Brief Genetics Report

Molecular Scanning of the Insulin Receptor Substrate 1 Gene in Subjects With Severe Insulin Resistance

Detection and Functional Analysis of a Naturally Occurring Mutation in a YMXM Motif

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Despite recent major advances in the understanding of insulin signaling, the molecular mechanisms of human insulin resistance are still unclear. To date, the only known molecular defects causing inherited syndromes of severe insulin resistance are those of the insulin receptor gene (1). However, in patients with syndromes of severe insulin resistance other than the Donohue (also known as Leprechaunism) and Rabson-Mendenhall syndromes, only 10–15% have been found to have insulin receptor mutations (2,3). It therefore seems likely that the presence of defects in signaling molecules downstream of the insulin receptor may contribute to insulin resistance in some of these patients.

The insulin receptor substrate (IRS)-1 was first identified as a central component of the insulin signal transduction pathway (4) and has been shown to be required for mediating both the metabolic and mitogenic responses to insulin (5). More recently, IRS-1 has also been shown to be involved in the signaling pathways emanating from various growth factor and cytokine receptors (5). IRS-1 contains 21 potential tyrosine phosphorylation sites, with 6 in YMXM motifs and 3 in YXXM motifs, as well as over 30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases thought to modulate insulin action (6,7). IRS-1 undergoes phosphorylation of several tyrosines by the activated insulin receptor, including residues 613, 633, 942, and 990 in human IRS-1, which are in YMXM motifs (8) (numbers of amino acids are given according to Nishiyama et al. [9]). Tyrosine-phosphorylated IRS-1 then serves as a multisite docking protein with amino acid sequence motifs around the phosphotyrosine residues mediating specific binding of SH2 domain–containing proteins (10). These include the adaptor proteins Grb2, Nck, and CrkII, the protein tyrosine phosphatase SHP2, the tyrosine kinase Fyn, and the dual specificity lipid/protein kinase phosphatidylinositol 3-kinase (PI 3-kinase) (5).

Because of the prominent role of IRS-1 in the early stages of the insulin signal transduction pathway, investigators have examined the IRS-1 gene in cohorts of patients with type 2 diabetes. To date, 11 amino acid polymorphisms have been identified. This study represents the first to report the examination of the IRS-1 gene in a cohort of patients with severe insulin resistance (n = 15).

Molecular scanning of the IRS-1 gene was performed by polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP) analysis and direct nucleotide sequencing (11). In total, six variant SSCP patterns were detected in this study. Of these, four silent polymorphisms at codons 90, 235, 805, and 894 have been previously reported, as has the mutation Gly972Arg (found in 1 of 15 subjects), which has been reported to cause impaired insulin signaling in transfection studies (12,13). In addition, a novel missense mutation (ATG > GTG), changing Met to Val, was identified in heterozygous form at codon 614 in one patient, a 17-year-old Caucasian female with type A insulin resistance. The patient presented at age 13 with severe acanthosis nigricans on the neck and axillae, facial hirsutism, and oligomenorrhea. Initial biochemical evaluation revealed normal fasting plasma glucose but markedly elevated fasting plasma insulin (703 pmol/l). The patient subsequently developed NIDDM and is treated with diet and metformin. In cultured dermal fibroblasts from the patient, similar levels of expression of both wild-type and mutant IRS-1 alleles were demonstrated by RT-PCR (data not shown).

Restriction fragment length polymorphism analysis (the mutation abolishes an *Nsp*I site) revealed that none of the proband's clinically and biochemically normal relatives carried the mutation (n = 3). The proband's father was found to be heterozygous for the mutation, but had recently deceased with no

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CHO, Chinese hamster ovary; IR, insulin receptor; IRS, insulin receptor substrate; M614V, Met614Val; PCR, polymerase chain reaction; PI 3-kinase, phosphatidylinositol 3-kinase; SSCP, single-stranded conformation polymorphism.

