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Differential Signaling by Insulin Receptor Substrate 1 (IRS-1) and IRS-2 in IRS-1-Deficient Cells

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Mice made insulin receptor substrate 1 (IRS-1) deficient by targeted gene knockout exhibit growth retardation and abnormal glucose metabolism due to resistance to the actions of insulin-like growth factor 1 (IGF-1) and insulin (E. Araki et al., Nature 372:186–190, 1994; H. Tamemoto et al., Nature 372:182–186, 1994). Embryonic fibroblasts and 3T3 cell lines derived from IRS-1-deficient embryos exhibit no IGF-1-stimulated IRS-1 phosphorylation or IRS-1-associated phosphatidylinositol 3-kinase (PI 3-kinase) activity but exhibit normal phosphorylation of IRS-2 and Shc and normal IRS-2-associated PI 3-kinase activity. IRS-1 deficiency results in a 70 to 80% reduction in IGF-1-stimulated cell growth and parallel decreases in IGF-1-stimulated S-phase entry, PI 3-kinase activity, and induction of the immediate-early genes c-*fos* and *egr-1* but unaltered activation of the mitogen-activated protein kinases ERK 1 and ERK 2. Expression of IRS-1 in IRS-1-deficient cells by retroviral gene transduction restores IGF-1-stimulated mitogenesis, PI 3-kinase activation, and c-*fos* and *egr-1* induction in proportion to the level of reconstitution. Increasing the level of IRS-2 in these cells by using a retrovirus reconstitutes IGF-1 activation of PI 3-kinase and immediate-early gene expression to the same degree as expression of IRS-1; however, IRS-2 overexpression has only a minor effect on IGF-1 stimulation of cell cycle progression. These results indicate that IRS-1 is not necessary for activation of ERK 1 and ERK 2 and that activation of ERK 1 and ERK 2 is not sufficient for IGF-1-stimulated activation of c-*fos* and *egr-1*. These data also provide evidence that IRS-1 and IRS-2 are not functionally interchangeable signaling intermediates for stimulation of mitogenesis despite their highly conserved structure and many common functions such as activating PI 3-kinase and early gene expression.

Insulin and insulin-like growth factor 1 (IGF-1) elicit diverse biological effects by binding to and activating their endogenous tyrosine kinase receptors (17, 40). The major substrates of both the insulin receptor and the IGF-1 receptor tyrosine kinases are the closely related high-molecular-weight proteins insulin receptor substrates 1 and 2 (IRS-1 and -2), which become rapidly phosphorylated on multiple tyrosine residues following ligand stimulation (22, 34, 35). These phosphorylated substrates then bind proteins containing Src homology 2 (SH2) domains, such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (3), growth factor receptor binding protein 2 (Grb-2), which links signaling via SOS to activation of the Ras complex (5, 32), and protein tyrosine phosphatase SHPTP-2/Syp (33). In addition, depending on the cell type, IGF-1 and insulin receptor can phosphorylate some lower-molecular-weight substrates, such as Shc (11, 25, 27, 28), p60 (15, 18, 21, 43), and Gab1 (14), which link to one or another of these same pathways. Together these intermediate signals stimulate a variety of different downstream biological effects, including mitogenesis, gene expression, glucose transport, and glycogen synthesis.

The importance of IRS-1 vis-à-vis other substrates has been inferred from experiments in which IRS-1 has been overexpressed in different cell lines or its levels have been reduced by using antisense mRNA or anti-IRS-1 antibody microinjection techniques (29, 41). More importantly, in mice made deficient for IRS-1 by homologous recombinant gene targeting, insulin and IGF-1 stimulation of glucose metabolism and growth are reduced by 50 to 60%, despite the fact that IRS-2 and the other proteins can act as alternative substrates of the insulin receptor kinase (26). This finding raises the important question of whether IRS-1 plays a unique role in insulin and IGF-1 signaling or is simply one of several alternative substrates which may mediate hormone action.

In the present study, we investigated the role of IRS-1 in IGF-1-mediated signaling by developing embryonic fibroblast and 3T3 cell lines from IRS-1-deficient (IRS-$1^{-/-}$) and control mice. These cells were then used to study the mechanisms of IGF-1 signaling, as well as reconstitution by IRS-1 or IRS-2 introduced by retrovirus-mediated gene transfer. Our data reveal that the IRS-$1^{-/-}$ cells exhibit three classes of IGF-1-stimulated responses: (i) those that do not require IRS-1 (mitogen-activated protein [MAP] kinase activation); (ii) those that are impaired by IRS-1 deficiency and can be reconstituted by either IRS-1 or IRS-2 (PI 3-kinase activation and immediate-early gene induction); and (iii) those that are impaired by IRS-1 deficiency, can be reconstituted by IRS-1, but are not fully reconstituted by IRS-2 (mitogenesis).

MATERIALS AND METHODS

Materials. Human recombinant IGF-1 was a generous gift from Chiron Inc. (Emeryville, Calif.). Polyclonal antibodies to IRS-2 and p85, monoclonal antibody to phosphotyrosine, and a plasmid containing the IRS-2 cDNA were a generous gift from M. F. White (Joslin Diabetes Center, Boston, Mass.). The cDNAs for murine c-*fos* and human *egr-1* were provided by B. Spiegelman and S. M. Kharbanda (Dana-Farber Cancer Institute, Boston, Mass.). Antibodies to IRS-1 and MAP kinase were prepared as described previously (7). Polyclonal antisera to Shc and Grb-2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, Calif.). Medium and supplements were purchased from Gibco Inc. (Gaithersburg, Md.), except for fetal bovine serum (FBS), which was from Sigma Chemical Co. (St. Louis,

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Mo.). Nitrocellulose used for Western blots and nylon membranes used for Northern blots were from Schleicher & Schuell, Inc. (Keene, N.H.).

Cell culture. Female mice from breeding pairs of IRS-$1^{+/-}$ mice were sacrificed at day 16.5 postconception. Embryos were dissected from the uterus, and extra embryonic membranes, as well as viscera and heads, were removed. The embryo bodies were soaked overnight in 0.25% trypsin-EDTA at 4°C. Meanwhile, the genotype of the embryos was determined by using DNA extracted from the removed tissues by PCR as previously described (2). The following day, the tryptic digest was activated at 37°C for 15 min and then inactivated by the addition of 10 ml of Dulbecco modified Eagle medium (DMEM) with 10% FBS. Cells were then suspended by extensive pipetting, counted, and plated at a density of $10^6/cm^2$ in DMEM supplemented with 10% FBS. Adherent cells were allowed to reach confluency once and then passaged according to the 3T3 protocol of Todaro and Green (38). Briefly, cells were plated at a density of 10^6/10-cm-diameter dish. After 72 h cells were trypsinized, counted, and replated at 10^6/dish in new 10-cm-diameter dishes (one passage). This procedure was continued for 20 passages, after which cells established permanent lines. When cell numbers dropped below 10^6 during this procedure, cells were plated onto 60- or 30-cm-diameter dishes at the appropriate density. Once established, the 3T3 cells were split 1:10 to 1:20 every third day and never allowed to reach confluency except as specified for experiments.

