Human Insulin Receptor Substrate-1 (IRS-1) Polymorphism G972R Causes IRS-1 to Associate with the Insulin Receptor and Inhibit Receptor Autophosphorylation*

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The most commonly detected polymorphism in human insulin receptor substrate-1 (IRS-1), a glycine to arginine change at codon 972 (G972R), is associated with an increased risk of Type 2 diabetes and insulin resistance. To determine the molecular mechanism by which this polymorphism may be linked to insulin resistance, we produced recombinant peptides comprising amino acid residues 925–1008 from IRS-1 that contain either a glycine or arginine at codon 972 and the two nearby tyrosine phosphorylation consensus sites (EY\(^941\)MLM and DY\(^989\)MTM), which are known binding sites for the p85\(^\alpha\) regulatory subunit of phosphatidylinositol 3-kinase. The wild type peptide could be phosphorylated at these sites in vitro by purified insulin receptor. Introduction of the G972R polymorphism into the peptide reduced the amount of tyrosine phosphorylation by >60%. Pull-down experiments indicated that there was an association between the IRS-1-(925–1008) peptide and the insulin receptor that was markedly enhanced by the presence of the G972R polymorphism. The use of additional overlapping fragments localized this interaction to domains between residues 950–986 of IRS-1 and residues 966–1271 of the insulin receptor, containing the tyrosine kinase domain of the receptor. In addition, the IRS-1-(925–1008) G972R peptide acted as a competitive inhibitor of insulin receptor and insulin-like growth factor-1 receptor autophosphorylation. Taken together, these data indicate that the G972R naturally occurring polymorphism of IRS-1 not only reduces phosphorylation of the substrate but allows IRS-1 to act as an inhibitor of the insulin receptor kinase, producing global insulin resistance.

Type 2 diabetes is a complex disease involving both impaired insulin secretion and peripheral insulin resistance impacted by genetics and the environment (1). Insulin receptor substrate-1 (IRS-1)\(^1\) functions as one of the key downstream signaling molecules in both the insulin receptor and the insulin-like growth factor-1 receptor signaling pathways (IGF-1R). Thus genetic changes in IRS-1 may potentially contribute toward the development of insulin resistance. Screening of IRS-1 has identified a number of genetic variants, the most common of these being a glycine to arginine change at codon 972 (G972R) (Fig. 1) (2). This polymorphism is found in ~5% of the normal population and 10% of Type 2 diabetics (3) and carriers of the G972R variant have a 25% increased risk for developing Type 2 diabetes (4).

The G972R polymorphism appears to be associated with obesity because it is found more commonly among obese populations compared with lean populations (5), and decreased insulin sensitivity has been observed in obese carriers of the polymorphism (6). Normal glucose-tolerant subjects carrying the G972R change have also been shown to have decreased insulin secretion (7). In addition, an association with an increased risk of developing coronary artery disease has been reported in people with this polymorphism (8–10), and two recent studies have also demonstrated an association between this polymorphism and Type 1 diabetes (11, 12).

After autophosphorylation of the insulin receptor, the receptor kinase is activated and phosphorylates IRS-1 and other intracellular substrates. This signaling molecule then acts as a docking protein for multiple Src homology-2 domain (SH2)-containing proteins, including PI 3-kinase, Grb-2, and SHP2 (13). In vitro studies in 32D(II)R cells have demonstrated that when the G972R polymorphism was expressed, it caused impaired insulin-stimulated signaling along the PI 3-kinase pathway (14). Other studies in different tissues have shown altered glucose metabolism in muscle over-expressing IRS-1 G972R (15), defects in insulin secretion and increased susceptibility to apoptosis in islets from G972R carriers (16–18), and impaired regulation of Akt/endothelial nitric-oxide synthase activation in endothelial cells from G972R carriers (19), demonstrating that this polymorphism has detrimental effects in multiple insulin-sensitive tissues.

