MOLECULAR AND CELLULAR BIOLOGY, Sept. 1995, p. 4711–4717 Vol. 15, No. 9
0270-7306/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Insulin Receptor Substrate 1 Rescues Insulin Action in CHO Cells Expressing Mutant Insulin Receptors That Lack a Juxtamembrane NPXY Motif

DAXIN CHEN,[1] DEBRA J. VAN HORN,[1] MORRIS F. WHITE,[2] AND JONATHAN M. BACKER[1]*

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461,[1] and Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215[2]

Received 29 March 1995/Returned for modification 17 April 1995/Accepted 26 May 1995

Insulin signals are mediated through tyrosine phosphorylation of specific proteins such as insulin receptor substrate 1 (IRS-1) and Shc by the activated insulin receptor (IR). Phosphorylation of both proteins is nearly abolished by an alanine substitution at Tyr-960 (A960) in the β-subunit of the receptor. However, overexpression of IRS-1 in CHO cells expressing the mutant receptor (A960 cells) restored sufficient tyrosine phosphorylation of IRS-1 to rescue IRS-1/Grb-2 binding and phosphatidylinositol 3′ kinase activation during insulin stimulation. Shc tyrosine phosphorylation and its binding to Grb-2 were impaired in the A960 cells and were unaffected by overexpression of IRS-1. Although overexpression of IRS-1 increased IRS-1 binding to Grb-2, ERK-1/ERK-2 activation was not rescued. These data suggest that signaling molecules other than IRS-1, perhaps including Shc, are critical for insulin stimulation of $p21^{ras}$. Interestingly, overexpression of IRS-1 in the A960 cells restored insulin-stimulated mitogenesis and partially restored insulin stimulation of glycogen synthesis. Thus, IRS-1 tyrosine phosphorylation is sufficient to increase the mitogenic response to insulin, whereas insulin stimulation of glycogen synthesis appears to involve other factors. Moreover, IRS-1 phosphorylation is either not sufficient or not involved in insulin stimulation of ERK.

The biological effects of insulin on target tissues are initiated by its binding to the insulin receptor, a heterotetrameric integral membrane protein which contains an intrinsic insulin-stimulated tyrosine kinase activity (15, 48). Insulin receptor substrate 1 (IRS-1) is a key mediator of the signaling events that lie downstream from the insulin receptor (41). IRS-1 is phosphorylated on multiple tyrosine residues in insulin-stimulated cells and is also phosphorylated during stimulation of responsive cells with insulin-like growth factor I (IGF-I), interleukin 4, and growth hormone (25, 38, 45). Tyrosine-phosphorylated IRS-1 binds with high affinity to Src homology 2 (SH2) domains found in various enzymes and adapter molecules, including phosphatidylinositol 3′ kinase (PI 3′-kinase), Grb-2, the proto-oncogene Nck, and the tyrosine-specific phosphatase SHPTP-2 (2, 13, 18, 21, 37, 39). The specificity of insulin signaling presumably arises from the cohort of downstream elements which interact with IRS-1, as well as from the activation of IRS-1-independent pathways such as tyrosine phosphorylation of Shc (28, 36).

Given its interactions with multiple signaling molecules, IRS-1 may play a pleiotropic role in insulin signaling. Several studies have shown that IRS-1 is necessary for mitogenic responses to insulin and IGF-I in responsive cells (31, 46, 47). Moreover, overexpression of IRS-1 increases insulin-stimulated mitogenesis in Chinese hamster ovary (CHO) cells and in IRS-1-deficient 32-D myeloid progenitor cells (40, 46). In contrast, the role of IRS-1 in the regulation of classic metabolic responses to insulin is less clear. Reducing IRS-1 expression in rat adipose cells by using an antisense ribozyme impairs insulin-stimulated glucose transporter translocation, and disruption of the IRS-1 gene in mice causes elevated fasting insulin levels and mild insulin resistance (1, 30, 42). These data suggest that IRS-1 plays a role in glucose homeostasis, although other pathways may be involved (19).

To examine the role of IRS-1 in distinct mitogenic and metabolic responses to insulin, we used CHO cells expressing mutant insulin receptors that lack a tyrosine phosphorylation site, Tyr-960, in the juxtamembrane of the receptor β-subunit (12, 49). We have previously shown that receptors containing substitutions or deletions of the $NPXY_{960}$ motif are unable to mediate tyrosyl phosphorylation of IRS-1 in CHO cells. Mutations at this site also impair the generation of mitogenic and metabolic responses to insulin (4, 49). However, unlike receptors with mutations in the ATP binding site of the receptor, these mutant receptors undergo normal autophosphorylation at other sites in the insulin receptor β-subunit, and solubilized receptors have normal kinase activity towards peptide substrates in vitro (4, 8, 22, 49). The inability of the mutant receptors to phosphorylate IRS-1 in intact cells may result from a decreased affinity for endogenous substrates (4, 27, 49).

The approach used in these studies is to rescue various insulin responses in cells (A960 cells) expressing an insulin receptor with a Tyr-960→Ala mutation (IR_{A960}) by overexpressing IRS-1, thereby providing the mutant receptors with an excess of substrate. If successful, this strategy should selectively enhance IRS-1-dependent biological responses, without affecting pathways mediated by phosphorylation of Shc or other substrates. Our results demonstrate that overexpression of IRS-1 in the A960 cells increased insulin-stimulated IRS-1 phosphorylation, formation of IRS-1/Grb-2 complexes, PI 3′-kinase activation, and DNA synthesis, whereas glycogen synthesis was only moderately increased. In contrast, Shc tyrosyl phosphorylation, formation of Shc/Grb-2 complexes, and ERK-1/ERK-2 activation were not increased. These data suggest that IRS-1 mediates many but not all insulin responses in

* Corresponding author. Mailing address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461. Phone: (718) 430-2153. Fax: (718) 829-8705. Electronic mail address: Backer@Mailserver.aecom.yu.edu.

CHO cells. In addition, we conclude that the NPXY motif in the insulin receptor β-subunit is important for IRS-1 and Shc phosphorylation in intact cells but is not essential at high concentrations of IRS-1.

MATERIALS AND METHODS

Transfection of CHO cells and selection of mutant cell lines. All CHO cells are derivatives of the K1 clone, which contains approximately 3,000 endogenous insulin receptors and 50,000 endogenous IGF-1 receptors per cell (8, 9). CHO cells expressing wild-type human insulin receptors (exon 11-minus isoform [44]) (IR cells) or IR_{A960} and cells expressing rat IRS-1 (IRS-1 cells) have been previously described (5, 40). A960 cells were transfected with a plasmid containing the gene for resistance to histidinol and the rat IRS-1 cDNA under the control of the cytomegalovirus promoter (pCMVhis/IRS-1) as previously described (40) to produce two clones, Cl-23 and Cl-57. Histidinol-resistant clones were screened by Western blotting (immunoblotting) for expression of IRS-1 by using an antipeptide antibody to IRS-1 (41).

