A Common Amino Acid Polymorphism in Insulin Receptor Substrate-1 Causes Impaired Insulin Signaling

Evidence from Transfection Studies

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Abstract

Insulin receptor substrate-1 (IRS-1) is the major cytoplasmic substrate of the insulin and IGF-1 receptors. Recent studies have identified multiple sequence variants of IRS-1, especially in patients with non–insulin-dependent diabetes mellitus. In the present study, we have examined insulin-stimulated processes in 32D(IR) cells, a myeloid progenitor cell stably overexpressing the insulin receptor, transfected with wild-type human-IRS-1 or the most common human variant of IRS-1 in which glycine 972 is replaced by arginine. As compared to wild-type IRS-1, insulin stimulation of cells transfected with mutant IRS-1 exhibited a 32% decrease in incorporation of [^{3}H]thymidine into DNA ($P = 0.002$), a 36% decrease in IRS-1 associated phosphatidylinositol (PI) 3-kinase activity ($P = 0.004$) and a 25% decrease in binding of the p85 regulatory subunit of PI 3-kinase to IRS-1 ($P = 0.002$). There was also a tendency for a decrease in Grb2 binding to IRS-1 and insulin-stimulated mitogen-activated protein kinase activity, however, these were not statistically significant. The changes occurred with no change in insulin receptor or IRS-1 tyrosine phosphorylation. These data indicate that the mutation in codon 972 in IRS-1 impairs insulin-stimulated signaling, especially along the PI 3-kinase pathway, and may contribute to insulin resistance in normal and diabetic populations. (*J Clin. Invest.* 1996. 97:2569–2575.) Key words: diabetes mellitus • IRS-1 • insulin resistance • genetics • PI 3-kinase

Introduction

The primary substrate of the insulin receptor in most tissues is insulin receptor substrate-1 (IRS-1).[1] By genetic analysis of insulin-resistant non–insulin-dependent diabetes mellitus (NIDDM) patients and normal individuals, we recently discovered four polymorphisms in IRS-1 (1). Two of these were silent, while two predicted amino acid substitutions. One of these was at codon 513 (numbered according to Nishiyama and Wands, reference 2) and resulted in a proline for alanine substitution; the other was at codon 972 and predicted a replacement of glycine by arginine. Several subsequent studies (3–9) in different ethnic groups have confirmed these and other polymorphisms in IRS-1, and when grouped together show that the overall frequency of the codon 972 variant is 10.7% in NIDDM patients versus 5.8% in control subjects ($P < 0.02$), suggesting that this genetic variant is a potential contributor to the insulin resistance in NIDDM (7). Moreover, in a recent study of the IRS-1 polymorphism in a random sample of 380 young, healthy adults, the IRS-1 polymorphism at codon 972 in its heterozygous form was shown to potentiate obesity-linked insulin resistance (10). Multivariate analysis substantiated that the combination of obesity and the codon 972 variant was associated with a 50% reduction in insulin sensitivity ($P = 0.0008$). The same obese subjects were characterized by a clustering of metabolic cardiovascular risk factors with elevated fasting levels of plasma glucose, serum triglyceride, plasma tPA, and plasma PAI-1 activity. One lean male homozygous for the codon 972 mutation had impaired insulin sensitivity and developed transient diabetes after a 24-h dexamethasone load (10).

Despite the multiple population studies investigating polymorphisms in IRS-1, the effect of these sequence variations on insulin signaling has not been determined. IRS-1 is tyrosine phosphorylated on multiple sites after stimulation by insulin, insulin-like growth factor-1 (IGF-1), and IL-4 (11). These phosphorylation sites then serve as docking sites for several proteins possessing SH2 domains. Thus, phosphorylated IRS-1 binds to and stimulates phosphatidylinositol (PI) 3-kinase, SHPTP-2, and via GRb2-SOS the Ras and mitogen-activated protein (MAP) kinase pathways (11–18). Although Gly^{972} is not directly located in a phosphorylation site, it is conserved in human, rat, and murine IRS-1 and lies between two potential sites of tyrosine phosphorylation involved in binding the p85 subunit of PI 3-kinase. The aim of the present study was to determine if and/or how cells expressing the variant IRS-1 would change insulin-stimulated signaling. Since cells taken from the patients are usually heterozygous for IRS-1 mutation and also possess IRS-2, complicating any analysis using patient cells, for these experiments we used cultured cells lacking both IRS-1 and -2 which were transfected with normal and mutants IRS-1s, as well as the normal human insulin receptor.

Methods

Reagents. Restriction endonucleases were obtained from New England BioLabs Inc., Beverly, MA. Leupeptin, aprotinin, DTT, PMSF, protein kinase inhibitor, and myelin basic protein were from Sigma Chemical Co., St. Louis, MO and protein A Sepharose 6MB from Pharmacia, Uppsala, Sweden. Immobilon polyvinylidne diflouride (PVDF) transfer membranes were from Millipore Corp., Bedford,

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1. *Abbreviations used in this paper:* IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; NIDDM, non–insulin-dependent diabetes mellitus; PI 3-kinase, phosphatidylinositol 3-kinase.