In this study we have investigated the detailed molecular mechanism by which this polymorphism may be linked to insulin resistance. The G972R polymorphism is found near the C terminus of IRS-1 flanked by two tyrosine phosphorylation consensus sites (EY\(^941\)MLM and DY\(^989\)MTM), which are known binding sites for the p85\(^\alpha\) regulatory subunit of PI 3-kinase (Fig. 1). A series of recombinant fragments from this region of human IRS-1 were produced with either a glycine or an arginine at codon 972. These included fragments containing amino acid residues 925–1008, both of the flanking tyrosine phosphorylation consensus sites and overlapping fragments containing residues 950–1027 or 910–986, which contain either the downstream (DY\(^989\)MTM) or the upstream (EY\(^941\)MLM) tyrosine phosphorylation consensus sites, respectively (Fig. 2). These

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\(^{1}\)The abbreviations used are: IRS, insulin receptor substrate; IGF-1, insulin-like growth factor-1; IGFR1, IGF-1 receptor; IR, insulin receptor; WT, wild type; PI, phosphatidylinositol; PTB, phosphotyrosine binding domain; PH, pleckstrin homology domain.

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fragments were used in in vitro experiments with purified insulin receptor to investigate the mechanism by which the G972R polymorphism contributes to altered insulin signaling.

**EXPERIMENTAL PROCEDURES**

**Cloning of the IRS-1 Fragments—DNA fragments (nucleotides 3793–4044, 3886–4101, and 3748–3975 of wild type human IRS-1 cDNA) corresponding to amino acid residues 925–1008, 950–1027, and 910–986 respectively were amplified using standard PCR techniques with Pfu polymerase (Stratagene, La Jolla, CA). Each of the primers contained a restriction site (BamHI in the forward primer, HindIII in the reverse primer) at the 5’ end for cloning purposes. After digestion, the amplified DNA was cloned into BamHI/HindIII-cleaved pQE30 vector (Qiagen, Valencia, CA). Plasmid pQE30 contains a sequence encoding a His tag located upstream of the cloned fragments. The G972R polymorphism donor cDNA (CGG → CGG) was introduced in each of the wild type plasmids using PCR-based site-directed mutagenesis, and the clones were sequenced to confirm introduction of the polymorphism. The DNA was used to transform *Escherichia coli* strain M15 containing a lac repressor plasmid (pREP4, Qiagen).

**Production of Recombinant IRS-1 Fragments—**Recombinant clones were grown at 37 °C in LB containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. Expression of the recombinant protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a concentration of 1 mM at the end of the log phase. Growth was continued for an additional 4 h. Cells were lysed in loading buffer (100 mM NaH₂PO₄, 1 mM dithiothreitol, and 50 mM Tris, 300 mM NaCl, and 10 mM imidazole, pH 8) on ice, sonicated to reduce viscosity, and clarified by centrifugation at 10,000 rpm for 30 min. The cleared lysate was incubated with Talon cobalt metal-affinity resin-protein complexes were washed three times with loading buffer supplemented with 50 mM NaH₂PO₄, 10 mM sodium orthovanadate, 100 nM sodium fluoride, 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium orthophosphate, 10 mM sodium orthophosphate, 10 mM sodium sulfate, and 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and complete protease inhibitor tablets (one per 50 ml, pH 7.6, Roche Applied Science). After sonication, the cell lysate was centrifuged at 3,000 rpm for 5 min, and the cleared lysate was loaded onto a wheat germ agglutinin-agarose column (Amersham Biosciences) and passed through three times. The column was washed exhaustively with 50 mM HEPES, 150 mM NaCl, and 0.1% Triton X-100, pH 7.6, and the IRS-1 fragment was eluted from the column using wash buffer supplemented with 0.6 M N-acetylglycoursamine (Sigma).