Quantitation of IRS-1 tyrosyl phosphorylation and expression. Expression and phosphorylation of IRS-1 in CHO lines were determined by incubating cells grown in six-well dishes (approximately 1.2×10^6 per well) in the absence or presence of insulin for 3 min at 37°C and then lysing the cells in boiling sample buffer. Solubilized proteins (approximately 70 μg of protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (7.5% resolving), transferred to nitrocellulose, and analyzed by immunoblotting with rabbit polyclonal antibodies to IRS-1 or monoclonal antibodies to phosphotyrosine (Upstate Biotechnology [UBI]) as previously described (2). Proteins were visualized with a peroxidase-coupled second antibody and ECL reagents (Amersham). IRS-1 expression and phosphorylation were quantitated by scanning densitometry (Molecular Dynamics). Quantitation of IRS-1 by immunoblotting was validated in control experiments with different concentrations of recombinant IRS-1 or immunoprecipitated IRS-1 from CHO IRS-1 cells (data not shown).

PI 3′-kinase assays. CHO cells were grown to 80% confluence in 10-cm dishes. Quiescent cells (approximately 8×10^6 per dish) were stimulated with insulin (5 nM) for 10 min at 37°C, washed, and solubilized. PI 3′-kinase was immunoprecipitated from the lysates with a rabbit polyclonal antibody to residues 321 to 724 of the 85-kDa regulatory subunit as previously described (3). After absorption to protein A-Sepharose beads (Pharmacia), PI 3′-kinase activity was determined as described by Ruderman et al. (32). All determinations were made in triplicate.

Immunoprecipitations and Western blotting. Quiescent CHO cells grown in 10-cm dishes (8×10^6 cells per dish) were incubated in the presence or absence of 100 nM insulin for 5 min and lysed as previously described (3). IRS-1 and Shc immunoprecipitations were performed with monoclonal anti-Shc antibodies (Transduction Laboratories) or monoclonal anti-IRS-1 antibodies (26). The anti-Shc and anti-IRS-1 antibodies were coupled to protein G-Sepharose (Pharmacia) with dimethylpimelimidate prior to use. Immunoblotting was performed with monoclonal anti-Grb-2 antibody (Transduction Laboratories) or antiphosphotyrosine antibody.

ERK-1/ERK-2 assays. Quiescent or insulin-stimulated cells grown in 10-cm dishes (8×10^6 cells per dish) were lysed in 1 ml of boiling 1% SDS–10 mM Tris-HCl (pH 7.4) and sonicated briefly. One hundred microliters of lysate was diluted into 1% Triton X-100–0.5% Nonidet P-40–10 mM Tris (pH 7.4)–150 mM NaCl–1 mM EDTA–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid]–200 μM sodium orthovanadate–200 μM phenylmethylsulfonyl fluoride (final concentrations). ERK-1/ERK-2 was immunoprecipitated with a monoclonal antibody that recognizes both the p42 and p44 isoforms of mitogen-activated protein (MAP) kinase (Transduction Laboratories). After absorption with protein G-Sepharose (Pharmacia), the immunoprecipitates were washed and proteins were eluted, separated by SDS-PAGE, and immunoblotted with monoclonal antiphosphotyrosine antibody. Alternatively, ERK activity was assayed by incubating quiescent cells grown in 10-cm dishes (approximately 8×10^6 cells per dish) for 5 min in the absence or presence of 100 nM insulin for 5 min and then rapid chilling and lysis in cold 10 mM KPO_4 (pH 7.05) containing 1 mM EDTA, 0.5% Nonidet P-40, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg each of aprotinin and leupeptin per ml. After removal of insoluble material by centrifugation, ERK was immunoprecipitated with a polyclonal antibody which recognizes both p42 and p44 isoforms (UBI). After absorption with protein A-Sepharose, the pellets were washed and assayed as described elsewhere (26) by using 2 μg of myelin basic protein per assay. Reaction products were separated by SDS-PAGE (12.5% resolving) and incorporation of $[^{32}P]$phosphate into myelin basic protein was quantitated on a Molecular Dynamics Phosphorimager.

Incorporation of $[^3H]$thymidine into DNA. Thymidine incorporation was measured as previously described (7, 14). CHO cells were plated at low density in 24-well plates and incubated without serum for 3 days. The cells (approximately 10^5 per well) were stimulated with the indicated concentrations of insulin or 10% fetal bovine serum (FBS) for 15 h and incubated in medium containing 1 μCi of $[^3H]$thymidine (New England Nuclear, Boston, Mass.) per ml at 37°C for 1 h. The cells were washed in phosphate-buffered saline and lysed in 1 mg of SDS per ml, and DNA was precipitated in 10% (final concentration) cold trichloroacetic acid overnight. The DNA was collected on glass fiber filters (Whatman), washed in 10% cold trichloroacetic acid, and counted in a liquid scintillation counter. All determinations were made in triplicate. Fifty-percent effective doses (ED_{50}s) are presented as means and standard errors of the means of ED_{50}s from individual experiments and are approximate in that several of the curves are not maximal at 100 nM insulin.

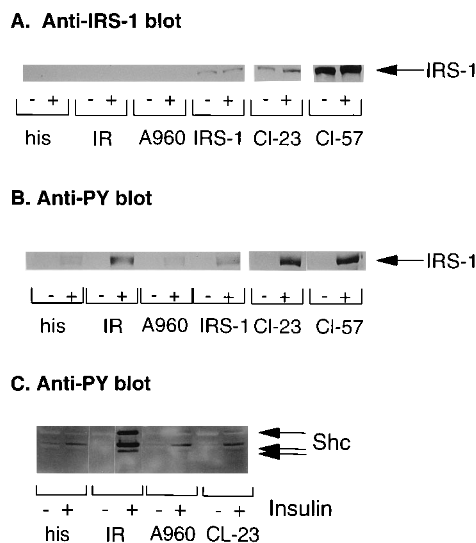

FIG. 1. Tyrosyl phosphorylation of IRS-1 in cells expressing wild-type and mutant insulin receptors. CHO cells expressing only endogenous receptors (his), endogenous receptors plus IRS-1 (IRS-1), wild-type insulin receptors (IR), mutant insulin receptors (A960), or mutant receptors plus IRS-1 (Cl-23 and Cl-57) were incubated without (−) or with (+) 100 nM insulin for 2 min and then lysed in Laemmli sample buffer and boiled. After brief sonication, proteins were separated by SDS-PAGE (7.5% resolving), transferred to nitrocellulose, and blotted with anti-IRS-1 (A) or antiphosphotyrosine (anti-PY) (B) antibodies. (C) Anti-Shc immunoprecipitates from quiescent or insulin-stimulated cells were immunoblotted with monoclonal antiphosphotyrosine antibodies. Bands were detected with horseradish peroxidase-labeled second antibody and ECL reagents.

