MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 2509–2517 Vol. 16, No. 5
0270-7306/96/$04.00+0
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The *Drosophila* Insulin Receptor Activates Multiple Signaling Pathways but Requires Insulin Receptor Substrate Proteins for DNA Synthesis

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Received 24 October 1995/Returned for modification 1 December 1995/Accepted 9 February 1996

The *Drosophila* insulin receptor (DIR) contains a 368-amino-acid COOH-terminal extension that contains several tyrosine phosphorylation sites in YXXM motifs. This extension is absent from the human insulin receptor but resembles a region in insulin receptor substrate (IRS) proteins which binds to the phosphatidylinositol (PI) 3-kinase and mediates mitogenesis. The function of a chimeric DIR containing the human insulin receptor binding domain (hDIR) was investigated in 32D cells, which contain few insulin receptors and no IRS proteins. Insulin stimulated tyrosine autophosphorylation of the human insulin receptor and hDIR, and both receptors mediated tyrosine phosphorylation of Shc and activated mitogen-activated protein kinase. IRS-1 was required by the human insulin receptor to activate PI 3-kinase and $p70^{s6k}$, whereas hDIR associated with PI 3-kinase and activated $p70^{s6k}$ without IRS-1. However, both receptors required IRS-1 to mediate insulin-stimulated mitogenesis. These data demonstrate that the DIR possesses additional signaling capabilities compared with its mammalian counterpart but still requires IRS-1 for the complete insulin response in mammalian cells.

Many plasma membrane receptors regulate cellular processes through protein tyrosine kinases. The human insulin receptor (IR), like the receptors for epidermal growth factor and platelet-derived growth factor, contains intrinsic tyrosine kinase activity and undergoes tyrosine autophosphorylation during ligand stimulation (26). However, unlike the epidermal growth factor and platelet-derived growth factor receptors, the human IR does not associate strongly with signaling proteins containing Src homology 2 (SH2) domains (19, 22). Instead, the IR phosphorylates IR substrate (IRS) proteins (IRS-1 and IRS-2) on multiple tyrosine residues (19, 30). Many of the signaling pathways activated by the human IR require the presence of an IRS protein (19): IRS-1 binds and activates phosphatidylinositol (PI) 3-kinase, Grb-2/Sos, SH-PTP2, and nck (34); IRS-1 also mediates insulin-stimulated $p70^{s6k}$, probably through the activation of PI 3-kinase (5, 18). IRS-1 also plays a role in the stimulation of glucose uptake by insulin (1). However, not all insulin-stimulated pathways require IRS proteins, as Shc is phosphorylated directly by the human IR and then binds Grb-2/Sos, providing one of the pathways to activate $p21^{ras}$ and the mitogen-activated protein (MAP) kinase cascade (18, 19).

The *Drosophila* IR (DIR) is similar to the human IR, except that it is larger because of approximately 300 additional amino acids at both the NH_2 terminus and the COOH terminus (27). The overlapping portions of the α and β subunits of the DIR are approximately 30 and 44% identical to the human IR, respectively (Fig. 1A). The DIR binds human insulin with slightly decreased affinity, but its signaling capacity is similar to that of the human IR, suggesting that essential functions in the β subunit were conserved from insects to mammals (8, 37). The DIR is essential for *Drosophila* development and appears to be required for the epidermis and the nervous system (8).

The COOH-terminal extension of the DIR β subunit (DIR tail) is not present in the human IR and in some instances is cleaved from the receptor in *Drosophila* cell lines (8). The DIR tail has similarity to a region in IRS-1 that is required for insulin-stimulated DNA synthesis (Fig. 1A). Moreover, the DIR tail contains three YXXM motifs (Tyr-1941, Tyr-1957, and Tyr-1978) which align with tyrosine phosphorylation sites in IRS-1 (Fig. 1B). Because of this homology, the DIR should mediate signaling pathways that ordinarily require the phosphorylation of an IRS protein. In order to investigate the function of the additional signaling capacity of the DIR, we expressed a chimeric DIR molecule containing the extracellular domain of the human IR (hDIR) in 32D cells. Similar to the human IR, hDIR mediates Shc phosphorylation and MAP kinase activation during insulin stimulation; but unlike the human IR, hDIR associates with PI 3-kinase and activates $p70^{s6k}$ without IRS proteins. Surprisingly, hDIR does not mediate insulin-stimulated mitogenesis without IRS-1. We conclude that activation of Shc/$p21^{ras}$/MAP kinase and PI 3-kinase/$p70^{s6k}$ are not sufficient to mediate insulin-stimulated DNA synthesis in 32D cells. While the DIR has an extended signaling capacity, IRS proteins provide additional signaling elements that are absent from the homologous DIR tail; an