**In Vitro Phosphorylation Assays—**Purified full-length insulin receptor (kind gift of Jongsun Lee, Joslin Diabetes Center) was incubated with either 80 or 60 min at room temperature and was then supplemented with 50 μM ATP, 10 mM MgCl₂, and 8 mM MnCl₂ (final concentrations). Recombinant IRS-1 fragments were added, and a time course of phosphorylation was performed. To investigate the inhibition of insulin receptor autophosphorylation, we incubated recombinant IRS-1 fragments with the insulin-activated insulin receptor prior to the addition of 50 μM ATP, 10 mM MgCl₂, and 5 mM MnCl₂, and performed a time course. At each time point, the reaction was stopped by the addition of Laemmli’s sample buffer containing 100 mM dithiothreitol, and the samples were analyzed by SDS-PAGE.

**RESULTS**

**In Vitro Phosphorylation of the IRS-1-(925–1008) Fragment Is Reduced by the G972R Polymorphism—**In vivo association of IRS-1 via the PTB domain (Fig. 1) with Tyr⁹⁴⁶ of the insulin receptor is normally required for optimal phosphorylation of IRS-1 by the insulin receptor (20, 21). The IRS-1-(925–1008) wild type and G972R His-tagged fusion proteins, which contain both of the flanking tyrosine phosphorylation consensus sites (Fig. 2), were incubated with purified full-length human insulin receptor, and a time course of tyrosine phosphorylation was performed. Under the conditions used, the wild type (WT) recombinant fragment was able to undergo tyrosine phosphorylation in vitro (Fig. 3) despite the absence of the PTB domain in this fragment. Introduction of the G972R polymorphism into the IRS-1-(925–1008) fragment reduced the level of phosphorylation seen by >60% (Fig. 3). Mass spectrometry of the WT and G972R phosphopeptides confirmed that phosphorylation was significantly reduced at Tyr⁹⁴¹ in the EY⁹⁴¹LM phosphorylation consensus site upstream of the polymorphism compared with the WT (data not shown). The phosphopeptide containing the DY⁹⁸⁹MTM consensus site could not be detected under the conditions used for mass spectrometry; therefore, the extent of reduction at this site could not be determined.

**The IRS-1-(925–1008) G972R Fragment Associates with the Insulin Receptor—**Pull-down experiments were performed to investigate any association between the insulin receptor and IRS-1-(925–1008). The WT and G972R His-tagged fragments were incubated with insulin-activated insulin receptor followed by pull-down experiment with Talon metal-affinity resin specific for the His tag. Immunoblotting of the metal-affinity resin-protein complexes for the insulin receptor indicates that there was an association between the insulin receptor and the IRS-1-(925–1008) fragments (Fig. 4) and that this was markedly enhanced when the fragment contained the G972R polymorphism.

**The IRS-1-(925–1008) G972R Fragment Inhibits Autophosphorylation of the Insulin Receptor and the IGF-1R—**To deter-
mine the mechanism by which the phosphorylation of the IRS-
1-(925–1008) G972R fragment was reduced, we preincubated
the insulin receptor with the IRS-1-(925–1008) fragments prior
to the addition of ATP, and we performed a time course of
insulin receptor autophosphorylation after the addition of ATP.
Fig. 5 illustrates that, in the presence of the IRS-1-(925–
1008), WT fragment autophosphorylation of the insulin recep-
tor was robust and increased over time. In the presence of the
IRS-1-(925–1008) G972R fragment, the autophosphorylation of
the insulin receptor was markedly reduced (Fig. 5), demon-
strating that the IRS-1-(925–1008) G972R fragment acts as a
competitive inhibitor of insulin receptor autophosphorylation.

This effect occurred in a dose-dependent manner with decreasing
insulin receptor autophosphorylation as increasing molar
concentrations of IRS-1-(925–1008) G972R were added (Fig. 6).
The IRS-1-(925–1008) G972R fragment was also able to significantly reduce autophosphorylation of the IGF-1R compared with the IRS-1-(925–1008) WT fragment (Fig. 5B), demonstrating that the IRS-1 G972R may also act as a competitive inhibitor of IGF-1R activity.