Incorporation of $[^{14}C]$glucose into glycogen. Glucose incorporation into glycogen was measured as previously described (14, 43). CHO cells were grown to 80% confluence in six-well plates (approximately 10^6 cells per well). The cells were starved for 3 h in low (0.04%)-glucose media, and then stimulated with various concentrations of insulin for 30 min at 37°C and for an additional 1 h in the presence of 10 μCi of $[^{14}C]$glucose per ml. The cells were washed in phosphate-buffered saline and solubilized by the addition of 0.4 ml 20% KOH for 1 h at 37°C. The solubilized cells were transferred to glass tubes, the wells were washed with an additional 0.5 ml of water, and the combined samples were boiled for 20 min. After the addition of 0.1 ml of 5% Na_2SO_4 containing 1 mg of glycogen per ml, the samples were precipitated overnight on ice with 2.5 ml of ethanol. The precipitates were collected on glass filters (Whatman) and washed with 66% ethanol, and the filters were counted in a scintillation counter. All determinations were made in triplicate. ED_{50}s are presented as means and standard errors of the means of ED_{50}s from individual experiments and are approximate in that several of the curves are not maximal at 100 nM insulin.

RESULTS

Overexpression of IRS-1 restores its phosphorylation by IR_{A960}. Tyr-960 is a phosphorylation site of the insulin receptor β-subunit but does not directly regulate its tyrosine kinase activity (4, 12, 49). However, substitution of Tyr-960 with alanine or phenylalanine, or deletion of the surrounding 12 amino acids, impairs IRS-1 and Shc phosphorylation during insulin stimulation (4, 49, 52). Insulin-stimulated IRS-1 and Shc tyrosyl phosphorylation was barely detected in control CHO cells (his), which express approximately 3,000 endogenous receptors per cell (Fig. 1B and 1C). Overexpression of wild-type insulin receptor (10^6 receptors per cell) increased tyrosine phosphorylation of endogenous IRS-1 nearly fourfold during insulin

TABLE 1. IRS-1 expression and insulin-stimulated biological responses in CHO cells expressing wild-type or mutant insulin receptors

Cells	IRS-1		% Activity (basal)[a]		[^{3}H]thymidine incorporation[a]			Glycogen synthesis[a,b]	
	% Expression[c]	% Tyrosyl phosphorylation[d]	PI 3′-kinase	MAP kinase	Basal (%)	With FBS (%)	ED_{50} (nM)[e]	Basal (%)	ED_{50} (nM)[e]
his	ND[f]	100	100 ± 5	100 ± 19	100 ± 2	1,613 ± 224	11.25 ± 1.3	100 ± 3	17.7 ± 4.1
IR	ND	414	145 ± 33	178 ± 55	159 ± 12	1,964 ± 332	0.98 ± 0.8	91 ± 15	0.4 ± 0.1
IRS-1	100	190	157 ± 27	ND	107 ± 4	1,612 ± 456	7.0 ± 0.8	75 ± 8	18.7 ± 5.7
A960	ND	59	139 ± 20	115 ± 13	78 ± 6	770 ± 121	10.0 ± 1.6	119 ± 13	23.3 ± 1.4
Cl-23	254	593	245 ± 28	89 ± 8	65 ± 8	580 ± 163	1.3 ± 0.2	82 ± 9	5.3 ± 1.3
Cl-57	1,656	796	385 ± 13	ND	63 ± 7	611 ± 202	3.0 ± 0.6	78 ± 5	4.2 ± 0.4

[a] From two to four separate experiments. To facilitate the pooling of data from different experiments, activities were normalized to the signal in unstimulated his cells in each experiment. This procedure normalizes for variation in the absolute magnitude of radioactivity incorporated in different experiments but does not affect the relative levels of incorporation in different cell lines in each experiment.

[b] [^{14}C]glucose incorporation.

[c] Detected by immunoblotting with anti-IRS-1 antibodies, quantitated by scanning densitometry, and normalized to the level of expression in IRS-1 cells. Expression of endogenous IRS-1 in his, IR, and A960 cells could not be determined.

[d] Detected by immunoblotting with antiphosphotyrosine antibodies, quantitated by scanning densitometry, and normalized to the level of phosphorylation in his cells. Exposures were chosen so as to detect IRS-1 phosphorylation in the his cells without the linear range of the film being exceeded.

[e] The data were pooled from three or four experiments and are approximate, as the biological responses did not reach a well-defined maximal value in all cases.

[f] ND, not determined.

stimulation (Fig. 1B and Table 1). As previously shown, expression of IR_{A960} in CHO cells did not enhance and may even reduce IRS-1 phosphorylation during insulin stimulation (Fig. 1B and Table 1) (16, 49). Similarly, insulin-stimulated Shc tyrosyl phosphorylation was enhanced in the IR cells, whereas its phosphorylation in A960 cells was not increased relative to control cells (Fig. 1C) (52). These results confirm that Tyr-960 is important for IRS-1 and Shc tyrosyl phosphorylation in insulin-stimulated CHO cells.

Recombinant IRS-1 is phosphorylated normally by partially purified IR_{A960} s in detergent solution (1a), suggesting that the catalytic activity of the receptor is approximately normal. However, coupling of IR_{A960} to IRS-1 in intact CHO cells is clearly impaired. To restore IRS-1-dependent insulin responses in A960 cells, we overexpressed rat IRS-1. Immunoblots revealed significantly increased expression of IRS-1 in two clones (Cl-23 and Cl-57) (Fig. 1A and Table 1). The level of insulin-stimulated tyrosyl phosphorylation of IRS-1 in IR cells was compared with those in Cl-23 and Cl-57 by immunoblotting with antiphosphotyrosine antibody. The levels of phosphorylated IRS-1 in both Cl-23 and Cl-57 were similar to or greater than that in the IR cells (Fig. 1B; Table 1). In contrast, the impaired tyrosyl phosphorylation of Shc in A960 cells was not affected by overexpression of IRS-1 (Fig. 1C). These results suggest that the wild-type insulin receptor efficiently phosphorylates the low level of IRS-1 and Shc in IR cells. (Overexpression of IRS-1 in IR cells causes an extremely elevated level of IRS-1 phosphorylation [40]. However, these cells are not relevant to this study.) Although IR_{A960} inefficiently phosphorylated endogenous IRS-1 and Shc, the elevated levels of IRS-1 in Cl-23 and Cl-57 restored tyrosyl phosphorylation of IRS-1. We conclude that the NPXY motif in the insulin receptor β-subunit is not required for phosphorylation of IRS-1 at high levels of substrate.

IRS-1 expression restores signaling by IR_{A960}. IRS-1 binds to multiple signaling proteins that contain SH2 domains, including PI 3′-kinase, Grb-2, SHPTP-2, Nck, and possibly other molecules involved in the pleotropic responses to insulin (48). To determine whether elevated expression of IRS-1 in A960 cells rescues these binding interactions, we measured PI 3′-kinase activation and Grb-2 binding to IRS-1. PI 3′-kinase activity was low in the control cells (his). Insulin caused a 3.5-fold stimulation of PI 3′-kinase in IR cells, whereas stimulation was minimal in A960 cells (Fig. 2). In contrast, overexpression of IRS-1 in the A960 cells (Cl-23 and Cl-57) increased basal PI 3′-kinase activity (Table 1) and restored insulin stimulation of PI 3′-kinase to the levels in IR cells, consistent with the increased level of IRS-1 tyrosyl phosphorylation (Fig. 2; Table 1). Thus, overexpression of IRS-1 restored the ability of the mutant IR_{A960} to activate PI 3′-kinase, an enzyme whose regulation by insulin is directly coupled to IRS-1 phosphorylation (2).

