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Role of IRS-1–GRB-2 Complexes in Insulin Signaling

MARTIN G. MYERS, JR.,[1] LING-MAI WANG,[2] XIAO JIAN SUN,[1] YITAO ZHANG,[1] LYNNE YENUSH,[1] JOSEPH SCHLESSINGER,[3] JACALYN H. PIERCE,[2] AND MORRIS F. WHITE[1]*

Research Division, Joslin Diabetes Center and Harvard Medical School, Boston, Massachusetts 02215[1]; Laboratory of Cellular and Molecular Biology, National Institutes of Health, Bethesda, Maryland 20892[2]; and Department of Pharmacology, NYU School of Medicine, New York, New York 10016[3]

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GRB-2 is a small SH2- and SH3 domain-containing adapter protein that associates with the mammalian SOS homolog to regulate p21^{ras} during growth factor signaling. During insulin stimulation, GRB-2 binds to the phosphorylated Y_{895}VNI motif of IRS-1. Substitution of Tyr-895 with phenylalanine (IRS-1^{F-895}) prevented the IRS-1–GRB-2 association in vivo and in vitro. The myeloid progenitor cell line, 32-D, is insensitive to insulin because it contains few insulin receptors and no IRS-1. Coexpression of IRS-1 or IRS-1^{F-895} with the insulin receptor was required for insulin-stimulated mitogenesis in 32-D cells, while expression of the insulin receptor alone was sufficient to mediate insulin-stimulated tyrosine phosphorylation of Shc and activation of p21^{ras} and mitogen-activated protein (MAP) kinase. The Shc–GRB-2 complex formed during insulin stimulation is a possible mediator of p21^{ras} and MAP kinase activation in IRS-1-deficient 32-D cells. Interestingly, IRS-1, but not IRS-1^{F-895}, enhanced the stimulation of MAP kinase by insulin in 32-D cells expressing insulin receptors. Thus, IRS-1 contributes to the stimulation of MAP kinase by insulin, probably through formation of the IRS-1–GRB-2 complex at Tyr-895. Our results suggest that the Shc–GRB-2 complex and the activation of p21^{ras}-dependent signaling pathways, including MAP kinase, are insufficient for insulin-stimulated mitogenesis and that the essential function(s) of IRS-1 in proliferative signaling is largely unrelated to IRS-1–GRB-2 complex formation.

IRS-1 is a 131-kDa protein that undergoes tyrosine phosphorylation during stimulation of cells with insulin, insulin-like growth factor 1 (IGF-1), and interleukin 4 (IL-4) and immediately associates with signaling molecules which contain Src homology 2 domains (SH2 proteins) (23, 25, 36, 37, 40, 41). Overexpression of IRS-1 increases the proliferative response of Chinese hamster ovary cells to insulin and IGF-1 (36), whereas reduction of the IRS-1 level reduces the response (42). Moreover, 32-D myeloid progenitor cells are insensitive to insulin, IGF-1, and IL-4 because they lack IRS-1 or a functionally related molecule called 4PS (41). Expression of IRS-1 in 32-D cells rescues the mitogenic response to IGF-1 and IL-4, whereas coexpression of IRS-1 and insulin receptors (IRs) rescues the insulin response (40, 41). Thus, IRS-1 appears to play an essential role for some insulin responses by linking the receptor tyrosine kinase to key SH2 proteins.

IRS-1 contains more than 20 potential tyrosine phosphorylation sites, several of which bind specifically to the SH2 domains in various signaling proteins, including phosphatidylinositol (PtdIns) 3′-kinase, GRB-2, Syp, and nck (17, 18, 33, 37). The SH2 domains in PtdIns 3′-kinase preferentially bind to phosphopeptides containing Y-460, Y-608, Y-939, and Y-987, whereas GRB-2 and SH-PTP2 bind phosphopeptides containing Y-895 and Y-1172, respectively (35). Tyrosine phosphorylation of IRS-1 acts as the switch to control these interactions, whereas the surrounding amino acid sequences select the individual elements (26, 37). The divergent structure and function of SH2 proteins which bind to IRS-1 suggest that multisite phosphorylation of IRS-1 mediates the pleiotropic insulin response by simultaneously engaging several downstream signaling pathways (15, 26, 37). The IRS-1–SH2 protein interaction may regulate the activity of the SH2 proteins or target them to specific cellular locations (2, 14, 24).

GRB-2, a 23-kDa SH2 protein, binds to phosphotyrosine in Y(P)XNX motifs found in certain activated growth factor receptors or their substrates (33, 34). During insulin stimulation, GRB-2 does not bind to the IR; however, both IRS-1 and Shc are tyrosine phosphorylated during insulin stimulation and bind GRB-2. GRB-2 links tyrosine kinase receptors to mitogen-activated protein kinase (MAPK) and cellular proliferation by mediating the activation of p21^{ras} (9, 11, 19–21, 29, 31). GRB-2 appears to activate p21^{ras} by forming a stable complex through its SH3 domains to a proline-rich region in a *ras*-specific GDP/GTP exchange factor, mammalian *Son of Sevenless* (mSOS) (9, 19, 20, 29, 31, 33). Microinjection of GRB-2 and p21^{ras} into NIH 3T3 fibroblasts increases DNA synthesis, whereas injection of either protein alone has no effect (20). Moreover, overexpression of GRB-2 in rat L6 myoblasts increases the stimulation of MAPK by insulin (32). Thus, GRB-2 functions on the signaling pathway between the IR and MAPK and the binding of IRS-1 to GRB-2 may be important for this process (8, 32, 33).

In order to investigate the role of the IRS-1–GRB-2 interaction in insulin-stimulated mitogenesis, we created a site-directed mutant of IRS-1 in which Tyr-895, the putative binding site for GRB-2 on IRS-1 (33, 35), was replaced with phenylalanine (IRS-1^{F-895}). This mutation removed a major tyrosine phosphorylation site on IRS-1 and inhibited the association of IRS-1 and GRB-2 in vitro and in vivo. Expression of IRS-1, but not IRS-1^{F-895}, increased the activity of MAPK during insulin stimulation, suggesting that IRS-1–GRB-2 complexes contribute to this signal. However, p21^{ras} GTP loading and partial MAPK activation occurred in the absence of IRS-1 in 32-D cells, suggesting that other pathways—possibly involving Shc–GRB-2 complexes—are active. Together, our results clearly show that stimulation of mitogenesis by insulin requires a unique contribution from IRS-1 not

* Corresponding author. Mailing address: Research Division, Joslin Diabetes Center, 1 Joslin Pl., Boston, MA 02215. Phone: (617) 732-2578. Fax: (617) 732-2593.

involving the IRS-1–GRB-2 complex. Furthermore, insulin-stimulated Shc–GRB-2 complex, $p21^{ras}$ GTP loading, and the stimulation of MAPK activity are not sufficient for insulin-stimulated mitogenesis.

MATERIALS AND METHODS

Generation of IRS-1^{F-895} cDNA. The cDNA for rat IRS-1 in pBluescript (37) was used as a template for PCR-mediated oligonucleotide-directed mutagenesis with the mutagenic primers 5′ ACT/CCC/GAA/TTC/AAT/ATT/CAC/AAA/TTC/TCC/TGG 3′ and 5′ CTA/CTC/ATT/ACC/AAG/GTC 3′ (13). The rat IRS-1 cDNA and mutant PCR product were digested with *Eco*RI and *Bam*HI, and the mutant PCR fragment was inserted in place of the wild-type sequence. Presence of the desired mutation was confirmed by sequencing the recombinant molecule. The cDNA for IRS-1^{F-895} was subcloned into the pCMV his eukaryotic expression vector (37) and pBlueBac (24). Selection of recombinant IRS-1^{F-895}-expressing baculoviruses was previously described, except that the linearized *Autographa californica* nuclear polyhedrosis virus genome was used in conjunction with cationic liposome-mediated transfection (Invitrogen) (24).