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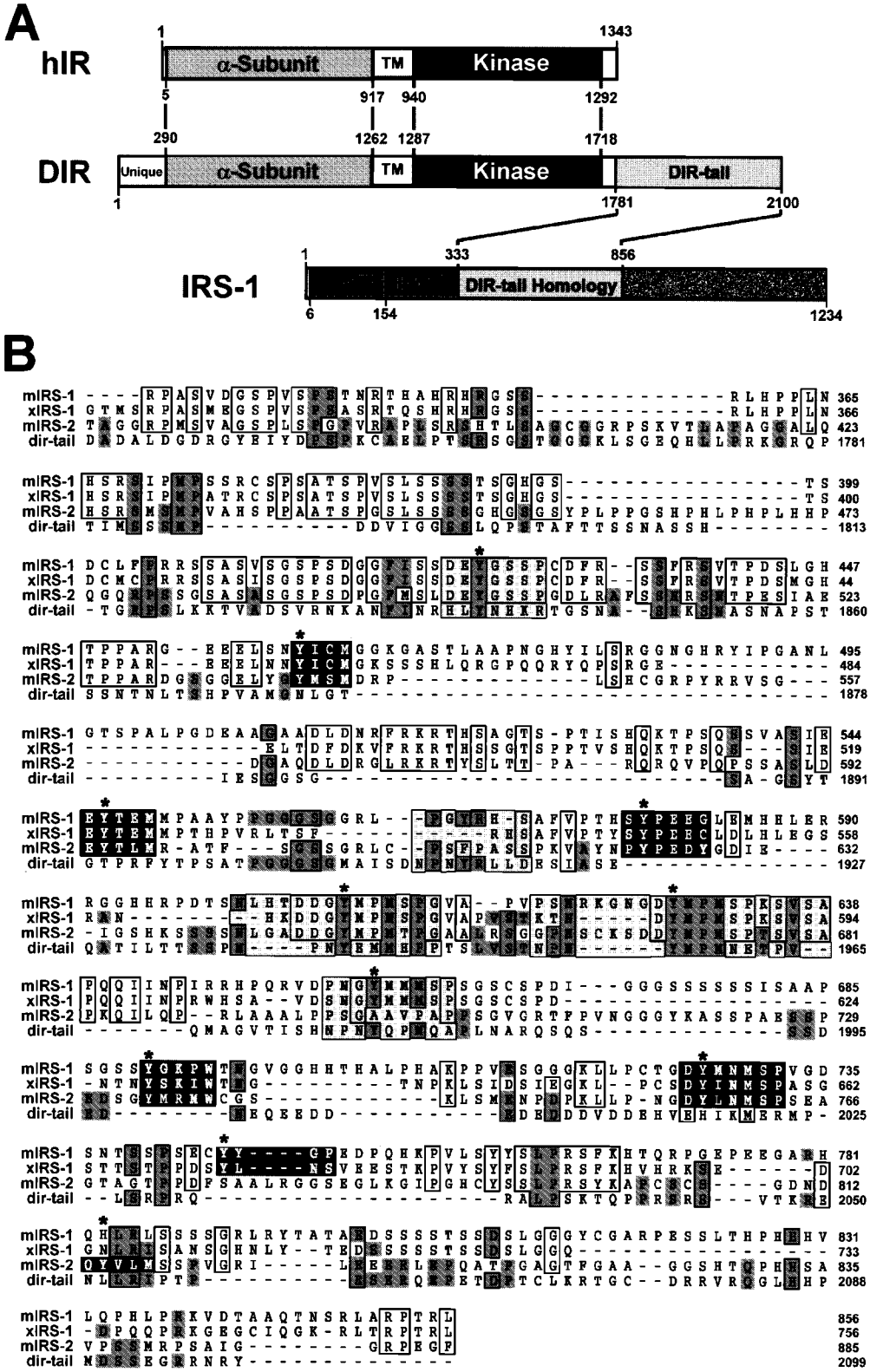

FIG. 1. Alignment of the human and *Drosophila* IRs with IRS proteins. (A) A schematic representation of the human IR (hIR) and DIR is shown along with IRS-1. The locations of the α subunits, transmembrane domains (TM), and kinase domains are shown. The region of homology between the DIR tail and IRS-1 is shown. (B) The amino acid sequences of mouse (m) IRS-1 and IRS-2 and *Xenopus* (x) IRS-1 are aligned with COOH-terminal extension of the DIR. This represents an optimal alignment determined with the PILEUP program (Genetics Computer Group, Inc.). Potential tyrosine phosphorylation motifs occurring in all four sequences are highlighted in light grey; amino acid identities between the DIR and either IRS-1 or IRS-2 are highlighted in dark grey; tyrosine phosphorylation motifs found only in the IRS protein are highlighted in black. Asterisks indicate potential tyrosine phosphorylation sites.

IRS-related protein may remain to be identified in *Drosophila melanogaster.*

MATERIALS AND METHODS

Expression of hDIR and IRS-1 in 32D cells. 32D cell lines were grown and maintained in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum and 5% WEHI conditioned medium to provide interleukin-3 (IL-3) (32, 33). Cell lines expressing IR and/or IRS-1 have previously been described (33). CsCl-purified pCMV^{his}hDIR, pCMV^{his}IRS-1, and pCMV^{neo}hDIR DNA was introduced into 32D cells by electroporation, and transformants were selected and grown in 2 to 5 mM histidinol or 750 mg of G418 per liter as previously described (32, 33). Cells expressing similar amounts of hDIR, IR, and IRS-1 were selected by analyzing lysates from equivalent number of cells by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antiphosphotyrosine (αPY), anti-IRS-1 (αIRS-1), and anti-DIR (αDIR) antibodies. In some cases, insulin binding was measured to ensure that the expression of receptors at the cell surface was nearly equal.

Insulin binding. Various 32D cell lines were made quiescent in Dulbecco modified essential medium (Sigma) for 4 h at 37°C. Cells (10^6) were washed and resuspended in binding buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM EDTA, 15 mM sodium acetate, 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 mM glucose, and 0.1 mM phenylmethylsulfonyl fluoride. [^{125}I]insulin (20,000 cpm/point) was added in the presence and absence of excess unlabeled competitor (at a final concentration of 100 nM). Binding was carried out at 4°C for 4 h. Cells were then collected, unbound label was removed, and bound radioactivity was determined by scintillation counting.

Immunoprecipitation. 32D cell lines were collected by low-speed centrifugation and made quiescent in Dulbecco modified essential medium (Sigma) for 2 to 4 h at 37°C. Cells were stimulated with insulin (100 nM) for 5 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), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40, 10% glycerol, 10 mg of aprotinin per ml, 10 mg of leupeptin per ml, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. 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 dithiothreitol and resolved by SDS-PAGE. Gels were transferred to nitrocellulose membranes, blocked, and probed as described elsewhere (15). Blots were incubated with Renaissance chemiluminescent reagents (NEN) and exposed to Kodak X-AR film or, for ^{125}I-protein A-probed materials, dried and exposed to Kodak X-AR film or detected and quantified on a Molecular Dynamics PhosphorImager.

Incorporation of [^{3}H]thymidine into DNA in 32D cells. Insulin-stimulated thymidine incorporation was assayed as previously described (33). Briefly, cells in log-phase growth were washed, and 2×10^5 cells were seeded into 1 ml of medium in each of 24 wells containing RPMI 1640 medium with 10% fetal bovine serum alone or containing various concentrations of insulin or IL-3-containing conditioned media (WEHI). Cells were grown for 48 h at 37°C. [^{3}H]thymidine (ICN) was added to a final concentration of 0.5 mCi/ml, and incubation was continued for 2 h. Cells were collected onto glass microfiber filters and lysed, and unincorporated nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

PI 3-kinase activity. 32D 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% Nonidet P-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 PI (Avanti) sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (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 plate (Merck) which had been coated with 1% potassium oxalate. Thin-layer chromatography 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.