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MA. Antibodies to the COOH-terminal peptide of IRS-1 were raised as previously described (19). Antibodies to baculovirus produced rat-IRS-1 and phosphotyrosine antibody (4G10) were provided by M.F. White (Joslin Diabetes Center, Boston, MA). Anti–rat PI 3-kinase antibody, rabbit antiserum to p85, and Grb2 antibody were from Upstate Biotechnology Inc., Lake Placid, NY. Anti-MAP kinase antibodies were prepared as previously described (20). Thymidine, [methyl-^{3}H], nucleoside triphosphate [^{32}P], and enhanced chemiluminescence reagents were from NEN-DuPont, Wilmington, DE. ^{125}I-protein A was from ICN Biomedicals Costa Mesa, CA. Human recombinant insulin was from Boehringer Mannheim Biochemica, Mannheim, Germany, and phosphotidylinositol was from Avanti Polar Lipids, Alabaster, AL. The retroviral pBABE-Puromycin expression vector and the BOSC23 packaging cell line were gifts from Dr. D. Baltimore (Massachusetts Institute of Technology, Cambridge, MA).

Cell culture. The 32D cell line is a myeloid progenitor cell which is dependent on IL-3 for its proliferation and survival (21, 22). 32D cells do not proliferate upon exposure to IL-4 or insulin, although 32D cells have $\sim$ 1,200 IL-4 and $\sim$ 500 insulin receptors. This lack of sensitivity to IL-4 and insulin can be explained by a lack of the insulin and IL-4 receptor substrates IRS-1 and IRS-2 (21). 32D cells were grown in 5% CO_2 in RPMI 1640 containing 10% FBS and 5% WEHI-conditioned medium. WEHI cells produce IL-3, and the medium extracted from confluent flasks of WEHI cells is used as an IL-3 supplement for the 32D cells.

Construction of IRS-1 cDNAs. The cDNA construct containing the mutation in IRS-1 was created by site-directed mutagenesis by overlap extension using the polymerase chain reaction (23). Two complimentary oligonucleotide primers containing the nucleotide substitution at the first position of codon 972 were used for the PCR to create the nucleotide substitution. cDNA containing the full-length open reading frame of human IRS-1 in pBluescript was used as a template. The PCR fragment containing the codon 972 variant in IRS-1 was digested with BclI and NheI and inserted in place of the wild-type sequence. The new construct was confirmed by nucleotide sequencing (Sequenase 2.0; U.S. Biochemical Corp., Cleveland, OH).

Transfection of IRS-1 cDNA to 32D cells. The IRS-1 cDNAs containing wild-type or mutant IRS-1 were digested at the EcoRI and SalI sites and inserted into the retroviral pBABE-puromycin expression vector (24, 25) at the same sites. The BOSC23 packaging cell line, was maintained in DME containing 10% FBS at 37°C in a humidified atmosphere of 10% CO_2 (25). BOSC23 cells (2×10^6 in a 60-mm dish) were transfected by calcium phosphate coprecipitation with 10 μg of pBABE/wtIRS-1 or pBABE/R^{972}IRS-1. The medium was removed 48 h after transduction, and 4×10^6 32D or 32D(IR) cells (a cell line overexpressing insulin receptor, line 286.5w3) were added to the dish for 24 h, then transferred to a clean dish. After 24 h, the cells were spun down and selected in 2 μg/ml puromycin. Stable cell lines were maintained in RPMI 1640 containing 10% FBS, 5% WEHI at 37°C in a humidified atmosphere of 5% CO_2. Equivalent numbers of cells from each cell line were collected and lysed for 10 min in 1 ml of lysis buffer to give final concentrations of 20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 10% glycerol, 10 μg/ml aprotinin 10 μg/ml leupeptin, 100 μM sodium orthovanadate (Na_3VO_4), and 1 mM PMSF. Insoluble material was removed by centrifugation in a microfuge for 10 min at 4°C. The supernatant was denatured by boiling in Laemmli sample buffer containing 100 mM DTT and separated by SDS PAGE. Gels were transferred to Immobilon PVDF transfer membranes, blocked, and probed with the indicated antibody (see below). The blots were washed and then incubated with 2 μCi of ^{125}I-protein A [30 μCi/μg], washed again, and imaged on a Molecular Dynamics Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA).

Expression of insulin receptors and IRS-1. Expression of IRS-1 in 32D(IR) cells after retroviral transduction and selection was an efficient and reproducible process. The amounts of insulin receptor and IRS-1 protein expressed and quantitated by immunoblotting were similar among the cell lines (Table I). Similar levels of expression were obtained for cells expressing the codon 513 IRS-1 mutant in 32D(IR) cells (results not shown).

