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YMXM Motifs and Signaling by an Insulin Receptor Substrate 1 Molecule without Tyrosine Phosphorylation Sites

MARTIN G. MYERS, JR.,[1] YITAO ZHANG,[1] GLADYS A. I. ALDAZ,[1] TIMOTHY GRAMMER,[2] ERIN M. GLASHEEN,[1] LYNNE YENUSH,[1] LING MEI WANG,[3] XIAO JIAN SUN,[1] JOHN BLENIS,[2] JACALYN H. PIERCE,[3] AND MORRIS F. WHITE[1]*

Research Division, Joslin Diabetes Center and Program in Biological and Biomedical Sciences,[1] and Department of Cell Biology,[2] Harvard Medical School, Boston, Massachusetts 02115, and Laboratory of Cell and Molecular Biology, National Institutes of Health, Bethesda, Maryland 20892[3]

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Tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) by the activated receptors for insulin, IGF-1, and various cytokines creates binding sites for signaling proteins with Src homology 2 domains (SH2 proteins). Determining the role of specific SH2 proteins during insulin signaling has been difficult because IRS-1 possesses as many as 18 potential tyrosine phosphorylation sites, several of which contain redundant motifs. Using 32D cells, which contain no endogenous IRS proteins, we compared the signaling ability of an IRS-1 molecule in which 18 potential tyrosine phosphorylation sites were replaced by phenylalanine (IRS-1^{F18}) with two derivative molecules which retained three YMXM motifs (IRS-1^{3YMXM}) or the two COOH-terminal SHP2-Fyn binding sites (IRS-1^{YCT}). During insulin stimulation, IRS-1^{F18} failed to undergo tyrosine phosphorylation or mediate activation of the phosphotidylinositol (PI) 3′-kinase or p70^{s6k}; IRS-1^{YCT} was tyrosine phosphorylated but also failed to mediate these signaling events. Neither IRS-1^{3YMXM} nor IRS-1^{YCT} mediated activation of mitogen-activated protein kinases. IRS-1^{F18} and IRS-1^{YCT} partially mediated similar levels of insulin-stimulated mitogenesis at high insulin concentrations, however, suggesting that IRS-1 contains phosphotyrosine-independent elements which effect mitogenic signals, and that the sites in IRS-1^{YCT} do not augment this signal. IRS-1^{3YMXM} mediated the maximal mitogenic response to insulin, although the response to insulin was more sensitive with wild-type IRS-1. By contrast, the association of IRS-1^{3YMXM} with PI 3′-kinase was more sensitive to insulin than the association with IRS-1. Thus, the binding of SH2 proteins (such as PI 3′-kinase) by YMXM motifs in IRS-1 is an important element in the mitogenic response, but other elements are essential for full mitogenic sensitivity.

The IRS proteins (IRS-1 and IRS-2) are endogenous cellular substrates of the insulin and insulin-like growth factor 1 (IGF-1) receptor tyrosine kinases, as well as substrates for various cytokine receptors which activate various members of the Janus kinase family (22, 41, 42, 47, 48). Tyrosine phosphorylation plays a central role in the mechanism of IRS protein signaling by providing docking sites for proteins containing isoforms of the Src homology 2 (SH2) domain (25, 38, 39). Since the phosphorylation sites in IRS-1 are situated in various amino acid sequence motifs, IRS-1 engages numerous SH2 proteins, including p85, Grb-2, Fyn, Nck, and SHP2 (SHPTP2, Syp, and PTP1D) (10, 15, 36). IRS proteins contain several phosphorylation sites in YXXM motifs, which are selective binding sites for the SH2 domains in the regulatory subunits (p85 and p55^{PIK}) of phosphatidylinositol (PI) 3′-kinase (22, 28, 41). During insulin stimulation, IRS proteins mediate the direct activation of PI 3′-kinase, which appears to play essential roles in the activation of the p70^{s6k} and the akt kinase (21, 22) and the serine phosphorylation of eIF4e and PHAS-I as well as in mediating various biological responses including vesicle movement (including glucose transporter translocation), mitogenesis, general protein synthesis, receptor endocytosis, and chemotaxis, among other things (22, 25, 48).

While the plethora of signaling events apparently mediated by PI 3′-kinase raises questions about whether PI 3′-kinase is a regulator of specific cellular events or whether it is a global potentiator of cellular signaling, it is obviously an important molecule in the transmission of downstream signals by tyrosine kinases. The 110-kDa PI 3′-kinases are coupled to tyrosine kinases by regulatory adapter molecules (p85 and p55^{PIK}) which use their SH2 domains to bind tyrosyl-phosphorylated YMXM motifs (14, 28). The 110-kDa catalytic domain (p110) is activated by the binding of these tyrosyl-phosphorylated YMXM motifs (2). In addition to its ability to phosphorylate PI, p110 also phosphorylates certain proteins on serine residues (3, 8, 14, 17). The SH2 domains in p85 may bind PI as well as phosphotyrosine, providing a dual-specificity docking molecule during signal generation (30).

SHP2 is an SH2 domain-containing tyrosine phosphatase which associates with IRS-1 via the COOH-terminal autophosphorylation sites, Tyr-1172 and Tyr-1222 (34b, 38). SHP2 has been implicated in the control of mitogenesis and mitogen-activated protein kinase activation by insulin in a variety of studies employing microinjection and overexpression of inactive forms, SH2 domains, and antibodies (27, 37). Fyn is a member of the Src family of tyrosine kinases which contain an SH2 and an SH3 domain. We have recently shown that Fyn associates with IRS-1 via the COOH-terminal tyrosine phosphorylation sites, Tyr-1172 and Tyr-1222, although the physiological function of this association is unknown (40).

Because of the number and redundancy of tyrosine phosphorylation sites in IRS proteins, determining which sites mediate relevant biological responses has been difficult. Mutation at single tyrosine phosphorylation sites or deletion of portions of IRS-1 (20, 23) have not clarified the downstream pathways

* Corresponding author. Mailing address: Research Division, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. Phone: (617) 732-2578. Fax: (617) 732-2593. Electronic mail address: Whitemor@joslab.harvard.edu.

of IRS signaling. To resolve the mechanism of IRS signaling, we removed 18 potential tyrosine phosphorylation sites from IRS-1 by site-directed mutagenesis and compared its signaling potential with wild-type IRS-1, IRS-1^{3YMXM} (which retains three phosphorylation sites: Tyr-608, Tyr-628, and Tyr-658) and IRS-1^{YCT} (which retains Tyr-1172 and Tyr-1222). The three sites in IRS-1^{3YMXM} are clustered together in the middle of the IRS-1 molecule and have the same surrounding sequence (YMXMSP). Tyr-608 and Tyr-628 are known tyrosine phosphorylation sites which bind p85, and Tyr-658 is a likely site of tyrosine phosphorylation and p85 binding (38, 41). Tyr-1172 and Tyr-1222 are also known tyrosine phosphorylation sites on IRS-1 (38); these sites bind and activate SHP2 (27, 37), as well as binding Fyn in vitro and in vivo with unknown consequences (40).

As expected, IRS-1^{F18} was not tyrosine phosphorylated during insulin stimulation of 32D cell lines; surprisingly, it mediated, albeit weakly, insulin-stimulated mitogenesis. IRS-1^{YCT} underwent tyrosine phosphorylation but failed to signal differently than IRS-1^{F18} in our assays. By contrast, IRS-1^{3YMXM} retained much of the signaling potential of wild-type IRS-1 in 32D cells. Thus, IRS-1 mediates phosphotyrosine-independent signals during insulin stimulation of 32D cells, and the presence of phosphotyrosine specifically in YMXM motifs strongly enhances this response.

MATERIALS AND METHODS

Cell lines. 32D cell lines were maintained in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS) and 5% conditioned medium from WEHI-3 cells (a source of interleukin-3 [IL-3]) (48). Cell lines expressing the insulin receptor (IR), IRS-1, and both IR and IRS-1 have been described (23, 48). Cell lines expressing IRS-1 (or IRS-1 mutants) were maintained in medium containing 5 to 10 mM histidinol (Sigma). 32D cell lines were made quiescent for experiments by incubation in unsupplemented Dulbecco's minimal essential medium for 2 to 4 h.