Grb-2 binding to IRS-1 requires phosphorylation of Tyr-895 (26). As expected, Grb-2 binding to IRS-1 was not detected in IRS-1 immunoprecipitates from insulin-stimulated control cells (his). However, Grb-2 was readily detected as a sharp 24-kDa band in anti-IRS-1 immunoprecipitates from insulin-stimulated IR cells (Fig. 3A). Grb-2 was barely detected in anti-IRS-1 immunoprecipitates from unstimulated or insulin-stimulated A960 cells, in agreement with the reduced IRS-1 tyrosyl phosphorylation in these cells. However, overexpression of IRS-1 in A960 cells (clone Cl-23) restored association

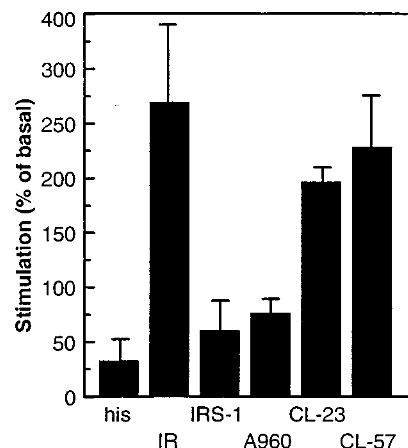

FIG. 2. Insulin stimulation of PI 3′-kinase in cells expressing wild-type and mutant insulin receptors. CHO cells expressing insulin receptors and IRS-1 as indicated were stimulated with 5 nM insulin for 10 min at 37°C. The cells were lysed, and PI 3′-kinase was immunoprecipitated and assayed in vitro. All determinations were done in triplicate, and data were pooled from three separate experiments. The stimulation over basal activity is expressed as a percentage of basal activity in each cell line. Basal activities are listed in Table 1.

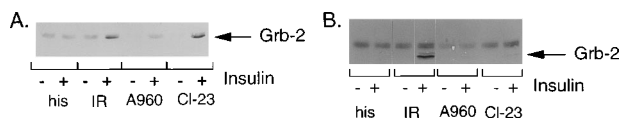

FIG. 3. Insulin-stimulated binding of Grb-2 to IRS-1 and Shc. Anti-IRS-1 (A) or anti-Shc (B) immunoprecipitates from quiescent or insulin-stimulated cells were immunoblotted with monoclonal anti-GRB-2 antibody. Bands were detected with horseradish peroxidase-labeled second antibody and ECL reagents. The diffuse band present in most lanes is due to the mouse immunoglobulin G light chain from the monoclonal primary antibodies, which cross-react with the anti-mouse second antibody used to visualize Grb-2.

of Grb-2 with IRS-1 to the level in IR cells, reflecting the increased tyrosyl phosphorylation of IRS-1.

Shc binding to Grb-2 was measured by immunoprecipitation with anti-Shc antibody, followed by immunoblotting with anti-Grb-2 antibody. The formation of Shc/Grb-2 complexes correlated well with the observed levels of Shc tyrosyl phosphorylation. Grb-2 was strongly detected in anti-Shc immunoprecipitates from insulin-stimulated IR cells, whereas it was undetectable or barely detectable in immunoprecipitates from insulin-stimulated his, A960, or Cl-23 cells (Fig. 3B). Thus, overexpression of IRS-1 in A960 cells provides a way to study IRS-1-dependent signaling in the background of low Shc activity.

Insulin stimulation of ERK-1/ERK-2. Both Shc and IRS-1 bind Grb-2 in insulin-stimulated cells, an observation possibly implicating them in the activation of $p21^{ras}$ and the p42/p44 MAP kinase cascade (6, 36). We examined the activation of ERK-1/ERK-2 in CHO cells by measuring tyrosine phosphorylation of ERK (p42/p44) and its kinase activity in specific immunoprecipitates. As previously shown (16, 50), both insulin-stimulated tyrosine phosphorylation of p42/p44 and ERK activity were significantly increased in IR cells (Fig. 4). However, expression of the mutant IR_{A960} did not enhance insulin stimulation of ERK activity and only slightly increased p42/p44 phosphorylation in agreement with the decreased binding of Grb-2 by Shc and IRS-1 in these cells and with previous studies (16, 52). Surprisingly, overexpression of IRS-1 in the A960 cells (Cl-23 or Cl-57) did not restore insulin-stimulated ERK activity or p42/p44 tyrosine phosphorylation to the level in IR cells (Fig. 4), even though Grb-2 binding to IRS-1 was increased (Fig. 3A). Similar results were seen with a gel shift assay used to measure insulin-stimulated ERK phosphorylation (data not shown). Since Shc tyrosine phosphorylation and binding to Grb-2 in A960 cells were not increased by overexpression of IRS-1, our data are consistent with the hypothesis that Shc may be the principal pathway regulating the p42/p44 MAP kinases in CHO cells.

Insulin stimulation of thymidine incorporation into DNA. To determine whether overexpression of IRS-1 rescues insulin-stimulated mitogenesis, we measured insulin-stimulated incorporation of [^{3}H]thymidine into DNA in the A960 cells and in A960 cells overexpressing IRS-1 (Cl-23 and Cl-57) (Fig. 5). Basal and FBS-stimulated thymidine incorporation in each line and ED_{50}s are listed in Table 1. As previously shown, overexpression of wild-type insulin receptors in the IR cells increased the sensitivity of insulin-stimulated thymidine incorporation 10-fold over the response in control cells (ED_{50}, 1.0 ± 0.08 versus 11.3 ± 1.3 nM) but had little effect on the magnitude of the response (Fig. 5A). In contrast, overexpression of IRS-1 in the IRS-1 cells increased the magnitude of the response but not the sensitivity to insulin (approximate ED_{50}, 7 ± 0.7 nM), as these cells express only endogenous receptors (Fig. 5A).