Table I. IRS-1 and Insulin Receptor Protein Levels in Transfected 32D(IR) Cells

Cell line	IRS-1 expression	IR expression
	%	%
WT-1	100	100
WT-2	97	92
972-1	166	95
972-2	167	78

Protein expression was measured by performing SDS-PAGE followed by IRS-1 immunoblotting of cell lysates from 32D(IR) cells transfected with pBABE/IRS-1 constructs. Immunoblots were quantitated using a Phosphorimager. The amount of protein is expressed as percent of control (WT-1).

Incorporation of [^{3}H]thymidine into DNA in 32D(IR) cells. Cells in log phase growth were washed twice and 2×10^5 cells were seeded in 1 ml of medium into each of 24 wells containing RPMI with 10% FBS alone or containing various concentrations of insulin or 5% WEHI (26). Cells were grown for 48 h in a 37°C incubator. [^{3}H]thymidine was added to a final concentration of 0.5 μCi/ml, and the incubation continued for 3 h. Cells were collected onto glass microfiber filters. The cells were lysed, and unincorporated nucleotide was removed by washing with water. The filters were counted in scintillation fluid for 1 min.

In vivo IRS-1 phosphorylation and associated PI 3-kinase activity. The cell lines were grown to confluency whereupon 5×10^7 cells were collected by low-speed centrifugation and then starved for 4 h in DME at 37°C. Cells were stimulated with 100 nM insulin for 10 min, collected, and lysed in 1 ml of ice-cold lysis buffer containing final concentrations of 50 mM Hepes, pH 7.6, 1% Triton X-100 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM Na_3VO_4, 1 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, and 4 mM EDTA for 10 min at 4°C. The tubes were centrifuged for 10 min, and supernatants containing 750 μg total protein were incubated with anti-IRS-1 COOH-terminal antibody (6 μg/ml) for 75 min. Immune complexes were collected with 60 μl of a 50% slurry of protein A sepharose. The immunoprecipitates were washed three times in PBS containing 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM Na_3VO_4, three times in 0.5 M LiCl, 0.1 M Tris, pH 7.5, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM Na_3VO_4, and twice in 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM Na_3VO_4. The pellets were resuspended in 50 μl of 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 100 μM Na_3VO_4, 10 μl of 100 mM $MgCl_2$, and 10 μl of 2 μg/μl phosphotidylinositol, and finally sonicated in 10 mM Tris, pH 7.5, containing 1 mM EGTA. The PI 3-kinase reaction was initiated by addition of 5 μl of 880 μM ATP, 20 mM $MgCl_2$ containing 35 μCi [^{32}P]ATP (3,000 Ci/mmol) per tube. After vortexing vigorously for 10 min, the reaction was stopped by addition of 20 μl 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1). The samples were centrifuged for 10 min and the lower organic phase spotted onto a silica gel TLC plate previously coated with 1% potassium peroxalate. TLC plates were developed in $CHCl_3$:methanol:H_2O:NH_4OH (ratio 120:94:23.2:4), dried, and visualized by autoradiography. Phosphorylated phosphatidylinositol was quantitated with the Phosphorimager.

Immunoprecipitation and immunoblotting. Each cell line was stimulated for 2 min for quantitating IRS-1 phosphorylation and p85 binding or for 0, 1, 3.5, or 10 min for determination of Grb2 binding, and lysed as described above. Supernatants were immunoprecipitated with anti–IRS-1 antibody (1:100) and 750 μg of total protein was re-

solved by SDS-PAGE and transferred to PVDF paper. The IRS-1 immunoprecipitates for measuring p85 binding were blotted with p85 antibody (1:1,000 dilution). Aliquots of IRS-1 immunoprecipitates for quantitating IRS-1 phosphorylation were blotted with rat-IRS-1 antibody and with a phosphotyrosine antibody (1:500) followed by blotting with anti–mouse IgG antibody (1:1,000) raised in rabbit. The blots were then incubated with 2 μCi of ^{125}I-protein A [30 μCi/μg]. Detection and quantitation were performed on a Phosphorimager as previously described (27). IRS-1 immunoprecipitates for determination of IRS-1/Grb2 association were blotted with Grb2 antibody (1:300), detected by immunoblotting with anti–rabbit IgG coupled to peroxidase, and developed with enhanced chemiluminescence reagents as described by manufacturer. These blots were quantitated by scanning densitometry.

MAP kinase assay. Serum-starved cells were treated with 10^{-7} M insulin at 37°C for 5 min, lysed, and immunoprecipitated with anti-MAP kinase antibody followed by protein A sepharose incubation as previously described. The immunoprecipitates were washed three times with lysis buffer and twice with 40 mM Hepes, pH 8.0, 10 mM $MgCl_2$ (kinase reaction buffer). The reactions were initiated by adding kinase reaction buffer containing 0.25 mg/ml myelin basic protein, 2 μM protein kinase inhibitor, and 50 μM [γ-^{32}P]ATP, in a final vol of 40 μl. The reactions were allowed to proceed for 10 min at 30°C then stopped by the addition of 20 μl of 3× Laemmli buffer containing 300 mM DTT. The mixtures were boiled for 5 min and subjected to SDS-PAGE in 12.5% polyacrylamide gels.