Plasmids and transfection. To reconstitute IRS-1 expression in IRS-$1^{-/-}$ 3T3 cell lines, a retroviral expression vector was constructed by inserting the open reading frame of human IRS-1 from phIRS-30 into the *Sal*I and *Eco*RI sites of pBABE. The IRS-2 retroviral vector was constructed by inserting a blunt-ended *Hin*dIII-*Bam*HI fragment from pBSIRS-2 encompassing the entire murine IRS-2 coding sequence into the SNAB I site in pBABE. The correct orientation of the insert was monitored by *Cla*I digestion and partial sequence analysis. BOSC 23 cells were transiently transfected by using the calcium precipitation technique with 20 μg of plasmid DNA per 10-cm-diameter dish. Cells were refed 12 to 16 h after transfection, and Polybrene (8 μg/ml)-supplemented virus-containing supernatant was transferred to the target cells 48 h after transfection. After an overnight infection period, target cells were refed. Selection was begun by using 2.5 μg of puromycin (Sigma) per ml 48 h after infection.

Immunoprecipitation and Western blot analysis. For stimulation of IGF-1-mediated responses, cells were serum deprived overnight in medium containing 0.1% bovine serum albumin (BSA) and then exposed to IGF-1 unless noted otherwise at a final concentration of 10 nM in DMEM medium supplemented with 0.1% BSA for the indicated times. Protein extracts were prepared by using buffer A (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 20 mM $Na_4P_2O_2$, 100 mM NaF, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml) for 15 min at 4°C, and insoluble protein was removed by centrifugation at 12,000 rpm in a microcentrifuge. Protein content was determined by the method of Bradford. The extract was then resolved directly in SDS-polyacrylamide gels after boiling in Laemmli SDS sample buffer or subjected to immunoprecipitations with the indicated antibodies. For immunoprecipitation, 500 μg of cellular protein was incubated with the indicated antibodies for 2 h at 4°C. Immunocomplexes were collected by adding 70 μl of a 50% slurry of protein A-Sepharose (Pharmacia Inc., Piscataway, N.J.) in phosphate-buffered saline (PBS) for 1 h at 4°C. After two washes in buffer A containing 0.1% SDS, protein complexes were liberated from beads by boiling in SDS sample buffer for 3 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Immunodetection was performed after the membranes were blocked for 2 h at room temperature in 20 mM Tris-HCl (pH 7.5)–137 mM NaCl–0.05% Tween 20–3% BSA by incubation with the appropriate antibody at the indicated concentration for 2 h at room temperature. Specifically bound primary antibodies were detected with either ^{131}I-protein A and autoradiography or peroxidase-coupled secondary antibody and enhanced chemiluminescence detection as noted in the figure legends.

In vitro kinase reactions. Two hundred micrograms of cellular protein was subjected to immunoprecipitation with a polyclonal antiserum to ERK 1 and ERK 2, and immunocomplexes were collected with protein A-Sepharose beads. After two washes in lysis buffer and two washes in 50 mM HEPES–10 mM $MgCl_2$ (pH 7.4), beads were resuspended in 30 μl of reaction buffer, and the reactions were started by addition of 2 μCi of [γ-^{32}P]ATP, 100 μM ATP, 2 μM protein kinase C inhibitor (Sigma), and 5 μg of myelin basic protein (MBP) (Sigma). Reactions were allowed to proceed for 15 min at 30°C and stopped by addition of 12 μl of 5× Laemmli sample buffer; the reaction mixtures were boiled for 5 min, resolved by SDS-PAGE (12% gel), electroblotted onto polyvinylidene difluoride membranes, and exposed to autoradiography. Bands of labeled substrates were quantified with a Molecular Dynamics densitometer.

For in vitro kinase reaction assays of PI 3-kinase activity, quiescent cells were stimulated for 10 min with 10 nM IGF-1 in DMEM containing 0.1% BSA. After three washes with ice-cold PBS, cells were lysed in buffer A for 10 min at 4°C and cleared by centrifugation at 12,000 rpm in a microcentrifuge at 4°C, and the protein content of the supernatant was determined. Then 250 μg of cellular protein was subjected to immunoprecipitation with the indicated antibodies for 2 h at 4°C, and immunocomplexes were collected by addition of 60 μl of 50% protein A-Sepharose in PBS. After centrifugation, bound complexes were washed three times with PBS containing 1% Nonidet P-40, three times with 0.5 M LiCl–0.1 M Tris (pH 7.5), and twice in reaction buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA). After brief centrifugation at 12,000 rpm, beads were resuspended in a mixture containing 50 μl of reaction buffer, 10 μl of 100 mM $MgCl_2$, and 10 μl of phosphatidylinositol (2 μg/μl) and sonicated in 10 mM Tris (pH 7.5), containing 1 mM EGTA. Reactions were initiated by addition of 5 μl of a solution containing 880 μM ATP, 20 mM $MgCl_2$, and 35 μCi of [^{32}P]ATP (3,000 Ci/mmol) per tube. After vortexing for 10 min at room temperature, the reactions were stopped by addition of 20 μl of 8 N HCl and 160 μl of $CHCl_3$-methanol (1:1). The samples were briefly centrifuged, and the lower organic phase was spotted on silica gel thin-layer chromatography plates. The plates were developed in $CHCl_3$-methanol-H_2O-NH_4OH (120:94:23:2.4), dried, and visualized by autoradiography. Phosphorylated phosphatidylinositol was quantitated with a Molecular Dynamics densitometer.

Northern blot analysis. Northern blot analysis was performed according to standard techniques in denaturing formaldehyde-containing agarose gels (30). After stimulation with 10 nM IGF-1 for the indicated times, total cellular RNA was isolated by using RNAzol B; 10 to 20 μg of RNA was subjected to electrophoresis in 1% agarose gels. Ethidium bromide staining of the gels confirmed equal loading and integrity of the RNA. After transfer to nylon membrane (Schleicher & Schuell), blots were hybridized with probes for the full-length cDNA to the mouse c-*fos* gene or the human *egr-1* gene, which had been previously labeled by using a random prime labeling kit (Amersham Inc., Cleveland, Ohio). After an overnight incubation at 42°C, blots were washed three times in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 20 min each time at room temperature and for 30 min in 0.1× SSC–0.1% SDS at 52°C. Filters were air dried and subjected to autoradiography.

[^{3}H]thymidine incorporation into DNA. Cells were plated at a density of 2×10^5/well in 24-well dishes. After 1 day, the medium was changed to DMEM with 0.1% insulin-free BSA for 72 h. The cells were then stimulated with the indicated substances for 16 h and pulsed with 2 μCi of [*methyl*-^{3}H]thymidine (NEN Inc., Woburn, Mass.) per well for 1 h at 37°C. After two washes with ice-cold PBS, cells were lysed for 1 h in 0.1% SDS. Trichloroacetate-precipitable DNA was then counted for incorporated radioactivity. All assays were performed in triplicate.

RESULTS

Growth of IRS-1-deficient cells is severely impaired. Primary embryonic fibroblasts were derived from wild type (+/+), heterozygous (+/−), and mutant (−/−) mice at embryonic day 16.5. As expected, homozygous −/− cell lines had no immunoreactive IRS-1 protein, and cells derived from heterozygous embryos showed a ~50% reduction in IRS-1 expression (Fig. 1A). Expression of IRS-2 did not change in the absence of IRS-1 (Fig. 1A), consistent with observations for other tissues taken from the IRS-1 knockout mice (26).