Construction and expression of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT}. The cDNA for IRS-1^{F18} was produced by a modification of PCR-mediated site-directed mutagenesis by using the rat IRS-1 cDNA in pBluescript as the template (23). After creating a DNA fragment containing a single-point mutation, as described (23), the fragment was used as a template for a second round of PCR-mediated mutagenesis, and so on until the fragment contained mutations which replaced all tyrosines of interest with phenylalanine, including Tyr-147, -426, -460, -526, -578, -608, -628, -658, -727, -745, -746, -895, -939, -987, -999, -1010, -1172, and -1222. Mutant-containing PCR fragments were then subcloned into rat IRS-1 in pBluescript. The presence of desired mutations and the absence of adventitious mutations was confirmed by DNA sequencing following subcloning of PCR fragments, and again following reconstruction of the entire IRS-1^{F18} cDNA. The IRS-1^{3YMXM} cDNA was derived by subcloning the *Nco*I fragment containing wild-type sequences for tyrosines 608, 628, and 658 into the IRS-1^{F18} cDNA. IRS-1^{YCT} was derived from IRS-1^{F18} by joining the region containing the mutant sites 939 to 1010 with the region containing the wild-type sites 1172 and 1222 by PCR and subcloning the resultant hybrid fragment into the IRS-1^{F18} cDNA by using *Eco*RI and *Aat*II. The presence of the desired sequences was determined by nucleotide sequencing, and the cDNAs were subcloned into pCMVhis for expression by using *Sac*I and *Sal*I (41).

Antibodies and growth factors. Affinity-purified rabbit polyclonal antiphosphotyrosine antibodies (αPY) and polyclonal rabbit antibodies raised against baculovirus-produced IRS-1 protein were previously described (18, 49). In some cases, antibodies raised against the pleckstrin homology (PH) domain of IRS-1 expressed as a glutathione *S*-transferase fusion protein (25a) were used to immunoprecipitate IRS-1 isoforms. Rabbit antisera against $p70^{s6k}$ have been previously described (7), as have αp85α antibodies (21). Insulin was from Calbiochem (La Jolla, Calif.).

PI 3′-kinase activity. In vitro phosphorylation of PI was carried out in immune complexes as described previously (33). Quiescent cells were incubated with various concentrations of insulin for 10 min, washed once with ice-cold phosphate-buffered saline (PBS), and lysed. The cells were solubilized as described (33) and incubated with antibody for 1 to 2 h at 4°C. Immune complexes were precipitated from the supernatant with Protein A Sepharose (Pharmacia) and washed as described (33). Immune complexes were incubated with PI (Avanti) and [γ-^{32}P]ATP for 10 min at 22°C. The reactions were stopped with 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1) and centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography plate (Merck) which had been coated with 1% potassium oxalate (33). Thin-layer chromatography plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and then visualized and quantitated on a Molecular Dynamics Phosphorimager.

Immunoblotting. Proteins in cell lysates prepared as for PI 3′-kinase assays or from immunoprecipitates similarly prepared were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to nitrocellulose membranes (Schleicher & Schuell) in Towbin buffer containing 0.02% SDS and 20% methanol (45). Membranes were blocked, probed, and developed as previously described (7, 23). Blots were exposed to Kodak XAR film or imaged on a Molecular Dynamics Phosphorimager.

In vitro assays for $p70^{s6k}$ kinase activity. Quiescent cells were stimulated for 30 min and collected by centrifugation. Cells were lysed in ice-cold 10 mM potassium phosphate–1 mM EDTA (pH 7.05) containing 0.5% Nonidet P-40, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (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 at 10,000 × *g* for 10 min. Antibodies against $p70^{s6k}$ (αp70) were added for 2 h and collected on Protein A Sepharose beads for 1 h at 4°C. Immunoprecipitates were washed (7, 11) and incubated with [γ-^{32}P]ATP (50 μM final concentration; 20 μCi per reaction) containing 20 μg of 40S ribosomes per reaction (7, 11); the ribosomes were purified as previously described (7). Assays were incubated for 15 min at room temperature and were stopped by the addition of 2× Laemmli sample buffer. Samples were denatured by boiling and phosphorylated substrates were analyzed by SDS-PAGE.

Incorporation of [^{3}H]thymidine into DNA of 32D cells. Insulin-stimulated thymidine incorporation was assayed as previously described (23, 48). Briefly, cells in log phase growth were washed and seeded into RPMI 1640 with 10% FBS alone, 5% IL-3-containing WEHI-3 conditioned medium, or with various concentrations of insulin. Cells were grown for 48 h at 37°C. [^{3}H]thymidine (ICN) was added to a final concentration of 0.5 μCi/ml and incubation was continued for 3 h. Cells were collected onto glass microfiber filters and lysed, and unincorporated nucleotides were removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

Assay for long-term growth of 32D cell lines. 32D cell lines growing logarithmically in RPMI 1640 supplemented with 10% FBS and 5% WEHI-3 conditioned medium were washed and split 1:100 into RPMI 1640 containing 10% FBS and 100 nM insulin. When cells became confluent (>10^6 cells per ml), they were split 1:100 into the same medium. Ability to grow long term in insulin was scored by the ability to become confluent in insulin-containing medium following three successive 1:100 splits.

RESULTS

Expression of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT}. IRS-1^{F18} was prepared by sequential site-directed mutagenesis substituting phenylalanine for 18 potential tyrosine phosphorylation sites lying outside of the PH domain of IRS-1 (Fig. 1A). To examine the role of specific tyrosine-phosphorylated motifs in IRS-1 signaling, we also constructed IRS-1^{3YMXM} (which retained three YMXM motifs at Tyr-608, Tyr-628, and Tyr-658) and IRS-1^{YCT} (which retained two other sites, Tyr-1172 and Tyr-1222) (Fig. 1A). These residues are known phosphorylation sites in IRS-1 which bind to the SH2 domains in p85 (IRS-1^{3YMXM}) and SHP2 and Fyn (IRS-1^{YCT}) (38). We expressed IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} in 32D cells (Fig. 1B) or 32D cells overexpressing human IR (32D^{IR}) (Fig. 1C). 32D cells constitute a powerful system for the analysis of IRS-1 function since they contain no endogenous IRS proteins and require exogenous expression of these proteins for many insulin-stimulated signaling events (21, 25). 32D and 32D^{IR} cell lines expressing similar levels of IRS-1, IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} were identified by immunoblotting (Fig. 1B and C). All three mutants migrated at the appropriate molecular weight on SDS-PAGE, suggesting that they were stably expressed and serine phosphorylated under basal conditions. These results suggest that the multiple Y→F mutations do not grossly alter the behavior of IRS-1.

Phosphorylation of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} during insulin stimulation. We analyzed phosphorylation of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} by immunoblotting lysates of insulin-stimulated 32D cells lines with αPY (Fig. 2A and C). Consistent with the absence of IRS-1 from 32D cells, no tyrosine phosphorylation of IRS-1 was detected in parental