Antibodies and growth factors. Affinity-purified rabbit polyclonal anti-PY antibodies have been previously described (44). The anti-IRS-1 antibodies were mouse monoclonal 1M92-7 for immunoblotting (UBI 05-190) (25) and rabbit polyclonal antibody prepared against baculovirus-produced rat IRS-1 for immunoprecipitations (UBI 06-165) (24). Anti-GRB-2 antibodies were protein A-purified rabbit polyclonal antibodies directed against amino acids 51 to 152 of GRB-2 expressed as a glutathione *S*-transferase (GST) fusion protein (33). Anti-MAPK antisera were previously described (5). Immunoprecipitating anti-p85 antibodies were rabbit antibodies directed against GST-p85 (2). The anti-Shc and immunoblotting anti-p85 antibodies were purchased from UBI (Lake Placid, N.Y.). For some experiments, anti-Shc and anti-IRS-1 antibodies were coupled to protein A-Sepharose 6 MB (Pharmacia) with dimethylpimelimidate (Pierce) (12).

In vitro phosphorylation and phosphopeptide mapping of IRS-1 and IRS-1^{F-895}. Wheat germ agglutinin-purified IR (WGA-IR) was prepared from CHO/IR cells (43). Baculovirus-expressed IRS-1 and IRS-1^{F-895} were produced and phosphorylated in the presence of activated WGA-IR and [γ-^{32}P] ATP as previously described (24) before being denatured in Laemmli sample buffer containing 100 mM dithiothreitol (DTT). For phosphopeptide mapping, IRS-1 from 2-h in vitro phosphorylation reactions was boiled in Laemmli sample buffer containing 10 mM DTT, incubated with excess iodoacetamide (Pierce) for 30 min, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were transferred to nitrocellulose and exposed to X-ray film. Bands corresponding to IRS-1 were excised, blocked, and digested with sequencing grade trypsin (Stratagene) as previously described (35). Tryptic peptides were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) with a HI-Pore reverse-phase RP-318 column eluted at a flow rate of 0.5 ml/min with 0.055% trifluoroacetic acid modified with 75% acetonitrile–0.05% trifluoroacetic acid solution (35). Eluted radioactivity was collected in 0.5-ml fractions and quantitated (Cerenkov counts) in a Beckman LS 1801 scintillation counter.

Association of IRS-1 and IRS-1^{F-895} with SH2 domain fusion proteins. GST or GST fusion proteins (1 μg) containing the SH2 domains of p85 (24) or GRB-2 (33) were resolved by SDS–15% PAGE and transferred to nitrocellulose. Membranes were washed four times in 10 mM Tris-HCl (pH 7.4) containing 250 mM NaCl, 0.05% Tween 20, and 10 mM 2-mercaptoethanol before being blocked overnight in 10 mM Tris-HCl (pH 7.4) containing 250 mM NaCl, 10 mM 2-mercaptoethanol, and 5% nonfat dry milk (Carnation) (block buffer). IRS-1 and IRS-1^{F-895} were phosphorylated for 2 h in vitro as described above, denatured, reduced, carboxymethylated, and incubated with the membranes in block buffer overnight at 4°C. Unbound nucleotide and IRS-1 were removed by extensive washing in 10 mM Tris-HCl (pH 7.4) containing 250 mM NaCl, 10 mM 2-mercaptoethanol, and 0.01% Tween 20. Bound IRS-1 was detected by imaging with a PhosphorImager (Molecular Dynamics).

Expression of IRS-1^{F-895} in 32-D cells. 32-D cell lines were grown and maintained in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum and 5% conditioned medium (IL-3 containing) from WEHI cells (40, 41). CsCl-purified pCMVhisIRS-1^{F-895} was introduced into 32-D cells and 32-D cells expressing the IR (line 286.5w3) by electroporation, and transformants were selected and grown in 2 to 5 mM histidinol as previously described (40, 41). Cells expressing similar amounts of IRS-1 and IRS-1^{F-895} were selected by analyzing lysates from equivalent numbers of cells by SDS–7.5% PAGE and immunoblotting with anti-IRS-1 antibodies.

Immunoprecipitation. 32-D cell lines were collected by low-speed centrifugation and made quiescent in Dulbecco modified Eagle medium (Sigma) for 2 to 4 h at 37°C. Cells were stimulated with insulin (100 nM) for 1 min before being diluted threefold in ice-cold phosphate-buffered saline (PBS), collected by centrifugation, and lysed in 1 ml of ice-cold lysis buffer containing 20 mM Tris (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40 (NP-40), 10% glycerol, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation, and supernatants were incubated with antibody for 1 to 2 h at 4°C before being collected with protein A-Sepharose 6 MB (Pharmacia) for 1 h at 4°C. Immunoprecipitations with protein A-coupled antibodies were performed for 2 h at 4°C. Immunoprecipitates were washed three times in lysis buffer before being denatured in Laemmli sample buffer and resolved by SDS-PAGE.

Immunoblotting. Immunoprecipitated proteins or cell lysates (prepared as described above for immunoprecipitates) were denatured by boiling in Laemmli sample buffer containing 100 mM DTT and were resolved by SDS-PAGE. Gels were transferred to nitrocellulose membranes, blocked, and probed as previously described (23). For detection by enhanced chemiluminescence (ECL; Amersham), blots were incubated for 30 to 60 min with affinity-purified goat anti-rabbit or goat anti-mouse antibodies (1:2,000) (Cappel). Blots were washed four or five times in wash buffer, dried, and exposed to Kodak X-AR film or imaged on a Molecular Dynamics PhosphorImager.

Incorporation of [^{3}H]thymidine into DNA in 32-D cells. Insulin-stimulated thymidine incorporation was assayed as previously described (41). Briefly, cells in log-phase growth were washed, and 2×10^5 cells were seeded into 1 ml of medium into each of 24 wells containing RPMI with 15% fetal bovine serum alone or containing various concentrations of insulin or IL-3-containing conditioned media (WEHI). Cells were grown for 48 to 72 h in 37°C incubators. [^{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; cells were lysed and unincorporated

nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

In vitro assay for activation of MAPK. Quiescent cells were stimulated with insulin, collected as described above, and lysed in ice-cold 10 mM KPO_4–1 mM EDTA (pH 7.05) containing 0.5% NP-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 DTT, 0.1 mM PMSF, and aprotinin and leupeptin at 10 μg/ml each. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Anti-p44 MAPK antibodies were added for 2 h and collected on protein A-Sepharose beads for 1 h at 4°C. Immunoprecipitates were washed twice in buffer A (10 mM Tris-HCl [pH 7.2] containing 1% NP-40, 0.5% deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM DTT, 0.1 mM PMSF), twice in buffer B (10 mM Tris-HCl [pH 7.2] containing 1 M NaCl, 0.1% NP-40, 1 mM Na_3VO_4, 2 mM DTT, and 0.1 mM PMSF), and once in 50 mM Tris-HCl (pH 7.2) containing 150 mM NaCl before being suspended in 20 μl of 1.5× kinase buffer (4). Assays were started by the addition of [γ-^{32}P]ATP (50 μM final concentration, 20 μCi per reaction mixture) and 2 μg of myelin basic protein (MBP; Sigma) per reaction mixture. Assay mixtures were incubated for 15 min at room temperature. Reactions were terminated by the addition of Laemmli sample buffer, and phosphorylated MBP was resolved by SDS-PAGE. ^{32}P incorporated into MBP was quantitated on a PhosphorImager.

