated by phorbol esters, unlike other PKC isoforms (20). Previous studies have indicated that platelet-derived growth factor activates PKCλ, another atypical PKC, through a mechanism involving PI3K (1) and that IL-4, acting through PI3K, causes PKCζ translocation from the cytosolic to the membrane fraction (34). In the case of insulin,

it has been shown that the levels of both classical and atypical PKCs decrease in cytosolic locations and increase in membrane locations after insulin treatment of rat adipocytes (25). PKCζ is unlikely to be downstream of Akt, however, since phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate directly activate PKCζ (50). It is more likely that PI3K activates Akt and PKCζ independently, in both cases secondary to the synthesis of phosphatidylinositides in the plasma membrane.

A number of previous studies have shown an effect of phorbol esters and nonspecific PKC inhibitors on the phosphorylation of members of the eIF4 group initiation factors (reference 27 and references therein). By contrast, there have been relatively few studies on the involvement of PKC in general protein synthesis. In Swiss 3T3 fibroblasts, down-regulation of classical PKC with phorbol esters prevented the insulin response as did a general PKC inhibitor (70). However, in the 32D cell system (IR/IRS-1 cells), pretreatment with phorbol esters only slightly diminished the insulin response (data not shown). This discrepancy may be due to differences in the cell types employed. In any case, the interpretation of experiments in which PKC is down-regulated is complicated by the fact that down-regulation of PKC also inhibits the ability of insulin to stimulate GRB-2/SOS and the consequent downstream activation of MAPK (60). As discussed below, MAPK is required for insulin-stimulated protein synthesis, independent of the IRS-1 signaling system.

The experiments presented here indicate that insulin-stimulated general protein synthesis occurs through atypical and not classical PKCs. First, we show that wortmannin inhibits insulin-stimulated protein synthesis (43) (Fig. 1A, column 5), yet the stimulation by TPA plus insulin is not blocked by wortmannin (Fig. 1A, column 7). This indicates that the TPA signal is not proceeding through PI3K but rather through a parallel pathway. Second, classical PKCs are at very low levels in 32D cells (Fig. 2). Third, Gö 6976 and bisindolylmaleimide, which inhibit all PKC isoforms except the atypical ones (42), failed to prevent the insulin-stimulated response, while a general PKC inhibitor was effective (Fig. 1B). Yet all three inhibitors prevented TPA-stimulated protein synthesis (Fig. 1C). We conclude that, although classical PKCs can stimulate general protein synthesis, the pathway is parallel to that of insulin. The atypical PKCζ, on the other hand, is an essential link in the insulin pathway to general protein synthesis.

It is noteworthy that insulin is still required in cells expressing constitutively active PKCζ but lacking IRS-1 (Fig. 4A), suggesting that there is at least one IRS-1-independent pathway emanating from the IR that is required for general protein synthesis in conjunction with PKCζ. The results presented here indicate that the activation of MAPK is one of these IR-mediated events, despite the fact that IRS-1-mediated activation of MAPK does not play a significant role in insulin-stimulated protein synthesis (43). Depletion of MAPK with an antisense oligonucleotide prevented insulin-stimulated protein synthesis (Fig. 5). This is similar to the observation that a specific inhibitor of MEK prevents the stimulation of protein synthesis in aortic smooth-muscle cells by growth factors (62). The precise mechanistic interpretation of the MAPK depletion experiment, however, is not clear. Presumably, targets downstream of MAPK have a positive effect on protein synthesis, but more complicated scenarios involving phenotypic changes that may result from long-term (48- to 76-h) depletion of MAPK activity cannot be ruled out.

We conclude that the insulin signal bifurcates at PI3K (Fig. 8), one branch leading to general protein synthesis (through PKCζ) and one, to growth-regulated protein synthesis (through Akt). Although more complex explanations of the data are possible, a consistent, straightforward model is that PI3K generates phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate in the plasma membrane and that both Akt and PKCζ are activated by these phospholipids. Our inability to obtain viable cell lines stably expressing a dominant-negative form of PKCζ (7) is consistent with a requirement of general protein synthesis for cell survival (data not shown). The downstream targets of PKCζ are expected to be factors equally required for translation of all mRNAs but are not yet known.