Previous studies have shown that IGF-1 is only a weak mitogenic stimulus on its own but can potently promote cell growth in the presence of low concentrations of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (31). When primary embryonic cultures from wild-type mice were grown in conditioned medium containing PDGF, EGF, and IGF-1, the cell number increased by 200% within 48 h. By contrast, IRS-$1^{+/-}$ cells showed only a 100% increase of cell number, and IRS-$1^{-/-}$ cells responded with less than a 50% increase over the same period (Fig. 1B). Treatment with only PDGF and EGF did not induce growth in any of the cell lines tested, whereas 10% FBS stimulated growth by 450 to 500% in all three cell lines despite reduced or absent IRS-1 expression. These data indicate that reduced IRS-1 expression results in a reduced growth response to IGF-1 stimulation but has no effect on other growth stimuli.

To further analyze the impact of the IRS-1 null mutation on cell growth, we established immortal cell lines by using a 3T3 protocol (38). During this period of repetitive splitting to low density, IRS-$1^{-/-}$ cells exhibited reduced growth compared to cells from wild-type embryos, even in the presence of 10% FBS. Growth rates from the fourth 3T3 passage were significantly lower than those of IRS-$1^{+/+}$ cells (Fig. 1C). As a result, from six independent populations of each genotype passaged according to the 3T3 protocol, permanent cell lines could be derived from all wild-type cells, whereas only three of the six IRS-$1^{-/-}$ populations recovered from crisis to become immortalized cell lines. Like the parental fibroblasts, these IRS-$1^{-/-}$

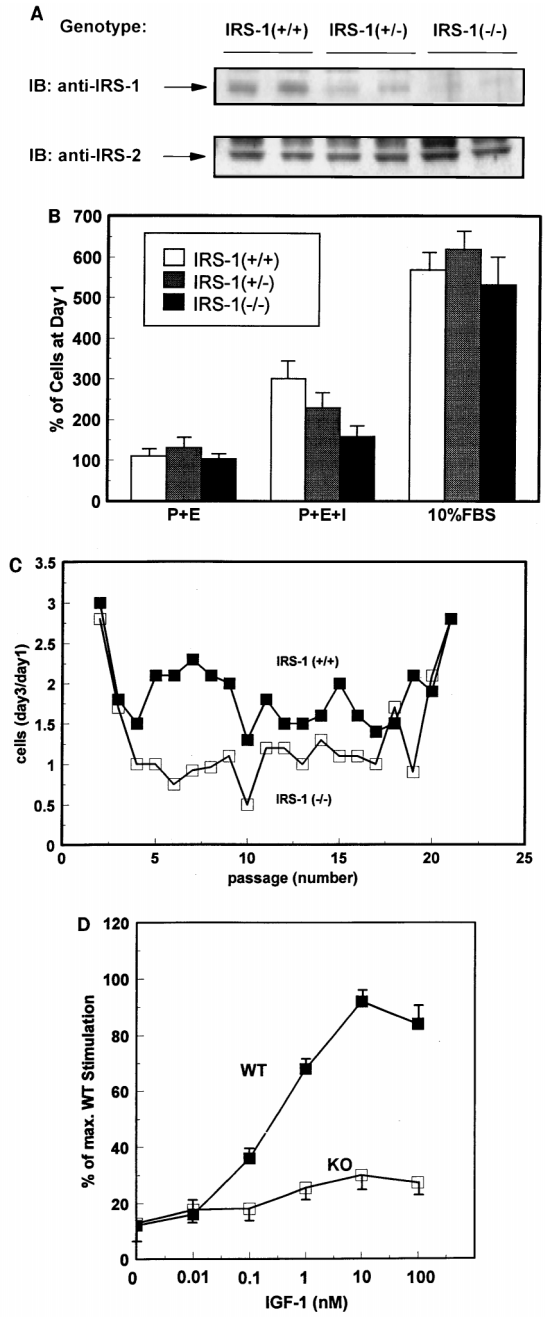

FIG. 1. Lack of IRS-1 causes an impaired mitogenic response to IGF-1. (A) Cell lysates from primary embryonic fibroblasts of IRS-$1^{+/+}$, IRS-$1^{+/-}$, and IRS-$1^{-/-}$ embryos were separated by SDS-PAGE, followed by Western blot (immunoblot [IB]) analysis using antisera specific to IRS-1 (upper panel) and IRS-2 (lower panel). (B) Primary mouse embryonic fibroblasts from wild-type (WT), IRS-$1^{+/-}$, and IRS-$1^{-/-}$ embryos were plated at 10^5/30-mm-diameter dish in DMEM with 10% FBS. The following day, cells were washed with PBS and medium was changed to DMEM supplemented with either 0.1% BSA, 5 ng of EGF (Gibco) per ml, and 20 ng of PDGF-BB (Gibco) per ml (P+E) or P+E with additional IGF-1 (50 ng/ml) or medium supplemented with 10% FBS. Cells were counted after an additional 48 h in culture. Results are expressed as percentages of cells present on day 1. Data represent means ± standard errors of the means from three independent experiments, each performed in duplicate. (C) Growth characteristics of IRS-$1^{+/+}$ and IRS-$1^{-/-}$ cells during passages performed according to the 3T3 protocol. Cells were passaged as described in Materials and Methods. Growth characteristics are shown for an exemplary line derived from IRS-$1^{+/+}$ cells and one line of IRS-$1^{-/-}$ primary embryonic fibroblasts. Results are expressed as cells present 72 h after plating/cells initially plated. (D) IGF-1 stimulation of [^{3}H]thymidine incorporation into DNA. Quiescent IRS-$1^{+/+}$ (wild-type [WT]) and IRS-$1^{-/-}$ (knockout [KO]) 3T3 cells were stimulated with various concentrations of IGF-1 for 14 h at 37°C. Incorporation of [^{3}H]thymidine into DNA was assessed as described in the text. Determinations were done in triplicate, and the data are means ± standard errors of the means for four separate experiments. Results are expressed as percentages of maximum stimulation in wild-type cells.

3T3 cell lines exhibited decreased mitogenic potential due to a failure of progression to S phase. Thus, IGF-1-stimulated incorporation of [^{3}H]thymidine into DNA was decreased by 70% in IRS-$1^{-/-}$ 3T3 cells compared to cells from wild-type embryos at all concentrations of IGF-1 (Fig. 1D).

Expression and phosphorylation of IRS-2 in IRS-1-deficient cells. To investigate whether the absence of IRS-1 expression produced any compensatory alterations in the expression of the closely related IRS-2 protein, we performed Western blot analysis on whole-cell extracts from IRS-$1^{+/+}$ and IRS-$1^{-/-}$ immortalized cell lines. As noted for the primary fibroblasts, an antibody specific to the C-terminal region of IRS-1 did not detect IRS-1 in the cells carrying the disrupted IRS-1 gene (Fig. 2A). Western blots probed with anti-IRS-2 antibodies indicated equivalent levels of IRS-2 in these cells (Fig. 2A). Likewise, there were equal levels of Shc, p85, and Grb2 (see below), indicating that other potential substrates and SH2 domain-containing proteins do not undergo compensatory changes in expression due to the absence of IRS-1. Immunoprecipitate from cell lysates of wild-type and IRS-$1^{-/-}$ cells, using anti-IRS-2 antibodies followed by Western blot analysis with an antiphosphotyrosine antibody, indicated that IRS-2 became phosphorylated on tyrosine residues upon IGF-1 stimulation in both IRS-$1^{+/+}$ and IRS-$1^{-/-}$ cells (Fig. 2B). Quantitation of six independent experiments showed no significant difference in IRS-2 phosphorylation in IRS-$1^{-/-}$ cells compared to wild-type cells.