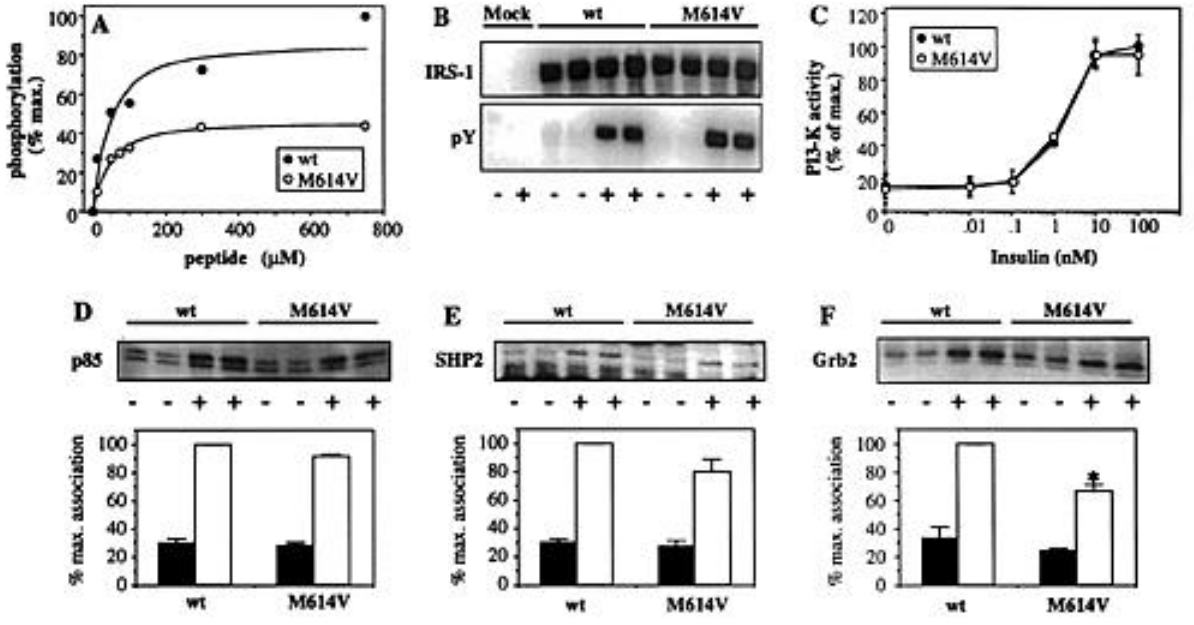

FIG. 1. Characterization of the M614V IRS-1 mutant. *A*: Decreased phosphorylation of a M614V IRS-1 peptide by the insulin receptor in vitro. Synthetic wild-type (RRTLHTDDGYMPMSPGVA) and M614V IRS-1 (RRTLHTDDGYVPMSPGVA) peptides were incubated with immunocaptured-activated insulin receptors in the presence of [γ-^{32}P]ATP and the incorporation of ^{32}P was determined. Results shown are standardized to the maximum incorporation of ^{32}P for the wild-type peptide and represent the mean from three independent experiments performed in duplicate. *B*: Expression and insulin-stimulated phosphorylation of epitope-tagged wild-type and M614V IRS-1. CHO.IR cells were transfected with pIRS1.wt or pIRS1.M614V encoding epitope-tagged wild-type (wt) or M614V IRS-1, respectively. After overnight serum deprivation, cells were left untreated (–) or treated (+) with 100 nmol/l insulin for 5 min. Cells were harvested and lysates were immunoprecipitated using anti-Myc antibody. After SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride membrane and probed with anti–IRS-1 antibody or anti-phosphotyrosine antibody, as indicated, and [^{125}I]-labeled secondary antibody. Results were visualized and quantified using a Phosphorimager. Expression and phosphorylation of wild-type and M614V IRS-1 were comparable in repeated experiments (n = 4) and the blots shown are representative. *C*: Insulin-stimulated PI-3 kinase activity associated with epitope-tagged wild-type and M614V IRS-1 are shown. Immune complexes were prepared as in *B* and assayed for their ability to phosphorylate phosphatidylinositol. Reaction products were resolved by thin layer chromatography and results quantified using a Phosphorimager. Results shown are standardized to the response seen at 100 nmol/l insulin stimulation in cells transfected with epitope-tagged wild-type IRS-1. The data are the mean ± SE from three independent experiments, each performed in duplicate. Insulin-stimulated association of p85 (*D*), SHP2 (*E*), and Grb2 (*F*) with epitope-tagged wild-type and M614V IRS-1 are shown. Upper panels show representative blots of immunoprecipitates as in *B* except membranes were probed with anti-p85 antibody (both α [upper band] and β [lower band] isoforms are recognized by the anti-p85 antibody) (*D*), anti-SHP2 antibody (*E*), or anti-Grb2 antibody (*F*). Lower panels show quantitation of p85 (*D*), SHP2 (*E*), and Grb2 (*F*) association with epitope-tagged wild-type and M614V IRS-1. Results are standardized to the association seen with epitope-tagged wild-type IRS-1 after insulin stimulation. The data are the mean ± SE from three independent experiments, each performed in duplicate. Untreated (□) or treated with 100 nmol/l insulin for 5 min (■). *P < 0.01.

information about his ante-mortem metabolic status being available. A total of 131 unrelated U.K. Caucasian subjects, including 71 patients with type 2 diabetes and 60 control subjects, were studied without identification of the mutation.

