Mutation of Tyrosine 960 within the Insulin Receptor Juxtamembrane Domain Impairs Glucose Transport but Does Not Inhibit Ligand-mediated Phosphorylation of Insulin Receptor Substrate-2 in 3T3-L1 Adipocytes*

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CSF-1 is equipotent to insulin in its ability to stimulate 2-[3H]deoxyglucose uptake in 3T3-L1 adipocytes expressing the colony stimulating factor-1 receptor/insulin receptor chimera (CSF1R/IR). However, CSF-1-stimulated glucose uptake and glycogen synthesis is reduced by 50% in comparison to insulin in 3T3-L1 cells expressing a CSF1R/IR mutant at Tyr960 (CSF1R/IRA960). CSF-1-treated adipocytes expressing the CSF1R/IRA960 were impaired in their ability to phosphorylate insulin receptor substrate 1 (IRS-1) but not in their ability to phosphorylate IRS-2. Immunoprecipitation of IRS proteins followed by Western blotting revealed that the intact CSF1R/IR co-precipitates with IRS-2 from CSF-1-treated cells. In contrast, the CSF1R/IRA960 co-precipitates poorly with IRS-2. These observations suggest that Tyr960 is important for interaction of the insulin receptor cytoplasmic domain with IRS-2, but it is not essential to the ability of the insulin receptor tyrosine kinase to use IRS-2 as a substrate. These observations also suggest that in 3T3-L1 adipocytes, tyrosine phosphorylation of IRS-2 by the insulin receptor tyrosine kinase is not sufficient for maximal stimulation of receptor-regulated glucose transport or glycogen synthesis.

A complete understanding of insulin action requires the identification of the intracellular pathways that regulate insulin-stimulated growth, development, and metabolism. Molecular analysis of structures within the insulin receptor cytoplasmic domain that are mediators of insulin-stimulated signal transduction pathways has provided one strategy for the study of insulin-sensitive intracellular signaling. The usual approach has been to create mutations within specific receptor sequences that result in the selective disruption of some insulin-regulated pathways, while leaving others intact. Deletion mutagenesis of the insulin receptor cytoplasmic domain has generated insulin receptors with altered biological properties (1–4) and led to the suggestion that different regions of the insulin receptor cytoplasmic domain play distinct roles in modulating the biological effects of insulin. Most mutations of the insulin receptor cytoplasmic domain that altered autophosphorylation sites within the cytoplasmic domain (5–8) furthered the notion that tyrosine phosphorylation and the tyrosine kinase encoded within the receptor β subunit are essential components of normal insulin action. Previously, structure/function analysis required the introduction of mutated insulin receptors into cells expressing low levels of endogenous insulin receptors, e.g. Chinese hamster ovary or Rat-1 fibroblast cell lines (for a review, see Ref. 9) to minimize the background signal generated by insulin activation of endogenous receptors. However, the relative insensitivity of fibroblast cell lines to insulin treatment has made them less than ideal models for studying the action of insulin on intermediary metabolism.

Conversely, insulin-responsive cell types are excellent systems in which to study insulin action, but the fact that they express large numbers of endogenous insulin receptors is an impediment to the expression and analysis of mutated recombinant insulin receptors. To circumvent this concern, the extracellular ligand binding domain of the human CSF-11 receptor (10) was spliced to the transmembrane and cytoplasmic domains of the human insulin receptor (11–13) to construct a chimeric receptor (CSF1R/IR). Recently, we showed that the CSF1R/IR has CSF-1-dependent enzymatic and biological properties expected of the insulin receptor tyrosine kinase in insulin-responsive cells (14). Deletion of 12 amino acids within the juxtamembrane domain of the CSF1R/IR inhibits CSF-1-stimulated phosphorylation of IRS-1 and Shc (14) and blocks CSF1R/IR-mediated glucose uptake (15) but does not block the ability of the chimeric receptor to stimulate the differentiation of 3T3-L1 cells into adipocytes (14) or promote cell survival in the presence of an apoptotic stimulus (15). In the experiments presented here we examined the signaling properties of a CSF1R/IR construct bearing a single mutation of the tyrosine at the position corresponding to Tyr960 of the normal human insulin receptor to alanine. Our data demonstrate that the Ala960 mutation partially inhibits the ability of the CSF1R/IR

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The abbreviations used are: CSF-1, colony stimulating factor-1; CSF1R/IR, colony stimulating factor-1 receptor/insulin receptor chimera; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; DMEM, Dulbecco’s modified Eagle medium; GLUT4, insulin-responsive isofrom of the glucose transporter; PI, phosphatidylinositol.
to stimulate glucose uptake and blocks the ability of the chimeric receptor to stimulate the phosphorylation of IRS-1 but not IRS-2. Despite the ability of the CSF1R/IR to phosphorylate IRS-2, co-precipitation studies indicate that the Ala^690 mutation decreases the ability of the insulin receptor cytoplasmic domain to interact with IRS-2.