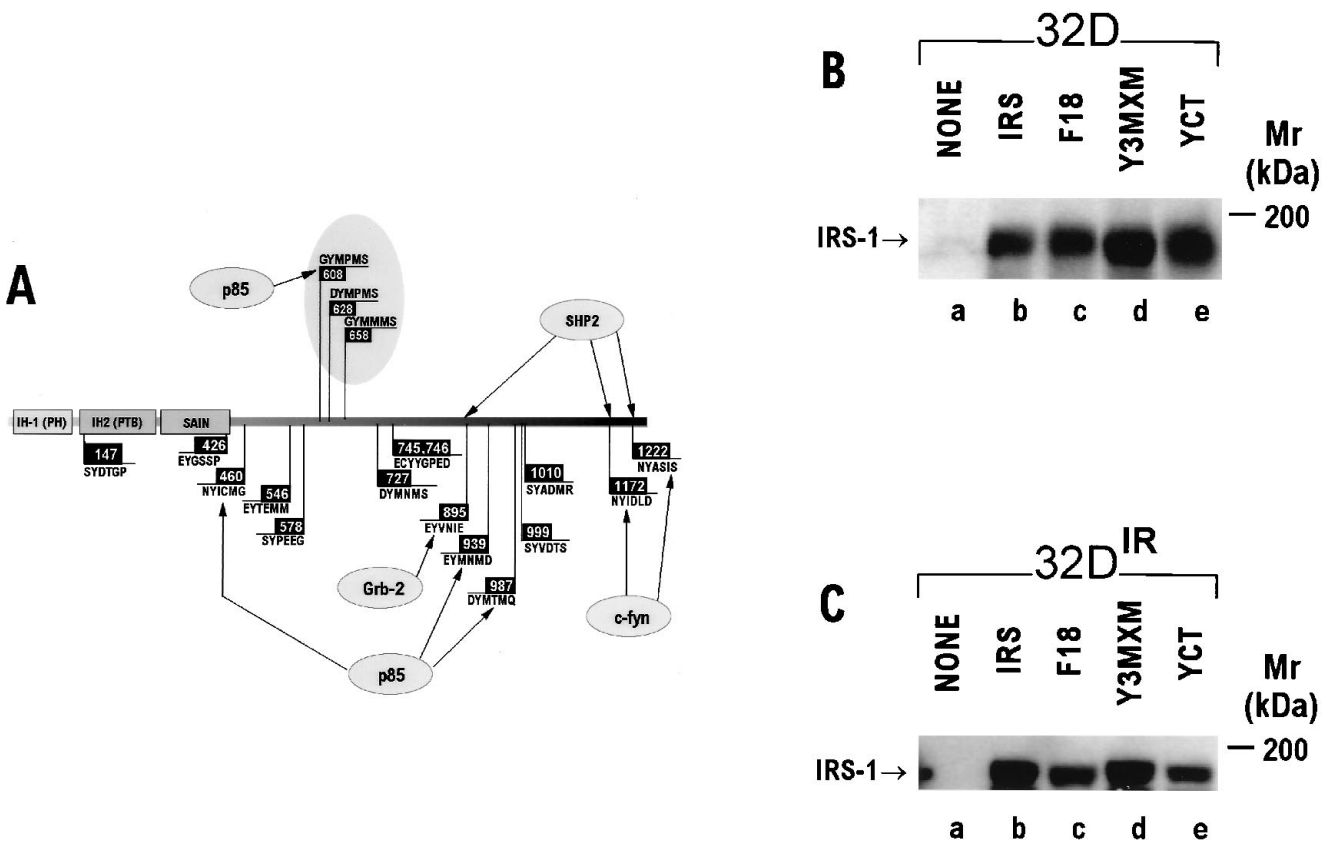

FIG. 1. (A) Generation of IRS-1^{F18} and IRS-1^{3YMXM}. Shown is a model of IRS-1 including tyrosine phosphorylation sites and the SH2 domain-containing proteins which bind them. We replaced the tyrosines in the 18 potential tyrosine phosphorylation sites outside of the PH domain in IRS-1 with phenylalanine (IRS-1^{F18}) by PCR-mediated, site-directed mutagenesis. IRS-1^{3YMXM} is a derivative of IRS-1^{F18} in which Tyr-608, Tyr-628, and Tyr-658 (all of which lie close together in YMXMSP motifs and which are predicted to bind PI 3′-kinase) were restored. IRS-1^{YCT} is an IRS-1^{F18} derivative with Tyr-1172 and Tyr-1222 restored. The tyrosines replaced are shown along with the PH domain (IH-1^{PH}), the phosphotyrosine-binding domain (IH-2^{PTB}), and the so-called SAIN region. Also shown are several SH2 domain-containing proteins and their binding sites on IRS-1. (B and C) Expression and tyrosine phosphorylation of IRS-1, IRS-1^{F18} and IRS-1^{3YMXM} in 32D cell lines. 32D cells (B) and 32D cells overexpressing the human IR (C) (lanes a) were transfected with the cDNAs for IRS-1 (lanes b), IRS-1^{F18} (lanes c), IRS-1^{3YMXM} (lanes d), IRS-1^{YCT} (lanes e), and selected expressing clones by immunoblotting with αIRS-1 antibodies. The migration of IRS-1 isoforms and the 200-kDa molecular mass marker are shown.

32D or $32D^{IR}$ cells (Fig. 2A and C); insulin-stimulated Shc tyrosine phosphorylation was detectable in all $32D^{IR}$ cell lines (Fig. 2C). Insulin stimulated IRS-1 tyrosine phosphorylation in 32D/IRS-1 cells, and this insulin effect was even more apparent in $32D^{IR}$/IRS-1 cells. However, IRS-1^{F18} was not tyrosine phosphorylated during insulin stimulation of 32D/F18 or $32D^{IR}$/F18 cells (Fig. 2A and C). Thus, IRS-1^{F18} no longer contains tyrosine phosphorylation sites recognized by the IR.

IRS-1^{3YMXM} was tyrosine phosphorylated in both 32D/3YMXM and $32D^{IR}$/3YMXM cells, and IRS-1^{YCT} was tyrosine phosphorylated in 32D/YCT and $32D^{IR}$/YCT cells (Fig. 2A and C). The tyrosyl phosphorylation of IRS-1^{3YMXM} and IRS-1^{YCT} was detectably lower than that of wild-type IRS-1, consistent with the removal of 15 and 16 potential tyrosine phosphorylation sites, respectively, from these molecules (Fig. 2A and C). Since insulin-stimulated phosphorylation was detected in IRS-1^{3YMXM} and IRS-1^{YCT} but not in IRS-1^{F18}, it is likely that no tyrosine phosphorylation sites remain in IRS-1^{F18}.

IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} signaling to PI 3′-kinase and $p70^{s6k}$. Tyrosine phosphorylation of specific motifs on IRS-1 recruits various SH2 proteins to IRS-1 (36, 38). Since phosphorylated YMXM motifs bind PI 3′-kinase, we tested the association of PI 3′-kinase with IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} during insulin stimulation (Fig. 2B and D). As previously shown, no PI 3′-kinase activity was detected in αIRS-1 immunoprecipitates from 32D or $32D^{IR}$ cells, consistent with the inability of human IR to bind p85 (21). In cells expressing IRS-1, however, large amounts of IRS-1-associated PI 3′-kinase activity was detected and the amount of PI 3′-kinase bound was increased in $32D^{IR}$/IRS-1 cells, consistent with the increased tyrosine phosphorylation of IRS-1 in these cells. No PI 3′-kinase activity was associated with IRS-1^{F18} or IRS-1^{YCT} during insulin stimulation of 32D or $32D^{IR}$ cells expressing these mutants, consistent with their lack of phosphorylated YMXM motifs (Fig. 2B and D). In contrast, IRS-1^{3YMXM} bound PI 3′-kinase, confirming that tyrosine-phosphorylated YMXM motifs in an IRS-1 context bind PI 3′-kinase during insulin stimulation (19, 39, 41).

PI 3′-kinase is activated by association with IRS-1 during insulin stimulation (2, 21). In vitro, this activation is mediated by the association of peptides containing phosphorylated YMXM motifs with the SH2 domains of p85 (2, 19); maximal activation occurs when both SH2 domains are occupied (31). In order to determine whether IRS-1^{F18} and IRS-1^{3YMXM} similarly mediate activation of PI 3′-kinase during insulin stimulation, we assayed the PI 3′-kinase activity recovered in αp85 immunoprecipitates from $32D^{IR}$ cell lines (Fig. 3A). There was