The Met614Val (M614V) mutation occurs at the Y+1 position of a YMXM motif of IRS-1, which has been implicated as a key site for the recruitment and activation of PI-3 kinase (14). The methionine residues at the Y+1 and Y+3 positions facilitate efficient phosphorylation of the tyrosine by the insulin receptor (15,16). We therefore hypothesized that decreased phosphorylation of tyrosine 613 in the mutant IRS-1 might result in a reduction of insulin-stimulated association and activation of PI-3 kinase.

To address this, we first examined the ability of wild-type and mutant IRS-1 peptides to act as substrates for the insulin receptor in an in vitro tyrosine kinase assay using immunocaptured insulin receptors (17). Compared with the wild-type peptide, the mutant peptide showed a marked reduction in phosphorylation by the insulin receptor (Fig. 1*A*), with a reduction in V_{max} yet unchanged K_m (V_{max} 95.5 ± 9 vs. 46.9 ± 1 arbitrary units; K_m 49.2 ± 20 vs. 39.1 ± 3 μmol/l peptide). The mechanism for the reduced V_{max} is unclear, but it supports the hypothesis that the mutation could affect phosphorylation of this site in IRS-1.

Having confirmed that the M614V mutation is likely to render this particular YMXM motif in IRS-1 a poor substrate for the insulin receptor tyrosine kinase, the M614V IRS-1 mutant was reconstructed. To facilitate immunoprecipitation of exogenous IRS-1, the Myc epitope (EQKLISEEDL) was introduced at the penultimate codon of both the wild-type and mutant IRS-1 cDNAs by PCR-based methods. Transient transfections were then performed in Chinese hamster ovary (CHO) cells stably overexpressing the insulin receptor (IR). The levels of expression and insulin-stimulated tyrosine phosphorylation were indistinguishable between wild-type and M614V IRS-1, as determined by immunoprecipitation using anti-Myc antibody and Western blotting using anti-IRS-1 or anti-phosphotyrosine antibodies, respectively (Fig. 1*B*). The levels of PI-3 kinase activity associated with wild-type or M614V IRS-1 were then measured in anti-Myc immunopre-

cipitates from transfected cells (18). Surprisingly perhaps, insulin-stimulated PI-3 kinase activity associated with wild-type or M614V IRS-1 was indistinguishable (Fig. 1*C*), as was the association of the p85 regulatory subunit (Fig. 1*D*). We therefore examined the association of wild-type and M614V IRS-1 with other SH2 domain–containing proteins. There was no significant difference between SHP2 association with wild-type and M614V IRS-1 (Fig. 1*E*). However, the association of Grb2 with M614V IRS-1 was moderately but significantly reduced ($P < 0.01$) after insulin stimulation, compared with its association with wild-type IRS-1 (Fig. 1*F*). It is difficult to reconcile this finding with the location of the putative binding site of Grb2 on IRS-1, which has previously been shown to be around the YVNI motif at tyrosine 897 (8).

In summary, we report the first study of the structure of the IRS-1 gene in a group of patients with severe insulin resistance and have demonstrated that mutations in IRS-1 are not common in this disorder. A mutation in the Y+1 amino acid in the YMXM motif centered on tyrosine 613 was detected in one patient. While this mutation impairs the ability of mutant IRS-1 peptide to act as a substrate for the insulin receptor in an in vitro tyrosine kinase assay, the normal insulin-stimulated PI-3 kinase association suggests that this may not be the case for the full length M614V IRS-1 protein. Alternatively, reduced phosphorylation of tyrosine 613 in the M614V IRS-1 molecule may not be sufficient to reduce the association and activation of PI-3 kinase in this system. Surprisingly, insulin-stimulated association of Grb2 with M614V IRS-1 was significantly impaired. One possible interpretation of this finding is that the mutation may affect the overall conformation of the molecule.

While the association of this mutation with extreme insulin resistance remains intriguing, conclusive proof of causality will require further studies in more insulin-sensitive transgenic systems.

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