In vitro assays for MAP and p70^{s6k} kinase activities. Quiescent cells were stimulated with 100 nM insulin (5 min for MAP kinase assays or 30 min for p70^{s6k} assays, unless otherwise noted) and collected as described above. Cells were lysed in ice-cold 10 mM potassium phosphate–1 mM EDTA (pH 7.05) containing 0.5% Nonidet P-40, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mg each of aprotinin and leupeptin per ml. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Anti-MAP kinase or anti-p70^{s6k} antibodies were added, incubated for 2 h, and collected on protein A-Sepharose beads for 1 h at 4°C. Immunoprecipitates were washed and incubated with [γ-^{32}P]ATP (50 mM final concentration; 20 mCi per reaction) containing 2 mg of myelin basic protein per reaction (for MAP kinase assays) or 20 mg of 40S ribosomes per reaction (for p70^{s6k} assays) as described previously (6, 18, 36). Assay mixtures were incubated for 15 min at room temperature, and reactions were stopped by the addition of 2× Laemmli sample buffer. Samples were denatured by boiling, and phosphorylated substrates were analyzed by SDS-PAGE.

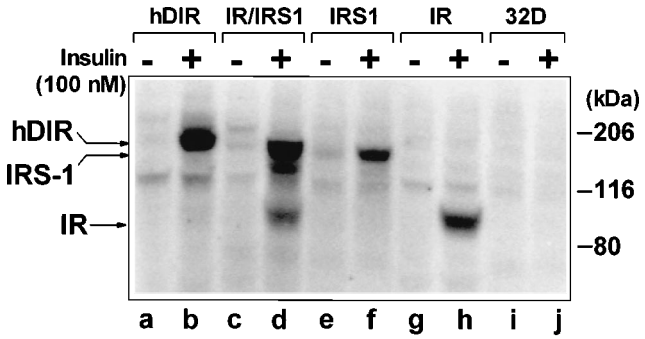

FIG. 2. Expression and phosphorylation of hDIR in 32D cells. The 32D cell lines were starved and then incubated in the presence (+) or absence (−) of 100 nM insulin for 5 min. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed for tyrosine phosphorylation by Western blotting (immunoblotting) using αPY (35).

RESULTS

Expression and insulin-stimulated tyrosine phosphorylation of hDIR in 32D cells. In order to analyze the signaling capabilities of the DIR, we placed it under control of human insulin by constructing a chimeric molecule composed of amino acids 1 to 915 of the human IR and amino acids 1322 to 2162 of the β subunit of the DIR, which includes the transmembrane domain, the kinase domain, and the 368-amino-acid COOH-terminal extension. This chimeric receptor (hDIR) was expressed in 32D cells, which contain few murine IRs and no detectable IRS proteins (33).

In order to determine whether hDIR undergoes normal insulin-stimulated tyrosyl phosphorylation, 32D cell lines expressing hDIR or the human IR with or without IRS-1 were analyzed by immunoblotting with αPY. Insulin had no effect on tyrosine phosphorylation in untransfected 32D cells, whereas human insulin stimulated tyrosine phosphorylation of the β subunit in 32D^{hDIR} cells (190 kDa) and 32D^{IR} cells (95 kDa) (Fig. 2). As previously shown, insulin weakly stimulated tyrosine phosphorylation of recombinant IRS-1 in 32D/IRS-1 cells because of the activation of the few endogenous IRs; by contrast, insulin strongly stimulated tyrosine phosphorylation of IRS-1 in 32D^{IR}/IRS-1 cells (Fig. 2). Thus, hDIR was activated by human insulin in 32D cells, as previously shown in other cell systems (37).

Association of the PI 3-kinase with hDIR. To compare the association of PI 3-kinase with hDIR and the human IR in 32D cells, we immunoprecipitated these receptors with an antibody against the extracellular domain of the human IR (αIR). Very little PI 3-kinase activity was detected in αIR immunoprecipitates from parental 32D cells. A small amount of PI 3-kinase activity was immunoprecipitated with αIR from the 32D^{IR} cells, but there was an insignificant effect of insulin (Fig. 3A).