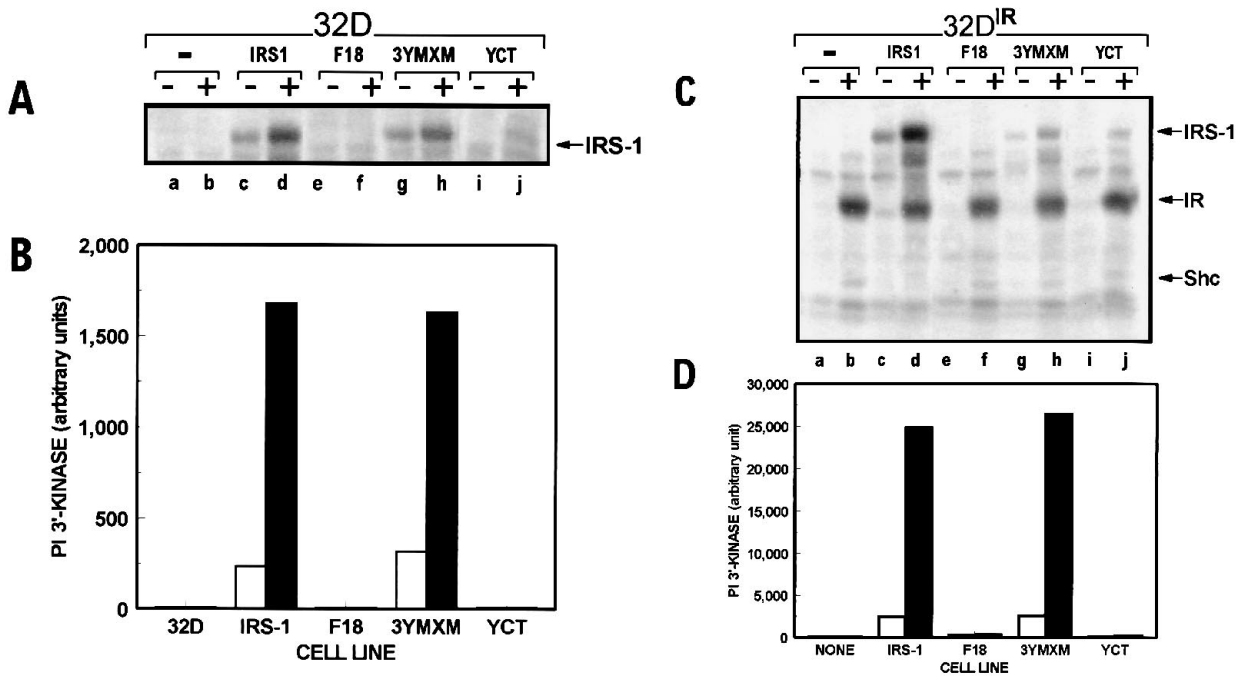

FIG. 2. Tyrosine phosphorylation of IRS-1, IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} in 32D (A) and $32D^{IR}$ (C) cell lines. Parental 32D or $32D^{IR}$ cells (lanes a and b) or cell lines expressing IRS-1 (lanes c and d), IRS-1^{F18} (lanes e and f), IRS-1^{3YMXM} (lanes g and h), and IRS-1^{YCT} (lanes i and j) were incubated in the absence (−) or presence (+) of 100 nM insulin for 2 min and lysed. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting with αPY antibodies. The migration of IRS-1, IR, and Shc are noted. Association of PI 3′-kinase with IRS-1 isoforms in 32D (B) and $32D^{IR}$ (D) cell lines. The indicated 32D cell lines were incubated in the absence (open bar) or presence (solid bar) of 100 nM insulin for 2 min and lysed. Lysates were immunoprecipitated with αIRS-1 antibodies. Immune complexes were collected on Protein A Sepharose, washed, and assayed for associated PI 3′-kinase activity. Activity was quantified on a Phosphorimager and is expressed in arbitrary units.

no activation of PI 3′-kinase by insulin in $32D^{IR}$ cells, commensurate with the lack of IRS proteins in these cells and the inability of the IR to bind directly to p85 in this cell background (21). Insulin did not activate PI 3′-kinase in $32D^{IR}$/F18 cells, in which the mutant IRS-1^{F18} molecule lacked sites for interaction with PI 3′-kinase. However, insulin activated a three- to fourfold increase in PI 3′-kinase in $32D^{IR}$/IRS-1 and $32D^{IR}$/3YMXM cells, demonstrating that IRS-1^{3YMXM} fully

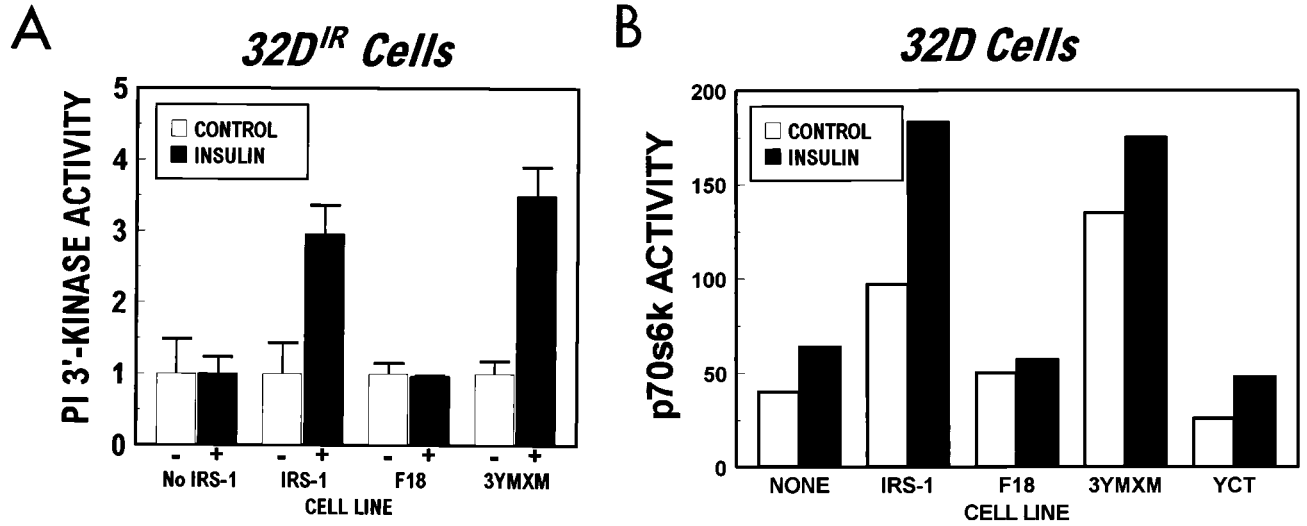

FIG. 3. (A) Activation of PI 3′-kinase by IRS-1 isoforms. Control $32D^{IR}$ cell lines or $32D^{IR}$ cell lines expressing IRS-1, IRS-1^{F18}, or IRS-1^{3YMXM} were starved and stimulated for 10 min with 100 nM insulin. Lysates were prepared and PI 3′-kinase was immunoprecipitated with αp85. Immunoprecipitates were washed and assayed for PI 3′-kinase and activity was quantified on a Phosphorimager. Experimental results were performed in triplicate and are expressed as mean ± standard errors of the mean. These results are representative of two similar experiments. (B) Stimulation of $p70^{s6k}$ activation by insulin in 32D cell lines. The indicated 32D cell lines were incubated in the absence or presence of 100 nM insulin for 30 min and lysed. Lysates were immunoprecipitated with α$p70^{s6k}$ antibodies, washed, and assayed for kinase activity by using 40S ribosomes as a substrate. Activity was quantified on a Phosphorimager and is expressed in arbitrary units. This assay is representative of two independent assays with multiple clones of each cell line.

TABLE 1. Proliferative signaling by IRS-1 isoforms in 32D cell lines

Cell line[a]	Thymidine incorporation[b]	Long-term growth[b,c]
32D	−	−
$32D^{IR}$	−	−
32D/IRS-1	−	−
$32D^{IR}$/IRS-1	+++	+++
$32D^{IR}$/F18	+	−
$32D^{IR}$/3YMXM	++	+++
$32D^{IR}$/YCT	+	−
$32D^{IR}$/F147	+++	+++
$32D^{IR}$/F608	+++	+++
$32D^{IR}$/F628	+++	+++
$32D^{IR}$/F658	+++	+++
$32D^{IR}$/F895	+++	+++
$32D^{IR}$/F1172	+++	+++
$32D^{IR}$/F1172, 1222	+++	+++

[a] 32D cell lines were analyzed for their ability to undergo mitogenesis (see Fig. 4) and grow long-term in 100 nM insulin. As well as comparing cells expressing IRS-1, IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} (boldface), we have included $32D^{IR}$ cell lines expressing site-directed point mutations of IRS-1 in which Tyr-147, Tyr-608, Tyr-628, Tyr-658, Tyr-895, Tyr-1172 (individually) or Tyr-1172 and Tyr-1222 (in combination) were replaced by phenylalanine.

[b] +++, maximal response; ++, less sensitive maximal response; +, blunted response; −, no response.

[c] Long-term growth was scored by the ability to withstand three 1:100 splits in insulin-containing media without IL-3.

activates PI 3′-kinase in these cells at high levels of insulin stimulation (Fig. 3A).