**EXPERIMENTAL PROCEDURES**

3T3-L1 cells expressing the CSF1R/IR were described previously (14). Antibodies Ca-1 and CT-1 directed against the cytoplasmic domain of the human insulin receptor (17) were a gift from Ken Siddle, Cambridge. Antibodies to CSF1R were a gift from L. sweet (18). Antibodies to IRS-2 were derived from Dr. S. Cohen (19), and antibodies to IRS-1 were the product of Transduction Laboratories. Dulbecco’s modified Eagle medium (DMEM), penicillin, streptomycin, and neomycin antibiotics, G418, restriction endonucleases and LipofectAMINE were purchased from Life Technologies, Inc. Fetal bovine serum was purchased from Atlanta Biologicals. Peritoneal macrophages were from Calbiochem. Dexamethasone and 3-isobutyl-1-methylxanthine were from Sigma. Human recombinant CSF-1 was a gift from Genetics Institute.

**Construction and Expression of the CSF1R/IR and the CSF1R/IRA960—Construction of the CSF1R/IR and the CSF1R/IRA960**

The CSF1R/IR chimera containing an alanine substitution for Tyr^252 in the cytoplasmic domain of the human insulin receptor (20) was constructed by replacing the SpeI/BglII fragment from the CSF1R/IR with the corresponding fragment from pSGHIRcA960 (19). Expression of the CSF1R/IR and the CSF1R/IRA960 cDNAs was accomplished by subcloning the chimeric cDNAs into the vector pEF-1 (14). Transfection of 3T3-L1 preadipocytes was performed with LipofectAMINE according to the manufacturer’s instructions. Cells expressing the CSF1R/IR were selected with 800 μg/ml G418 and isolated by flow cytometry with a monoclonal antibody against the extracellular domain of the human CSF-1 receptor (20) (Oncogene Science) and fluorescent secondary antibody. Flow cytometry was performed by the University of Nebraska Medical Center Flow Cytometry Core Laboratory.

**Cell Culture—3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. All cells were incubated at 37 °C in 5% CO₂. Where indicated, 2 mM glutamine. Cells were washed in glycogen assay buffer (140 ml of 0.1M NaOH. Carrier glycogen (1 mg) was added, and the samples were boiled for 30 min. Glycogen was precipitated with 70% ethanol overnight at −20 °C. Each sample was centrifuged at 2100 × g, washed once with 70% ethanol, and resuspended in water. The amount of radioactivity incorporated into glycogen was determined by scintillation counting.

**Immunoprecipitation and Western Blot Analysis—Quiescent cells were stimulated with 10 nm CSF-1 for 10 min or left untreated, washed once with ice-cold phosphate-buffered saline containing 0.1 mm sodium orthovanadate, and lysed in buffer (50 mM HEPES, pH 7.8, 1% Triton X-100, 10 mM NaF, 2 mM Na₃VO₄, 10 mM sodium inorganic pyrophosphate, 5 mM EDTA, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation (14). The pellet was resuspended in 100 μl of 10 mM insulin for 2 min. The media was aspirated, and the cell monolayers were washed once with ice-cold phosphate-buffered saline containing 0.1 mm Na₃VO₄. The proteins were solubilized for 10 min in lysis buffer (40 mM Tris-HCl, pH 7.4, 2 mM Na₃VO₄, 20 mM NaF, 10 mM sodium inorganic pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin) containing 1% Triton X-100. Cell lysates containing equal amounts of protein were incubated with the indicated antibodies for 2 h at 4 °C. Protein G-Sepharose (25 μl) was added to each immune complex, and incubation was continued for 2 h at 4 °C. The immunoprecipitates were washed twice with each of the following buffers: (a) phosphate-buffered saline, pH 7.4, containing 1% Triton X-100, (b) 100 mM Tris, pH 7.4, 0.5 mM LiCl, 0.1 mM Na₃VO₄, and (c) 50 mM HEPES, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.1 mM Na₃VO₄. The pellets were resuspended in 30 μl of 30 mM HEPES, 30 mM MgCl₂, 0.4 mM EGTA. The kinase reactions were started by the addition of 15 μl of sonicated substrate (0.6 mg/ml PI in 30 mM HEPES and 0.4 mM EGTA) and 10 μCi of [γ⁻²³¹]ATP (6000 Ci/mmol/tube). After mixing for 10 min at room temperature, the reactions were stopped by the addition of 14 μl of 9 γ/ml HCl and then extracted once with methanol:chloroform (1:1). The samples were briefly separated by centrifugation, and the lower organic phase (20 μl) was spotted on silica gel 60A plates (Merck) that had been pretreated with 1% potassium oxalate at 100 °C. The plates were developed in chloroform/acetone/methanol/acetic acid/water (35:15:12:8), dried, and detected by autoradiography. Phosphorylated PI was quantified with a Molecular Dynamics densitometer.