Insulin stimulation of thymidine incorporation in the A960 cells was similar to that in control his cells (approximate ED_{50}, 10 ± 1.6 nM), although basal incorporation was lower (Fig. 5B; Table 1). Overexpression of IRS-1 in A960 cells (clones Cl-23 and Cl-57) had little effect on basal and FBS-stimulated thymidine incorporation levels (Table 1). However, IRS-1 expression increased insulin-stimulated thymidine incorporation

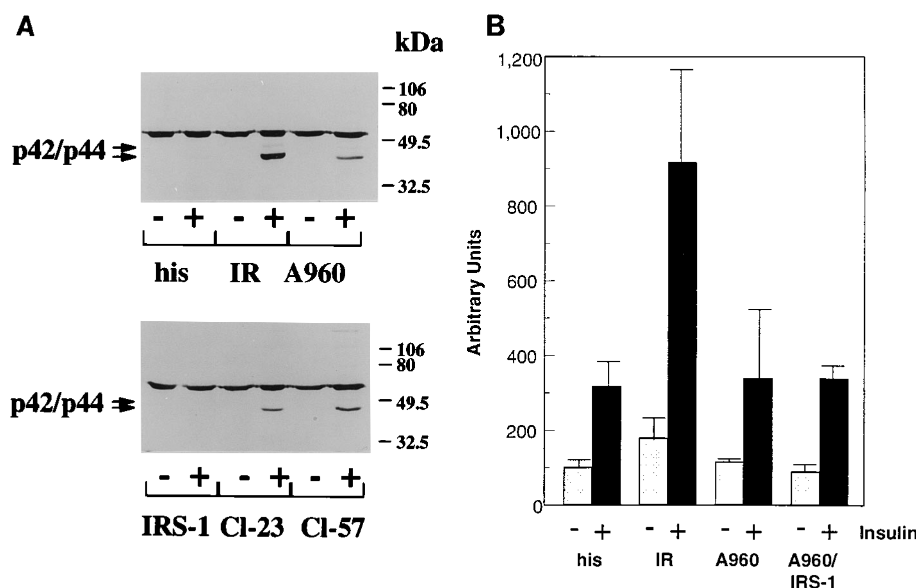

FIG. 4. Insulin stimulation of MAP kinase activity. (A) Anti-MAP kinase immunoprecipitates were prepared under denaturing conditions from quiescent or insulin-stimulated cells. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting with antiphosphotyrosine antibody. Bands were detected with horseradish peroxidase-labeled second antibody and ECL reagents. (B) Anti-MAP kinase immunoprecipitates were prepared from quiescent (open bars) or insulin-stimulated (solid bars) cells. In vitro kinase activity toward myelin basic protein was determined as described in the text. The data from two separate experiments were pooled, with the MAP kinase activity present in quiescent control cells (his) being used to normalize activities in different experiments. Basal activities are listed in Table 1.

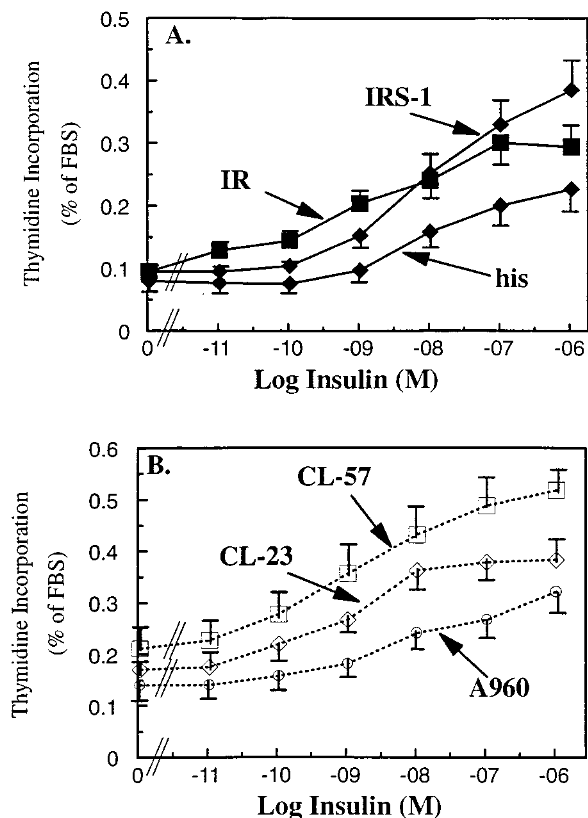

FIG. 5. Insulin stimulation of [^{3}H]thymidine incorporation into DNA. Quiescent CHO cells expressing insulin receptors and IRS-1 as indicated were stimulated with various concentrations of insulin or with 10% FBS for 15 h at 37°C. The incorporation of [^{3}H]thymidine during an additional 1-h incubation was measured as described in the text. All determinations were done in triplicate, and the data are means with standard errors of the means for four separate experiments. Data are plotted as fractions of the signal obtained with 10% FBS in each cell line. (A) his, IR, and IRS-1; (B) A960, Cl-23, and Cl-57. Basal and FBS-stimulated activities are listed in Table 1.

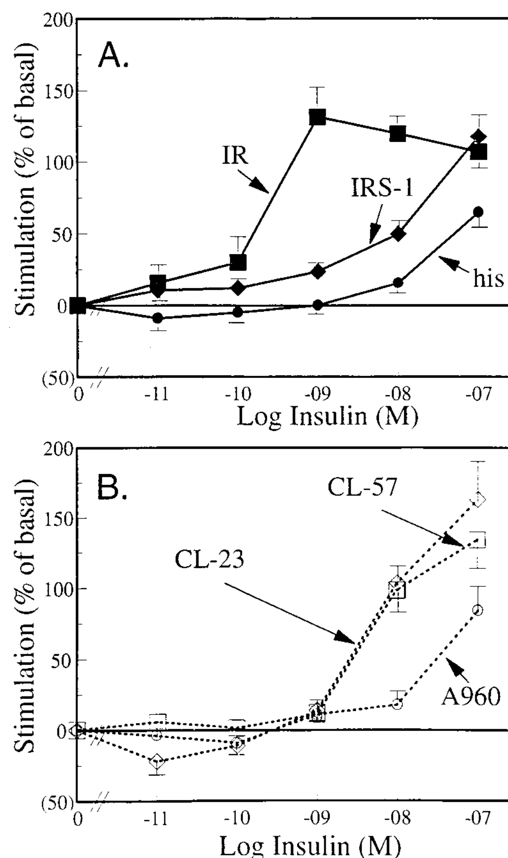

FIG. 6. Insulin stimulation of [^{14}C]glucose incorporation into glycogen. CHO cells expressing insulin receptors and IRS-1 as indicated were starved in 0.04% glucose for 3 h, stimulated with various concentrations of insulin for 30 min, and then incubated with [^{14}C]glucose in the presence of insulin for an additional 1 h. The incorporation of [^{14}C]glucose into glycogen was measured as described in the text. All determinations were done in triplicate, and the data are means with standard errors of the means for three separate experiments. Stimulation over basal activity is expressed as a percentage of basal glucose incorporation in each cell line. (A) his, IR, and IRS-1; (B) A960, Cl-23, and Cl-57. Basal activities are listed in Table 1.

(Fig. 5B). The sensitivities to insulin in Cl-23 (ED_{50}, 1.3 ± 0.2 nM) and Cl-57 (ED_{50}, 3.0 ± 0.6 nM) were similar to that in IR cells (ED_{50}, 1.0 ± 0.8 nM), and the magnitude of the response was increased in Cl-57. Thus, overexpression of IRS-1 restored insulin-stimulated mitogenic responses in cells expressing the mutant IR_{A960}.