Since IRS-1 is required for activation of $p70^{s6k}$ during insulin signaling (21), and PI 3′-kinase is implicated in the activation of this enzyme (5, 6), we investigated the ability of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT} to mediate activation of $p70^{s6k}$ (Fig. 3B). Insulin had no effect on $p70^{s6k}$ activity in 32D or $32D^{IR}$ cells, which do not express IRS-1, as previously reported (21); as expected, the $p70^{s6k}$ was not activated by insulin in 32D/F18 or 32D/YCT cells. Insulin stimulated $p70^{s6k}$ in 32D/IRS-1 and 32D/3YMXM cells, however. Furthermore, $p70^{s6k}$ activity was increased over the control level even in the unstimulated state in cell lines expressing IRS-1 or IRS-1^{3YMXM} in this experiment, suggesting that even very low levels of appropriate tyrosine phosphorylation is required for the activation of $p70^{s6k}$. Thus, tyrosine phosphorylation of YMXM motifs in IRS-1 is sufficient for insulin-stimulated activation of $p70^{s6k}$, but phosphorylation of tyrosine not in YMXM motifs is not, consistent with the suggested role for PI 3′-kinase in $p70^{s6k}$ activation (5, 6).

Insulin-stimulated DNA synthesis and cell growth. Coexpression of the IR and IRS-1 is required for the long-term growth and survival of 32D cells in insulin (defined by recovery from three successive 1:100 splits from confluence). We assayed the ability of our mutant IRS-1 molecules to mediate long-term insulin-supported proliferation (Table 1). Insulin failed to support the long-term growth of 32D, $32D^{IR}$, and 32D/IRS-1 cell lines, as previously reported (48); however, $32D^{IR}$/IRS-1 and $32D^{IR}$/3YMXM cells survived and grew under these conditions. Insulin failed to support the long-term survival of $32D^{IR}$/F18 and $32D^{IR}$/YCT cells (Table 1). Thus, the nontyrosine-phosphorylated IRS-1^{F18} and tyrosine phosphorylation of Tyr-1172 and Tyr-1222 did not mediate long-term growth in insulin, but the presence of YMXM motifs on the IRS-1 backbone rescued this response.

As well as being necessary for long-term growth in insulin, IRS-1 expression with the IR is also required for shorter term insulin-stimulated mitogenesis in 32D cells (48). In order to compare the mitogenic signaling potential of IRS-1^{F18}, IRS-1^{3YMXM}, and IRS-1^{YCT}, we assayed 48-h insulin-stimulated [^{3}H]thymidine incorporation in 32D cell lines (Fig. 4A). There was no stimulation of thymidine incorporation by insulin in $32D^{IR}$ cells, as previously reported (23, 48). In cells expressing both IR and IRS-1, insulin stimulated a full mitogenic effect with a half-maximal stimulation occurring with 0.3 nM insulin. While insulin did not stimulate maximal mitogenesis in $32D^{IR}$/F18 or $32D^{IR}$/YCT cells, these cell lines performed similarly in this assay, attaining 30 to 50% of the full mitogenic response at 100 nM insulin. The $32D^{IR}$/F18 cell line with greater mitogenic signaling expresses higher levels of IRS-1^{F18} (data not shown). IRS-1^{3YMXM} mediated a full mitogenic response in $32D^{IR}$/3YMXM cells, although the concentration of insulin required for half-maximal thymidine incorporation was increased by 10-fold (3 nM) compared with cells expressing wild-type IRS-1. Thus, IRS-1^{F18} retained mitogenic potential, although the presence of three YMXM motifs restored the maximal mitogenic signaling potential of IRS-1, albeit with slightly less sensitivity to insulin than wild-type IRS-1. The addition of the COOH-terminal Tyr-1172 and Tyr-1222 to IRS-1^{F18} did not apparently affect signaling, suggesting that these sites and the SH2 domain-containing proteins that they bind are not important mediators of insulin-stimulated mitogenic signaling.

The diminished sensitivity of IRS-1^{3YMXM} mitogenic signaling compared with wild-type IRS-1 at low insulin concentrations could arise from diminished binding of PI 3′-kinase to IRS-1^{3YMXM} (due to the reduced total number of YXXM and YMXM motifs on IRS-1^{3YMXM} compared with wild-type IRS-1) or its inability to bind other SH2 proteins. We therefore assayed the association of PI 3′-kinase with IRS-1 and IRS-1^{3YMXM} during stimulation with various concentrations of insulin (Fig. 4B). This analysis demonstrated that the binding of PI 3′-kinase by IRS-1^{3YMXM} was indistinguishable from that of IRS-1 at most insulin concentrations, but at very low concentrations of insulin (10^{-10} M), IRS-1^{3YMXM} bound more PI 3′-kinase than wild-type IRS-1. Thus, while PI 3′-kinase clearly plays a major role in the mitogenic signal mediated by IRS-1, additional elements are required for the fully sensitive insulin response.

DISCUSSION

During insulin stimulation, the tyrosine kinase in the β subunit of the IR is activated by autophosphorylation and mediates the phosphorylation of the IRS proteins (IRS-1 and IRS-2), Shc (29, 32, 35, 41, 50), and *vav* (46). Tyrosine-phosphorylated motifs on these substrate proteins create tight binding sites for the SH2 domains of downstream signaling proteins. Thus, IRS proteins, which contain 15 to 20 potential phosphorylation sites, nucleate the formation of multisubunit signaling complexes (22), and are crucial mediators of a variety of insulin-stimulated events, including mitogenesis, anti-apoptosis (52), and glucose transport (1, 39, 43, 48).

In order to determine which element(s) of IRS-1 is required for various aspects of insulin signaling, we have previously examined the role of individual tyrosine residues in IRS-1 by replacing them with phenylalanine. In this manner we have individually examined tyrosine residues Tyr-608, Tyr-628, and Tyr-658 (which reside in YMXM motifs responsible for the engagement of PI 3′-kinase), Tyr-895 (which engages GRB-2), and Tyr-1172 and Tyr-1222 (which are responsible for the engagement of the tyrosine phosphatase, SHP2, and the Src family tyrosine kinase, Fyn) (23, 34a, 38, 40). Although mutation of Tyr-895 reduced signaling to mitogen-activated protein

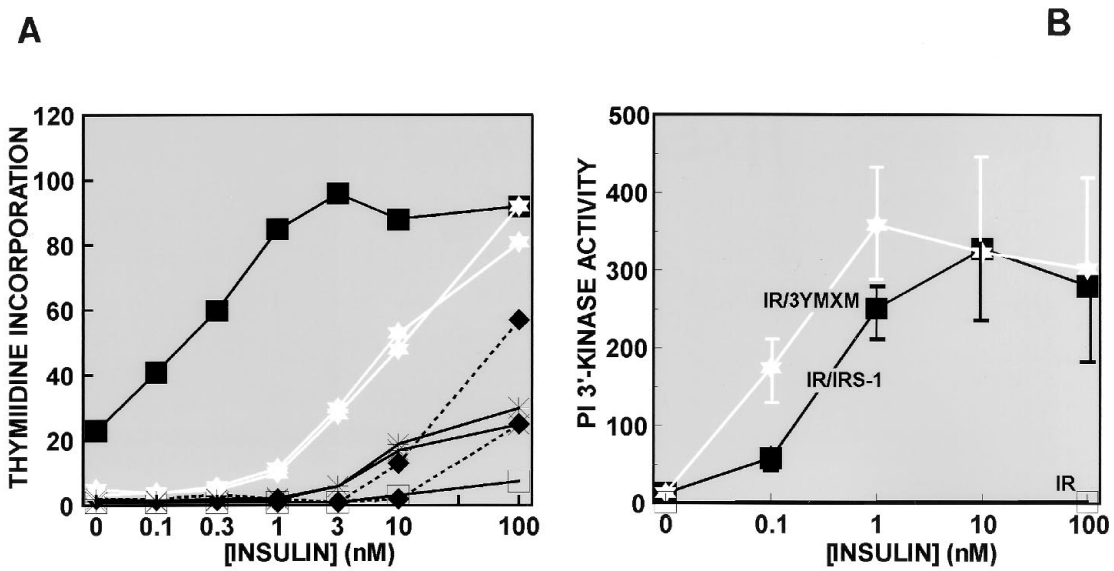

FIG. 4. (A) Mitogenic signaling by IRS-1 isoforms in 32D/IR cell lines. $32D^{IR}$ cell lines were washed and incubated in the presence of IL-3 or various concentrations of insulin for 48 h. [^{3}H]thymidine was added and incubation continued for 3 additional h. Cells were harvested on glass microfiber filters, washed, and counted with scintillation fluid. Results are expressed relative to maximal stimulation obtained with IL-3. Two independent clones of each of the IRS-1 isoform-expressing cell types were used and are graphed independently (open boxes, $32D^{IR}$ cells; closed boxes, $32D^{IR}$/IRS-1 cells; diamonds, $32D^{IR}$/F18 cells; white stars, $32D^{IR}$/3YMXM cells; and asterisks, $32D^{IR}$/YCT cells). Each datum point is the average of triplicate determinations, and this assay is representative of multiple independent experiments with multiple clones of each cell line. (B) PI 3′-kinase association with IRS-1 and IRS-1^{3YMXM} at various concentrations of insulin. $32D^{IR}$ cell lines expressing IRS-1 or IRS-1^{3YMXM} were starved and incubated in the presence of various concentrations of insulin for 10 min before being harvested and lysed. Lysates were immunoprecipitated with αIRS-1. Immunoprecipitates were washed and assayed for associated PI 3′-kinase activity, which was quantified on a Phosphorimager. Determinations were made in triplicate and data are means ± standard errors of the mean. These data are representative of two similar experiments.

kinases (23), none of these individual mutations had a measurable effect upon IRS-1-mediated mitogenic signaling in 32D cells (Table 1).