**RESULTS**

**Tyrosine Phosphorylation of the CSF1R/IR and CSF1R/IRA960 Chimeras—Quiescent 3T3-L1 cells were incubated in the presence or absence of CSF-1, and aliquots of cell lysates containing equal amounts of protein were immunoprecipitated with Ca-1 antibody directed against the cytoplasmic domain of the insulin receptor. Treatment of 3T3-L1 preadipocytes expressing the CSF1R/IR chimera with CSF-1 for 10 min resulted in the tyrosine phosphorylation of the 170-kDa chimeric receptor protein (Fig. 1, 2nd lane). The same treatment of 3T3-L1 cells expressing the CSF1R/IRA960 chimera resulted in a slightly reduced extent of the tyrosine phosphorylation of the mutant receptor (Fig. 1, 4th lane) compared with the intact chimeric receptor, which is consistent with prior studies (8). The intact and mutated chimeras were expressed equally, as determined by Western blot with Ca-1 antibody (Fig. 1). The same effect of CSF-1 on phosphorylation of the CSF1R/IR and CSF1R/IRA960 was observed in fully differentiated 3T3-L1 adipocytes (data not shown).**

**CSF-1 Stimulates Glucose Uptake in 3T3-L1 Adipocytes Ex-
pressing the CSF1R/IR—Differentiation of 3T3-L1 preadipocytes into adipocytes results in an increase in expression of the GLUT4 and in an increase in insulin-stimulated glucose transport (23, 24). 3T3-L1 adipocytes or 3T3-L1 adipocytes expressing CSF1R/IR were treated for 20 min with a maximally effective insulin concentration (100 nM) or with CSF-1 at the indicated concentrations before the addition of 2-[^3H]deoxyglucose (Fig. 2). After 10 min, the 2[^3H]deoxyglucose uptake was measured by scintillation counting. Insulin stimulated an 8–10-fold increase in 2[^3H]deoxyglucose uptake in 3T3-L1 adipocytes (Fig. 2). Tyrosine autophosphorylation of the CSF1R/IR is maximal at 10 nM and half-maximal at 1 nM CSF-1 (14). 3T3-L1 adipocytes expressing the CSF1R/IR were able to increase 2[^3H]deoxyglucose uptake approximately 7.5-fold in response to 10 nM CSF-1 and to a slightly lesser extent when 1 nM CSF-1 was used (Fig. 2). In contrast, 10 nM CSF-1 had no effect on glucose uptake in 3T3-L1 control adipocytes (Fig. 2). These data suggest that physiological concentrations CSF-1 are able to activate the CSF1R/IR and stimulate glucose uptake in 3T3-L1 adipocytes.

Effect of the CSF1R/IRA960 on Glucose Transport and Glycogen Synthesis—Tyr^{960} in the insulin receptor juxtamembrane domain is essential for the ability of the receptor kinase to phosphorylate IRS-1 and couple to downstream effectors (1, 25). The tyrosine in the CSF1R/IR corresponding to Tyr^{960} in the insulin receptor was changed to alanine, and the resulting CSF1R/IRA960 chimera was tested for its ability to stimulate glucose uptake upon stimulation with different doses of CSF-1. CSF-1 was equipotent to insulin in its ability to stimulate maximal glucose uptake in 3T3-L1 adipocytes expressing the CSF1R/IR (Fig. 3A). At submaximal concentrations, CSF-1 appears more potent than insulin in its ability to stimulate glucose uptake and glycogen synthesis (Fig. 3). This may result from the fact that the expression of CSF1R/IR is slightly higher than endogenous insulin receptors in the transfected 3T3-L1 adipocytes (data not shown). In adipocytes expressing the CSF1R/IRA960, glucose uptake in response to 100 nM CSF-1 was no greater than that observed when the same cells were treated with 1 nM insulin (Fig. 3A). However, glucose uptake stimulated by 1 nM and 10 nM CSF-1 was unaffected by the Ala^{960} mutation (Fig. 3A).