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## REFERENCES

1. **Akimoto, K., R. Takahashi, S. Moriya, N. Nishioka, J. Takayanagi, K. Kimura, Y. Fukui, S. Osada, K. Mizuno, S. Hirai, A. Kazlauskas, and S. Ohno.** 1996. EGF or PDGF receptors activate atypical PKC lambda through phosphatidylinositol 3-kinase. EMBO J. **15:**788–798.
2. **Azpiazu, I., A. R. Saltiel, A. A. DePaoli-Roach, and J. C. Lawrence.** 1996. Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. J. Biol. Chem. **27:**5033–5039.
3. **Barbet, N. C., U. Schneider, S. B. Helliwell, I. Stansfield, M. F. Tuite, and M. N. Hall.** 1996. TOR controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell **7:**25–42.
4. **Barve, S. S., D. A. Cohen, A. DeBenedetti, R. E. Rhoads, and A. M. Kaplan.** 1994. Mechanism of differential regulation of IL-2 murine Th1 and Th2 cell subsets. J. Immunol. **152:**1171–1181.
5. **Baserga, R.** 1990. The cell cycle: myths and realities. Cancer Res. **50:**6769–6771.
6. **Beretta, L., A. C. Gingras, Y. V. Svitkin, M. N. Hall, and N. Sonenberg.** 1996. Rapamycin blocks the phosphorylation of 4E-BP and inhibits cap-dependent initiation of translation. EMBO J. **15:**658–664.
7. **Berra, E., M. T. Diaz-Meco, I. Dominguez, M. M. Municio, L. Sanz, J. Lozano, R. S. Chapkin, and J. Moscat.** 1993. Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell **74:**555–563.
8. **Berra, E., M. T. Diaz-Meco, J. Lozano, S. Frutos, M. M. Municio, P. Sanchez, L. Sanz, and J. Moscat.** 1995. Evidence for a role of MEK and MAPK during signal transduction by protein kinase C zeta. EMBO J. **14:**6157–6163.
9. **Bommer, U.-A., A. Lazaris-Karatzas, A. DeBenedetti, P. Nurnberg, R. Benndorf, H. Bielka, and N. Sonenberg.** 1994. Translational regulation of the mammalian growth-related protein P23: involvement of eIF-4E. Cell. Mol. Biol. Res. **40:**633–641.
10. **Brown, E. J., and S. L. Schreiber.** 1996. A signalling pathway to translational control. Cell **86:**517–520.
11. **Brunn, G. J., J. Williams, C. Sabers, G. Wiederrecht, J. C. Lawrence, and R. T. Abraham.** 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and Ly294002. EMBO J. **15:**5256–5267.
12. **Burgering, B. M., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature **376:**599–602.
13. **Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis, and C. R. Kahn.** 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol. Cell. Biol. **14:**4902–4911.
14. **Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis.** 1994. PDGF- and insulin-dependent pp70$^{S6K}$ activation mediated by phosphatidylinositol-3-OH kinase. Nature **370:**71–75.
15. **Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis.** 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of signaling by the 70 kd S6 protein kinase. Cell **69:**1227–1236.
16. **Cross, D. A. E., D. R. Alessi, J. R. Vandenheede, H. E. McDowell, H. S. Hundal, and P. Cohen.** 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem. J. **303:**21–26.
17. **De Benedetti, A., B. Joshi, J. R. Graff, and S. G. Zimmer.** 1994. CHO cells transformed by the translation factor eIF4E display increased c-Myc expression, but require overexpression of Max for tumorigenicity. Mol. Cell. Differ. **2:**347–371.