In order to trace the signaling pathways controlled by IRS-1, we constructed IRS-1^{F18} by replacing 18 potential tyrosine phosphorylation sites with phenylalanine and generated the derivative IRS-1^{3YMXM}, which retains three YMXMSP motifs (Tyr-608, Tyr-628, and Tyr-658) in the middle of the IRS-1 molecule. These sites are expected to create tight binding sites for PI 3′-kinase when phosphorylated (2, 19, 36, 41) (indeed, Tyr-608 and Tyr-628 are known to become tyrosine phosphorylated and bind p85). We also generated IRS-1^{YCT}, which retains Tyr-1172 and Tyr-1222 (the binding sites for SHP2 and Fyn) (38, 40). IRS-1^{3YMXM} was also stably expressed but, unlike IRS-1^{F18}, became tyrosine phosphorylated during insulin stimulation.

We analyzed the phosphoamino acid content of IRS-1 and the IRS-1 mutants during insulin stimulation. Unfortunately, even wild-type IRS-1 immunoprecipitated from insulin-stimulated $32D^{IR}$ cells contains so much more phosphoserine than phosphotyrosine that the levels of phosphotyrosine are virtually undetectable by this assay; therefore, phosphoamino acid analysis becomes meaningless as a method to demonstrate that IRS-1^{F18} is not tyrosine phosphorylated.

While we cannot entirely rule out the possibility that IRS-1^{F18} attains some level of insulin-stimulated tyrosine phosphorylation undetectable by Western blotting (immunoblotting) (with several monoclonal and polyclonal αPY antibodies), this is unlikely for several reasons. First, while the three tyrosine phosphorylation sites in IRS-1^{3YMXM} or the two different sites in IRS-1^{YCT} are readily detectable, no tyrosine phosphorylation is detectable in IRS-1^{F18}, and thus any remaining phosphorylated site(s) would likely be phosphorylated at a much lower stoichiometry than the sites in IRS-1^{3YMXM} and IRS-1^{YCT}. Second, although IRS-1^{F18} mediates a small amount of mitogenic signaling, it mediates neither activation of PI 3′-kinase nor $p70^{s6k}$, two sensitive downstream pathways dependent upon tyrosyl phosphorylation of IRS-1. Third, there remain no reasonable tyrosine phosphorylation sites in IRS-1^{F18}, as none have an acidic amino acid within five residues upstream or downstream of the target phosphorylation site (41). Three tyrosine residues in the PH domain with nearby acidic residues were retained in this construct; however, on the basis of the structure of the PH domain, these are unlikely to be available for phosphorylation by the tyrosine kinase.

While IRS-1^{F18} and IRS-1^{YCT} were unable to engage PI 3′-kinase or mediate activation of $p70^{s6k}$, IRS-1^{3YMXM} mediated both of these signaling events. These results are consistent with the requirement for tyrosine-phosphorylated YMXM motifs to bind the SH2 domains of PI 3′-kinase (2, 24) and with the putative role of PI 3′-kinase in the activation of $p70^{s6k}$ (4, 6). Also, IRS-1^{3YMXM} mediates the full mitogenic response to insulin and potentiates long-term growth, although the sensitivity to insulin is slightly reduced. Impaired association of PI 3′-kinase with IRS-1^{3YMXM} does not appear to account for the differences in signaling between IRS-1^{3YMXM} and IRS-1, since IRS-1^{3YMXM} binds quantitatively more PI 3′-kinase than the wild-type IRS-1 at low doses of insulin. It is possible that the increased PI 3′-kinase binding by IRS-1^{3YMXM} reflects the absence from this molecule of a tyrosine phosphorylation site(s) which engages negative signals. The binding of SHP2 to the COOH-terminal autophosphorylation sites Tyr-1172 and Tyr-1222 in wild-type IRS-1 represents one such possibility.

It is possible, however, that there is a qualitative difference in the interaction of these two IRS-1 molecules with PI 3′-kinase that we are unable to detect in our assays; for instance, the amount of PI 3′-kinase bound to IRS-1^{3YMXM} could reflect more IRS-1 molecules, which each bind a single PI 3′-kinase

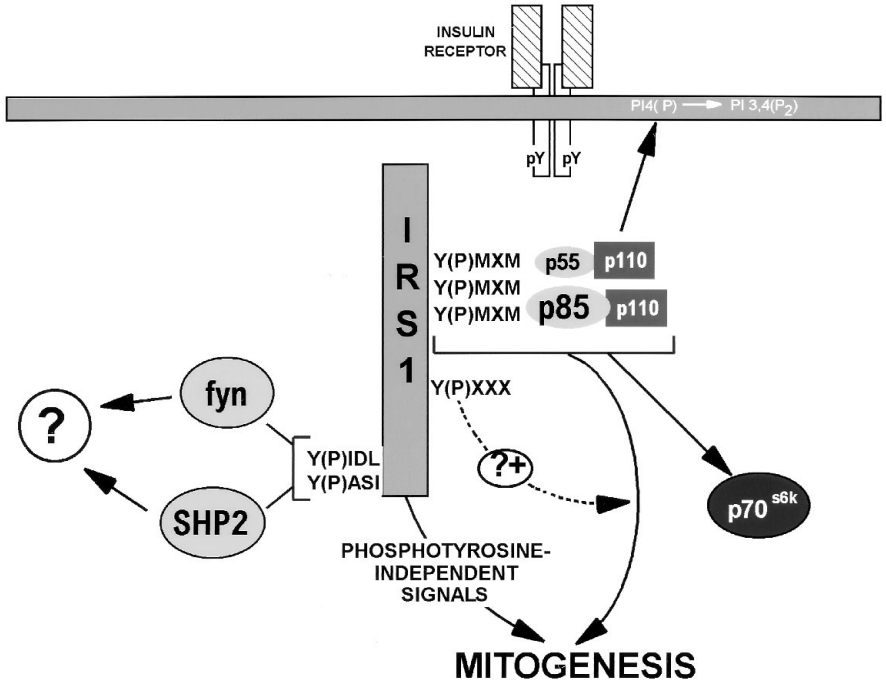

FIG. 5. Model of IRS-1-mediated signaling events. During insulin stimulation, IRS-1 is phosphorylated by the insulin receptor. This phosphorylated IRS-1 binds SH2 domain-containing signaling proteins and mediates downstream signals, such as mitogenesis. The binding of molecules such as the p85-p110 or p55-p110 PI 3′-kinase complex to tyrosine-phosphorylated YMXM motifs activates $p70^{s6k}$ and enables the full mitogenic signal at high doses of insulin. The COOH-terminal SHP2-Fyn binding tyrosine phosphorylation sites do not appear to play a role in mitogenic signal transmission. The binding of other SH2 proteins to distinct sites may modulate the PI 3′-kinase signal. Furthermore, IRS-1 mediates some mitogenic signals in the absence of tyrosine phosphorylation and PI 3′-kinase binding, suggesting that this molecule contains signaling potential beyond docking SH2 domains.

molecule, as opposed to fewer molecules of wild type IRS-1, which each bind multiple PI 3′-kinase molecules. The more likely possibility, however, is that another signaling pathway which complements or enhances PI 3′-kinase signaling is engaged by other sites in IRS-1.