To determine whether the mutation of Tyr^{960} inhibited only the ability of the chimera to stimulate maximal glucose uptake, we examined glycogen synthesis in 3T3-L1 adipocytes expressing CSF1R/IR and CSF1R/IRA960. In cells expressing the CSF1R/IR, glycogen synthesis stimulated by CSF-1 resembled the dose-dependent increases in synthesis observed with insulin (Fig. 3B). As seen with glucose uptake, however, CSF-1 was unable to stimulate maximal glycogen synthesis in cells expressing the CSF1R/IRA960 above the level of synthesis observed with 1 nM insulin (Fig. 3B).

CSF-1-stimulated Tyrosine Phosphorylation of IRS-1 and IRS-2 in 3T3-L1 Adipocytes Expressing the CSF1R/IR and CSF1R/IRA960—The ability of the Ala^{960} mutation to blunt but not abolish CSF-1-stimulated glucose uptake and glycogen synthesis in adipocytes expressing the CSF1R/IRA960 indicated that the juxtamembrane mutation did not completely uncouple the CSF1R/IR from downstream effectors that regulate these biological activities. IRS-1 is readily detectable in adipocytes (26) permitting examination of the ability of the CSF1R/IR and CSF1R/IRA960 to tyrosine-phosphorylate this endogenous substrate of the insulin receptor kinase. Cells were left untreated or treated with CSF-1 and lysed, and IRS-1 was immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine antibodies. CSF-1 stimulated tyrosine phosphorylation of IRS-1 in adipocytes expressing the intact CSF1R/IR (Fig. 4). Consistent with previous studies (27), substitution of Tyr^{960} with alanine-blocked CSF-1 stimulated tyrosine phosphorylation of IRS-1 in adipocytes expressing the CSF1R/IRA960 (Fig. 4). Stripping and reprobing of the blot also revealed comparable levels of expression of IRS-2 in each immunoprecipitate from each cell line (Fig. 4, middle panel). In contrast, CSF-1-treated 3T3-L1 adipocytes expressing the CSF1R/IRA960 were comparable with 3T3-L1 adipocytes expressing the CSF1R/IR in their ability to phosphorylate IRS-2 (Fig. 4). Stripping and reprobing of the blot also revealed comparable levels of expression of IRS-2 in each immunoprecipitate from each cell line (Fig. 4).

Interaction of IRS-2 with the CSF1R/IR and the CSF1R/ IRA960—A second tyrosine-phosphorylated protein co-precipitated with IRS-2 from CSF-1-treated cells (Fig. 4). To determine the identity of this protein, the CSF1R/IR or the CSF1R/
IRA960 were left untreated or treated with 10 nM CSF-1, and lysates were immunoprecipitated with antibodies to IRS-2 or to the carboxyl terminus of the insulin receptor cytoplasmic domain (CT-1). IRS-2 was phosphorylated in CSF-1-treated cells expressing the CSF1R/IR or the CSF1R/IRA960. However, the second tyrosine-phosphorylated protein co-precipitated with IRS-2 in cells expressing the CSF1R/IR but did not co-precipitate with IRS-2 in cells expressing the CSF1R/IRA960 (Fig. 5). Reprobing of the blots with CT-1 antibody revealed that the CSF1R/IR, but not the CSF1R/IRA960, coprecipitated with phosphorylated IRS-2 protein.

CSF-1-stimulated Activation of PI 3' Kinase in 3T3-L1 Adipocytes Expressing the CSF1R/IR or the CSF1R/IRA960—PI 3’ kinase is activated by the insulin receptor via association with tyrosine-phosphorylated intermediate proteins including IRS-1 (28). To further investigate the role of the juxtamembrane Tyr960 in downstream signaling by the insulin receptor, we examined the ability of the CSF1R/IRA960 to activate PI 3' kinase associated with IRS-1 and IRS-2 or with tyrosine-phosphorylated proteins. 3T3-L1 adipocytes expressing the CSF1R/IR or the CSF1R/IRA960 were serum-deprived for 5 h and then treated with 10 nM CSF-1 or with 100 nM insulin for 2 min. The cells were lysed and immunoprecipitated with antibodies to IRS-1, IRS-2, or phosphotyrosine. In adipocytes expressing the CSF1R/IR, insulin and CSF-1 stimulated PI 3' kinase activity in anti-IRS-1 immunoprecipitates as well as in anti-IRS-1 or anti-IRS-2 immunoprecipitates (Fig. 6). In contrast, CSF-1 was unable to stimulate PI 3' kinase in anti-IRS-1 immunoprecipitates from adipocytes expressing the CSF1R/IRA960 (Fig. 6). CSF-1 stimulated PI 3' kinase activity in anti-IRS-2 and anti-phosphotyrosine immunoprecipitates from adipocytes expressing the CSF1R/IRA960 (Fig. 6), consistent with its ability to stimulate the phosphorylation of IRS-2.