Insulin stimulation of glycogen synthesis was examined by measuring the incorporation of [^{14}C]glucose into glycogen (Fig. 6; Table 1). Insulin weakly stimulated glycogen synthesis in the control his cells (ED_{50}, 17.7 ± 4.1 nM), whereas expression of wild-type insulin receptors increased the magnitude of the response nearly 2-fold and increased the sensitivity of the response nearly 50-fold (ED_{50}, 0.4 ± 0.1 nM) (Fig. 6A; Table 1). Overexpression of IRS-1 alone caused a similar increase in the magnitude of insulin-stimulated glycogen synthesis but had no effect on the sensitivity of the response (ED_{50}, 18.7 ± 5.7 nM) (Fig. 6A; Table 1). A960 cells were similar to the control cells (ED_{50}, 23.3 ± 1.4 nM), confirming our previous results (4, 49). Interestingly, overexpression of IRS-1 in the A960 cells (Cl-23 and Cl-57) showed an increase in the magnitude of insulin-stimulated glycogen synthesis which was greater than that in IRS-1 cells (Fig. 4B). The responses to insulin in both Cl-23 (ED_{50}, 5.3 ± 1.3 nM) and Cl-57 (ED_{50}, 4.1 ± 0.3 nM) were significantly more sensitive than that in control cells (ED_{50}, 17.7 ± 4.1 nM) but were still 10-fold less sensitive than that in the IR cells (ED_{50}, 0.4 ± 0.1 nM) (Table 1). Thus, overexpression of IRS-1 partially rescued insulin-stimulated glycogen synthesis in cells expressing the mutant IR_{A960}.

DISCUSSION

We have used a mutant insulin receptor, lacking a tyrosine phosphorylation site at Tyr-960 in the cytoplasmic juxtamembrane region of the receptor, to identify insulin-stimulated responses downstream from IRS-1. IR_{A960}s undergo normal insulin-stimulated autophosphorylation at sites in the regulatory and C-terminal domains of the insulin receptor, and partially purified receptors possess normal phosphotransferase activity toward peptide substrates (49) or recombinant IRS-1 (1a) in vitro. However, when expressed in CHO cells, the mutant receptor is defective for tyrosyl phosphorylation of IRS-1 and Shc and is unable to mediate insulin-stimulated biological responses (16, 24, 49). Tyr-960 is located in an amino acid motif, LxxxxNPxYxSxSD, which also appears to be required for phosphorylation of IRS-1 by the IGF-I receptor (Tyr-757) and the interleukin 4 receptor (Tyr-495) (17). Recent studies using the yeast two-hybrid system suggest that Tyr-960 is required for a specific interaction between the in-

sulin receptor and the amino terminus of IRS-1, and substitution of Tyr-960 with Phe disrupts this interaction (27). The reduced ability of IR_{A960} to mediate tyrosine phosphorylation of IRS-1 and Shc in intact cells may relate to a disruption of this enzyme-substrate interaction, effectively reducing the availability of substrate to the receptor. In agreement with this hypothesis, we find that overexpression of IRS-1 in A960 cells (Cl-23 and Cl-57) restores IRS-1 phosphorylation to levels similar to that in cells expressing wild-type receptors. We conclude that the NPXY motif of the insulin receptor β-subunit contributes to the sensitivity of interactions with IRS-1 but is not essential at high levels of substrate.

Restoration of IRS-1 tyrosyl phosphorylation in Cl-23 and Cl-57 rescues insulin stimulation of PI 3′-kinase. These data confirm our previous hypothesis that IRS-1 is the principal activator of PI 3′-kinase in insulin-stimulated CHO cells (2). Overexpression of IRS-1 in the A960 cells also rescues insulin-stimulated mitogenic responses, as the sensitivity of insulin-stimulated thymidine incorporation in these cells is similar to that in IR cells. These data are consistent with studies that have implicated IRS-1 in the regulation of cell growth by insulin (31, 40, 46, 47). It should be noted that overexpression of IRS-1 alone in CHO cells increases the magnitude of both insulin-stimulated PI 3′-kinase activity and incorporation of thymidine into DNA at high insulin concentrations (2, 40). However, overexpression of IRS-1 has little effect on the sensitivity of these insulin-stimulated responses, as IRS-1 cells possess only 3,000 endogenous insulin receptors per cell (2, 8, 40). Thus, the increased activation of PI 3′-kinase at low insulin levels (5 nM) and the increased sensitivity of insulin-stimulated mitogenesis in Cl-23 and Cl-57 arise from the phosphorylation of IRS-1 by the mutant IR_{A960}s.

IRS-1 possesses multiple tyrosine phosphorylation sites and during insulin stimulation interacts with various SH2 domain-containing proteins including Grb-2, SHPTP-2, Nck, and perhaps other factors that may be important for mitogenesis. Grb-2 is an SH2/SH3 adapter protein that associates with the guanine nucleotide exchange factor SOS to regulate $p21^{ras}$ GTP loading (23, 35). This signaling pathway provides one means of regulating ERK-1/ERK-2 through the activation of c-Raf or other serine kinases (10). Although Grb-2 binding to IRS-1 is increased by overexpression of IRS-1 in A960 cells, this does not lead to an increase in insulin-stimulated ERK activity. In contrast, expression of IRS-1 in 32-D myeloid progenitor cells significantly increases ERK activation in a manner that is dependent on the phosphorylation of the Tyr-895 Grb-2 binding site (26). It is not clear why the observed increase in IRS-1/Grb-2 binding in Cl-23 does not lead to increases in ERK activation. The precise pathway used by the insulin receptor to regulate the p42/p44 MAP kinases may vary between cells and depend on the relative competition between Shc and IRS-1 for Grb-2, as has been suggested by Yamauchi and Pessin (51). Alternatively, recent studies with insulin-stimulated adipocytes suggest that IRS-1 and $p21^{ras}$, the target of the Grb-2/SOS complex, may be in different subcellular compartments (20).

The inability of IRS-1 to rescue insulin-stimulated ERK activation points to Shc, and perhaps other signaling molecules, as an essential element in the effect of insulin on this signaling pathway. Tyrosyl phosphorylation of Shc and its binding to Grb-2 are both decreased in A960 cells (this study; 52) and are unaffected by overexpression of IRS-1. These data suggest that insulin-stimulated tyrosyl phosphorylation of Shc, and the subsequent formation of Shc/Grb-2 complexes, may be a major mechanism for insulin stimulation of the p42/p44 MAP kinase cascade in CHO cells. These findings are consistent with data for other cell types (11, 29, 33, 34). However, it is possible that IRS-1 might be coupled to ERK activation in combination with Shc and/or another signaling pathway that is not activated in the Cl-23 and Cl-57 cells.

Overexpression of IRS-1 in the A960 cells partially restores insulin stimulation of glycogen synthesis. The sensitivity of insulin-stimulated glycogen synthesis is increased four- to sixfold in Cl-23 and Cl-57 relative to A960 cells. However, the response is still 10-fold less sensitive than that in cell expressing wild-type receptors. These data suggest that tyrosyl phosphorylation of IRS-1 may play some role in this biological response to insulin but it cannot fully rescue the effects of the mutation at Tyr-960. If activation of ERK-1/ERK-2 is involved in the regulation of glycogen synthesis in CHO cells, as suggested for skeletal muscle (11), then a partial effect on glycogen synthesis is consistent with our results. Alternatively, it is important to note that, although overexpression of IRS-1 in A960 cells increases the net level of IRS-1 tyrosyl phosphorylation, the stoichiometry of IRS-1 phosphorylation may be lower than that in cells expressing the wild-type receptor. Thus, the simultaneous recruitment of multiple signaling molecules to the same molecule of IRS-1 is an important component of insulin signaling that may not be replicated by IRS-1 overexpression. Similarly, although the PI 3′-kinase and Grb-2 phosphorylation sites are utilized normally in the Cl-23 and Cl-57 cells, the partial rescue of glycogen synthesis could be due to decreased phosphorylation of some other specific phosphorylation site in IRS-1.