PtdIns 3′-kinase activity. 32-D cell lines were grown, stimulated, lysed, and immunoprecipitated as for immunoprecipitations (see above). Immune complexes were precipitated from the supernatant with protein A-Sepharose (Pharmacia) and washed successively in PBS containing 1% NP-40 and 2 mM Na_3VO_4 (three times), 100 mM Tris-HCl (pH 7.5) containing 500 mM LiCl and 2 mM Na_3VO_4 (three times), and 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 2 mM Na_3VO_4 (two times). The pellets were resuspended in 50 μl of 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA and combined with 10 μl of 100 mM $MnCl_2$ and 10 μl of 2-μg/μl PtdIns (Avanti) sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EGTA. The phosphorylation reaction was started by adding 10 μl of 440 μM ATP containing 30 μCi of [γ-^{32}P]ATP. After 10 min at 22°C, the reaction was stopped with 20 μl of 8 N HCl and 160 μl of $CHCl_3$-methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography (TLC) plate (Merck) which had been coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$-CH_3OH-H_2O-NH_4OH (60:47:11.3:2), dried, and visualized and quantitated on a Molecular Dynamics PhosphorImager.

p21^{ras} GTP loading assay. Cells were grown to 10^6 cells per ml before being starved and labeled for 5 to 6 h in phosphate-free RPMI 1640 (Gibco) medium supplemented with 0.3 to 1.0 mCi of $^{32}P_i$ (NEN) per ml. Cells were stimulated with insulin for 3 min, diluted in ice-cold PBS, and collected by centrifugation. Cells were lysed in MILD buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 2 mM NaF, 5 mM Na_3VO_4, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml) containing 2.5 μg of Y13-259 anti-p21^{ras} antibody (Oncogene Science). Free nucleotides were removed by incubation with activated charcoal for 30 min, and immune complexes were collected with rabbit anti-rat antibodies prebound to protein A–Sepharose CL-4B (Pharmacia) for 30 min before being washed five times in MILD buffer and once in PBS. Labeled nucleotides were eluted for 3 min in 1 M potassium phosphate (pH 3.4) at 85°C and resolved on phosphocellulose TLC plates in the same buffer. To ensure that the visualized nucleotides were specifically p21^{ras} bound, controls in which the specific anti-p21^{ras} antibody was omitted were performed.

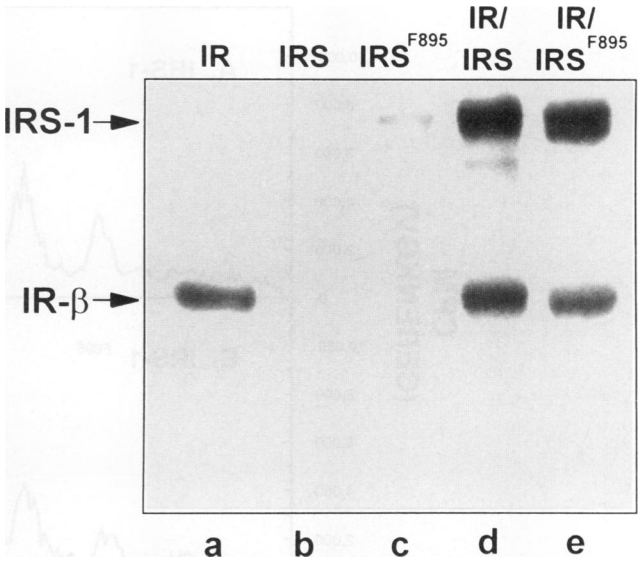


FIG. 1. IR-dependent phosphorylation of IRS-1^{F-895} in vitro. Baculovirus-expressed IRS-1 (lanes b and d) and IRS-1^{F-895} (lanes c and e) were incubated with [γ-^{32}P]ATP in the presence (lanes d and e) or absence (lanes b and c) of activated IR for 30 min. Lane a, activated IR alone. Samples were boiled in Laemmli sample buffer, separated by SDS–7.5% PAGE, and visualized by imaging on a PhosphorImager. Migration of normal and mutant IRS-1 and the IR β-subunit is indicated.

RESULTS

Association of GRB-2 with IRS-1 and IRS-1^{F-895}. We expressed IRS-1 and IRS-1^{F-895} in Sf9 cells infected with recombinant baculovirus containing the wild-type and mutant IRS-1. During incubation with [γ-^{32}P]ATP, neither IRS-1 nor IRS-1^{F-895} was significantly phosphorylated (Fig. 1, lanes b and c); however, after addition of the activated IR, both IRS-1 and IRS-1^{F-895} were strongly phosphorylated on multiple tyrosine residues (Fig. 1). We digested the [^{32}P]IRS-1 and [^{32}P]IRS-1^{F-895} with trypsin and separated the [^{32}P]phosphopeptides by reverse-phase HPLC (Fig. 2). One doublet peak which eluted in fractions 164 to 172 from the IRS-1 digest was absent from the phosphopeptide elution profile of IRS-1^{F-895}; this tryptic phosphopeptide was previously shown by direct sequencing to contain Tyr(P)-895 (35).

Tyr(P)-895 may be the principal site for the association of GRB-2 with IRS-1 (33, 35) since it is the only tryptic phosphopeptide that specifically binds to the SH2 domain of GRB-2 (35). We used a membrane binding assay to measure directly the association between the SH2 domain of GRB-2 and IRS-1^{F-895}. GST fusion proteins containing the SH2 domain of GRB-2 (GST-SH2^{GRB2}) or p85 (GST-SH2^{p85n}) were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with recombinant [^{32}P]IRS-1 and [^{32}P]IRS-1^{F-895} (Fig. 3). Neither IRS-1 nor IRS-1^{F-895} bound GST alone, whereas IRS-1 bound to both GST-SH2^{p85n} and GST-SH2^{GRB-2} (Fig. 3A). However, IRS-1^{F-895} bound only to GST-SH2^{p85n}, suggesting that Tyr(P)-895 was required for high-affinity binding of IRS-1 to GRB-2 but not to p85. These

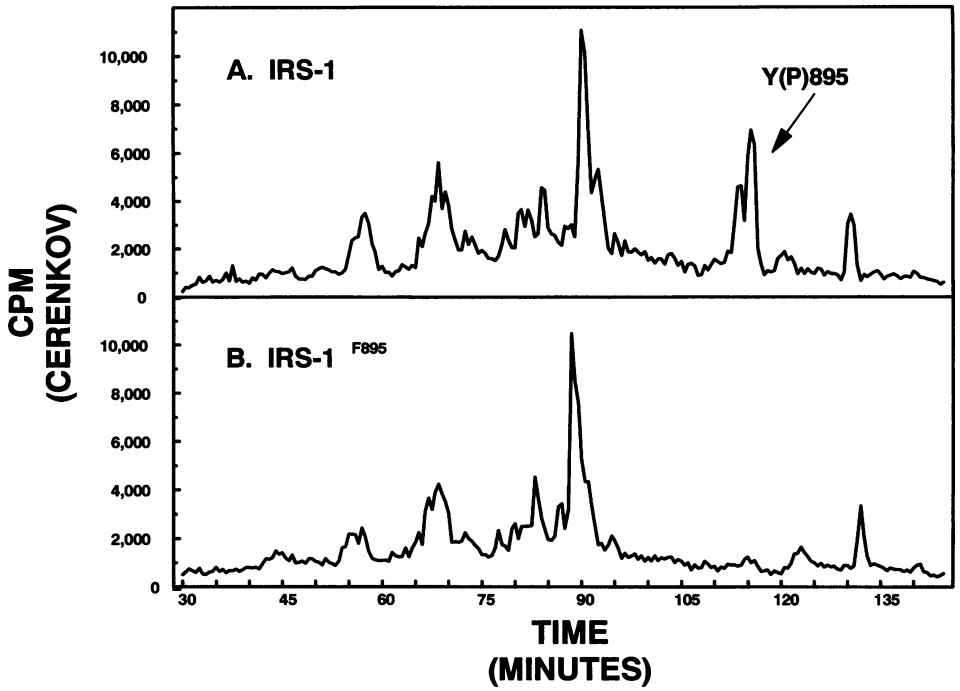


FIG. 2. Tryptic phosphopeptide map of IRS-$1^{F\text{-}895}$. Baculovirus-produced IRS-1 and IRS-$1^{F\text{-}895}$ were phosphorylated for 2 h with WGA-IR in the presence of [γ-^{32}P]ATP for 2 h in vitro, reduced, carboxymethylated, and analyzed by SDS–7.5% PAGE. Phosphorylated IRS-1 was transferred to nitrocellulose, visualized, and digested with trypsin, and phosphopeptides were analyzed by reverse-phase HPLC. Eluting fractions were collected, and phosphopeptides were detected by counting in a scintillation counter. The peak containing the Y(P)-895-containing phosphopeptide is indicated.