Retroviral expression of IRS-1 reconstitutes IGF-1 signaling in IRS-$1^{-/-}$ cells. If the loss of IRS-1 expression is responsible for the observed reduction in IGF-1-stimulated growth, then reconstitution of IRS-1 expression would be predicted to correct most, if not all, of these signaling defects. For this goal, we used retrovirus-mediated gene transfer. This method enabled us to achieve levels of IRS-1 expression that were 60 to 70% of that seen in wild-type cells (Fig. 2A). We also examined whether IRS-2 overexpression is capable of correcting the growth-deficient phenotype of IRS-1-deficient cells by transduction with a retrovirus vector containing murine IRS-2. Infection with this vector resulted in IRS-2 expression ~2.5-fold above endogenous levels (Fig. 2A). Both the exogenously expressed IRS-1 and the overexpressed IRS-2 underwent tyrosine phosphorylation in response to IGF-1 stimulation in proportion to the increased level of protein (Fig. 2B).

IGF-1-stimulated activation of PI 3-kinase is reduced in IRS-1-deficient cells. An important component of the IRS-1- and IRS-2-mediated response to IGF-1 which has been shown previously to contribute to a mitogenic response is docking of the p85 regulatory subunit of PI 3-kinase to IRS-1 and IRS-2 and activation of the p110 catalytic subunit. Consistent with the lack of IRS-1 expression and phosphorylation after IGF-1 stimulation, no p85 could be detected in anti-IRS-1 precipitates in IRS-$1^{-/-}$ cells (Fig. 2B), while coprecipitation of p85 with IRS-2 was unaltered, consistent with the unaltered expression and phosphorylation of IRS-2 in IRS-1-deficient cells (Fig. 2B). Expression of IRS-1 from the retroviral vector restored IRS-1–p85 association in IRS-$1^{-/-}$ cells in proportion to the level of IRS-1 protein (Fig. 2B). Likewise, retroviral overexpression of IRS-2 increased IGF-1-stimulated p85 docking to IRS-2 (Fig. 2B) without affecting the amount of p85

associating with IRS-1 (Fig. 2B). In both cases, the total cellular content of p85 was unaltered by retroviral transduction (data not shown).

PI 3-kinase activity paralleled the results for p85 association. Thus, there was a loss of activation of PI 3-kinase in anti-IRS-1 precipitates in IRS-1-deficient cells (Fig. 3A and B), and IRS-1 expression restored this defect, whereas IRS-2 overexpression had no effect on IRS-1 associated PI 3-kinase activity (Fig. 3A and B). Coincident with the unaltered phosphorylation and binding of p85 to IRS-2 in IRS-1-deficient cells, PI 3-kinase activities immunoprecipitated with IRS-2 were similar in IRS-$1^{+/+}$ and IRS-$1^{-/-}$ cells (Fig. 3A and B). To assess the relative contributions of IRS-1 and IRS-2 to the total IGF-1-stimulated activation of PI 3-kinase, we performed in vitro kinase reactions on antiphosphotyrosine immunoprecipitates. As shown in Fig. 3A and B, the amount of PI 3-kinase activity recovered in IRS-1-deficient cells was reduced by 70%. Given the fact that the only detectable phosphoproteins associating with p85 in these cells appear to be IRS-1 and IRS-2 (Fig. 3C), these data indicate that IRS-1 accounts for about 70% of the IGF-1 receptor-mediated activation of PI 3-kinase in embryonic fibroblasts. Again IRS-1 expression in IRS-$1^{-/-}$ cells was capable of restoring both IRS-1-associated and phosphotyrosine-associated PI 3-kinase activity in response to IGF-1 stimulation (Fig. 3A and B, top and bottom panels). Although overexpression of IRS-2 led to no increase in IRS-1-associated PI 3-kinase, there was a 2.5-fold increase in IRS-2-associated PI 3-kinase activity and a similar reconstitution of total antiphosphotyrosine-associated activity as in the IRS-1-reconstituted cells (Fig. 3A and B, middle and bottom panels).

IGF-1-stimulated activation of MAP kinases is unaltered in IRS-1-deficient cells. A second important pathway activated by IGF-1 and involved in gene expression and the mitogenic response is the activation of the MAP kinase signaling cascade. Upon receptor activation, phosphorylated Shc, IRS-1, and IRS-2 bind to the SH2 domain of the adapter molecule Grb-2, which in turn binds the guanine nucleotide exchange factor SOS, thus activating the small GTP binding protein Ras. Activation of Ras then initiates a signaling cascade of serine threonine kinases, including Raf, MEK, ERK 1, and ERK 2. To assess the impact of the loss of IRS-1 expression on the final step in this pathway, activation of ERK 1 and ERK 2, we performed in vitro kinase assays using anti-MAP kinase immunoprecipitates and MBP as a substrate. Somewhat surprisingly, the MAP kinases ERK 1 and ERK 2 were activated to the same extent in response to IGF-1 in both wild-type and IRS-1-deficient cells (Fig. 4A and B). Activation of ERK 1 and ERK 2 by IGF-1 also followed the same time course in response to IGF-1 stimulation and exhibited identical dose-response curves (Fig. 4C). Western blot analyses of anti-MAP kinase immunoprecipitates revealed the presence of equal levels of the enzyme in lysates from both cell lines (Fig. 4A, upper panel). These data indicate that IRS-1, although capable of binding Grb-2 and activating the MAP kinases, is not necessary for the IGF-1-stimulated activation of ERK 1 and ERK 2 in these cells.

IGF-1-stimulated association of Grb-2 to Shc and IRS-2. Shc is another substrate of the insulin and IGF-1 receptors and has been shown to bind Grb-2 upon ligand stimulation, thereby mediating the activation of the Ras-MAP kinase pathway. Given the unaltered activation of ERKs in the absence of IRS-1, we addressed whether expression or Grb-2 association of Shc might be increased in the absence of IRS-1 and thus compensate for the loss of IRS-1-mediated signaling. Western blot analyses of cell lysates from wild-type and IRS-$1^{-/-}$ cells revealed similar expression levels of Shc and Grb-2 in the two

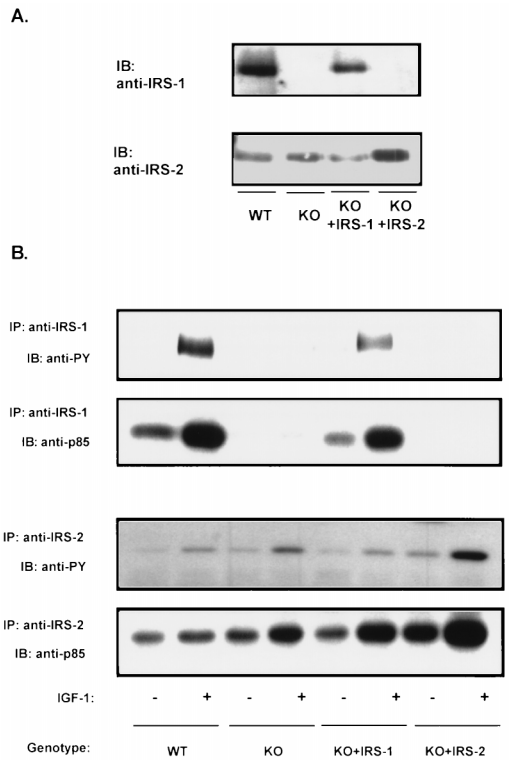

FIG. 2. Expression, tyrosine phosphorylation, and association of IRS-1 and -2 with p85 in various cell lines. (A) Cell extracts from IRS-$1^{+/+}$ (wild-type [WT]) cells, IRS-$1^{-/-}$ (knockout [KO]) cells, and IRS-$1^{-/-}$ cells in which either IRS-1 [KO+IRS-1] or IRS-2 [KO+IRS-2] was expressed from a retrovirus were separated by SDS-PAGE and analyzed by Western blotting (immunoblotting [IB]) for IRS-1 (upper panel) or IRS-2 (lower panel) expression. (B) Cell extracts from cell lines were subjected to immunoprecipitation (IP) with IRS-1- or IRS-2-specific antiserum, followed by Western blotting with an antiphosphotyrosine (anti-PY) monoclonal antibody or an anti-p85 antiserum. The experiment shown is representative of multiple experiments. The IGF-1-stimulated phosphorylation of IRS-2 in knockout cells was 105% of that seen in wild-type cells (mean of six independent experiments). IRS-1 expression and IGF-1-stimulated tyrosine phosphorylation in KO+IRS-1 cells reached 70% of levels in wild-type cells. IRS-2 expression and tyrosine phosphorylation in KO+IRS-2 cells were 250% of levels in wild-type cells.