Statistics. Differences between the cell lines were tested by the unpaired or paired Student's *t* test where appropriate. Data in figures are given as mean±standard error of the mean.

Results

Incorporation of [^{3}H]thymidine into DNA. 32D cell lines require IL-3 and serum for growth, and maximal proliferation of the cells is obtained by maintenance in FBS and IL-3. Thus, for each cell line, the incorporation of [methyl-^{3}H]thymidine into DNA of transfected and nontransfected 32D and 32D(IR) cells, respectively, was measured at insulin concentrations ranging from 0 to 1,000 nM and expressed as a percentage of maximal proliferation stimulated by IL-3. Nontransfected 32D cells with a low number of endogenous insulin receptors did not proliferate upon exposure to insulin and 32D(IR) cells stably transfected with human insulin receptor showed only a minimal mitogenic response to insulin (Fig. 1). Likewise, transfection of 32D cells with IRS-1 alone conferred only modest increase in insulin-mediated incorporation of thymidine into DNA, with a maximal increase of 20% above basal (results not shown). However, coexpression of insulin receptor and IRS-1 resulted in a significant response to insulin with a maximum of 70% of the IL-3 response. Insulin-stimulated thymidine incorporation progressed in the same fashion for every IRS-1 expressing cell line, although the cell lines expressing the Gly→Arg substitution of codon 972 had an average 32% decrease in maximal insulin-stimulated mitogenesis. Similar results were observed in two independent cell lines expressing the mutant IRS-1. A paired Student's *t* test for all points from 0.01 to 1,000 nM demonstrated a significant difference (P = 0.002) between cell lines expressing mutated and wild-type-IRS-1. By contrast, cells expressing the codon 513 mutation showed no decrease in proliferation compared to wild-type expressing cell lines (results not shown).

PI 3-kinase activity in IRS-1 immunoprecipitates from insulin stimulated 32D(IR) cells expressing wildtype-IRS-1 or R^{972} IRS-1. To determine the mechanism of the reduced insulin effect on DNA synthesis in the presence of the mutant IRS-1, a series of early steps in insulin action was studied. PI 3-kinase activity was measured by counting ^{32}P incorporated into phosphatidylinositol in vitro using anti-IRS-1 immunoprecipitates.

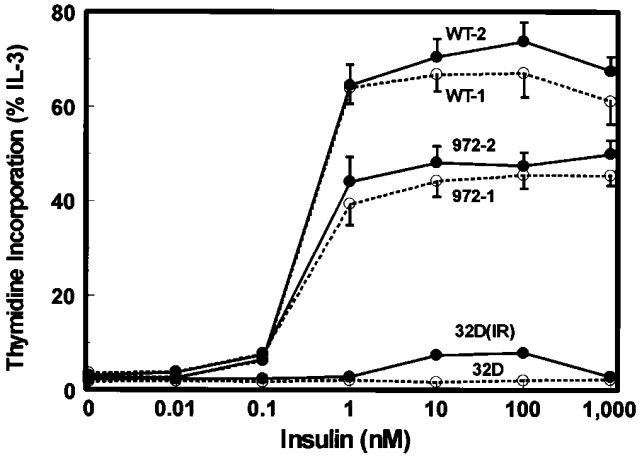

Figure 1. DNA synthesis in response to insulin in 32D and 32D(IR) cells expressing wild-type IRS-1 or mutated R^{972}IRS-1. [^{3}H]thymidine incorporation into DNA was measured 48 h after insulin stimulation as described in Methods. The data points represent the mean of three independent experiments each done in triplicate. Results are expressed as percentage of [^{3}H]thymidine uptake of the cell lines stimulated with IL-3.