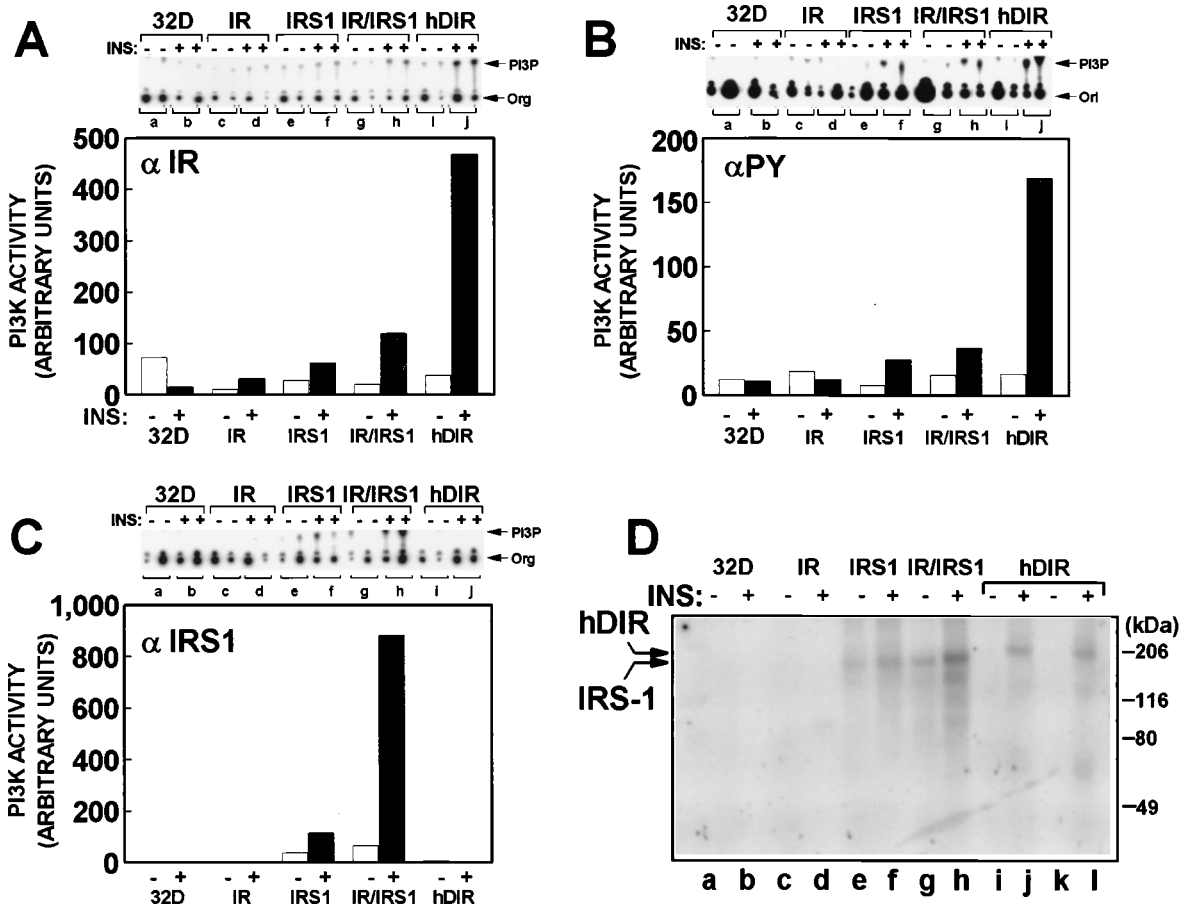

FIG. 3. Association of PI 3-kinase with insulin-stimulated phosphoproteins in 32D cell lines. PI 3-kinase (PI3K) activity was measured in αIR, αPY, and αIRS-1 immunoprecipitates prepared from 32D cell lines before (−) or after (+) stimulation with 100 nM insulin (INS) for 5 min. Samples were normalized for protein content, and immunoprecipitates were washed and assayed for associated PI 3-kinase activity as described in Materials and Methods. [^{32}P]phosphate incorporated into PI was visualized and quantified on a PhosphorImager. The absolute amount of activity is not directly comparable between these independent assays. Similar results were observed in three separate experiments. (D) The association of tyrosine phosphorylated proteins with p85 were measured in αp85 immunoprecipitates by immunoblotting with αPY. Two independent clones of 32D^{hDIR} cells were examined.

In contrast, insulin strongly stimulated the association of the PI 3-kinase with hDIR in αIR immunoprecipitates (Fig. 3A); similar results were obtained with αPY immunoprecipitates (Fig. 3B). As expected, the level of activity was similar in cells expressing either IRS-1 alone or both the receptor and IRS-1 (18). Insulin stimulated the association of PI 3-kinase with IRS-1 in 32D/IRS-1 and 32D^{IR}/IRS-1 cells, as previously shown (18); however, αIRS-1 did not immunoprecipitate PI 3-kinase from 32D, 32D^{IR}, or 32D^{hDIR} cells, confirming the absence of IRS-1 in these cells (Fig. 3C). Consistent with these results, IRS-1 and hDIR, but not the human IR, associated with p85 during insulin stimulation (Fig. 3D).

Activation of PI 3-kinase. PI 3-kinase is activated during association with tyrosine phosphorylated IRS-1 (2, 16, 25). In order to determine whether hDIR activates PI 3-kinase upon binding, we measured its activity in αp85 immunoprecipitates before and after insulin stimulation (Fig. 4A). The antibody against p85 used in these experiments has a minimal effect on PI 3-kinase activity and can be used to observe insulin stimulation of PI 3-kinase (18). Insulin stimulated PI-3 kinase activity approximately 3-, 10-, and 7-fold in 32D/IRS-1, 32D^{IR}/IRS-1, and 32D^{hDIR} cells, respectively; however, there was no detectable activation of PI 3-kinase in 32D or 32D^{IR} cells (Fig. 4A), as previously described (18). Moreover, since p85 is not tyrosine phosphorylated during insulin stimulation (2), we conclude provisionally that PI 3-kinase was activated by association of its SH2 domains with hDIR or IRS-1.

Tyrosine phosphorylation of Shc and activation of MAP kinase by hDIR. The human IR stimulates tyrosine phosphorylation of Shc and activates MAP kinase in 32D cells independently of IRS-1 (18). To examine this signaling pathway during activation of hDIR, Shc was immunoprecipitated from various insulin-stimulated 32D cells and detected by αPY immunoblotting. Insulin has no effect on Shc phosphorylation in 32D cells, whereas Shc was tyrosine phosphorylated in 32D^{IR}, 32D^{hDIR}, and 32^{IR}/IRS-1 cells (Fig. 4B). Shc did not form a stable complex with either human IR or hDIR under our experimental conditions (20).

Previous studies suggest that phosphorylated Shc engages Grb2/SOS, which stimulates GTP loading of p21^{ras}, which in turn activates the MAP kinase cascade (29). This pathway comprises at least one component of the mitogenic signal (7, 9, 23). We measured the activity of MAP kinase in specific immunoprecipitates from each of the 32D cell lines (Fig. 4C). During insulin stimulation, the human IR activated MAP kinase in the absence of IRS-1; insulin failed to activate MAP kinase in 32D and 32D/IRS-1 cells, confirming that a low level of IR in these cells was not sufficient to engage the cascade.