cell lines (Fig. 5A). Moreover, the amounts of Grb-2 coimmunoprecipitated with Shc upon IGF-1 stimulation were similar in IRS-$1^{+/+}$ and IRS-$1^{-/-}$ cell lines (Fig. 5B). To assess the potential contribution of IGF-1-stimulated association of Grb-2 to IRS-2 to the maintained activation of MAP kinase in IRS-1-deficient cells, we performed anti-Grb-2 Western blot analyses on anti-IRS-2 immunoprecipitates from wild-type and IRS-1-deficient cells. As shown in Fig. 5C and D, there was no significant difference in the amount of Grb-2 coimmunoprecipitated with IRS-2 upon IGF-1 stimulation in wild-type and IRS-1-deficient cells. Thus, IGF-1-stimulated MAP kinase activation was preserved in the IRS-1-deficient cells without any measurable increase in Shc- or IRS-2-mediated signaling pathway.

Immediate-early gene expression in IRS-1-deficient cells. Since the expression of proto-oncogenes, such as c-*fos* and *egr-1*, has been implicated in embryonic growth and differentiation, we investigated the role of IRS-1 in IGF-1-stimulated immediate-early gene expression. As shown in the Northern blots in Fig. 6A, IRS-1-deficient cells showed a markedly reduced expression of c-*fos* in response to IGF-1 over a 60-min time course. Quantitation of several independent experiments

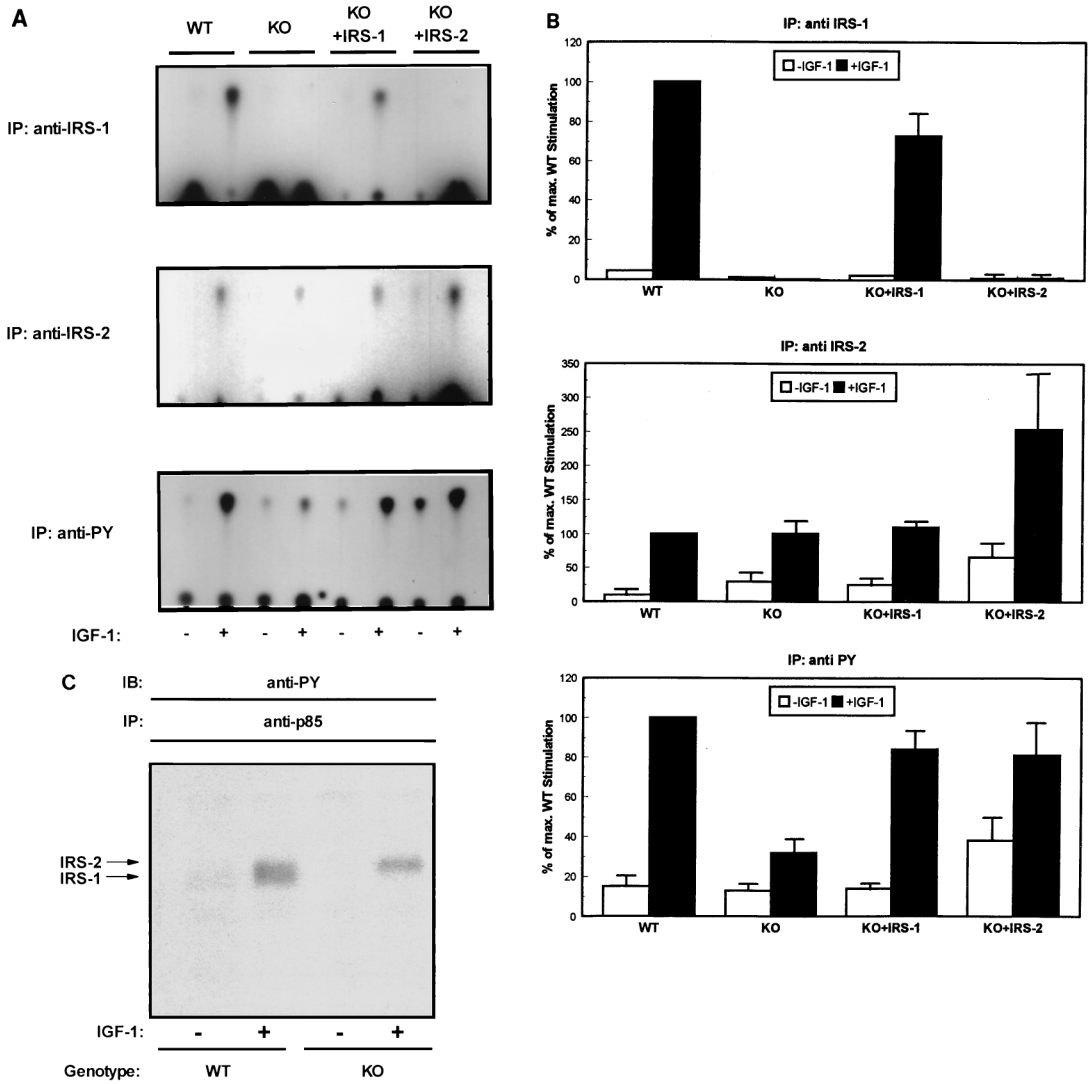

FIG. 3. IGF-1-stimulated activation of PI 3-kinase. Quiescent cells were stimulated with 10 nM IGF-1 in DMEM with 0.1% BSA for 10 min at 37°C. In vitro kinase assays of cell lysates were performed as described in Materials and Methods on anti-IRS-1, anti-IRS-2, and antiphosphotyrosine (anti-PY) immunoprecipitates. (A) Results of a representative experiment. (B) Cells lines are designated as in Fig. 2. IP, immunoprecipitation. Quantitation of three independent experiments, each performed with duplicate determinations. Results are expressed as percentages of maximum stimulation seen in IRS-$1^{+/+}$ cells and represent means ± standard errors of the means. (C) Immunoprecipitations from cells either unstimulated (−) or stimulated for 10 min with 10 nM IGF-1 (+) were performed with an antiserum to p85 and resolved by SDS-PAGE followed by Western blot (immunoblot [IB]) analysis with an antiphosphotyrosine monoclonal antibody. Arrows indicate the migration of proteins immunoreactive with IRS-1- and IRS-2-specific antisera.

revealed a 60% reduction of c-*fos* induction in IRS-$1^{-/-}$ cells compared to wild-type cells (Fig. 6B). Similar results were observed for IGF-1-stimulated *egr-1* expression (Fig. 7B). By contrast, when both cell lines were stimulated with 10% FBS, expression of c-*fos* and *egr-1* was induced to the same magnitude, indicating that IGF-1-mediated gene expression was specifically impaired in these cells (data not shown).