$IRS\text{-}1^{F18}$ and $IRS\text{-}1^{YCT}$ do not mediate long-term growth in insulin in $32D^{IR}$ cells. Surprisingly, however, $IRS\text{-}1^{F18}$ mediates some mitogenic signaling. At insulin concentrations between 50 and 100 nM, 32^{IR}-F18 cells exhibited 30 to 50% of the DNA synthesis of wild-type expressing cells. Thus, $IRS\text{-}1^{F18}$ mediates a mitogenic signal, which gives rise to a partial mitogenic phenotype even in the absence of other important signals, such as those mediated by YMXM motifs. The mitogenic phenotype of $IRS\text{-}1^{YCT}$ is similar to that of $IRS\text{-}1^{F18}$, suggesting that Tyr-1172 and Tyr-1222 are not able to mediate positive or negative mitogenic signals in 32D cells. This conclusion is consistent with the lack of any observed signaling phenotype in $IRS\text{-}1^{YCT}$, in which only Tyr-1172 and Tyr-1222 are replaced by phenylalanine (Table 1).

Three conclusions arising from this analysis are illustrated in Fig. 5: (i) a nontyrosine-phosphorylated IRS-1 molecule can mediate biological signals, (ii) the C-terminal SHP2 and Fyn binding sites do not contribute to the mitogenic signal, and (iii) insulin-stimulated mitogenesis occurs through an IRS-1 molecule which contains only one type of tyrosine phosphorylation site, i.e., the YMXM motif. It is unlikely that the three tyrosine residues which we restored in $IRS\text{-}1^{3YMXM}$ would mediate signaling in a manner different from that of any of the other YMXM motifs in IRS-1, since these sites can be mutagenized individually or even deleted without destroying the ability of IRS-1 to signal in 32D cells (25b).

The nature of the phosphotyrosine-independent signals mediated by IRS^{F18} are at present unclear, as is their role in a normal, fully functional IRS-1 molecule. Our data from deletion mutants of IRS-1 suggest that sequences in the middle third of IRS-1 mediate signals important for generation of the full mitogenic response (25c). It is possible that these signals function independently of IRS-1 tyrosine phosphorylation, although further studies are required to determine whether this is actually the case.

The rescue of the full mitogenic and long-term growth response and $p70^{s6k}$ signaling by YMXM motifs, however, suggests a role for PI 3′-kinase in mediating insulin-stimulated mitogenesis and $p70^{s6k}$ activation. The SH2 domains of the p85 regulatory subunit bind phosphotyrosine residues lying in this motif (36). It is possible that other SH2 proteins may bind to these sequences, however. Furthermore, this result gains support from the analysis of mitogenic and $p70^{s6k}$ signaling in other systems, such as the platelet-derived growth factor receptor and polyoma middle T antigen (6, 9, 10, 14). In these systems, YMXM motifs are also important for the stimulation of mitogenesis. Furthermore, inhibitors of PI 3′-kinase enzymatic function interfere with insulin- and platelet-derived growth factor-stimulated mitogenesis and $p70^{s6k}$ in multiple cell types (4, 6, 14). The weight of these multiple lines of evidence suggests that PI 3′-kinase is, indeed, a key functionary in these signaling pathways. However, the IRS-1-independent activation of PI 3′-kinase and $p70^{s6k}$ by the YMXM-motif-containing tail of the *Drosophila* IR is not sufficient to mediate insulin-stimulated mitogenesis in 32D cells (51). Since mitogenic signaling by the *Drosophila* IR can be rescued by coexpression of IRS-1, IRS-1 must contribute essential non-PI 3′-kinase elements to the mitogenic signal (51). Moreover, the finding that insulin-stimulated PI 3′-kinase activity is more

sensitive in cells expressing IRS-1^{3YMXM} is consistent with the notion that additional signals are engaged through non-YMXM motifs of IRS-1.

As well as being a requisite player in mitogenic signaling and regulation of $p70^{s6k}$, PI 3′-kinase is thought to control movement of vesicles (including movement of GLUT4 to membranes for insulin-stimulated glucose transport), chemotaxis, and transporter endocytosis (4, 13, 14, 16, 22). How PI 3′-kinase performs these functions remains for the most part a mystery. While the lipid products are thought to mediate the downstream effects of PI 3′-kinase, the only demonstrated function for them is the activation of certain protein kinase C isoforms in vitro (44). The p110 catalytic subunit has multiple homologs, including the mammalian TORs (whose function is also poorly understood) and the *Saccharomyces cerevisiae* VPS34 (14, 34). VPS34 is a PI 3′-kinase required for effective targeting of vacuolar enzymes in yeast, suggesting that PI 3′-kinase may play an important role in vesicular movement. The p110 subunit possesses serine kinase activity as well as the better known PI 3′-kinase activity, however, and this may effect downstream signaling (8).

There exist multiple isoforms of both PI 3′-kinase subunits and the differences among their signaling function are poorly understood (12, 14, 26, 28). The recent cloning of a novel regulatory subunit isoform ($p55^{PIK}$) lacking the N-terminal SH3 domain and BCR-like domain (28) underscores another unknown in PI 3′-kinase signaling, i.e., the function of these N-terminal domains in p85.

In summary, we have demonstrated that IRS-1 transmits a signal in the absence of tyrosine phosphorylation and that PI 3′-kinase binding is restored by addition of YMXM motifs, but not other motifs, to an IRS-1 molecule without tyrosine phosphorylation sites. The ability of IRS-1 to mediate a full mitogenic response and activate $p70^{s6k}$ parallels binding of PI 3′-kinase, suggesting that these effects are PI 3′-kinase dependent. Future studies will hopefully elucidate the nature of the phosphotyrosine-independent signal(s) mediated by IRS-1, enhancing our understanding of insulin signaling and IRS-1 function.

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REFERENCES

1. **Araki, E., M. A. Lipes, M. E. Patti, J. C. Bruning, B. Haag III, R. S. Johnson, and C. R. Kahn.** 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature (London) **372:**186–190.
2. **Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. The phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11:**3469–3479.
3. **Carpenter, C. L., K. R. Auger, B. C. Duckworth, W.-M. Hou, B. Schaffhausen, and L. C. Cantley.** 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. **13:**1657–1665.
4. **Cheatham, R. 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.
5. **Chen, R. H., C. Abate, and J. Blenis.** 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc. Natl. Acad. Sci. USA **90:**10952–10956.
6. **Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis.** 1994. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. Nature (London) **370:**71–75.
7. **Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis.** 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinase. Cell **69:**1227–1236.
8. **Dhand, R., I. Hiles, G. Panayotou, S. Roche, M. J. Fry, I. Gout, N. F. Totty, O. Truong, P. Vicendo, K. Yonezawa, M. Kasuga, S. A. Courtneidge, and M. D. Waterfield.** 1994. PI-3-kinase is a dual specificity enzyme - autoregulation by an intrinsic protein-serine kinase activity. EMBO J. **13:**522–533.
9. **Escobedo, J. A., D. R. Kaplan, W. M. Kavanaugh, C. W. Turck, and L. T. Williams.** 1991. A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine. Mol. Cell. Biol. **11:**1125–1132.
10. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signalling pathways. Cell **69:**413–423.
11. **Fingar, D. C., S. F. Hausdorff, J. Blenis, and M. J. Birnbaum.** 1993. Dissociation of pp70 ribosomal protein S6 kinase from insulin-stimulated glucose transport in 3T3-L1 adipocytes. J. Biol. Chem. **268:**3005–3008.
12. **Hiles, I. D., M. Otsu, S. Volinna, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N. F. Totty, J. J. Hsuan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. Cell **70:**419–429.
13. **Joly, M., A. Kazlauskas, F. S. Fay, and S. Corvera.** 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science **263:**684–687.
14. **Kapeller, R., and L. C. Cantley, Jr.** 1994. Phosphatidylinositol 3-kinase. Bio. Essays **16:**565–576.
15. **Koch, C. A., D. J. Anderson, M. F. Moran, C. A. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252:**668–674.
16. **Kundra, V., J. A. Escobedo, A. Kazlauskas, H. K. Kim, S. G. Rhee, L. T. Williams, and B. R. Zetter.** 1994. Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. Nature (London) **367:**474–476.
17. **Lam, K., C. L. Carpenter, N. B. Ruderman, J. C. Friel, and K. L. Kelly.** 1994. The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. J. Biol. Chem. **269:**20648–20652.
18. **Miralpeix, M., X. J. Sun, J. M. Backer, M. G. Myers, Jr., E. Araki, and M. F. White.** 1992. Insulin stimulates tyrosine phosphorylation of multiple high molecular weight substrates in FAO hepatoma cells. Biochemistry **31:**9031–9039.
19. **Myers, M. G., Jr., J. M. Backer, X. J. Sun, S. E. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M. F. White.** 1992. IRS-1 activates the phosphatidylinositol 3′-kinase by associating with the src homology 2 domains of p85. Proc. Natl. Acad. Sci. USA **89:**10350–10354.
20. **Myers, M. G., Jr., T. C. Grammer, J. Brooks, E. M. Glasheen, L. M. Wang, X. J. Sun, J. Blenis, J. H. Pierce, and M. F. White.** 1995. The pleckstrin homology domain in IRS-1 sensitizes insulin signaling. J. Biol. Chem. **270:**11715–11718.
21. **Myers, M. G., Jr., T. C. Grammer, L. M. Wang, X. J. Sun, J. H. Pierce, J. Blenis, and M. F. White.** 1994. IRS-1 mediates PI 3′-kinase and $p70^{s6k}$ signaling during insulin, IGF-1 and IL-4 stimulation. J. Biol. Chem. **269:**28783–28789.
22. **Myers, M. G., Jr., X. J. Sun, and M. F. White.** 1994. The IRS-1 signaling system. Trends Biochem. Sci. **19:**289–294.
23. **Myers, M. G., Jr., L.-M. 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.
24. **Myers, M. G., Jr., and M. F. White.** 1993. The new elements in insulin signaling. Insulin receptor substrate-1 and proteins with SH2 domains. Diabetes **42:**643–650.
25. **Myers, M. G., Jr., and M. F. White.** 1995. New frontiers in insulin receptor substrate signaling. Trends Endocrinol. Metab. **6:**209–215.