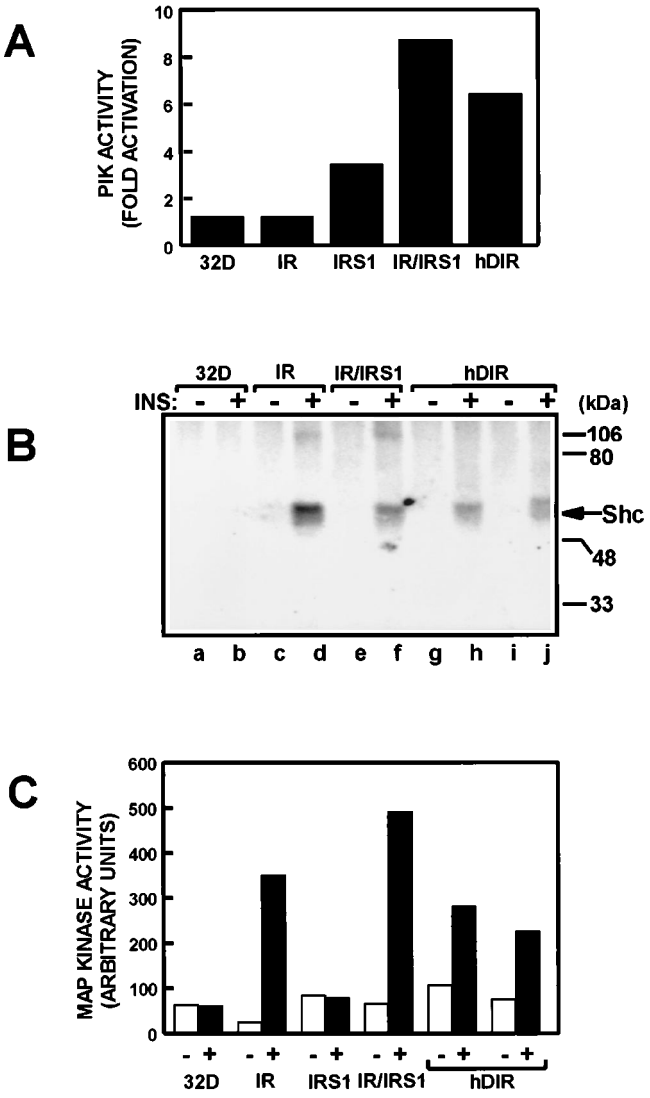

FIG. 4. Activation of PI 3 kinase and MAP kinase and the phosphorylation of Shc by insulin in 32D cell lines. 32D cell lines were stimulated with 100 nM insulin (INS) for 5 min, lysed, normalized for protein content, and immunoprecipitated with αp85 antibodies (A), Shc antibodies (B), or MAP kinase antibodies (C). (A) The αp85 immunoprecipitates were washed, and PI 3-kinase (PIK) activity was quantitated in an in vitro kinase assay. Data are triplicate determinations and plotted as fold stimulation for each cell line during insulin stimulation. (B) Lysates were immunoprecipitated with αShc antibodies, separated by SDS-PAGE, and blotted with αPY antibodies. Migration of molecular mass standards is indicated, as is the migration of Shc. (C) MAP kinase was precipitated and assayed for its ability to phosphorylate myelin basic protein in vitro. Data are duplicate determinations, and phosphate incorporation was quantified on a PhosphorImager. Two independent cell lines expressing equivalent amounts of hDIR were used in these studies. Similar results were observed in two independent experiments.

Consistent with the hypothesis (20), insulin stimulated MAP kinase activity in $32D^{hDIR}$ cells two- to threefold, which was slightly lower than the activation of MAP kinase in $32D^{IR}$ cells. This difference is consistent with the lower level of Shc phosphorylation observed in the $32D^{hDIR}$ cells (Fig. 4B). The strongest activation of MAP kinase occurred in the $32D^{IR}$/IRS-1 cells, as both Shc and the IRS-1/GRB-2 complex contribute to this response (18). Thus, the human IR and hDIR mediate MAP kinase activation during insulin stimulation, and this response is independent of IRS proteins.

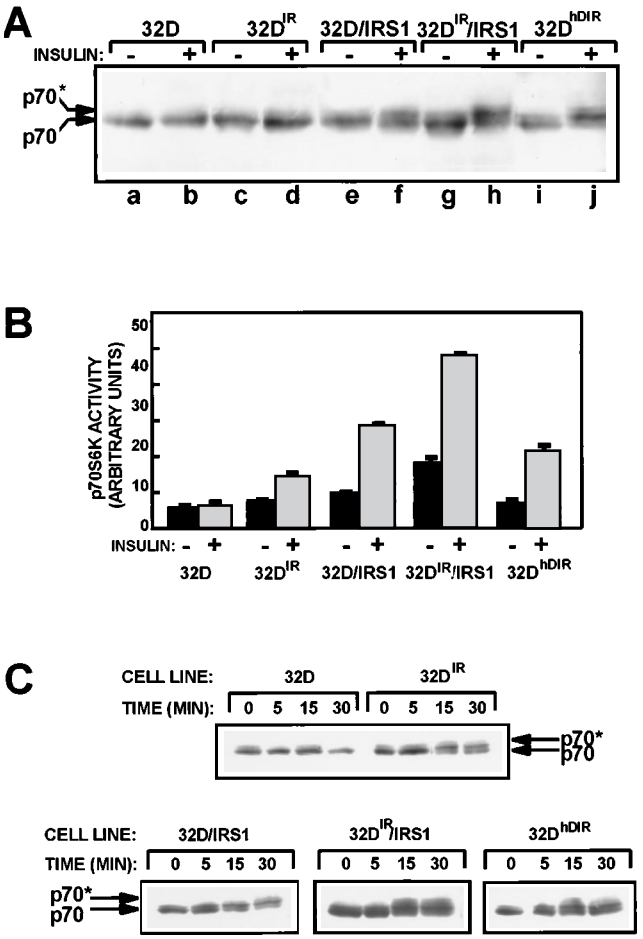

FIG. 5. Activation of $p70^{s6k}$ in 32D cells. (A) Gel retardation assay. 32D cell lines were incubated in the presence of 100 nM insulin for 30 min and lysed. Lysates were resolved by SDS-PAGE and immunoblotted with $\alpha p70^{s6k}$ antibodies. p70 and p70* indicate the migration of unphosphorylated and phosphorylated $p70^{s6k}$, respectively. This gel is representative of three independent experiments. (B) In vitro kinase assay. 32D cell lines were incubated in the absence (−) or presence (+) of insulin for 30 min and lysed. $p70^{s6k}$ was precipitated and assayed for its ability to phosphorylate 40S ribosomes in vitro. Data are from duplicate determinations, and phosphate incorporation was quantified on a PhosphorImager. Error bars represent the standard error for each datum point. (C) Time course of $p70^{s6k}$ activation. Various 32D cell lines were starved and stimulated with 100 nM insulin for the indicated times. Lysates were resolved by SDS-PAGE and immunoblotted with $\alpha p70^{s6k}$ antibodies.

Phosphorylation and activation of $p70^{s6k}$ by hDIR in 32D cells. $p70^{s6k}$ is activated by a series of phosphorylation events which can be monitored by retarded migration of $p70^{s6k}$ during SDS-PAGE; only the most highly phosphorylated (slowly migrating) forms are activated (6). Several studies suggest that PI 3-kinase mediates the effect of insulin and other growth factors on $p70^{s6k}$ (4, 5, 18); however, recent studies indicate that PI 3-kinase may not be sufficient (10, 14). Our previous data suggest that IRS-1 is required for the insulin response, which presumably occurs through the activation of PI 3-kinase (18).

Insulin had very little effect on the migration of $p70^{s6k}$ in 32D and $32D^{IR}$ cells, confirming that the human IR alone cannot activate this pathway (Fig. 5A). However, the more highly phosphorylated forms of $p70^{s6k}$ were clearly present after insulin stimulation of 32D/IRS-1 and $32D^{hDIR}$ cells; the strongest effect of insulin occurred in $32D^{IR}$/IRS-1 cells (Fig. 5A). Thus, hDIR effectively mediated the phosphorylation of

$p70^{s6k}$ in a manner similar to that of IRS-1: the common event appears to be the activation of PI 3-kinase.

The catalytic activity of $p70^{s6k}$ was measured in immunoprecipitates by an in vitro kinase assay using 40S ribosomes as the substrate. As previously shown, the human IR poorly stimulated $p70^{s6k}$ activity; however, $p70^{s6k}$ was activated two- to threefold in 32D/IRS-1, $32D^{IR}$/IRS1, and $32D^{hDIR}$ cells (Fig. 5B). Phosphorylation of $p70^{s6k}$ occurred with approximately the same time course in cells expressing IRS-1 or hDIR (Fig. 5C), suggesting that both molecules utilize the same pathway.

