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

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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 p21WAF1. 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 tyrosines 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 NPXY960 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 (IR960A) 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
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 were grown in six-well dishes (approximately 1.2 × 10⁶ 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 reagent (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).

PI3-kinase assays. CHO cells were grown to 80% confluence in 10-cm dishes. Quiescent cells (approximately 8 × 10⁶ per dish) were serum starved for 8 h in serum-free DMEM containing 1% glucose, washed, and solubilized. PI3-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), PI3-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 (approximately 1.2 × 10⁶ 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 (Abcam). 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 × 10⁶ 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 × 10⁶ cells per dish) for 5 min in the presence or absence of 100 nM insulin for 5 min and then rapid chilling and lysis in cold 10 mM KPO₂ (pH 7.0) containing 1 mM EDTA, 0.5% Nonidet P-40, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM diithiothreitol, 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 [32P]phosphate into myelin basic protein was quantitated on a Molecular Dynamics Phosphorimager.

Incorporation of [3H]thymidine into DNA. Thymidine incorporation was measured as described (7, 14, 49). CHO cells were plated at low density in 24-well plates and incubated without serum for 3 days. The cells (approximately 10⁶ 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 (ED₅₀) are presented as means and standard errors of the means of ED₅₀s from individual experiments and are approximate in that several of the curves are not maximal at 100 nM insulin.

Incorporation of [14C]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 10⁶ 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 μM of [14C]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% Na₂SO₄ 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. ED₅₀s are presented as means and standard errors of the means of ED₅₀s 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 IR₉₆₀. 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 tyrosine 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 (10⁶ receptors per cell) increased tyrosine phosphorylation of endogenous IRS-1 nearly fourfold during insulin stimulation.

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 secondary antibody and ECL reagents.

SAFETY PRECAUTIONS

We used standard safety precautions for handling chemicals and biological samples. All work was conducted in a biosafety cabinet, and chemical spills were cleaned up with appropriate absorbents. Biological samples were handled with appropriate personal protective equipment (gloves, laboratory coats, and masks).
TABLE 1. IRS-1 expression and insulin-stimulated biological responses in CHO cells expressing wild-type or mutant insulin receptors

<table>
<thead>
<tr>
<th>Cells</th>
<th>IRS-1</th>
<th>% Activity (basal)</th>
<th>[% Tyrosyl phosphorylation]</th>
<th>[% Expression]</th>
<th>PI 3'-kinase</th>
<th>MAP kinase</th>
<th>[1H]thymidine incorporation</th>
<th>Glycogen synthesisa,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>his</td>
<td>ND</td>
<td>100</td>
<td>100 ± 5</td>
<td>100 ± 19</td>
<td>100 ± 2</td>
<td>1.613 ± 224</td>
<td>11.25 ± 1.3</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>IR</td>
<td>ND</td>
<td>414</td>
<td>145 ± 33</td>
<td>178 ± 55</td>
<td>159 ± 12</td>
<td>1.964 ± 332</td>
<td>0.98 ± 0.8</td>
<td>91 ± 15</td>
</tr>
<tr>
<td>IRS-1</td>
<td>100</td>
<td>190</td>
<td>157 ± 27</td>
<td>ND</td>
<td>107 ± 4</td>
<td>1.612 ± 456</td>
<td>7.0 ± 0.8</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>A960</td>
<td>ND</td>
<td>59</td>
<td>139 ± 20</td>
<td>115 ± 13</td>
<td>78 ± 6</td>
<td>770 ± 121</td>
<td>10.0 ± 1.6</td>
<td>119 ± 13</td>
</tr>
<tr>
<td>Cl-23</td>
<td>254</td>
<td>593</td>
<td>245 ± 28</td>
<td>89 ± 8</td>
<td>65 ± 8</td>
<td>580 ± 163</td>
<td>1.3 ± 0.2</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>Cl-57</td>
<td>1,656</td>
<td>796</td>
<td>385 ± 13</td>
<td>ND</td>
<td>63 ± 7</td>
<td>611 ± 202</td>
<td>3.0 ± 0.6</td>
<td>78 ± 5</td>
</tr>
</tbody>
</table>

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.

aND, not determined.

*b[3H]glucose incorporation.

**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.

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.

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.
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 p21ras 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 [3H]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 ED50s 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 (ED50, 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 ED50, 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 ED50, 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. 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 [14C]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} 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 substitu-
tion of Tyr-960 with Phe disrupts this interaction (27). The
reduced activity of IR<sub>A960</sub> to mediate tyrosine phosphoryla-
tion 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 an A960 cells
(Cl-23 and Cl-57) restores IRS-1 phosphorylation to levels
similar to that in cells expressing wild-type receptors. We con-
clude 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 insulinsti-
mulated 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 sen-
titivity 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 insu-
lin levels (5 nM) and the increased sensitivity of insulin-stim-
mulated mitogenesis in Cl-23 and Cl-57 arise from the phos-
phorylation of IRS-1 by the mutant IR<sub>A960</sub>

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 per-
haps 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<sub>ras</sub>
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 pro-
genator 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 re-
cepotor 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-stimu-
lated adipocytes suggest that IRS-1 and p21<sub>ras</sub>, the target of
the Grb-2/SOS complex, may be in different subcellular com-
partments (20).

The inability of IRS-1 to rescue insulin-stimulated ERK
activation points to Shc, and perhaps other signaling mole-
cules, as an essential element in the effect of insulin on this
signaling pathway. Tyrosyl phosphorylation of Shc and its bind-
ing 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
flow of 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 six-
fold 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 phos-
phorylation 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 in-
creases 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 simulta-
neous recruitment of multiple signaling molecules to the same
molecule of IRS-1 is an important component of insulin sig-
naling that may not be replicated by IRS-1 overexpression.
Similarly, although the PI 3'-kinase and Grb-2 phosphoryla-
sion 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<sub>A960</sub> rescues insulin-stimu-
lated 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 phos-
phorylation. Our data support the idea that the full insulin
response is a composite of multiple downstream elements with
variable sensitivities.

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