**DISCUSSION**

These data demonstrate that the juxtamembrane phosphorylation site Tyr960 within the insulin receptor cytoplasmic domain is an essential determinant for the tyrosine phosphorylation of IRS-1 but not for the phosphorylation of IRS-2 by the insulin receptor kinase (Fig. 4). Phosphorylation of IRS-2 by the CSF1R/IRA960 is functional as it stimulates the activation of PI 3' kinase bound to IRS-2 (Fig. 6). The Ala960 mutation blocks the ability of the chimeric CSF1R/IR to maximally stimulate glucose uptake and glycogen synthesis but does not impair stimulation of glucose transport by submaximal amounts of CSF-1 (Fig. 3). If IRS-2 is capable of mediating insulin-stimulated GLUT4 translocation (29), then these data suggest that interaction of IRS-2 with the insulin receptor juxtamembrane domain may not be a required event to induce glucose uptake. The ability of the CSF1R/IRA960 to phosphorylate IRS-2 and to stimulate glucose transport may explain why ectopic expression of IRS-1 domains that interact with the insulin receptor juxtamembrane domain were able to block IRS-1 (28).
insulin-stimulated mitogenic effects mediated by IRS-1 but did not inhibit insulin-stimulated GLUT 4 translocation or glucose transport (30, 31). The data presented here demonstrate that competitive inhibition of interaction with the juxtamembrane domain is not required for tyrosine phosphorylation of IRS-2. These data also suggest an important but not exclusive role for Tyr960 and IRS-1 in signaling by the insulin receptor kinase (14, 15, 30, 31). The conclusion that other mechanisms, in addition to the phosphorylation of IRS-1, specifically contribute to insulin-stimulated glucose uptake is consistent with several forms of evidence. Microinjection of antibodies and peptides or the exogenous expression of IRS-1 domains disrupt protein interaction with the insulin receptor juxtamembrane region (30, 31). IRS-1−/− mice show mild resistance to insulin and impaired glucose tolerance (32, 39). Adipocytes isolated from IRS-1−/− mice are impaired but not completely resistant to insulin-stimulated glucose uptake (33). IRS-2−/− mice also demonstrate peripheral insulin resistance, but the severity of the phenotype is compounded by a coinciding lack of pancreatic β-cell compensation (34).

The Ala960 mutation does appear to affect the interaction of the insulin receptor cytoplasmic domain with IRS-2. Western blotting revealed that the CSF1R/IR co-precipitated with IRS-2, but the CSF1R/IRA960 was not detected in anti-IRS-2 immunoprecipitates. These data indicate that the juxtamembrane domain is a point of interaction between the insulin receptor cytoplasmic domain and IRS-2 as demonstrated previously (35, 36). The observation that IRS-2 is still tyrosine-phosphorylated upon activation of the CSF1R/IRA960 may emphasize the importance of additional domains of interaction between the insulin receptor cytoplasmic domain and IRS-2. Recent observations that IRS-1 and IRS-2 are distributed differently in 3T3-L1 cells and that they translocate from intracellular membrane compartments to cytosol after insulin stimulation (37) also raises the possibility of differential interactions between the insulin receptor cytoplasmic domain and these intracellular substrates.

Two-hybrid analyses (35, 36) have demonstrated that IRS-2 interacts with phosphotyrosines in the insulin receptor kinase activation loop through a region termed the kinase regulatory loop binding domain (38). Furthermore, it has been suggested that phosphorylation of Tyr624 and Tyr628 within the kinase regulatory loop binding domain of IRS-2 may inhibit interaction of this domain with the insulin receptor kinase (38). If these data are correct, then the phosphorylation-induced decrease in affinity between the insulin receptor and IRS-2 might explain the inability to co-precipitate IRS-2 and the CSF1R/IRA960 (Fig. 5).

The insulin receptor has multiple intracellular substrates (39–45) that may serve as distinct signaling intermediates for the pleiotropic effects of insulin. The ability of the insulin
receptor kinase to mediate diverse signals through distinct intracellular substrates may be determined not only by the phosphorylation state of the substrate but by the nature of the signaling complex formed upon interaction between the receptor and each substrate. The data presented here demonstrate the utility of the chimeric CSF1R/IR for probing the molecular details of the interactions between the insulin receptor kinase and its various intracellular effectors.

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