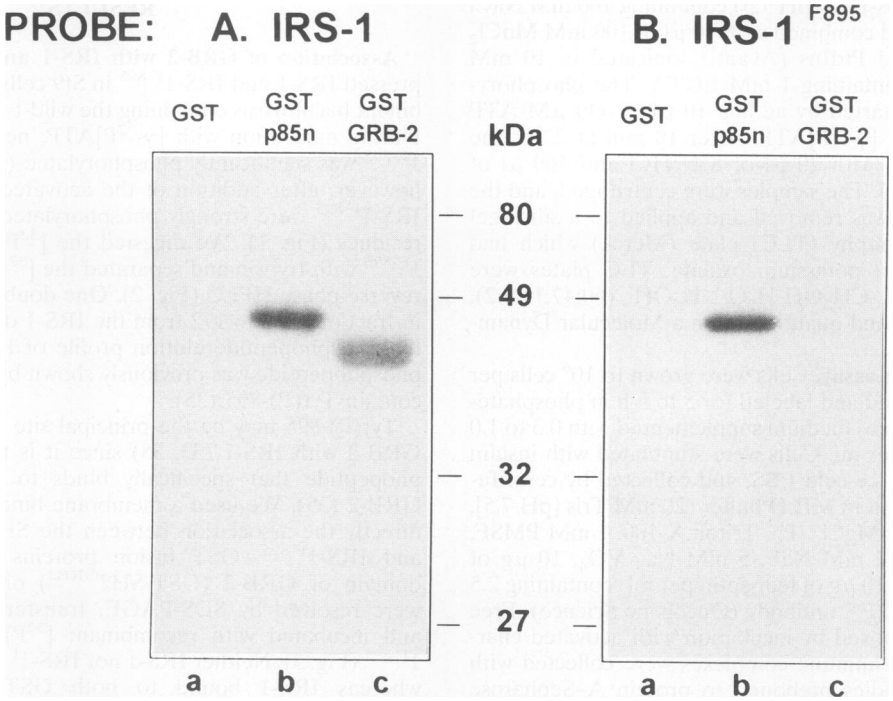


FIG. 3. Binding of phosphorylated IRS-1 and IRS-$1^{F\text{-}895}$ to SH2 domain-GST fusion proteins. GST alone (lanes a), GST-p85α^{nSH2} (lanes b), or GST–GRB-2^{SH2} (lanes c) was resolved by SDS–15% PAGE and transferred to nitrocellulose. Filters were incubated with [^{32}P]IRS-1 (A) or [^{32}P]IRS-$1^{F\text{-}895}$ (B) and washed. Bound IRS-1 was detected by imaging with a PhosphorImager. Migration of molecular mass standards is indicated.

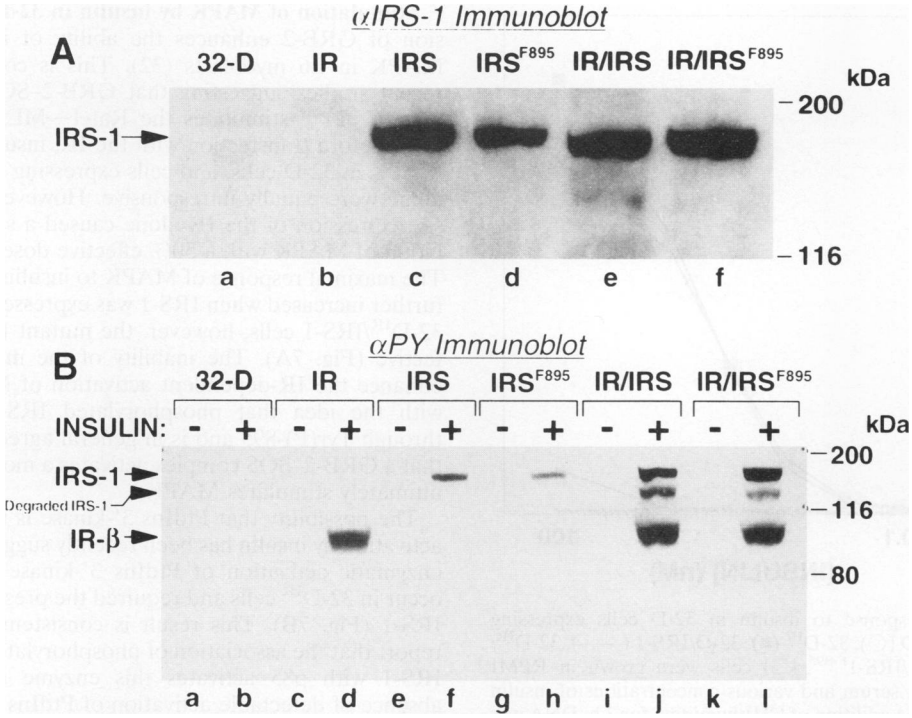


FIG. 4. Expression and phosphorylation of IRS-1 and IRS-1^{F-895} in 32-D cells. (A) Lysates of 32-D (lane a), 32-D^{IR} (lane b), 32-D/IRS-1 (lane c), 32-D/IRS-1^{F-895} (lane d), 32-D^{IR}/IRS-1 (lane e), and 32-D^{IR}/IRS-1^{F-895} (lane f) cells were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-IRS-1 (αIRS-1). (B) Lysates of unstimulated (lanes a, c, e, g, i, and k) or insulin-stimulated (lanes b, d, f, h, j, and l) 32-D (lanes a and b), 32-D^{IR} (lanes c and d), 32-D/IRS-1 (lanes e and f), 32-D/IRS-1^{F-895} (lanes g and h), 32-D^{IR}/IRS-1 (lanes i and j), or 32-D^{IR}/IRS-1^{F-895} (lanes k and l) cells were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PY (αPY). Migration of molecular mass standards, IRS-1/IRS-1^{F-895}, and IR β-subunit is indicated. The 130-kDa phosphoprotein in lanes from insulin-stimulated 32-D^{IR}/IRS-1 and 32-D^{IR}/IRS-1^{F-895} cells represents a partial degradation product of IRS-1 (lanes j and l) (26a).

results confirm our original conclusion that Tyr(P)-895 is the principal site for association between GRB-2 and IRS-1.

Expression and tyrosine phosphorylation of IRS-1^{F-895} in 32-D cells. We expressed IRS-1 and IRS-1^{F-895} in 32-D cells (32-D/IRS-1 and 32-D/IRS-1^{F-895}) which contain few IRs and no endogenous IRS-1 (41). Cell lines expressing approximately equal amounts of protein were identified by immunoblotting with anti-IRS-1 (Fig. 4A). Before coexpression of the human IR, insulin weakly stimulated tyrosine phosphorylation of both IRS-1 and IRS-1^{F-895}, confirming that 32D cells contain a small number of endogenous IRs (Fig. 4B). However, the level of insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1^{F-895} was significantly increased by the coexpression of the IR in 32-D^{IR}/IRS-1 and 32-D^{IR}/IRS-1^{F-895} cells (Fig. 4).