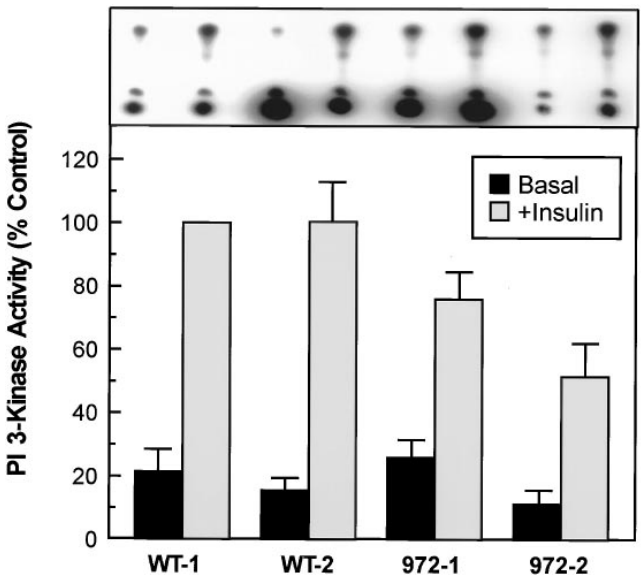

Figure 2. IRS-1 associated PI 3-kinase activity in 32D(IR) cells transfected with wild-type IRS-1 or R^{972}IRS-1. (*Top*) PI-3-^{32}P produced during PI 3-kinase assay in anti–IRS-1 immunoprecipitates from two independent cell lines expressing wild-type IRS-1 (*WT-1* and *WT-2*) and two independent cell lines expressing R^{972}IRS-1 (*972-1* and *972-2*) as analyzed by thin layer chromatography and autoradiography. (*Bottom*) PI-3-kinase activity was determined as shown in top panel and quantified using a Phosphorimager. Data represent three independent experiments each in duplicate and are expressed as percentage of control (*WT-1*). Basal PI 3-kinase activity for WT = 21.3±4.5 and R^{972}IRS-1 = 21.0±4.3. Insulin-stimulated PI 3-kinase activity for WT = 102.5±5.1 and R^{972}IRS-1 = 66.8±9.1 (P = 0.004) expressed as mean±SEM.

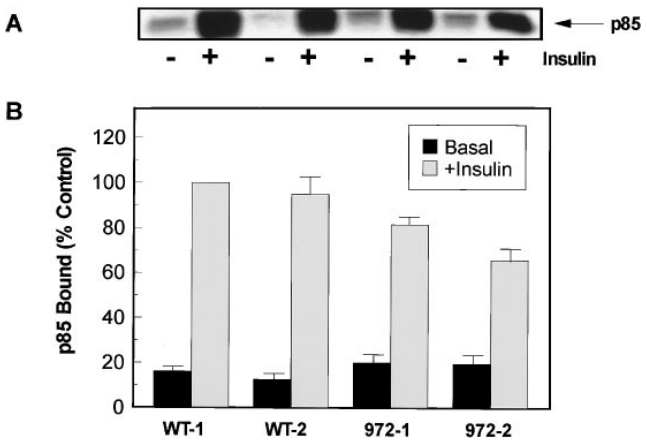

Figure 3. Binding of p85 of PI 3-kinase to IRS-1 in 32D(IR) cells expressing wild-type IRS-1 or R^{972}IRS-1. (*A*) Supernatants from transfected 32D(IR) cells were immunoprecipitated with IRS-1 antibody and immunoblotted with p85 antibody. (*B*) p85 protein levels in IRS-1 immunoprecipitates quantified using a Phosphorimager. Data represent four independent experiments expressed as percent of control (*WT-1*). Basal p85 binding for WT = 14.3±2 and R^{972}IRS-1 = 19.8±2.2. Insulin stimulated p85 binding for *WT* = 97.5±4.5 and R^{972}IRS-1 = 73.6±4.2 (P = 0.002) expressed as mean±SEM.

A representative autoradiogram of a TLC-plate is shown in Fig. 2 (*top*). Quantitation of the radioactivity incorporated into phosphatidylinositol revealed that the cells expressing wild-type and the mutated R^{972}IRS-1 had a similar low level of basal PI 3-kinase activity (Fig. 2, bottom). Insulin stimulation of cells expressing wild-type-IRS-1 increased the IRS-1-associated PI 3-kinase activity by 4.8-fold after insulin stimulation. In R^{972} IRS-1 expressing cells, maximal insulin-stimulated activity was decreased by 36% as compared to supernatants from cells expressing wild-type–IRS-1 (P = 0.004).

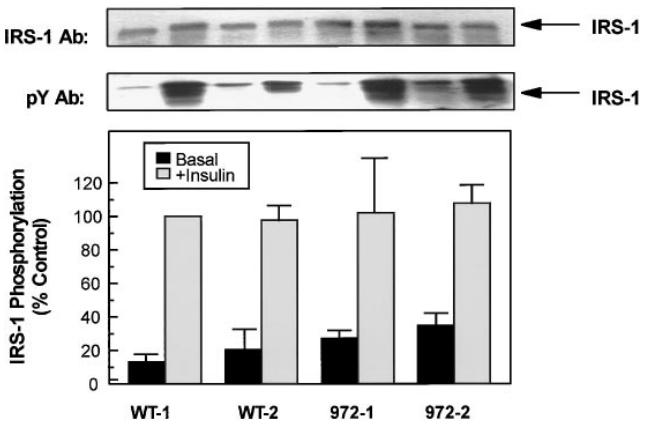

Figure 4. Phosphorylation of wild-type IRS-1 and R^{972}-1 expressed in 32D(IR) cells. (*Top*) Immunoblots of equal amounts of total protein from IRS-1 immunoprecipitates with anti-IRS-1 antibody. (*Middle*) Blotting of equal amounts of total protein from IRS-1 immunoprecipitates with anti-phosphotyrosine (*pY*) antibody. (*Bottom*) IRS-1 phosphorylation quantified using a Phosphorimager. Data represent four independent experiments expressed as percent of control (*WT-1*). Basal IRS-1 phosphorylation for WT = 16.3±5.9 and R^{972}IRS-1 = 30.9±5.3. Insulin stimulated IRS-1 phosphorylation for WT = 99.0±4.2 and R^{972}IRS-1 = 104.9±14.2 expressed as mean±SEM.