25a. **Myers, M. G., Jr., and M. F. White.** Unpublished data.

25b. **Myers, M. G., Jr., and M. F. White.** Unpublished data.

25c. **Myers, M. G., Jr., and M. F. White.** 1996. Unpublished data.

26. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruis-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle-T/$pp60^{c\text{-}src}$ complexes and PI3-kinase. Cell **65:**91–104.
27. **Pluskey, S., T. J. Wandless, C. T. Walsh, and S. E. Shoelson.** 1995. Potent stimulation of SH-PTP2 phosphatase activity by simultaneous occupancy of both SH2 domains. J. Biol. Chem. **270:**2897–2900.
28. **Pons, S., T. Asano, E. Glasheen, M. Miralpeix, Y. Zhang, T. L. Fisher, M. G. Myers, Jr., X. J. Sun, and M. F. White.** 1995. The structure and function of $p55^{PIK}$ reveal a new regulatory subunit for phosphatidylinositol 3- kinase. Mol. Cell. Biol. **15:**4453–4465.
29. **Pronk, G. J., J. McGlade, G. Pelicci, T. Pawson, and J. L. Bos.** 1993. Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins. J. Biol. Chem. **268:**5748–5753.
30. **Rameh, L. E., C. S. Chen, and L. C. Cantley.** 1995. Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association

with tyrosine-phosphorylated proteins. Cell **83:**821–830.
31. **Rordorf-Nikolic, T., D. J. Van Horn, D. Chen, M. F. White, and J. M. Backer.** 1995. Regulation of phosphatidylinositol 3-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85 kDa regulatory subunit. J. Biol. Chem. **270:**3662–3666.
32. **Rosen, O. M.** 1987. After insulin binds. Science **237:**1452–1458.
33. **Ruderman, N., R. Kapeller, M. F. White, and L. C. Cantley.** 1990. Activation of phosphatidylinositol-3-kinase by insulin. Proc. Natl. Acad. Sci. USA **87:** 1411–1415.
34. **Schu, P. V., T. Kaoru, M. J. Fry, J. H. Stack, M. D. Waterfield, and S. D. Emr.** 1993. Phosphatidylinositol 3-kinase encoded by yeast *VPS34* gene essential for protein sorting. Science **260:**88–91.
34a. **Shi, P., M. G. Myers, Jr., and M. F. White.** 1995. Unpublished data.
34b. **Shi, P., M. G. Myers, Jr., and M. F. White.** 1996. Unpublished data.
35. **Skolnik, E. Y., C. H. Lee, A. G. Batzer, L. M. Vicentini, M. Zhou, R. J. Daly, M. G. Myers, Jr., J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger.** 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS-1 and Shc: implications for insulin control of ras signalling. EMBO J. **12:**1929–1936.
36. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and J. C. Cantley.** 1993. SH2 domains recognized specific phosphopeptide sequences. Cell **72:**767–778.
37. **Sugimoto, S., T. J. Wandless, S. E. Shoelson, B. G. Neel, and C. T. Walsh.** 1994. Activation of the SH2-containing protein tyrosine phosphatase, SH-PTP2, by phosphotyrosine-containing peptides derived from insulin receptor substrate-1. J. Biol. Chem. **269:**13614–13622.
38. **Sun, X. J., D. L. Crimmins, M. G. Myers, Jr., M. Miralpeix, and M. F. White.** 1993. Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol. Cell. Biol. **13:**7418–7428.
39. **Sun, X. J., M. Miralpeix, M. G. Myers, Jr., E. M. Glasheen, J. M. Backer, C. R. Kahn, and M. F. White.** 1992. The expression and function of IRS-1 in insulin signal transmission. J. Biol. Chem. **267:**22662–22672.
40. **Sun, X. J., S. Pons, T. Asano, M. G. Myers, Jr., E. Glasheen, and M. F. White.** 1996. The fyn tyrosine kinase binds IRS-1 and forms a distinct signaling complex during insulin stimulation. J. Biol. Chem. **271:**10583–10587.
41. **Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, B. J. Goldstein, and M. F. White.** 1991. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London) **352:**73–77.
42. **Sun, X. J., L. M. Wang, Y. Zhang, L. P. Yenush, M. G. Myers, Jr., E. Glasheen, W. S. Lane, J. H. Pierce, and M. F. White.** 1995. Role of IRS-2 in insulin and cytokine signalling. Nature (London) **377:**173–177.
43. **Tamemoto, H., T. Kadowaki, K. Tobe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, H. Sekihara, S. Yoshioka, H. Horikoshi, Y. Furuta, Y. Ikawa, M. Kasuga, Y. Yazaki, and S. Aizawa.** 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature (London) **372:**182–186.
44. **Toker, A., M. Meyer, K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley, Jr.** 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_3. J. Biol. Chem. **269:**32358–32367.
45. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
46. **Uddin, S., S. Katzav, M. F. White, and L. C. Platanias.** 1995. Insulin-dependent tyrosine phosphorylation of the *vav* proto-oncogene product in cells of hematopoietic origin. J. Biol. Chem. **270:**7712–7716.
47. **Uddin, S., L. P. Yenush, X. J. Sun, M. E. Sweet, M. F. White, and L. C. Platanias.** 1995. Interferon-alpha engages IRS-signaling proteins to regulate the phosphatidylinositol 3-kinase. J. Biol. Chem. **270:**15938–15941.
48. **Wang, L. M., M. G. Myers, Jr., X. J. Sun, S. A. Aaronson, M. F. White, and J. H. Pierce.** 1993. IRS-1: essential for insulin and IL-4-stimulated mitogenesis in hematopoietic cells. Science **261:**1591–1594.
49. **White, M. F., and J. M. Backer.** 1991. Preparation and use of antiphosphotyrosine antibodies to study structure and function of insulin receptors. Methods Enzymol. **201:**65–79.
50. **White, M. F., R. Maron, and C. R. Kahn.** 1985. Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature (London) **318:**183–186.
51. **Yenush, L., R. Fernandez, M. G. Myers, Jr., T. C. Grammer, X. J. Sun, J. Blenis, J. H. Pierce, J. Schlessinger, and M. F. White.** 1996. The *Drosophila* insulin receptor activates multiple signaling pathways but requires insulin receptor substrate proteins for DNA synthesis. Mol. Cell. Biol. **16:**2509–2517.
52. **Yenush, L., and M. F. White.** 1995. Unpublished data.