To determine whether IRS-1 and GRB-2 interact in 32-D cells and to determine whether Tyr(P)-895 of IRS-1 is required for this interaction in vivo, we immunoprecipitated IRS-1 from the transfected 32-D cells and analyzed for the association of

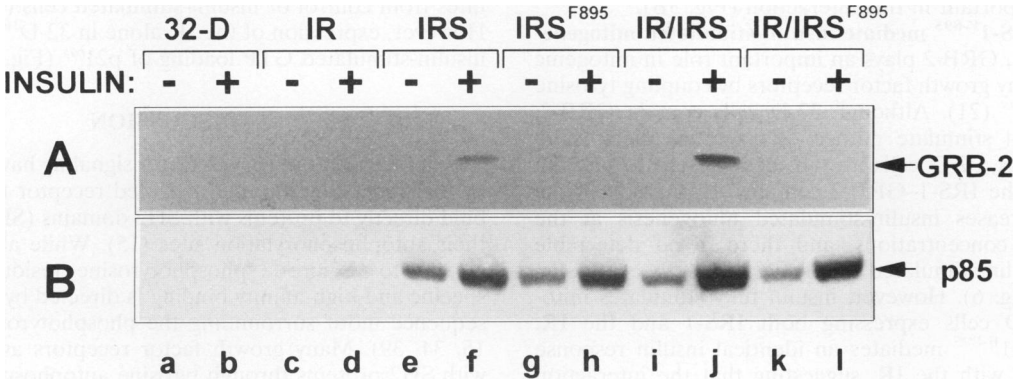


FIG. 5. Binding of IRS-1 and IRS-1^{F-895} to SH2 domain-containing proteins in 32-D cells. 32-D (lanes a and b), 32-D^{IR} (lanes c and d), 32-D/IRS-1 (lanes e and f), 32-D^{IR}/IRS-1 (lanes g and h), or 32-D^{IR}/IRS-1^{F-895} (lanes i and j) cells were stimulated with insulin and lysed. Immunoprecipitates were prepared with anti-IRS-1 antibodies, resolved by SDS-PAGE, and transferred to nitrocellulose. Membranes were blocked and probed with anti-GRB-2 (A) and anti-p85 (B). The migrations of GRB-2 and p85 are indicated.

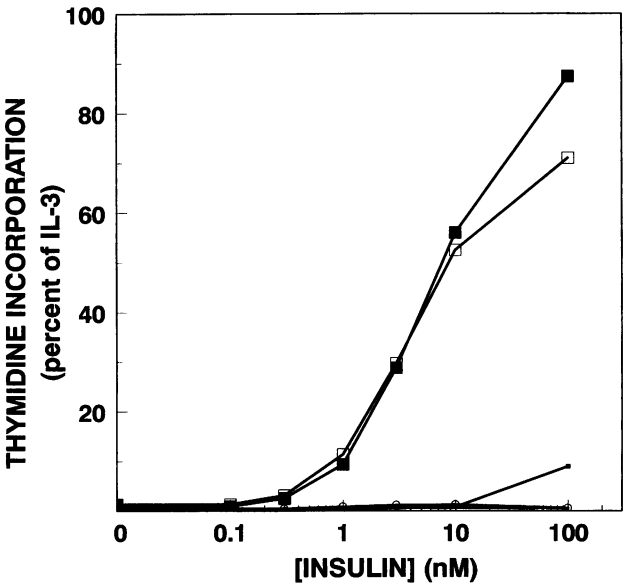


FIG. 6. Mitogenic response to insulin in 32-D cells expressing IRS-1 or IRS-1^{F-895}. 32-D (○), 32-D^{IR} (■), 32-D/IRS-1 (——), 32-D^{IR}/IRS-1 (■), and 32-D^{IR}/IRS-1^{F-895} (□) cells were grown in RPMI medium containing 10% serum and various concentrations of insulin or IL-3 for 48 h before the addition of [^{3}H]thymidine for 3 h. DNA was collected onto glass microfiber filters, and free nucleotide was washed away. The amount of nucleotide incorporated into DNA was quantitated by scintillation counting and normalized to incorporation in IL-3-treated cells.

GRB-2 by immunoblotting with anti-GRB-2 (Fig. 5A). No GRB-2 was detected in anti-IRS-1 immunoprecipitates from cells which did not express IRS-1 (32-D and 32-D^{IR}), whereas GRB-2 associated with anti-IRS-1 immunoprecipitates from insulin-stimulated 32-D/IRS-1 or 32-D^{IR}/IRS-1 cells (Fig. 5A). The GRB-2–IRS-1 association was dependent on tyrosine phosphorylation of IRS-1, since no GRB-2 was detected in unstimulated cells and more GRB-2 was associated with the more highly phosphorylated IRS-1 from 32-D^{IR}/IRS-1 cells than with the IRS-1 from 32-D/IRS-1 cells (Fig. 5A). GRB-2 was not bound to IRS-1^{F-895} in 32-D/IRS-1^{F-895} or 32-D^{IR}/IRS-1^{F-895} cells, demonstrating that mutation of Y-895 interferes with the association of IRS-1 and GRB-2 in the 32-D cells. In contrast, p85 binds normally to IRS-1^{F-895}, confirming that Y-895 is not important in this interaction (Fig. 5B).

IRS-1 and IRS-1^{F-895} mediate insulin-stimulated mitogenesis in 32-D cells. GRB-2 plays an important role in mitogenic signaling by many growth factor receptors by coupling tyrosine kinases to p21^{ras} (21). Although 32-D cells contain GRB-2, insulin does not stimulate mitogenesis because there is no IRS-1 (41). Thus, 32-D cells are an ideal system to test the importance of the IRS-1–GRB-2 complex. Expression of the IR weakly increases insulin-stimulated mitogenesis at the highest insulin concentrations, and there is no detectable increase in insulin-stimulated mitogenesis in cells expressing IRS-1 alone (Fig. 6). However, insulin fully stimulates mitogenesis in 32-D cells expressing both IRS-1 and the IR. Moreover, IRS-1^{F-895} mediates an identical insulin response when expressed with the IR, suggesting that the interaction between GRB-2 and IRS-1 is not required for insulin-mediated mitogenesis in 32-D cells. Similar results were obtained with cell lines expressing various levels of IRS-1 and IRS-1^{F-895} (data not shown).

Stimulation of MAPK by insulin in 32-D cells. Overexpression of GRB-2 enhances the ability of insulin to stimulate MAPK in L6 myoblasts (32). This is consistent with many recent studies suggesting that GRB-2–SOS-mediated activation of p21^{ras} stimulates the Raf-1→MEK→MAPK cascade (21). Before transfection with the IR, insulin had no effect on MAPK in 32-D cells, and cells expressing IRS-1 or IRS-1^{F-895} alone were equally unresponsive. However, unlike mitogenesis, expression of the IR alone caused a strong insulin stimulation of MAPK with a 50% effective dose of 1 nM (Fig. 7A). The maximal response of MAPK to insulin in 32-D^{IR} cells was further increased when IRS-1 was expressed with the IR in the 32-D^{IR}/IRS-1 cells; however, the mutant IRS-1^{F-895} was ineffective (Fig. 7A). The inability of the mutant IRS-1^{F-895} to enhance the IR-dependent activation of MAPK is consistent with the idea that phosphorylated IRS-1 engages GRB-2 through Tyr(P)-895 and is in general agreement with the idea that a GRB-2–SOS complex activates a molecular cascade that ultimately stimulates MAPK.

The possibility that PtdIns 3′-kinase is upstream of MAPK activation by insulin has been recently suggested. However, the enzymatic activation of PtdIns 3′-kinase by insulin did not occur in 32-D^{IR} cells and required the presence of both IR and IRS-1 (Fig. 7B). This result is consistent with our previous report that the association of phosphorylated YXXM motifs of IRS-1 with p85 activates this enzyme (2). Moreover, the absence of detectable activation of PtdIns 3′-kinase in 32-D^{IR} cells suggests that it is not involved in the IRS-1-independent activation of MAPK by insulin.