IRS-1 expression, phosphorylation, and binding of p85 of PI 3-kinase. To determine if the reduced PI 3-kinase activity in cells expressing the 972 mutant resulted from a diminished interaction between insulin receptor and R^{972} IRS-1 or from decreased binding of p85α to R^{972} IRS-1, equal amounts of total protein were immunoprecipitated with anti-IRS-1 antibody and subjected to SDS-PAGE and immunoblotting with p85 antibody (Fig. 3 *A*). As previously described, insulin induced binding of p85 to IRS-1. Quantitation of the protein bands confirmed that after insulin stimulation, binding of p85 protein to IRS-1 in insulin-stimulated wild-type cells was increased 6.8-fold. In cells expressing mutated R^{972}IRS-1 binding of p85 to IRS-1 was decreased by 25% (P = 0.002) as compared to cells expressing wild-type protein. The decrease was not due to a decrease in IRS-1 protein or its phosphorylation. Western blots of lysates from insulin-stimulated cells and from nonstimulated cells revealed equal amounts of IRS-1 protein (Fig. 4, top). Phosphorylation of tyrosine residues on IRS-1, as measured by phosphotyrosine immunoblots, also showed no change after 2 min of insulin stimulation (Fig. 4, *middle*).

Insulin-stimulated binding of Grb2 and MAP-kinase activity. Insulin stimulation of 32D(IR) cells transfected with IRS-1 results in a IRS-1-Grb2 association and activation of MAP-kinase (18). To determine whether the amino acid variant had any influence on the association between Grb2 and IRS-1, IRS-1 immunoprecipitates were analyzed for Grb2 binding by immunoblotting with a Grb2 antibody. Fig. 5 *A* shows a time course of IRS-1 tyrosine phosphorylation and Grb2 association. With both wild-type and mutant IRS-1, tyrosine phosphorylation, and Grb2 binding were maximal within 1 min of insulin stimulation and sustained for at least 10 min. Quantita-

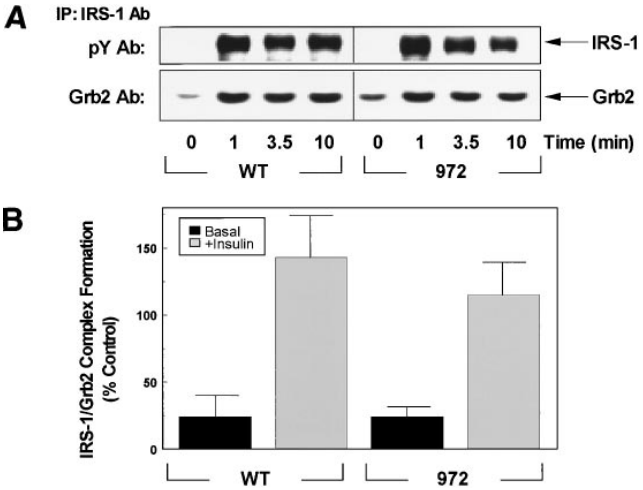

Figure 5. Time course of IRS-1 tyrosine phosphorylation and IRS-1/Grb2 complex formation. (*A*) IRS-1 was immunoprecipitated after insulin stimulation, and the resulting precipitates were analyzed by SDS-PAGE and blotting with anti-phosphotyrosine (*pY*) antibody (*top*) or anti-Grb2 antibody (*bottom*) in 32D(IR) cells expressing wild-type or R^{972}IRS-1 after 0,1,3.5, and 10 min of insulin stimulation. (*B*) Grb2 protein levels in IRS-1 immunoprecipitates were quantitated using a Phosphorimager. Data represent two independent experiments expressed as percentage of control (*WT-1*). Basal Grb2 binding for WT = 24.4±12.3 and R^{972}IRS-1 = 24.4±7.5. Insulin stimulated Grb2 binding for WT = 142.7±30.9 and R^{972}IRS-1 = 114.9±23.9 expressed as mean±SEM.

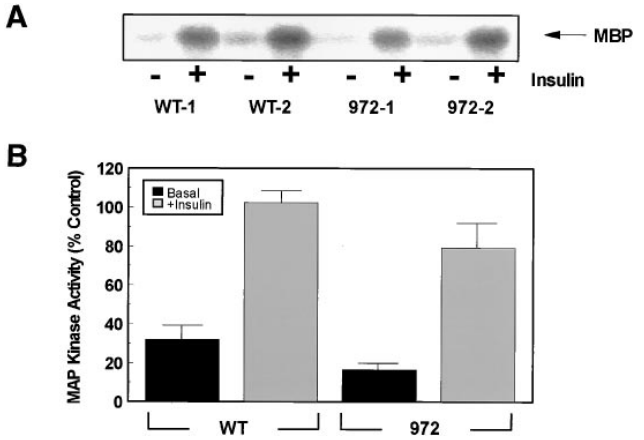

Figure 6. MAP-kinase activation in lysates from cells expressing wild-type IRS-1 or R^{972}IRS-1. (*A*) Phosphorylation of myelin basic protein (*MBP*) in MAP-kinase immunoprecipitates from nonstimulated or insulin-stimulated cells expressing wild-type IRS-1 and R^{972}IRS-1. (*B*) MAP-kinase activity measured as the phosphorylation and quantitated using a Phosphorimager. Data represent two independent experiments expressed as percent of control (*WT-1*). Basal MAP-kinase activation for WT = 31.9±8.2 and R^{972}IRS-1 = 16.3±3.3. Insulin stimulated MAP-kinase activity for WT = 102.2±4.9 and R^{972}IRS-1 = 79.1±10.5.