To further prove the specificity of the phenotype observed for the absence of IRS-1, we performed Northern blots on knockout cells in which IRS-1 was expressed from the retrovirus. As with PI 3-kinase activity, expression of IRS-1 reconstituted both c-*fos* and *egr-1* expression in response to IGF-1 in IRS-1-deficient cells by about 70%, i.e., in proportion to the level of reconstitution (Fig. 7A and B). Likewise, overexpression of IRS-2 was capable of restoring the defects in c-*fos* and *egr-1* expression due to the lack of IRS-1. These data indicate the existence of a common pathway(s) mediated by both IRS-1 and IRS-2, independent of the activation of MAP kinases, which is necessary for IGF-1-stimulated activation of these immediate-early genes.

Expression of exogenous IRS-1 restores IGF-1-stimulated S-phase entry in IRS-$1^{-/-}$ cells. Finally, to address whether reconstituted IRS-1 expression or overexpression of IRS-2 in IRS-$1^{-/-}$ cells was capable of restoring IGF-1-stimulated cell cycle progression, as it reconstitutes PI 3-kinase activity and immediate-early gene expression, we determined IGF-1-stimulated incorporation of [*methyl*-^{3}H]thymidine into DNA in wild-type and IRS-$1^{-/-}$ cells and in IRS-$1^{-/-}$ cells in which IRS-1 and IRS-2 were expressed by retroviral transduction. In parallel with other responses, IGF-1-stimulated [^{3}H]thymidine labeling was decreased 70% in IRS-$1^{-/-}$ cells, and expression of IRS-1 in the deficient cells restored the ability of the cells to enter S phase by about 65% (Fig. 8), i.e., in proportion to the level of reconstitution of IRS-1 expression. By contrast, in

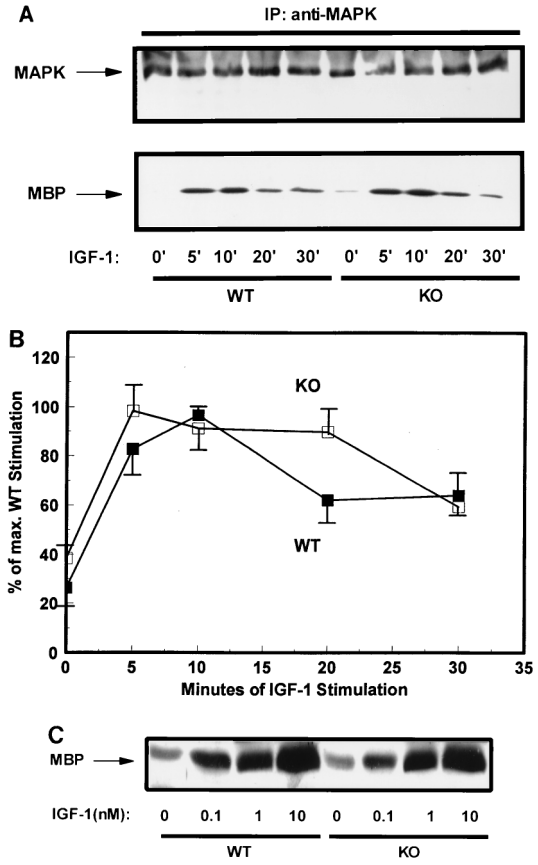

FIG. 4. IGF-1-stimulated activation of MAP kinases. (A) Cell extracts from wild-type (WT) and IRS-$1^{-/-}$ (knockout [KO]) 3T3 cell lines were prepared after stimulation of serum-deprived cells with 10 nM IGF-1 for the indicated times (minutes). MAP kinase (MAPK) activity was then assayed on immunoprecipitates (IP), using MBP as a substrate as described in the text (bottom panel). After SDS-PAGE in 12% gels and electroblotting onto polyvinylidene difluoride membranes, filters were cut and the MBP-containing part was exposed to film. The upper part of the filter, containing immunoprecipitated MAP kinases, was subjected to Western blot analysis with an anti-ERK antiserum (upper panel), indicating the presence of similar levels of MAP kinase in the immunoprecipitates. (B) Quantitation of six independent experiments. Results, expressed as percentages of maximum stimulation in wild-type cells, are means ± standard errors of the means. (C) Cell extracts were obtained from wild-type and knockout cells which had been serum deprived overnight and then either left untreated or treated with the indicated concentration of IGF-1 for 10 min. MAP kinase activity was assayed as described for panel A. Shown is the result of a typical experiment representative of four independent experiments with similar results.

multiple experiments, overexpression of IRS-2 reconstituted IGF-1-stimulated S-phase progression to only 40% of the control level, despite its ability to reconstitute more completely IGF-1-stimulated effects on PI 3-kinase activity and immediate-early gene expression. This is also reflected by the fold stimulation of DNA synthesis caused by IGF-1. While IGF-1 can stimulate DNA synthesis 6.3-fold in wild-type cells, this effect is reduced to 1.7-fold in IRS-1-deficient cells. IRS-1 expression restores DNA synthesis to a 3.4-fold stimulation, while IRS-2 overexpression is able to cause only a 2.7-fold stimulation.

To further address the importance of IGF-1-stimulated PI 3-kinase activity on mitogenesis, we performed the same assay using the synthetic PI 3-kinase inhibitor LY 294002. When cells were preincubated with this inhibitor 20 min prior to study, IGF-1 was not longer able to stimulate S-phase progression in any of the cell lines (Fig. 8). Thus, IGF-1-stimulated PI 3-kinase activity is necessary, but not sufficient, for IGF-1-stimulated cell cycle progression, since reconstitution of PI 3-kinase activity by IRS-2 overexpression had only a minor effect on mitogenesis.

DISCUSSION

A variety of studies have demonstrated the importance of the IGF-1 receptor in mediating embryonic growth and development in response to both IGF-1 and IGF-2 (31). While mice homozygous for a null allele in the insulin receptor gene exhibit normal intrauterine growth (1), those deficient in IGF-1, IGF-2, or the IGF-1 receptor show about a 50% reduction in birth weight and, in the latter case, die shortly after birth from respiratory failure due to muscular hypoplasia (4, 19). IRS-1 has been shown to act as a primary substrate for both the insulin and IGF-1 receptors. Like IGF-1 receptor-deficient mice, IRS-1 null mice exhibit IGF-1 resistance and intrauterine growth retardation. In addition these animals exhibit resistance to the metabolic actions of insulin (2, 36). Reduction of endogenous IRS-1 by expression of antisense mRNA or microinjection of anti-IRS-1 antibodies also reduces the mitogenic response to insulin and IGF-1 in cultured cells (29, 41). In the present study, we investigated the molecular mechanisms leading to IGF-1 resistance in IRS-$1^{-/-}$ embryos by comparing IGF-1 action in cell lines derived from wild-type and IRS-$1^{-/-}$ embryos.

We find that the growth response of both primary embryonic fibroblasts and stable 3T3 cell lines to IGF-1 is severely impaired in IRS-1-deficient cells and that this is the result of a failure to enter S phase. Several intermediate signaling events, including the activation of PI 3-kinase and the Ras-MAP kinase pathway, have been implicated in insulin- and IGF-1-stimulated mitogenesis. Although the Ras-MAP kinase pathway can be stimulated through IRS-1 by binding of Grb-2 to phosphorylated IRS-1 (5, 32), the activation of ERKs was unaltered in the absence of IRS-1, probably as a result of signaling through the Shc–Grb-2 pathway. Thus, in undifferentiated embryonic mesoderm, Grb-2 binding to IRS-1 does not appear to be essential for IGF-1-stimulated activation of MAP kinases.