18. **De Benedetti, A., and R. E. Rhoads.** 1990. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. Proc. Natl. Acad. Sci. USA **87:**8212–8216.
19. **De Haro, C., R. Mendez, and J. Santoyo.** 1996. The eIF-2α kinases and the control of protein synthesis. FASEB J. **10:**1378–1387.
20. **Dekker, L. V., and P. J. Parker.** 1994. Protein kinase C—a question of specificity. Trends Biochem. Sci. **19:**73–77.
21. **Diaz-Meco, M. T., J. Lozano, M. M. Municio, E. Berra, S. Frutos, L. Sanz, and J. Moscat.** 1994. Evidence for the *in vitro* and *in vivo* interaction of Ras with protein kinase C zeta. J. Biol. Chem. **269:**31706–31710.
22. **Diggle, T. A., S. K. Moule, M. B. Avison, A. Flynn, E. J. Foulstone, C. G. Proud, and R. M. Denton.** 1996. Both rapamycin-sensitive and -insensitive pathways are involved in the phosphorylation of the initiation factor-4E-binding protein (4E-BP1) in response to insulin in rat epididymal fat cells. Biochem. J. **316:**447–453.
23. **Dworkin, M. B., A. Shruthkowski, M. Baumgarten, and E. Dworkin-Rastl.** 1984. The accumulation of prominent tadpole mRNAs occurs at the beginning of neurulation in *Xenopus laevis* embryos. Dev. Biol. **106:**289–295.
24. **Fagan, R. J., A. Lazaris-Karatzas, N. Sonenberg, and R. D. Rozen.** 1991. Translational control of ornithine aminotransferase. Modulation by initiation factor eIF-4E. J. Biol. Chem. **266:**16518–16523.
25. **Farese, R. V., M. L. Standaert, A. J. Francois, K. Ways, T. P. Arnold, H. Hernandez, and D. R. Cooper.** 1992. Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. Biochem. J. **288:**319–323.
26. **Feigenblum, D., and R. J. Schneider.** 1996. Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. Mol. Cell. Biol. **16:**5450–5457.
27. **Flynn, A., and C. G. Proud.** 1996. Insulin and phorbol ester stimulate initiation factor eIF-4E phosphorylation by distinct pathways in Chinese hamster ovary cells overexpressing the insulin receptor. Eur. J. Biochem. **236:**40–47.
28. **Flynn, A., and C. G. Proud.** 1996. Insulin-stimulated phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway. FEBS Lett. **389:**162–166.
29. **Franke, T. F., D. R. Kaplan, L. C. Cantley, and A. Toker.** 1997. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science **275:**665–668.
30. **Franke, T. F., S. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis.** 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81:**727–736.
31. **Gilman, M.** 1994. Preparation of cytoplasmic RNA from tissue culture cells, p. 4.1.1–4.1.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. Greene Publishing Associates, Inc., and John Wiley and Sons, Inc., New York, N.Y.
32. **Gschwendt, M., H. Leibersperger, W. Kittstein, and F. Marks.** 1992. Protein kinase C zeta and eta in murine epidermis. FEBS Lett. **307:**151–155.
33. **Haghighat, A., S. Mader, A. Pause, and N. Sonenberg.** 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J. **14:**5701–5709.
34. **Ikizawa, K., K. Kajiwara, Y. Basaki, T. Koshio, and Y. Yanagihara.** 1996. Evidence for a role of phosphatidylinositol 3-kinase in IL-4-induced germline C epsilon transcription. Cell. Immunol. **170:**134–140.
35. **Jefferies, H. B. J., C. Reinhard, S. C. Kozma, and G. Thomas.** 1994. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc. Natl. Acad. Sci. USA **91:**4441–4445.
36. **Kevil, C., P. Carter, B. Hu, and A. DeBenedetti.** 1995. Translational enhancement of FGF-2 by eIF-4 factors, and alternate utilization of CUG and AUG codons for translation initiation. Oncogene **11:**2339–2348.
37. **Kimball, S. R., T. C. Vary, and L. S. Jefferson.** 1994. Regulation of protein synthesis by insulin. Annu. Rev. Physiol. **56:**321–348.
38. **Lane, H. A., A. Fernandez, N. J. Lamb, and G. Thomas.** 1993. $p70^{S6K}$ function is essential for G1 progression. Nature **363:**170–172.
39. **Lazaris-Karatzas, A., K. S. Montine, and N. Sonenberg.** 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature **345:**544–547.
40. **Lin, T., X. Kong, T. A. J. Haystead, A. Pause, G. Belsham, N. Sonenberg, and J. C. Lawrence.** 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science **266:**653–656.
41. **Lin, T. A., X. Kong, A. R. Saltiel, P. J. Blackshear, and J. C. Lawrence, Jr.** 1995. Control of PHAS-I by insulin in 3T3-L1 adipocytes. Synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway. J. Biol. Chem. **270:**18531–18538.
42. **Martiny-Baron, G., M. G. Kazanietz, H. Mischak, P. M. Blumberg, G. Kochs, H. Hug, D. Marme, and C. Schachtele.** 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. **268:**9194–9197.
43. **Mendez, R., M. G. Myers, M. F. White, and R. E. Rhoads.** 1996. 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. Mol. Cell. Biol. **16:**2857–2864.
44. **Merrick, W. C.** 1992. Mechanism and regulation of eukaryotic protein synthesis. Microbiol. Rev. **56:**291–315.