tion of multiple experiments indicated a trend toward a lower level of Grb2-IRS-1 association, however, they did not reach statistical significance. To assess MAP kinase activity, lysates from insulin-stimulated and nonstimulated cells were immunoprecipitated with a MAP-kinase antibody and activity was measured by myelin basic protein phosphorylation in vitro. Again, cells transfected with mutated IRS-1 tended to be lower in MAP-kinase activity as compared to cells transfected with wildtype-IRS-1, however this change was also not statistically significant (Fig. 6).

Discussion

Analysis of the gene encoding IRS-1 has revealed several mutations resulting in amino acid substitutions. The most prevalent amino acid change in IRS-1 is a glycine to arginine change at codon 972. The occurrence of this amino acid polymorphism has been examined in more than 1,500 NIDDM patients and control subjects from different ethnic groups. Overall, the frequency of the Gly^{972}→Arg amino acid substitution is ∼ 6% in normal populations and is increased in NIDDM patients by about twofold (7), suggesting that mutation of the IRS-1 gene may act as a risk factor predisposing to NIDDM. Equilibrium glucose infusion rates during a euglycemic clamp in both NIDDM patients and controls with the codon 972 variant are lower than those in comparable groups without IRS-1 mutations (28). In nondiabetic obese individuals, the codon 972 mutation, in association with obesity, is associated with a potentiation of the obesity-linked insulin resistance (10). However, functional studies were needed to confirm whether there is a direct impact of this genetic variant on insulin sensitivity or whether the amino acid polymorphism is just a marker for a linked genetic factor.

IRS-1 is considered the major insulin receptor substrate in most insulin-sensitive tissues. IRS-1 is required for the insulin-mediated cellular effects including activation of PI 3-kinase (29), p70 S6 kinase (11), and for the cellular events leading to mitogenesis (30). The importance of IRS-1 in in vivo insulin signaling has been most directly demonstrated in mice made deficient in IRS-1 using targeted gene knockout by homologous recombination (31, 32). IRS-1–deficient mice have a 50% reduction in intrauterine growth, impaired glucose tolerance, and a decrease in insulin and IGF-1–stimulated glucose uptake. The residual insulin/IGF-1 action in these mice is linked to the presence of IRS-2 (33). This second insulin receptor substrate has an overall identity to IRS-1 of 43% and is also expressed in many tissues (34). IRS-2 shows increased insulin-stimulated tyrosine phosphorylation in IRS-1–deficient mice and under these biological conditions acts as an alternative substrate for the insulin receptor. 32D cells lack both IRS-1 and IRS-2 and, thus, are ideal to demonstrate the insulin-mediated response transmitted through these substrates after transfection with either wild-type or mutant IRS-1.

Our results confirm that expression of wild-type IRS-1 in 32D(IR) cells restores the proliferative response to insulin as previously shown (21). Expression of IRS-1 containing the mutation in codon 972 similarly results in insulin-stimulated mitogenesis, however, the maximal incorporation of thymidine into DNA is decreased by 32% as compared to cells expressing wild-type IRS-1. In an attempt to elucidate at which step the insulin-mediated signal is decreased, we examined several insulin-signaling proteins. As noted above, tyrosine phosphorylated IRS-1 associates with PI 3-kinase which in turn acts as an important upstream element in signaling to glucose transporter translocation, p70 S6 kinase, DNA synthesis, and glycogen synthesis (35, 36). We therefore determined the PI 3-kinase activity directly associated with IRS-1 by performing the PI 3-kinase assays on IRS-1 immunoprecipitates. After insulin stimulation there is a 5-fold increase in IRS-1–associated PI 3-kinase activity in cells expressing wild-type IRS-1. Cells expressing mutated R^{972}IRS-1 have a 36% reduction in IRS-1-associated PI 3-kinase activity when compared to cells transfected with wild-type IRS-1. This decrease in PI 3-kinase activity is a consequence of a diminished interaction between PI 3-kinase and IRS-1, which is reduced by ∼ 25% in cells expressing R^{972} IRS-1.