The ability of insulin to stimulate MAPK in 32-D cells expressing the IR alone was surprising and led us to identify alternative IRS-1-independent pathways to activate GRB-2–SOS. One possibility is insulin-stimulated tyrosine phosphorylation of Shc, which we previously assumed to be IRS-1 dependent. Shc is not tyrosine phosphorylated during insulin stimulation of untransfected 32-D cells; however, expression of the IR alone is sufficient to mediate insulin-stimulated Shc tyrosine phosphorylation and its association with GRB-2 (Fig. 8A). Expression of IRS-1 or IRS-1^{F-895} has no detectable effect on the phosphorylation of Shc or its association with GRB-2 (Fig. 8A). Insulin-stimulated tyrosine phosphorylation of Shc provides a plausible alternative pathway to activate the GRB-2–SOS complex and stimulate GTP loading of p21^{ras} in the absence of IRS-1. We tested this hypothesis by measuring GTP loading of p21^{ras} in ^{32}P-labeled 32-D and 32-D^{IR} cells before and after insulin stimulation. Untransfected 32-D cells revealed no detectable GTP loading of p21^{ras} in immunoprecipitates from control or insulin-stimulated cells (Fig. 8B and C). However, expression of the IR alone in 32-D^{IR} cells mediated insulin-stimulated GTP loading of p21^{ras} (Fig. 8B and C).

DISCUSSION

Several aspects of growth factor signaling have been clarified by the understanding that activated receptor tyrosine kinases bind directly to proteins with SH2 domains (SH2 proteins) via their autophosphorylation sites (15). While all SH2 domains appear to require a phosphotyrosine residue for binding, specific and high-affinity binding is directed by the amino acid sequence motif surrounding the phosphotyrosine residue (8, 15, 34, 39). Many growth factor receptors associate directly with SH2 proteins through tyrosine autophosphorylation sites located in intrinsic domains of the receptor or tightly associated subunits. However, although the IR contains multiple tyrosine autophosphorylation sites, it weakly binds known SH2 proteins (26). In contrast, IRS-1, a principal substrate of the IR

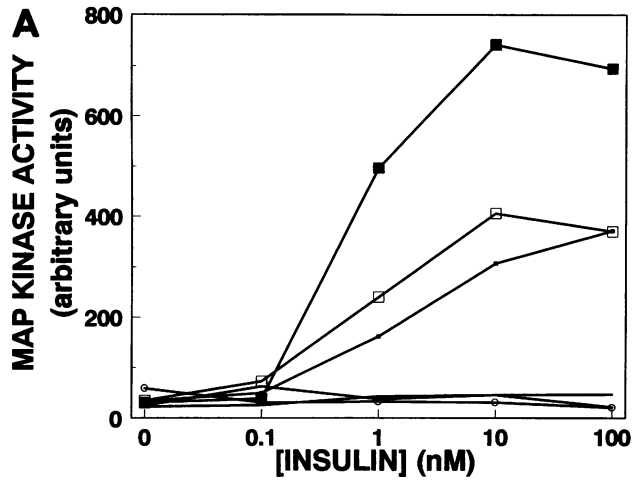
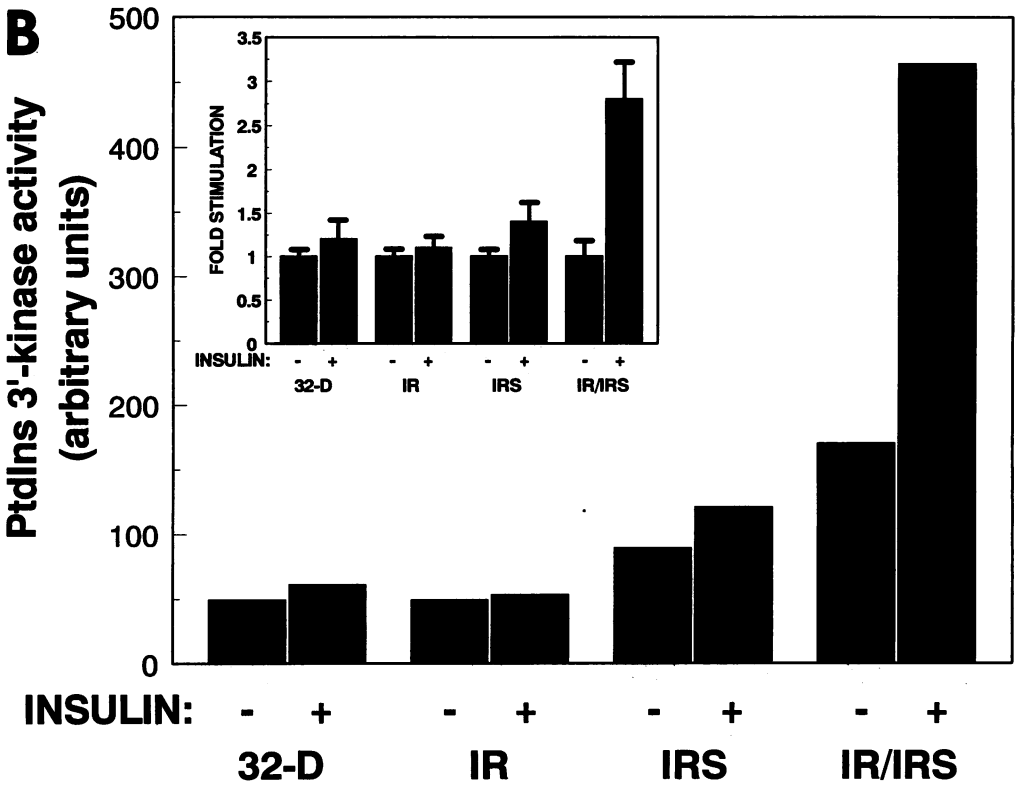


FIG. 7. Activation of MAPK and PtdIns 3′-kinase during insulin stimulation of 32-D cell lines. 32-D cell lines were stimulated for 5 min with the indicated concentrations of insulin and lysed. (A) MAPK was immunoprecipitated with anti-MAPK and collected on protein A-Sepharose beads. Activity of MAPK was determined by incubation of the washed immunoprecipitates with MBP and [γ-^{32}P]ATP. Labeled MBP was resolved by SDS-PAGE, and incorporated ^{32}P was quantitated on a PhosphorImager. Similar results were obtained in two independent experiments. Symbols are as for Fig. 6, except as follows: ——, 32-D and 32-D/IRS-1 cells; ○, IRS-1^{F-895} cells. (B) PtdIns 3′-kinase was immunoprecipitated with an antibody against p85α, and activity of immunoprecipitated PtdIns 3′-kinase was assayed in vitro. Data were quantitated on a PhosphorImager. The datum points were performed in triplicate. (Inset) Data are represented as fold stimulation, with error bars indicating standard errors of the mean.



tyrosine kinase, becomes tyrosine phosphorylated during insulin stimulation and binds at least five known SH2 proteins, including p85α, p85β, GRB-2, SH-PTP2, and nck (17, 18, 33, 37, 43a). IRS-1 is required for several insulin-mediated biochemical and cellular effects, including activation of PtdIns 3′-kinase (2, 24, 37), activation of the serine/threonine kinase p70^{s6k} (24a), maturation of *Xenopus* oocytes (6), and cellular mitogenesis (36, 41). It is thought, therefore, that the tyrosine phosphorylation of IRS-1 and its association with SH2 proteins are key elements in the insulin signaling cascade (26).

We have identified eight tyrosine phosphorylation sites on IRS-1, including sites which are preferred binding sites for SH2 domains in p85 (Tyr-608 and Tyr-939), SH-PTP2 (Tyr-1172), and GRB-2 (Tyr-895) (35). Thus, the mutation of specific tyrosine phosphorylation sites should selectively remove binding sites for distinct or limited sets of SH2 proteins. In this report, substitution of Tyr-895 with phenylalanine deletes a single phosphorylation site from IRS-1. Since IRS-1^{F-895} fails to associate with GRB-2 SH2 domains in a membrane binding assay and in intact cells, Tyr-895 appears to be the principal GRB-2 binding site in IRS-1. Tyr-895 is located within a YVNI motif, in agreement with another report suggesting that the YXNX motif forms a preferred binding site for the SH2 domains of GRB-2 (34).