45. **Minich, W. B., M. L. Balasta, D. J. Goss, and R. E. Rhoads.** 1994. Chromatographic resolution of *in vivo*-phosphorylated and nonphosphorylated translation initiation factor eIF-4E. Demonstration of increased cap affinity of the phosphorylated form. Proc. Natl. Acad. Sci. USA **91:**7668–7672.
46. **Morgenstern, P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
47. **Myers, M. G., T. C. Grammer, L. Wang, X. J. Sun, J. H. Pierce, J. Blenis, and M. F. White.** 1994. Insulin receptor substrate-1 mediates phosphatidylinositol 3′-kinase and $p70^{S6K}$ signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. J. Biol. Chem. **269:**28783–28789.
48. **Myers, M. G., L. Wang, X. J. Sun, Y. Zhang, L. Yenush, J. Schlessinger, J. H. Pierce, and M. F. White.** 1994. Role of IRS-1–GRB-2 complexes in insulin signaling. Mol. Cell. Biol. **14:**3577–3587.
49. **Myers, M. G., Y. Zhang, E. Glasheen, T. Grammer, L. Yenush, L. M. Wang, X. J. Sun, J. Vlenis, J. H. Pierce, and M. F. White.** 1996. YMXM-motifs and signaling by an IRS-1 molecule without tyrosine phosphorylation sites. Mol. Cell. Biol. **16:**4147–4155.
50. **Nakanishi, H., K. A. Brewer, and J. H. Exton.** 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. **268:**13–16.
51. **Nielsen, F. C., L. Ostergaard, J. Nielsen, and J. Christiansen.** 1995. Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. Nature **377:**358–362.
52. **Ouwens, D. M., G. C. van der Zon, G. J. Pronk, J. L. Bos, W. Moller, B. Cheatham, C. R. Kahn, and J. A. Maassen.** 1994. A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and $p21^{ras}$-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. J. Biol. Chem. **269:**33116–33122.
53. **Pause, A., G. J. Belsham, A. Gingras, O. Donze, T. Lin, J. C. Lawrence, and N. Sonenberg.** 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature **371:**762–767.
54. **Price, N., and C. Proud.** 1994. The guanine nucleotide-exchange factor, eIF-2B. Biochimie **76:**748–760.
55. **Redpath, N. T., E. J. Foulstone, and C. G. Proud.** 1996. Regulation of translation elongation factor-2 by insulin via rapamycin-sensitive signalling pathway. EMBO J. **15:**2291–2297.
56. **Rhoads, R. E.** 1991. Protein synthesis, cell growth, and oncogenesis. Curr. Opin. Cell Biol. **3:**1019–1024.
57. **Rhoads, R. E.** 1993. Regulation of eukaryotic protein synthesis by initiation factors. J. Biol. Chem. **268:**3017–3020.
58. **Rosenwald, I. B., R. Kaspar, D. Rousseau, L. Gehrke, P. Leboulch, J. J. Chen, E. V. Schmidt, N. Sonenberg, and I. M. London.** 1995. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and posttranscriptional levels. J. Biol. Chem. **270:**21176–21180.
59. **Sale, E. M., P. G. P. Atkinson, and G. J. Sale.** 1995. Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin serum stimulation of DNA synthesis. EMBO J. **14:**674–684.
60. **Schubert, C., K. Carel, D. DePaolo, W. Leitner, and B. Draznin.** 1996. Interactions of protein kinase C with insulin signaling. J. Biol. Chem. **271:**15311–15314.
61. **Selden, R. F.** 1994. Analysis of RNA by northern hybridization, p. 4.9.1–4.9.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. Greene Publishing Associates, Inc., and John Wiley and Sons, Inc., New York, N.Y.
62. **Servant, M. J., E. Giasson, and S. Meloche.** 1996. Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. J. Biol. Chem. **271:**16047–16052.
63. **Shantz, L. M., and A. E. Pegg.** 1994. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res. **54:**2313–2316.
64. **Sonenberg, N.** 1993. Translation factors as effectors of cell growth and tumorigenesis. Curr. Opin. Cell Biol. **5:**955–960.
65. **Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita.** 1986. Staurosporine, a potent inhibitor of phospholipid/$Ca^{++}$ dependent protein kinase. Biochem. Biophys. Res. Commun. **135:**397–402.
66. **Terada, N., J. J. Lucas, A. Szepesi, R. A. Franklin, J. Domenico, and E. W. Gelfand.** 1993. Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle. J. Cell. Physiol. **154:**7–15.
67. **Terada, N., H. R. Patel, K. Takase, K. Kohno, and A. C. Nairn.** 1994. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc. Natl. Acad. Sci. USA **91:**11477–11481.
68. **Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley.** 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-$P_2$

and PtdIns-3,4,5-$P_3$. J. Biol. Chem. **269:**32358–32367.
69. **Wang, L., M. G. Myers, X. J. Sun, S. A. Aaronson, M. White, and J. H. Pierce.** 1993. IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. Science **261:**1591–1594.
70. **Welsh, G. I., and C. G. Proud.** 1993. Evidence for a role for protein kinase-C in the stimulation of protein synthesis by insulin in Swiss 3T3-fibroblasts. FEBS Lett. **316:**241–246.
71. **Whalen, S. G., A. C. Gingras, L. Amankwa, S. Mader, P. E. Branton, R. Aebersold, and N. Sonenberg.** 1996. Phosphorylation of eIF-4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. J. Biol. Chem. **271:**11831–11837.
72. **Yamauchi, K., and J. E. Pessin.** 1994. Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling. J. Biol. Chem. **269:**31107–31114.