Insulin-stimulated DNA synthesis in 32D cells. Having demonstrated that hDIR possesses a broader signaling potential than the human IR, including the activation of PI 3-kinase and $p70^{s6k}$ without IRS-1/IRS-2, we investigated whether hDIR mediates an insulin-stimulated mitogenic response. As shown previously (18, 33), $32D^{IR}$/IRS-1 cells proliferate vigorously in response to insulin, whereas 32D cells or 32D cells expressing only the human IR or IRS-1 do not proliferate during insulin stimulation (Fig. 6B). Cell lines which bound approximately the same amount of insulin were analyzed for insulin-stimulated [^{3}H]thymidine incorporation (Fig. 6). Even though the $32D^{hDIR}$ cells bound more insulin than the responsive $32D^{IR}$/IRS-1 cells and mediate several IRS-dependent pathways, insulin did not stimulate [^{3}H]thymidine incorporation in $32D^{hDIR}$ cells. Thus, IRS proteins contribute additional signals required for the mitogenic response during insulin stimulation.

In contrast to our results, previous studies suggest that the *Drosophila* and human IRs possess identical signaling potential in mammalian cells which contain IRS proteins (37). To determine whether hDIR utilizes IRS proteins to mediate insulin-stimulated DNA synthesis, 32D cells which express both hDIR and IRS-1 were prepared. Three different clones of 32D cells expressing various levels of hDIR were isolated, as well as one $32D^{hDIR}$/IRS-1 cell line that coexpresses hDIR and IRS-1 (Fig. 7A and B). To investigate whether hDIR phosphorylated IRS-1 in an insulin-dependent manner, we immunoprecipitated IRS-1 and detected its tyrosine phosphorylation with αPY. Both the human IR and the hDIR phosphorylated IRS-1 in an insulin-dependent manner and to similar extents (Fig. 7C).

Coexpression of IRS-1 restored insulin-stimulated mitogenic signaling in $32D^{hDIR}$/IRS-1 cells. The mitogenic response in $32D^{hDIR}$/IRS-1 cells attained the same maximal activation at high doses of insulin, but the response was less sensitive than that of the $32D^{IR}$/IRS-1 cells (Fig. 7D). These results suggest that IRS-1 is essential for insulin-dependent mitogenic signaling in 32D cells and that activation of PI 3-kinase, $p70^{s6k}$, Shc/Grb-2/Sos, $p21^{ras}$, and MAP kinase by hDIR was not sufficient.

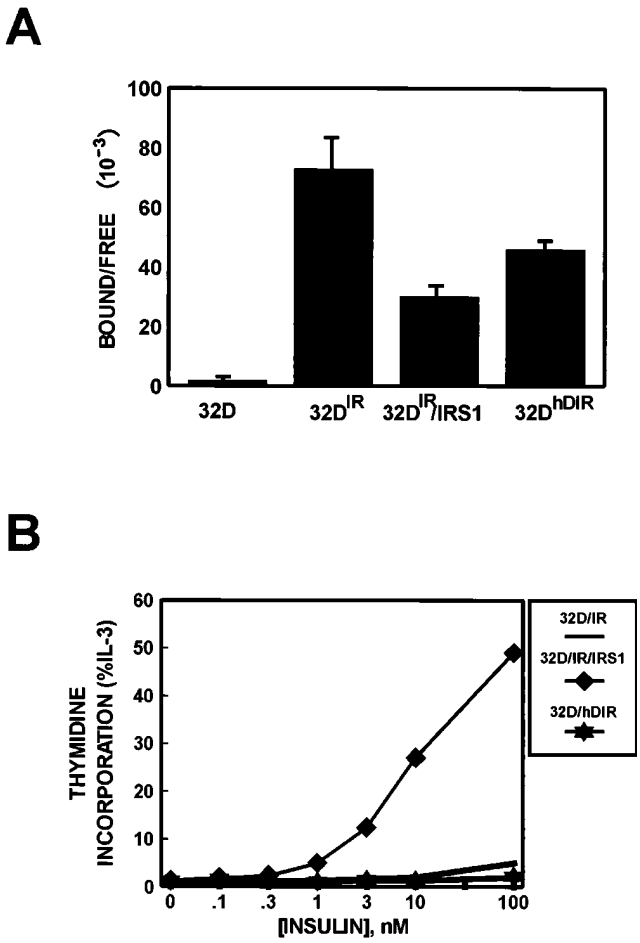

FIG. 6. Insulin binding and insulin-stimulated thymidine incorporation into DNA in 32D cell lines. (A) Various 32D cell lines were assayed for maximum insulin binding as described in Materials and Methods. Each point represents the average of triplicate determinations; the graph depicts the averaged results of three separate experiments. Error bars represent the standard error for each datum point. (B) 32D cell lines were grown in the absence or presence of increasing concentrations of insulin for 48 h. [^{3}H]thymidine was then added to the medium and incubated for 3 h. Cells were collected on glass microfiber filters and lysed, and unincorporated label was removed, as described in Materials and Methods. Data are the averages of triplicate determinations and expressed as percent IL-3 (positive control) stimulation. Similar results were observed in five separate experiments.

DISCUSSION

During insulin stimulation, the DIR activates signaling pathways in murine 32D cells which ordinarily require the phosphorylation of IRS-1 by the mammalian IR. The intracellular portion of the DIR β subunit is similar to the β subunit of the human IR, especially around the tyrosine autophosphorylation sites in the regulatory and juxtamembrane regions, and was shown previously to display a similar signaling capacity (37). The broader signaling potential of the DIR revealed by our results is apparently due to the 368-amino-acid extension at the COOH terminus (DIR tail). The DIR tail is not present in mammalian IRs, and it contains several significant similarities with a region of IRS-1 that mediates insulin-stimulated PI 3-kinase, $p70^{s6k}$, and DNA synthesis (20a). Importantly, this region contains several potential tyrosine phosphorylation sites in YXXM motifs, which align with Tyr-608, Tyr-628, and Tyr-658 in IRS-1 (Fig. 1B). In IRS-1, these tyrosine residues are phosphorylated and strongly bind to the SH2 domains in p85, which activates PI 3-kinase and leads to the stimulation of $p70^{s6k}$ kinase (4, 5, 18). Without an IRS protein (IRS-1 or IRS-2), the mammalian IR cannot engage these pathways. In contrast, the DIR activates PI 3-kinase and $p70^{s6k}$ without IRS-1, as the DIR tail compensates for the absence of IRS proteins in 32D cells.