IRS-1^{F-895} is expressed normally and binds p85 normally in vivo and in vitro during insulin-stimulated tyrosine phosphorylation. Thus, the failure of IRS-1^{F-895} to bind GRB-2 is probably not a result of nonspecific disruption of IRS-1 secondary or tertiary structure. Tryptic phosphopeptide mapping confirms that all sites on IRS-1^{F-895} are phosphorylated normally, with the exception of the removed site. The Y-895→F mutation appears to specifically block the associa-

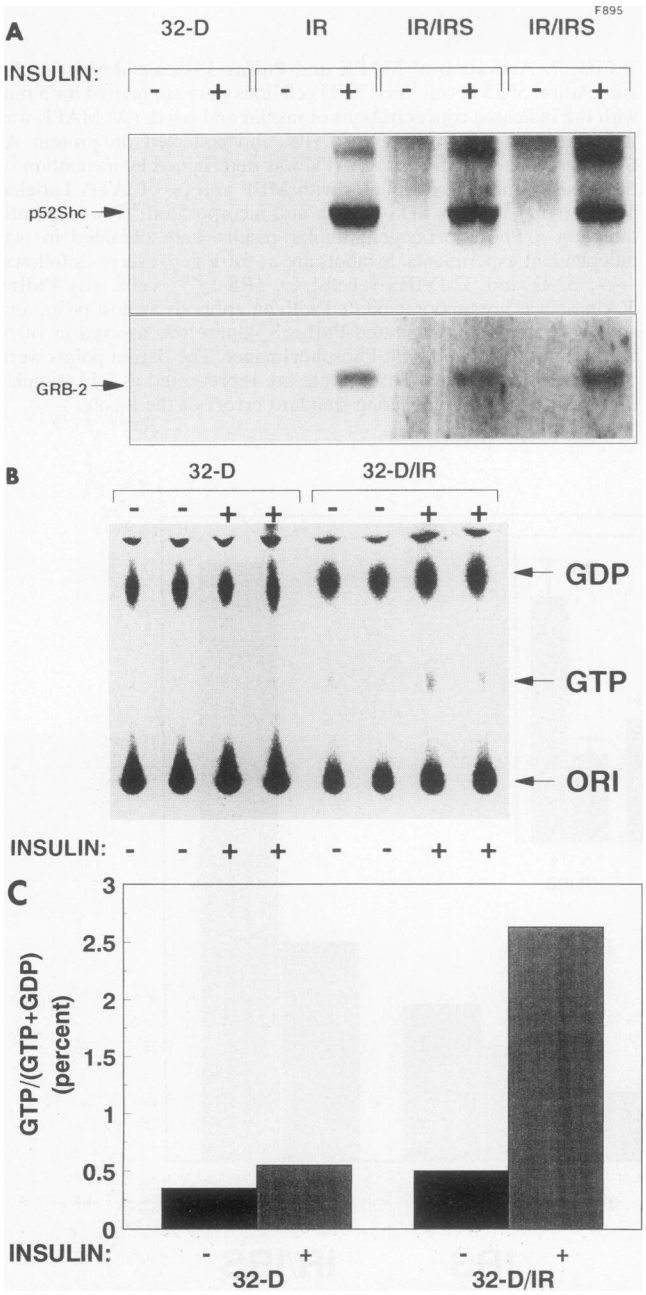


FIG. 8. Formation of Shc–GRB-2 complexes and GTP loading of p21^{ras} are independent of IRS-1 expression in 32-D cells. (A) Tyrosine phosphorylation of Shc and formation of Shc–GRB-2 complexes. 32-D, 32-D^{IR} (IR), 32-D^{IR}/IRS-1 (IR/IRS), or 32-D^{IR}/IRS-1^{F-895} (IR/IRS^{F-895}) cells were left unstimulated (−) or were stimulated with insulin for 10 min (+) and lysed. Immunoprecipitates were prepared with anti-Shc antibodies, resolved by SDS-PAGE, and transferred to nitrocellulose. Membranes were blocked and probed with anti-PY and anti-GRB-2, as indicated. The identity of Shc was confirmed by reblotting the decayed membrane with anti-Shc antibodies (not shown). The migrations of Shc and GRB-2 are indicated. (B) p21^{ras} GTP loading. 32-D and 32-D^{IR} cells were labeled with $^{32}P_i$ for 5 to 6 h, incubated in the absence (−) or presence (+) of insulin for 3 min, and lysed. Free nucleotides were removed by binding with activated charcoal, and p21^{ras} was immunoprecipitated. Bound nucleotides were eluted from washed immunoprecipitates and analyzed by TLC. Location of the origin (ORI) and the migration of GDP and GTP are indicated. (C) Results from panel B were quantitated as follows: GTP and GDP were quantitated on a PhosphorImager, and background and nonspecific binding to nonimmunocomplexes were subtracted. The GTP/(GTP + GDP) ratio was calculated and plotted.

tion of GRB-2 with IRS-1, since the p85 subunit of PtdIns 3′-kinase associates not only with normal IRS-1 following insulin stimulation but also with IRS-1^{F-895} as well. Together, these results confirm that Tyr-895 is an IRS-1 phosphorylation site in vivo (35) and demonstrate that Tyr-895 is the unique site which functions in the IRS-1–GRB-2 interaction in vivo and in vitro.

GRB-2 exists in a complex with mSOS, the mammalian homolog of the *Drosophila Son of Sevenless* gene product (3, 9, 19, 27, 29, 31). mSOS is a guanine nucleotide exchange factor for p21^{ras} which resides in a complex with GRB-2 (3, 9, 29). The association of the GRB-2–mSOS complex with tyrosyl phosphoproteins is thought to mediate guanine nucleotide exchange on p21^{ras} (3, 9, 19, 27, 29, 31). Considerable evidence implicates p21^{ras} and the activation of p21^{ras} by the GRB-2–mSOS complex in mitogenic signaling. Mutations in p21^{ras} which cause constitutive GTP loading are associated with the uncontrolled proliferation of many cell types in the absence of exogenous growth factors (30). Furthermore, while microinjection of GRB-2 and p21^{ras} individually into fibroblasts results in no change in DNA synthesis, coinjection of GRB-2 and p21^{ras} results in a marked increase in this measure of cell proliferation (20). Moreover, expression of mutant p21^{ras} molecules (Ras^{N-17}) that are incapable of undergoing GTP loading interfere with normal growth factor-stimulated mitogenesis (22). Thus, p21^{ras} appears to play an essential role in growth factor-stimulated mitogenesis.

We suggested previously that the binding of IRS-1 to GRB-2 mediates insulin-stimulated mitogenesis by activating the p21^{ras} signaling pathway (33). However, our present results demonstrate that IRS-1–GRB-2 interactions are not required for insulin-stimulated mitogenesis in 32-D cells. Thus, for GRB-2–phosphoprotein interactions to be important for insulin proliferative signaling in these cells, other pathways must exist to engage the GRB-2–mSOS complex. Since Shc proteins are known to be tyrosine phosphorylated and to associate with GRB-2 in response to insulin in several cell types, we examined this pathway in 32-D cells as an alternative mechanism to engage GRB-2–mSOS during insulin stimulation. Our results show that expression of IRs alone mediates insulin-stimulated tyrosyl phosphorylation of p52^{shc} and its association with GRB-2. Moreover, it is unlikely that the Shc signaling pathway requires expression of IRS-1, since Shc and IRS-1 do not associate by a variety of criteria and expression of IRS-1 does not affect the phosphorylation of Shc in 32-D cells (26a). Therefore, it is possible that GRB-2 phosphoprotein interaction is required for insulin-mediated mitogenesis in 32-D cells and that the requirement for F-895 in IRS-1 is hidden by the Shc–GRB-2 interaction in 32-D^{IR}/IRS-1^{F-895} cells (Fig. 9). However, since expression of IRS-1 is required for mitogenesis, it is also evident that Shc–GRB-2 interactions are not sufficient for insulin-stimulated cell proliferation.