Localization of the IRS-1-(925–1008)-Insulin Receptor Interaction—To determine the sites of interaction between the IRS-1-(925–1008) fragments and the insulin receptor, purified fragments of the insulin receptor were utilized. IR-(966–1271) (InsR active, Upstate Biotechnology) includes insulin receptor residues 966–1271, spanning the juxtamembrane region of the β-subunit through the tyrosine kinase domain but not including Tyr960 (Fig. 7A, i). IR-(947–1343) (IRK, BioMol) is a larger insulin receptor construct that contains residues 947–1343 and that also spans the tyrosine kinase domain and does contain Tyr960 (Fig. 7A). In vitro phosphorylation assays were performed, and the IRS-1-(925–1008) G972R fragment was shown to inhibit autophosphorylation of both IR-(966–1271) (Fig. 7B, i) and IR-(947–1343) (Fig. 7B, ii). This demonstrates that the interaction between the insulin receptor and the IRS-1-(925–1008) fragment occurs between residues 966–1271 of the insulin receptor, most likely in the kinase domain, and does not require Tyr960 for the interaction.

To localize the site of interaction on the IRS-1-(925–1008) fragment and to determine the importance of the two flanking phosphorylation sites (EY941MLM and DY989MTM), a new set of overlapping IRS-1 fragments were produced, also as His-tagged fusion proteins. A WT fragment containing IRS-1 residues 950–1027 (IRS-1(950–1027)) and the single tyrosine phosphorylation site, DY989MTM, was produced along with the corresponding G972R-containing fragment (Fig. 2). In addition, a WT fragment, containing IRS-1 residues 910–986 (IRS-1(910–986)) and the other tyrosine phosphorylation site, EY941MLM, was also produced along with the corresponding G972R-containing fragment (Fig. 2). In vitro phosphorylation assays were performed using the IR-(947–1343) construct containing the insulin receptor kinase and the new overlapping fragments. The IRS-1(950–1027) G972R and IRS-1(910–986) G972R fragments were both able to inhibit autophosphorylation of IR-(947–1343) (Fig. 8, B and C) in a similar manner to IRS-1-(925–1008) G972R (Fig. 8A). This demonstrates that residues 950–986 of IRS-1, which are common to all of the IRS-1 fragments, are required for this interaction.

DISCUSSION

The aim of these experiments was to provide an understanding at a molecular level of how the most common human polymorphism in IRS-1, the G972R polymorphism, may be contributing to insulin resistance and diabetes. This study has demonstrated that the G972R variant alters the ability of the recombinant IRS-1-(925–1008) fragment to be phosphorylated at specific tyrosine residues (EY941MLM and DY989MTM) flanking the polymorphism, which normally form high affinity binding sites for the p85α regulatory subunit of PI 3-kinase. Reduced phosphorylation at these sites and lowered IRS-1-associated PI 3-kinase activ-
ity. This is consistent with the decrease in IRS-1-associated PI 3-kinase activity that Almind et al. (14) observed in 32D(IR) cells expressing the full-length IRS-1 G972R molecule. However, no significant decrease in insulin-stimulated tyrosine phosphorylation of the full-length IRS-1 G972R molecule was observed compared with the wild type molecule, suggesting that phosphorylation at other sites was likely intact and demonstrating the importance of studies like this one using small recombinant fragments from IRS-1 to determine the specific effect of the G972R polymorphism.

Our results are in agreement with several other studies in muscle and adipocytes that have shown decreased binding of p85α to IRS-1 G972R, reduced IRS-1-associated PI 3-kinase activity, and decreased glycogen synthase kinase-3 phosphorylation in these tissues. Taken together with the present study, these studies suggest that the IRS-1 G972R polymorphism might be contributing to insulin resistance by impairing the ability of insulin to activate the IRS-1/PI 3-kinase/Akt/glycogen synthase kinase-3 signaling pathway in these two vitally important insulin-sensitive tissues. This would lead to multiple defects in processes including glucose transport, glucose transporter translocation and glycogen synthesis in muscle (15), and insulin secretion and cell survival in the β-cell (16–18).

Type 1 diabetes is characterized by rapid decline and loss of β-cell function. Studies from the Sesti laboratory (16, 17) showing that the G972R variant impairs human pancreatic function and survival in vitro may provide a possible link between Type 1 and Type 2 diabetes. Because Type 2 diabetes involves other tissues including liver, fat, and brain, it would also be interesting to investigate the effect of this polymorphism in these other tissues.