hDIR and the human IR share an ability to engage Shc and mediate its tyrosine phosphorylation (20, 28). Shc contains two modules to bind phosphotyrosine, a COOH-terminal SH2 domain and an NH_2-terminal PY binding domain (3, 23). The PY binding domain of Shc recognizes PY in various NPXY motifs (28, 31). The human IR contains one NPEY motif in the juxtamembrane region which is essential for Shc phosphorylation (38). The DIR contains a homologous NPFY motif in its juxtamembrane region which may mediate a similar interaction

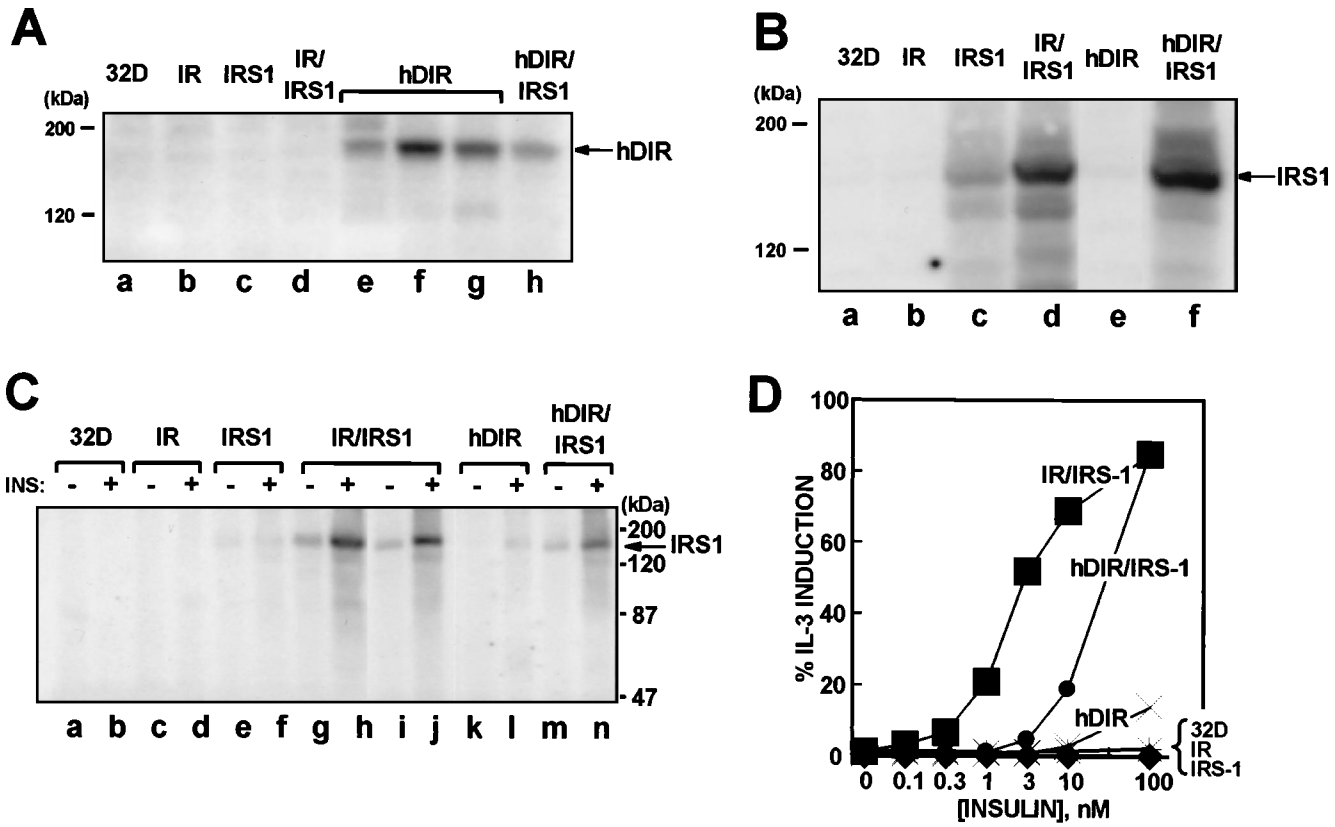

FIG. 7. Tyrosine phosphorylation of IRS-1 by hDIR in 32D cells. 32D cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody raised against the COOH-terminal 100 amino acids of the DIR (αDIR) (A) or αIRS-1 (B). (C) Insulin-stimulated tyrosine phosphorylation of IRS-1 in various 32D cells. Cells were serum starved for 4 h and stimulated with 100 nM insulin (INS) for 5 min, immunoprecipitated with an αIRS-1 antibody (JD63), washed, resolved by SDS-PAGE, and immunoblotted with an αPY antibody. (D) Insulin-stimulated mitogenic activity of 32D cells. [^{3}H]thymidine incorporation was determined with various 32D cell lines as described in the legend to Fig. 6. Data are the averages of triplicate determinations and expressed as percent IL-3 (positive control) stimulation.

(8, 27); however, four more NPXY motifs which may also contribute to the recognition of Shc exist in the DIR tail (Fig. 1B).

Insulin-stimulated phosphorylation of the YVNI motif in Shc mediates the association of Shc with the SH2 domain in Grb-2, an adapter molecule which regulates the guanine nucleotide exchange factor mSos (29). One consequence of Shc phosphorylation is the increased binding of GTP to p21^{ras} followed by the activation of MAP kinase (13). Although the phosphorylation of Shc and the activation of MAP kinase are independent of IRS proteins, this pathway is relatively insensitive, as Shc requires relatively high levels of the human IR to be phosphorylated during insulin stimulation (20). However, without IRS-1 these high receptor levels cannot mediate insulin-stimulated DNA synthesis in 32D cells, indicating that the phosphorylation of Shc and the subsequent events are not sufficient for this insulin response.

In 32D cells that express IRS-1, insulin stimulates PI 3-kinase and p70^{s6k} at comparatively low IR levels and under conditions in which Shc phosphorylation and MAP kinase activation are undetectable (18, 20). Thus, the coupling of the receptor to IRS-1 is more sensitive than its coupling to Shc. Most reports point to an important role for PI 3-kinase and p70^{s6k} for growth factor-stimulated DNA synthesis and mitogenesis (5, 12). However, the expression of IRS-1 alone in 32D cells does not mediate insulin-stimulated DNA synthesis. In contrast, the combination of high IR and IRS-1 levels mediates insulin-stimulated DNA synthesis in 32D cells, apparently because both IRS-1-dependent (PI 3-kinase and p70^{s6k}) and -independent (Shc, p21^{ras}, and MAP kinase) pathways are engaged.