The GRB-2–mSOS complex, as an activator of p21^{ras}, is implicated as an upstream regulator of MAPK by growth factor receptors (45). Activated p21^{ras} has been shown to bind Raf-1 (7, 38), a serine/threonine kinase that stimulates MEK (16, 35). Activated MEK phosphorylates the threonine and tyrosine residues on MAPK that are required for activation (1, 28). This general model may apply to insulin signaling, since overexpression of GRB-2 in L6 myocytes leads to increased activation of MAPK during insulin stimulation (32). Moreover,

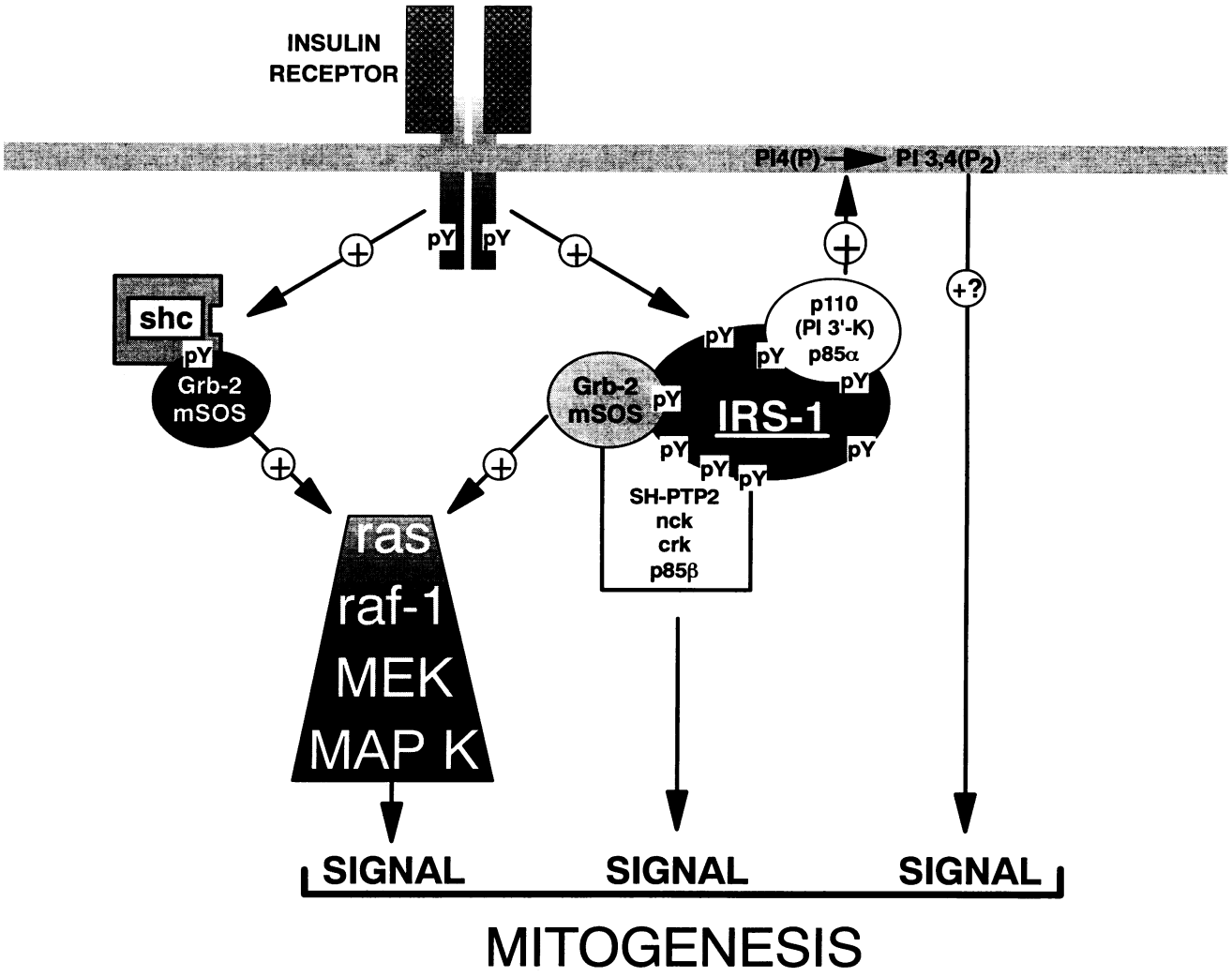


FIG. 9. The role of GRB-2–phosphoprotein interactions in insulin-stimulated mitogenesis. Insulin stimulation of IR-expressing cells results in the tyrosine phosphorylation of Shc, which associates with GRB-2, and IRS-1, which associates with GRB-2, p85, and other SH2 proteins. Both the Shc–GRB-2 and the IRS-1–GRB-2 complexes regulate the $p21^{ras}$→MAPK pathway. This pathway appears to be required but not sufficient for mitogenesis. The full mitogenic response to insulin requires another signaling contribution unique to IRS-1. PI, PtdIns; PI4(P), PtdIns 4-phosphate; PI 3,4 (P_2), PtdIns 3,4-bisphosphate.

overexpression of IRS-1, but not IRS-$1^{F\text{-}895}$, enhances insulin-stimulated MAPK in 32-D cells, suggesting that a complex between IRS-1 and GRB-2–mSOS can be important. Since GRB-2–mSOS can be engaged through Shc during insulin stimulation of 32-D cells, at least two pathways are available (Fig. 9). However, the relative importance of Shc or IRS-1 to insulin-stimulated MAPK may vary from tissue to tissue, depending on the expression and activity of each pathway. Furthermore, PtdIns 3′-kinase does not operate upstream of MAPK, since activation of PtdIns 3′-kinase requires expression of IRS-1, but MAPK activation occurs in 32-D^{IR} cells which do not overexpress IRS-1.

Since insulin-stimulated mitogenesis is dependent on IRS-1, we conclude that engagement of GRB-2–mSOS by Shc and the activation of $p21^{ras}$ and MAPK are not sufficient. Moreover, activation of $p21^{ras}$ and MAPK, specifically by the IRS-1–GRB-2 complex, is not required, as the mitogenic response of 32-D^{IR}/IRS-$1^{F\text{-}895}$ cells is indistinguishable from that of 32-D^{IR}/IRS-1 cells. However, activation of $p21^{ras}$ in 32-D^{IR}/IRS-1 cells appears to be required for insulin-stimulated mitogenesis, because induction of the dominant negative $p21^{ras}$ is inhibitory (28a). Thus, the crucial element(s) that IRS-1 draws into the insulin signaling pathway does not involve GRB-2 but likely involves other SH2 proteins which associate with IRS-1, such as the PtdIns 3′-kinase, nck, or SH-PTP2.

The model in Fig. 9 summarizes our current view of the IRS-1 signaling system. At the present time, we recognize two pathways from the IR: one is IRS-1 independent, while the other centers on IRS-1. Insulin independently stimulates the phosphorylation of Shc and IRS-1, which associate with GRB-2–mSOS and activate the $p21^{ras}$→Raf-1→MEK→MAPK signaling pathway. However, the relative contributions of the IRS-1 and Shc complexes will undoubtedly be tissue specific. By binding and regulating multiple SH2 proteins, IRS-1 stimulates other signaling pathways, one or more of which collaborate with the $p21^{ras}$ pathway to mediate a crucial mitogenic signal. These IRS-1-dependent pathways include SH-PTP2,

nck and crk, or PtdIns 3′-kinase, which provides an important mitogenic signal in other systems (10).

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