In summary, we have shown that overexpression of IRS-1 in CHO cells expressing the mutant IR_{A960} rescues insulin-stimulated mitogenic responses and activation of PI 3′-kinase. However, overexpression of IRS-1 does not restore insulin stimulation of the p42/p44 MAP kinases, despite increases in IRS-1 binding to Grb-2, and only partially restores insulin stimulation of glycogen synthesis. Thus, in CHO cells, ERK activation correlates best with Shc phosphorylation, whereas regulation of PI 3′-kinase, DNA synthesis, and, to a lesser extent, glycogen synthesis appear to be linked to IRS-1 phosphorylation. Our data support the idea that the full insulin response is a composite of multiple downstream elements with variable sensitivities.

ACKNOWLEDGMENTS

We thank Erin Glasheen for assistance in the transfections, Allison Wisk for contributions to several pilot experiments, and George Orr and Jeff Segal for helpful discussions.

This work was supported by grants to J.M.B. from the National Institutes of Health (DK-44541), the Juvenile Diabetes Foundation, and the Alexandrine and Alexander Sinsheimer Fund.

REFERENCES

1. **Araki, E., M. A. Lipes, M.-E. Patti, J. C. Bruning, B. Haag III, R. S. Johnson, and C. R. Kahn.** 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature (London) **372:**186–189.

1a. **Backer, J. M.** Unpublished observations.

2. **Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. The phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11:**3469–3479.

3. **Backer, J. M., M. G. Myers, Jr., X.-J. Sun, D. J. Chin, S. E. Shoelson, M. Miralpeix, and M. F. White.** 1993. Association of IRS-1 with the insulin receptor and the phosphatidylinositol 3′-kinase. Formation of binary and ternary signaling complexes in intact cells. J. Biol. Chem. **268:**8204–8212.

4. **Backer, J. M., G. G. Schroeder, D. A. Cahill, A. Ullrich, K. Siddle, and M. F. White.** 1991. The cytoplasmic juxtamembrane region of the insulin receptor: a critical role in ATP binding, endogenous substrate phosphorylation, and insulin-stimulated bioeffects in CHO cells. Biochemistry **300:**6366–6372.

5. **Backer, J. M., S. E. Shoelson, M. A. Weiss, Q. X. Hua, R. B. Cheatham, E. Haring, D. C. Cahill, and M. F. White.** 1992. The insulin receptor juxtamem-

brane region contains two independent tyrosine/b-turn internalization signals. J. Cell Biol. **118:**831–839.