The role of IRS-1 in MAP kinase activation may differ from tissue to tissue, since Yamauchi et al. have found reduced insulin-stimulated activation of MAP kinase in muscle of IRS-1-deficient mice but a normal response in liver (42). Several studies using cell culture systems support our current findings. In the hematopoietic progenitor cell 32D, which lacks both the insulin receptor and IRS-1, expression of the insulin receptor allows for insulin-stimulated activation of MAP kinase, but simultaneous expression of IRS-1 is required for stimulation of mitogenesis (23). Moreover, Chen et al. found that in cells expressing the mutant insulin receptor A960, overexpression of IRS-1 reconstitutes insulin-stimulated IRS-1–Grb-2 binding without rescuing insulin-stimulated activation of MAP kinases (8). In previous studies using adipocytes, we (7) and others (12) demonstrated that IRS-1–Grb-2 complex formation occurs in the low-density microsome fraction, whereas Shc–Grb-2 binding occurs at the plasma membrane near the site of Ras activation. Therefore, not only the tissue specific distribution of Shc but also the subcellular localization of Grb-2 complexes might serve different functions. Whatever the mechanism, it is clear that in embryonic fibroblasts, IGF-1-stimulated MAP kinase activation is independent of the presence of IRS-1, while the mitogenic response is severely impaired.

Both insulin and IGF-1 stimulation involves activation of PI

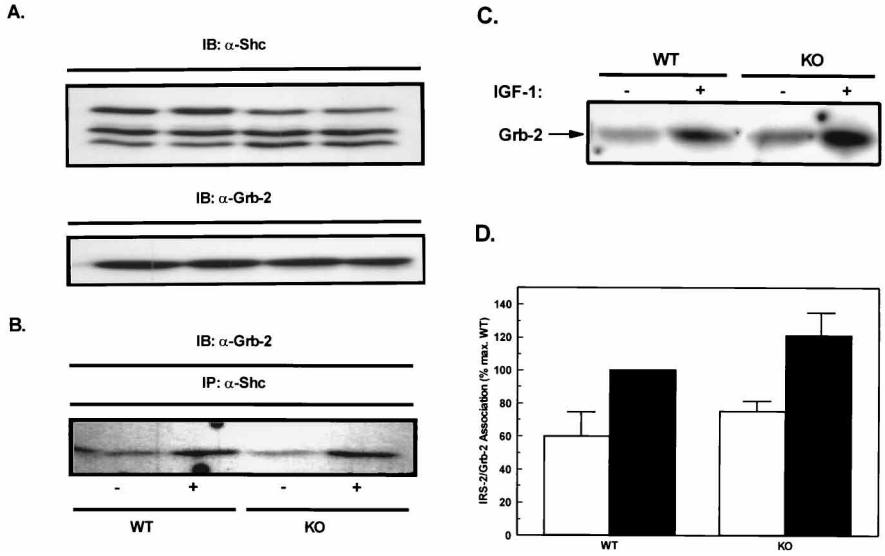

FIG. 5. IGF-1-stimulated association of Grb-2 with Shc and IRS-2. (A) Cell lysates from unstimulated (−) and IGF-1-stimulated (+) IRS-$1^{+/+}$ (wild-type [WT]) and IRS-$1^{-/-}$ (knockout [KO]) 3T3 cells were separated by SDS-PAGE and subjected to Western blot (immunoblot [IB]) analysis using antisera to Shc (upper panel) and Grb-2 (lower panel). (B) Anti-Shc immunoprecipitates (IP) from quiescent and IGF-1-stimulated cells were immunoblotted with a polyclonal anti-Grb-2 antiserum. The experiment shown is representative of multiple experiments with similar results. (C) Anti-IRS-2 immunoprecipitates from quiescent and IGF-1-stimulated cells were immunoblotted with a polyclonal anti-Grb-2 antiserum. Shown is the result obtained in a typical experiment. (D) Quantification of three independent experiments performed as outlined for panel C. Results are expressed as percent signal intensities seen in the stimulated wild-type cell line.

3-kinase. Studies using microinjection of antibodies against the p85 regulatory subunit of PI 3-kinase or p85 SH2 domain fusion proteins have shown that PI 3-kinase is required for insulin-stimulated mitogenesis in 3T3-L1 cells and IGF-1-stimulated oocyte maturation (9, 10, 16). Likewise, inhibition of insulin-stimulated PI 3-kinase activation by use of a chemical inhibitor of the enzyme blocks cell cycle progression without affecting activation of MAP kinases (6). In the current study, we find that despite the fact that both IRS-1 and IRS-2 can bind to and activate the p85 subunit of PI 3-kinase, IRS-1 plays the dominant role in activation of the enzyme in embryonic fibroblasts. Thus, both reintroduction of IRS-1 and overexpression of IRS-2 restore total antiphosphotyrosine-associated PI 3-kinase activity. Both proteins also support IGF-1 stimulation of immediate-early gene expression to the same extent. Despite this, IRS-2 overexpression fails to rescue IGF-1-stimulated entry into S phase to a level comparable to that found upon overexpression of IRS-1. These data clearly indicate that although IRS-2 can act as a substrate of the IGF-1 receptor kinase in vivo and mediate some biological effects with efficiency comparable to that of IRS-1, there must be additional pathways, specific for IRS-1, required for IGF-1-stimulated mitogenesis.

Immediate-early gene products often encode transcription factors which have been implicated in cellular growth and differentiation. Waters et al. (41) have shown that expression of IRS-1 antisense RNA causes a reduction of insulin-stimulated activity of a luciferase gene under control of the c-*fos* serum response element (SRE), the same region which has been shown to confer insulin sensitivity to the c-*fos* promoter (37). Our data also provide evidence that expression of the endogenous c-*fos* gene depends on IRS-1. These findings are of even greater interest considering that IGF-1 stimulation of MAP kinases is normal in IRS-$1^{-/-}$ cells. The c-*fos* SRE contains binding sites for two major families of transcription factors: one is the serum response factor, and the other belongs to the family of Ets-related transcription factors, including Elk-1 (for a review, see reference 39). Several studies have shown that MAP kinase-dependent phosphorylation of Elk-1 initiates a ternary complex formation involving the SRE and serum response factor, resulting in transactivation of the SRE (20). Our data provide evidence for a MAP kinase-independent,

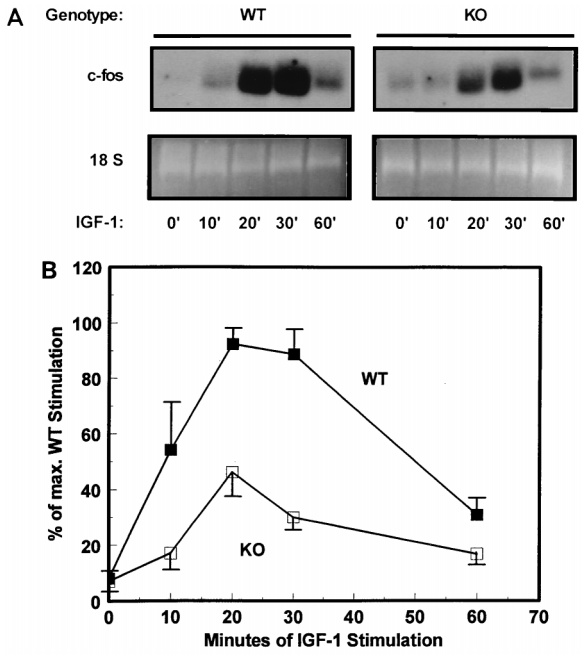

FIG. 6. IGF-1-stimulated induction of c-*fos* mRNA is reduced in the absence of IRS-1. (A) Northern blot analysis of RNA extracted from wild-type (WT) and knockout (KO) cells stimulated with 10 nM IGF-1 for the indicated times (minutes). Blots were hybridized with a probe to the full-length cDNA of c-*fos* as described in the text. The upper panel shows the autoradiograph of a typical experiment. The lower panel shows the ethidium bromide-stained agarose gel, confirming integrity of the RNA and equal loading. (B) Quantitation of the results from four independent experiments. Data are expressed as mean percentages of maximum stimulation in wild-type cells ± standard errors of the means.