In addition to decreased phosphorylation of the fragment, we found that the IRS-1-(925–1008) G972R fragment associates with the insulin receptor directly and inhibits autophosphorylation of the receptor. We have shown that this association occurs between residues 966 and 1271 of the insulin receptor, the region of the receptor β-subunit that includes the tyrosine kinase autophosphorylation domain, and between residues 950 and 986 of IRS-1. Normally after ligand binding and autophosphorylation of the insulin receptor, IRS-1 associates with the receptor via the PH and PTB domains in the N terminus of IRS-1 (Fig. 1). The PH domain targets the molecule to the plasma membrane, and the PTB domain associates with phosphorylated Tyr960 in the juxtamembrane region of the receptor (22). This interaction is transient and occurs with low affinity; therefore it is unexpected that the IRS-1-(925–1008) fragment containing the G972R polymorphism and lacking both the PH and PTB domains associates with the insulin receptor kinase domain, a region not normally involved in insulin receptor/IRS-1 interactions, with such high affinity.

We have also demonstrated that the IRS-1-(925–1008) G972R fragment is able to inhibit autophosphorylation of the closely related IGF-1R, which also signals through IRS-1. It is reasonable to speculate that the mechanism of inhibition would be similar to that for the insulin receptor because the tyrosine kinase domains of the two receptors are highly homologous.

The question remains as to whether the reduced phosphorylation seen is associated with a direct effect of the G972R polymorphism because of conformational changes in IRS-1 or a change in charge state related to the substitution of glycine 972 for arginine or whether it is associated with an indirect effect of the polymorphism because of inhibition of insulin receptor tyrosine kinase autophosphorylation by IRS-1 G972R. The G972R polymorphism may interfere with the ATP-binding site at Lys1018 in the insulin receptor tyrosine kinase domain. The substituted Arg972 in IRS-1 has charge characteristics similar to those of Lys1018, and it has been shown previously that if ATP binding is abolished at this site, tyrosine kinase activity is lost (23–25). It seems likely that all of these mechanisms may contribute. Furthermore, this indicates how, even in a het-

![Fig. 8. Inhibition of insulin receptor autophosphorylation by overlapping IRS-1 fragments.](image-url)
erozygous state, the G972R polymorphism may alter insulin sensitivity. Co-crystallization of the IRS-1-(925–1008) G972R fragment with the insulin receptor tyrosine kinase domain would give detailed information about the altered interaction between the two proteins and would provide insight into how the G972R polymorphism enhances the association. Because of the ability to bind with high affinity to the insulin receptor and to inhibit insulin receptor and IGF-1R autophosphorylation, the IRS-1-(925–1008) G972R peptide could also be a potentially useful tool to inhibit receptor function in vivo.

Type 2 diabetes is a complex polygenic disorder, and the true extent of the genes involved is unclear (26). Polymorphisms that cause only a modest change in gene or protein function may become clinically significant when expressed along with other genetic or acquired defects. Genetic alterations have been reported in numerous insulin-signaling proteins in addition to IRS-1 including the insulin receptor (27), IRS-2 (28–30), IRS-4 (31), and p85α (32, 33), as well as other modulators of insulin signaling including peroxisome proliferator-activated receptor-γ (34, 35) and peroxisome proliferator-activated receptor coactivator-1 (PGC-1) (36, 37). A combination of changes in multiple genes/proteins involved in insulin signaling may lead to the end-disease phenotype. Therefore the IRS-1 G972R polymorphism may be just one contributing factor of many that predispose an individual to Type 2 diabetes.

Taken together, these data indicate that the primary defect of the G972R polymorphism is the increased association of IRS-1 G972R with the insulin receptor and the inhibition of insulin receptor autophosphorylation leading to reduced downstream signaling along the PI 3-kinase pathway. These effects in combination with changes in other genes encoding insulin-signaling proteins may contribute to global insulin resistance.

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