Expression of the DIR in 32D cells provides a unique test of this hypothesis. hDIR mediates the tyrosine phosphorylation of Shc and the activation of MAP kinase during insulin stimulation, similar to the human IR (20); it also provides insulin-sensitive pathways for the stimulation of the PI 3-kinase and p70^{s6k} in the absence of IRS proteins. Since hDIR stimulates both IRS-1-dependent and IRS-1-independent pathways, it was expected to mediate insulin-stimulated DNA synthesis without IRS-1. However, DNA synthesis in 32D^{hDIR} cells is insensitive to insulin, indicating that the phosphorylation of Shc and the activation of PI 3-kinase, p70^{s6k}, and MAP kinase are not sufficient. Since the coexpression of IRS proteins and hDIR mediates insulin-stimulated DNA synthesis in 32D cells, additional signals provided by IRS-1 are required.

Other studies investigating the signaling capacity of the *Drosophila* and mammalian IRs have suggested that there are no differences in function (37). However, these studies are confounded by the presence of endogenous IRS proteins in the cell systems used. As demonstrated previously and in this study, the *Drosophila* receptor phosphorylates IRS-1 in response to insulin and the engagement of IRS-1 propagates a mitogenic response in cells that express both hDIR and IRS-1. By using the 32D cell system, which contains no endogenous IRS proteins, we demonstrate the signaling capabilities of hDIR in the absence of IRS proteins.

Additional explanations for the inability of the DIR to mediate DNA synthesis without IRS-1 include the possibility that sufficient activation of the PI 3-kinase/$p70^{s6k}$ was not attained; however, this explanation is unlikely, as hDIR activates nearly the same amount of PI 3-kinase as the combination of human IRs and IRS-1 (10- and 7-fold, respectively) and with approximately the same time course (data not shown). The geometry and/or the subcellular distribution of the IRS protein signaling complex formed by a highly tyrosine phosphorylated IRS-1 may also be important to produce the insulin signal. However, the DIR functions similarly to several mammalian growth factor receptors, like the epidermal growth factor and platelet-derived growth factor receptors, which directly bind to SH2 proteins and mediate DNA synthesis in 32D cells (24). Thus, the tail of the DIR may lack an essential signaling element present in IRS proteins, such as an ability to engage other proteins, which are normally recruited by other mammalian growth factor receptors or by the IRS proteins.

Insulin-stimulated DNA synthesis in cells expressing IRS-1 and either hDIR or the human IR reaches the same maximal level of activation but is approximately 10-fold less sensitive with hDIR. This loss of sensitivity may be due to a change in the binding affinity of IRS-1 for this receptor. Two mechanism are employed by the human IR to engage the IRS protein: the pleckstrin homology domain ($IH1^{PH}$) provides part of the interaction, and the PY binding domain ($IH2^{PTB}$) provides the other (17). The mechanism used by the $IH1^{PH}$ domain is unknown but could be inoperative with the DIR. The function of the $IH2^{PTB}$ domain is better understood. It recognizes the phosphorylated NPXY motif in the juxtamembrane region of the human IR. Moreover, alignment of various receptors which engage IRS-1 suggests that a longer motif (LXXXX**NPXY**SX SD) contributes to this interaction (11). Recent work using recombinant $IH2^{PTB}$ domains from IRS-1 and IRS-2 reveals a preference for the phosphorylated LYASS**NPEY**LSASD motif (37a). Although the NPXY motif is conserved in the juxtamembrane region of the DIR, the surrounding sequence is different (MNTEV**NPFY**ASMQY), which may change the binding affinity. Moreover, the presence of four additional NPEY motifs in the DIR tail may engage the IRS proteins but position them less effectively for phosphorylation by the receptor tyrosine kinase.

IRS-1 contains many other functional features, including additional tyrosine phosphorylation sites that may contribute important signals to the insulin response (Fig. 1). The $IH1^{PH}$ domain and the $IH2^{PTB}$ domain could provide downstream interactions that are absent from the DIR; however, this is unlikely, since IRS proteins lacking both domains are still more effective than hDIR (37a). Recent experiments with $IRS\text{-}1^{F18}$ (an IRS-1 molecule that is incapable of tyrosine phosphorylation because of the substitution of 18 tyrosine phosphorylation sites with phenylalanine) suggest that phosphotyrosine-independent signals are also involved (21). Insulin has no effect on PI 3-kinase or $p70^{s6k}$ activity in $32D^{IR}/IRS1^{F18}$ cells but partially mediates DNA synthesis at high insulin concentrations (50 to 100 nM). This pathway may be required to stimulate mitogenesis in cells expressing the DIR. Our future analysis of $IRS\text{-}1^{F18}$ together with hDIR will provide more insight into this question and provide an opportunity to further resolve the unique signal mediated by IRS-1.

The similarity between the DIR tail and a portion of the mammalian IRS proteins is interesting from an evolutionary point of view. The DIR resembles many mammalian growth factors, as it directly binds signaling proteins with SH2 domains. During evolution, the IRS-like region may have separated from the receptor and acquired additional functions such as the pleckstrin homology domains, the PY binding domain, and additional tyrosine phosphorylation motifs, especially in the COOH-terminal region. The finding that the murine IRS-2 gene is located close to the IR on chromosome 8 supports this hypothesis (30a).

The selective advantage for the use of IRS proteins is not understood. IRS proteins dissociate the intracellular signaling complex from the endocytic pathways of the activated receptor. However, IRS proteins also provide a means for signal amplification by eliminating the stoichiometric constraints encountered by most receptors which directly recruit SH2 proteins to their autophosphorylation sites. The existence of two IRS proteins and possibly other related substrates provides additional signaling diversity. Moreover, replication of the IRS-signaling elements as intrinsic domains in various receptors is redundant; the shared use of IRS proteins by multiple receptors provides an efficient way to share common and multifunctional signaling elements.

ACKNOWLEDGMENTS

We thank Erin Glasheen and Yitao Zhang for excellent technical assistance. We also thank Montse Miralpeix and Tomo Asano for help in the production of αp85 antibodies.

This work was supported by DK 38712, DK 43808 (M.F.W.), and CA 46595 (J.B.). J.B. is an established investigator of the American Heart Association and a recipient of HMS Funds for Discovery. L.Y. and M.G.M. were partially supported by the Albert J. Ryan Foundation at Harvard Medical School.

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