The exact reason for the decrease in IRS-1/p85 binding is not clear. The association of IRS-1 and PI 3-kinase is mediated by binding of SH2 domains of the 85-kD regulatory subunit (p85α) to tyrosyl phosphorylated YMXM motifs of IRS-1 (12). Although some activation of PI 3-kinase occurs by the occupancy of only one SH2 domain, full activation of PI 3-kinase requires occupancy of both SH2 domains (37). Codon 972 of IRS-1 is located between two YMXM sites (Y^{939} and Y^{987}) which when phosphorylated are known to bind p85 (11). A diminished binding of p85 to IRS-1 containing the Gly→Arg substitution could be due to an effect of the mutation on the tertiary structure of IRS-1. It is also possible that the amino acid substitution prevents the interaction of one of the YMXM motifs with the SH2 domain or prevents the binding of both domains at the same time, thus reducing the affinity of p85 for IRS-1.

While the IRS-1 amino acid substitution has a negative effect on p85 binding, this protein modification does not have an impact on the interaction of the insulin receptor kinase with IRS-1, which results in phosphorylation of IRS-1. Indeed, insulin-stimulated tyrosine phosphorylation of IRS-1 is the same in

all cell lines. Insulin receptor autophosphorylation is also similar. These results are not surprising since codon 972 is not directly within a tyrosine phosphorylation site nor within the more NH_2-terminal regions of IRS-1, which are thought to be involved in the interaction with the insulin receptor kinase (38). Since the exact stoichiometry of phosphorylation is difficult to determine, it is possible that there is a decrease in phosphorylation of one or more tyrosine residues in the cells expressing R^{972}IRS-1 which is undetected by the methods used in this study. Ultimately, it may be relevant to map the phosphorylation sites, as well as study the structure of the variant molecules.

The tendency toward a decrease in Grb2 association and MAP kinase activation in cells expressing the mutant IRS-1 might suggest a more general conformational change in IRS-1 due to the amino acid substitution; however, neither of these biochemical measures were statistically different in cells expressing mutant IRS-1. Whether more sensitive assays could define some alterations in these pathways remains to be determined. In either case, our results suggest that the Grb2-Ras-MAP kinase pathway is less influenced by the codon 972 amino acid substitution than the pathway leading to PI 3-kinase activation, in which two YMXM motifs of IRS-1 are involved.

This study is the first functional study of a naturally occurring amino acid polymorphism in IRS-1. The mutant studied has been shown to be more frequent in persons with NIDDM than normals and is associated with an accentuation of the insulin resistance in obese, otherwise healthy, young adults (10). Ura et al. (28) have also found the 972 variant of IRS-1 to be increased in Japanese with NIDDM, and in this setting associated with an $\sim$ 25% further decrease in insulin sensitivity. A recent study by Goodyear et al. (40) has also suggested that the deficiency in insulin action in human obesity may result from a decrease in IRS-1 related PI 3-kinase signaling, but in this case the decrease appears to be in IRS-1 protein expression. IRS-1 phosphorylation and PI 3-kinase activation have also been shown to be defective in several animal models of diabetes and insulin resistance (41). Since most patients with NIDDM have a normal IRS-1 molecule or are only heterozygous for these mutations, other factors must contribute to the insulin resistance. These include insulin receptor down-regulation (40), defects in GLUT4 glucose transporter translocation (40), alterations of plasma membrane lipids (42), elevated expression of TNF-α in adipose tissue (43), increased expression of the membrane glycoprotein PC-1 (44), and overexpression of the Ras-related cytosolic protein rad (45). The current study clearly established that the 972 variant of IRS-1 may also confer cellular insulin resistance, in part through a defective interaction between the mutant IRS-1 and the regulatory subunit of PI 3-kinase. Since PI 3-kinase activation appears essential in several insulin-mediated intracellular processes, including translocation of the insulin-responsive glucose transporter, Glut 4, to the plasma membrane (35), defects in PI 3-kinase could lead to insulin resistance in glucose metabolism.

In nonobese humans, however, the impact of IRS-1 mutations may be somewhat limited by the presence of alternative pathways of signaling. In the IRS-1 knockout mouse, insulin and IGF-1 signaling is reduced $\sim$ 50%; however, there is significant residual signaling via IRS-2 (33). Likewise, we have previously reported a young healthy and lean male who carries the codon 972 variant on both alleles. Although he exhibited insulin resistance, low fasting serum insulin levels, and a low acute insulin response, his glucose tolerance was only minimally abnormal (10). However, after a 24-h dexamethasone load he developed transient diabetes. Taken together with the data of the present study, we suggest that sequence variation in IRS-1 may contribute significantly to insulin resistance, but alone may be insufficient to result in clinical diabetes mellitus. Since IRS-1 is ubiquitously expressed in insulin and IGF-1–sensitive tissues, including the α- and β-cells of the islets of Langerhans, however, it is possible that the presence of the codon 972 variant may also affect islet cell differentiation, maturation, or function, and thereby diminish the β-cells response. Indeed, Rothenberg et al. have shown that glucose-stimulated insulin secretion by pancreatic β-cells seems to be modulated by autocrine activation of the insulin-signaling pathway involving tyrosine phosphorylation of IRS-1 and activation of PI 3-kinase (46). Thus, in humans, the defective interaction between IRS-1 and PI 3-kinase in the codon 972 variant may contribute to both the peripheral tissue insulin resistance and the impaired insulin secretion.

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