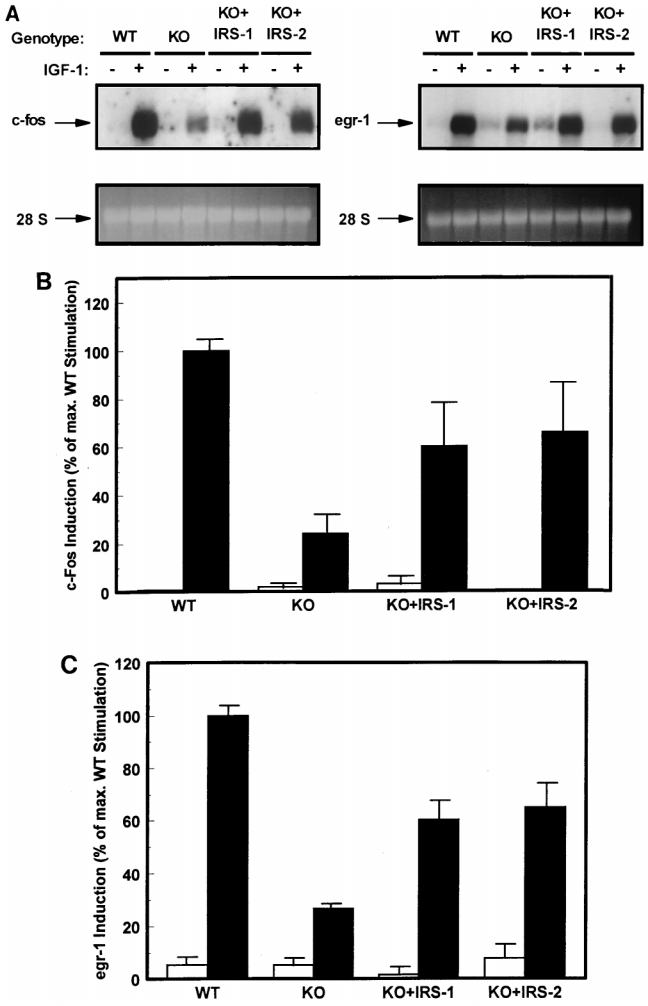

FIG. 7. Expression of IRS-1 and overexpression of IRS-2 rescue IGF-1-stimulated c-*fos* and *egr-1* induction in IRS-$1^{-/-}$ cells. (A) RNA was extracted from wild-type [WT] cells, IRS-$1^{-/-}$ (knockout [KO] cells, and IRS-$1^{-/-}$ cells in which IRS-1 [KO+IRS-1] or IRS-2 [KO+IRS-2] had been stably expressed from a retroviral vector. RNA from cells which had been left untreated (−) or stimulated with 10 nM IGF-1 for 30 min (+) was separated in agarose gels. After transfer, the blots were probed for c-*fos* (left) or *egr-1* (right). The upper panel shows the autoradiograph of an exemplary experiment; the lower panel demonstrates the ethidium bromide-stained gel. (B) Quantitation of four independent experiments probed for c-*fos* as shown in the left portion of panel A. (C) Quantitation of four independent experiments probed for *egr-1* as shown in the right portion of panel A. Data are expressed as percentages of maximum stimulation seen in wild-type cells (means ± standard errors of the means).

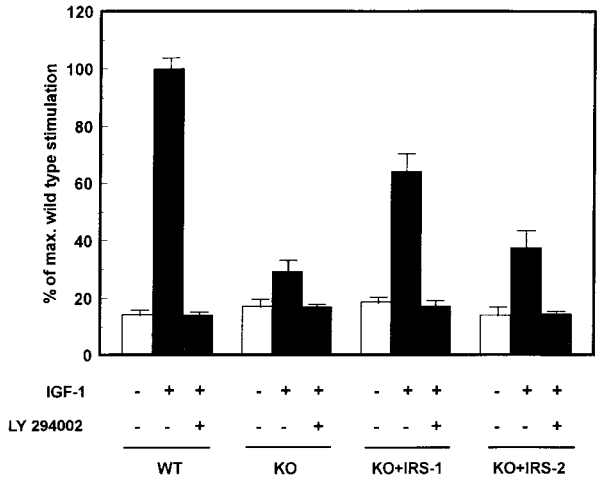

FIG. 8. Effect of IRS-1 or IRS-2 overexpression on cell cycle progression in IRS-$1^{-/-}$ (knockout [KO]) cells. Quiescent cells were stimulated with 10 nM IGF-1 for 16 h and pulsed with 2 μCi of [^{3}H]thymidine/2 × 10^5 cells for 1 h at 37°C. When the PI 3-kinase inhibitor LY 294002 was used, cells were preincubated with 20 μM LY 294002 for 20 min prior to IGF-1 stimulation. Cells were lysed and assayed for incorporation of radioactivity into trichloroacetate-precipitable DNA. Results are means ± standard errors of the means of six independent experiments (with or without IGF-1) and three independent experiments with the PI 3-kinase inhibitor, expressed as percentages of maximum stimulation in wild-type (WT) cells.

IRS-1- or IRS-2-dependent pathway activating the c-*fos* promotor. A likely candidate for this pathway is PI 3-kinase through IRS-1 and -2, since previous studies have shown that microinjection of anti-p85 antibodies blocks insulin-stimulated c-*fos* activation (16). Recently Hill et al. have shown the involvement of the Rho family of GTPases (RhoA, Rac-1, and CDC42Hs) in Elk-1-independent activation of the c-fos SRE (13). Interestingly, these GTPases appear to be downstream of the activation of PI 3-kinase in the insulin signaling cascade (24). Thus, the cell lines described here provide a unique tool with which to further define pathways for IRS-1-dependent c-*fos* expression.

In conclusion, we have demonstrated three classes of IGF-1 action in embryonic fibroblasts. Class I represents actions which do not require IRS-1 and is exemplified by MAP kinase activation. Class II consists of actions deficient in IRS-1-deficient cells which can be reconstituted by either expression of IRS-1 or overexpression of IRS-2; this would include IGF-1-stimulated PI 3-kinase activity and immediate-early gene expression. Class III actions are those that are deficient in IRS-$1^{-/-}$ cells, can be reconstituted by IRS-1, but are not fully reconstituted by overexpression of IRS-2, such as mitogenesis. The molecular mechanisms for such complementarity could include differences in SH2 domain partners for IRS-1 and IRS-2, differences in cellular localization, or differences in other associated signaling proteins. Further study will be required to define the exact nature of these differences.

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