6. **Baltensperger, K., L. M. Kozma, A. D. Cherniack, J. K. Klarlund, A. Chawla, U. Banerjee, and M. P. Czech.** 1993. Binding of the Ras activator Son of sevenless to insulin receptor substrate-1 signaling complexes. Science **260:** 1950–1952.
7. **Bollum, F. J.** 1968. Filter paper disk techniques for assaying radioactive macromolecules. Methods Enzymol. **12:**169–173.
8. **Chou, C. K., T. J. Dull, D. S. Russell, R. Gherzi, D. Lebwohl, A. Ullrich, and O. M. Rosen.** 1987. Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. J. Biol. Chem. **262:**1842–1847.
9. **Condorelli, G., R. Bueno, and R. J. Smith.** 1994. Two alternatively spliced forms of the human insulin-like growth factor I receptor have distinct biological activities and internalization kinetics. J. Biol. Chem. **269:**8510–8516.
10. **Davis, R. J.** 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. **268:**14553–14556.
11. **Dent, P., A. Lavoinne, S. Nakielny, F. B. Caudwell, P. Watt, and P. Cohen.** 1990. The molecular mechanisms by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature (London) **348:**302–307.
12. **Feener, E. P., J. M. Backer, G. L. King, P. A. Wilden, X. J. Sun, C. R. Kahn, and M. F. White.** 1993. Insulin stimulates serine and tyrosine phosphorylation in the juxtamembrane region of the insulin receptor. J. Biol. Chem. **268:**11256–11264.
13. **Freeman, R. M., J. Plutzky, and B. G. Neel.** 1992. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. Proc. Natl. Acad. Sci. USA **89:**11239–11243.
14. **Hofmann, C., M. F. White, and J. Whittaker.** 1989. Human insulin receptors expressed in insulin-insensitive mouse fibroblasts couple with existent cellular effector systems to confer insulin-sensitivity and responsiveness. Endocrinology **124:**257–264.
15. **Hubbard, S. R., L. Wei, L. Ellis, and W. A. Hendrickson.** 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature (London) **372:**746–754.
16. **Kaburagi, Y., K. Momomura, R. Yamamoto-Honda, K. Tobe, Y. Tamori, H. Sakura, Y. Akanuma, Y. Yazaki, and T. Kadowaki.** 1993. Site-directed mutagenesis of the juxtamembrane domain of the human insulin receptor. J. Biol. Chem. **268:**16610–16622.
17. **Keegan, A. D., K. Nelms, M. White, L.-M. Wang, J. H. Pierce, and W. E. Paul.** 1994. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. Cell **76:**811–820.
18. **Koch, A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252:**668–674.
19. **Kozma, L. M. K. Baltensperger, J. Klarlund, A. Porras, E. Santos, and M. P. Czech.** 1993. The Ras signaling pathway mimics insulin action on glucose transporter translocation. Proc. Natl. Acad. Sci. USA **90:**4460–4464.
20. **Kozma, L. M., and M. P. Czech.** 1994. Divergent cellular localization pathways for IRS-1 versus Shc in adipocytes. Diabetes **43**(Suppl. 1)**:**2A. (Abstract.)
21. **Lee, C.-H., W. Li, R. Nishimura, M. Zhou, A. G. Batzer, M. G. Myers, Jr., M. F. White, J. Schlessinger, and E. Y. Skolnik.** 1993. Nck associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells. Proc. Natl. Acad. Sci. USA **90:**11713–11717.
22. **McClain, D. A., H. Maegawa, J. Lee, T. J. Dull, A. Ullrich, and J. M. Olefsky.** 1987. A mutant insulin receptor with defective tyrosine kinase displays no biological activity and does not undergo endocytosis. J. Biol. Chem. **262:** 14663–14671.
23. **McCormick, F.** 1993. Signal transduction: how receptors turn Ras on. Nature (London) **363:**15–16.
24. **Murakami, M. S., and O. R. Rosen.** 1991. The role of insulin receptor autophosphorylation in signal transduction. J. Biol. Chem. **266:**22653–22660.
25. **Myers, M. G., Jr., X. J. Sun, B. Cheatham, B. R. Jachna, E. M. Glasheen, J. M. Backer, and M. F. White.** 1993. IRS-1 is a common element in insulin and IGF-1 signaling to the phosphatidylinositol 3′-kinase. Endocrinology **132:**1421–1430.
26. **Myers, M. G., Jr., L.-M. Wang, X. J. Sun, Y. Zhang, L. Yenush, J. Schlessinger, J. H. Pierce, and M. F. White.** 1994. Role of IRS-1–GRB-2 complexes in insulin signaling. Mol. Cell. Biol. **14:**3577–3587.
27. **O'Neill, T. J., A. Craparo, and T. A. Gustafson.** 1994. Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. Mol. Cell. Biol. **14:**6433–6442.
28. **Pronk, G. J., A. M. M. de Vries-Smits, L. Buday, J. Downward, J. A. Maassen, R. H. Medema, and J. L. Bos.** 1994. Involvement of Shc in insulin- and epidermal growth factor-induced activation of p21*ras*. Mol. Cell. Biol. **14:** 1575–1581.
29. **Pruett, W., Y. Yuan, E. Rose, A. G. Batzer, N. Harada, and E. Y. Skolnik.** 1995. Association between GRB2/Sos and insulin receptor substrate 1 is not sufficient for activation of extracellular signal-regulated kinases by interleukin-4: implications for Ras activation by insulin. Mol. Cell. Biol. **15:**1778–1785.
30. **Quon, M. J., A. J. Butte, M. J. Zarnowski, G. Sesti, S. W. Cushman, and S. I. Taylor.** 1994. Insulin receptor substrate 1 mediates the stimulatory effect of insulin on GLUT-4 translocation in transfected rat adipose cells. J. Biol. Chem. **269:**27920–27924.
31. **Rose, D. W., A. R. Saltiel, M. Majumdar, S. J. Decker, and J. M. Olefsky.** 1994. Insulin receptor substrate 1 is required for insulin-mediated mitogenic signal transduction. Proc. Natl. Acad. Sci. USA **91:**797–801.
32. **Ruderman, N., R. Kapeller, M. F. White, and L. C. Cantley.** 1990. Activation of phosphatidylinositol-3-kinase by insulin. Proc. Natl. Acad. Sci. USA **87:** 1411–1415.
33. **Sasaoka, T., B. Draznin, J. W. Leitner, W. J. Langlois, and J. M. Olefsky.** 1994. Shc is the predominant signaling molecule coupling insulin receptors to activation of guanine nucleotide releasing factor and p21*ras*-GTP formation. J. Biol. Chem. **269:**10734–10738.
34. **Sasaoka, T., D. W. Rose, B. H. Jhun, A. R. Saltiel, B. Draznin, and J. M. Olefsky.** 1994. Evidence for a functional role of Shc proteins in mitogenic signaling induced by insulin, insulin-like growth factor-1, and epidermal growth factor. J. Biol. Chem. **269:**13689–13694.
35. **Schlessinger, J.** 1993. How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. **18:**273–275.
36. **Skolnik, E. Y., A. Batzer, N. Li, C.-H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis, and J. Schlessinger.** 1993. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science **260:**1953–1955.
37. **Skolnik, E. Y., C.-H. Lee, A. Batzer, L. M. Vicentini, M. Zhou, R. Daly, M. J. Myers, Jr., J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger.** 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of *ras* signalling. EMBO J. **12:**1929–1936.
38. **Souza, S. C., G. P. Frick, R. Yip, R. B. Lobo, L.-R. Tai, and H. M. Goodman.** 1994. Growth hormone stimulates tyrosine phosphorylation of insulin receptor substrate-1. J. Biol. Chem. **269:**30085–30088.
39. **Sun, X. J., D. L. Crimmins, M. G. Myers, Jr., M. Miralpeix, and M. F. White.** 1993. Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol. Cell. Biol. **13:**7418–7428.
40. **Sun, X. J., M. Miralpeix, M. G. Myers, Jr., E. M. Glasheen, J. M. Backer, C. R. Kahn, and M. F. White.** 1992. The expression and function of IRS-1 in insulin signal transmission. J. Biol. Chem. **267:**22662–22672.
41. **Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, B. J. Goldstein, and M. F. White.** 1991. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London) **352:**73–77.
42. **Tamemoto, H., T. Kadowaki, K. Tobe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, H. Sekihara, S. Yoshioka, H. Horikoshi, Y. Furuta, Y. Ikawa, M. Kasuga, Y. Yazaki, and S. Aizawa.** 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature (London) **372:**182–186.
43. **Thomas, J. A., K. K. Schlender, and J. Larner.** 1968. A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. Anal. Biochem. **25:**486–499.
44. **Ullrich, A., J. R. Bell, E. Y. Chen, R. Herrera, L. M. Petruzzeli, T. J. Dull, A. Gray, L. Coussens, Y.-C. Liao, M. Tsubokawa, A. Mason, P. H. Seeburg, C. Grunfeld, O. M. Rosen, and J. Ramachandran.** 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature (London) **313:**756–761.
45. **Wang, L.-M., A. D. Keegan, W. Li, G. E. Lienhard, S. Pacini, J. S. Gutkind, M. G. Myers, Jr., X.-J. Sun, M. F. White, S. A. Aaronson, W. E. Paul, and J. H. Pierce.** 1993. Common elements in interleukin 4 and insulin signaling pathways in factor-dependent hematopoietic cells. Proc. Natl. Acad. Sci. USA **90:**4032–4036.
46. **Wang, L.-M., M. G. Myers, Jr., X.-J. Sun, S. A. Aaronson, M. F. White, and J. H. Pierce.** 1993. IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. Science **261:**1591–1594.
47. **Waters, S. B., K. Yamauchi, and J. E. Pessin.** 1993. Functional expression of insulin receptor substrate-1 is required for insulin-stimulated mitogenic signaling. J. Biol. Chem. **268:**22231–22234.
48. **White, M. F., and C. R. Kahn.** 1994. The insulin signaling system. J. Biol. Chem. **269:**1–4.
49. **White, M. F., J. N. Livingston, J. M. Backer, V. Lauris, T. J. Dull, A. Ullrich, and C. R. Kahn.** 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell **54:**641–649.
50. **Wilden, P. A., and C. R. Kahn.** 1994. The level of insulin receptor tyrosine kinase activity modulates the activities of phosphatidylinositol 3-kinase, microtubule-associated protein, and S6 kinases. Mol. Endocrinol. **8:**558–567.
51. **Yamauchi, K., and J. E. Pessin.** 1994. Enhancement or inhibition of insulin signaling by insulin receptor substrate 1 is cell context dependent. Mol. Cell. Biol. **14:**4427–4434.
52. **Yonezawa, K., A. Ando, Y. Kaburagi, R. Yamamoto-Honda, T. Kitamura, K. Hara, M. Nakafuku, Y. Okabayashi, T. Kadowaki, Y. Kaziro, and M. Kasuga.** 1994. Signal transduction pathways from insulin receptors to Ras. Analysis by mutant insulin receptors. J. Biol. Chem. **269:**4634–4640.